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Novel in situ methodology to observe the interactions of chemotherapeutical Pt drugs with DNA under physiological conditions


The binding of the antitumor drug cisplatin with DNA was determined by means of in situ resonant inelastic X-ray scattering (RIXS) spectroscopy. Because of the penetrating properties of hard X-rays, we could determine, under physiological conditions, the identity and number of platinum complexes present. In situ RIXS revealed that under physiological conditions, water molecules replace chloride ligands owing to drug hydration. The subsequent interaction with DNA, led to the bonding of the aqua complexes into the DNA structure with simultaneous loss of the coordinating water and chloride ion. The data analysis reveals that Pt is coordinated by two adjacent guanines giving cis-[Pt(NH)₂(d(GpG)-N7(1),-N7(2))] upon losing its coordinating water or chloride ligands.

Introduction

Platinum(II) drugs and in particular cis-diaminedichloroplatinum(II) (cisplatin) are used routinely in oncology to treat testicular, ovarian, head and neck tumors, and most recently bladder, cervical, and small cell lung tumors. Other platinum drugs have been developed and marketed, such as carboplatin, [cis-diammine-1,1'-cyclobutanedicarboxylato]platinum(II), which is less toxic than cisplatin but possesses the same spectrum of antitumor activity and oxaplatin, used in combination therapy with 5-fluourouracil cisplatin but possesses the same spectrum of antitumor activity and oxaplatin, used in combination therapy with 5-fluourouracil for colon cancer. Satraplatin, picoplatin, nedaplatin, and triplatin have been developed to varying extents. For stereochemical reasons, the trans isomer cannot form intrastrand crosslinks. The X-ray structure of the adduct of cisplatin bonded to the dinucleotide d(GpG) was published in 1985, and later studies confirmed the formation of intrastrand crosslinks (cis-[Pt(NH)₂(d(GpG)-N7(1),-N7(2))] in interactions with DNA. Binding of DNA to platinum drugs, particularly cisplatin, has been studied by a plethora of methods, including indirect methods such as nuclear magnetic resonance (NMR), circular dichroism, biochemical methods, theoretical calculations, and kinetic measurements, or directly by crystal structure determination of isolated adducts. However, the direct observation of the coordination of platinum(II) compounds to DNA under physiological conditions is rare.

195Pt NMR spectroscopy gives direct information about the platinum coordination but requires enrichment of the 195Pt isotope thereby modifying the drug composition. Atom-based analytical techniques, such as electron microscopy, X-ray analysis, synchrotron radiation induced X-ray emission, micro-X-ray absorption near edge structure spectroscopy, and X-ray fluorescence, are able to map the cellular distribution of the elements with high accuracy but are limited in respect to chemical speciation, e.g. unable to distinguish between intact and reacted drug. Extended X-ray absorption fine structure (EXAFS) is a technique able to probe local environment of the platinum ion in solution. However, it requires concentrated samples and/or relatively long acquisition times, from several hours to days, in order to attain statistically meaningful
The very low solubility of cisplatin (1-2 mg/mL at room temperature)\textsuperscript{11} prevents the use of concentrated solutions. Furthermore, EXAFS is a structural technique with relatively low chemical resolution, i.e., unable to resolve between Pt bonded to O or C or N due to similarity in bond distance and atomic number.\textsuperscript{19}

Results and discussion

Figure 2 shows the RIXS maps measured \textit{in situ} around the Pt L\textsubscript{3}-edge for cisplatin in water, buffer, and with DNA. The central resonance, located at incident beam energy of 11565 eV, reflects the density of unoccupied Pt 5d electronic states. It should be mentioned that RIXS spectrum of solid cisplatin was the same as cisplatin in deionized water. We note that higher intensities in the presented RIXS planes correspond to higher un-occupancy values in the 5d orbitals, which are sensitive to the type of coordinating ligand, bonding strength and angle. According to Nørskov’s model,\textsuperscript{24} the availability of valence orbitals to form chemical bonds depends on their electron occupancy and energy. Consequently, a change in the platinum binding site can be easily detected by measuring its density of states in the 5d orbital. To better understand the experimental RIXS maps, FEFF9.0 calculations\textsuperscript{25} were performed. Crystal structure data were used as input files for the calculations when available, such for the cases cis-[PtCl\textsubscript{2}(NH\textsubscript{3})\textsubscript{2}](CUKRAB) and cis-[Pt(NH\textsubscript{3})\textsubscript{2}(d(GpG)-N7(1),-N7(2))] (CCDC 562451). The structures were retrieved from the Cambridge Crystallographic Data Centre.\textsuperscript{26} Since we were unable to retrieve the crystal structure of the cisplatin hydration products, a planar structure containing a water molecule \{cis-[PtCl(NH\textsubscript{3})\textsubscript{2}(H\textsubscript{2}O)]\} or two equidistant water molecules \{cis-[Pt(NH\textsubscript{3})\textsubscript{2}(H\textsubscript{2}O\textsubscript{2})\}\textsuperscript{2} instead of the chloride ions was optimized \textit{in silico}.

![Schematic representation of the experimental setup used to acquire \textit{in situ} RIXS maps.](image1.png)

Figure 3 shows the RIXS map difference (\(\Delta\)RIXS) for cisplatin in water, buffer, and with DNA. The experimental result could be fitted with two possible structures, namely \textit{cis}-[PtCl(NH\textsubscript{3})\textsubscript{2}(H\textsubscript{2}O)]\textsuperscript{+} with a chloride ion at 2.20±0.05 Å from the platinum center, and a diaqua complex \{cis-[Pt(NH\textsubscript{3})\textsubscript{2}(H\textsubscript{2}O\textsubscript{2})\]\textsuperscript{2} with water molecules located at 2.20±0.05 Å from the platinum center. The optimized structure related to the loss of a single chloride, has the remaining water molecule at around 2.21 Å from the platinum center.
A comparison with reported structural data and calculations favour interpretation as cis-[PtCl(NH$_3$)$_2$(OH$_2$)$_2$]+. Thus, the Pt-Cl bond length corresponds with those (2.292(4)-2.3599(4) Å) of a range of square planar Pt$^{IV}$ complexes with a cis-PtCl(OH$_2$)$_2$ unit, Pt-Cl of cisplatin (2.3088(10); 2.313(2) Å), calculated values for [PtCl(NH$_3$)$_2$(OH)$_2$]$^+$ (2.289-2.325 Å), and is consistent with EXAFS data (but which has large errors) for cisplatin in a hydrated liposome. The Pt-OH$_2$ bond length is more deviant from values (2.071-2.129(2) Å)$^{27}$ for the same cis-PtCl(OH$_2$)$_2$ complexes and that calculated for [PtCl(NH$_3$)$_2$(OH)$_2$]$^+$ (2.124-2.129 Å). However, if assigned as [Pt(NH$_3$)$_2$(OH)$_2$]$^{2+}$, agreement with Pt-O (2.036(5)-2.052(5) Å) of square planar complexes with a cis-Pt(OH$_2$)$_2$ array is worse.$^{28}$ A calculated value for [PtCl(NH$_3$)$_2$(OH)$_2$]$^{2+}$ (2.121-2.128 Å) is closer, but does not account for effects of solution by water.

Since mono- and diaqua complexes induce similar effects on Pt $5d$ density of states it is difficult to judge which of the structures is prevalent and/or more reactive. Baik et al.$^{29a}$ and Cepeda et al.$^{29b}$ mentioned that both structures are likely to play a role in the reaction with DNA but kinetic evidence favours cis-[PtCl(NH$_3$)$_2$(H$_2$O)]$^+$ as the key intermediate.$^{16a,16b}$ The present results show that RIXS measurements coupled with theoretical calculations can provide structural data for platinum drugs under physiological conditions with high precision and at much lower concentrations than is possible with EXAFS. The approach is particularly valuable when crystal structures are available, as is the case for [PtCl(NH$_3$)$_2$]$^+$ and [Pt(NH$_3$)$_2$(OH)$_2$]$^{2+}$.

Figure 4 shows the $\Delta$-RIXS resulting from the addition of DNA to the aquated cisplatin. It should be mentioned that DNA is the primary intracellular target and therefore the reason to use it as the model system.$^{17}$ RIXS analysis shows a clear change in the unoccupied Pt $5d$ density of states, consistent with bond formation. The difference in the resultant RIXS map can be assigned to a single species. The species is related to hydrated Pt(NH$_3$)$_2$ units bond to the DNA via the N(7) position of adjacent guanines, according to FEFF9.0 calculations, a result which is consistent with data published for isolated crystals.$^{10}$ It should be noted that the difference observed in the measured RIXS map could not be fitted with hydrated cisplatin bonded to a single N(7) position. The single bonding site was modeled by cleaving one of the Pt-N(7) bond and replacing it with either Cl or H$_2$O located at different bond distances.

If all the hydrated cisplatin species is bonded to the DNA, a $+145\%$ signal change at an excitation energy of 11568 eV is expected, and since we detected only one species after DNA addition, the effectiveness of drug bonding after 24 h can be estimated by simply dividing the measured signal difference (in this case +95%) by the theoretical expectation (145%), which equates to 65% of the drug being bonded to the DNA after 24 h incubation. Similar values were reported by Gonnet et al.$^{29a}$ who applied HPLC, and also by van der Veer et al.$^{29b,c}$ who used NMR to determine the drug binding sites. The calculation assumes that an insignificant amount of drug was lost due to beam damage, a conclusion that is reasonable because we stirred the solution, and there is an excess of bonding sites because we used an excess of DNA. The remaining 35% relates to residual aquated cisplatin complex.
The hydration of cisplatin and consequent formation of mono- and/or di-aqua complexes led to a striking decrease in the unoccupied 5d states of Pt, reflected in Figure 3 by a negative signal difference and a significant decrease in the whiteline intensity (direct measure of 5d empty states) in respect to cisplatin (Figure 5 (top panel)). The change can be rationalized in terms of orbital contributions. In the case of cisplatin, the whiteline is primarily composed from a hybridization of Pt d-orbitals with Cl p-orbitals with a minor contribution of N p-orbitals from the amine groups. Substitution of the chloride ligands by water led to a drastic decrease of whiteline intensity because of the removal of the Cl p-orbital contribution and the appearance of a contribution due to the O p-orbitals from the water molecules. There seems to be a weaker hybridization between Pt d-orbitals and O p-orbitals compared with Pt d-orbitals and Cl p-orbitals, i.e., less overlap of the orbitals. This suggests that water molecules are not strongly bonded to Pt and therefore can be replaced, justifying the highly reactive nature of the aqua complexes toward nucleophilic centers of biomolecules. 

It should be mentioned that hydration of cisplatin occurred under physiological conditions but not in demineralized water.

Incubation of the hydrolyzed complex in physiological solution with DNA led to an increase of the 5d empty states of Pt and a shift to higher energy of the main resonance (Figure 5 (top panel)). The result is consistent with the binding of the drug, confirmed by FEFF9.0 theoretical calculations (Figure 4). The N(7) atoms of the imidazole rings of guanine and adenine located in the major groove of the DNA double helix are the most accessible and reactive nucleophilic sites. Therefore it is understandable that aquaplutinium complexes coordinate preferentially to the N(7) position of guanines adjacent to each other. In the process its two coordinating water molecules, and/or coordinating water and chloride ions are released. The absence of other discernible species confirms cis-[Pt(NH$_2$)$_2$(d(GpG)-N7(1),N7(2))] as the main adduct. Electronically speaking, the coordination to N(7) atoms resulted in a significant increase in the empty states of Pt and a strong hybridization of the N p-orbitals of the ligands (Figure 5 (bottom panel)) since there is a clear overlap between Pt d-orbitals and N p-orbitals. The significant increase in empty states of Pt suggests strong electronic donation from Pt, i.e., strong involvement of Pt valence states in the Pt-N bond. Consequently, the newly formed bonds are significantly stronger that Pt-Cl (cisplatin) and Pt-O (mono- and diaqua complexes), leading to the shift to higher energy.

Conclusions

In conclusion, the present work shows the applicability of in situ RIXS combined with FEFF9.0 theoretical calculations to validate and/or elucidate the mechanism of action of cisplatin under physiological conditions. The reported RIXS study suggested that cisplatin aquates in a buffer solution leading to the formation of a mono- and a diaqua complex. The aqua complexes are substituted by the N(7) position of guanines adjacent to each other, in an equal manner. We estimate that 65% of the drug bonded to the DNA after 24 h incubation. The bond strength and identity of the coordinating group could be interpreted on the basis of electronic orbital contributions.

In our view, RIXS and HR-XAS methodology is a welcome addition to the plethora of strategies used to establish reaction mechanisms of antitumor drugs since it can be carried out in situ and even in vivo due to the large penetration depth of hard X-ray without the need for concentration, extraction or crystallization of reaction products. These properties allow not only the determination of structural considerations in equilibrium but more importantly, enables time-resolved studies to monitor reaction kinetics and the amount of drug bonded, in real time, which is important in the estimation of drug dosage. It should be mentioned, that the emphasis of this work is to demonstrate that the technique possesses sufficient sensitivity and resolution to perform chemical speciation under in situ conditions before embarking on time-resolved studies. The methodology can be used to elucidate potential competitive and/or promoting factors along with the influence of the DNA-type, and drug selectivity. Finally, the method should be very useful in assessing if new metal containing drugs, including Pt ones, target DNA.

Experimental section

Calf Thymus DNA (25 mg/mL), in an aqueous buffer (20 mM HEPES solution), was incubated with 4mM of cisplatin at 37 °C for 24 hours before the measurement. The same concentrations of cisplatin in 20 mM HEPES buffer solution and on deionized water
were studied as references. Sigma Aldrich supplied DNA and HEPES. Institute of Drug Technology, Victoria, Australia, provided a sample of commercial cisplatin.

The Pt L edge RIXS maps were performed at the SuperXAS beamline at the Swiss Light Source, Paul Scherrer Institute, Switzerland. The X-ray beam delivered by the 2.9 Tesla super-cooled bending magnet was collimated by an spherically bent Rh mirror. The collimated X-rays were monochromatized by means of a double Si (111) crystal monochromator and focused by a toroidal bent Rh mirror. The beam was focused down to 100 µm and the photon flux on the sample was 7–8 Å photons/s. A 4 µm thick Pt foil was used to calibrate the energy. X-ray detection was performed using a wavelength-dispersive spectrometer, in the von Hamos geometry. The X-rays emitted from the sample were diffracted by a Ge (660) cylindrically bent crystal with a radius of curvature of 25 cm, and detected via a 2D Pilatus detector. The spectrometer was operated in the vertical scattering geometry. The acquisition time per map was ca. 10 min, and 6 maps were collected continuously to prevent beam damage and sample homogeneity throughout.

Theoretical calculations of the RIXS and HR-XAS spectra were performed with FEFF9.0, which is an ab initio self-consistent multiple-scattering code able to simulate simultaneously the excitation spectra and electronic structure. Crystal structure data were used as input files either from previously published data cis-[PtCl(NH3)2] (CUKRAB) and [cis-[Pt(NH3)2{d(GpG)4N7(1),4}]2-] (Hamos geometry). The Pt L edge RIXS maps were performed at the SuperXAS beamline at the Swiss Light Source, Paul Scherrer Institute for providing access to the SuperXAS beamline.

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Notes and references


