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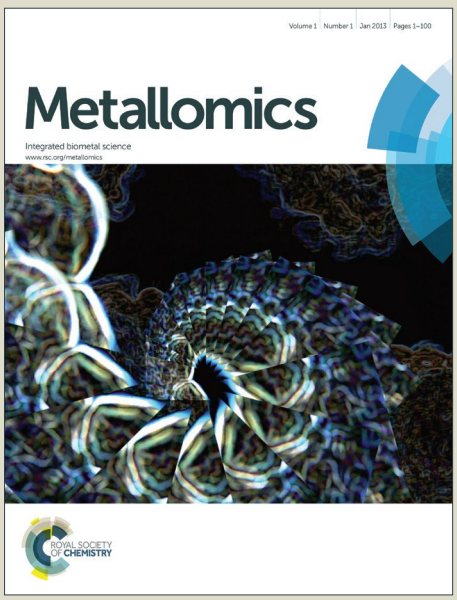

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LA-ICP-MS imaging in multicellular tumor spheroids – a novel tool in preclinical development of metal-based anticancer drugs

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Abstract

A novel application of advanced elemental imaging offers cutting edge *in vitro* assays with more predictive power on the efficacy of anticancer drugs in preclinical development compared to two dimensional cell culture models. We propose LA-ICP-MS analysis of multicellular spheroids, which are increasingly being used as three dimensional (3D) models of tumors, for improving *in vitro* evaluation of anticancer metallodrugs. The presented strategy is very well suited for screening drug-tumor penetration, a key issue for drug efficacy.

A major advantage of tumor spheroid models is that they enable to create tissue-like structure and function. With respect to 2D culture on the one hand and *in vivo* models on the other, multicellular spheroids thus show intermediate complexity, still allowing high repeatability and adequate through-put for drug research. This strongly argues for the use of spheroids as bridging models in preclinical anticancer drug development.

Probing the lateral platinum distribution within these tumor models allows visualizing penetration depth and targeting of platinum-based complexes. In the present study, we show for the first time that spatially-resolved metal accumulation in tumor spheroids upon treatment with platinum compounds can be appropriately assessed. The optimized LA-ICP-MS setup allowed discerning the platinum localization in different regions of the tumor spheroids upon compound treatment at biologically relevant (low micromolar) concentrations. Predominant platinum accumulation was observed at the periphery as well as in the center of the spheroids. This corresponds to the proliferating outermost layers of cells and the necrotic core, respectively, indicating enhanced platinum sequestration in these regions.

Introduction

A first selection step of experimental metal-based anticancer drugs relies on *in vitro* testing (with regard to cytotoxic potency, cellular uptake and cellular trafficking), mostly in 2D monolayer cultures of cancer cells that are generally exposed to uniform drug concentrations under uniform conditions.[1] In this context, metallodrug accumulation is conventionally studied by wet chemical techniques such as inductively coupled plasma-mass spectrometry (ICP-MS).[2] However, these approaches bear the limitation that only information on the average metal accumulation is obtained in a cell model that does not resemble the complex tumor microenvironment and might have poor predictive power, as already shown for platinum-based drugs.[3] Alternatively, multicellular tumor spheroids have emerged as attractive and innovative preclinical model for screening of novel drug candidates.[4] They have proven to be well suitable for mimicking certain features of solid tumors (e.g. necrotic core, hypoxic regions etc.) as well as important molecular, biochemical and physiological characteristics of *in vivo* tissue.[4,5] A careful evaluation of the localization of metal-based compounds within multicellular tumor spheroids can add valuable information to tests of biological activity in these models, thereby strongly enhancing their potential as a screening tool.[6]

So far, bioimaging studies in metallodrug research were rather focused on analytical method development. The main characteristics and applications of analytical imaging techniques (X-ray-based techniques, nanoSIMS, MALDI-MS etc.) in this research area were reviewed by other authors.[2,7,8] Among the developed bioimaging techniques, laser ablation (LA) in combination with ICP-MS has a leading role in the field, allowing elemental mapping in different biological systems with sufficiently high spatial resolution power (with current improvements potentially in the submicrometer range)[9,10] and outstanding sensitivity (low µg/g range depending on the

element)[11,12] of this configuration. Bioimaging by LA-ICP-MS has proven to be very well suited to assess the spatially-resolved metal distribution in histologically heterogeneous structures.[10,13] However, the question whether drug development would be significantly advanced by such bio-imaging tools remained elusive so far. The major goal of getting insights into the mode of action and the occurrence of side-effects (such as nephrotoxicity or ototoxicity) in organs upon treatment with metal-based drugs was not achieved so far.[10,13,14] This goal could only be reached by designing proper predictive experiments focusing on specific questions relevant to the drug response. Drug tumor penetration is exactly such a pre-clinically relevant question to be addressed. The suitability of ICP-MS and LA-ICP-MS for studying the penetration depth of metal-based anticancer drugs into tumor tissue has already been shown.[13,15,16] Hence, the next step would be to implement predictive *in vitro* model experiments, minimizing the number of *in vivo* experiments.

Therefore, the present study aimed to introduce LA-ICP-MS as a powerful and sensitive tool for elemental mapping of platinum-based compounds in two different multicellular spheroid models grown from human tumor cell lines. The laser parameters were optimized to yield a lateral resolution that allows elucidating the platinum accumulation in different substructures of tumor spheroids (~ 450 µm diameter) upon treatment with three different platinum(IV) compounds at biologically relevant (low micromolar) concentrations.

Experimental

Compounds and spheroid culture

Compounds **1**, **2** and satraplatin (Figure 1) were synthesized according to literature procedures.[17] All platinum(IV) compounds were dissolved in minimum essential medium (MEM) to stock solutions of 2 mM and then diluted in MEM to the required concentrations.

For spheroid production, CH1/PA-1 ovarian teratocarcinoma (obtained from CRC Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, UK) and HCT116 colon carcinoma cells (from Institute of Cancer Research, Medical University of Vienna, Austria) were harvested from culture flasks by trypsinization and seeded in MEM and RPMI1640 medium, respectively, into non-cell culture treated round bottom 96-well plates (Nunclon™ Sphera™, purchased from VWR) in densities of 1×10^4 (CH1/PA-1) and 2×10^3 (HCT116) viable cells/well, whereupon single spheroids per well are growing within 7 days to a diameter of > 450 µM. For this purpose, cultures were maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. For characterization of the spheroids regarding growth, development of hypoxia and necrosis see a previous article.[19]

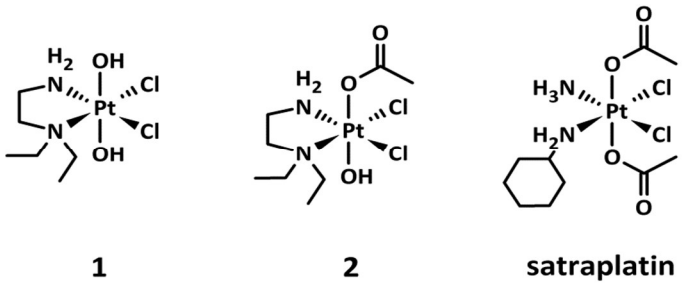

Figure 1. Structural formulae of platinum(IV) complexes **1**, **2** and satraplatin under investigation

Sample preparation for LA-ICP-MS measurements

Spheroids were treated with compounds **1**, **2** and satraplatin in concentrations of 1-5 µM for 96 hours (in the wells of Nunclon™ Sphera™ plates used for their production) by adding to each well 100 µl of the respectively medium containing appropriate drug concentrations. Spheroids were harvested by pipetting and transferred into 1.5 mL Eppendorf tubes (10–20 spheroids per tube) and washed 3x with PBS. PBS was removed completely and Eppendorf tubes were filled up with 0.5–1 mL Tissue Tek (Sakura, Netherlands) and stored at –80 °C. For LA-ICP-MS measurements, samples were cryosectioned into slices of 20 µm thickness with a cryotom (Microm HM 550, Thermo Fischer), placed onto glass slides and air dried.

Bioimaging in multicellular tumor spheroids by LA-ICP-MS

Laser ablation was performed with a solid state laser (Nd:YAG) at a wavelength of 213 nm (NWR 213, ESI, Fremont, CA, USA) to obtain spatially-resolved platinum distribution in tumor spheroid samples. An optical sample map of each tumor spheroid was generated prior to the measurement. The laser beam path was equipped with a square-shaped laser spot table ensuring a constant delivery of energy onto the moving sample throughout the entire diameter of the laser beam. The output laser energy was tuned separately for every sample in order to ensure complete ablation of the sample material. Ablation was performed with parallel line scans, a frequency of 20 Hz, a spot size of 10 µm and a scan speed of 10 µm/s, which can cause some image distortion. The ablated sample material was transferred to the ICP-MS instrument with helium (quality 5.0) at a flow rate of 400 mL/min. Laser ablation data were recorded with Triple Quadrupole ICP-MS Agilent 8800 (Agilent Technologies, Tokyo, Japan) equipped with nickel cones and operated at an RF power of 1350 W. Argon was used as plasma gas (15 l min^{-1}) and as

carrier gas with a flow rate of ~1.1 l min^{-1}. The ^{195}Pt isotope was recorded with a dwell time of 0.1 s. The performance of the ICP-QQQ-MS was monitored daily with a solution-based tuning procedure. To maximize sensitivity and ensure low oxide formation (ThO/Th < 0.5%) with laser ablation conditions, a NIST 612 Trace Element in Glass CRM was ablated. The generated files were imported into Iolite (Iolite Version 2.5)[18] as an add-on to Igor Pro (Wavemetrics, Igor Pro 6.34A). The platinum distribution maps were generated with the data reduction scheme 'Trace_Elements' including blank subtraction and no smoothing of the visualization.

Results and Discussion

Two different tumor spheroid models (HCT116 and CH1/PA1) regularly developing necrotic and hypoxic regions[19] were used to study the spatially-resolved platinum accumulation by LA-ICP-MS in three dimensional tumor culture systems upon treatment with biologically relevant concentrations of platinum(IV) complexes.

Optimization of the LA-ICP-MS setup

An important consideration in LA-ICP-MS bioimaging experiments is the choice of an optimal sample preparation method to preserve the structural as well as the chemical integrity of the cells and to avoid the occurrence of artifacts (such as the loss or redistribution for analytes). In order to minimize these unwanted effects introduced by sample preparation, cryosectioning of the tumor spheroids was chosen for our LA-ICP-MS bioimaging experiments. Hambley and co-workers have compared cryofixation and formalin fixation of DLD-1 spheroids as sample preparation strategies. They observed rearrangement of unbound platinum species in formalin-fixed spheroids upon incubation with cisplatin and platinum detection by X-ray fluorescence computed micro-tomography (XRF-CT).[20] A similar observation was made for bioimaging of organs, where a decrease in elemental concentrations in rat heart upon formalin fixation was shown indicating elemental leaching processes.[21] As a next step, the laser output energy and the laser shot frequency were tuned separately for every tumor spheroid to enable complete ablation of the sample material. In order to cope with the small size of the spheroids (diameter size range between 400 to 500 μm) and the levels of platinum accumulation, the laser ablation parameters had to be optimized to improve lateral resolution without compromising the sensitivity of the platinum signal. Elemental bioimaging LA-ICP-Q-MS experiments usually employ scan speeds

where the distance traversed in one second is equal to or less than the diameter of the laser beam.[22] As both parameters determine the lateral resolution and sensitivity, they were gradually decreased in our experiments with parallel monitoring of the platinum signal to find the optimal setting. The sensitivity of the platinum signal was found to be sufficient with the smallest laser spot size (with a square shape) of 10 µm available in the used laser system and a corresponding scan speed of 10 µm/s. This is in accordance with the employed laser ablation parameters of Karst et al. for the analysis of a palladium-based photosensitizer in tumor spheroids by means of LA-ICP-MS.[23] In order to estimate the spatial resolution of the experimental setting, ablation was performed in parallel, horizontal line scans. The lateral resolution is vertically limited by the spot size to 10 µm and horizontally (along the line) by the scan speed and dwell time (10 µm/s and 0.1 s, respectively). We chose to record only one isotope (^{195}Pt) resulting in a horizontal spatial resolution of ~0.5 µm at a frequency of 20 Hz. Thus, the theoretical limit equals a pixel size of 10 x 0.5 µm with our experimental setting.

Bioimaging in multicellular tumor spheroid models by LA-ICP-MS

The platinum distribution in the HCT116 and CH1/PA1 tumor spheroids after treatment with the respective platinum compounds was determined with LA-ICP-MS using the optimized setup and correlated with the grey-scaled images taken prior to the ablation of the samples (Figure 2). The LA-ICP-MS measurements revealed a heterogeneous platinum distribution in the tumor spheroid sections for both cell lines with a strong platinum accumulation in specific areas (indicated by red color). Platinum enrichment occurs for all three compounds in the HCT116 cell line predominantly at the periphery of the tumor spheroid sections and in the spheroid core. The spheroid periphery resembles tumor tissue in close proximity to a blood vessel and the actively

dividing cells in these parts are accumulating the platinum compounds to a high extent. The high levels of platinum in the central region are most likely co-localizing with necrotic core that had been shown to be present within HCT116 spheroids of the kind employed in this study.[19] For example for the HCT116 tumor spheroid treated with satraplatin, different substructures can be observed already in the grey-scale image taken of the spheroid section prior to ablation, indicating most probably necrotic matter (Figure 2A). Once deep penetration is achieved, binding partners for platinum are likely to be more readily accessible due to the substantial loss of the compartmentalization (and thus diffusion barriers) within the necrotic core. The three platinum compounds under investigation showed a significant degree of platinum drug and/or metabolite sequestration in this region. Hambley et al. have pointed out the importance of using tumor spheroids containing necrotic cores[20] as they are a characteristic feature in progression of solid tumors and the predominant platinum accumulation in this region could serve as important criterion in the selection of promising platinum-based drug candidates.

For CH1/PA1 spheroids treatment with compounds **1** and **2** resulted again in platinum enrichment predominantly at the periphery of the spheroids (Figure 2). Compound **1** showed to accumulate platinum additionally in the central region of the spheroid (most likely corresponding to the necrotic core), whereas for compound **2** this was not observed. A specific platinum accumulation pattern was not visible for satraplatin in the CH1/PA1 tumor spheroid. However, the platinum distribution was heterogeneous with the presence of platinum hotspots in different parts of the tumor spheroid. Nevertheless, a clear platinum penetration into the CH1/PA1 spheroids was demonstrated for all three platinum(IV) compounds. This capacity of deep penetration indicates an increased chance of damaging quiescent (non-proliferating) tumor cells and may be taken as one criterion for compound selection in preclinical drug evaluation studies.

Taken together, these data demonstrate the applicability of LA-ICP-MS to study the spatially resolved platinum distribution in the heterogeneous structures of multicellular tumor spheroids.

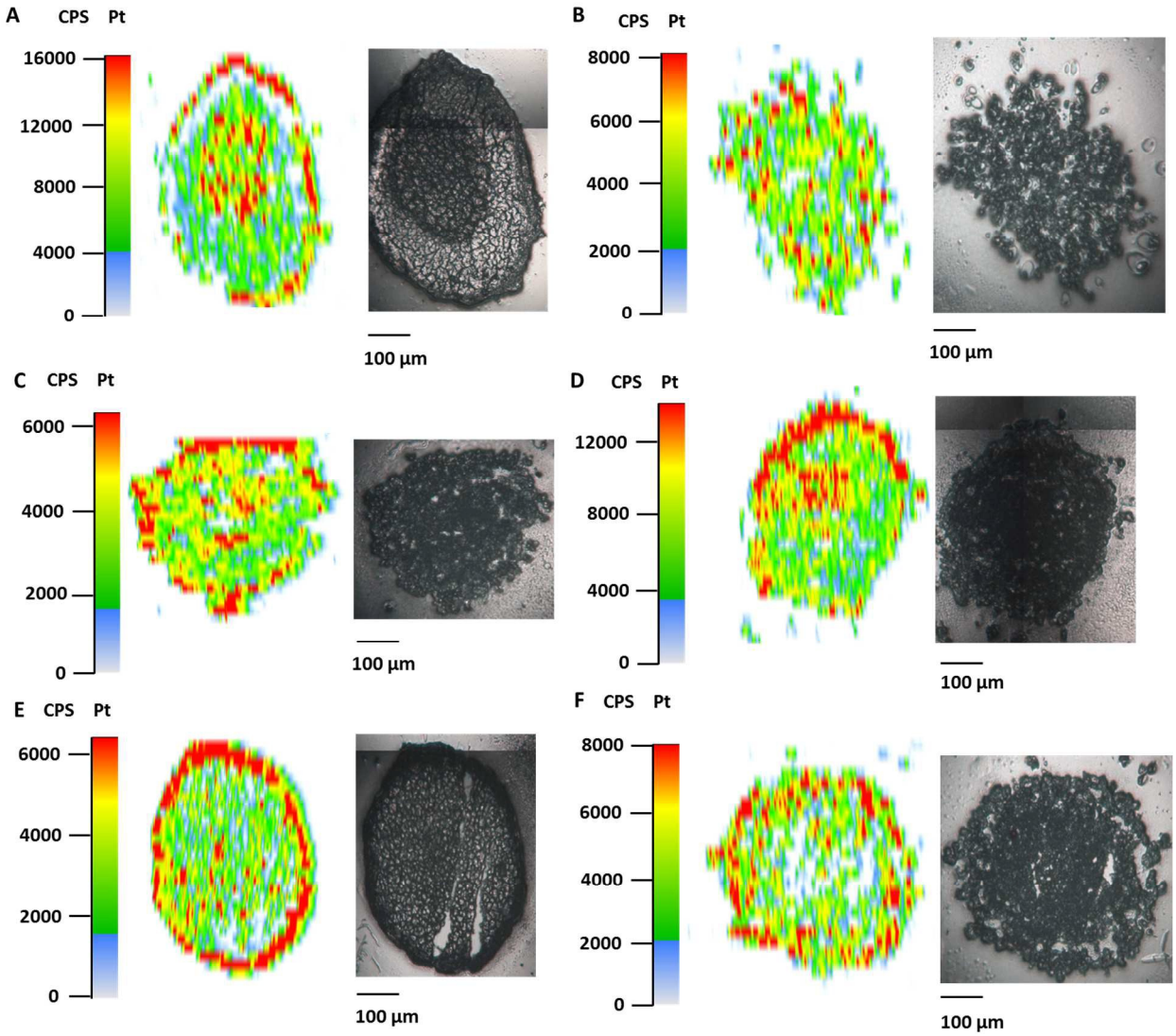

Figure 2. Platinum distribution in cryosections of HCT116 and CH1/PA1 tumor spheroids upon treatment (1–5 µM) with platinum(IV) complexes measured by LA-ICP-MS. HCT116 tumor spheroids were treated with (A) satraplatin, (C) compound **1** and (E) compound **2**, CH1/PA1 spheroids were treated with (B) satraplatin, (D) compound **1** and (F) compound **2**.

Conclusions

The application of the state of the art elemental imaging by LA-ICP-MS to the innovative preclinical *in vitro* multicellular tumor spheroid model provided a new tool for studying drug accumulation, a key issue to be addressed during drug development. The proof-of–principle experiments showed that the sensitivity and the achieved spatial resolution (~ 10 μm) allowed assigning platinum hotspots to the periphery of the spheroids as well as to the necrotic core. The experiments were carried out without compromising preclinical relevant conditions (regarding drug concentration). This unique combination of advanced imaging technique and tumor model paves the way for future quantitative studies on drug uptake into tumor spheroids, by the preparation of matrix-matched calibration standards.

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