**Intracellular Localization of an Osmocenyl-Tamoxifen Derivative in Breast Cancer Cells Revealed by Synchrotron Radiation X-ray Fluorescence Nanoimaging**

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**Abstract:** A series of tamoxifen-like metallocifens of the group-8 metals (Fe, Ru, and Os) has strong antiproliferative activity on the triple-negative breast cancer cells (MDA-MB-231). To shed light on the mechanism of action of these molecules, synchrotron radiation X-ray fluorescence nanoimaging studies were performed on cells exposed to osmocenyl-tamoxifen (Oc-OH-Tam) to disclose its intracellular distribution. High-resolution mapping of the lipophilic Oc-OH-Tam in cells revealed its preferential accumulation in the endomembrane system. This is consistent with the ability of the amino nitrogen chain of the compounds to be protonated at physiological pH and responsible for electrostatic interactions between Oc-OH-Tam and membranes. A comprehensive scenario is proposed that provides new insight into the cellular behavior and activation of Oc-OH-Tam and advances the understanding of its mechanism of action.

Breast cancer remains the most prevalent form of cancer in women.[1] Hormone independent, triple-negative breast cancers suffer from genetic heterogeneity and remain difficult to cure, with very few treatment options left.[2] Tamoxifen-like metallocifens have been developed, which result from the replacement of the β-aromatic ring in 4-hydroxytamoxifen by a metalloenic entity (Fe, Ru, or Os).[3–5] The main benefit of these complexes is their antiproliferative activity against both hormone-dependent and -independent cancer cells. There is strong evidence suggesting that tamoxifen-like metallocifens are pro-drugs. They are rapidly converted in cells into electrophilic quinone–methides that further react selectively with thiols and selenols via a 1,8-Michael addition.[6] As such, they were shown to inhibit thioredoxin reductase (TrxR), a selenoenzyme involved in the cellular redox balance, in vitro and in Jurkat cells.[7,8] Here we use state-of-the-art synchrotron radiation X-ray fluorescence (SR-XRF) nanoimaging[9–11] to quantitatively trace the intracellular distribution of metal-based drugs at biologically relevant concentrations in a label-free fashion. The metaldrug, and the endogenous elements in MDA-MB-231 cells, exposed for 1 h or 24 h to 2 μM Oc-OH-Tam, were mapped.

Oc-OH-Tam was selected since it contains the exogenous element Os and displays physico-chemical and biological properties close to those of ferrocifen. The high-resolution synchrotron X-ray nanoprobe ID16A-NI available at ESRF, Grenoble, France, was used, which offers a nanobeam down to 13 nm at 33.6 keV[12] and 30 nm at 17 keV (Supporting Information, Figure S1). The unique high X-ray flux of 4 × 10<sup>11</sup> photons per second offered by this XRF nanoprobe allows the detection of metal-based drugs at biologically relevant concentration (low micromolar range).[10,11] 2D SR-XRF images were first recorded on chemically fixed cancer cells using 17 keV energy X-rays according to the workflow shown in Figure S2 in the Supporting Information. Chemical fixation has previously been applied, with success, to SR-XRF cell imaging,[11,12] although chemical fixation has been reported to affect diffusible ions like chloride and potassium.[13]

To minimize element leakage, cells were fixed as quickly as possible and immediately dried upon completion of aldehyde fixation, as suggested by Jin and co-workers[14,15] and as recently used for the intracellular study of TiO<sub>2</sub> nanoparticles.[16] While most cells were scanned at 50 nm step size, some SR-XRF maps were also recorded at 35 nm step size as shown in Figure 1 C.E. Cells (Figure 1 A) exposed to vehicle (0.2% DMSO v/v) showed the expected homogeneous distribution of potassium (K). As expected, no Os signal was detected in control cells.
The intrinsic presence of Os in the structure of Oc-OH-Tam allows its direct mapping via the SR-XRF emission spectrum along with physiological elements, such as K, Zn or S (Figure 1D). The partial spectral overlapping between the Zn K-lines and the Os L-lines was successfully deconvolved by using the PyMca X-ray fluorescence toolkit.\[14\] Using entire elements rather than single peaks as a model, the contributions of the different elements are reliably separated. While the self-absorption of the Os and Zn signals is negligible, the self-absorption of the light elements (P, S, K) is estimated and accounted for during the spectral fitting with the PyMca program. Figure 1B,C shows images of MDA-MB-231 cells exposed to 2 \(\mu\)M Oc-OH-Tam for 1 h, a concentration slightly lower than its IC\(_{50}\), measured as 2.7 \(\mu\)M after 4 days incubation.\[3\] Although the nuclear membrane could not be resolved, the Os was found within a confined region (full width at half maximum in the order of 125 nm; Supporting Information, Figure S3), a pattern likely consistent with an nuclear envelope localization. Nanoanalysis of Os revealed a large perinuclear localization that likely corresponds to the endoplasmic reticulum (ER). Indeed, the ER is a well-described organelle, including interconnected membrane structures (tubules, vesicles, and cisternae) continuous to the outer nuclear-envelope and organized as an intricate perinuclear network.

Previous multimodal optical imaging studies of a rhenium(I) carbonyl derivative of mestranol in MDB-MB-231 cells showed preferential accumulation in another highly membranous organelle namely, the golgi apparatus,\[15\] while recently, a mixed ferrocenyl/rhenium(I) carbonyl tamoxifen derivative was located, by nano-IR imaging, mainly in the nucleus of MDA-MB-231 cells.\[16\] This contrasts dramatically with the cellular distribution of another anticancer cationic half-sandwich Os(II) complex, which was found mainly located in the mitochondria by the same nanoimaging technique and cellular fractionation coupled to inductively coupled plasma-mass spectrometry (ICP-MS) analysis.\[11\]

Chemically fixed cells were further analyzed by 3D X-ray fluorescence nanoimaging. While 2D-XRF imaging informs on the projected elemental distribution across the cell thickness, 3D XRF provides depth information and discriminates between intracellular, surface and extracellular locations of elements (both endogenous and exogenous). XRF nanotomography requires acquisition times of approximately 24 h for 26 projections over 150 degrees (missing wedge effects). Such a thin sample over a flat substrate is analytically challenging and far from ideal for tomography. Nevertheless, this experiment showed that Os had a similar distribution as that of the fluorescent probe ER-Tracker\[2\] Blue-White DPX applied before chemical fixation (Figure 2A).

Furthermore, 3D XRF imaging confirmed the perinuclear distribution of Os, as well as a likely nuclear envelope location, with depth resolution (Figure 2B,C). Although the cell is very thin after chemical fixation and drying, 3D rendering of the distribution of Zn and Os in a whole cell supports a clear intracellular distribution of Oc-OH-Tam (Figure 2B,C and Supporting Information, Movie 1).

The effect of the exposure time on the intracellular distribution of Oc-OH-Tam was also explored. This is of fundamental importance for understanding the pharmacology of this novel organometallic Os-based compound. XRF mapping of Oc-OH-Tam at a biologically relevant concentration
(2 μm) and on a short time scale (1 h), during which no cytotoxicity is observed, demonstrated that the complex readily reached the targeted intracellular compartments owing to its high lipophilicity (Log P<sub>ow</sub> = 4.3). After 24 h incubation, the intracellular distribution of Os displayed a similar pattern, that is, concentrated within membranous regions, such as the ER, as well as the nuclear envelope region (Figure 3A).

Next, the elemental quantification analysis highlighted that the average intracellular areal mass density of Os was already rather elevated after 1 h exposure to 2 μM Oc-OH-Tam. While the Zn areal mass density remained stable (p = 0.17 for 24 h treatment compared to the control or p = 0.058 for 24 h treatment compared to 1 h treatment), the Os areal mass density increased by about 2.3-fold after 24 h incubation, as compared to the level after 1 h exposure, reaching an average value of 0.4 μg cm<sup>-2</sup>, although this was found to be insignificant (p = 0.137) (Figure 3B). Intracellular sulfur-containing molecules, such as glutathione, metallothioneins and thioredoxins, have been previously reported to bind to platinum-based metallodrugs.[18] This finding could apply to other metallodrugs and could explain the large increase in average sulfur concentration observed after 24 h of exposure to Oc-OH-Tam. Despite imaging a reasonably high number of cells with the synchrotron nanoprobe, a large range of areal mass density was observed. Furthermore, cell growth was not synchronized and some physiological parameters may vary from cell to cell that may account for the variability in the measured areal mass density of the randomly imaged, chemically fixed cells.

To examine whether the Oc-OH-Tam disturbed the structure and function of the ER, the potential ER stress response was investigated via measuring the release of Ca<sup>2+</sup>. The total Ca<sup>2+</sup> (mainly Ca bound to proteins) can be detected by X-ray fluorescence imaging, but this method cannot account for the dynamics of free Ca<sup>2+</sup>. Free Ca<sup>2+</sup> was imaged by optical fluorescence microscopy using the dedicated probe, Fluo-4. Ca<sup>2+</sup> imaging of live cells exposed to Oc-OH-Tam for up to 1 h showed that the basal level of Fluo-4 fluorescence was not affected, indicating that it did not disrupt the cytosolic concentration of free Ca<sup>2+</sup>. This result suggests that Oc-OH-Tam was unable to trigger the release of Ca<sup>2+</sup> from internal stores nor induce an influx of Ca<sup>2+</sup> from the extracellular environment, and hence did not induce ER stress development after a short exposure to Oc-OH-Tam (Supporting Information, Figure S4). This finding was also supported by the insignificant change in gene-specific mRNA expression related to ER stress markers (GPR78, IRE1, and CHOP) after 1 h exposure to Oc-OH-Tam (Supporting Information, Figure S5),[17] along with a higher level of mRNA related to AFT6 and GPR78, indicative of an ER stress response.

The ER network is strongly associated with microtubules. Indeed, ER tubules dynamically extend or contract along microtubules, which is essential for the structure and functions of this organelle. After microtubule disruption with the antimiotic agent nocodazole, the ER has been shown to retract back towards the cell center.[19] Therefore, redistribution of Os should be observed upon successive incubation of Oc-OH-Tam for 24 h and nocodazole. XRF nanoimaging clearly shows this expected redistribution of Os, providing further support for the preferential accumulation of Oc-OH-Tam in the ER (Figure 3A).

**Figure 2.** 3D X-ray fluorescence imaging of an MDA-MB-231 cell incubated for 1 h with 2 μM Oc-OH-Tam. A) The epifluorescence optical microscopy image of the cell in PBS prior to fixation allows the localization of the ER labeled with ER-Tracker™ Blue-White DPX fluorescent dye (shown in green false color); the cell morphology after chemical fixation is shown using X-ray phase contrast imaging prior to the acquisition of XRF maps. Selected elements (phosphorus (P), sulfur (S), zinc (Zn), and Os) are presented that report on the intracellular distribution of Oc-OH-Tam (projection at normal incidence, size of the scan is 31 μm horizontal × 21 μm vertical). Selected projections (5 from 26 in total) for XRF tomography acquired with 100 nm step size and 50 ms dwell time. All projections can be found in Figure S6 in the Supporting Information. C) Although the cell is very thin after chemical fixation and drying, a 3D rendering of zinc (magenta) and osmium (green) in the cell clearly confirms the intracellular distribution of the Oc-OH-Tam compound. Color calibration bars in μg cm<sup>-2</sup>.

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Finally, the recent implementation of cryogenic capability at the ESRF ID16A nanoprobe allowed 3D XRF imaging, at 120 nm voxel resolution, of MDA-MB-231 cells under frozen hydrated conditions. To our knowledge, this experiment had not previously been attempted, although previous work on peptide nanoconjugate distributions in HeLa cells was reported using 23 projections covering a total angular range of 138° and a scanning step size of 200 nm. As expected, 3D cryo-XRF analysis of frozen hydrated cells demonstrated a much better preservation of the cell volume and distribution of diffusible ions, as depicted by the potassium distribution (Figure 3C). 3D imaging of Os corroborated a similar perinuclear and nuclear contour distribution (Figure 3C and Supporting Information, Movie 2). It also showed a very high concentration of Oc-OH-Tam in vesicular structures. It can be speculated that the highly lipophilic Oc-OH-Tam targets membrane components that are further exchanged among organelles of the endomembrane system and the plasma membrane.

Overall, these results show that Oc-OH-Tam undergoes fast cellular uptake in MDA-MB-231 cells with preferential accumulation in the endomembrane system, encompassing ER, nuclear envelope, and vesicular structures, such as endosomes and lysosomes. Cell biology studies recently disclosed the ability of tamoxifen-like metalloicnens to induce redox imbalance in Jurkat cells owing to TrxR inhibition, eventually triggering mitochondria-mediated apoptosis. The driving force for this process was assumed to arise from the intracellular oxidation of metalloicnens, affording highly electrophilic quinone–methides. Indeed, these quinone–methides could be generated in vitro in the presence of enzymatic oxidation systems, such as HRP, rat liver microsomes, and human recombinant cytochrome P450 (CYP450). Taken together, these data let us propose the following scenario to explain the anticancer activity of this family of compounds. Oc-OH-Tam is rapidly taken up by cells owing to its lipophilic nature. It travels, via...

Figure 3. A) X-ray fluorescence maps of osmium (Os), phosphorus (P), sulfur (S), and zinc (Zn) in whole MDA-MB-231 breast cancer cells after 24 h exposure to 2 μM Oc-OH-Tam (top) and in whole MDA-MB-231 breast cancer cells after 24 h exposure to 2 μM Oc-OH-Tam, followed by 5 h exposure to 5 μM of the microtubule-disrupting drug nocodazole (bottom). Images were obtained as raster scan at 50 × 50 nm² step size and 50 ms dwell time. B) Average areal mass density (μg cm⁻²) for S, Os, and Zn for control (N = 7), 1 h exposure to Oc-OH-Tam (N = 9), and 24 h exposure to Oc-OH-Tam (N = 6). The comparison of the averages shows significant greater Os concentration after 1 h or 24 h exposure to Oc-OH-Tam compared to control cells, p < 0.05, Kruskal–Wallis test with Dunn’s multiple comparison test. C) 3D rendering of the potassium (K; blue) and Os (magenta) distributions in a frozen hydrated MDA-MB-231 cell obtained after 1 h exposure to 2 μM Oc-OH-TAM. The cryo-XRF nanotomography required 28 projections over 150 deg., a voxel resolution of 120 nm, 50 ms dwell time, and approximately 24 h analysis. Color calibration bars in μg cm⁻². Scale bar 5 μm.
vesicles, to the ER, which acts as a reservoir, where it is converted to the quinone–methide via oxidation by CYP450. Indeed, the ER is the main location for the expression of CYP450 enzymes in cancer cells, such as MDA-MB-231.[22] The quinone–methide is then able to diffuse to other organelles including the mitochondria and inhibit TrxR, eventually resulting in cell death.

In conclusion, we revealed and quantified the intracellular distribution of an osmocyanin-tamoxifen derivative in triple negative breast cancer cells. Coupled with cell biology and optical fluorescence microscopy results, we propose a comprehensive mode of action of this new family of metallo-drugs. This study was possible thanks to the impressive progress in synchrotron-nanoprobe imaging that has now reached the relevant concentrations. In particular, the advent of cryo-XRF nanoimaging and correlative microscopy to unambiguously identify specific organelles, opens exciting perspectives in the understanding of the time-dependent uptake and behavior of metallo-foci in the cellular environment.

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Conflict of interest

The authors declare no conflict of interest.

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