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Electronic Supporting Information For:

A texaphyrin-oxaliplatin conjugate that overcomes both pharmacologic and molecular mechanisms of cisplatin resistance in cancer cells

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Synthesis

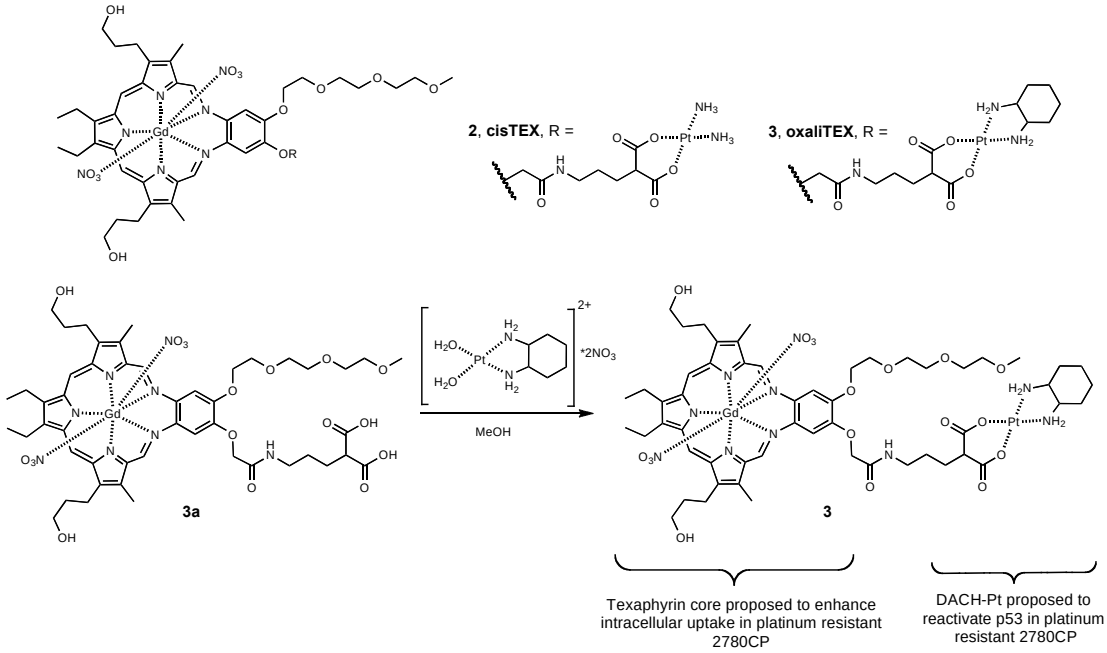

Figure S1. Texaphyrin-Platinum Conjugates **2** (cisTEX) and **3** (oxaliTEX).

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Methods and Materials

General Procedures

Anhydrous solvents and starting materials were purchased from Fisher Scientific and used without further purification unless otherwise specified. The texaphyrin conjugates, both before and after treatment with Pt(II), were purified on RP-tC18 SPE (Waters Sep-Pak) columns containing 10 g of C-18. HPLC analyses were performed on a Shimadzu Analytical/Preparative HPLC system equipped with PDA detector and a Zorbax XDB 5 μM 5 x 100 mm column. Texaphyrin-platinum conjugate **3** was subject to HPLC analysis on a C-18 column using 100 mM $NaNO_3$ and MeCN (10% MeCN to 100% MeCN over 20 min) as the eluent and detection at 472 nm. Mass spectrometric analyses were carried out in the University of Texas at Austin Mass Spectrometry Facility. Low-resolution and high-resolution electrospray mass spectrometric (ESI-MS) analyses were carried out using a Thermo Finnigan LTQ instrument and a Qq-FTICR (7 Telsa) instrument, respectively. Elemental analyses were performed by Atlantic Microlabs Inc. The FDA-approved platinum complexes, cisplatin and oxaliplatin were purchased from Fisher Scientific. Stock solutions were prepared by dissolving these complexes in normal saline (cisplatin) or ultrapure water (oxaliplatin). The stock solutions were sterilized by passing through 0.22 μm filters and platinum concentrations were confirmed by FAAS.[S1]

Cell Lines and Antibodies

The A2780 line was established from a patient's biopsy prior to initiation of any chemotherapeutic regimen.[S2] The resistant cell line, 2780CP, used in this study, was established from A2780 cells, as described previously.[S3] The two cell lines have wild-

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type p53 genotype and/or function. Cells were grown in RPMI containing 10% fetal calf serum and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin). Human specific p53 antibody DO-1 was obtained from Oncogene Research Products (Cambridge, Mass.). Phospho-specific p53 antibody (Ser-15:9284S) was purchased from New England Biolabs (Beverly, MA). Monoclonal p21 antibody (C24420) was obtained from Transduction Laboratories (Lexington, KY) and monoclonal β-actin antibody (AC-15) was acquired from Sigma Chemical Co. (St. Louis, MO).

Preparation and Synthesis of oxaliTEX (3)

Preparation of [(1*R*,2*R*)-cyclohexane-1,2-diamine](diiodo)platinum(II)(DACHPtI_2): To an ice cold, magnetically stirred solution of 5.00 g (12.05 mmol) of K_2PtCl_4 (Acros Organics) and 20.0 g (10 equiv, 120.5 mmol) of KI in ultrapure water was added dropwise 1.38 g (12.05 mmol) of (1*R*,2*R*)-cyclohexane-1,2-diamine in 5.0 mL ultrapure water. The solution was allowed to stir for 1 hour and the resulting light yellow precipitate was filtered and washed sequentially with 10 mL water, 5 mL cold acetone, and 10 mL diethyl ether. The resulting amorphous solid was dried under vacuum overnight to produce 6.2 g (11.05 mmol, 92%) of DACHPtI_2.

In situ preparation of [(1*R*,2*R*)-cyclohexane-1,2-diamine](diaquo)platinum(II)-(DACHPt•2H_2O): To a suspension of 1.0 g (1.78 mmol) of DACHPtI_2 in 50 mL of ultrapure water was added 6.0 g (3.56 mmol) of $AgNO_3$. The suspension was allowed to stir under ambient conditions in the dark for 48 hours. The resulting pale yellow suspension was filtered and the filtrate (a ~35 mM solution of DACHPt•2H_2O) was used

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directly for platination of the texaphyrin derivative as described below.

Platination of Texaphyrin: To a stirring solution of 100 mg (0.08 mmol) of the gadolinium(III) complex of 4,5-diethyl-16-acetoxycarbamoyl-3-propyl-[propanediato]-17-(2-[2-(2-methoxyethoxy)ethoxy]ethoxy)-9,24-bis(3-hydroxypropyl)-10,23-dimethyl-13,20,25,26,27-pentaazapentacyclo[20.2.1.$1^{3,6}$.$1^{8,11}$.$0^{14,19}$]heptacosa-1,3,5,7,9,11(27),12,14,16,18,20,22(25),23-tridecaene (**3a**)[S4] in 50 mL of methanol was added 11.8 mL (~5.0 equiv, 0.41 mmol) of the DACHPt•2H_2O solution prepared as described above. The resulting solution was allowed to stir under ambient conditions in the dark for 24 h, and was monitored by HPLC. Once a single HPLC peak was observed, the reaction was diluted with 250 mL of 100 mM KNO_3. The aqueous solution was added to a 10 g C-18 SPE cartridge. To the cartridge was added 100 mL of 15% MeCN/100 mM KNO_3 to elute off any highly water-soluble impurities. Following this, conjugate **3** was eluted off by treating the cartridge with 100-200 mL of 30% MeCN/100 mM KNO_3. This process was then repeated with a new cartridge. The solution containing **3** was then diluted with 500 mL of 100 mM KNO_3 and added to a new 10 g C-18 SPE cartridge. The cartridge was then washed with 100 mL of ultrapure water to remove any residual salts. Conjugate **3** was then eluted with 25 mL methanol and the solvent was evaporated under reduced pressure to produce 74 mg (0.05 mmol, 61%) of a dark green solid. HPLC (monitoring at 472 nm): Tr 9.8 min 95%. MS (ESI) $(M–NO_3)^+$ 1453.42, $(M-2NO_3)^{2+}$ 695.92 . HRMS(ESI) calculated for $C_{55}H_{75}GdN_8O_{12}Pt$: 1392.43932; found: 1392.43822. Anal. Calcd for $C_{55}H_{75}GdN_8O_{12}Pt•2NO_3•4H_2O$: C 41.58%, H 5.27% N 8.82%; found : C 41.92%; H 5.66%; N 8.42%.

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In vitro Anti-proliferative Activity

The proliferation of exponential phase cultures of A2780 and 2780CP cells was assessed by tetrazolium dye reduction.[S5] In brief, tumor cells were seeded in 96-well microliter plates at 500 and 1000 cells/well, respectively, and allowed to adhere overnight in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat inactivated fetal bovine serum, and antibiotics (200 U/cm^3 penicillin and 200 μg/cm^3 streptomycin). Stock solutions of oxaliplatin (ultrapure H_2O), cisplatin (normal saline) or conjugates **2** and **3** (methanol) were formulated in the indicated solvent for maximum stability and then diluted in medium for secondary stocks of 40-1000 μM depending on the cell line being tested. Secondary stock solutions were serially diluted in medium and immediately added to wells, whereupon plates were incubated at 37^{o}C under a 5% CO_2/95% air atmosphere. After 5 days, the tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical) was added to each well, the plates incubated at 37 ^{o}C, whereupon the medium was removed, the formazan product dissolved in DMSO and absorbance values at 560-650 nm were measured using a microplate reader (Molecular Devices, Sunnyvale, CA). Absorbance values were corrected for background and then normalized to wells containing untreated cells to allow plate-to-plate comparisons. The growth inhibition data were fitted to a sigmoidal dose-response curve to generate IC_{50}, which is the drug concentration inhibiting cell growth by 50%. The IC_{50} is presented as mean ± standard deviation.

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Determination of Free Platinum in Fetal Bovine Serum Incubated with Pt Complexes.

Representative platinum complexes were added to non-heat inactivated FBS samples at final drug concentrations of 5 μg/mL. Samples were then incubated at 37ºC. Aliquots were taken and diluted with 4x the original volume of cold methanol so as to precipitate proteins, which were removed by centrifugation. The supernatant was then subjected to flameless atomic absorption spectrometry (FAAS), which allowed for the determination of % free platinum, which is defined as (Free Platinum in Sample)/(Free Platinum in Sample at T_0) x 100%.

Determination of intracellular Platinum and Isolation of Platinated DNA adducts

Tumor cells (~5 x 10^6 cells) were incubated with 200 μM of the Pt complex in question for 4 h. The cells were then washed with ice-cold PBS, trypsinized, and pelleted by centrifugation. Cells were re-suspended in 200 μL PBS and 200 μL of ATL buffer (Qiagen) and then heated in a water bath (56 °C) for 1 h. The lysed suspension was then diluted with H_2O and subjected to flameless atomic absorption spectrophotometry (FAAS) for Pt determination. The Pt levels in the lysate were normalized to total protein concentration, which was determined through the Bradford assay using a Bio-Rad assay kit.

For the isolation and quantification of platinated DNA, 5 x 10^6 cells were incubated with 200 μM of the Pt complex in question for 4 h and processed, as above. Pelleted cells were then resuspended in 200 μL PBS and subjected to DNA isolation and purification using a

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Qiagen DNeasy Blood and Tissue Kit. The isolated platinated DNA was quantified by UV-Vis spectroscopy and the number of Pt-adducts were determined through FAAS.

Determination of Apoptosis through FACS Analysis

Tumor cells (2-3 x 10^6) were plated overnight and then incubated with media containing platinum drug concentrations at 5 x IC_{50} level. Control cells were treated with vehicle only. At defined time-points, the media was collected and the cells were washed with PBS. The PBS washing was collected and the attached cells were then subjected to 1.0 mM EDTA. The loosened cells were passed through a 40 μm cell strainer. All media and washings were collected, pelleted by centrifugation (3 min @ 300 g) and washed twice with cold PBS. The cells were once again pelleted and suspended in Annexin V binding buffer (BD Pharmingen) at a final concentration of 2 x 10^6 cells/mL. To 1.5 mL centrifuge tubes was added 100 μL cell suspension followed by 5 μL Annexin V-FITC and propidium iodide (PI) solution. Control samples of unstained cells, cells stained with only Annexin V-FITC, and cells stained with only PI were also prepared. Samples were allowed to incubate in the dark for 15 min. After incubation, each sample was diluted with 300 μL of Annexin V binding buffer, and 250 μL of each sample was added to one well of a 96-well plate. Samples were then subjected to FACS analysis using a Millipore Guava easyCyte 8 and analyzed using Guava inCyte software.

Western Immunoblot Analyses.

Western analyses were conducted following continuous exposure to drug concentrations at 2.5-5 x IC_{50} levels. For these studies, ovarian cancer cells were exposed to oxaliTEX

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(conjugate **3**) and oxaliplatin in media at 37°C for 6, 12, and 24-hour time periods. The cells were then washed with ice-cold PBS, removed from plates with a cell-scraper and pelleted by centrifugation (3 min @ 300 g). The cells were then lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.2% NaN_3, 1.0% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) containing 1.0 mM sodium vanadate, and 100 μg/mL aprotinin via sonication (MSE Soniprep 150) for 5 half-second pulses on ice. Cell lysates were centrifuged at 14,000 rpm for 15 min at 5 °C followed by the determination of their protein concentration by the Lowry procedure. Equal protein aliquots of cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose membranes in a wet-transfer unit at 5°C overnight. The membranes were blocked for 2 h using blocking buffer (5% milk in TBS and incubated with specific antibodies) at room temperature. Proteins were detected with HRP-conjugated secondary antibodies (Goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugate) and visualized using the enhanced chemiluminescence assay (Amersham Biosciences, England). Quantification of bands using the ImageJ software allowed assessment of the increase in proteins observed relative to controls.

S1. Z. H. Siddik, F. E. Boxall, K. R. Harrap, *Anal. Biochem.*, 1987, **163**, 21.

S2. A. K. Godwin, A. Meister, P. J. O'Dwyer, C. S. Huang, T. C. Hamilton, M. E. Anderson, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 3070.

S3. Z. H. Siddik, B. Mims, G. Lozano, G. Thai, *Cancer Res.*, 1998, **58**, 698.

S4. J. F. Arambula, J. L. Sessler, M. E. Fountain, W. Wei, D. Magda, Z. H. Siddik, *Dalton Trans.*, 2009, **48**, 10834.

S5. T. Mosmann, *J. Immunol. Methods*, 1983, **65**, 55.