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Formation of Carbonato and Hydroxo Complexes in the Reaction of Platinum Anticancer Drugs with Carbonate

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The second-generation Pt^{II} anticancer drug carboplatin is here shown to react with carbonate, which is present in blood, interstitial fluid, cytosol, and culture medium, to produce platinum−carbonato and −hydroxo complexes. Using [^{1}H−^{15}N] HSQC NMR and ^{15}N-labeled carboplatin, we observe that *cis*-[Pt(CBDCA-*O*)(OH)$(NH_3)_2]^-$, *cis*-[$Pt(OH)_2(NH_3)_2$], *cis*-[$Pt(CO_3)(OH)(NH_3)_2]^-$, and what may be *cis*-[$Pt(CO_3)(NH_3)_2$] are produced when **1** is allowed to react in 23.8 mM carbonate buffer. When ^{15}N-labeled carboplatin is allowed to react in 0.5 M carbonate buffer, these platinum species, as well as other hydroxo and carbonato species, some of which may be dinuclear complexes, are produced. Furthermore, we show that the carbonato species *cis*-$[Pt(CO_3)(OH)(NH_3)_2]^-$ is also produced when cisplatin is allowed to react in carbonate buffer. The study outlines the conditions under which carboplatin and cisplatin form carbonato and aqua/hydroxo species in carbonate media.

Introduction

Platinum drugs are believed to exert their biological effects by interacting with genomic DNA and other cellular targets.[1,2] Carboplatin, *cis*-diammine(cyclobutane-1,1-dicarboxylato)platinum(II), **1** in Figure 1, is a second-generation Pt^{II} anticancer drug that was developed by Barnett Rosenberg and colleagues to improve upon the clinical performance of cisplatin, *cis*-diamminedichloroplatinum(II), **2** in Figure 1.[1,3] Carboplatin, which is less oto-, neuro-, and nephrotoxic than cisplatin,[1,4,5] contains a bidentate dicarboxylate chelate-leaving ligand, making it much less reactive than cisplatin,[6] which contains two monodentate chloride-leaving ligands.

Carbonate, CO_3^{2-}, which is in equilibrium with hydrogencarbonate, HCO_3^-; carbonic acid, H_2CO_3; and dissolved carbon dioxide, CO_2,[7] is found in high concentrations in the blood, interstitial fluid, and cytosol.[8,9] Extensive work by investigators has shown that carbonato complexes can form via the rapid addition of dissolved carbon dioxide to a metal hydroxo species (reaction (a) of Figure 2);[10−14] second-order rate constants of $k = 37-590\ M^{-1}\ s^{-1}$ at 25 °C have been reported for this reaction with cobalt, chromium, iridium, rhodium, and zinc complexes.[10] The important feature of this reaction is that it does not involve a metal−ligand bond-breaking step, so addition rates are largely governed by the nucleophilicity of the hydroxo ligand and other factors. Whereas the immediate product of this reaction is a hydro-

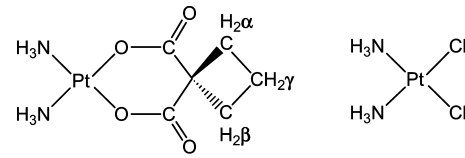

Figure 1. Structures of carboplatin **1** and cisplatin **2**.

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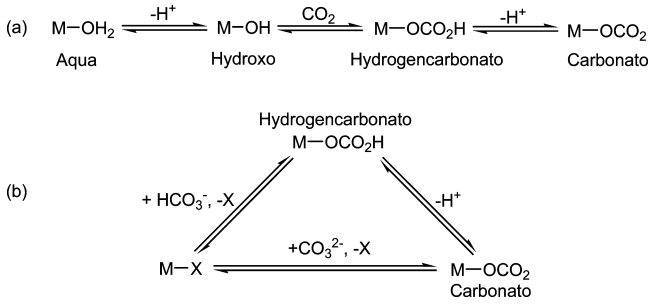

Figure 2. Mechanism of formation of transition-metal carbonato complexes. (a) Carbon dioxide addition to a metal hydroxo species and (b) nucleophilic attack by carbonate/hydrogencarbonate ion.

gencarbonato complex, depending on the pK_a for deprotonation of the hydrogencarbonato complex and the pH of the reaction medium, the product could exist as either a hydrogencarbonato or carbonato species or an equilibrium mixture of both. Carbonato complexes can also be formed by the attack of carbonate or hydrogencarbonate at the metal center in a ligand-displacement reaction (reaction (b) of Figure 2).[10−15] This reaction requires metal−ligand bonds to be broken and re-formed, and, thus, reaction rates are strongly influenced by the substitution kinetics of the metal ion involved.[16] The literature shows that, although some hydrogencarbonato complexes are stable and have been characterized by X-ray analysis,[17] certain hydrogencarbonato complexes of cobalt, iridium, and rhodium[10,15] are unstable and loose CO_2 with rate constants of 0.25−4.40 s^{-1} at 25 °C.

Since carbonate has three oxygen atoms that can serve as donor atoms to a metal ion, this ligand exhibits rich coordination chemistry. For example, depending on the nature of the ligand cis to a monodentate carbonato group, bound carbonate can undergo intramolecular ring closure to produce a bidentate carbonato species.[10] Also, if the concentration of the metal ion is relatively high, the polydentate nature of the carbonate ligand allows for the formation of multinuclear complexes having metal ions bonded to the carbonate oxygen atoms.

The major platinum drugs, cisplatin and carboplatin, are most commonly introduced into the blood by intravenous injection. Since blood contains ∼25 mM carbonate,[9] the possibility exists that these drugs could react with carbonate in vivo to produce complexes that circulate in the blood and, in part, give rise to the antitumor effects of these compounds in therapy. The effect of carbonate on carboplatin may especially be important because the rates of reaction of the drug with other substances, for example, chloride and phosphate, are slower than with carbonate.[18] We have already shown that carbonate displaces the CBDCA ligand of **1**, producing species that are more cytotoxic than intact carboplatin in vitro.[18,19]

In an attempt to uncover the possible role of carbonate in the mechanism of action of cisplatin and carboplatin, we have studied the reactivity of these drugs in carbonate buffer and in cell culture media, and we and others examined the effect of carbonate on the binding of the drugs to DNA.[18−25] Here we use [^{1}H−^{15}N] HSQC NMR and ^{15}N-labeled carboplatin and cisplatin to more extensively investigate the reaction of these drugs in carbonate buffer media, finding that some of our initial assignments concerning the products formed require revision.

Experimental Section

Materials. K_2PtCl_4 (99.99%), KI (ACS Reagent), $^{15}NH_4Cl$ (98+% ^{15}N), KOH, $AgNO_3$ (99+%), and 1,1-cyclobutanedicarboxylic acid (H_2CBDCA) (99%), which were used to prepare ^{15}N-labeled carboplatin,[18] were purchased from Sigma-Aldrich (St. Louis, MO). $NaHCO_3$ (>99%) and D_2O (99.96+% D) were also purchased from Sigma-Aldrich (St. Louis, MO).

[^{1}H−^{15}N] HSQC NMR Spectroscopy. Spectra were recorded on a Bruker Avance 500 MHz NMR equipped with a 5 mm triple-axis probe. Peak volumes in arbitrary units for all peaks of appreciable intensity, excluding ^{195}Pt satellites, were calculated using Bruker software. The ^{15}N chemical shifts were referenced externally to 1 M $(^{15}NH_4)_2SO_4$ in 95:5 H_2O/D_2O, which was acidified to pH ∼1 by the addition of H_2SO_4, and the ^{1}H chemical shifts were referenced externally to TSP in a pH 7.15, 23 mM sodium carbonate buffer. The appropriate volume of a freshly prepared stock solution of ^{15}N-labeled **1** in water was added to a solution to give a final concentration of 110 μM drug in 23.8 mM or 0.5 M $NaHCO_3$, pH 8.6, in a final volume of 920 μL, and HSQC NMR was collected (data acquisition ∼1 h). Although this pH is not the physiological value, rate and spectra data under other conditions through the physiological range can be found in an earlier publication.[18] To determine speciation, freshly prepared 5 mM ^{15}N-labeled carboplatin was allowed to react in 23.8 mM or 0.5 M carbonate buffer, pH 8.4 or 8.6, respectively, at 37 °C for 20 or 45 h. After this time, 46 μL of D_2O was added to 874 μL of each sample, and HSQC NMR spectra were obtained (acquisition time per spectrum ∼0.39 h). Also, 184 μL of a solution containing 5 mM ^{15}N-labeled carboplatin aged in 0.5 M carbonate buffer, pH 8.6, at 37 °C for 20 h was added to a solution containing 690 μL of water and 46 μL of D_2O; the final pH was ∼8.5, and an HSQC NMR spectrum was obtained. The reaction of 5 mM, freshly prepared ^{15}N-labeled carboplatin in 23.8 mM or 0.5 M carbonate buffer, pH 8.4 or 8.6, respectively, at 37 °C was also followed using HSQC NMR over ∼45 h. Successive NMR measurements (data acquisition ∼0.39 h) were recorded in 5% D_2O at 37 °C. To determine speciation in a solution containing cisplatin in carbonate, [^{1}H−^{15}N] HSQC NMR spectra of a solution

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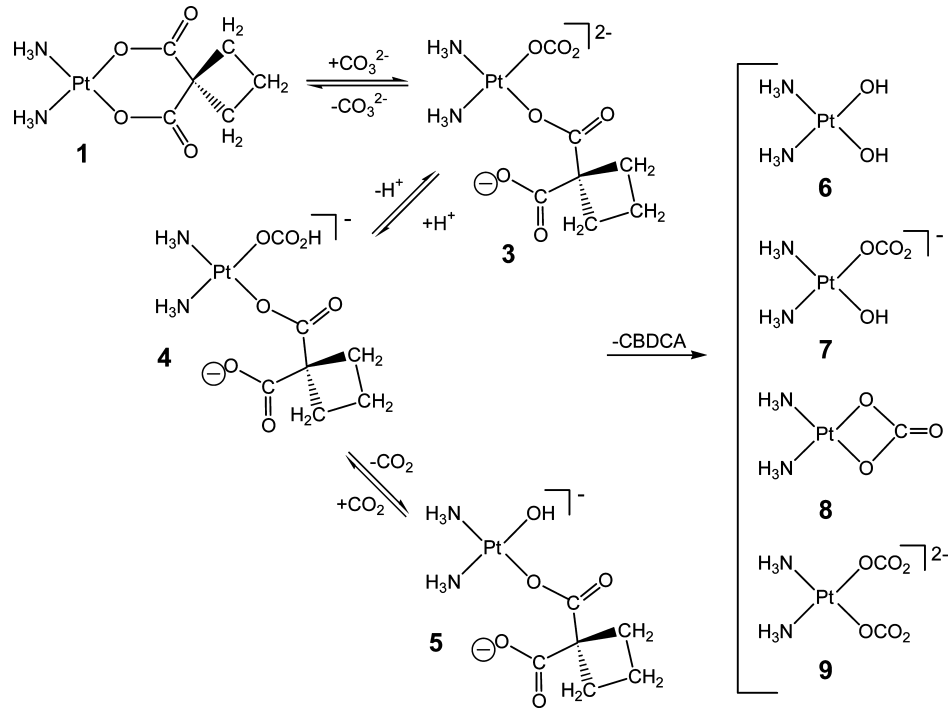

Figure 3. Scheme showing the reaction of carboplatin in carbonate buffer.

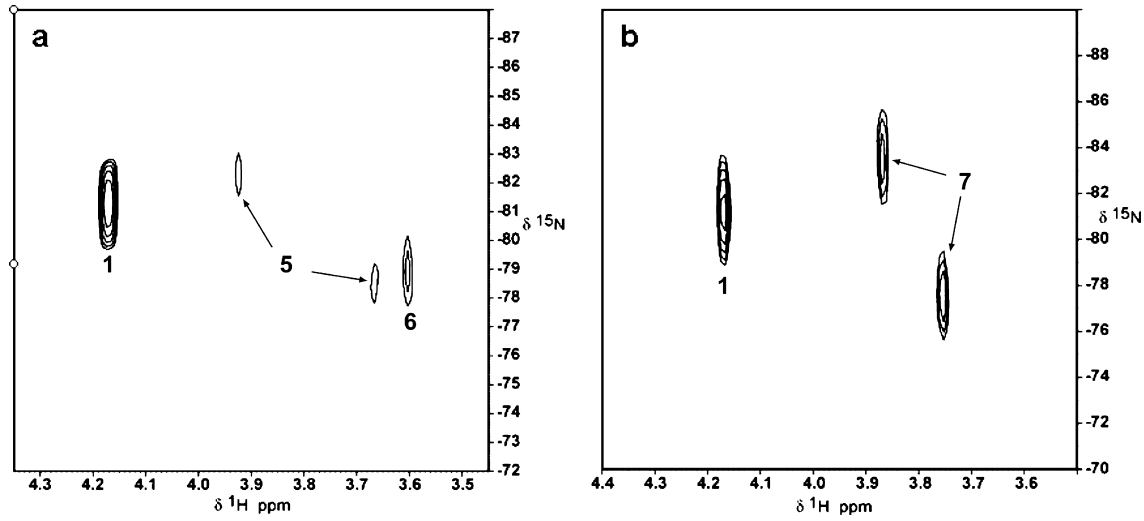

Figure 4. [^{1}H−^{15}N] HSQC NMR spectra of 110 μM (at $t = 0$) ^{15}N-labeled carboplatin in (a) 23.8 mM, pH 8.6, at 37 °C, obtained at $t = 12.6$ h and in (b) 0.5 M carbonate, pH 8.6, at 37 °C, obtained at $t = 10$ h.

containing 2 mM ^{15}N-labeled cisplatin in 5 mM carbonate and 1 mM NaCl, pH ∼7.4, at 37 °C at various times, up to 72 h, were obtained. Due to the poor buffering capacity of the solution and the formation of products, the pH of the solution slowly becomes slightly acidic with time, and the pH after 72 h is ∼6.8.

Results and Discussion

The reaction of 110 μM ^{15}N-labeled **1** in 23.8 mM carbonate buffer results in a decrease in the integrated intensity of the peak for **1** at ^{1}H/^{15}N $\delta = 4.17/-81.3$ and the appearance of new cross-peaks with ^{15}N chemical shifts consistent with N trans to O.[26] We, initially, observed the appearance of two new signals that were assigned to the ring-opened complex *cis*-[Pt(CO$_3$)(CBDCA-*O*)(NH$_3$)$_2$]$^{2-}$ (**3**).[18] However, after further investigation, we determined that three new peaks arise during the reaction of clinically relevant concentrations of carboplatin with carbonate over a 20 h time period. These signals have been assigned to *cis*-[Pt(OH)(CBDCA-*O*)(NH$_3$)$_2$]$^-$ (**5**) and *cis*-[Pt(OH)$_2$(NH$_3$)$_2$] (**6**) (Figures 3 and 4, panel (a)). By comparison with the ^{15}N chemical shift parameters of *cis*-[PtCl(CBDCA-*O*)(NH$_3$)$_2$]$^-$,[27] an acetato complex,[28] and a carboplatin acid hydrolysis product,[18] the peak at ^{1}H/^{15}N $\delta = 3.92/-82.3$ is N trans to O(CBDCA) of *cis*-[Pt(OH)(CBDCA-*O*)(NH$_3$)$_2$]$^-$ (**5**),

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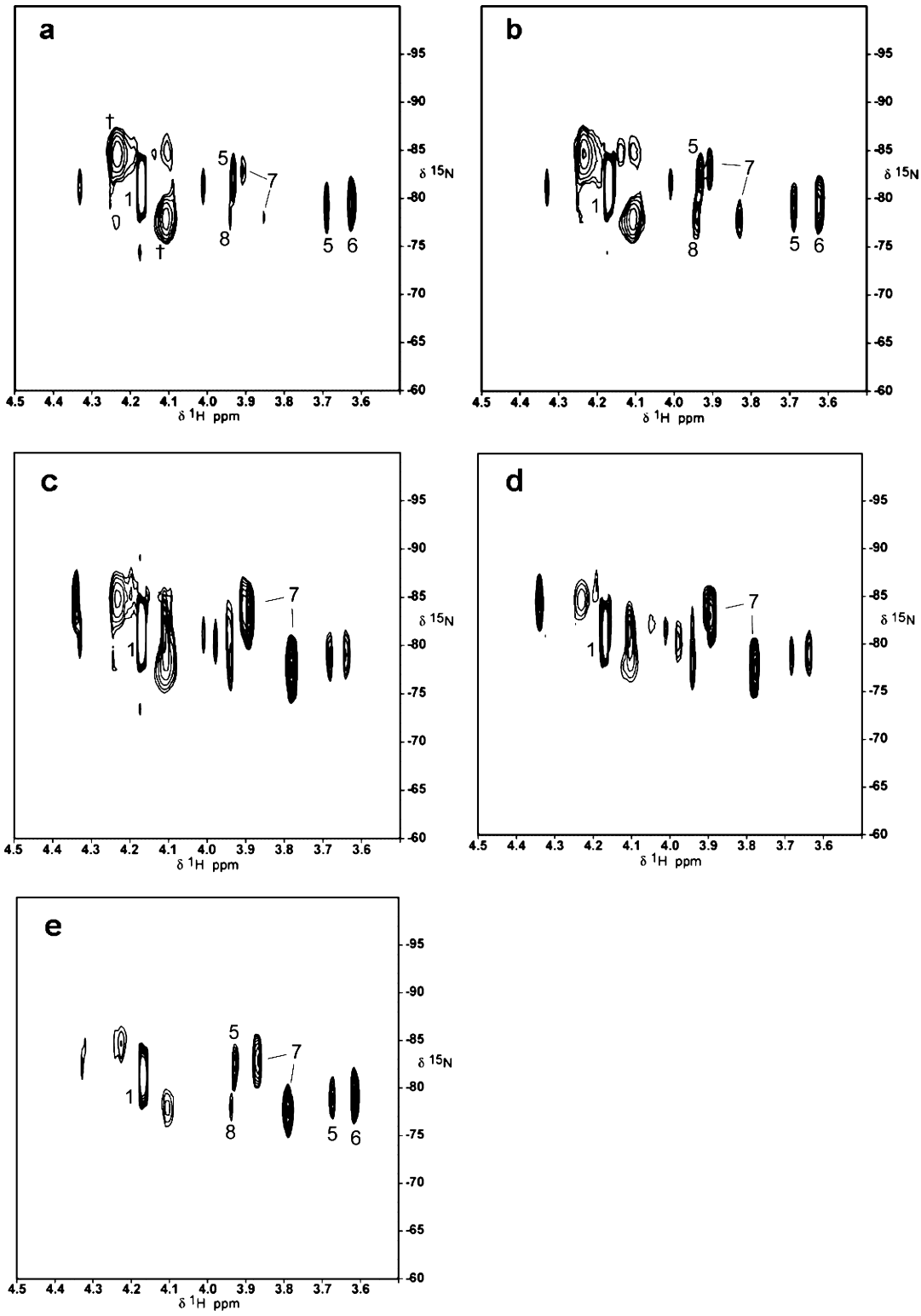

Figure 5. [^{1}H−^{15}N] HSQC NMR spectra of solutions containing 5 mM ^{15}N-labeled carboplatin aged in 23.8 mM or 0.5 M carbonate. Spectra of ^{15}N-labeled carboplatin aged in 23.8 mM, pH 8.4, at 37 °C for (a) 20 h or (b) 45 h or in 0.5 M carbonate, pH 8.6, at 37 °C for (c) 20 h or (d) 45 h are shown. (e) Spectrum of a solution containing ^{15}N-labeled carboplatin aged in 0.5 M carbonate, pH 8.6, at 37 °C for 20 h, after a 1:5 dilution in water. Surrounding the peak for carboplatin **1** in panel (a) are two peaks labeled with daggers (†) due to coupling with ^{195}Pt and a series of other minor peaks that appear to be artifacts of the HSQC NMR measurement (see text). Spectra were obtained after the addition of D_2O (5% final volume).

whereas the peak at ^{1}H/^{15}N $\delta = 3.67/-78.5$ is assigned to N trans to O(OH$^-$) of **5**. The peak at ^{1}H/^{15}N $\delta = 3.60/-78.9$ has been assigned to N trans to O(OH$^-$) of *cis*-[Pt(OH)$_2$(NH$_3$)$_2$] (**6**), which, at pH 8.6, is consistent with what has been reported in the literature for this complex.[28]

By measuring the rate of disappearance of the HSQC NMR peak for **1**, the pseudo-first-order rate constant for the disappearance of ^{15}N-labeled carboplatin in 0.5 M carbonate buffer, pH 8.6, at 37 °C is $k_1 = (4.95 \pm 0.93) \times 10^{-6}$ s^{-1}, which is consistent with that obtained using ^{1}H NMR.[19] The

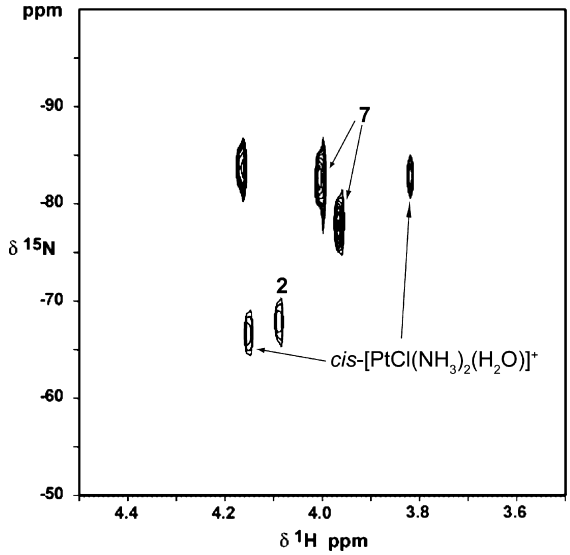

Figure 6. [^{1}H−^{15}N] HSQC NMR spectrum of a solution containing 2 mM ^{15}N-labeled cisplatin in 5 mM carbonate and 1 mM NaCl after 72 h (pH ~7.4 at $t = 0$, the beginning of the NMR data collection time).

Table 1. ^{1}H and ^{15}N Chemical Shifts of the Ammine Ligands in *cis*-Diammine Pt^{II} Complexes Produced in the Reaction of 5 mM Carboplatin in 23.8 mM Carbonate

complex	δ (^{1}H)	δ (^{15}N)	trans ligand
cis-[Pt(CBDCA-*O*,*O*′) $(NH_3)_2$] (**1**)	4.17	−81.3	(O)CBDCA
cis-[Pt(CBDCA-*O*)(OH)$(NH_3)_2]^-$ (**5**)	3.93	−82.5	(O)CBDCA
	3.69	−79.2	(O)OH
cis-[Pt$(OH)_2(NH_3)_2$] (**6**)	3.62	−79.4	(O)OH
cis-[Pt(CO_3)(OH)$(NH_3)_2]^-$ (**7**)	3.91	−83.2	(O)CO_3
	3.83	−78.0	(O)OH
[Pt$(CO_3)(NH_3)_2$] (**8**)	3.94	−78.2	(O)CO_3

reaction of ^{15}N-labeled **1** in 0.5 M carbonate buffer, pH 8.6, at 37 °C results in a decrease in the intensity of the peak for **1** and the appearance of new peaks at ^{1}H/^{15}N $\delta = 3.87/-83.7$ and 3.75/−77.6 (Figure 4, panel (b)). These peaks that appear to increase at approximately the same rate and have ^{15}N chemical shifts consistent with N trans to O[26] are assigned to *cis*-[Pt(CO_3)(OH)$(NH_3)_2]^-$ (**7**). The signal at ^{1}H/^{15}N $\delta =$ 3.87/−83.7 is assigned to N trans to O(CO_3^{2-}) of **7**, whereas that at 3.75/−77.6 is assigned to N trans to O(OH^-) of **7**. The latter assignment is based on the assignment of N trans to O(OH^-) of **5**. The cross-peaks produced when a physiologically relevant concentration of **1** is allowed to react in 23.8 mM or 0.5 M carbonate buffer are assigned to either hydroxo or carbonato species, on the basis of observations made using 5 mM **1** and [^{1}H−^{15}N] HSQC (see below) and ^{13}C NMR.[19,29]

To observe as many signals as possible in the HSQC NMR experiments, the concentration of **1** was increased to 5 mM and was aged in 23.8 mM or 0.5 M carbonate buffer, pH 8.4 or 8.6, respectively, at 37 °C. These spectra are shown in Figure 5, and peak assignments for the 23.8 mM carbonate system are listed in Table 1. The signals at ^{1}H/^{15}N $\delta = 3.93/-82.5$ and 3.69/−79.2 are assigned to *cis*-[Pt(CBDCA-*O*)(OH)$(NH_3)_2]^-$ (**5**) (Figure 3). Since we previously showed that the attacking nucleophile in the reaction of **1** in carbonate buffer is carbonate ion, CO_3^{2-}, by measuring pseudo-first-order rate constants,[18] the initially formed product in this reaction must be *cis*-[Pt(CO_3)(CBDCA-*O*)$(NH_3)_2]^{2-}$ (**3**). However, since the ^{13}C NMR data do not support the presence of this species at this point in the reaction[29] and the HSQC NMR show the presence of a product that has OH^- trans to NH_3, the compound in solution has been assigned to *cis*-[Pt(CBDCA-*O*)(OH)$(NH_3)_2]^-$ (**5**). In view of the documented instability of hydrogencarbonato complexes,[10−15] **5** probably forms through the protonation of **3** to form **4** that loses CO_2 to produce **5** (Figure 3). It is also possible that the hydrogencarbonato ligand is displaced by a hydroxide ion, but since this would involve a metal−ligand bond-breaking step that is expected to be slow and there is no evidence for a carbonato species at this point in the reaction from ^{13}C NMR, the mechanism involving loss of CO_2 is favored. The monohydroxo complex *cis*-[Pt(CBDCA-*O*)(OH)$(NH_3)_2]^-$ (**5**) is also produced in the reaction of **1** with OH^-;[29,30] however, this reaction appears to be less important than the reaction of carboplatin with carbonate when using physiologically relevant concentrations of **1**, based on k_1 values obtained using [^{1}H−^{15}N] HSQC NMR.[18,29] This Pt ring-opened species then further reacts to give *cis*-[Pt$(OH)_2(NH_3)_2$] (**6**), which has a strong peak at ^{1}H/^{15}N $\delta = 3.62/-79.4$. The carbonato species *cis*-[Pt(CO_3)(OH)$(NH_3)_2]^-$ (**7**), with signals at ^{1}H/^{15}N $\delta = 3.91/-83.2$ and 3.83/−78.0, and what appears to be *cis*-[Pt$(CO_3)(NH_3)_2$] (**8**), with a signal at 3.94/−78.2, also form in this reaction. The signals at ^{1}H/^{15}N $\delta = 4.33/-81.2$ and 4.01/−81.5 are artifacts of decoupling in the NMR experiment.[31,32] All other peaks, excluding the ^{195}Pt satellites, appear to be artifacts of the [^{1}H−^{15}N] HSQC NMR experiment and have been observed in earlier experiments of this type.[31−35] The observed rate constant for the reaction of 5 mM ^{15}N-labeled **1** in 23.8 mM carbonate buffer, pH 8.4, at 37 °C is $k_1 = (0.46 \pm 0.36) \times 10^{-6}$ s^{-1}, which is smaller than that observed for the reaction of 110 μM **1** in 23.8 mM carbonate.[18] This may be due to the formation of dimers or higher-order oligomers in solution when the concentration of **1** is high. Oligomerization of carboplatin, which was observed by Liu et al.,[36] could produce structures that may block the attack of carbonate ion, leading to low ring-opening rates for the drug in 23.8 mM carbonate.

[^{1}H−^{15}N] HSQC NMR spectra of solutions containing 5 mM ^{15}N-labeled **1** aged in 0.5 M carbonate buffer, pH 8.6, at 37 °C are shown in Figure 5, panels (c) and (d). The spectra shown here are slightly more complicated than those obtained for the reaction of 5 mM ^{15}N-labeled **1** in 23.8 mM carbonate. Signals that are similar in position to those that

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arise when ^{15}N-labeled **1** is allowed to react in 23.8 mM carbonate buffer, as well as other cross-peaks with ^{15}N chemical shifts consistent with N trans to O,[35] appear during this reaction. From ^{13}C NMR spectroscopy,[19] three platinum−carbonato species are produced in this reaction. Thus, one of these ^{1}H/^{15}N cross-peaks may be due to the dicarbonato species *cis*-$[Pt(CO_3)_2(NH_3)_2]^{2-}$ (**9**). Also, it is possible to form dinuclear species that have ^{15}N chemical shifts similar in position to the signals shown here.[35] A 1:5 dilution in water of a solution containing 5 mM ^{15}N-labeled **1** aged in 0.5 M carbonate for 20 h is shown in Figure 5, panel (e). After dilution, the signal associated with the dihydroxo species appears to increase in relative intensity. Since dilution would shift the equilibrium in favor of aqua/hydroxo species, this behavior is expected.

The NMR peaks associated with *cis*-$[Pt(CO_3)(OH)(NH_3)_2]^-$ (**7**) also arise when cisplatin is allowed to react with carbonate (Figure 6). The signal for cisplatin is located at ^{1}H/^{15}N δ = 4.09/−68.0, and those at 4.15/−67.0 and 3.82/−83.0 are assigned to the monochloro/monohydroxo species at pH ∼6.8.[28] These were previously assigned to a carbonato complex; however, based on ^{13}C NMR,[19,21] this is not the case. The signals at ^{1}H/^{15}N δ = 4.01/−83.0 and 3.77/−77.0 are assigned to *cis*-$[Pt(CO_3)(OH)(NH_3)_2]^-$ (**7**), whereas that at 4.17/−84.0 is most likely a dinuclear species with no coordinated carbonate, based on ^{13}C NMR.[19,21] Additional spectra can be found in the Supporting Information.

Earlier work on the reaction of cisplatin in carbonate buffer[21] produced a product having a ^{13}C NMR resonance at 167.0 ppm that was initially assigned to *cis*-$[Pt(CO_3)_2(NH_3)_2]^{2-}$ (**9**). It has been determined that this assignment is incorrect and that it is more reasonable to assign this ^{13}C NMR resonance, on the basis of [^{1}H−^{15}N] HSQC NMR data for both cisplatin and carboplatin, to *cis*-$[Pt(CO_3)(OH)(NH_3)_2]^-$ (**7**). The reaction of **1** in carbonate yields a complex with a chemical shift at 166.9 ppm,[19] which is similar in position to that obtained when cisplatin is allowed to react in carbonate;[21] thus, this peak is attributed to *cis*-$[Pt(CO_3)(OH)(NH_3)_2]^-$ (**7**). The resonance at 169.2 ppm in the ^{13}C NMR spectrum of carboplatin aged in 23.8 mM carbonate[19] is associated with the second carbonato species that forms in solution, which may be *cis*-$[Pt(CO_3)(NH_3)_2]$ (**8**).

Conclusions

Using [^{1}H−^{15}N] HSQC NMR and ^{15}N-labeled platinum anticancer drugs, we observe that *cis*-$[Pt(CO_3)(OH)(NH_3)_2]^-$ (**7**) is produced when either carboplatin or cisplatin reacts in carbonate buffer. When carboplatin reacts in 23.8 mM carbonate buffer, *cis*-$[Pt(CBDCA\text{-}O)(OH)(NH_3)_2]^-$ (**5**), *cis*-$[Pt(OH)_2(NH_3)_2]$ (**6**), *cis*-$[Pt(CO_3)(OH)(NH_3)_2]^-$ (**7**), and what may be *cis*-$[Pt(CO_3)(NH_3)_2]$ (**8**) are produced. When ^{15}N-labeled carboplatin is allowed to react in 0.5 M carbonate buffer, these platinum species, as well as other hydroxo and carbonato species, some of which may be dinuclear complexes, are formed in the reaction. The study outlines the conditions under which carboplatin and cisplatin form carbonato and aqua/hydroxo species in carbonate media.

Supporting Information Available: Additional figure and references. This material is available free of charge via the Internet at http://pubs.acs.org..

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