Microfocus x-ray fluorescence mapping of tumour penetration by an organo-osmium anticancer complex

Carlos Sanchez-Cano,* Isolda Romero-Canelón, Kalotina Geraki, and Peter J. Sadler*

Electronic Supplementary information (ESI)

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Experimental
Complexes \([\text{Os}({\eta}^6-\text{p-cym})(\text{Azpy-NMe}_2)]\text{PF}_6\) \((1)\), and \([\text{Os}({\eta}^6-\text{p-cym})(\text{Azpy-NMe}_2)\text{Cl}]\text{PF}_6\) \((1-\text{Cl})\) were synthesised and characterised as described previously.\(^1\)

Cell culture
Human ovarian carcinoma cells (A2780) were obtained from the European Collection of Cell Cultures (ECACC) and were grown using RPMI-1640 medium supplemented with 10% v/v of foetal calf serum, 1% v/v of 2 mM glutamine and 1% v/v penicillin/streptomycin. Cells were grown as adherent monolayers at 310 K in a 5% CO\(_2\) humidified atmosphere and passaged at approximately 70-80% confluence.

*In vitro* Growth Inhibition Assay in 2D models
Briefly, A2780 ovarian cells were seeded in 96-flat-well plates. The cells were pre-incubated in drug-free media at 310 K for 72 h before adding different concentrations of the complexes 1 and 1-Cl. The drug exposure period was 16, 24 or 48 h. The SRB assay was used to determine cell viability.\(^2\) Absorbance measurements of the solubilised dye allowed the determination of viable treated cells compared to untreated controls. IC\(_{50}\) values (concentrations which caused 50% of cell growth inhibition), were determined as duplicates of triplicates in two independent sets of experiments and their standard deviations were calculated.

Growth of 3D spheroid models and *in vitro* growth Inhibition assays
A2780 ovarian cancer cells were seeded in U-bottom 96-well plates with repellent surface at various densities (e.g. between 1000 and 5000 cells/well) and allowed to grow for 5 days in drug-free medium. Dimensions and structures of the formed spheroids was monitored by light microscopy. For tumour inhibition assays, A2780 ovarian cancer cells were seeded at a density of 2500 cells/well in a U-bottom 96-well plate with repellent surface and allowed to grow for 3 days. Spheroids were exposed to various concentrations of complexes 1 and 1-Cl for 16, 24 or 48 h. After this time, cell survival was determined using the CellTiter-Glo 3D Luminescent Cell Viability Assay from Promega according to the manufacturer’s instructions. IC\(_{50-\text{sph}}\) values (concentrations which caused 50% of cell growth inhibition), were determined as duplicates of triplicates in two independent sets of experiments and their standard deviations were calculated.

Spheroids for SXRF experiments
A2780 ovarian cancer cells were seeded at a density of 2500 cells/well in round-bottom 96-well plates with repellent surface and allowed to grow for 3 days. Spheroids were exposed to \(\frac{1}{2}\text{IC}_{50}\) concentrations of complex 1 \((0.7 \mu\text{M})\) for 16, 24 or 48 h. Then, they were fixed with 2% glutaraldehyde in sodium cacodylate buffer at pH 7.6 (Agar Scientific) for 1 h, and washed 3x with PBS. Finally, spheroids were dehydrated with graded levels of ethanol (20-100% ethanol), and infiltrated with 100% propylene oxide for 1 h, followed by a 1:1 mixture of propylene oxide and EPON resin for 6 h. This was replaced with several changes of 100% resin over 18 h before curing for 24 h at 333 K. Blocks were trimmed and sectioned on a Leica Ultracut E ultramicrotome (Leica Microsystems). 500 nm thick sections were deposited on sapphire discs. Untreated spheroids were prepared similarly as negative controls.
XRF mapping of spheroid sections

XRF experiments were performed on the I18 beamline at the DIAMOND synchrotron light source. Irradiation of the samples was carried out with 12 keV energy using a beam focused at 2x2 μm². Detection was performed using a 6-element silicon drift detector (SGX). Scan step size was fixed at 10x10 μm² (dwell time 1 s) for coarse scans and 2x2 μm² (dwell time 1 s) for fine scans. The raw maps were processed using the free PyMCA software. Concentration maps were produced assuming a matrix of 500 nm thick soft tissue (Fig. S5), while the quantitative calibration was determined using a thin film X-ray fluorescence 7-element reference sample (AXO Dresden GmbH; Table S1). The concentration maps were further processed using FIJI ImageJ package with the EDFread plugin.

XAS acquisition

XAS spectra were collected in areas identified in the XRF maps as having the highest concentration of Os. Spectra were collected at ambient temperature with a focused beam (2x2 μm²) in fluorescence mode using a 6-element silicon drift detector (SGX). Acquisition started 70 eV before the expected L3 edge and finished 180 eV after (10.80-11.05 keV), and used 1 eV step-size with 2 s accumulation.

References

Table S1. Elemental area density of a 7-element RF4-200-S1749 reference sample (according to the supplier, AXO Dresden GmbH). The calibration was done at 12 keV excitation energy using a beam focused at 2x2 μm² (Scan step size 2x2 μm²; dwell time 1s).

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<th>Element</th>
<th>Emission Lines</th>
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<tr>
<td>Pb</td>
<td>L</td>
<td>7.61±0.96</td>
</tr>
<tr>
<td>La</td>
<td>L</td>
<td>11.01±0.62</td>
</tr>
<tr>
<td>Pd</td>
<td>L</td>
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<tr>
<td>Mo</td>
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<tr>
<td>Cu</td>
<td>K</td>
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<tr>
<td>Fe</td>
<td>K</td>
<td>5.04±0.87</td>
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<tr>
<td>Ca</td>
<td>K</td>
<td>19.31±1.10</td>
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Fig. S1. Bright field images and diameters of 3D A2780 ovarian carcinoma spheroids generated after 5 days incubation of different initial cell densities (1000-5000 cells/well). Statistical significance was evaluated using Welch’s t-test (* p < 0.05, ** p < 0.01, *** p < 0.001).
Fig. S2. Antiproliferative activity (IC$_{50}$ μM) of A2780 ovarian carcinoma A) 3D spheroids or B) cell monolayer cultures, after treatment with 1, 1-Cl, or cisplatin using various drug exposure times (16, 24, or 48h).
Fig. S3. Effect of incubation time (expressed as the ratio $IC_{50\cdot16h}/IC_{50\cdot48h}$) on the antiproliferative activity ($IC_{50}$ μM) of 1, 1-Cl, or cisplatin on A2780 ovarian carcinoma cell monolayer cultures and 3D spheroids.
Fig. S4. Normalised SXRF spectrum from a section (500 nm thick) of an A2780 human ovarian carcinoma spheroid treated with 0.7 μM 1 (½IC_{50}) for 48 h. Raster scan: 2×2 μm² step size, 1 s dwell time. The spectrum was fitted using PyMca,¹ and the contribution of selected elements is displayed: Zn K x-ray emission lines, and Os L X-ray emission lines. A) 0-10 keV energy range; B) 7.5-10 keV energy range.
**Fig. S5.** Bright field images of a control and two A2780 human ovarian carcinoma spheroid sections (500 nm thick) after treatment of the spheroids with 0.7 μM 1 (½×IC₅₀) for 0, 16, 24 or 48 h. Yellow squares in bright field images indicate areas of the spheroid studied using SXRF.

**Fig. S6.** SXRF elemental maps of Os in the control and two A2780 human ovarian carcinoma spheroid sections shown in Fig. S5 (500 nm thick) after treatment of the spheroids with 0.7 μM 1 (½×IC₅₀) for 0, 16, 24 or 48 h. Raster scan: 2x2 μm² step size, 1 s dwell time. Scale bar 100 μm. Calibration bar in ng mm⁻². Red areas in SXRF elemental maps indicate the limits of the spheroids.
**Fig. S7.** SXRF elemental maps of Ca in the control and two A2780 human ovarian carcinoma spheroid sections shown in Fig. S5 (500 nm thick) after treatment of the spheroids with 0.7 μM 1 (½xIC₅₀) for 0, 16, 24 or 48 h. Raster scan: 2x2 μm² step size, 1 s dwell time. Scale bar 100 μm. Calibration bar in ng mm⁻². Red areas in SXRF elemental maps indicate the limits of the spheroids.

**Fig. S8.** SXRF elemental maps of Zn in the control and two A2780 human ovarian carcinoma spheroid sections shown in Fig. S5 (500 nm thick) after treatment of the spheroids with 0.7 μM 1 (½xIC₅₀) for 0, 16, 24 or 48 h. Raster scan: 2x2 μm² step size, 1 s dwell time. Scale bar 100 μm. Calibration bar in ng mm⁻². Red areas in SXRF elemental maps indicate the limits of the spheroids.
Fig. S9. Osmium penetration into A2780 human ovarian carcinoma spheroid after treatment with 0.7 μM 1 (½xIC₅₀) for 0, 16, 24 or 48 h. (A) Spheroids areas containing Os (indicated as black dots) (B) Percentage of spheroid area containing Os.
**Fig. S10.** Total (pg/section) Os (A), Zn (B) or Ca (C), and ratios Os/Zn (D) or Os/Ca (E) in sections (500 nm thick) of A2780 human ovarian carcinoma spheroid treated with 0.7 μM 1 (½xIC_{50}) for 0, 16, 24 or 48 h. Quantities of the different elements were obtained from the respective SXRF elemental maps (Raster scan: 2x2 μm^2 step size, 1 s dwell time).