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Activation of Carboplatin by Carbonate

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Carboplatin, [Pt(NH$_3$)$_2$(CBDCA-*O*,*O*′)], **1**, where CBDCA is cyclobutane-1,1-dicarboxylate, is in wide clinical use for the treatment of ovarian, lung, and other types of cancer. Because carboplatin is relatively unreactive toward nucleophiles, an important question concerning the drug is the mechanism by which it is activated in vivo. Using [^{1}H,^{15}N] heteronuclear single quantum coherance spectroscopy (HSQC) NMR and ^{15}N-labeled carboplatin, we show that carboplatin reacts with carbonate ion in carbonate buffer to produce ring-opened products, the nature of which depends on the pH of the medium. The assignment of HSQC NMR resonances was facilitated by studying the reaction of carboplatin in strong acid, which also produces a ring-opened product. The HSQC NMR spectra and UV-visible difference spectra show that reaction of carboplatin with carbonate at pH > 8.6 produces mainly *cis*-[Pt(NH$_3$)$_2$(CO$_3^{-2}$)(CBDCA-*O*)]$^{-2}$, **5**, which contains the mono-dentate CBDCA ligand and mono-dentate carbonate. At pH 6.7, the primary product is the corresponding bicarbonato complex, which may be in equilibrium with its decarboxylated hydroxo analogue. The UV-visible absorption data indicate that the pK_b for the protonation of **5** is ∼8.6. Thus, the reaction of carboplatin with carbonate produces a mixture of ring-opened species that are anions at physiological pH. HSQC NMR studies on ^{15}N-labeled carboplatin in RPMI culture media containing 10% fetal bovine serum with and without added carbonate suggest that carbonate is the attacking nucleophile in culture media. However, because the rate of reaction of carbonate with carboplatin at physiological pH is small, NMR peaks for ring-opened carboplatin were not detected with HSQC NMR. The rate of disappearance of carboplatin in culture medium containing 9×10^8 Jurkat cells is essentially the same as that in carbonate buffer, indicating that the ring-opening reaction is not affected by the presence of cells. This work shows that carbonate at concentrations found in culture media, blood, and the cytosol readily displaces one arm of the CBDCA ligand of carboplatin to give a ring-opened product, which at physiological pH is a mixture of anions. These ring-opened species may be important in the uptake, antitumor properties, and toxicity of carboplatin.

Introduction

Carboplatin, [Pt(NH$_3$)$_2$(CBDCA-*O*,*O*′)][1] (**1** in Figure 1), where CBDCA is cyclobutane-1,1-dicarboxylate, is a second-generation Pt^{2+} anticancer drug, which is less oto- and nephrotoxic than *cis*-diamminedichloroplatinum(II) (cisplatin), **2** (*1*, *2*). Both cisplatin and carboplatin have cis ammonia ligands, but in the latter, the two chloride ligands of cisplatin are replaced by a dicarboxylate chelate ring (Figure 1). Because a bidentate chelate ring is difficult to displace in substitution reactions (*3*), carboplatin is much less reactive than cisplatin. Studies show that the rate constant for the reaction of carboplatin with water is very small, $k_1 < 10^{-8}$ s^{-1} (*4*), which likely precludes hydrolysis as a means of activating the drug in vivo.

In exploring ways to displace the chelate ring of carboplatin, Sheldrick and co-workers (*5*) used HPLC, NMR, and capillary electrophoresis−mass spectrometry to show that the thioether group in a tripeptide can displace the bidentate dicarboxylate of carboplatin. The introduction of the thioether into the coordination sphere of the platinum (Pt) ion labilized the ammonia molecule trans to the thioether, which ultimately led to the replacement of all of the ligands of carboplatin with donors from the peptide. Sadler and co-workers (*6*) showed that the reaction of carboplatin with methionine is rapid ($k = 2.7 \times 10^{-3}$ M^{-1} s^{-1}) and that it produces a stable ring-opened species, *cis*-[Pt(CBDCA-*O*)(NH$_3$)$_2$(L-HMet-*S*)], which has a long half-life. The authors suggested that this reaction may play a role in the biological activity of carboplatin. While a thioether group can readily displace the bidentate chelate ring of carboplatin, the resulting Pt−methionine adduct is thermodynamically stable making transfer of the Pt to a target via breakage of the Pt−S bond difficult (*3*). For example, a *cis*-(NH$_3$)$_2$Pt(II) moiety, which is bound to the terminal Met1 of ubiquitin, cannot be transferred

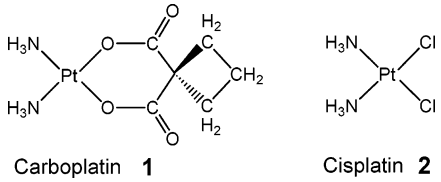

Figure 1. Structures of carboplatin and cisplatin.

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[1] Abbreviations: carboplatin, *cis*-diammine(cyclobutane-1,1-dicarboxylato)platinum(II) Pt(NH$_3$)$_2$(CBDCA-*O*,*O*′); CBDCA, cyclobutane-1,1-dicarboxylate; H$_2$CBCDA, cyclobutane-1,1-dicarboxylic acid; cisplatin, *cis*-diamminedichloroplatinum(II); dH$_2$O, distilled deionized water; D$_2$O, deuterium oxide; FBS, fetal bovine serum; Pt, platinum; HSQC, heteronuclear single quantum coherance spectroscopy.

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to a potential DNA target, 5′-GMP (*7*). However, for certain structures containing both a thioether group and a DNA base, the initially formed Pt−S adduct can rearrange to the Pt−base adduct, suggesting that reaction of carboplatin with a thioether may be a means of activating the drug in vivo (*8*).

In an effort to show that carboplatin may not require activation, Sadler and co-workers (*9*) used NMR to measure the rate of reaction of the drug with 5′-GMP. They found that carboplatin slowly reacts with the nucleotide, $t_{1/2} \sim 46$ h, to produce *cis*-[Pt(NH_3)$_2$(CBDCA-*O*)(5′-GMP)], suggesting that the drug could directly attack DNA in vivo.

Carbonate is an important ligand for the uptake and toxicity of Cd^{2+}, Pb^{2+}, and Ti^{4+} (*10*−*12*), and it may play a role in the biological activity of Pt antitumor agents. Carbonate, which is in equilibrium with bicarbonate, carbonic acid, and dissolved carbon dioxide (*13*), is ubiquitous in biological systems, and it is found in high concentration (∼24 mM) in blood (*14*). Metal complexes having bound carbonate (carbonato complexes) can be made by ligand displacement reactions involving carbonate ion, or they can be formed by the rapid addition of carbon dioxide to a metal-bound hydroxide ligand (*15*, *16*).

The potential of carbonate as an activating ligand for Pt was explored by Chaney and co-workers (*17*) who demonstrated that a six-membered malonate chelate ring bound to Pt^{2+} could be readily displaced in carbonate buffer. Because the resulting carbonato complex was unstable, the authors suggested that bicarbonate/carbonate ion may be a nonenzymatic pathway for the formation of biologically active Pt complexes.

Earlier, we used [^{1}H,^{15}N] heteronuclear single quantum coherance spectroscopy (HSQC) NMR and ^{15}N-labeled cisplatin to show that a metabolite of the drug that forms in RPMI culture medium is rapidly taken up and/or modified by Jurkat leukemia cells (*18*). Further investigation by us using one-dimensional and two-dimensional NMR and UV-vis absorption spectroscopy revealed that the metabolite is a Pt carbonato complex (*19*), which is formed when cisplatin aquates in a medium containing carbonate.

In this report, we use ^{15}N-labeled carboplatin, [^{1}H,^{15}N] HSQC NMR, and UV-vis spectroscopy to show that carbonate ion in carbonate buffer readily displaces the CBDCA chelate ring of carboplatin to give a complex having mono-dentate carbonate and the mono-dentate CBDCA ligand. The compound is characterized from its HSQC NMR chemical shift data and by comparison of the corresponding aquo complex produced in the acid hydrolysis of carboplatin. Studies done in the pH range from 6.7 to 9.3 show that the mono-carbonato compound is in a proton equilibrium with its bicarbonate form and possibly its decarboxylated hydroxo form. In all studies, therapeutically relevant carboplatin concentrations were used.

The ability of carbonate to react with carboplatin in culture media is examined by measuring the rate of disappearance of ^{15}N-labeled carboplatin using [^{1}H,^{15}N] HSQC NMR. Although the amount of ring-opened product produced in culture media is too small to be detected with HSQC NMR, the rate of disappearance of **1** is dependent on the presence of carbonate in the medium, suggesting that carbonate ion is the attacking nucleophile under these conditions as well. Studies done in the presence of Jurkat cells show that the rate of disappearance of **1** is not affected by cells in the medium. Because carboplatin reacts with carbonate in culture media, a carboplatin−carbonato complex may also be present in the blood of patients receiving the drug. To the extent that speciation in blood is important for biodistribution, carbonato complexes may play a role in the uptake, antitumor properties, and toxicity of carboplatin.

Materials and Methods

Materials. K_2PtCl_4, H_2CBDCA, and $^{15}NH_4Cl$ used in the synthesis of ^{15}N-labeled carboplatin were purchased from Sigma-Aldrich (St. Louis, MO). The $NaHCO_3$ (>99%) was from Sigma-Aldrich, and the fetal bovine serum (FBS) and RPMI-1640 with (15-040) and without (50-020) $NaHCO_3$ were from Mediatech (Herndon, VA). The $HClO_4$ (60%) and deuterium oxide (D_2O) (99.9%) were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich, respectively, and used without purification.

Synthesis of ^{15}N-Carboplatin. To a solution containing 0.659 g (1.36 mmol) of *cis*-Pt($^{15}NH_3$)$_2$I$_2$ (*20*) in 5 mL of distilled deionized water (dH_2O) was added 5 mL of a solution containing 0.485 g (2.85 mmol) of silver nitrate in 5 mL of dH_2O. The resulting solution was stirred overnight in the dark after which time the AgI that formed was removed by filtration through diatomaceous earth. To the filtrate was added 0.215 g (1.50 mmol) of 1,1-cyclobutanedicarboxylic acid, and the pH of the resulting solution was adjusted to 5.0 using 10 M KOH. After it was stirred for 3 h, the solvent was removed in a vacuum (∼30 °C) and the white solid was washed with 5 mL of cold water, ethanol, and ether and dried to yield 27.8% based on *cis*-Pt($^{15}NH_3$)$_2$I$_2$. The ^{1}H NMR spectrum of ^{15}N-carboplatin in D_2O [referenced internally to, DSS, $Me_3Si(CH_2)_3SO_3Na$] at 25 °C, pH 5.0, exhibits signals at 2.880 (triplet, 4H, $^3J_{H-H} = 8.0$ Hz) and 1.895 (quintet, 2H, $^3J_{H-H} = 8.0$ Hz) (*4*, *9*).

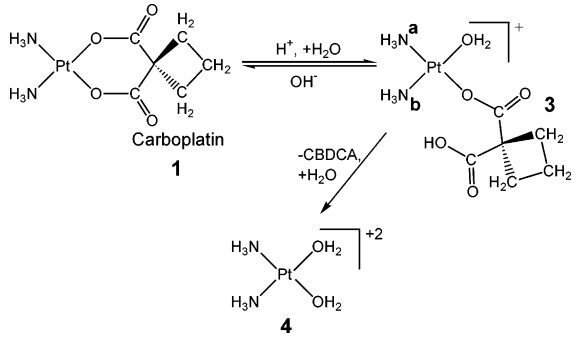
[^{1}H,^{15}N] HSQC NMR Spectroscopy. HSQC NMR spectra were recorded on a Bruker Advance 500 MHz NMR equipped with a 5 mm triple axis probe in the earlier described manner (*18*). Each kinetic run consisted of successive NMR measurements (each 60 min in length, ns = 48). Peak volumes in arbitrary units 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 ($^{15}NH_4$)$_2$SO$_4$ in 95/5 H_2O/D_2O, which was acidified to pH ∼ 1 by the addition of H_2SO_4. The ^{1}H chemical shifts were referenced externally to $Me_3SiCD_2CD_2CO_2Na$, TSP, in a pH 7.15, 23 mM sodium carbonate buffer.

Spectra were recorded in 95/5 H_2O/D_2O at 37 °C. Freshly prepared stock solutions of the labeled drug in water were prepared, and appropriate volumes were added to solutions to give a final concentration of drug of 110 μM in a final volume of 920 μL. The pH 8.6, 23.8 mM and pH 8.6, 10 mM carbonate buffers were prepared by dissolving the appropriate amount of $NaHCO_3$ in water. The pH 7.5, 23.8 mM and pH 7.5, 10 mM carbonate buffers as well as the pH 6.7, 23.8 mM and pH 6.7, 10 mM carbonate buffers were prepared in the identical manner with the addition of the appropriate amount of 3 N $HClO_4$. The "normal medium" used in the NMR studies was RPMI-1640 (pH 7.2) plus 10% FBS. To this was also added 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM S-glutamine. The pH of the "medium without carbonate", RPMI (50-020), 10% FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM S-glutamine, was adjusted to pH 7.5 by addition of 3 N perchloric acid. Studies were also done with "FBS alone", which were done in 95/5 H_2O/D_2O containing 10% FBS, pH 7.5.

UV−Visible Absorption Spectroscopy. The UV-vis absorption spectra at 37 °C were obtained using a Cary 50 spectrophotometer equipped with a thermostated cell holder in a 1 cm path length cell. Absorption spectra were collected every hour for 30 h in the wavelength range of 500−190 nm, using a scan rate of 60 nm/min. Difference spectra were obtained by subtracting the spectrum immediately after addition of **1** to the buffer ($t = 0$ spectrum) from spectra taken at various times. Each difference spectrum was fitted to a sum of Gaussians using PeakFit (v. 4) to give band positions and areas. The area of a particular band (identified by position) was then plotted vs time.

Cell Studies. Carboplatin (58 μL from a 37.8 mM stock solution of **1** in water) was added to 20 mL of medium (RPMI + 10% FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM S-glutamine) containing 900 million Jurkat cells to give a final concentration of 110 μM **1** in the culture medium**.** The cells were kept under standard conditions in a humidified, 37 °C, 5% CO_2

Scheme 1. Ring Opening of Carboplatin in Acid



incubator. At various times, a 916 μL aliquot of cells plus medium was removed from the incubation flask and the cells were separated from the medium by centrifugation (1400*g*). To the medium supernatants, volume $\sim$ 875 μL, was added $\sim$45 μL of D_2O to give a final volume of 920 μL. The solution, referred to as "medium exposed to Jurkat cells," was placed in a capped 5 mm NMR tube, and an $^1H-^{15}N$ HSQC NMR spectrum of the sample was obtained. The elapsed time from the point of removal of the aliquot from the incubation flask to the end of the NMR data collection time (duration, 0.5 h) for each time point was $\leq$0.8 h. Cell viabilities were measured by light microscopy, using a hemacytometer under standard trypan blue conditions (*21*). Cell viabilities taken at 0, 6, and 25 h after addition of drug were 96.9, 96.9, and 0%, respectively.

Acid Hydrolysis of Carboplatin. Freshly prepared stock solutions of labeled **1** in water were prepared, and appropriate volumes were added to a solution of 95/5 H_2O/D_2O to give a final concentration of 220 μM carboplatin in a final volume of 920 μL. The solution was acidified by the addition of $HClO_4$ to give a hydrogen ion concentration of 0.03 M, and [^1H,^{15}N] HSQC NMR spectra, at 37 °C, were obtained approximately every 0.33 h. At 1.8 h after the addition of acid (t = 1.8 h), the pH of the solution was adjusted to $\sim$2.2 by the addition of 10 M KOH, and further HSQC NMR spectra were obtained at 0.33 h intervals out to t = 3.3 h. Assuming that peak intensities were proportional to concentrations and knowing that the sum of the concentrations of the three species observed was 220 μM, we derived the species concentrations at each time point, before and after the addition of KOH.

Results

Ring Opening of Carboplatin. [^1H,^{15}N] HSQC NMR. The hydrolysis of carboplatin in acid, Scheme 1, was followed using [^1H,^{15}N] HSQC NMR (*22*). Allowing **1** to stand in 0.03 M $HClO_4$ at 37 °C resulted in a decrease in the HSQC NMR intensity for peak **1** (at ^1H,^{15}N, δ = 4.17/$-$81.3) and the appearance of two new peaks at 4.26/$-$82.3 and 4.31/$-$87.8 (Table 1). At later times, an additional peak at 4.50/$-$86.5 was also observed in the spectra. The [^1H,^{15}N] HSCQ NMR spectrum 1.3 h after addition of acid is shown in Figure 2a. Because the peaks at 4.26/$-$82.3 and 4.31/$-$87.8 increase in intensity at the same rate, they have been assigned to the ring-opened product, *cis*-[Pt(NH_3)$_2$(CBDCA-*O*)(H_2O)]$^+$, **3**, which has nonequivalent ammonia molecules. By comparison with ^{15}N chemical shift data for *cis*-[Pt(NH_3)$_2$(CBDCA-*O*)Cl]$^-$ (*9*) and a Pt^{2+} compound containing a bound acetate ligand (*22*), the peak at 4.26/$-$82.3 is N trans to O (CBDCA), **3a**, while the peak at 4.31/$-$87.8 is assigned to N trans to O (H_2O), **3b**. The peak at 4.50/$-$86.5, observed at later times, which has an ^{15}N chemical shift similar to that of **3b**, has been assigned to *cis*-[Pt(NH_3)$_2$(H_2O)$_2$]$^{+2}$, **4** (*23*). (Peak assignments are summarized in Table 1.) The presence of this complex was also shown by earlier studies, which employed UV-visible spectroscopy to measure the kinetics of acid hydrolysis of carboplatin (*24*).

Table 1. ^1H and ^{15}N Chemical Shifts of the Ammine Ligands in *cis*-Diammine Pt(II) Complexes

complex	δ (^1H)	δ (^{15}N)	trans ligand
Pt(NH_3)$_2$(CBDCA-*O*,*O*′) (**1**)	4.17	$-$81.3	(O)CBDCA
[Pt(NH_3)$_2$(CBDCA-*O*)(H_2O)]$^+$ (**3**)	4.26	$-$82.3	(O)CBDCA
	4.31	$-$87.8	(O)H_2O
[Pt(NH_3)$_2$(H_2O)$_2$]$^{+2}$ (**4**)	4.50	$-$86.5	(O)H_2O
[Pt(NH_3)$_2$(CBDCA-*O*)(CO_3)]$^{-2}$ (**5**)[a,b]	3.93	$-$82.3	(O)CBDCA
	3.61	$-$79.2	(O)CO_3^{-2}
[Pt(NH_3)$_2$(CBDCA-*O*)Cl]$^-$ [c]	3.93	$-$84.3	(O)CBDCA
	4.12	$-$68.7	Cl^-
[Pt(NH_3)$_2$(CBDCA-*O*)(HPO_4)][d]	3.99	$-$83.3	(O)CBDCA
	3.86	$-$79.2	(O)HPO_4^{-2}

[a] Mixture of **5**, **6**, and possibly **7** at pH 8.6. [b] The observed chemical shift parameters at pH 7.5 are 3.94/$-$82.5 and 3.64/$-$79.6. [c] Ref *9*. [d] Protonation state and number of bound phosphate ligands not determined; ref *9*.

Further evidence that the initially formed product in the hydrolysis reaction is the ring-opened complex was obtained by changing the pH of the solution. As shown in Figure 2b,c, when the pH is raised to 2.2 by the addition of 10 M KOH, the peaks for **3** disappear and the peak for **1** intensifies. Thus, the deprotonation of the unbound carboxyl group of **3** induces ring closure, producing more **1**, and that **3** indeed has an attached mono-dentate CBDCA ligand. In Figure 2c, peak intensities have been converted to concentrations, using the sum of the peak intensities for **3a**,**b** to obtain the concentration of the monoaquo species **3**. The concentration of carboplatin, species **1**, decreases with time (linear fit shown in Figure 2c) until base is added at 1.8 h, after which it increases. For t < 1.8 h, the concentration of **3** increases at about the same rate as the concentration of species **1** decreases; after 1.8 h, it decreases rapidly to below the detection limit of the NMR measurement, confirming that **3** forms from **1** in acid solution (ring opening), and **3** can be converted to **1** (ring closing) if the pH is raised. The concentration of the diaquo species **4** increases slowly with time throughout the entire time period indicating that, while most of **3** returns to **1**, additional **4** also forms.

The reaction of **1** in 23.8 mM carbonate buffer (pH 8.6) results in the slow decrease in the intensity of the peak for **1** (kinetics discussed below) and the appearance of two new peaks at $^1H/^{15}N$, δ = 3.93/$-$82.3 and 3.61/$-$79.2 (Figure 3a). Because the intensities of both product peaks increase at the same rate and their ^{15}N chemical shifts are consistent with N trans to O (*22*), the carboplatin product that forms in carbonate buffer has been assigned to the ring-opened complex, *cis*-[Pt(NH_3)$_2$(CO_3^{-2})(CBDCA-*O*)]$^{-2}$, **5** (Scheme 2). However, as discussed below, this compound is in a proton equilibrium with its protonated bicarbonate form, **6**, and possibly also its decarboxylated hydroxo analogue, **7** (*13*, *15*, *16*, *25*). By comparison with the ^{15}N chemical shift parameters of *cis*-[Pt(NH_3)$_2$(CBDCA-*O*)Cl]$^-$ (*9*), an acetato complex (*22*), and **3**, the peak at $^1H/^{15}N$, δ = 3.93/$-$82.3 in Figure 3a, is N trans to O (CBDCA), **5a**, while the peak at $^1H/^{15}N$, δ = 3.61/$-$79.2, has been assigned to N trans to O (CO_3^{-2}), **5b**. When the pH of the 23.8 mM carbonate buffer is lowered to 7.5, the rate of conversion of **1** to the ring-opened product is reduced and **5** can only be detected after $\sim$18 h. Because lowering the pH from 8.6 to 7.5 would lower the concentration of carbonate ion, which is the attacking nucleophile in the reaction, a reduction in the rate of formation of **5** at lower pH is expected. In addition to slowing the reaction, lowered pH also results in protonation of the bound carbonate to bicarbonate (Scheme 2). The resulting bicarbonato complex could spontaneously decarboxylate to the corresponding hydroxo form, **7**, or it could retain the bound bicarbonate (*13*, *15*, *16*, *25*). As will be evident below, the UV

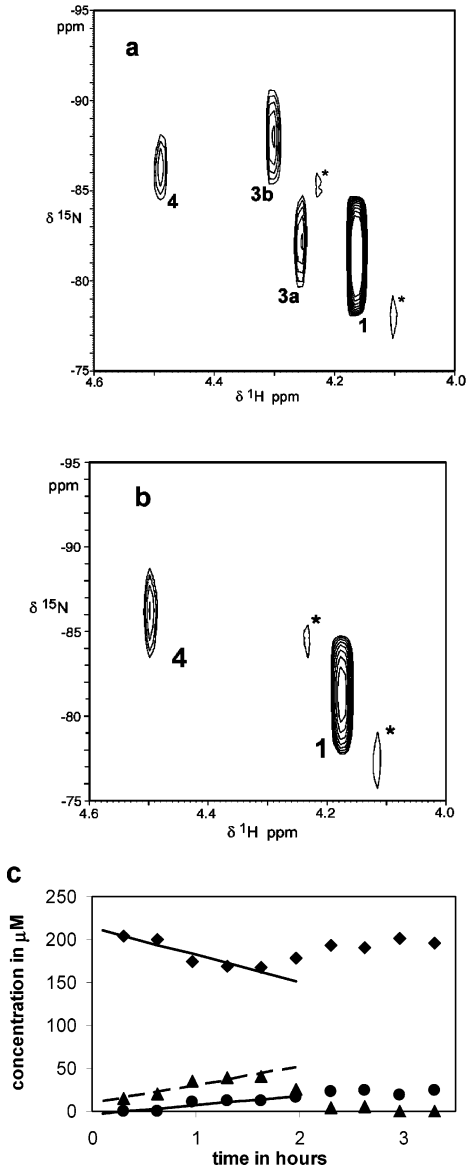


Figure 2. (a) [^{1}H,^{15}N] HSQC NMR spectra of 220 μM ^{15}N-carboplatin 1.3 h after the addition of 0.03 M $HClO_4$ (t = 1.3 h). Asterisks show the ^{195}Pt satellites of **1**. At t = 1.8 h, 10 M KOH was added to the solution in part a to adjust the pH to 2.2. (b) HSQC NMR of solution at pH 2.2 at t = 3 h. Asterisks show the ^{195}Pt satellites of **1**. (c) Concentrations of ^{15}N-carboplatin species (original concentration, 220 μM) as functions of time, derived from HSQC NMR intensities in solution. The solution was originally 0.03 M $HClO_4$; addition of KOH at 1.8 h changed the pH to 2.2. Diamonds and solid line, carboplatin; triangles and dashed line, *cis*-$[Pt(NH_3)_2(CBDCA\text{-}O)(H_2O)]^+$ (the sum of the intensities for both nonequivalent ammonia peaks is used); and circles and gray line, *cis*-$[Pt(NH_3)_2(H_2O)_2]^{+2}$. Lines are linear fits to the first five points.

absorption studies show that the apparent pK_b for the protonation of **5** is ~8.6, so, at pH 7.5, the ring-opened product exists as a mixture of **5**, **6**, and possibly **7**, which are in rapid equilibrium with each other on the NMR time scale.

UV−Visible Absorption Studies. The absorption spectrum of 500 μM carboplatin in 23.8 mM carbonate buffer, pH 7.5, is shown in Figure 4. The compound exhibits a strong band at 203 nm (ϵM = 4600 M^{-1} cm^{-1}) and a shoulder at 229 nm (ϵM = 2300 M^{-1} cm^{-1}), which because of their intensities appear to be charge-transfer transitions (*3*). The compound also exhibits a much weaker band at 330 nm (ϵM = 40 M^{-1} cm^{-1}), which appears to be due to the $d-d$ transitions of the square planar Pt^{2+} ion. After several hours in carbonate buffer, carboplatin

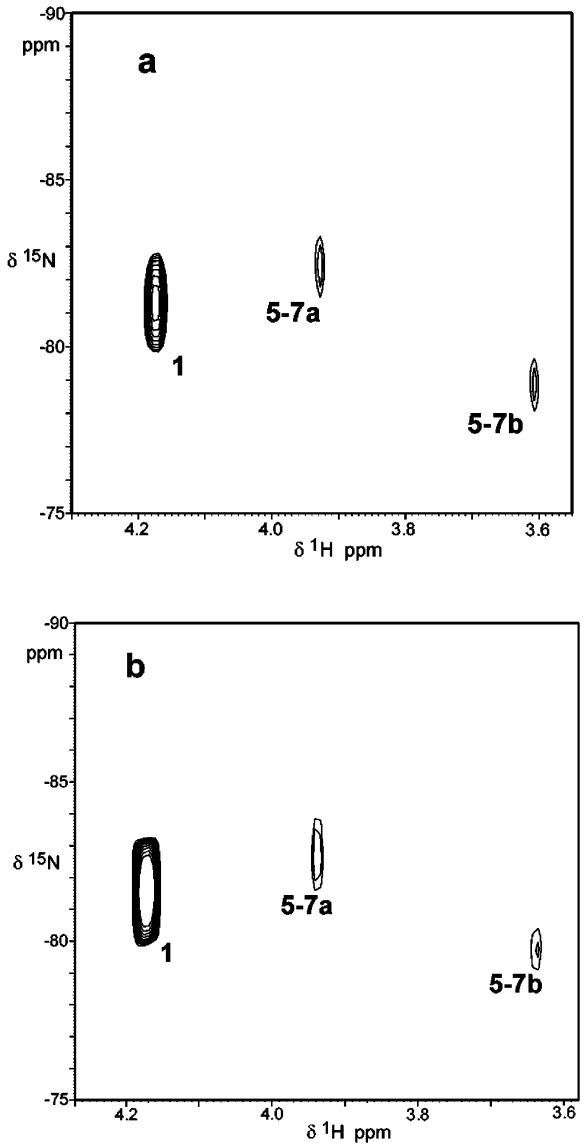


Figure 3. [^{1}H,^{15}N] HSQC NMR spectra of 110 μM ^{15}N-carboplatin in 23.8 mM carbonate solution. (a) pH 8.6, t = 15.6 h; (b) pH 7.5, t = 18.6 h.

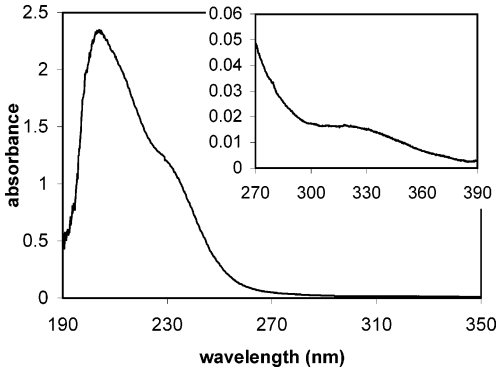
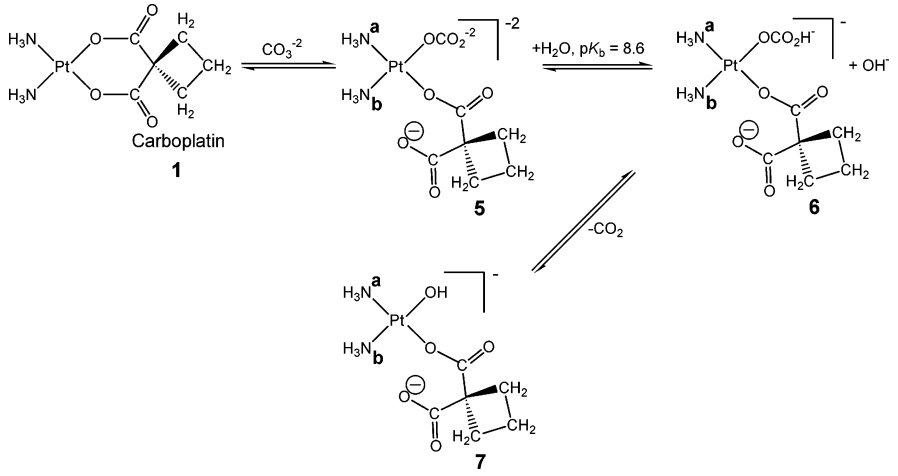


Figure 4. Absorption spectrum of 500 μM carboplatin in 23.8 mM carbonate buffer, pH 7.5.

exhibits small but reproducible spectral changes indicating that some of the drug has reacted with carbonate.

Figure 5, left side, shows difference spectra for the wavelength region, λ = 200−300 nm, at five values of pH (6.7, 7.5, 8.1, 8.6, and 9.3) for the reaction of 500 μM **1** in 23.8 mM carbonate buffer. Each graph shows difference spectra for the times t = 6, 12, 18, 24, and 30 h. The difference spectrum at

Scheme 2. Ring Opening of Carboplatin by Carbonate Ion



time *t* is obtained by subtracting the absorption spectrum at time 0 from the absorption spectrum at time *t*. There is considerable noise in a difference spectrum because the two spectra being subtracted are very similar so that their difference is much smaller than either. For clarity, we have shown in Figure 5 the fits of the difference spectra to a sum of Gaussian bands (left panel). The areas of individual bands as a function of reaction time are also shown (right panel).

In each case, there is a relatively narrow peak at about 210 nm and a small broader peak at about 270 nm. For pH 6.7 and pH 7.5, there is another peak between 215 and 225 nm. For pH 8.1, pH 8.6, and pH 9.3, there is a negative peak at about 239 nm. Linear fits have been made to peak areas vs time and are shown on the right side of Figure 5.

In all cases, the areas of the peak near 210 nm are well-fit to linear functions of time, $R^2 \geq 0.910$. At pH 6.7, the areas for the peak at 215−225 nm are fit ($R^2 = 0.992$) to a linear function of time, $-0.0998 + 0.0448t$. For the peak near 270 nm, only the area for the 3 minus 0 difference spectra was measurable. At pH 7.5, the areas for the peak at 215−225 nm are fit ($R^2 = 0.933$) to $0.133 + 0.023t$, and the areas for the peak at 270 are fit ($R^2 = 0.841$) to $0.0945 + 0.0131t$.

The negative peak for pH 8.1 and higher, appearing at 239 nm, must arise from the depletion of carboplatin, since carboplatin exhibits an absorption at ∼230 nm (Figures 4 and 5). When the areas of this peak at pH 8.1 are fit to a linear function of time, $-0.0942 + 0.0019t$, the $R^2 = 0.454$. Thus, the value of the slope is very uncertain. The same is true for the broad peak near 270 nm, for which peak areas were only measurable for eight out of 10 difference spectra.

At pH 8.6, the negative peak near 239 nm is much more pronounced than it is at pH 8.1. This suggests that the rate of disappearance of carboplatin increases with increasing pH. The areas of the 239 nm peak are fit ($R^2 = 0.974$) to $-0.2085 - 0.0159t$. At pH 9.3, the peak at 239 nm grows even more negative. The areas of the 239 nm peak are well-fit ($R^2 = 0.938$) to a linear function of time, $-0.7042 - 0.1129t$.

In the cases where peak areas can be measured reliably from the UV difference spectra, it is found that ratios of peak areas are constant in time. For example, the ratio of the areas of the peaks at pH 6.7 is 0.47 ± 0.07 (average ± standard deviation), and at pH 7.5, the ratio of the sum of the areas of the high-energy peaks to the area of the 270 nm peak is 2.4 ± 0.2. The constancy of a peak ratio implies either that both peaks come from the same product or, if they come from different products, that the products are produced simultaneously from the same reactant. (If one product is transformed into the other by a rapid proton exchange, it is equivalent to simultaneous production.) At pH 8.6, the ratio of the 210 nm peak to the 270 nm peak is 4.0 ± 0.4 and the ratio of the 210 nm peak to the 239 nm peak is -3.0 ± 0.2. The latter implies that the species giving rise to the 210 nm peak arises from the species whose depletion is responsible for the negative peak at 239 nm (carboplatin).

Determination of the pK_b for the 5 to 6 Equilibrium. From the UV-visible results, it is clear that, depending on pH, ring opening produces one of two products. The products are likely a carbonate adduct (**5**) at high pH and a bicarbonate adduct (**6**) at low pH. The latter may decarboxylate to the corresponding hydroxo form (**7**), with different UV absorption peaks. Because **5** differs from **6** by loss of a proton, it is of interest to determine the pK_b of **5**. Normally, this would be done by plotting absorption intensity for a given wavelength, taken from a difference plot, vs pH, with all difference plots being obtained at the same *t*. In the present case, however, one must take into account the pH dependence of the rate of the ring-opening reaction. From the rate of decrease of the carboplatin peak intensity in NMR, one can find the first-order rate constant k_1 for this reaction (see Table 2). A plot of log k_1 vs pH for 10 mM carbonate is well-fit by log $k_1 = -9.25 + 0.422$ pH, and, for 23.8 mM carbonate, log $k_1 = -8.54 + 0.363$ pH. This predicts that the ratios of the rates at pH 7.5, 8.1, 8.6, and 9.3 to the rate at pH 6.7 are approximately 2.1, 3.5, 5.6, and 10.5, respectively. So, if one compares absorption intensities for different pH values at some fixed wavelength, one should compare the 30 h difference spectrum for pH 6.7 with the 14 h difference spectrum for pH 7.5, the 9 h difference spectrum for pH 8.1, the 5 h difference spectrum for pH 8.6, and the 3 h difference spectrum for pH 9.3.

Absorbances were obtained from the difference spectra for each of the above times for 12 different wavelengths ranging from 210 to 240 nm. Each set of data, corresponding to a specific wavelength, was fit to a titration curve using Excel Solver, giving a K_b value of ∼8.6. The corresponding pK_a for deprotonation of **5** is 5.4.

Reaction of 1 in Different Media. Figure 6 shows carboplatin concentrations, obtained from HSQC NMR intensities, as functions of time for three 110 μM solutions, with least-squares linear fits. The three solutions were carboplatin in water at pH 7.5, carboplatin in 10 mM carbonate buffer at pH 8.6, and carboplatin in 23.8 mM carbonate buffer at pH 8.6. Figure 7 shows similar information for three 110 μM solutions containing different cell growth media conditions. These were

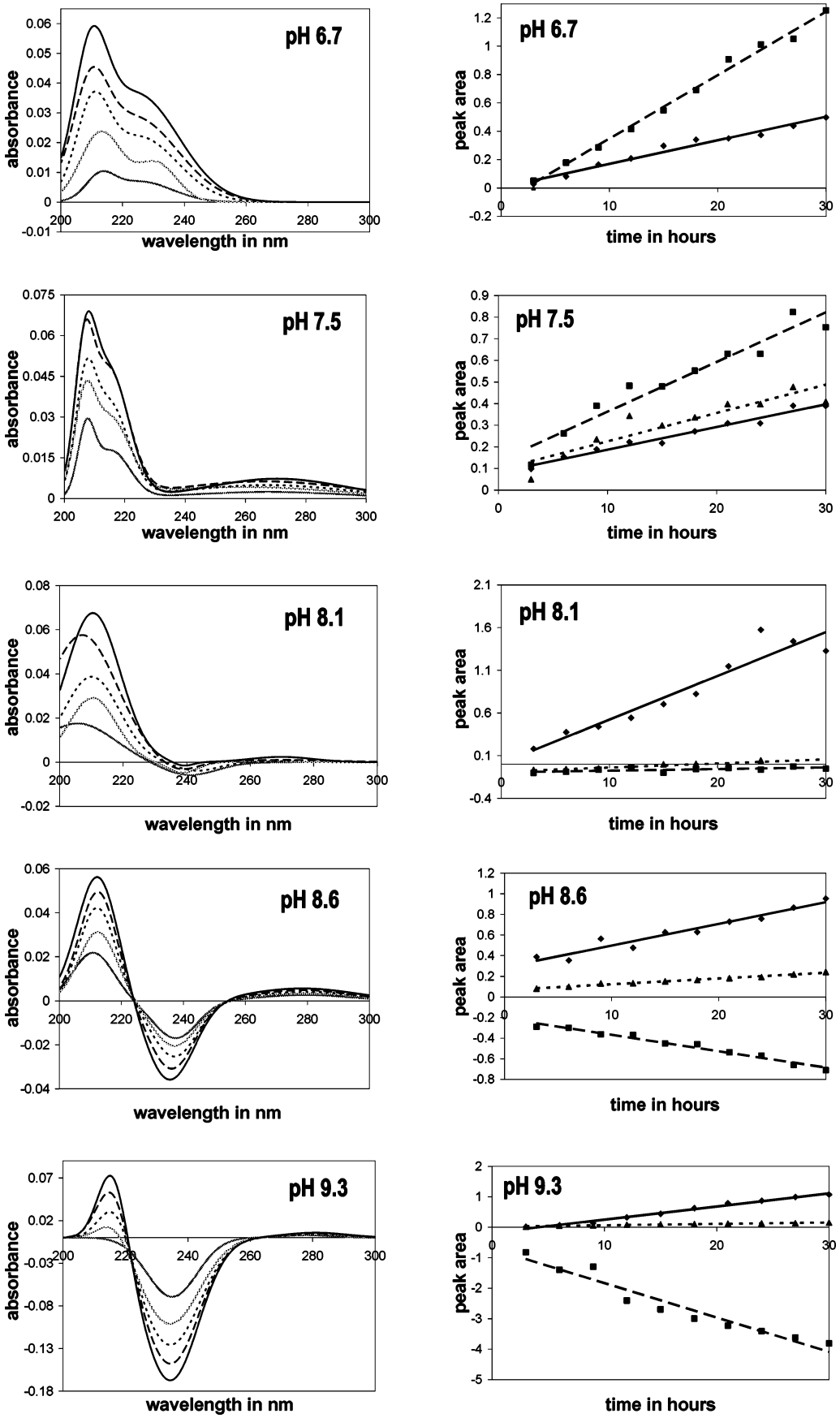


Figure 5. Shown in the left panel are UV-visible difference spectra at pH 6.7, 7.5, 8.1, 8.6, and 9.3. Each plot shows the difference between the spectra at times $t = 30$ (top difference curve at 210 nm), 24, 18, 12, and 6 h (bottom difference curve at 210 nm) and the spectrum at $t = 0$ h. For clarity, the fit to a sum of Gaussians is shown rather than the original experimental data, which shows considerable scatter. Shown in the right panel are peak areas plotted vs time, with linear fits to the data. The peak at 210 nm is diamonds and solid line; the peak at 215−225 nm for pH 6.7 and pH 7.5 and at 239 nm for pH ≥ 8.1 is squares and large dash line; and the peak at 270 nm is triangles and small dash line.

Table 2. Pseudo-First-Order Rate Constants for the Reaction of Carboplatin in Various Media Obtained Using HSQC NMR

	k_1 (s^{-1})	standard error
water, pH 7.5	5.09×10^{-7}	4.01×10^{-7}
10 mM carbonate, pH 6.7	3.88×10^{-7}	6.39×10^{-7}
23.8 mM carbonate, pH 6.7	6.54×10^{-7}	3.30×10^{-7}
10 mM carbonate, pH 7.5	7.87×10^{-7}	3.28×10^{-7}
23.8 mM carbonate, pH 7.5	2.04×10^{-6}	8.10×10^{-7}
10 mM carbonate, pH 8.6	2.44×10^{-6}	1.31×10^{-6}
23.8 mM carbonate, pH 8.6	3.36×10^{-6}	3.10×10^{-7}
normal medium[a]	3.08×10^{-6}	2.02×10^{-7}
medium minus carbonate[a]	2.25×10^{-6}	2.52×10^{-7}
FBS, pH 7.5	1.79×10^{-6}	1.77×10^{-7}
with Jurkat cells	2.82×10^{-6}	5.94×10^{-7}
140 mM chloride, pH 7[b]	7.7×10^{-7}	
100 mM phosphate, pH 7[b]	4.3×10^{-7}	2.0×10^{-8}

[a] See Materials and Methods for complete description of media and conditions. [b] Ref *9*.

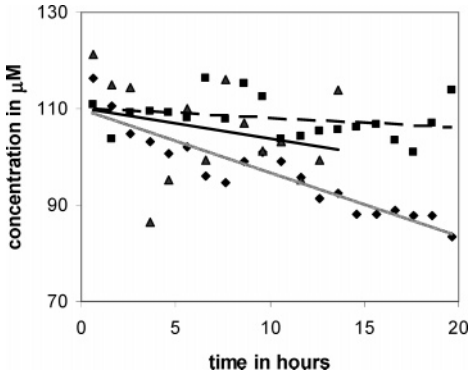


Figure 6. Concentration of ^{15}N-carboplatin (original concentration, 110 μM) as a function of time derived from HSQC NMR intensities, with linear fits to the data. Carboplatin in water at initial pH 7.5 is squares and dashed line; carboplatin in 10 mM carbonate buffer at pH 8.6 is triangles and solid line; and carboplatin in 23.8 mM carbonate buffer at pH 8.6 is diamonds and gray line.

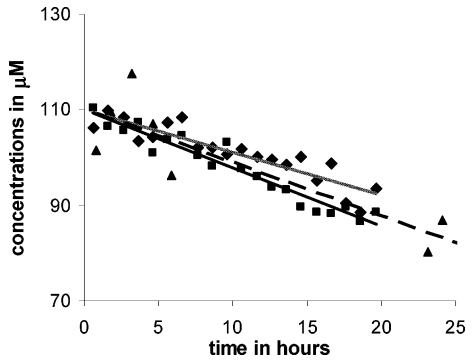


Figure 7. Concentration of ^{15}N carboplatin in media (original concentration, 110 μM) as a function of time, derived from HSQC NMR intensities, with linear fits to the data. Carboplatin in medium is squares and solid line; carboplatin in medium minus carbonate is diamonds and gray line; and carboplatin in medium with Jurkat cells is triangles and dashed line. See Materials and Methods for complete description of media and conditions.

the following: normal medium, RPMI + 10% FBS; normal medium without carbonate, RPMI + 10% FBS minus $NaHCO_3$; and normal medium exposed to Jurkat cells. In both Figures 6 and 7, HSQC peak intensities have been converted to concentrations by performing a least-squares linear fit, obtaining the *y*-intercept I_0, and multiplying all intensities by (110 μM)/I_0.

In Figure 6, the top line is for water at pH 7.5, the second line is for 10 mM carbonate at pH 8.6, and the bottom line is for 23.8 mM carbonate at pH 8.6. Because of increased scatter in the data, only 14 (instead of 20) data points were used for

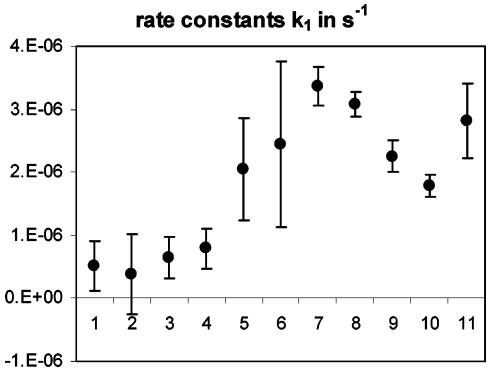


Figure 8. Pseudo-first-order rate constants derived from decay rate of the ^{1}H−^{15}N HSQC NMR peak for ^{15}N-carboplatin, with statistical errors from linear fits. Key: 1, in water at pH 7.5; 2, in 10 mM carbonate at pH 6.7; 3, in 23.8 mM carbonate at pH 6.7; 4, in 10 mM carbonate at pH 7.5; 5, in 23.8 mM carbonate at pH 7.5; 6, in 10 mM carbonate at pH 8.6; 7, in 23.8 mM carbonate at pH 8.6; 8, in "medium"; 9, in "medium minus carbonate"; 10, in "FBS alone"; and 11, in "medium with Jurkat cells". See Materials and Methods for the definitions of the terms in quotations.

the 10 mM carbonate run. It can be seen that k_1 is close to zero for water but appreciable for the two carbonate solutions. In fact, at pH 6.7, 7.5, and 8.6, k_1 for 23.8 mM carbonate is, on average, twice the k_1 for 10 mM carbonate. This concentration dependence suggests that attack of a species in carbonate buffer (most likely carbonate ion) is responsible for the ring opening, which removes carboplatin. In Figure 7, the top line is for 110 μM carboplatin in medium without carbonate at a pH of 7.5, the bottom line is for carboplatin in normal medium, and the middle line is for carboplatin in medium exposed to Jurkat cells. The three slopes are fairly close together, but the slope for normal medium is ~1.5 that for medium without carbonate. This confirms that carbonate in the medium is responsible for the reaction that converts carboplatin to products. The rate of decrease of carboplatin in medium exposed to Jurkat cells is about the same as that in normal medium not exposed to cells. This shows that Jurkat cells have no effect on the ability of substances in the medium (carbonate) to decrease the concentration of **1**. In none of the studies involving culture media (and FBS alone), which were done at pH 7.2−7.5, were HSQC NMR peaks for products observed. This is most likely due to the fact that the rate of ring opening slows when the pH is lowered and a small amount of product would be difficult to detect with the NMR instrumentation used.

By dividing the slope of a linear fit by the *y* intercept, one obtains the pseudo-first-order decay constant k_1 for the disappearance of carboplatin. Note that although the ring-opening reaction is really second-order (first-order in both carbonate and carboplatin), it behaves like a first-order reaction because carbonate is not consumed in the reaction. The values obtained for k_1 are given in Table 2, with the statistical errors from the linear fits, and are also plotted in Figure 8 for easier visual comparison.

If carbonate is the attacking reagent, k_1 should be proportional to $[CO_3^{2-}]$, the concentration of carbonate ion. This can be calculated for a given pH and total carbonate concentration, since the equilibrium constants

$$K_1 = \frac{[HCO_3^-][H^+]}{[CO_2]}$$

and

$$K_2 = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]}$$

are known as: $K_1 = 9.33 \times 10^{-7}$, p$K_1 = 6.35$; and $K_2 = 1.59 \times 10^{-10}$, p$K_2 = 10.33$ (*26*). The total carbonate concentration is $[CO_2] + [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}]$; note that $[H_2CO_3]$ is negligibly small (*25*, *26*).

The proportionality to carbonate concentration at fixed pH has already been demonstrated. In Figure 9, six values of k_1 are plotted vs $[CO_3^{2-}]$, calculated as above, on a semilog plot for clarity. The k_1 values were determined from the observed rates from the HSQC NMR data (Table 2 and Figure 8) for 23.8 and 10 mM carbonate at pH 6.7, 7.5, and 8.6. The statistical errors associated with linear fits of the data, shown in Figure 9, are large, which is not unexpected, given the difficulty in measuring accurate rate constants at the low concentrations of carboplatin used. The best-fit line, also shown, is $k_1 = 9.3 \times 10^{-7} + 0.00612\ [CO_3^{2-}]$ ($R^2 = 0.79$), the small value of R^2 being due to the scatter in the k_1 values. Considering that the independent variable $[CO_3^{2-}]$ ranges over 3 orders of magnitude, the fit is quite satisfactory.

Discussion

As is evident from Table 2 and Figure 8, increasing the pH at a constant total carbonate concentration or increasing the total carbonate concentration at a constant pH leads to an *increase* in the rate of reaction of **1** in carbonate buffer. The k_1 values for 10 mM carbonate at three pH values follow log (k_1) $= -9.25 + 0.422$ pH ($R^2 = 0.998$), and the k_1 values for 23.8 mM carbonate obey log (k_1) $= -8.54 + 0.363$ pH ($R^2 = 0.901$). Also, the ratio of k_1 for 23.8 mM carbonate to k_1 for 10 mM carbonate is equal to 1.7, 2.6, and 1.4 for pH = 6.7, 7.5, and 8.6, respectively. Because the actual concentration of carbonate ion increases when either total carbonate or pH increases (*13*), the attacking nucleophile in the ring-opening reaction of carboplatin is carbonate ion. This is confirmed by plotting k_1 vs $[CO_3^{2-}]$ (see Figure 9) and showing the data fit the linear function, $k_1 = 9.3 \times 10^{-7}\ s^{-1} + 6.1 \times 10^{-3}\ s^{-1}\ M^{-1}\ [CO_3^{2-}]$.

Sadler and co-workers (*9*) obtained pseudo-first-order rate constants for reaction of carboplatin in 140 mM chloride and 100 mM phosphate, pH 7.0, 37 °C, of 7.7×10^{-7} and $4.3 \times 10^{-7}\ s^{-1}$, respectively. Because the pseudo-first-order rate constant for the reaction of carboplatin in 23.8 mM carbonate, pH 7.5, 37 °C, is much larger (2.04×10^{-6}, Table 2), carbonate ion is probably the nucleophile activating carboplatin (opening the ring) in vivo. This conclusion is also supported by the observed rates of disappearance of carboplatin in medium in the presence and absence of carbonate (Table 2 and Figure 7).

Sadler and co-workers (*9*) measured the pseudo-first-order rate constant for the hydrolysis of carboplatin in water. Because they were unable to detect changes in the ^{1}H NMR spectra of the compound after 2 weeks at 37 °C, pH 7, they reported a value of k_1 for the hydrolysis of $< 5 \times 10^{-9}\ s^{-1}$, which was consistent with an earlier study by Canovese et al. (*4*), which suggested that k_1 is $< 1 \times 10^{-8}\ s^{-1}$. As is evident from Table 2, the rate constant for the reaction of carboplatin in water measured by HSQC NMR in this study is $(5.09 \pm 4.01) \times 10^{-7}\ s^{-1}$, pH 7.5, which is significantly larger than that found in the earlier studies. Because our study was done using a low concentration of carboplatin (110 μM) and the pH was not controlled (no buffer was added), it may be that changes in pH, occurring as the reaction takes place, influence the rate of disappearance of **1** during the 20 h NMR experiment. Significant variations in measured hydrolysis rates for **1** in unbuffered media have been previously reported (*9*, *27*).

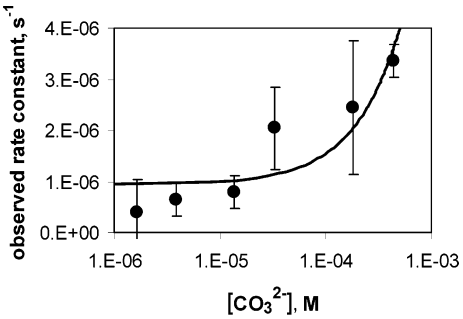


Figure 9. Observed rate constant for the disappearance of carboplatin obtained from HSQC NMR data plotted vs true carbonate concentration. The best-fit line, $R^2 = 0.79$, is $k_1 = 9.3 \times 10^{-7} + 0.00612\ [CO_3^{2-}]$.

As shown in Scheme 2, the initially formed mono-carbonato complex **5** can be protonated to form the mono-dentate bicarbonato complex, **6**. Because the pK_b for the protonation of **5** determined from the UV-visible absorption data is ~8.6, the compound is partially protonated (as **6**) at physiological pH. Evidence from the literature suggests that **6** could remain as a mono-dentate bicarbonato complex, or it could decarboxylate to the hydroxo complex, **7** (*13*, *15*, *16*, *25*). A mono-dentate bicarbonato−zinc complex is believed to be important in the dehydration of HCO_3^- catalyzed by human carbonic anhydrase II (*28*, *29*), and a similar species could be among the ring-opened products for carboplatin. Thus, at physiological pH, compound **5** appears to be in equilibrium with **6** and possibly also its decarboxylated hydroxo form, **7**. Even though the unbound carboxylate of the CBDCA ligand is deprotonated at physiological pH and it could presumably attack the Pt to reform carboplatin, ring closure for **5**−**7** does not occur. Most likely, this is due to the poor leaving groups in cis positions (anions), which would need to be displaced in the closure reaction. As shown in Scheme 1 and Figure 2, if the group cis to the mono-dentate CBDCA ligand is H_2O, which is generated in the acid hydrolysis of **1**, release of the proton from the unbound carboxylate results in water displacement from Pt and ring closure to reform carboplatin.

The UV-visible difference spectra show that the nature of the ring-opened product formed in 23.8 mM carbonate buffer depends on pH (Figure 5). At pH 9.3, the negative band at 239 nm increases with time more rapidly than at pH 8.6 and much more rapidly than at pH 8.1, indicating that the concentration of compound **1** is decreasing and that the mixture of products has a relatively weak absorption at this wavelength. At pH 6.7, or at pH 7.5, the band at 215−225 nm becomes strongly positive and grows with time, indicating that the product predominantly formed at lower pH has a strong absorption at this wavelength. Mahal and van Eldik (*15*) have reported that a CT band near 260 nm (the absorption maximum was not reported) for a series of Pd(II) complexes having mono-dentate carbonate decreases in intensity as pH is lowered. The decrease in intensity was attributed to the elimination of intramolecular hydrogen bonding between the bound carbonate and an adjacent amine hydrogen of the complex as the carbonate ligand is destroyed by acid. As is evident from Figure 5, lowering the pH leads to an increase in intensity in this region. However, because **5** has two ligands, CO_3^{2-} and CBDCA, which can potentially form intramolecular hydrogen bonds with adjacent amine hydrogen atoms, the effects of pH on the CT transition of the ring-opened products are difficult to predict.

As is evident from Table 2, the NMR peak for carboplatin disappears at approximately the same rate in culture media and 23.8 mM carbonate, implying that carbonate is the attacking nucleophile, and the reaction shown in Scheme 2 takes place. However, ring-opened products are not detected with HSQC NMR in culture media. This contrasts with what is found with cisplatin, which rapidly reacts with carbonate in culture media to produce amounts of *cis*-$[Pt(NH_3)_2(CO_3^{-2})Cl]^-$ readily detected with HSQC NMR (*18*, *19*). The reason for no observation of product peaks in the 20 h NMR experiment for carboplatin probably relates to the mechanism of formation of carbonato complexes for cisplatin and carboplatin in carbonate media. For cisplatin, aquation occurs much more rapidly than with carboplatin, to produce *cis*-$[Pt(NH_3)_2(H_2O)Cl]^+$ and *cis*-$Pt(NH_3)_2(OH)Cl$, which, at pH 7.4, are in a rapid proton equilibrium with one another. While both of these compounds can potentially react with components in media to produce carbonato species, the mono-hydroxo complex probably reacts with dissolved carbon dioxide within milliseconds (*26*) to give the monocarbonato compound, *cis*-$[Pt(NH_3)_2(CO_3^{-2})Cl]^-$ (*18*, *19*). The reaction is rapid because reaction of CO_2 with a hydroxo ligand does not require a metal−ligand bond-breaking step, which would be slow. For cisplatin, the initial amount of mono-aquo complex present in the clinical formulation, and the rapid rate of production of more mono-aquo complex from the dichloro compound, allow *cis*-$[Pt(NH_3)_2(CO_3^{-2})Cl]^-$ to rapidly build up and persist in the HSQC NMR spectrum in culture media for 20 h. The hydrolysis rate of carboplatin is much slower than that of cisplatin, so formation of a carbonato complex by reaction of carbon dioxide with a hydroxo species is not possible. As outlined above, compound **5** forms by the attack of carbonate ion on carboplatin, which is much slower than the route used by cisplatin to form a carbonato complex. Thus, the carboplatin ring-opened product is slow to form in culture media, making detection of the product by HSQC NMR difficult.

We have shown that, at physiological pH, **1** reacts with carbonate ion at concentrations found in culture media and blood to produce a small amount of ring-opened products, all of which are anions. If produced under conditions of therapy, they could be taken up by cells and distributed to various organs of the body. In the studies done in the presence of a high density of Jurkat cells, the rate of disappearance of carboplatin in media was the same as that in media without cells or in 23.8 mM carbonate buffer (pH 8.6) (Table 2 and Figure 8). Thus, the cells do not significantly affect the reaction of the medium with carboplatin, from which we infer that carbonate in the medium is mainly responsible for the disappearance of **1** even when cells are present. As expected, continued exposure of Jurkat cells to carboplatin results in significant loss of cell viability and after 6 h a significant fraction of the population has died.

An important parameter in the administration of Pt drugs to patients is the AUC, which is the product of the exposure time to the drug and the concentration of the drug in the extracellular fluid (*30*, *31*). In calculating AUC, attention is not normally paid to what forms of the drug are present in the extracellular fluid but only to the total Pt content. It seems more appropriate to use the extracellular concentrations of the forms of the drug that are taken up by the cell and responsible for the antitumor effects. Although the rate of reaction of carboplatin with carbonate is about 2 orders of magnitude slower than the corresponding rate for cisplatin with carbonate (*18*, *19*), the elimination rate of **1** from the body is significantly slower than that of cisplatin (*32*, *33*). Combined with the fact that the concentration of **1** normally used in therapy is an order of magnitude greater than that of cisplatin (*32*, *33*), this may mean that, in terms of carbonato complexes (which may be the species entering the cell and producing the cytotoxic effects), AUC values could be similar for **1** and **2**. To what extent the AUC values of the carbonato complexes of carboplatin and cisplatin in cell culture studies and therapy correlate with the toxicity and antitumor effects of these drugs will require further investigation.

In the commonly accepted mechanism of action of cisplatin, the drug enters the cell as the dichloro form, which, because the chloride concentration inside the cell is low, aquates to produce the more reactive mono-aquo complex (*34*, *35*). The latter reacts with many components of the cell, but its interaction with DNA is believed to be the important event leading to cell death (*36*). Recently, transport into the cell has been shown to possibly involve trans membrane proteins and direct binding to Pt (*37*, *38*). The mechanism of action of carboplatin has been less studied than that of cisplatin, but carboplatin is believed to enter the cell either unmodified or possibly modified in the extracellular fluid by some nucleophile that displaces (opens) the CBDCA chelate ring. Like cisplatin, carboplatin treatment leads to platination of DNA, suggesting that the drugs have a common mechanism for the induction of apoptosis (*39*).

Relevant to the cytotoxic effects of Pt−carbonato complexes are the reaction conditions existing in the blood and cytosol. The intracellular concentration of carbonate for cells carrying out normal metabolism is approximately the same as that in blood, ∼24 mM (*14*), so Pt−carbonato complexes could be stable in plasma and, once inside the cell, also in the cytosol. It is well-known that thiols such as GSH inside the cell readily react with Pt drugs, producing Pt−GSH adducts, with consequences that have been widely discussed (*40*). Although GSH and other thiols are present in culture media, they seem to have little effect on the survival of *cis*-$[Pt(NH_3)_2(CO_3^{-2})(Cl)]^-$ in media (*18*). As was earlier suggested, this could be because thiols in media exist in disulfide form, which is less reactive toward Pt. An additional factor may be that, because **5**−**7** and *cis*-$[Pt(NH_3)_2(CO_3^{-2})(Cl)]^-$ are anions, attack by a thiolate, also an anion, is not favored, making the rate of Pt−S bond formation slower (*3*). Thus, anionic Pt−carbonato species could have longer than expected half-lives in plasma and the cytosol, giving them more time to produce their biological effects. Work in progress will determine if and to what extent the ring-opened carboplatin compounds are toxic to cells.

A central question concerning the carbonates formed by carboplatin and cisplatin is their potential to interact with nuclear DNA. In 1984, Fichtinger-Shepman et al. (*41*) reported that 20 mM $NaHCO_3$ reduces the amount of Pt bound to salmon sperm DNA to ∼one-third of that found when no buffer is present in the medium. Because cisplatin forms carbonato species in carbonate buffer (*18*, *19*), carbonato complexes can bind to DNA. Although the parallel study for carboplatin has not been reported, the results summarized in Scheme 2 suggest that carboplatin would behave similarly to cisplatin and that carboplatin carbonato compounds could also bind to DNA.

Clearly, characterizing the forms of the Pt drugs existing in biological media, the mechanisms by which they enter the cell, and the cellular sites of action are critical for understanding the antitumor effects and toxicities of these important drugs. We hope that this report will stimulate interest in the role of carbonate in the molecular mechanism of action of Pt anticancer drugs.

Conclusions

In this report, we show that carbonate ion at concentrations found in culture medium and blood readily displaces one arm of the CBDCA ligand of carboplatin to form a ring-opened product having bound carbonate. Using UV-visible absorption spectroscopy, we show that carboplatin reacts with carbonate in the pH range of 6.7−9.3, with the rate of the reaction and the nature of the product dependent on pH. Analysis of the UV-visible and NMR spectral data reveals that the compound formed at slightly basic pH is a ring-opened product having monodentate carbonate. We also show that the pK_b for protonation of this species is ∼8.6, so that reaction of carboplatin with carbonate at physiological pH produces a mixture of the carbonato and bicarbonato ring-opened products and, because the latter can spontaneously decarboxylate, the analogous hydroxo species may be present as well. An important similarity between cisplatin and carboplatin is that both drugs react with biologically relevant concentrations of carbonate to give negatively charged species. Because molecular charge affects biodistribution and reactivity of compounds in vivo, the existence of Pt−carbonato species under physiological conditions may help explain the uptake, antitumor properties, and toxicities of these important Pt drugs.

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References

(1) Boulikas, T., and Vougiouka, M. (2003) Cisplatin and platinum drugs at the molecular level. *Oncol. Rep. 10*, 1663−1682.

(2) Knox, R. J., Friedlos, F., Lydall, D. A., and Roberts, J. J. (1986) Mechanism of cytotoxicity of anticancer platinum drugs: Evidence that *cis*-diamminedichloroplatinum(II) and *cis*-diammine-(1,1-cyclobutanedicarboxylato) platinum(II) differ only in the kinetics of their interaction with DNA. *Cancer Res. 46*, 1972−1979.

(3) Housecroft, C. E., and Sharpe, A. G. (2004) *Inorganic Chemistry*, 2nd ed., Pearson Education Limited, Essex, England.

(4) Canovese, L., Cattalini, L., Chessa, G., and Tobe, M. L. (1988) Kinetics of the displacement of cyclobutane-1,1-dicarboxylate from diammine-(cyclobutane-1,1-dicarboxylato)platinum(II) in aqueous solution. *J. Chem. Soc., Dalton Trans.* 2135−2140.

(5) Kleine, M., Wolters, D., and Sheldrick, W. S. (2003) The influence of methionine-containing peptides on the reaction of carboplatin with 5′-guanosine monophosphate: A comparison with cisplatin. *J. Inorg. Biochem. 97*, 354−363.

(6) Barnham, K. J., Djuran, M. I., Murdoch, P., Ranford, J. D., and Sadler, P. J. (1996) Ring-opened adducts of the anticancer drug carboplatin with sulfur amino acids. *Inorg. Chem. 35*, 1065−1072.

(7) Peleg-Shulman, T., Najajreh, Y., and Gibson, D. (2002) Interaction of cisplatin and transplatin with proteins. Comparison of binding kinetics, binding sites and reactivity of the Pt-protein adducts of cisplatin and transplatin towards biological nucleophiles. *J. Inorg. Biochem. 91*, 306−311.

(8) Reedijk, J. (1999) Why does cisplatin reach guanine-N7 with competing S-donor ligands available in the cell? *Chem. Rev. 99*, 2499−2510.

(9) Frey, U., Ranford, J. D., and Sadler, P. J. (1993) Ring-opening of the anticancer drug carboplatin: NMR characterization of *cis*-[Pt(NH_3)$_2$-(CBDCA-O)(5′-GMP-N7)] in solution. *Inorg. Chem. 32*, 1333−1340.

(10) Cheong, J. H., Bannon, D., Olivi, L., Kim, Y., and Bressler, J. (2004) Different mechanisms mediate uptake of lead in a rat astroglial cell line. *Toxicol. Sci. 77*, 334−340.

(11) Guo, M., Sun, H., McArdle, H. J., Gambling, L., and Sadler, P. J. (2000) Ti^{IV} uptake and release by human serum transferring and recognition of Ti^{IV}-transferrin by cancer cells: Understanding the mechanism of action of the anticancer drug titanocene dichloride. *Biochemistry 39*, 10023−10033.

(12) Endo, T., Kimura, O., and Sakata, M. (1998) Bidirectional transport of cadmium across apical membrane of renal epithelial cell lines via H^+-antiporter and inorganic anion exchanger. *Toxicology 131*, 183−192.

(13) Acharya, A. N., Das, A. C., and Dash, A. C. (2004) Carbonato complexes: Models for carbonic anhydrase. *Adv. Inorg. Chem. 55*, 127.

(14) Gross, E., and Kurtz, I. (2002) Structural determinants and significance of regulation of electrogenic Na^+-HCO_3^- cotransporter stoichiometry. *Am. J. Physiol. Renal Physiol. 283*, F876−F887.

(15) Mahal, G., and van Eldik, R. (1985) Kinetics and mechanism of formation, aquation, and base hydrolysis reactions of a series of monodentate carbonato complexes of palladium(II). *Inorg. Chem. 24*, 4165.

(16) Palmer, D. A., van Eldik, R., and Harris, G. M. (1980) Kinetics and mechanism of aquation and formation reaction of carbonato complexes. 17. Carbon dioxide uptake by and decarboxylation of the cis-bis-(ethylenediamine)rhodium(III) system in aqueous solution. *Inorg. Chem. 19*, 1009.

(17) Mauldin, S. K., Plescia, M., Richard, F. A., Wyrick, S. D., Voyksners, R. D., and Chaney, S. G. (1988) *Biochem. Pharmacol. 37*, 3321−3333.

(18) Tacka, K. A., Szalda, D., Souid, A.-K., Goodisman, J., and Dabrowiak, J. C. (2004) Experimental and theoretical studies on the pharmacodynamics of cisplatin in Jurkat cells. *Chem. Res. Toxicol. 17*, 1434−1444.

(19) Centerwall, C. R., Goodisman, J., Kerwood, D. J., and Dabrowiak, J. C. (2005) Cisplatin carbonato complexes. Implications for uptake, antitumor effects, and toxicity. *J. Am. Chem. Soc. 127*, 12768−12769.

(20) Boreham, C. J., Broomhead, J. A., and Fairlie, D. P. (1981) A ^{195}Pt and ^{15}N NMR study of the anticancer drug, cis-diamminedichloroplatinum(II), and its hydrolysis and oligomerization products. *Aust. J. Chem. 34*, 659−664.

(21) Allison, D. C., and Riddolpho, P. (1980) Use of trypan blue assay to measure the deoxyribonucleic acid content and radioactive labeling of viable cells. *J. Histochem. 28*, 700−703.

(22) Berners-Price, S. J., and Appleton, T. G. (2000) In *Platinum Based Drugs in Cancer Chemotherapy* (Kelland, L. R., Farrell, N., Eds.) pp 3−35, Humana Press Inc., Totowa, NJ.

(23) Berners-Price, S. J., Frenkiel, T. A., Frey, U., Ranford, J. D., and Sadler, P. J. (1992) Hydrolysis products of cisplatin: pK_a determination via [^{1}H,^{15}N] NMR spectroscopy. *J. Chem. Soc. Chem Commun.*, 789−791.

(24) Hay, R. W., and Miller, S. (1998) Reactions of platinum(II) anticancer drugs. Kinetics of acid hydrolysis of cis-diammine(cyclobutane-1,1-dicarboxylato)platinum(II) "Carboplatin". *Polyhedron 17*, 2337−2343.

(25) Mao, Z.-W., Liehr, G., and van Eldik, R. (2001) Structural and mechanistic information on the reaction of bicarbonate with Cu(II) and Zn(II) complexes of tris(2-aminoethyl)amine. Identification of intermediate and product species. *J. Chem. Soc., Dalton Trans.*, 1593−1600.

(26) Palmer, D. A., and van Eldik, R. (1983) The chemistry of metal carbonato and carbon dioxide complexes. *Chem. Rev. 83*, 651−731.

(27) Allsopp, M. A., Sewell, G. J., Rowland, C. G., Riley, C. M., and Schowen, R. L. (1991) The degradation of carboplatin in aqueous solutions containing chloride or other selected nucleophiles. *Int. J. Pharm. 69*, 197−210.

(28) Mao, Z.-W., Liehr, G., and van Eldik, R. (2000) Isolation and characterization of the first stable bicarbonato complexes of bis(1,-10-phenanthroline)copper(II). Identification of Lipscomb- and Lindskog-like intermediates. *J. Am. Chem. Soc. 122*, 4839−4840.

(29) Silverman, D. N., and Lindskog, S. (1988) The catalytic mechanism of carbonic anhydrase: Implications of a rate-limiting protolysis of water. *Acc. Chem. Res. 21*, 30−36.

(30) Ozawa, S., Suglyama, Y., Mitsuhashi, Y., Kobayashi, T., and Inada, M. (1988) Cell killing action of cell cycle phase-nonspecific antitumor agents is dependent on concentration−time product. *Cancer Chemother. Pharmacol. 21*, 185−190.

(31) Calvert, A. H., Newell, D. R., Gumgrell, L. A., O'Reilly, S., Burnell, M., Boxall, F. E., Siddik, Z. H., Judson, I. R., Gore, M. E., and Wiltshaw, E. Carboplatin dosage: Prospective evaluation of a simple formaula based on renal function. *J. Clin. Oncol. 20*, 3533−3544.

(32) Van der Vijgh, W. J. (1991) Clinical pharmacokinetics of carboplatin. *Clin. Pharmacokinet. 21*, 241−261.

(33) Duffull, S. B., and Robinson, B. A. (1997) Clinical pharmacokinetics and does optimization of carboplatin. *Clin. Pharmacokinet. 33*, 161−183.

(34) Rosenberg, B. (1971) Some biological effects of platinum compounds. *Platinum Met. Rev. 15*, 42.

(35) Drobník, J., and Horáček, P. (1973) Specific biological activity of platinum complexes. Contribution to the theory of molecular mechanism. *Chem-Biol. Interact. 7*, 223.

(36) Wang, D., and Lippard, S. J. Cellular processing of platinum anticancer drugs. *Nat. Rev. Drug Discovery 4*, 307.
(37) Samimi, G., Safaei, R., Katano, K., Holzer, A. K., Rochdi, M., Tomioka, M., Goodman, M., and Howell, S. B. (2004) Increased expression of the copper efflux transporter ATP7A mediates resistance to cisplatin, carboplatin, and oxaliplatin in ovarian cancer cells. *Clin. Cancer Res. 10*, 4661−4669.
(38) Katano, K., Safaei, R., Samimi, G., Holzer, A., Tomioka, M., Goodman, M., and Howell, S. B. (2004) Confocal microscopic analysis of the interaction between cisplatin and the copper transporter ATP7B in human ovarian carcinoma cells. *Clin. Cancer Res. 10*, 4578−4588.
(39) Bloemink, M. J., and Reedijk, J. (1996) Cisplatin and derived anticancer drugs: Mechanism and current status of DNA binding. *Met. Ions Biol. Syst. 32*, 641−685.
(40) Siddik, Z. H. (2002) Biochemical and molecular mechanisms of cisplatin resistance. *Cancer Treat. Res. 112*, 263−284.
(41) Fichtinger-Schepman, A. M., vd Veer, J. L., Lohman, P. H., and Reedijk, J. (1984) A simple method fro the inactivation of monofunctionally DNA-bound cis-diamminedichloroplatinum (II). *J. Inorg. Biochem. 21*, 103−112.

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