Supporting Information

An integrin-targeted photoactivatable Pt(IV) complex as a selective anticancer pro-drug: synthesis and photoactivation studies

Albert Gandioso,[a] Evyenia Shaili,[b] Anna Massaguer,[c] Gerard Artigas,[a] Alejandro González-Cantó,[a] Julie A. Woods,[d] Peter J. Sadler,[b] and Vicente Marchán*[a]

[a]*Departament de Química Orgànica and IBUB, Universitat de Barcelona, Barcelona, E-08028, Spain. E-mail: vmarchan@ub.edu.*

[b]*Department of Chemistry, University of Warwick, Warwick, CV4 7AL, Coventry, UK.*

[c]*Departament de Biologia, Universitat de Girona, Campus Montilivi, E-17071 Girona, Spain.*

[d]*Photobiology Unit, Department of Dermatology, Ninewells Hospital, Dundee, DD1 9SY, UK.*

Table of contents

1. Experimental procedures

1.1. Materials and Methods.

Unless otherwise stated, common chemicals and solvents (HPLC grade or reagent grade quality) were purchased from commercial sources and used without further purification. Peptide grade DMF was purchased from Scharlau. Fmoc-protected amino acids, resins and coupling reagents for solid phase synthesis were obtained from Novabiochem, Bachem or Iris Biotech. Milli-Q water was directly obtained from a Milli-Q system equipped with a 5000-Da ultrafiltration cartridge. All the assayed compounds displayed a purity ≥95%, as determined by HPLC analysis.

Analytical reversed-phase HPLC analyses were carried out on a Jupiter Proteo column (250x4.6 mm, 4 μm, flow rate: 1 mL/min), using linear gradients of 0.045% TFA in H_2O (solvent A) and 0.036% TFA in ACN (solvent B). In some cases, small-scale purification was carried out using the same column. Large-scale purification was carried out on a Jupiter Proteo semipreparative column (250 x 10 mm, 10 μm, flow rate: 3 mL/min), using linear gradients of 0.1% TFA in H_2O (solvent A) and 0.1% TFA in ACN (solvent B). After several runs, pure fractions were combined and lyophilized.

ESI mass spectra (ESI-MS) were recorded on a Micromass ZQ instrument with single quadrupole detector coupled to an HPLC. High resolution electrospray mass spectra (HR ESI MS) were obtained on an Agilent 1100 LC/MS-TOF instrument.

1.2. Cell lines.

The SK-MEL-28 human melanoma, DU-145 human prostate carcinoma and MBA-MD-468 breast adenocarcinoma cell lines were from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco-BRL, Grand Island, NY, USA) and 100 U mL^-

[1] penicillin-streptomycin (Gibco-BRL) at 37˚C in a humidified atmosphere containing 5% CO_2. Cell growth and morphology were assessed using an inverted microscope.

1.3. Synthesis of Pt-c(RGDfK) conjugate (3).

To a solution of **2** (6.3 mg, 2.2 mol equiv.) and HATU (4.0 mg, 2.1 mol equiv.) in anhydrous DMF (0.5 mL), DIPEA (5.0 µL, 5.7 mol equiv.) was added. After stirring for 2 min at room temperature, the reaction mixture was added to peptide **4** (5.0 mg, 5.1 µmol) previously dissolved in anhydrous DMF (0.25 mL) and DIPEA (10 µL, 11.4 mol equiv.) under Ar atmosphere. After stirring for 2 h at room temperature under Ar, the solvent was evaporated *in vacuo* and the conjugate was purified by semipreparative HPLC (gradient from 0 to 80% B in 30 min, flow rate: 3 mL/min, R_t = 17.5 min). Overall yield (synthesis + purification): 3.9 mg of a pale yellow solid, 54 %. Characterization: R_t= 18.2 min (analytical gradient: 0 to 80 % in 30 min); HR ESI MS, positive mode: *m/z* 1302.4728 (calcd mass for $C_{47}H_{67}N_{18}O_{14}Pt$ $[M+H]^+$: 1302.4729).

1.4. Integrin expression analysis.

The expression of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins on the cell surface was analyzed by double immuno-fluorescence. The cells were incubated for 30 min at 4˚C with monoclonal antibodies against human $\alpha_V\beta_3$ (clone LM609) (Millipore, Temecula, CA) and $\alpha_V\beta_5$ integrin (sc-81632) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or medium alone as negative control. After washing with phosphate-buffered saline (PBS) (Gibco-BRL), cells were incubated for additional 30 min at 4˚C with the Alexa-Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA, USA). Next, the cell fluorescence was analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with CellQuest™ software (Becton Dickinson). 10.000 cells were analysed in each experiment. Fluorescence intensity was represented on a four orders of magnitude log scale (1-10.000).

1.5. Internalization experiments with Fluorescein-labeled c(RGDfK) (7).

The uptake efficiency of **7** by SK-MEL-28 and DU-145 cells was quantified by flow cytometry. 60.000 cells were seeded onto 24-well plates and allowed to attach for 24 h. Then, the cells were treated with either **7** at 10 or 50 μM, or medium alone as a control, for 1 h at 37ºC. After washing the cells three times with cold PBS (Gibco), the cells were harvested by trypsinization and the fluorescence of the cells, corresponding to the internalization of **7**, was analysed using a FACSCalibur (Becton Dickinson Immunocytometry Systems). The geometric mean fluorescence of 10.000 cells was determined with the CellQuestTM software (Becton Dickinson).

1.6. Phototoxicity studies.

Cell culture and other chemicals were obtained from Sigma-Aldrich Ltd (Poole, UK). Disposable sterile cell culture plastics were obtained from Greiner Bio-One (Cambridge, UK). All procedures were carried out in a specially adapted photobiology laboratory with ambient light levels measured below 1 lux (Solatell, UK). Phototoxicity was determined according to the OECD 432 guideline with some modification as described below.

For experiments, cells were mycoplasma free and maintained in antibiotic-free conditions in a humidified atmosphere of 5% CO_2/95% air. Cells were seeded at a density of 6-7 x 10^4cells/cm^2 in 96-well plates. Complexes were prepared immediately before use in Earle' Balanced Salt Solution (EBSS) and filter sterilized. Irradiations were performed in optically clear medium and experiments were controlled for light, complex, and handling. Visible light (5 J/cm^2) was delivered by a bank of TL03 fluorescent tubes (λ_{max}: 420 nm) with wavelengths shorter than 400 nm blocked by filtering. Irradiance was measured with a Gigahertz Optik meter calibrated to the source using a spectroradiometer (Bentham Instruments Ltd, UK; mean irradiance 1.3 mW/cm^2 ± 0.1). UVA (5 J/cm^2; λ_{max}: 365 nm) was delivered from a bank of 6ft fluorescent 15500/100W tubes with wavelengths <320 nm blocked. Irradiance was

measured with an International Light meter calibrated to the source using a spectroradiometer (Bentham Instruments Ltd, UK; mean irradiance 1.7 mW/cm^2 ± 0.1). Irradiances were measured through filters and cell culture plate lids. Sham-irradiated cells were treated identically and in parallel with irradiated cells, except that photons were blocked. The viability of DU-145 cells irradiated with visible light was 102.5 ± 6.7%; and of SK-MEL-28 cells was 101.3 ± 6.9%. For UVA this decreased to 71.8% and 85.1%, respectively.

Phototoxicity was determined by neutral red dye uptake 24 hours after irradiation at 540 nm in a Synergy™ 2 plate reader. The concentration of complex required to inhibit dye uptake by 50% (IC_{50} value) was calculated from the log-transformed cytotoxicity curves normalised to untreated cells (Graphpad Prism v.6). Goodness of fit was determined by the 95% confidence interval of the IC_{50} value, and the R2 value. Experiments were performed in triplicate, and independently repeated at least once on cells of differing passage number.

1.7. Platinum accumulation in cancer cells.

For platinum cellular uptake studies, about 1.0 $x10^6$ SK-MEL-28, DU-145 and MBA-MD-468 cells were plated in 100 mm Petri dishes and allowed to attach for 24 h. Next, the plates were exposed to *trans,trans,trans*-$[Pt(N_3)_2(OH)_2(py)_2]$ (**1**), *trans,trans,trans*-$[Pt(N_3)_2(OH)(succ)(py)_2]$ (**2**) or to the Pt-c(RGDfK) conjugate (**3**) at a 10 μM concentration. Additional plates were incubated with medium alone as negative control. After 1 h of incubation in the dark at 310 K, the cells were rinsed three times with cold PBS and harvested by trypsinization. The number of cells in each sample was counted manually in a haemocytometer using the trypan blue dye exclusion test. Then the cells were centrifuged to obtain the whole cell pellet for ICP-MS analysis. All experiments were conducted in triplicate.

1.8. ICP-MS analysis.

The whole cell pellets were dissolved in 500 µL of concentrated 72% v/v nitric acid, and the samples were then transferred into wheaton v-vials (Sigma-Aldrich) and heated in an oven at 373 K for 18 h. The vials were then allowed to cool, and each cellular sample solution was transferred into a volumetric tube and combined with washings with Milli-Q water (1.5 mL). Digested samples were diluted 5 times with Milli-Q to obtain a final HNO_3 concentration of approximately 3.6% v/v. Platinum content was analyzed on an ICP-MS Perkin Elmer Elan 6000 series instrument at the Centres Científics i Tecnològics of the Universitat de Barcelona. The solvent used for all ICP-MS experiments was Milli-Q water with 1% HNO_3. The platinum standard (High-Purity Standards, 1000 µg/mL $\pm$ 5 µg/mL in 5% HNO_3) was diluted with 1% HNO_3 to 20 ppb. Platinum standards were freshly prepared in Milli-Q water with 1% HNO_3 before each experiment. The concentrations used for the calibration curve were in all cases 0, 0.2, 0.4, 1, and 2 ppb. The isotope detected was ^{196}Pt and readings were made in triplicate. Rhodium was added as an internal standard at a concentration of 10 ppb in all samples.

1.9. Statistical analysis.

The statistical analysis was performed with the SPSS statistical software for Windows (version 15.0; SPSS Inc., Chicago, IL, USA). Quantitative variables were expressed as mean and standard desviation (SD).

2. Characterization of Pt-c(RGDfK) conjugate (3).

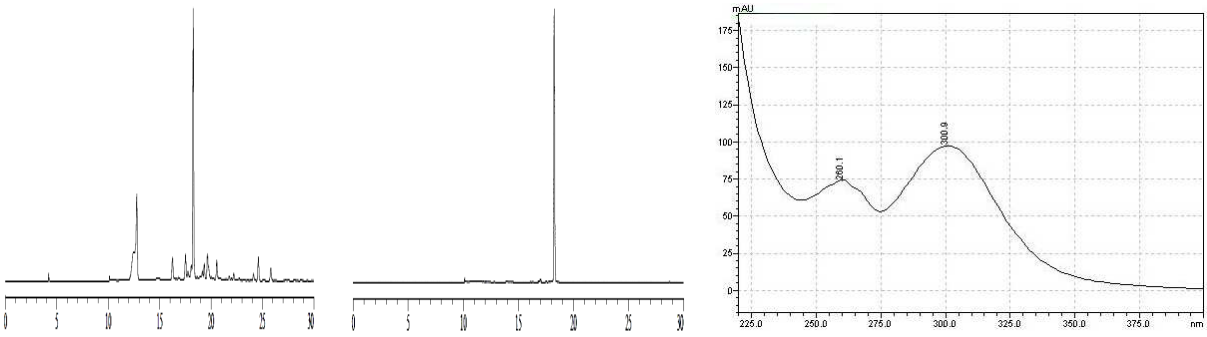

Fig. S1 Reversed-phase HPLC traces for Pt-c(RGDfK) conjugate (**3**): reaction crude (left) and purified (center), and UV-Vis spectrum of the compound in ACN/H_2O solvent (right).

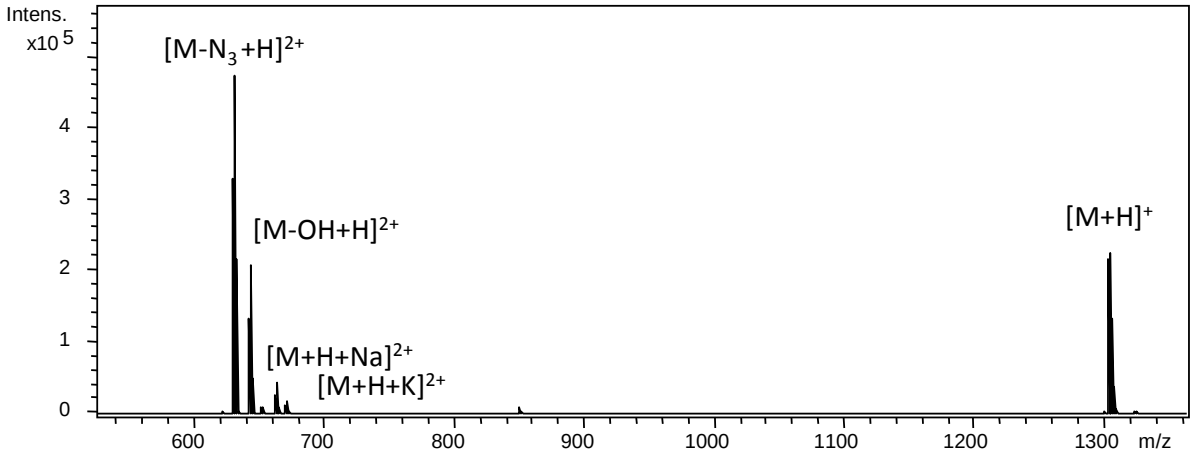

Fig. S2 High-resolution ESI MS of conjugate **3**.

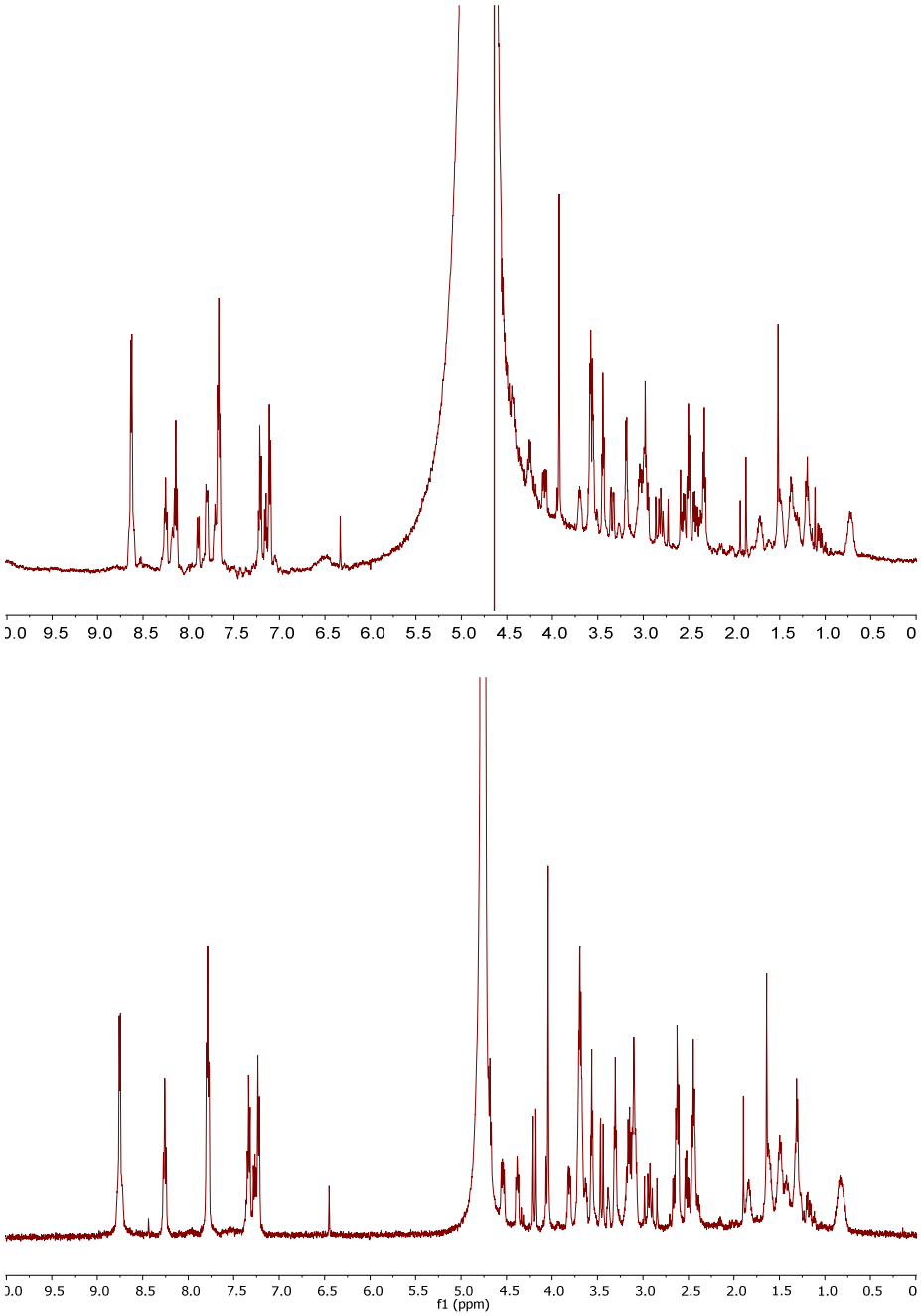

Fig. S3. 500 MHz ^{1}H NMR spectra of conjugate **3** in H_2O/D_2O 9:1 v/v (top) and in D_2O (bottom).

3. Photoactivation of conjugate 3.

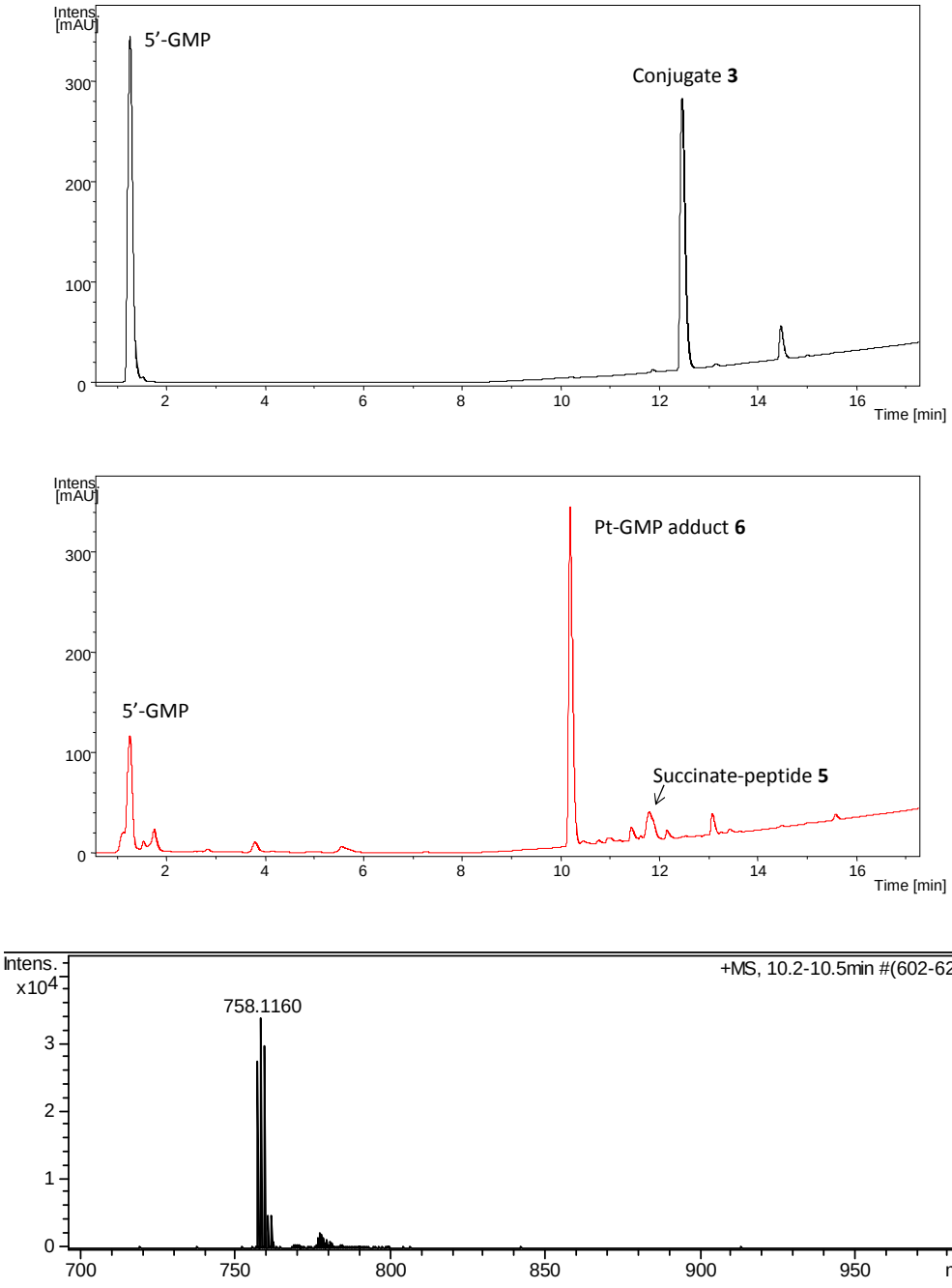

Fig. S4 Reversed-phase HPLC traces for the reaction between 5´-GMP and conjugate **3**: t= 0 (top) and after irradiation with blue light (λ_{irr}= 420 nm, 11 mW cm^{-2}, 45 min, 37ºC) (center). The sample was irradiated using the LZC-ICH2 photoreactor equipped with 420 nm lamps (Luzchem). High-resolution ESI MS of the Pt-GMP adduct **5** (bottom).

4. Cellular uptake of the fluorescein-labelled peptide.

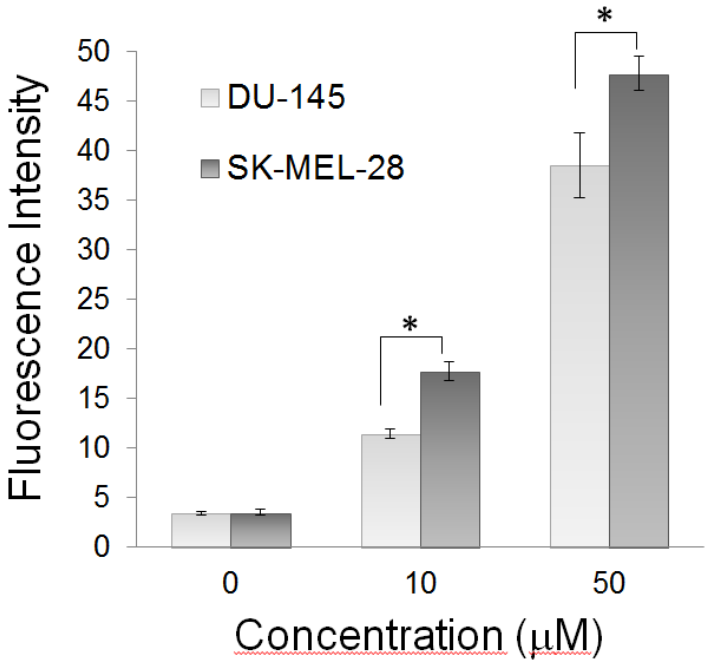

Fig. S5 Intracellular delivery efficiency of Fluo-c(RGDfK) (**7**) in SK-MEL-28 and DU-145 cells. Cells were incubated for 1 h with the peptide at 10 and 50 μM or with medium alone (0 μM) as a control. The fluorescence intensity of the cells, corresponding to the intracellular uptake of the peptides, was determined by flow cytometry. Each column in the graphs represents the mean fluorescence intensity of three independent experiments ± SD. * indicates statistically-significant differences ($P < 0.05$).

5. Cytotoxicity plots

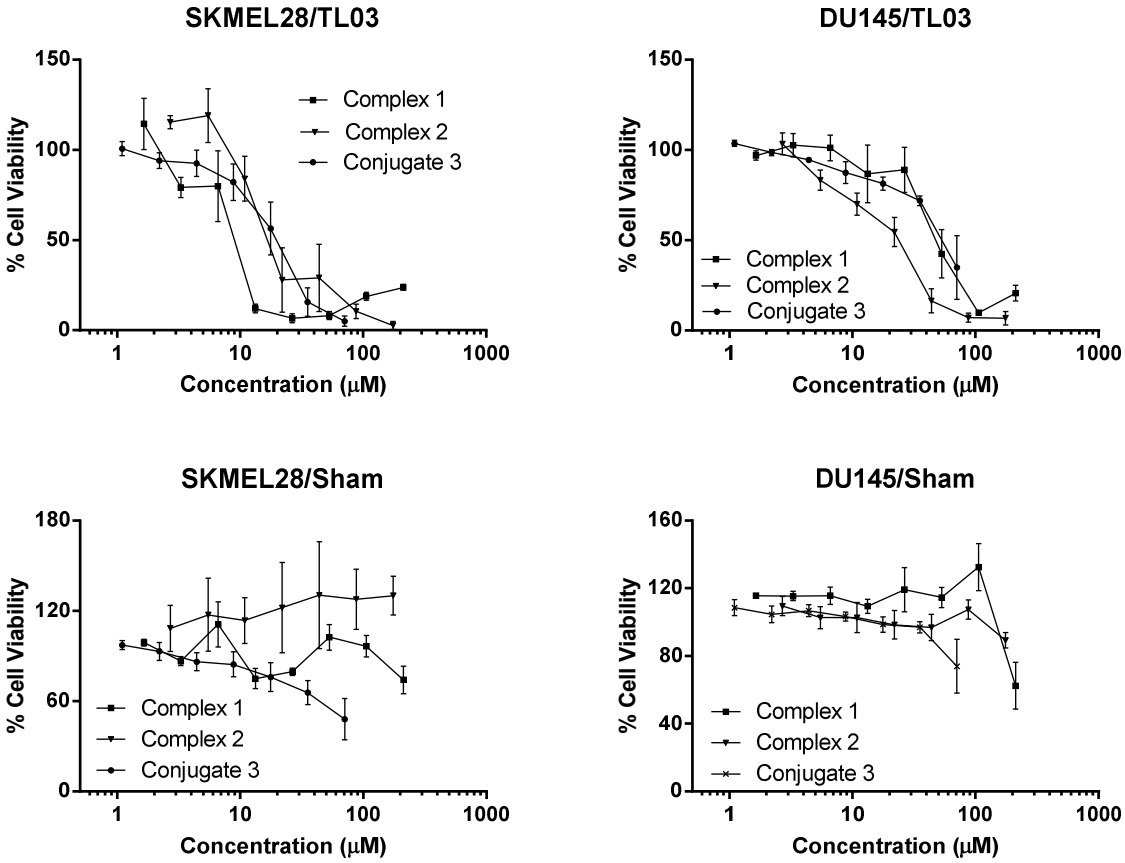

Fig. S6 Cytotoxicity plots with 5 J/cm^2 TL03 irradiation or with sham irradiation. Left hand panel: SK-MEL-28. Right hand panel: DU-145.