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COMMUNICATION

Stability of carboplatin and oxaliplatin in their infusion solutions is due to self-association

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Carboplatin and oxaliplatin are commonly used platinum anticancer agents that are sold as ready-to-use aqueous infusion solutions with shelf lives of 2 and 3 years, respectively. The observed rate constants for the hydrolysis of these drugs, however, are too large to account for their long shelf lives. We here use electrospray-trap mass spectrometry to show that carboplatin and oxaliplatin are self-associated at concentrations in their ready-to-use infusion solutions (~27 mM and 13 mM, respectively) and, as expected, when the drug concentration is reduced to more physiologically relevant concentrations (100 μM and 5 μM, respectively) the association equilibrium is shifted in favor of the monomeric forms of these drugs. Using ^{1}H NMR we measure the intensity of the NH resonance of the two symmetry-equivalent NH_3 molecules of carboplatin, relative to the intensity of the γ-methylene CH resonance, as a function of total drug concentration. Then, by fitting the data to models of different molecularity, we show that the association complex is a dimer with a monomer–dimer association constant of K (M^{-1}) = 391 ± 127. The work presented here shows that carboplatin and oxaliplatin mainly exist as association complexes in concentrated aqueous solution, a property that accounts for the long term stability of their ready-to-use infusion solutions, and that these association complexes may exist, to some extent, in the blood after injection.

Carboplatin, **1**, and oxaliplatin, **2**, Fig. 1, are used in the clinic to treat many types of cancer.[1] These platinum anticancer agents differ from cisplatin in that they both contain bidentate chelate leaving ligands.[1] For convenience of administration, these drugs are sold as ready-to-use infusion solutions, which in the case of carboplatin is a ~27 mM (10 mg ml^{-1}) solution of platinum complex dissolved in water alone, in water containing mannitol or dextrose or, in some cases, in water containing a small amount of the free ligand 1,1-cyclobutane dicarboxylate, CBDCA (*e.g.*, Paraplatin–AQ, Bristol-Myers Squibb Co.).[2,3] The ready-to-use infusion solution of oxaliplatin, Eloxatin (Sanofi-Aventis), is a

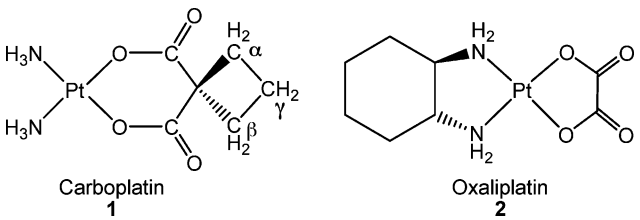

Fig. 1 Structures of carboplatin and oxaliplatin.

~13 mM (5 mg ml^{-1}) solution of the platinum complex in water.[4] In the clinic, the infusion solution of either drug is diluted to the required concentration, usually in 5% aqueous dextrose or glucose, and the resulting solution is administered into the blood of the patient in chemotherapy. An interesting property of these ready-to-use infusion solutions, and one that allows successful marketing of the product, is that their shelf lives are quite long (2 years for carboplatin and 3 years for oxaliplatin).[3,4] Analysis of ready-to-use infusion formulations of carboplatin showed that drug loss with time is relatively modest and independent of the presence of excess CBDCA ligand with losses ranging from 3.09% after 5 months to 7.28% after 78 months.[2] The degradation products of **1** in the infusion solutions are ring-opened structures and hydroxo/aquo species not having the dicarboxylate ligand.[2]

An interesting observation concerning the aqueous stability of carboplatin is that the reported pseudo-first-order rate constant for the hydrolysis of the drug in water at 37 °C spans an unusually large range, k ~5×10^{-7} s^{-1} to $k < 10^{-9}$ s^{-1}, and only the latter value is consistent with the observed high stability of the ready-to-use infusion solutions.[5–8] Earlier, we used 110 μM ^{15}N-labeled carboplatin and [^{1}H,^{15}N] HSQC NMR to obtain a hydrolysis rate constant for the drug of k ~5×10^{-7} s^{-1} (37 °C),[5] which agrees very well with the rate constant at 37 °C obtained by Brandšteterová and coworkers, who used HPLC and ~300 μM **1** to study the hydrolysis kinetics.[6] These investigators also reported that the hydrolysis rate decreases with temperature, with k ~10^{-8} s^{-1} at 25 °C.[6] In another study, Tobe and coworkers measured the hydrolysis rate constant of ~500 μM carboplatin finding that $k < 10^{-8}$ s^{-1} at 25 °C,[7] while Sadler and coworkers used ^{1}H NMR and a high concentration of **1**, 20 mM, to find that $k < 10^{-9}$ s^{-1} at 37 °C.[8] Collectively, these results show that the rate constant for hydrolysis of **1** is highly dependent on the starting concentration of drug in solution. Low concentrations, which mimic the concentration of carboplatin in the blood of patients receiving chemotherapy

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(~100 μM), produce large rate constants and high concentrations of **1** (20 mM), which simulate the concentration of drug in ready-to-use infusion solutions, give a small rate constant for hydrolysis. More recently, we found that the rate constant for the reaction of 5 mM ^{15}N-labeled carboplatin in 23.8 mM carbonate buffer (pH 8.4) at 37 °C is $k = \sim 5 \times 10^{-7}$ s^{-1},[9] which is approximately an order of magnitude smaller than that measured using 110 μM ^{15}N-labeled carboplatin.[5] The rate constants for the reaction of 5 mM and 110 μM **1** in 0.5 M carbonate buffer (pH 8.6), however, are the same.[9] While the literature on the hydrolysis of oxaliplatin is not extensive, Jerremalm, *et al.*[10] reported that the hydrolysis of 24 μM oxaliplatin at 37 °C in HEPES buffer is 1.2×10^{-6} s^{-1}, a value which is much too large to account for the long shelf life (3 years) of the 13 mM ready-to-use aqueous infusion solution of oxaliplatin.

In the case of the hydrolysis of **1** and **2** a dicarboxylate chelate ligand is displaced in a stepwise manner by the attacking nucleophile, which is water. Since it is well known that substitution reactions of square planar complexes of Pt(II) occur through an associative mechanism involving the formation of a 5-coordinate intermediate,[1] the first step in the ligand displacement reaction is the attack of a water molecule on Pt(II) *via* the *z*-axis of the complex (the ligands occupy the *x*, *y* plane). Consideration of the X-ray structure of carboplatin[11,12] shows that the α (β) methylene groups of the cyclobutane ring of the CBDCA ligand are positioned near the *z*-axis of the complex and, since the chelate ring is in motion in solution, the methylene hydrogen atoms could impede a water molecule from attacking the platinum ion *via* both the + and −*z* axis of the complex. While this potential blocking effect could lead to reduced hydrolysis rates for carboplatin, oxaliplatin, which has conformationally fixed DACH and oxalate ligands, is devoid of a similar structural feature that would restrict a water molecule from attacking the Pt(II) ion *via* the *z*-axis of the complex. All of the atoms of the oxalate chelate ring of oxaliplatin are nominally in the metal–donor plane, while the atoms of the chiral *trans* DACH ligand do not deviate far from this plane.

A striking feature of both **1** and **2** in the solid state, is that individual drug molecules are connected to each other through an extensive network of intermolecular hydrogen bonds in three dimensions which could play a role in the concentration dependence of the hydrolysis rate of these drugs.[11,13] In the case of carboplatin, the hydrogen bonds are between the ammonia molecules of one complex and the oxygen atoms of the dicarboxylate CBDCA ligand of neighboring complexes. For oxaliplatin, the H-bonds are between the amine hydrogen atoms of the DACH ligand of one complex and the oxalate oxygen atoms on adjacent complexes. Since elements of this hydrogen bond network could persist in concentrated aqueous solutions, association complexes of the monomeric forms of the compounds could be present in ready-to-use infusion solutions which, because of the steric constraints of the structure, may be the reason why high concentrations of these drugs are resistant to hydrolysis. From transition state theory, the presence of intermolecular associations would cause a large free energy of activation, $\Delta G^{\ddagger}$, leading to the activated complex which would reduce the rate of conversion of the intact drugs to their hydrolysis products through a decrease in the hydrolysis rate constant, *k*.[1]

We here use electrospray-trap mass spectrometry to show qualitatively that carboplatin and oxaliplatin self-associate at concentrations that exist in their ready-to-use infusion solutions (27 mM and 13 mM, respectively) but at lower, more physiologically relevant concentrations (100 μM and 5 μM, respectively), these platinum drugs do not self-associate to the same extent, Fig. 2 and 3. Positive electrospray ionization was performed on a Bruker 12 Tesla APEX-Qe FTICR-MS with an Apollo II ion source (COSMIC Lab, Norfolk, VA). Carboplatin, **1**, and oxaliplatin, **2**, were purchased as solids from Sigma–Aldrich (St. Louis, MO) and prepared in dH_2O. Peaks (*m*/*z*) corresponding to **1**H$^+$, **1**Na$^+$, (**1**)$_2$H$^+$ and (**1**)$_2$Na$^+$ can be seen and are labeled for clarity in the spectra of the 27 mM and 100 μM aqueous solutions of carboplatin, Fig. 2A and B, respectively. However, it is clear that in the 100 μM carboplatin solution the **1** dimer (*m*/*z* = 742) is much less predominant than it is in the 27 mM solution. In Fig. 3A, peaks for **2**H$^+$, **2**Na$^+$, (**2**)$_2$H$^+$, (**2**)$_2$Na$^+$ and (**2**)$_3$Na$^+$ are present in the spectrum of the 13 mM aqueous solution of oxaliplatin. However, of these four peaks, only **2**H$^+$, **2**Na$^+$ are present in the spectrum of the 5 μM oxaliplatin solution, Fig. 3B. Peak assignments for 27 mM carboplatin and 13 mM oxaliplatin are given in Table. 1. While it is possible that a dimer or a higher order association complex could occur *via* Pt–Pt interactions, as is the case of the platinum blues, the fact that the solutions are colorless and lack the characteristic

Table 1 Observed and calculated molecular masses for 27 mM carboplatin and 13 mM oxaliplatin

Peak	Carboplatin compound	Observed mass	Calculated mass
1	$[Pt(NH_3)_2(CBDCA)] + H^+$	367.6–376	372.2
2	$[Pt(NH_3)_2(CBDCA)] + Na^+$	387.1–395.0	394.2
3	$\{[Pt(NH_3)_2(CBDCA)]\}_2 + H^+$	740.1–747.1	743.5
4	$\{[Pt(NH_3)_2(CBDCA)]\}_2 + Na^+$	761.6–769.1	765.5
Peak	**Oxaliplatin compound**	**Observed mass**	**Calculated mass**
1	$[Pt(R,R\text{-DACH})(ox)] + H^+$	397.1–402.1	398.3
2	$[Pt(R,R\text{-DACH})(ox)] + Na^+$	419.0–423.1	420.3
3	$\{[Pt(R,R\text{-DACH})(ox)]\}_2 + H^+$	793.1–799.1	795.6
4	$\{[Pt(R,R\text{-DACH})(ox)]\}_2 + Na^+$	817.1–821.1	817.6
5	$\{[Pt(R,R\text{-DACH})(ox)]\}_3 + Na^+$	1212.2–1216.2	1214.8

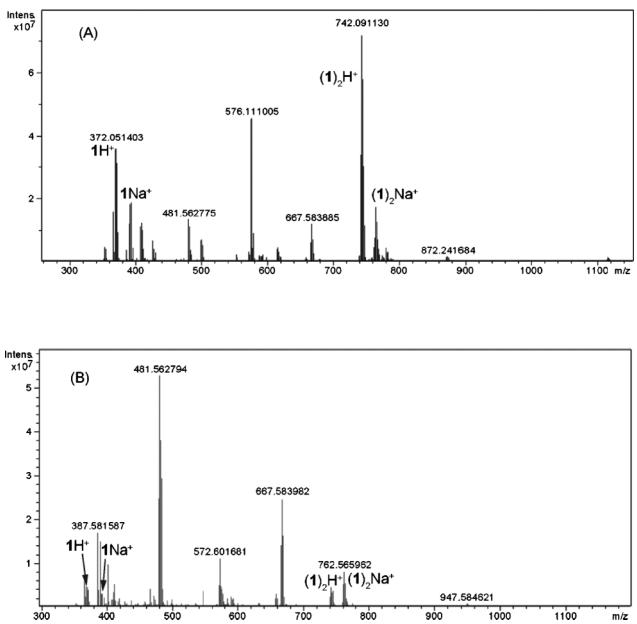

Fig. 2 Mass spectrum of 27 mM carboplatin, **1**, in water (A) and of 100 μM carboplatin, **1**, in water (B).

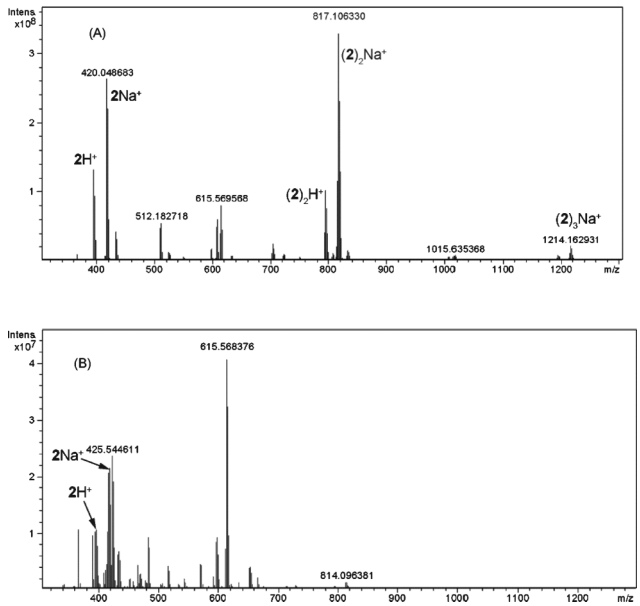

Fig. 3 Mass spectrum of 13 mM oxaliplatin, **2**, in water (A) and of 5 μM oxaliplatin, **2**, in water (B).

intense charge-transfer band of the Pt–Pt interaction indicates that close platinum contacts are absent in the associated species.

In 1996, Embree and coworkers detected the carboplatin dimer using liquid chromatography-mass spectrometry and ~300 μM carboplatin.[14] These investigators used formic acid in the mobile phase which, since formic acid would be expected to break hydrogen bonds, is evidence for the high stability of the dimer. Using mass spectrometry, Guo and coworkers observed the dimer, trimer, and tetramer forms of carboplatin in a ~3 mM aqueous solution of carboplatin,[15] and the carboplatin dimer was later observed by Vivekanandan *et al.* and by us.[16,17] Guo and coworkers suggested that these species could form in aqueous solution through self-association or they could be artifacts of the mass spectrometry measurement.[16] We also suggested that the inability of mesoporous silica to adsorb significant amounts of carboplatin from concentrated solutions of the drug was due to an associated form of the drug which is too large to enter the pore of the nanomaterial.[17]

Using freshly prepared stock solutions of **1** in 25 mM HEPES buffer, pH 7.4, H_2O/D_2O (90/10) we demonstrate that as the carboplatin concentration in solution is decreased, the ^{1}H NMR signal at δ 4.17 ppm, associated with the two symmetry-equivalent NH_3 molecules of **1**, decreases in intensity relative to the signal at 1.8 ppm (the γ-methylenes of the cyclobutane ring of **1**), Fig. 4. All 1D ^{1}H spectra were recorded at 25 °C on a Bruker Avance 500 MHz NMR equipped with a 5 mm inverse detection gradient probe. The pulse sequence used was Watergate water suppression with a sweep width of 12 ppm, relaxation delay of 10 s, and a delay for binomial water suppression of 200 μs. Sixteen scans were acquired for each concentration, and each spectrum was baseline corrected from 4.5 to 1.5 ppm. The integration module of Bruker's Topspin program was used to determine the relative intensities of the peaks at 4.17 ppm; the intensity of the peak at 1.8 ppm was calibrated to one for each concentration. In order to determine the fraction by which the NH_3 peak intensity is suppressed due to the pulse sequence, the excitation profile for the Watergate water suppression pulse

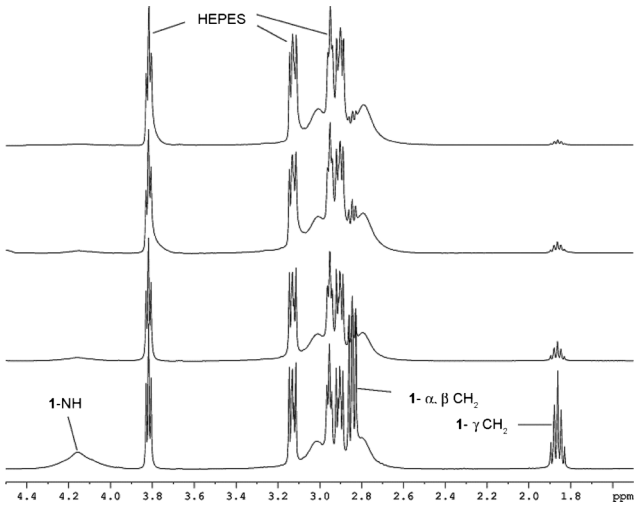

Fig. 4 ^{1}H NMR spectra of 27 mM, 5 mM, 2.5 mM and 1 mM (from bottom to top) carboplatin, **1**, in 25 mM HEPES buffer, pH 7.4, H_2O/D_2O (90/10). The proton resonances of HEPES occur in the range 2.6–3.8 ppm.

sequence was mapped. At ~280 Hz to the high field side of the transmitter offset (the location of the NH_3 resonance), a resonance is suppressed by 0.5. Thus, actual intensities for the NH_3 resonance were obtained by multiplying the observed peak intensities for the resonance by two. The estimated error in the measured intensities was determined to be ±10%.

The intensities of the peaks at 4.17 ppm (relative to the γ-methylene resonance) in 27 mM, 5 mM, 2.5 mM, and 1 mM carboplatin solutions are 2.44 (with and without HEPES buffer, data for the latter not shown), 1.88, 1.46 and 1.32, respectively, showing that a decrease in concentration of **1** causes a decrease in the intensity of the resonance at 4.17 ppm relative to the methylene peak. Observation of the ammonia NH resonances of **1** in water shows that these protons are in slow exchange on the NMR time scale, which is most likely due an association complex that blocks or slows the exchange of the protons with solvent.

The number of carboplatin molecules in the association complex, m, and the association equilibrium constant, K, can be estimated as follows: since the intensity of the 4.17 ppm peak, normalized to the 1.8 ppm peak, would be 3.0 if all the carboplatin were in the associated form (it is assumed that the NH's do not exchange with the small amount of deuterium present in the system), dividing the normalized intensity by 3.0 gives f, the fraction of the carboplatin that is in the complex. If the total carboplatin concentration is C_0, the concentration of the association complex is fC_0/m, and the concentration of unassociated monomer is $C_0 - fC_0$ since each complex contains m monomers. The association equilibrium is written $K = \frac{fC_0/m}{(C_0 - fC_0)^m}$

To determine m, we calculate K for each C_0, using m = 1, 2, 3 or 4, and determine which value of m yields the most constant value of K. The results are shown in Table 2.

For each value of m, we calculate the average and the standard deviation of the four values of K, and then obtain the relative standard deviation by dividing the standard deviation by the average. Constancy of K means small relative standard deviation. For m = 1, 2, 3 and 4, the relative standard deviations are 0.82, 0.32, 1.23, and 1.76. The clear choice is m = 2, suggesting that

Table 2 Calculations to determine *m*, number of carboplatin molecules in the association complex and *K*, equilibrium constant for complex formation

C_0 (mM)	Intensity[a]	f = Intensity[a]/3	K, $m = 1$	K, $m = 2$	K, $m = 3$	K, $m = 4$
27.0	2.44	0.814	4.38	436	5.78×10^4	8.64×10^6
5.0	1.88	0.626	1.67	448	1.60×10^5	6.40×10^7
2.5	1.46	0.486	0.95	368	1.91×10^5	1.11×10^8
1.0	1.32	0.440	0.79	702	8.35×10^5	1.12×10^9

[a] Intensity is the intensity of the NH_3 resonance of carboplatin relative to the γ-methylene resonances of the drug.

the association complex is a carboplatin dimer, consistent with the earlier work of Embree and coworkers.[14] The calculated value of the association constant K for the dimer is the average ± the standard deviation, *i.e.* K (M^{-1}) = 391 ± 127. With this value of K, the concentration of dimer in the 27 mM ready-to-use infusion solution is 11.0 mM, and more than 81% of the carboplatin is in the dimeric association complex.

The NH ^{1}H NMR signal at δ 4.17 ppm is the same resonance employed in 2D [^{1}H, ^{15}N] HSQC NMR studies involving a physiologically relevant concentration of carboplatin in carbonate buffer,[9] and in culture medium containing human cancer cells.[18] Since culture medium is similar in composition to blood, the dimeric form of **1** may also be present in the blood of patients receiving carboplatin in chemotherapy.

Earlier, we measured the rate of reaction of carboplatin in carbonate buffer by monitoring the intensity of the NH peak with [^{1}H,^{15}N] NMR and, in a separate NMR study, the intensity of the α,β-methylenes of the cyclobutane ring with 1D ^{1}H NMR.[9] Since the intensity of the NH resonance in the HSQC NMR spectrum is from only the associated form and the intensity of the methylene proton resonance is from all of the forms, obtaining the same reaction rates from these different NMR experiments implies that concentration of the dimer is small relative to that of the monomer in the [^{1}H, ^{15}N] HSQC NMR experiment, *i.e.*, when the concentration of the dimer is negligibly small, intensity changes with time for both NMR experiments must be the same.

Formulating stable ready-to-use infusion solutions of the platinum anticancer drugs is a challenging task. Since these compounds exhibit their cytotoxic effects by losing coordinated ligands and reacting with DNA and protein targets in the cell, they are prone to reaction with nucleophiles, especially water, present in commercial formulations of the drugs. In the case of carboplatin and oxaliplatin, it was found that high concentrations of the drugs in water prevented hydrolysis thus allowing ready-to-use solutions of these agents to be made with "shelf lives" that were acceptable for commercial application. In the case of cisplatin, the integrity of the compound in ready-to-use infusion solutions is maintained by the co-addition of sodium chloride to give a salt concentration of 154 mM (normal saline) which prevents the dichloro form from converting to more toxic aqua species through Le Châtelier's principle. Interestingly, the crystal structures of the alpha and beta forms of cisplatin reveal elaborate three-dimensional hydrogen-bonded networks.[19] Intermolecular N–H–Cl hydrogen bonding in the solid state and the observation of [^{1}H, ^{15}N] HSQC NMR spectra for this drug under a variety of solution conditions, including culture media, suggest that self-association of cisplatin may be possible as well.[19,20]

From the data and arguments given above, we show that carboplatin exists in a monomer–dimer equilibrium and suggest that oxaliplatin is also self-associated in concentrated aqueous solution. While the nature of the intermolecular interaction is different, drug–drug self-association in water has been found for the anticancer drug doxorubicin,[21] and the serotonin $5HT_3$ receptor antagonist dolasetron, which is used to treat nausea and vomiting that sometimes follow chemotherapy.[22] Since self-association processes are inherently concentration dependent, a decrease in concentration, as would occur when drug is diluted prior to its administration to the patient, shifts equilibria in favor of the monomeric form. In fact, the packaging insert for Paraplatin states that when the formulation is diluted in either 5% dextrose in water or 0.9% NaCl, carboplatin is stable for only 8 h at rt.[23] While dilution would be expected to drive the equilibrium toward the monomeric form, association complexes of carboplatin, oxaliplatin and perhaps also cisplatin could be circulating in blood during chemotherapy, which would have far reaching implications for the transport, uptake and possibly the molecular mechanism of action of these platinum anticancer drugs.

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