Chemical basis for the detoxification of cisplatin-derived hydrolysis products by sodium thiosulfate

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ABSTRACT: Cisplatin remains the most widely used platinum-based anti-cancer drug and is included on the World Health Organization's list of essential medicines. Cisplatin also exhibits severe toxic side-effects, in particular damage to both the kidney and the inner ear, which are thought to derive primarily from hydrolysis products that are more toxic than cisplatin itself. Selective inactivation of these hydrolysis products has emerged as a feasible strategy to mitigate side effects and transform cisplatin into a better medicinal drug. Sodium thiosulfate is one of the most promising of currently considered mitigation agents, and co-administration of large quantities with cisplatin has been shown to considerably reduce toxic side effects in animals without abolishing useful anti-cancer cytotoxicity. The structural basis of this antagonism has, however, remained uncertain. We report herein the structural characterization of the reaction product of hydrolyzed cisplatin and thiosulfate in aqueous solution using X-ray absorption spectroscopy. This reveals the formation of the four-coordinate Pt(II) species $[\text{Pt}(\text{S}_2\text{O}_3)_4]^{6-}$ with Pt—S bond lengths of 2.30 Å. Our structural conclusions are supported by density functional theory calculations. More generally speaking, the structural characterization of this Pt-thiosulfate complex reinvigorates the principle strategy to reduce the toxicity of cisplatin (and possibly other platinum-based anticancer drugs) by co-administering appropriate ameliorating agents for direct benefits to patients.

1. Introduction

The platinum-based anti-cancer drugs cisplatin (*cis*-diammine dichloridoplatinum(II)) and carboplatin (*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II)) (Fig. 1) are a mainstay in the treatment of many cancer patients. Indeed, cisplatin is included on the World Health Organization's list of essential medicines [1]. However, inherent toxicity to healthy tissues can limit cisplatin's use [2]. In particular severe nephrotoxicity and ototoxicity have been reported, with approximately one-third of all cisplatin treated cancer patients developing irreversible hearing loss [3]. The development of strategies to minimize toxicity while leaving anti-cancer activity mostly uncompromised represents a promising area of research [2,4,5].

After the intravenous injection of cisplatin, platinum-containing hydrolysis products are generated in the bloodstream which are thought to be more toxic than the parent drug [6]. One possible strategy to mitigate the toxic side effects of cisplatin would be to selectively inactivate these hydrolysis products *in vivo* by co-administering so-called 'ameliorating agents' [7-16]. Provided that the parent anticancer drug remains in the blood circulation for a considerable amount of time, this approach could enable improved therapeutic indices (defined as the ratio of the intended therapeutic action compared to the unintended toxicity) of platinum-based anticancer drugs that are in clinical use [17]. To this end, a plethora of studies with mammalian model organisms and humans have indeed revealed that sulfur-containing agents, such as sodium thiosulfate, are particularly effective in eliminating the toxic side effects of cisplatin [18]. In fact, thiosulfate has been demonstrated to reduce the toxicity of cisplatin in mice [19-23], rabbits [24], rats [25,26], hamsters [27,28], dogs [29], guinea pigs [30-33], and humans [34-43]. Key to the clinical deployment of thiosulfate and related chemoprotective agents is mitigation of chemotherapeutic toxicity without abrogating anticancer activity, and several studies have found that the elapsed time between administration of the chemotherapeutic and the chemoprotective agents is critical in this regard [23,26].

Recent *in vitro* studies with mammalian blood plasma [44] have demonstrated that cisplatin may react with thiosulfate to give complexes that may contain Pt—S bonds [45]. Closely related studies have revealed that complexes with Pt—S bonds may also be formed in mammalian plasma when cisplatin and N-acetylcysteine [46], D-methionine [47] or L-glutathione [48] are added. To date, however, the chemistry of the thiosulfate complex(es) that are formed with cisplatin in blood plasma remains unclear. Here we address this by using X-ray absorption spectroscopy (XAS) and density functional theory (DFT) to examine the products of cisplatin and thiosulfate in aqueous solutions.

2. Experimental section

2.1 Chemicals and reagents

A sterile clinical preparation of cisplatin containing 1 mg mL⁻¹ *cis*-Pt(NH₃)₂Cl₂, 1 mg mL⁻¹ mannitol and 9 mg mL⁻¹ NaCl, was obtained from Hospira (Montreal, QC, Canada). Phosphate-buffered saline (PBS) buffer tablets and anhydrous sodium thiosulfate (>99.99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solid platinum compounds were obtained from Strem chemical company (Newburyport, MA, USA) and were of the highest quality available. PBS buffer was prepared by dissolving PBS tablets in deionized water as prescribed by the manufacturer, followed by pH adjustment and filtration through a 0.45 µm nylon-filter membrane (Mandel Scientific, Guelph, ON, Canada). A stock solution of Na₂S₂O₃ was prepared by dissolving 293 mg mL⁻¹ in PBS buffer, and the pH adjusted to neutrality.

2.2 Preparation of the platinum-thiosulfate complex for X-ray absorption spectroscopy analysis

The concentration of Pt in previous work employing HPLC-ICP-AES analysis was ~130 µM which is less than the ideal Pt concentration (~ 1 mM) for the analysis by X-ray absorption spectroscopy (XAS). The following procedure was used to produce the platinum-thiosulfate complex in PBS-buffer

analogous to the species observed in plasma at the 50 min. time point [45]. PBS buffer (50.0 mL) was spiked with 2.1 mL of the cisplatin stock solution and was incubated at 37 °C for 10 min, after which 1.5 mL of the sodium thiosulfate stock solution was added. The resulting mixture was incubated at 37 °C for 50 min and was frozen at -30 °C, after which it was concentrated by lyophilization and reconstitution with deionized water with 20% v/v glycerol to give a final concentration 1.0 mM Pt for XAS, with freezing in liquid nitrogen and storage at low temperatures until XAS measurements were made.

2.3 X-ray Absorption Spectroscopy (XAS)

The XAS measurements were conducted at the Stanford Synchrotron Radiation Lightsource (SSRL) with the SPEAR storage ring containing close to 500 mA at 3.0 GeV. Pt L_{III}-edge data were collected on the structural molecular biology XAS beamline 7-3, employing a Si(220) double-crystal monochromator and a rhodium-coated vertical collimating mirror upstream of the monochromator. Harmonic rejection was accomplished by setting the mirror angle to achieve an energy cutoff of 13 keV. Incident and transmitted X-ray intensities were monitored using nitrogen-filled ionization chambers with a sweeping voltage of 1.6 kV. X-ray absorption was measured at the Pt L_{III} edge as the Pt L α ₁₂ fluorescence excitation spectrum using an array of 30 germanium detectors [49] (Canberra Instruments, Meriden CT, USA) equipped with a Soller slit assembly and zinc filters to preferentially reject scattered radiation. Data acquisition used the program XAS Collect [50]. During XAS data collection, samples were maintained at a temperature of approximately 10 K using an Oxford instruments (Oxford Instruments, Abingdon, Oxford, UK) liquid helium flow cryostat. For each data set, six scans each of 35 min. duration were accumulated. Energy was calibrated by reference to the absorption of a Pt metal foil measured simultaneously with each scan, assuming a lowest energy inflection point of 11563.0 eV.

XAS data analysis was carried out using the EXAFSPAK suite of computer programs (<http://ssrl.slac.stanford.edu/exafspak.html>) as previously described [51]. The threshold energy E_0 of the Pt L_{III} EXAFS oscillations ($k = 0 \text{ \AA}^{-1}$) was assumed to be 11580.0 eV. X-ray absorption near-edge spectra were normalized to the Pt L_{III} edge-jump to give a per-platinum normalized absorption. The edge-jump was estimated by calculating the spline through the extended X-ray absorption fine structure (EXAFS) part of the spectrum and taking the spline value at E_0 as the edge jump. The EXAFS oscillations $\chi(k)$ were quantitatively analyzed by curve-fitting in the EXAFSPAK suite using *ab initio* theoretical phase and amplitude functions calculated using the program FEFF version 8.4 [52]. No smoothing, filtering or related operations were performed on the data.

2.4 Density functional theory (DFT) calculations

DFT calculations used the program Dmol³ and Biovia Materials Studio 2016 for geometry optimization [53,54] using the Perdew-Burke-Ernzerhof functional [55], both for the potential during the self-consistent field procedure, and for the energy. Dmol³ double numerical basis sets included polarization functions for all atoms, were spin-unrestricted, and all electron relativistic core treatments were used. Solvation effects were modeled by using the Conductor-like Screening Model (COSMO) [56] with the dielectric constant of water ($\epsilon = 78.39$).

3. Results and discussion

3.1. X-ray absorption near-edge spectra

X-ray absorption spectra, which arise from photo-excitation of a core electron, can be arbitrarily divided into two overlapping regions: the near-edge spectrum within approximately 50 eV of the absorption threshold energy (sometimes referred to as the X-ray absorption near-edge fine structure or XANES); and the extended X-ray absorption fine structure (EXAFS) which comprises oscillations in

the absorption on the high-energy side of the threshold. The EXAFS arises from photoelectron backscattering by nearby atoms and can give a local radial structure around the absorber atom [57]. Intense absorptions in the near-edge spectrum arise from Laporte-allowed ($\Delta l = \pm 1$) transitions of the core electron to bound states. This study used the Pt L_{III} edge with 2p_{3/2} excitation, the intense transitions of which are therefore all to levels with substantial d-orbital character. Since the cisplatin compound is formally Pt(II) with a 5d⁸ configuration, a dipole-allowed intensity corresponding to the two 5d holes should be observed in the near-edge spectrum. The near-edge portion of the XAS spectrum provides a probe of electronic structure being sensitive to both ligand environment and oxidation state [57]. Thus, electron-rich donors, such as sulfur should shift the near-edge to lower energies relative to harder ligands such as oxygen species, and higher oxidation state will shift the near-edge to higher energies.

The near-edge spectra of cisplatin with excess thiosulfate and cisplatin solution are shown in Fig. 2. The spectra of cisplatin are similar to those previously reported [58]. The near-edge spectrum of thiosulfate-treated cisplatin solution differs substantially from that of untreated cisplatin, indicating that a chemical transformation has occurred. The possibility of Pt—S bond formation with thiosulfate has already been suggested [45], and the presence of such bonds would superficially be expected to shift the near-edge to lower energy relative to untreated cisplatin. However, examination of Fig. 2 shows that the spectrum is instead shifted to higher energy, which at first glance might suggest the possibility of a higher oxidation state. We therefore considered the possibility that the formal oxidation state platinum had been altered in the reaction product. Platinum is known to exist in Pt(II), Pt(III) and Pt(IV) oxidation states, although the most commonly known are Pt(II) and Pt(IV). Thiosulfate can potentially act as an oxidizing agent or a reducing agent:

While thiosulfate's reducing capacity is certainly better known, in our case the former reaction might reasonably yield either Pt(III) or Pt(IV). Should this occur *in vivo* it would generate low levels of sulfide in blood plasma, which although toxic in excess is also generated endogenously in mammals and used in cell signaling [61], so that generation of small amounts would probably not be detrimental. Importantly, we observed no characteristic odor of H₂S during the reaction of cisplatin solutions with thiosulfate, and testing the same solutions with commercial H₂S test strips (lead acetate paper) gave negative results, indicating that no H₂S had been generated. Because of the possibility of an altered platinum oxidation state we examined a wider range of model compounds, the spectra of which are shown in Fig. 3, and the first inflection energies, calculated from the peak of the first derivative are listed in Table 1.

Fig. 3 includes the Pt L_{III} near-edge spectrum of α -PtO₂ which has approximately octahedrally coordinated Pt(IV) with a Pt—O bond-length of 2.299 Å [62] and this shows both greater peak intensity at the edge and a shift in edge position to higher energy (Fig. 3, Table 1). Hambley and co-workers have investigated the Pt L_{III} XAS of a number of six-coordinate Pt(IV) species and observed a more intense peak in the Pt L_{III} near-edge spectrum, consistent with the decreased occupancy of the 5d manifold for the higher oxidation state, and correspondingly greater intensity of the 2p_{3/2}→5d transitions [58,63,64]. These workers normalized their near-edge spectra with post-absorption edge minimum intensity in X-ray absorbance [58,63,64], but because this will be sensitive to the presence of EXAFS oscillations in the data we have used the well-established method of normalizing with the edge jump as estimated from the value of the spline at the threshold energy to give a per platinum normalized absorption. The spectra of solid cisplatin and the clinical preparation of cisplatin are very similar (Fig. 3), indicating that as expected no substantial hydrolysis or other degradation of *cis*-PtCl₂(NH₃)₂ has occurred in the clinical formulation. Examination of the first inflection energies (Table 1) indicates that cisplatin in fact has an unusually low energy first inflection, and that the L_{III}

first inflection energy of the thiosulfate reaction product is approximately in the middle of the range of inflections of the Pt(II) species investigated. This, together with the absence of H₂S in the reaction mixture solutions, suggests that the Pt(II) oxidation state of cisplatin is unchanged by reaction with thiosulfate.

3.2 Extended X-ray absorption fine structure

Fig. 4 shows the EXAFS oscillations of thiosulfate treated cisplatin, together with the EXAFS Fourier transforms and curve-fitting analysis. The parameters derived from curve-fitting are summarized in Table 2. The analysis shows that the platinum in the thiosulfate treated cisplatin is bound by four sulfur atoms at a mean interatomic distance R of 2.30 Å, with no other first-shell backscatterers and no significant outer shell scattering. The value determined for σ^2 , the mean square deviation in R , is 0.0032 Å². This parameter has contributions both from vibrational modes and from static disorder in the Pt—S bond lengths present. As this value is close to the computed vibrational σ^2 value of 0.0028 Å² [57] we can conclude that the four Pt—S bonds are essentially of uniform bond-length. Because of the large 400-fold excess of thiosulfate over Pt it seems probable that the observed tetra-sulfur coordination arises from four equivalent thiosulfato ligands.

3.3 Oxidation state of platinum

Consideration of the Pt L_{III} near-edge spectra and chemical considerations suggest that the reaction product is Pt(II) (see 3.1). The EXAFS-derived bond-lengths are also expected to be sensitive to the metal oxidation state. Examination of the Cambridge Crystal Structure Database (CSD) [65] shows that Pt(IV) complexes are relatively common, and as expected are nearly always six-coordinate. Insofar as cisplatin related species are concerned 32 of the 38 entries containing [*cis*-Cl₂Pt(NH₃)₂] coordination are formally Pt(IV) complexes, and all of these are six-coordinate with two additional

ligands to Pt. Of the total 1,162 entries in the CSD that are indicated as formally Pt(IV) entities, only two entries are indicated as a four-coordinate sulfur bound species [66,67] and one of these may actually be six coordinate as the metal has long bonds to other components in the unit cell [67]. Conversely, Pt(III) species, although less common in the database with 250 entries, are predominantly four coordinate square planar, and of 42 Pt(III) CSD entries with Pt—S coordination, 24 of these are 4 coordinate with square planar type geometry, mostly involving maleonitriledithiolato $[(\text{NC})_2\text{C}_2\text{S}_2]^{2-}$ or a related ligand [65]. The mean bond-length of these compounds is 2.26 Å ranging from 2.25 to 2.28 Å, somewhat shorter than our EXAFS-derived Pt—S bond-length of 2.30 Å. In contrast, there is an abundance of Pt(II) compounds in the CSD with 7,884 total entries denoted as formal Pt(II) species, 169 of which are square planar species bound by four sulfur containing ligands. The mean Pt—S bond-length of these Pt(II) sulfur-bound species is 2.31 Å, which is very close to our EXAFS-derived bond-length of 2.30 Å. The accuracy for EXAFS determined bond-lengths is traditionally said to be around 0.02 Å, but this value comes from early studies, before the advent of modern theoretical approaches [52] and modern analyses are thought to be considerably more accurate than this [57]. In agreement with this, analysis of the EXAFS for the solid Pt(II) species of Fig. 3 plus comparison with crystallographic values, gives an estimated bond-length accuracy of ± 0.008 Å. Taken together, the Pt L_{III} near-edge, the lack of observed production of H_2S , and EXAFS data support a Pt(II) oxidation state and there is no evidence for metal oxidation in the reaction product.

3.4 Density functional theory (DFT) calculations

DFT energy-minimized geometry-optimized structures were calculated for the *tetrakis*-thiosulfato-Pt(II) for both deprotonated thiosulfate $[\text{Pt}(\text{S}_2\text{O}_3)_4]^{6-}$ and for protonated thiosulfate $[\text{Pt}(\text{S}_2\text{O}_3\text{H})_4]^{2-}$. In acidic solutions thiosulfate will normally decompose to sulfite and elemental sulfur, but protonated thiosulfate ligands are known to be stable in metal complexes [68]. Initially, calculations were

constrained to C_{4h} point group symmetry, but lifting this constraint when starting with the C_{4h} symmetric converged geometry did not result in any significant change in the structures. The results of both of these calculations are shown in Fig. 5. The Pt—S bond-length for deprotonated and protonated thiosulfate are 2.347 and 2.317 Å, respectively. The protonated DFT structure shows hydrogen bonds between protonated oxygen atoms and the adjacent sulfur atoms in the PtS₄ plane, with predicted H····S interatomic distance of 2.019 Å. This hydrogen bonding lowers the total energy considerably, by some 62 kJ/mol, as estimated by rotating the four protonated oxygens so that they are out of the molecular plane and calculating the energy without optimization. Geometry optimizations of [Pt(S₂O₃)₄] with different formal Pt oxidation states show that bond-lengths decrease with increasing oxidation state by 0.029 Å between Pt(II) and Pt(III) and by 0.025 Å between Pt(III) and Pt(IV). DFT geometry optimization will give systematically different bond-lengths from experimental values, depending upon the detailed nature of the calculation. In our case, we estimate from calculations of crystallographically characterized species with low *R*-factors (i.e. ~3%) that DFT over-estimates bond-lengths by approximately 0.015 Å, which means that our DFT calculations of Pt—S bond-lengths of 2.317 Å and our EXAFS-derived bond-lengths of 2.30 Å are in excellent agreement.

In conclusion, the near-edge spectra and the lack of production of H₂S jointly indicate that the thiosulfate-treated cisplatin reaction product is a Pt(II) species, and both the EXAFS-derived coordination number of four and the EXAFS-derived Pt—S bond-lengths of 2.30 Å argue against any oxidation. We conclude that the complex studied is a *tetrakis*-thiosulfato-Pt(II) entity. The conditions employed for making the complex studied here were designed to produce a platinum-thiosulfate complex in PBS-buffer that is directly analogous to the species previously observed in plasma at the 50 min. time point [18]. Despite the very high levels of thiosulfate that are employed *in vivo*, the *tetrakis* complex may not actually form *in vivo*. Irrespective of the actual stoichiometry, however, our

experimental results indicate that the mode of binding of thiosulfate to platinum in the species studied is via the external sulfur atom, which is also likely to be the case in complexes with fewer bound thiosulfate groups. Our study therefore affords a structural basis for the inactivation of toxic hydrolysis products of cisplatin *in vivo*. The presumed mode of action is thus that thiosulfate sequesters and inactivates these hydrolysis products, leaving intact some un-sequestered platinum species with anti-cancer activity, and corresponding beneficial therapeutic effects. Future work will use XAS to address the chemical forms of Pt in blood following cisplatin administration with and without amelioration agents such as thiosulfate.

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Table 1. First Pt L_{III} near-edge spectra inflection energies.^a

Compound	First L _{III} inflection (eV)	Formal Pt oxidation state
platinum metal	11563.00	0
cisplatin- <i>solid</i>	11563.67	2
cisplatin- <i>solution</i>	11563.70	2
cisplatin-thiosulfate	11564.00	
<i>cis</i> -Cl ₂ Pt(SEt ₂) ₂	11564.12	2
Carboplatin- <i>solid</i>	11564.21	2
<i>cis</i> -Cl ₂ Pt(PEt ₃) ₂	11564.54	2
α -PtO ₂	11565.83	4

a. First inflection energies were estimated from the lowest energy peak in the first derivative spectrum, as calculated using EXAFSPAK

Table 2. Extended X-ray Absorption Fine Structure (EXAFS) curve-fitting analysis of thiosulfate – treated cisplatin solution.^a

Fit #	Interaction	N	R	σ^2	ΔE_0	F
<i>A</i>	Pt—S	4	2.302(2)	0.0032(1)	-10.4(5)	0.2639
<i>B</i>	Pt—S	5	2.302(2)	0.0042(1)	-10.2(5)	0.3085
<i>C</i>	Pt—S	4	2.302(3)	0.0036(1)	-7.0(9)	0.2835
	Pt—O	2	2.215(8)	0.0045(2)		

- a.* Coordination number N , interatomic distances R (Å), Debye-Waller factors σ^2 (Å²), and threshold energy shift ΔE_0 (eV). Values in parentheses represent the standard uncertainties between the curve fitting parameters and spectroscopy data, expressed as the last digit of each value, obtained from the diagonals of the variance-covariance matrices. Goodness of fit function F is defined as $F = \sqrt{\sum k^6 (\chi(k)_{calc} - \chi(k)_{expt})^2 / \sum k^6 \chi(k)_{expt}^2}$, where $\chi(k)_{calc}$ and $\chi(k)_{expt}$ are the calculated and experimental EXAFS, respectively, with k (Å⁻¹) being the photoelectron wave vector and the sum being over all data points in the spectrum.

Fig. 1. Schematic structures of cisplatin (*a*) and carboplatin (*b*), the two most prevalent anti-cancer drugs.

Fig. 2. Pt L_{III} X-ray absorption near-edge spectra of the clinical solution preparation of cisplatin (-----) and thiosulfate-treated cisplatin (———). The spectrum of the thiosulfate-treated sample can be seen to be shifted to higher relative energy.

Fig. 3. Pt L_{III} X-ray absorption near-edge spectra. The figure shows *a* α -PtO₂, *b* solid *cis*-Cl₂Pt(PEt₃)₂, *c* solid carboplatin, *d* solid *cis*-Cl₂Pt(SEt₂)₂, *e* thiosulfate-treated cisplatin, *f* aqueous cisplatin solution, *g* solid cisplatin, and *h* platinum metal foil. The first Pt L_{III} edge inflection energies of these spectra are summarized in Table 1.

Fig. 4. Pt L_{III} EXAFS spectra (*a*) and EXAFS Fourier transforms (*b*) of thiosulfate-treated cisplatin. In both the solid lines (———) show the experimental data and the broken lines (-----) show the best fits obtained with the parameters summarized in Table 2. EXAFS Fourier transforms are phase-corrected for Pt—S backscattering.

Fig. 5. Energy minimized geometry optimized structure of the postulated *tetrakis*-thiosulfato-Pt(II) complexes, for both deprotonated (*a*) [Pt(S₂O₃)₄]⁶⁻, and protonated ligands (*b*) [Pt(S₂O₃H)₄]²⁻, discussed in the text. The calculations were constrained to have *C*_{4h} point-group symmetry in order to both reduce the computation time for the geometry optimization and to increase stability of the refinement. When the symmetry constraints were lifted and geometry optimization repeated starting with the converged coordinates, the structures did not change significantly.