ESRF	**Experiment title:** Determination of oxidation states of an osmium antitumor drug in human ovarian carcinoma cancer cells	**Experiment number**: CH-4714
Beamline: ID16B-NA	**Date of experiment**: from: 4th March 2016 to: 9th March 2016	**Date of report**: 06/09/2015
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We aimed to characterise the intracellular redox behaviour of a candidate Os(II) anticancer drug, $[Os(\eta^6\text{-p-cym})(Azpy\text{-}NMe_2)I]PF_6$ **[1]**, where p-cym = p-cymene and Azpy-NMe_2 = 2-(p-[dimethylamino]phenylazo) pyridine, in cancer cells using a combination of nano-focused XRF and XANES at ID16B-NA. This potent anticancer complex has the ability to catabolise H_2O_2 producing $OH^{\cdot}$ radicals,[1] and shows interesting *in vitro* and *in vivo* antiproliferative properties due to alteration of the cellular redox balance (possibly involving mitochondrial pathways).[2,3,4] Recent nano-focused XRF experiments show that **1** concentrates in small elliptical areas (0.3-0.4 µm in length) within the cytoplasm of treated cells,[5] but did not provide information on the oxidation state of intracellular Os. This information will provide new insight into the redox nature of the complex inside cells, which 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 (cryofixed using a propane-ethane mixture or fixed with PFA). Untreated cells were prepared similarly as negative controls. The beam was focused to a 70x100 nm^2 size, and the frames placed in the experimental hatch of ID16B. XRF maps were collected to detect ROIs with high concentration of Os (energy setting used: 11.05 keV), using the three elements silicon drift detectors. Fast coarse scans

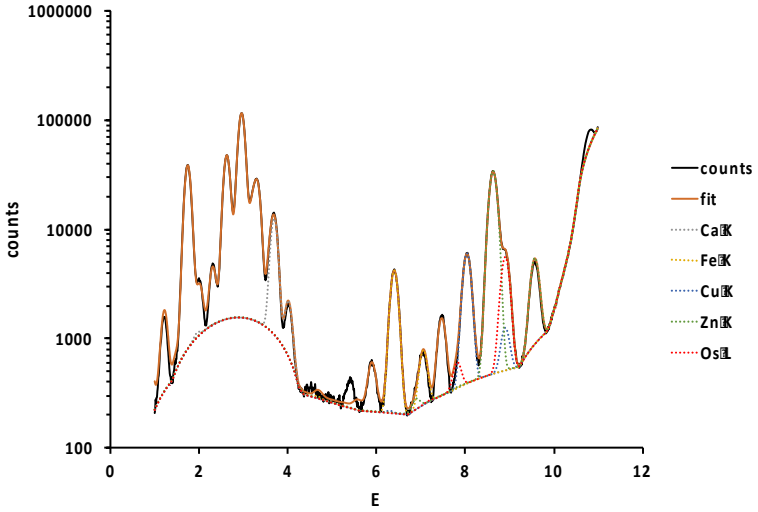

Fig. 1. XRF spectrum of cryofixed A2780 cell treated with 1 µM 1

were taken to identify interesting areas (step size 1x1 µm, dwell time 1000 ms), that were then studied using higher resolution (step size 100x100 nm^2, dwell time 1000 ms). As reported before, clear peaks corresponding to Os-L emission were observed in samples treated with **1** (Fig. 1), and maps confirmed that Os did not get localised in the same areas where high concentrations of Zn were found (Fig. 2). Our scans also showed that cryofixation preserved better intracellular Os and the structure of A2780 cells than PFA (not shown).

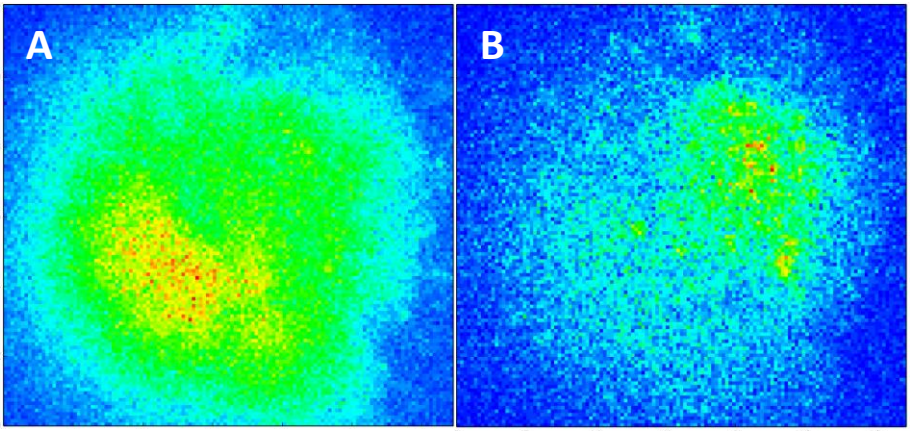

Fig 2. XRF maps of cryofixed A2780 cells treated 24 h with 1 µM 1 showing distribution of Zn (A) or Os (B). Step size 100x100 nm^2; dwell time 1000 ms.

After this we collected XAS spectra in areas with high concentration of Os, starting 50 eV before the expected edge and finishing 250 eV after (10.82-11.12 keV). We used 1 eV step size with 3s accumulation, and 4 acquisitions were collected for each experiments to increase the S/N ratio. A decrease in the intensity of the XAS spectra was normally observed after the first acquisition. Spectra were collected in all three samples (cryofixed, PFA fixed, or sections), and in different Os areas found in the same cell, yielding equivalent spectra.

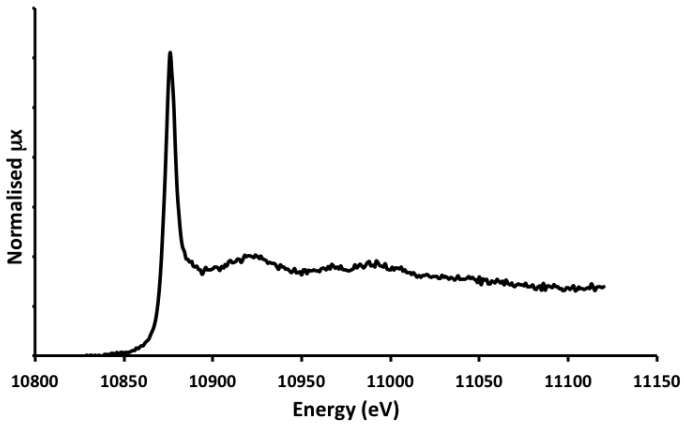

Fig 3. XAS spectrum of Os on cryofixed A2780 cells treated 24 h with 1 µM 1.

Averaging acquisitions collected over 24 h on cryofixed cells we improved dramatically S/N ratio (Fig 3). This allowed us to obtain a XAS spectra with well defined XANES and short EXAFS where fine structure could be observed.

Recent experiments have shown that **1** is a prodrug that get activated inside cells by the hydrolysis of the Os-I bond, generating the more reactive hydroxido species that can interact with different cellular components. This is catalysed by GSH and maybe other reducing agents.[1] The collected XAS spectrum has the potential to provide important information about the intracellular oxidation state of **1** but also its coordination sphere. Initially, XAS spectra of standards of Os^0 (Os powder), Os^{II} (**1**), Os^{III} ($OsCl_3$) and Os^{IV} ($[NH_4]_2OsCl_6$) were collected in ID16B-NA. Further standards of different analogues of **1** were collected in B18 (Diamond); Os^{II}-I (**1**-I); Os^{II}-Cl (**1**-Cl), Os^{II}-OH (**1**-OH), Os^{II}-S (**1**-NAC). Analysis of samples and standards is currently being performed, aiming to obtain information on the intracellular behaviour and speciation of **1**. This would lead to a better understanding of the mode of action of our Os drug candidate, and help in its progression towards phase I clinical trials.

1) Needham R, et al. *submitted*; 2) Fu Y, et al. *J Med Chem*. **2010**, *53*, 8192; 3) Shnyder SD, et al. *Med Chem Comm*. **2011**, *2*, 66; 4) Hearn JM, et al. *Proc. Natl. Acad. Sci. U.S.A.* **2015**, *112*, 3800; 5) Sanchez-Cano C, et al. *submitted* (*ESRF report CH-4283*).