ESRF	**Experiment title:** Organelle localisation of osmium-based antitumor drugs by antibody labelling using X-ray fluorescence nanoprobe	**Experiment number**: CH-4546
Beamline: ID16A-NI	**Date of experiment**: from: 10th November 2015 to: 16th November 2015	**Date of report**: 06/09/2016
Shifts: 18	**Local contact(s)**: Dr. Yang Yang	*Received at ESRF:*

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We aimed to assess organelle localisation of an Os(II) drug candidate, namely [Os(η^6-*p*-cym)(Azpy-NMe$_2$)I]PF$_6$ **[1]**, where *p*-cym = *p*-cymene and Azpy-NMe$_2$ = 2-(*p*-[dimethylamino]phenylazo) pyridine, by X-ray fluorescence nanoprobe (XRFN). This potent anticancer complex shows interesting *in vitro* and *in vivo* antiproliferative properties due to alteration of the cellular redox balance (possibly involving mitochondrial pathways).[1,2,3] Recent XRFN experiments show that **1** concentrates in small elliptical areas within the cytoplasm of treated cells (0.3-0.4 µm in length),[4] similar in size and shape to mitochondria of fast growing tumour cells (as A2780), but also to other cellular organelles (i.e. lysosomes, peroxisomes and endosomes). We intended to use NANOGOLD™-conjugated antibodies (targeting individually mitochondria, lysosomes, peroxisomes or endosomes) to identify the organelle targeted by **1** in A2780 using XRFN.[5] This correlative imaging is key to validate and further understand its mechanism of action.

A2870 ovarian carcinoma cells treated with 1 µM of **1** were prepared on 5x5 mm carbon frames with 500 nm silicon nitride windows (Silson Ltd, UK), both as epon embedded sections (500 nm thickness) and whole cells (fixed with PFA). Selected organelles were then stained with Au using secondary antibody labelling strategies. Untreated cells were prepared similarly as negative controls. The frames were placed under vacuum (in a sample holder with capacity for four frames) and irradiated individually using ID16A standard energy setting (17 KeV) with the beam focused to a 42x42 nm^2 size. XRF emission was collected using two six elements silicon drift diode detectors. Fast coarse scans were taken to identify

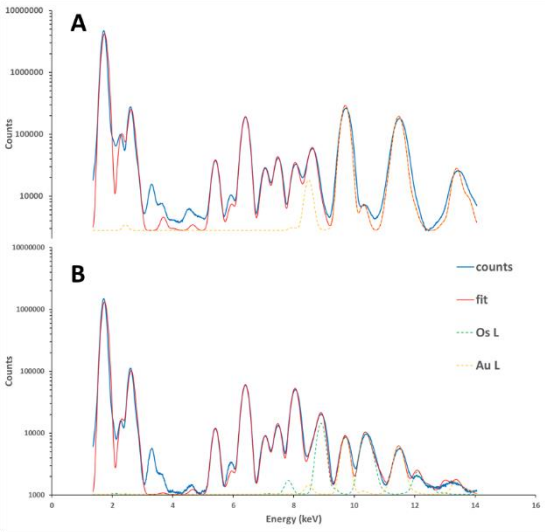

Fig 1. XRF spectra of PFA fixed A2780 cell treated with Au-Anti-Mitochondria antibodies (A), or 1 µM 1 + Au-Anti-Mitochondria antibodies (B).

interesting areas (step size 400x400 nm, dwell time 100 ms), that were then studied using higher resolution (step size 50x50, dwell time 50 ms). Phase constrast images were obtained to determine density of the samples (only in whole cells, a density of 500 nm was estimated for sections) for the processing of the images (that were normalised according to the beam flux).

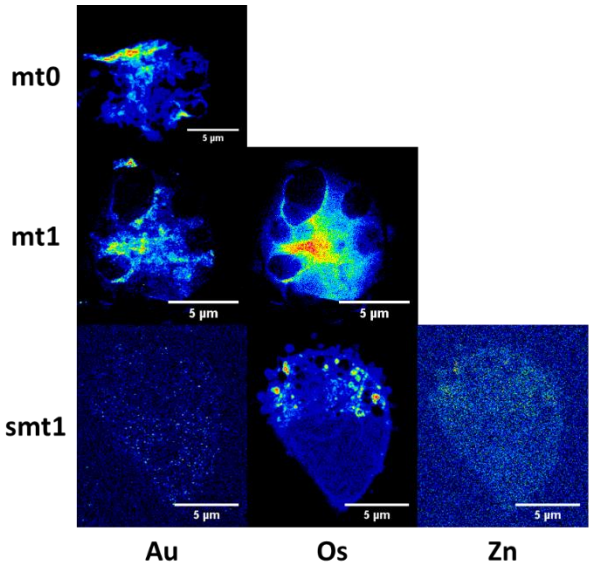

Fig2. XRF maps showing Au, Os or Zn of A2780 cells or sections treated with 0 (mt0) or 1 µM (mt1, cell; smt1, section) of 1 for 24 h. step size 50x50 nm^2. dwell time 50 ms.

A Distinct Au-L emission peaks were observed on XRF spectra obtained from whole cells after treatment with NANOGOLD^{TM}-antibody conjugates (Fig. 1). Clear peaks corresponding to Os-L emission were also observed in cells treated with **1** (Fig. 1; as reported before). Nevertheless, the (well established) protocols used for antibody labelling and Au staining proved to be too harsh for A2780 cells. As a result, we achieved good Au staining, but damaged greatly the cellular structure of our samples. This meant that the reported (elliptical) areas with high concentration of Os could not be recognised any longer (Fig. 2), hampering our attempts to assess the organelle localisation of Os. Phase contrast imaging of the samples confirmed that cells were damaged during the process (Fig. 3). Alternatively, epon embedded sections maintained well their cellular structure during antibody treatment, and the previously observed Os distribution was maintained. However, Au labelling did not work as expected, and yielded only marginal and unspecific labelling. Additionally, some biologically relevant elements were washed off from the section samples during the staining process (i.e. Zn; Fig. 2).

Overall, XRFN at ID16A worked as expected, providing us with elemental maps with the required spatial resolution and sensitivity. However, organelle labelling and retention of the cellular structures of the samples could not be achieved at the same time. This impeded us from determine the organelle localisation of **1** through colocalisation of Os-Au after labelling of endosomes, lysosomes, peroxisomes or mitochondria. New methods for antibody labelling and gold staining will need to be used in our samples and tested with alternative methods to XRF (i.e. TEM-EELS) before resubmition of the proposal is considered.

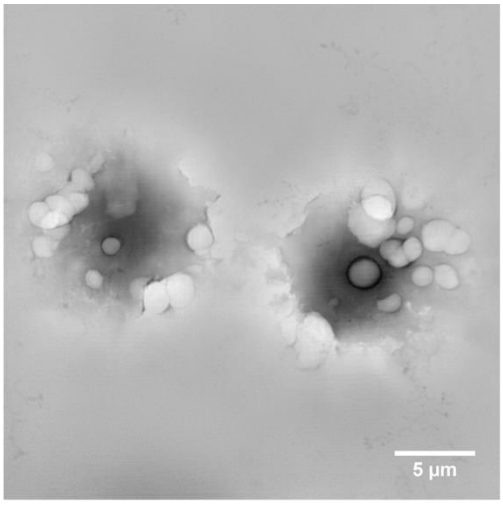

Fig 3. Phase contrast image of A2780 cells treated with 1 µM of 1 for 24 h. step size 15x15 nm^2.

1) Fu Y, et al. *J Med Chem.* **2010**, *53*, 8192; 2) Shnyder SD, et al. *Med Chem Comm.* **2011**, *2*, 66; 3) Hearn JM, et al. *Proc. Natl. Acad. Sci. U.S.A.*, **2015**, 112, E3800; 4) Sanchez-Cano C, et al. *submitted* (*ESRF report CH-4283*); 5) McRae R, et al. *J Struct Biol*. **2006**, *155*, 22.