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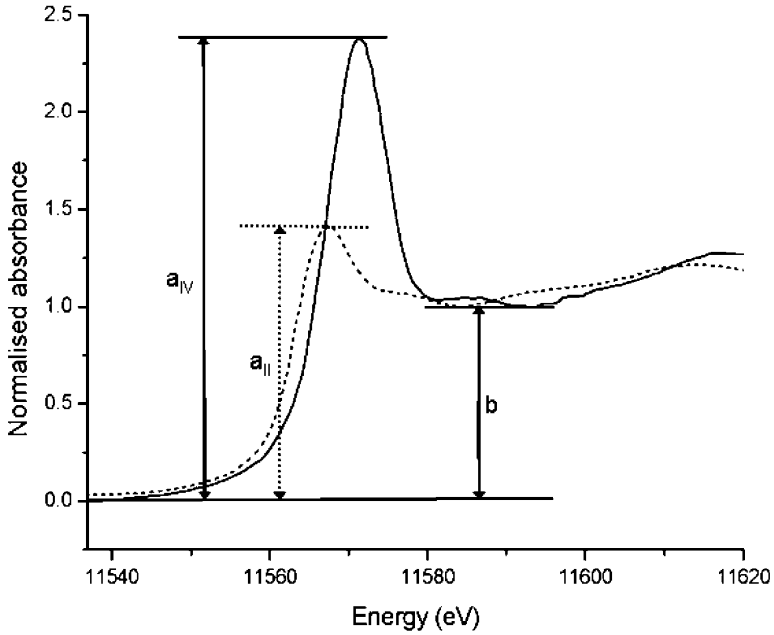

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XANES Determination of the Platinum Oxidation State Distribution in Cancer Cells Treated with Platinum(IV) Anticancer Agents

Matthew D. Hall,[†] Garry J. Foran,[‡] Mei Zhang,[§] Philip J. Beale,[⊥] and Trevor W. Hambley*,[†]

Centre for Heavy Metals Research, School of Chemistry, University of Sydney, Sydney, NSW 2006, Australia, Australian Synchrotron Research Program, c/- ANSTO, Private Mail Bag 1, Menai, N.S.W. 2234, Australia, Department of Radiation Oncology, Royal Prince Alfred Hospital, Camperdown, N.S.W. 2050, Australia, and Department of Medical Oncology, Sydney Cancer Centre, Royal Prince Alfred Hospital, Camperdown, N.S.W. 2050, Australia

Received April 5, 2003; E-mail: t.hambley@chem.usyd.edu.au

Platinum(IV) complexes are more inert than their platinum(II) analogues and consequently have potential advantages as anti-cancer agents, particularly if they arrive at their cellular target intact and are then reduced.[1,2] As a result, the rates and mechanism of the in vitro reduction of Pt(IV) complexes by biomolecules have been extensively investigated and recently reviewed.[3] However, no reliable spectroscopic method has been reported for following the reduction of Pt(IV) complexes in biological systems. Such information is essential if the properties of these complexes are to be tuned to achieve reduction and activation only where required. Current techniques used for monitoring oxidation states involve IR, HPLC, or MS identification of individual compounds,[4,5] methods which do not allow for facile in situ determination of the average or component oxidation states of a complex system. X-ray absorption near edge spectroscopy (XANES) has been used to provide information about the oxidation state of metal ions in a number of systems.[6,7] However, few X-ray absorption fine structure (XAFS) analyses or XANES investigations of Pt drugs have been reported,[8−10] and only one has been reported for Pt(IV).[11] Here we report a novel method for monitoring the platinum oxidation state and show that it is applicable to biological systems.

XANES spectra of the Pt L3-edge of therapeutically relevant Pt(II) and Pt(IV) complexes were collected in powder form and in solution at a temperature of 12 K at the Australian National Beamline Facility at the KEK Photon Factory, Tsukuba, Japan. The maxima of the first derivatives of the XANES spectra revealed the formal edge energy of the oxidation states, with the Pt(IV) edge appearing 2.2 eV higher than that for Pt(II). The XANES spectra of aqueous solutions containing varying proportions of Pt(II) and Pt(IV) complexes were collected to determine whether it was possible to correlate the known proportions with the XANES edge features, as a tool for monitoring reduction of Pt(IV) in complex systems.

The XANES spectra of Pt(II) and Pt(IV) differ in the height of their edges, with that for Pt(IV) being substantially greater. This is independent of the coordination sphere. By measuring the absorbance at *a* and *b* (Figure 1), we have found that for any of the complexes investigated here the ratio *a*/*b* is diagnostic of the oxidation state (Pt(II) 1.52 ± 0.08, Pt(IV) 2.51 ± 0.13). Significantly, mixtures of the two oxidation states in varying proportions resulted in *a*/*b* ratios intermediate between those of the individual oxidation states, and these ratios are linearly related to the proportions ($R^2 = 0.99788$, $P < 0.0001$) as shown in Figure 2. [Derived from XANES spectra of a series of solutions containing mixtures of *cis*-$[PtCl_2(NH_3)_2]$ (a Pt(II) complex) and *cis*,*trans*,*cis*-$[PtCl_2(OH)_2(NH_3)_2]$ (a Pt(IV) complex) in proportions ranging from 0:100 to 100:0.] This linear relationship allows for the monitoring of reactions of Pt(IV) complexes with reductants, and the relative proportions of each oxidation state can be determined simply and effectively. The error is approximately 5%, this being the observed variation in the *a*/*b* ratio for a range of Pt(II) and Pt(IV) standards.

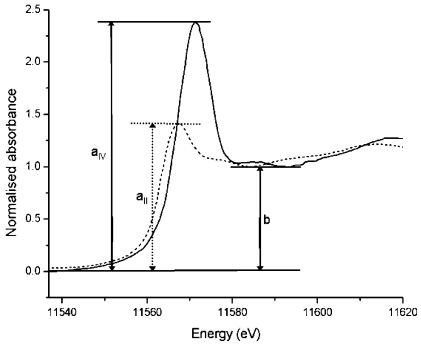

Figure 1. XANES spectra of Pt(II) (···) and platinum(IV) (—) complexes, showing the difference in peak heights for the two oxidation states, and the parameters *a* and *b* used in determining the ratio *a*/*b*.

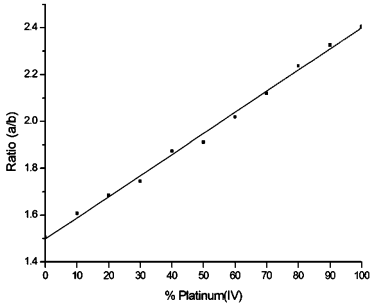

Figure 2. Linear fit of peak height ratio extracted from Pt(IV)/Pt(II) standard mixtures. Determining the white line height ratio showed a good linear fit ($R^2 = 0.99788$, $P < 0.0001$).

To monitor the cellular reduction of Pt(IV) complexes in parental A2780 ovarian cancer cells,[12] the cells were treated with either the Pt(II) complex cisplatin (*cis*-$[PtCl_2(NH_3)_2]$) or one of the three Pt(IV) complexes: *cis*-$[PtCl_4(NH_3)_2]$, *cis*,*trans*,*cis*-$[PtCl_2(OAc)_2(NH_3)_2]$ (OAc = CH_3COO^-), and *cis*,*trans*,*cis*-$[PtCl_2(OH)_2(NH_3)_2]$ at a concentration of 50 μM. Cells were isolated, washed, and freeze-dried at 2 and 24 h time points. The XANES spectra were collected at a temperature of 12 K, and the *a*/*b* ratio described above was determined for each sample.

The reduction from Pt(IV) to Pt(II), and the concomitant change in the edge height, is evident in Figure 3 which shows that the Pt XANES spectrum of cells treated with the *trans*-dihydroxo complex after 2 h has a more intense edge height compared to that in the 24

[†] University of Sydney.
[‡] Australian Synchrotron Research Program.
[§] Department of Radiation Oncology, Royal Prince Alfred Hospital.
[⊥] Department of Medical Oncology, Sydney Cancer Centre, Royal Prince Alfred Hospital.

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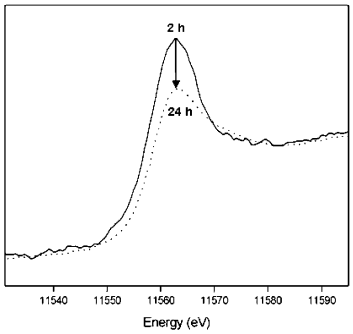

Figure 3. Normalized XANES spectra of frozen pellets of parental A2780 ovarian cancer cells incubated with *cis*,*trans*,*cis*-$[PtCl_2(OH)_2(NH_3)_2]$ after 2 h (—) and 24 h (···) showing a decrease in the white line height due to the cellular reduction of Pt(IV) over time.

Table 1. Proportion of Pt(IV) (±5%) Remaining after Incubation of Complexes with A2780 Ovarian Cancer Cells, and Their Reduction Potentials (E_p)

	treatment period		
complex	2 h	24 h	E_p (mV)
cis-$[PtCl_2(NH_3)_2]$	1%	−5%[a]	n/a
cis-$[PtCl_4(NH_3)_2]$	5%	−3%*	−260
cis,*trans*,*cis*-$[PtCl_2(OAc)_2(NH_3)_3]$	33%	2%	−635
cis,*trans*,*cis*-$[PtCl_2(OH)_2(NH_3)_3]$	54%	0%	−880

[a] Negative values have no physical significance, but are within the estimated error from zero.

h spectrum. The proportion of Pt(IV) by percentage present in the cells was calculated from the relationship derived using standard solutions (Figure 2) and is shown in Table 1. It is evident that after 2 h some of the Pt present in the cells is in the +IV oxidation state, and in the instance of *cis*,*trans*,*cis*-$[PtCl_2(OH)_2(NH_3)_2]$ approximately half remains in the higher oxidation state. After 24 h all are fully reduced to the +II oxidation state.

One of us has previously reported that for complexes of the type used in this study, the order of ease of reduction depends on the axial ligands, X, such that for complexes of the type *cis*,*trans*,*cis*-$[PtCl_2X_2(NH_3)_2]$, the ease of reduction is X = Cl > OAc > OH.[13] This order is reflected in the extent of cellular reduction of the three Pt(IV) complexes after 2 h with the *trans*-dichloro complex nearly fully reduced whereas the *trans*-diacetato and *trans*-dihydroxo complexes have a substantial proportion remaining as Pt(IV).

As expected, the control (untreated) samples did not give rise to a Pt L3 edge. While XANES spectra obtained from cells have been reported previously for other metal ions,[6] this is the first report of direct measurement of the relative proportions of two different oxidation states of a single element. That the Pt detected is primarily intracellular rather than located outside the cells has been established using elemental imaging of single cells treated with these complexes (Table 1).[14]

Pt(IV) drugs in preclinical trials have previously been detected intracellularly using HPLC,[15−17] although determination of the total Pt(IV) content in cells is not practical using HPLC as the level of protein-associated Pt cannot be assessed. The ability to monitor the fate of Pt(IV) drugs in situ is important and has not been achieved previously. The results validate the large quantity of research directed toward modification of axial ligands to tune the reduction potential and lipophilicity of Pt(IV) complexes.[3,13,18−20] Also, the notion of Pt(IV) complexes as unreactive prodrugs,[2] which are reduced to the active Pt(II) drug after entering the cell, is supported by the observation that all of the complexes are reduced after 24 h.

An equally important aspect of the reduction of these Pt(IV) complexes is that whereas reactions of *trans*-dihydroxo complexes with strong reductants such as cysteine and glutathione results in little or no reduction in vitro, consistent with their highly negative reduction potentials,[21] here we observe reduction of these same complexes. It is not clear what is responsible for the reduction, but it is clear that even highly inert Pt(IV) complexes are reduced in the cell on a time scale short enough for them to exert anticancer activity. These results are consistent with observations from clinical trials of iproplatin, a Pt(IV) complex with axial hydroxo ligands. A substantial proportion of iproplatin is expelled from the body unreduced, yet it is highly active.[4,22]

In conclusion, we have shown that both Pt(II) and Pt(IV) can be detected when present together in solution and that their proportions can be determined using the peak-height ratio of XANES spectra. The extent of intracellular reduction of Pt(IV) complexes was observed directly and found to correlate with previously reported reduction potentials. This technique will in the future be used to investigate the rate of reduction of prospective Pt(IV) drugs in tissues and tumors from treated animals. However, the potential applications of this technique are not limited to drug development, and it should also prove useful in many other areas of platinum chemistry.

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