The Direct Mapping of the Uptake of Platinum Anticancer Agents in Individual Human Ovarian Adenocarcinoma Cells Using a Hard X-ray Microprobe

Petr Ilinski, Barry Lai, Zhonghou Cai, Wenbing Yun, Daniel Legnini, Teresa Talarico, Marian Cholewa, Lorraine K. Webster, Glen B. Deacon, Silvina Rainone, Don R. Phillips, and Anton P. J. Stampfl

Abstract

Uptake of platinum-based anticancer compounds into individual human ovarian adenocarcinoma cells was measured using an X-ray microprobe. The uptake of cisplatin, a platinum-based compound, in drug-resistant cells is decreased by ~50% after 24 h, compared with the uptake of the drug in nonresistant cells over the same time period. The Pt103 derivative of the drug, in contrast, showed an increased uptake by an order of magnitude in resistant cells over the same time period. Increased uptake appears to allow Pt103 to overcome the resistance mechanism developed by the cell. This work additionally shows that the X-ray microprobe is able to directly quantify Pt drug uptake on a subcellular level and can measure the mass of Pt down to a detectable limit of 20 attograms of Pt (2 × 10^{-17} grams or 6 × 10^{4} Pt atoms) in 1 s. Such exquisite elemental sensitivity combined with high spatial resolution paves the way for quantitative submicron three-dimensional mapping of elemental distributions within individual cells.

Introduction

Cisplatin is one of the few chemotherapeutic, platinum-based compounds in clinical use for the treatment of cancer and used primarily for the treatment of testicular and ovarian tumors (1). Because tumors slowly develop resistance to cisplatin (and to all anticancer drugs), there is a need to find derivatives that continue to be active against tumors once cisplatin resistance has developed. Derivative Pt103, or Pt[N\text{p-HC}_{6}F_{4}]CH_{2}py\text{2}, was selected from an extensive series of cisplatin analogues (2) on the basis of its good activity against cisplatin-resistant tumors (3). Cellular studies of Pt103 were commenced several years ago in an attempt to define the chemical/biochemical properties that led to this activity against cisplatin-resistant tumors.

A critical aspect of this work was to establish if the cellular uptake and intracellular distribution of Pt103 were significantly different from that of the parent drug cisplatin. Although cellular uptake into tumor cells can be assessed by techniques, such as atomic absorption spectroscopy, and the distribution between nuclear and mitochondrial DNA can be determined using gene-specific DNA cross-linking as-says or by quantitative PCRs, a major technical advance would be to a spot size of 1 \mu m^2. The flux passing through this point was measured to be \( 1 \times 10^{10} \) photons/s, using an ion chamber in place of the sample (Fig. 1). The zone plate was located 71 meters from the undulator source, whereas the sample was placed 146.5 mm from the zone plate. To perform fluorescence microspectrometry, a specimen was mounted on a X-Y translation stage and placed at right angles to the incident beam. An energy-dispersive Ge detector was used to monitor fluorescence from the sample that was scanned across the focused X-ray spot. In this way, spatial maps of many different elements were acquired simultaneously. An incident X-ray energy of 11.7 keV, just above the 2p_{3/2} absorption edge of Pt, was used to produce the Pt L\alpha emission lines. At this energy, X-rays penetrate biological cells without significant absorption nor beam spreading; thus, no specimen thinning is required, and fluorescence maps of individual elements represent a two-dimensional projection of the volumetric distribution within the specimen.
Results

Elemental concentration within each cancer cell was obtained by comparison of the characteristic X-ray emission intensities to those from a suitable standard with uniform and known elemental concentration. Ten energy ranges spanning the characteristic X-ray lines of elements corresponding to P, S, Cl, K, Ca, Mn, Fe, Cu, Zn, and Pt were integrated in real time by software and stored as 10 ROI. The collection time at each pixel varied from 2 to 30 s depending on the total count rate of each detected signal. Part of the emission spectrum from a cancer cell is shown in Fig. 2. As can be seen, the characteristic X-ray line of interest, Pt L\text{\textsubscript{\alpha}}, overlaps with the Zn K\text{\textsubscript{\alpha}} line, so that some counts accumulated into the Pt L\text{\textsubscript{\alpha}} ROI were contaminated by Zn K\text{\textsubscript{\alpha}} emission. A corrected Pt count may be found by subtracting the number of counts generated by Zn K\text{\textsubscript{\alpha}} from the number of counts in the Pt L\text{\textsubscript{\alpha}} ROI. The number of Zn K\text{\textsubscript{\alpha}} counts can be calculated from the intensity of the Zn K\text{\textsubscript{\alpha}} line by knowing the ratio between the K\text{\textsubscript{\alpha}} and K\text{\textsubscript{\beta}} line intensities (6). The Zn K\text{\textsubscript{\beta}} ROI had, however, a contribution from the Cu K\text{\textsubscript{\beta}} line. The number of Cu K\text{\textsubscript{\beta}} counts is calculated by using the Cu K\text{\alpha} ROI, which does not overlap any other emission line. Finally, an expression to calculate the corrected number of counts in the Pt L\text{\textsubscript{\alpha}} line, $N_{\text{Pt}}$, can be defined as:

$$N_{\text{Pt}} = N_{\text{Pt ROI}} - 0.176 \times N_{\text{Zn K\alpha ROI}} + 0.0037 \times N_{\text{Cu K\alpha ROI}}.$$

where $N_{\text{Pt ROI}}$ is the total number of counts in the Pt L\text{\alpha} ROI, $N_{\text{Zn K\alpha ROI}}$ is the number of counts in the Zn K\text{\alpha} ROI, and $N_{\text{Cu K\alpha ROI}}$ is the number of counts in the Cu K\text{\alpha} ROI. Thus, if $N_{\text{Pt ROI}}$ is $\geq 20\%$ of $N_{\text{Zn K\alpha ROI}}$ and $\geq 0.5\%$ of $N_{\text{Cu K\alpha ROI}}$, and with sufficient counting statistics, a Pt signal can be reliably determined.

To calibrate the platinum concentration, the emission spectrum of SRM 1833 for X-ray fluorescence spectroscopy supplied by the National Bureau of Standards (7) was measured in the same geometrical configuration as for the cells. SRM 1833 does not contain platinum; the platinum concentration in the specimen was defined by comparison with the concentration of zinc in the standard. The relation between the intensity of characteristic X-ray emission from the specimen and elemental concentration within a thin sample is defined as (8):

$$I = I_0 G r \left(1 - \frac{1}{r} \omega f W l\right),$$

where $I_0$ is primary beam intensity, $G$ is a geometrical factor, $r$ is the absorption jump ratio of the corresponding atomic shell, $\omega$ is fluorescence yield, $f$ is the fraction of the characteristic X-ray line within the ROI to the entire emission series, $W$ is the weight fraction of the element in the specimen, $\rho$ is sample density, and $l$ is sample thickness. Factor $W l$ in Equation B is the surface density of the element in a specimen, which is defined below as $D$; therefore, when geometrical factors are the same, the ratio of zinc and platinum emission can be written as:

$D$ is the surface density of the element in a specimen.
The emission signal of the zinc Kα line was measured to be 84,100 counts/sec, whereas the zinc surface density of SRM 1833 was 3.9 μg/cm². For the same primary beam intensity and platinum surface density of 1 fg/μm², the platinum Lα line signal, according to Equation C, will be equal to 116 counts/s. With typical backgrounds of <10 counts/sec in the Pt Lα ROI, the XMP provides extremely high elemental sensitivity for platinum that can be detected down to a surface density (minimum detectable concentration) as low as 0.1 fg/μm² (or 10⁻⁸ gram/cm²) in 1 s. For a normal cell thickness of 10–20 μm, a surface density of 0.1 fg/μm² corresponds to a local platinum concentration of 5–10 ppm.

To obtain a distribution of the elements in a cell, samples were scanned within the plane using a step size of 1 μm in both the X and Y directions. One of the resulting platinum distributions is shown in Fig. 3. The total amount of platinum in the cell, given in units of fg, can be obtained by summation of the amount of platinum defined in each pixel:

\[ M = \sum_j M_j = \sum_j \frac{I_j}{116} A_j, \]

where \( M_j \) is the amount of platinum in a pixel \( j \), \( I_j \) is the platinum signal at this pixel, and \( A_j \) is the pixel area in units of μm². The pixel area was 1 μm², which is defined by the step size of the scan. The background was subtracted from the platinum distribution before summation. The level of the background was defined by averaging the pixel intensities along the cell boundary in Fig. 3. The amount of Pt was calculated for each cell and then averaged for a set of cells. Each set contained from three to four cells. Sources of error for the individual estimates of Pt mainly occurred from measurement errors of the Pt and Zn emission peaks and from the uncertainty in the Zn surface concentration of the SRM 1833 standard. The SD for individual measurements of Pt varied between 1.5 and 12%. The SD of platinum within batches of cells was also estimated to be between 3 and 53%. Most variation thus occurs between cells rather than from the uncertainty of individual measurements.

**Discussion**

As shown in Fig. 4, drug uptake in cancer cells that are resistant to cisplatin is reduced by ~50%, compared with uptake in normal cancer cells over a 24-h period. On the other hand, resistant cells show an increased uptake of the Pt103 derivative by up to an order of magnitude. The relative extent of uptake of Pt103 compared with cisplatin into single ovarian cells parallels results reported previously using atomic absorption spectroscopy on several million cells (3). The enhanced uptake of Pt103 may allow the drug to overcome the resistance mechanism induced by long-term exposure to cisplatin. Interestingly, Pt103 uptake was found to be largest in resistant cells over a 24-h period (Fig. 4), contrary to expectations and previous bulk measurements (3); nonresistant cells would be expected to have the highest uptake. Such a result highlights the difference between measurements performed on millions of cells as opposed to those performed on individual cells, as it appears from this work that measurable variation in uptake can indeed occur within cell batches.

This experiment successfully demonstrates the feasibility of using the XMP for single cell analysis, even for clinical doses of anticancer agents, which has not been possible previously with other microprobe analytical techniques (9, 10). Using the results from this study, the XMP technique provides a minimum detectable limit of 20 attograms (2 x 10⁻¹⁷ grams or 6 x 10⁴ atoms) for platinum in a beam spot of 1 (h) x 0.2 (V) μm² obtained in 1 s. In this study, a step size of 1 μm was chosen to map out the Pt distribution in the whole cell within a reasonable data collection time. The XMP, however, is capable of achieving a spatial resolution of ~150 nm (5) and may therefore be used for subcellular imaging in future studies when cell sections are used. For quantitative analysis, it is appropriate to use a minimum analyzable limit, which is defined as a concentration that can be measured with a SD of 10%, and that is at least six times higher than the minimum detectable limit (8). A minimum analyzable limit that

---

**Fig. 4.** Uptake of 10 μM cisplatin and Pt103 in human ovarian 2008 and cisplatin-resistant 2008/CDDP ovarian cells.

**Fig. 3.** Gray-scale map of the distribution of platinum in human ovarian 2008 cells treated with 10 μM Pt103 drug for 8 h.
can be achieved by XMP is \( \sim 100-1000 \) times lower than that currently measurable using state-of-the-art proton microprobes. Such sensitivity, on the level of several ppm, enables quantification of platinum and its spatial distribution directly in single cells. Sensitivity is even higher for transition metals, such as Cr, Fe, Ni, Cu, and Zn, by at least another order of magnitude. With additional improvements to optics and X-ray source, the microprobe will enable accurate spatial mapping of platinum and colocalization of other elements distributed throughout a single tumor cell, \( \text{e.g.,} \) by the reconstruction of XMP maps from a series of cell sections. In the near future, the XMP will be able to yield three-dimensional images of the distribution of platinum-derived anticancer drugs throughout a single tumor cell. Such spatial and elemental resolution will allow for direct quantification of drug distribution in the nuclear and mitochondrial compartments. And such subcellular information may 1 day allow the detailed mechanisms of drug uptake to be studied directly.

References

The Direct Mapping of the Uptake of Platinum Anticancer Agents in Individual Human Ovarian Adenocarcinoma Cells Using a Hard X-ray Microprobe


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/8/1776

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.