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# Role of Carbonate in the Cytotoxicity of Carboplatin

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Carboplatin, $[Pt(NH_3)_2(CBDCA\text{-}O,O')]$, **1**, where CBDCA is cyclobutane-1,1-dicarboxylate, is used against ovarian, lung, and other types of cancer. We recently showed (Di Pasqua et al. (2006) *Chem. Res. Toxicol. 19*, 139−149) that carboplatin reacts with carbonate under conditions that simulate therapy to produce carbonato carboplatin, *cis*-$[Pt(NH_3)_2(O\text{-}CBDCA)(CO_3)]^{2-}$, **2**. We use $^{13}C$ and $^{1}H$ NMR and UV−visible absorption spectroscopy to show that solutions containing carboplatin that have been aged in carbonate buffer under various conditions contain **1**, **2**, and other compounds. We then show that aging carboplatin in carbonate produces compounds that are more toxic to human neuroblastoma (SK-N-SH), proximal renal tubule (HK-2) and Namalwa-luc Burkitt's lymphoma (BL) cells than carboplatin alone. Moreover, increasing the aging time increases the cytotoxicity of the platinum solutions as measured by the increase in cell death. Although HK-2 cells experience a large loss in survival upon exposure to carbonato forms of the drug, they have the highest values of $IC_{50}$ of the three cell lines studied, so that HK-2 cells remain the most resistant to the toxic effects of the carbonato forms in the culture medium. This is consistent with the well-known low renal toxicity observed for carboplatin in therapy. The uptake rates for normal Jurkat cells (NJ) and cisplatin-resistant Jurkat cells (RJ), measured by inductively coupled plasma mass spectrometry (ICP-MS), are 16.6 ± 4.2 and 12.3 ± 4.8 amol of Pt $h^{-1}$ $cell^{-1}$, respectively, when exposed to carboplatin alone. However, when these cells are exposed to carboplatin that has been aged in carbonate media, normal Jurkat cells strongly bind/take up Pt at a rate of 14.5 ± 4.1 amol of Pt $h^{-1}$ $cell^{-1}$, while resistant cells strongly bind/take up 5.1 ± 3.3 amol of Pt $h^{-1}$ $cell^{-1}$. Collectively, these studies show that carboplatin carbonato species may play a major role in the cytotoxicity and uptake of carboplatin by cells.

## Introduction

Platinum drugs by themselves or in combination with other agents are used to treat many types of human cancer (*1*−*5*). These remarkable compounds are believed to exert their biological effects by interacting with genomic DNA, but other cellular targets may be attacked as well (*6*). Although research on the platinum drugs spans nearly four decades (*7*, *8*), the forms of the agents that circulate in the blood, the mechanism by which they enter the cell, and the main events that lead to drug-induced cell death are not known with certainty.

Carboplatin,[1] $[Pt(NH_3)_2(CBDCA\text{-}O,O')]$, where CBDCA is cyclobutane-1,1-dicarboxylate, **1**, Scheme 1, is a second-generation $Pt^{2+}$ anti-cancer drug, which is in wide clinical use for the treatment of ovarian, lung, and other types of cancer (*1*). Carboplatin contains a bidentate dicarboxylate chelate as a leaving ligand, which makes the drug much less reactive than its close analogue cisplatin, *cis*-$Pt(NH_3)_2Cl_2$, which contains two monodentate chlorides as leaving ligands. The reduced reactivity of **1** compared to cisplatin can easily be seen in the different hydrolysis rates of the two compounds. At neutral pH at 37 °C, the rate constant for the reaction of **1** with water, which involves displacement of one arm of the CBDCA chelate ring, is $k \approx 5 \times 10^{-7}$ $s^{-1}$, although smaller values of the hydrolysis rate constant have also been reported (*9*−*12*). By comparison, the rate constant for displacement of one of the chloro ligands of cisplatin with water at 35.5 °C is $1.8 \times 10^{-4}$ $s^{-1}$ (*13*), which is more than 2 orders of magnitude larger than for **1**. While it is easy to see that the substitution kinetics of cisplatin are fast enough to allow the drug to react with water and biological components under the conditions of therapy, the slower substitution kinetics of carboplatin have prompted investigators to focus on mechanisms by which the compound can be activated in vivo. Activation would involve the complete or partial displacement of the CBDCA ligand of **1** to give a product that is competent to react with DNA and other biological targets.

Since sulfur in a thiolate or thioether is considered a soft base and $Pt^{2+}$ is a soft acid (*14*), investigations have focused on the ability of cysteine and methionine, or peptides and proteins containing these amino acids, to react with carboplatin (*15*−*20*). As expected, these nucleophiles displace the CBDCA ligand at rates that are much larger than that for water to give a variety of different products. For example reaction of **1** with methionine proceeds with a second-order rate constant of $k = 2.7 \times 10^{-3}$ $M^{-1}$ $s^{-1}$ to initially give a ring-opened product having a monodentate CBDCA ligand and S-bonded methionine. This product is surprisingly stable to methionine chelate ring

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[1] Abbreviations: carboplatin, *cis*-diammine(cyclobutane-1,1-dicarboxylato)platinum(II), $Pt(NH_3)_2(CBDCA\text{-}O,O')$; CBDCA, cyclobutane-1,1-dicarboxylate; cisplatin, *cis*-diamminedichloroplatinum(II); $D_2O$, deuterium oxide; FBS, fetal bovine serum; PBS, phosphate-buffered saline; amol, attomole or $10^{-18}$ mol; $a_C$, activity of $CO_3^{2-}$; $\gamma_C$, activity coefficient of $CO_3^{2-}$.

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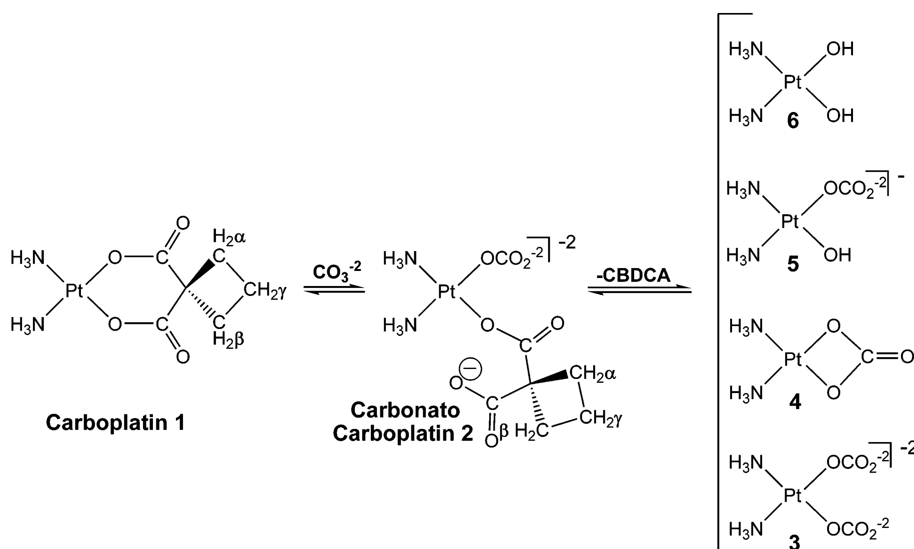
**Scheme 1. Reaction of Carboplatin in Carbonate Buffer**




closure and loss of CBDCA (*15*). Most studies have shown that the rate of reaction of carboplatin with nucleotides and purified polymeric DNA is slow, strongly suggesting that some form of activation is necessary if DNA platination is to occur under conditions of therapy (*21*, *22*). However, in the presence of sulfur-containing nucleophiles, the rate of binding of carboplatin to DNA is enhanced and the nature of the products formed is different than in the absence of these nucleophiles (*21*, *22*). A recent study by Henderson and co-workers (*23*) applied accelerator mass spectrometry to show that the rate of reaction of carboplatin with purified polymeric DNA is faster than the rate of reaction of the drug with dGMP (*12*), suggesting that that the drug may not require activation for facile reaction with DNA.

Culture media, blood, and the cytosol contain high concentrations of carbonate, which, because it is a good ligand for transition metal ions (*24*, *25*), may play an important role in the mechanism of action of carboplatin and other platinum drugs. Carbonate at pH 7.4 is mostly in the form of bicarbonate, $HCO_3^-$, but this form is in equilibrium with carbonate ($CO_3^{2-}$), carbonic acid ($H_2CO_3$), and dissolved $CO_2$. Extensive work has shown that carbonato complexes can form by the rapid addition of carbon dioxide to a metal hydroxo species or by a slower route involving ligand displacement by carbonate or bicarbonate (*24*).

Carbonato complexes, which were first reported by Werner and Goslings in 1903 (*26*), are known for a large number of metal ions (*24*, *25*, *27*) including $Pt^{2+}$. Complexes of the latter can be mononuclear with bidentate carbonate (*28*−*34*) or dinuclear with bridging carbonate (*35*) usually with phosphine ligands (*28*−*32*, *35*). However, 1,2-diaminocyclohexane (DACH) (*33*, *34*), the nonleaving ligand in the drug oxaliplatin (*3*), has also been incorporated into platinum carbonato complexes. These compounds can be made by metathesis reactions involving a *cis*-dichloro complex and silver carbonate (*30*, *33*, *34*), addition of carbon dioxide to hydrido (*28*), hydroxo (*29*, *35*), or dioxygen (*32*) species, or hydrolysis of *N*,*N*-diethylcarbamato compounds.[31] An unusual *cyclo*-tetranuclear complex of $Pt^{2+}$ having bridging carbonates and *cis*-ammonia molecules has also been reported to form as a minor product in the reaction of cisplatin with 2′-deoxyuridine (*36*). Although the mechanism by which the tetranuclear complex is formed is not known, atmospheric $CO_2$ is probably involved in the process (*29*). While square planar complexes of $Pd^{2+}$ having bound water readily form carbonato complexes in aqueous carbonate solutions (*37*) the corresponding complexes of $Pt^{+2}$ have been reported to be unreactive under the same conditions (*38*).

Ready-to-use carboplatin infusion solutions have a typical shelf life of ∼14 months during which time, depending on the storage temperature, a few percent is hydrolyzed to the ring-opened complex (*39*). Since blood contains ∼24 mM carbonate, 2.1 mM of which is aquated $CO_2$ at pH 7.4 (*24*), infusion of the drug solution into blood could result in the rapid formation of small amounts of monocarbonato species by reaction of a carboplatin aquo/hydroxo compound with dissolved $CO_2$. By comparison the ready-to-use cisplatin infusion solution, for example, Platinol, contains ∼5% aquated forms, mostly as the monoaquo complex (*13*). As was earlier shown by us (*9*), carbonate ion ($CO_3^{2-}$) accelerates the rate of disappearance of carboplatin in RPMI-1640 tissue culture medium, suggesting that carbonate could be activating the drug in therapy.

In 1988 Chaney and co-workers (*40*) showed that bicarbonate present in tissue culture medium could displace the malonate chelate ring of a $Pt^{2+}$ antitumor agent, suggesting that this may be a pathway to biologically active aquo platinum complexes. Although the products formed were analyzed by HPLC, carbonato species were not detected in the analysis. Lack of detection may have been due to the fact that the HPLC mobile phase was acidic, thus accelerating the destruction of any carbonato species formed in the reaction medium (*24*).

Remarkably, in nearly 40 years of physicochemical work on the platinum drugs, with a few exceptions (*40*−*42*), carbonate has not been present in the reaction medium. In attempting to uncover the possible role of carbonate in the mechanism of action of the platinum drugs, we found that a cisplatin−carbonato complex forms in tissue culture medium which contains carbonate (*43*), that the presence of carbonate in a binding buffer dramatically affects the type of cisplatin lesion which forms on DNA (*44*), and that Jurkat cells are able to selectively modify a cisplatin carbonato complex by an extracellular mechanism (*45*). In this report we further study the formation of carbonato complexes of carboplatin that form under low (23.8 mM) and high (0.5 M) carbonate conditions by $^{13}C$ and $^{1}H$ NMR and UV−visible spectroscopy. We also examine the cytotoxicities of both carboplatin and mixtures of **1** and carbonato species formed from the drug against neuroblastoma (SK-N-SH), proximal renal tubule (HK-2), and Namalwa-luc Burkitt's lymphoma (BL) cells. Since carbonato complexes may be present in therapy, we treat normal and cisplatin-resistant

Jurkat cells with solutions containing carbonato complexes and measure the rate at which the cells are able to take up and/or strongly bind platinum by inductively coupled plasma mass spectrometry (ICP-MS). The results of the study are consistent with the hypothesis that carbonato complexes of carboplatin can form during therapy and be the species responsible for the antitumor effects of the drug in vivo.

## Experimental Section

**Materials.** Carboplatin (10 mg/mL, 26.9 mM in water) was obtained from Bedford Laboratories (Bedford, OH) and used well before its expiration date and no later than 14 days after the initial injection of a needle into the septum-topped formulation bottle. Culture medium, minimum essential medium with Earle's salts and L-glutamine (EMEM) (10-010), RPMI-1640 (15-040), and fetal bovine serum (FBS) were from Mediatech (Herndon, VA). Keratinocyte-SFM (17005−042) was from Gibco Invitrogen Corp. (Carlsbad, CA). Cell counting kit 8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD). Carboplatin, $NaHCO_3$ (>99%), $NaH^{13}CO_3$ (98% enrichment), and $D_2O$ (99.9%) were purchased from Sigma−Aldrich (St. Louis, MO). $HClO_4$ (60%) was purchased from Fisher Scientific (Pittsburgh, PA).

**$^{13}$C NMR.** For $^{13}$C NMR, carboplatin (5 mM) was reacted in aqueous $NaH^{13}CO_3$, referred to as "aging", in either aqueous 23.8 mM carbonate at pH 8.4 for 45 h (solution 1), 0.5 M carbonate at pH 8.6 for 45 h (solution 2), or 0.5 M carbonate at pH 8.6 for 20 h (solution 3). After the aging period, 92 $\mu$L of $D_2O$ was added to 828 $\mu$L of each solution, solutions 1 and 2 were adjusted to pH 7.4 with $HClO_4$, and the resulting mixtures were introduced into plastic-capped 5 mm NMR tubes. Each NMR solution was immediately cooled to 4 °C in the probe of a Bruker Avance 500 NMR spectrometer to slow any reactions that were occurring, and $^{13}$C NMR data on the sample were collected. After the NMR experiment, the pH values of solutions 1 and 2 were 7.5 and 8.1, respectively. A one-pulse sequence with proton decoupling was used to acquire $^{13}$C NMR data. The spectral width was 220 ppm and the total relaxation period was 10 s. The free induction decay (FID) was Fourier-transformed by use of an exponential window function with line-broadening parameter set at 8 Hz. A one-pulse sequence with proton decoupling and presaturation of the bicarbonate peak was used to acquire the carbon spectrum of solution 3. The spectral width was 325 ppm with the offset on the bicarbonate peak at 160 ppm. The total relaxation period was 10 s with 2 s used for presaturation. The $^{13}CO_2$(aq) resonance at 125.3 ppm was used as an internal chemical shift standard.

**$^{1}$H NMR.** The solution for the $^{1}$H NMR spectra was prepared by reacting 5 mM **1** in aqueous 0.5 M $NaHCO_3$, pH 8.6, for 20 at 37 °C. After this time, an aliquot of 92 $\mu$L of $D_2O$ was added to 828 $\mu$L of the aged solution and a $^{1}$H NMR spectrum, at 37 °C, was obtained on a Bruker Avance 600 spectrometer. The $\alpha$ and $\beta$ methylene protons of the cyclobutane ring of carboplatin at 2.88 ppm (triplet, 4H, $^{3}J_{H-H} = 8.0$ Hz) were used as an internal chemical shift standard (*9*, *12*).

**UV-Visible Absorption Spectroscopy**. UV−visible absorption spectra were obtained on a Cary 50 spectrophotometer in a 1 cm path length cell at room temperature. A solution containing 5 mM carboplatin in 0.5 M $NaHCO_3$, pH 8.6, was incubated at 37 °C. At 0, 1.5, 22, and 45 h, a 300 $\mu$L aliquot of solution was removed and added to 2.7 mL of water, and its spectrum was recorded in the wavelength range of 500−190 nm (60 nm/min). The final concentrations of carboplatin and carbonate buffer in the spectrophotometer cell were 500 $\mu$M and 50 mM, respectively. A 50 mM carbonate buffer solution was used as a baseline. Difference spectra were obtained by subtracting the spectrum obtained immediately after addition of **1** to the buffer ($t = 0$ spectrum) from spectra taken at 1.5, 22, and 45 h. Each difference spectrum was fitted to a sum of Gaussians by use of PeakFit (v. 4) to obtain the smoothed difference spectrum (*9*).

After ~45 h, a small amount (<1%) of a dark insoluble material formed in the solutions containing 5 mM **1** and 0.5 M carbonate. This material, which could be removed by centrifugation, caused a slight monotonic increase in absorbance toward the blue region of the spectrum. This behavior, which is consistent with light scattering from suspended particulate matter, suggests that at long times a small amount of elemental platinum, $Pt^0$, is forming in the reaction mixture.

**Neuroblastoma and Proximal Renal Tubule Cell Studies.** Cell studies were carried out under standard conditions in a humidified, 37 °C, 5% $CO_2$ atmosphere. Human neuroblastoma (SK-N-SH) cells in culture medium (EMEM + 10% FBS, 100 $\mu$g/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM L-glutamine) and human proximal renal tubule (HK-2) cells in serum-free culture medium (Keratinocyte-SFM) were plated in 96-well plates, with each well containing ~6 × $10^3$ cells. The viabilities of SK-N-SH and HK-2 cells, determined prior to plating by light microscopy with a hemacytometer under standard trypan blue conditions (*46*), were 96% and 93%, respectively. Each experiment involved one control plate and three identical experimental plates. After being plated, the SK-N-SH and HK-2 cells, which were studied at different times, were given 24 h to adhere to the bottom of the wells. A control plate, which typically consisted of 12 wells of medium without cells and 12 wells of medium with cells (no drug), was used to determine the number of live cells at the beginning of an experiment ($t = 0$ was determined after the 24 h adhering time). Each experimental plate consisted of 6 wells containing medium without cells, 6 wells containing medium with cells, and a number of wells containing cells to which medium containing drug at a specific concentration/condition (≥50 $\mu$M) was added. The drug conditions for SK-N-SH cells were **1** aged in 23.8 mM or 0.5 M $NaHCO_3$ at 37 °C, pH 8.4 or 8.6, for 0 (**1**), 1.5, 22, or 45 h, while the conditions for HK-2 cells were **1** aged in 23.8 mM $NaHCO_3$ at 37 °C, pH 8.4, for 0 (**1**), 22, and 45 h.

The number of live cells in the wells of the control plate at the beginning of a particular experiment was determined by use of the cell counting kit, CCK-8 (Dojindo Molecular Technologies, Inc., Gaithersburg, MD). In this assay, 10 $\mu$L of a solution containing a water-soluble tetrazolium salt is added to each well of the control plate. If a specific cell is viable, the tetrazolium salt is absorbed by the cell and bioreduced to a formazan dye, which diffuses into the medium. After 1 h, the concentration of the formazan dye in the well, which is proportional to the number of live cells in the well, is determined by measuring the absorbance of the medium in the wells at 450 nm on a luminescence plate reader (Dynex Technologies). In order to obtain the concentration (absorbance) of the dye produced by live cells at the start of the experiment, $t = 0$ h, the absorbance at 450 nm of wells in the control plate without cells was subtracted from the absorbance of wells in the control plate having cells. Meanwhile, the medium above the cells in each experimental plate was replaced with drug-containing medium at a specific concentration/condition (6 wells per concentration/condition per plate). After 2 h, the drug-containing medium above the cells was removed, cells were washed with culture medium without drug, fresh medium was applied, and the cells were allowed to grow for 48 h. At the end of this period, the number of live cells was determined by the method described above.

Average $IC_{50}$ values, with standard deviations, were determined by fitting individual data sets (done on different days) to exponential curves ($R^2 = 0.95 \pm 0.04$ and $0.86 \pm 0.09$ for SK-N-SH and HK-2 cell studies, respectively) and averaging experiments. The neuroblastoma cells used in this experiment had an average doubling time of ~30 h. The proximal tubule cells had an average doubling time of ~33 h. In solutions containing carboplatin aged in 0.5 M carbonate buffer for 45 h, dark particulate material ($Pt^0$) could be recovered. This material was removed by centrifugation prior to drug incubation, but control experiments showed that the material is not toxic to cells.

**Namalwa-luc Burkitt's Lymphoma Cell Studies.** The Namalwa-luc Burkitt's lymphoma cell line, which expresses the luciferase gene under the control of the CMV promoter, was a gift from Dr.

Rosemary Rochford, SUNY Upstate Medical University (Syracuse, NY). Namalwa-luc Burkitt's lymphoma cell studies were carried out under standard conditions in a humidified, 37 °C, 5% $CO_2$ atmosphere. For each study, five 25 $cm^2$ flasks were prepared, each containing ~4 × $10^5$ cells/mL (94% viable) in 10 mL of culture medium (RPMI + 10% FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM L-glutamine). After a 2 h exposure of the cells in four of the flasks to various concentrations (≤75 μM) of either carboplatin or carboplatin aged in 0.5 M carbonate at 37 °C, pH 8.6, for 0 (**1**) or 22 h, the cells in all five flasks were washed twice by sedimenting the cells for 5 min at 450*g*, removing the medium, and resuspending the cells in 10 mL of fresh medium. After two washes and a 1:1 dilution with culture medium, 3 mL of each of the five conditions were placed into culture tubes with each tube containing ~1.6 × $10^5$ viable cells/mL. Cells were then allowed to grow for 48 h, after which time the number of viable cells in each culture tube was measured by use of trypan blue (*46*). Average $IC_{50}$ values were determined by fitting each data set to an exponential curve ($R^2 = 0.86 \pm 0.09$). The BL cells used in this experiment had an average doubling time of ~57 h.

**Platinum Uptake by Jurkat Cells and Cisplatin-Resistant Jurkat Cells.** Six samples, each containing 3.5 × $10^6$ cells suspended in 896 μL of culture medium (RPMI + 10% FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 2.0 mM L-glutamine), were used in each uptake study, and both normal Jurkat (NJ) and cisplatin-resistant Jurkat (RJ) cell lines were studied. The RJ cell line was a gift from Mr. Corey Centerwall, Syracuse University (Syracuse, NY). The NJ and RJ cells were ~92% and ~98% viable, respectively, at the beginning of each experiment. Viabilities were determined by light microscopy with a hemacytometer and trypan blue. After 5 h in a sealed tube, the viabilities of NJ and RJ cells were ~64% and ~90% viable, respectively. To each sample, 24 μL of either 5 mM **1** or 5 mM **1** aged in 23.8 mM $NaHCO_3$ pH 8.4, for 45 h at 37 °C was added, giving a final platinum concentration of 130 μM. Five samples of each condition were placed in capped Eppendorf tubes and placed in a 37 °C incubator, while the cells in one tube were immediately pelleted by centrifugation for 3 min at 200*g*. After centrifugation, culture medium was removed and cells were resuspended in 1 mL of phosphate-buffered saline (PBS). The cells were pelleted a second time by centrifugation, the PBS was removed, and the cells were digested for 48 h at 70 °C in 0.5 mL of 70% (v/v) nitric acid/water. This procedure was repeated every hour for a total of 5 h on the cells exposed to drug. The nitric acid solutions of digested cells were analyzed for platinum by ICP-MS (Department of Engineering, Syracuse University). The uptake rate per cell, in attomoles of Pt per hour per cell, was calculated from the total number of viable cells at $t = 0$ (3.5 × $10^6$) and the number of moles of platinum that each cell removed from the culture medium per hour.

## Results

As was earlier shown by us (*9*), the attacking nucleophile for the ring-opening reaction of carboplatin to give carbonato−carboplatin, **2**, is carbonate ion (Scheme 1). Once formed, **2** could produce a number of possible products, four of which are shown in Scheme 1. Because the species in the scheme are in equilibrium with each other through the addition or loss of $CO_2$ and the carbonato species can be protonated to their respective bicarbonato forms, which could either remain in solution or rapidly dicarboxylate to Pt aquo/hydroxo compounds, isolation of pure carbonato compounds was exceedingly difficult. After repeated unsuccessful attempts to isolate materials, we decided to generate carbonate species in solution by the reaction of **1** in carbonate buffer and determine the species present in the aged medium by use of spectroscopy. These solutions were then used to treat cells and study platinum uptake.

**$^{13}$C NMR.** $^{13}$C NMR spectra of carboplatin that has been aged in carbonate buffer under various conditions are shown in Figure 1. Although aging, that is, letting **1** react in carbonate medium, was carried out at 37 °C, the actual $^{13}$C NMR spectral data were collected at 4 °C. The lower temperature slowed the reaction kinetics, which helped improve the quality of spectra during the lengthy (20−40 h) NMR data collection period. Aging carboplatin (5 mM) in 23.8 mM $NaH^{13}CO_3$ at pH 8.4 or

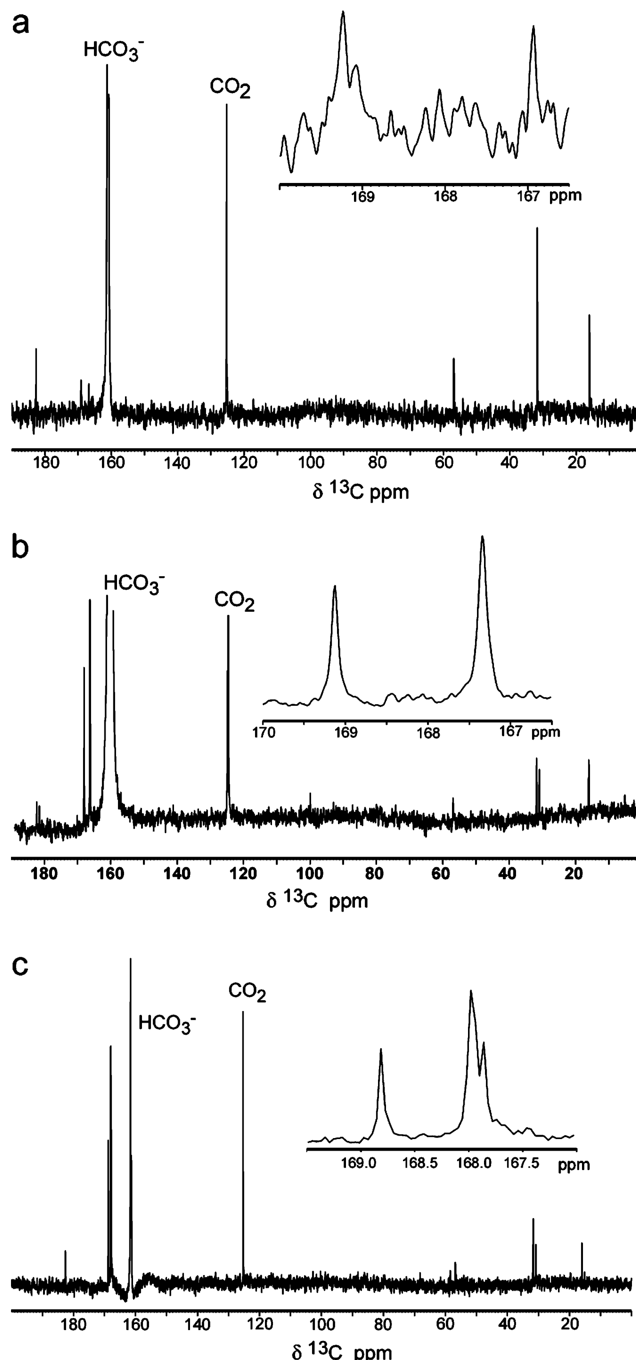



**Figure 1.** (a) $^{13}$C NMR spectrum of a solution containing 5 mM carboplatin aged in 23.8 mM $NaH^{13}CO_3$, pH 8.4, for 45 h at 37 °C. The spectrum shown was obtained after 10% $D_2O$ was added and the pH was adjusted to 7.4, at 4 °C. The final solution contained 4.5 mM **1** in 21.4 mM $NaH^{13}CO_3$. (b) $^{13}$C NMR spectrum of a solution containing 5 mM carboplatin aged in 0.5 M $NaH^{13}CO_3$, pH 8.6, for 45 h at 37 °C. The spectrum shown was obtained after 10% $D_2O$ was added and the pH was adjusted to 7.4, at 4 °C. The final solution contained 4.5 mM **1** in 0.45 M $NaH^{13}CO_3$. (c) $^{13}$C NMR spectrum of a solution containing 5 mM carboplatin aged in 0.5 M $NaH^{13}CO_3$, pH 8.6, for 20 h at 37 °C. The spectrum shown was obtained after the addition of 10% $D_2O$ at 4 °C. The final solution contained 4.5 mM **1** in 0.45 M $NaH^{13}CO_3$.

in 0.5 M $NaH^{13}CO_3$ at pH 8.6, for 45 h at 37 °C, followed by adjusting the pH of the solution to 7.4 and collecting NMR data at low temperature, gave the spectra shown in Figure 1a,b. Figure 1a shows two strong peaks at 161.2 and 125.3 ppm that are due to $HCO_3^-/CO_3^{2-}$ and $CO_2$(aq), respectively (*24*, *43*, *47*–*49*). Since the transfer of the proton between $HCO_3^-$ and $CO_3^{2-}$ is fast on the NMR time scale, bicarbonate and carbonate together give a single signal, the chemical shift of which depends on pH. Although gaseous $CO_2$ reacts with water to give carbonic acid, $H_2CO_3$, which enters into equilibrium with $HCO_3^-/CO_3^{2-}$, the kinetics of hydration of carbon dioxide is slow on the NMR time scale (*24*), which is the reason dissolved $CO_2$ appears as a distinct resonance at 125.3 ppm. By comparison with $^{13}C$ NMR spectra of carbonato complexes of $Zn^{2+}$ (*47*–*49*) and $Pt^{2+}$ (*31*, *43*), the signals at 169.2 and 166.9 ppm are assigned to platinum carbonato complexes that form in the reaction of **1** with $NaH^{13}CO_3$. Earlier work by us on the reaction of cisplatin in carbonate medium (*43*) produced a product having a $^{13}C$ NMR resonance at 167.0 ppm that was assigned to *cis*-$[Pt(NH_3)_2(CO_3)_2]^{2-}$ (**3**, Scheme 1). Since the reaction of **1** in carbonate yields a complex with a similar chemical shift, 166.9 ppm in Figure 1a, this species is likely also **3**. As is evident from the low intensity of the platinum carbonato resonances, not much of **1** has reacted with carbonate. Since the reaction is in its early stages and since the higher-field signal, at 166.9 ppm, is associated with a compound that forms in the cisplatin system (i.e., **3**), the resonance at 169.2 ppm is likely **2**, carbonato carboplatin.

Aging **1** in 0.5 M $NaH^{13}CO_3$ produces additional amounts of platinum–carbonato complexes (Figure 1b). These appear as strong resonances at 169.1 ppm (**2**) and 167.3 ppm (**3**), although, as will be evident below, the latter may be overlapped with the resonance of another species. From the weaker signals observed in Figure 1b, which are associated with natural-abundance $^{13}C$ in the CBDCA ligand, it is clear that both intact **1** and free CBDCA are present in solution. The resonances for the α and β carbons of the cyclobutane ring of **1** occur at 31.7 ppm, while the resonances for the same carbons in the free ligand, CBDCA, are observed at 30.9 ppm (*50*).

Figure 1c is the $^{13}C$ NMR spectrum of the reaction mixture after **1** is aged in 0.5 M $NaH^{13}CO_3$, pH 8.6, for 45 h at 37 °C and NMR data are obtained at 4 °C. The spectrum shows **2** at 168.8 ppm and two resonances at 168.0 and 167.9 ppm, as well as free CBDCA (α and β carbons) at 30.8 ppm. Since panels b and c refer to solutions differing in pH (7.4 for Figure 1b vs 8.6 for Figure 1c), the higher-field resonance appears to be an overlap of two platinum–carbonato species, one of which is likely **3**.

**$^1$H NMR.** The $^1H$ NMR spectrum of 5 mM **1** in 0.5 M carbonate buffer for 20 h at 37 °C is shown in Figure 2. The triplet ($^3J_{H-H}$ = 8.0 Hz) at 2.88 ppm is associated with the symmetry-equivalent methylene protons on the α and β positions of the cyclobutane ring of **1**, while the quintet ($^3J_{H-H}$ = 8.0 Hz) at 1.90 ppm is associated with the two methylene protons on the γ position of the four-membered ring (Scheme 1). The triplet in Figure 2 centered at 2.34 ppm ($^3J_{H-H}$ = 8.0 Hz) is due to the α and β protons of the CBDCA ligand of the ring-opened compound, carbonato–carboplatin, **2**, which is monodentate to $Pt^{2+}$ (Scheme 1). This assignment parallels that for the equivalent ring-opened compounds in which the $CO_3^{2-}$ ligand of **2** has been replaced with $HPO_4^{2-}$ (*12*). The quintet at 1.82 ppm is due to the two methylene protons on the γ position of the cyclobutane ring of **2**. When aged in carbonate buffer for 20 h, **2** further reacts with carbonate, releasing the free

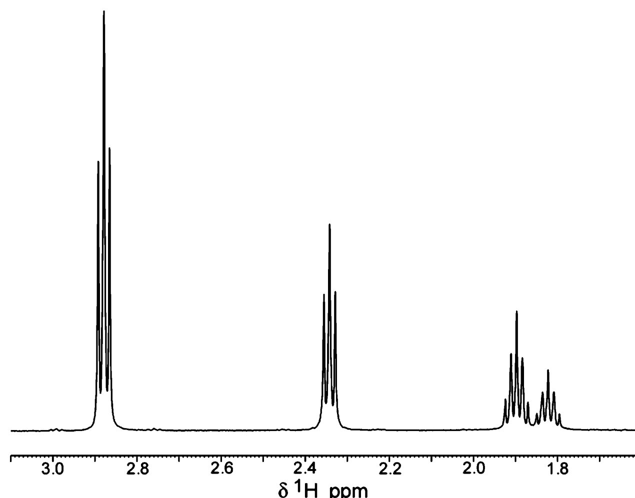



**Figure 2.** $^1H$ NMR spectrum of a solution containing 5 mM carboplatin aged in 0.5 M $NaHCO_3$, pH 8.6, for 20 h at 37 °C. The spectrum shown was obtained after the addition of 10% $D_2O$. The final solution contained 4.5 mM **1** in 0.45 M $NaHCO_3$.

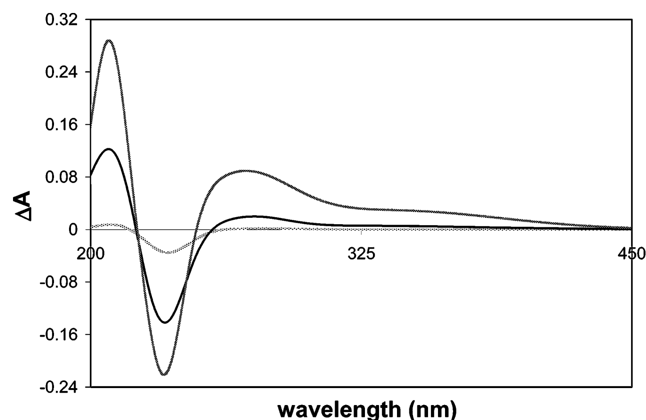



**Figure 3.** UV difference spectra of **1** in 0.5 M carbonate buffer, pH 8.6, at 1.5 h (lowest curve on left), 22 h (middle curve on left), and 45 h (highest curve on left).

CBDCA ligand to solution (Scheme 1). At the pH of the study, all of the methylene proton resonances of free $CBDCA^{2-}$ overlap with the corresponding resonances of **2** (*12*). From integration of the resonances in the spectrum, the composition of the solution at 20 h is 69% **1** and 31% ring-opened species. Using this information, we calculate a pseudo-first-order decay constant of $k_1 \approx 4.31 \times 10^{-6}$ s$^{-1}$ for the disappearance of **1** in 0.5 M carbonate buffer at 37 °C, pH 8.6, which is approximately 1.3 times as large as the rate constant for the disappearance of **1** in 23.8 mM carbonate under the same conditions.

**UV-Visible Absorption Spectra.** UV–visible absorption spectroscopy is useful for following the reaction of **1** in carbonate buffer. In analyzing the time course of the reaction, it is convenient to obtain difference spectra by subtraction of the spectrum obtained at "zero time" from spectra taken at later times. This gives the results shown in Figure 3. At $t$ = 1.5 h, a difference spectrum characteristic of a solution containing both **1** and **2** was obtained (*9*). The difference spectra at $t$ = 22 and 45 h show that the positive absorption at 200–210 nm, which is attributed to the formation of **2**, and the negative absorption at 230–240 nm, which is attributed to the depletion of **1**, become more pronounced with time, as does the positive absorption at ~270 nm (*9*). At later times, a positive absorption at ~350 nm, which also grows with time, is clearly visible. The positive

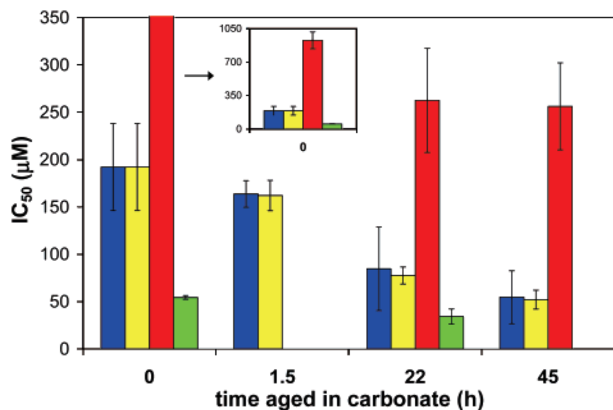



**Figure 4.** Calculated $IC_{50}$ values (micromolar), with statistical errors, where blue is **1** aged in 23.8 mM carbonate toward SK-N-SH, yellow is **1** aged in 0.5 M carbonate toward SK-N-SH, red is **1** aged in 23.8 mM carbonate toward HK-2, and green is **1** aged in 0.5 M carbonate toward BL. See Experimental Section for further details.

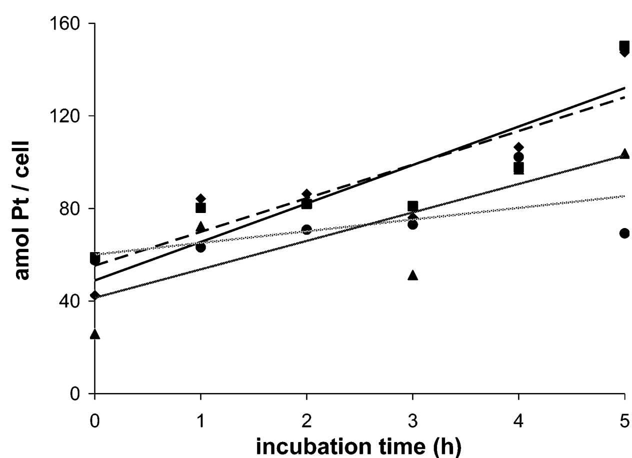



**Figure 5.** Platinum (in attomoles, $10^{-18}$ mol) taken up per normal Jurkat (NJ) or cisplatin-resistant Jurkat (RJ) cell, when exposed to carboplatin alone or aged carboplatin, as a function of time. Total concentration of platinum in the culture medium for each condition, 130 $\mu$M; aging conditions, 5 mM carboplatin, 23.8 mM $NaHCO_3$ for 45 h, pH 8.4, 37 °C. (◆, black line) Carboplatin alone + NJ cells; (■, dashed line) aged carboplatin + NJ cells; (▲, dark gray line) carboplatin + RJ cells; (●, light gray line) aged carboplatin + RJ cells. See Experimental Section for further details.

absorption at ~350 nm, which is probably of *d*−*d* origin, is shifted to lower energy than the corresponding absorption for **1** (*9*). This is consistent with a decrease in the magnitude of the crystal field when the bidentate dicarboxylate CBDCA chelate ring is opened and one or more of the coordination sites on $Pt^{2+}$ is replaced by carbonate (*14*, *51*, *52*).

**Cytotoxicity Studies**. Carboplatin and aged solutions of **1** in carbonate were used to treat human neuroblastoma (SK-N-SH), human proximal renal tubule (HK-2), and human Namalwa-luc Burkitt's lymphoma (BL) cells in culture. As is evident from Figure 4, the order of the $IC_{50}$ values for the three cell lines studied is BL < SK-N-SH < HK-2, which shows that carboplatin is most toxic to BL cells but least toxic to HK-2 cells. Since carboplatin is known to have low nephrotoxicity, this behavior is expected (*1*). Also evident from Figure 4 is that *increases* in the aging time of **1** in carbonate medium cause *decreases* in the measured $IC_{50}$ value for all three cell lines. This shows that the products produced by aging in carbonate are more toxic to cells than is carboplatin itself. Also evident from Figure 4 is that the fractional decrease in the $IC_{50}$ value produced by aging is greatest for HK-2 cells; that is, these cells are most affected by the presence of the carbonate reaction products. Interestingly, for SK-N-SH cells, aging for the same period of time in 23.8 mM or 0.5 M $NaHCO_3$ produces about the same $IC_{50}$ value.

**Table 1. Uptake Rates of Carboplatin and Solutions of Carboplatin plus Its Carbonate Reaction Products by Normal and Resistant Jurkat Cells**

| condition[a] | cell line[b] | rate of uptake[c] (amol of Pt $h^{-1}$ $cell^{-1}$) |
|---|---|---|
| **1** | NJ | 16.6 ± 4.2 |
| aged **1** | NJ | 14.5 ± 4.1 |
| **1** | RJ | 12.3 ± 4.8 |
| aged **1** | RJ | 5.1 ± 3.3 |

[a] Either 130 $\mu$M **1** or 130 $\mu$M **1** aged in 23.8 mM $NaHCO_3$ at 37 °C for 45 h. [b] NJ, normal Jurkat cells; RJ, Jurkat cells that have been made resistant to cisplatin. [c] Attomole (amol) = $10^{-18}$ mol.

**Platinum Uptake by Normal and Cisplatin-Resistant Jurkat Cells.** Figure 5 shows cellular Pt concentrations (determined by ICP-MS) as functions of time, with least-squares linear fits. As is evident from Table 1, the rates of platinum uptake by normal Jurkat (NJ) cells are the same for fresh carboplatin solutions and solutions aged in carbonate, that is, ~15 amol of Pt $h^{-1}$ $cell^{-1}$. However, for Jurkat cells that were made resistant to cisplatin by continued exposure to the drug, uptake of platinum from solutions that contain carbonate reaction products was much less than uptake from solutions containing **1** alone. This is consistent with RJ cells being more efficient than NJ cells in eliminating and/or preventing binding of platinum complexes that are "cisplatin-like" (carbonate reaction products) in nature.

## Discussion

The $^{13}C$ NMR spectra (Figure 1) clearly show that carbonato complexes are formed in the reaction of carboplatin in carbonate buffer. However, as is evident from Scheme 1, the system is complicated and even at early stages of the reaction more than one platinum−carbonato species is present in solution. Since the $^{13}C$ NMR chemical shift of the high-field resonance in Figure 1 (a, b) in carbonate buffer is nearly identical to the resonance of the carbonato species observed in the cisplatin system (*43*), it is assigned to *cis*-$[Pt(NH_3)_2(CO_3^{2-})_2]$ (**3**, Scheme 1). This implies that free CBDCA is present, which is confirmed by the natural-abundance $^{13}C$ NMR resonances for the ligand observed in Figure 1b. However, since aqueous $CO_2$ is in a hydrogen-ion-dependent equilibrium with all carbonato compounds, other species, some of which are shown in Scheme 1, are possible. The complexity of the system is evident in Figure 1c, which shows that an increase in pH causes the high-field signal to move to lower field and split into two resonances. The complex nature of aqueous solutions containing carbonato complexes has been recognized by van Eldik and co-workers (*47*−*49*), who reported that zinc−carbonato complexes that should give simple $^{13}C$ NMR patterns often yielded spectra having multiple resonances, indicating the presence of a number of species.

While $^1H$ NMR has high sensitivity, thus allowing short NMR data collection times, the proton resonances of free CBDCA and CBDCA monodentate to platinum, as in **2**, are overlapped at near-neutral values of pH (Figure 2). Sadler and co-workers (*12*) showed that it is possible to observe the difference between free and monodentate CBDCA by making the solution acidic. The fact that carbonato complexes would not survive at high hydrogen ion concentration precluded this approach as a means of distinguishing between free and monodentate CBDCA in carbonate medium. However, since resonances for **1** are not

overlapped with others in the spectrum (Figure 3), the kinetics of the reaction of **1** with carbonate can be measured easily from the $^1$H NMR signal integrations as a function of time. As earlier stated, this yielded a pseudo-first-order rate constant $k_1 \approx 4.31 \times 10^{-6}$ s$^{-1}$ for the disappearance of **1** in 0.5 M carbonate buffer at 37 °C, pH 8.6. This rate constant is only 1.3 times as large as the rate constant for the disappearance of **1** in 23.8 mM carbonate under the same conditions. Note, however, that the Debye−Huckel theory gives the following expression for $\gamma_C$ (activity coefficient of $CO_3^{2-}$):

$$\ln(\gamma_C) = \frac{-z^2 A\sqrt{I}}{1 + Ba\sqrt{I}}$$

Here, $z$ is ionic charge (−2), $I$ is the ionic strength (≈molarity for $NaHCO_3$ solution), $A = 1.17$(mol/kg)$^{-1/2}$, $B = 3.3 \times 10^9$ (mol/kg)$^{-1/2}$ m$^{-1}$, and $a$ is an ionic diameter. Assuming $a = 5 \times 10^{-10}$ m, this formula gives $\gamma_C = 0.562$ for 23.8 mM and 0.217 for 0.5 M, so that $a_C$, the activity of $CO_3^{2-}$, at 0.5 M is only ~8 times $a_C$ at 0.0238 M. Ion pairing and association effects, very important at high concentration, will reduce the ratio even further. The low ratio of rate constants in 0.5 and 0.0238 M bicarbonate is thus not entirely unexpected.

In addition to NMR, UV−visible difference spectroscopy is also useful for following the reaction of **1** in carbonate medium (Figure 3). Although this approach is quite sensitive to changes in the composition of the solution, it so far provides a largely qualitative assessment of the progress of the reaction. To be more quantitative, additional information on the molar extinction coefficients of the species present is required.

As earlier stated, the hypothesis that formed the basis for this study is that carboplatin is activated by carbonate and that carbonato species are responsible for the antitumor effects (cytotoxicity) of the drug in therapy. This hypothesis, which requires that the products of activation, carbonato complexes, be more lethal to cells than carboplatin alone, is supported by the data shown in Figure 4. Clearly, aging carboplatin in carbonate medium produces species that are more toxic to all cell lines studied than carboplatin alone. Moreover, increasing the aging time, which results in the formation of additional carbonato species, increases the toxicity of the platinum forms in solution that attack the cells. Human renal proximal tubule cells (HK-2) experience the greatest loss in survival upon exposure to the carbonato species formed after carboplatin is aged in carbonate at 37 °C for 22 h, as compared to carboplatin alone. However, as is evident from Figure 4, of the three cell lines studied, HK-2 cells have the highest values of $IC_{50}$, so that they remain the most resistant to the toxic effects of the carbonato forms in the culture medium. Since carboplatin is known to exhibit relatively low renal toxicity (*1*, *53*), the observed low toxicity of the carbonato forms toward HK-2 cells is consistent with the hypothesis that carbonato species could form in therapy and be the species responsible for the antitumor effects of the drug. The carbonato species could be circulating in the body, attacking other types of cells, but not be especially damaging to kidney cells. While spectroscopy shows that increasing the concentration of carbonate in the aging buffer containing carboplatin results in the formation of more ring-opened product in a given period of time, Figure 4 shows that low (23.8 mM) and high (0.5 M) carbonate−platinum solutions have almost the same $IC_{50}$ values for human neuroblastoma SK-N-SH cells. Although more carbonato species are formed at higher concentration, neither the concentrations nor the cytotoxicities of the species are known. Thus solutions aged under different conditions could in fact lead to the same cytotoxicities, as is apparently the case.

As is shown in Figure 5 and Table 1, normal Jurkat cells (NJ) and Jurkat cells that have been made resistant to cisplatin (RJ) both take up platinum. The uptake rates are about the same for both cell lines when exposed to carboplatin alone, but when exposed to carboplatin that has been aged in carbonate medium, normal Jurkat cells take up Pt more than twice as fast as resistant cells. Since carboplatin alone has only a small amount of carbonato species (see the statements on hydrolysis above) and aged carboplatin has a lot more, this suggests that resistant cells can act on the carbonato species to prevent it from entering cells.

Note that each cell takes up a very small fraction of the total platinum from the solution that is above it. For example, in these experiments each reaction tube contained 0.12 $\mu$mol of Pt in 920 $\mu$L of solution. As we show in this work, when exposed to carboplatin, a single normal Jurkat cell takes up 16.6 amol of Pt in 1 h, which represents $1.4 \times 10^{-10}$ of the total amount of Pt present in the solution above the cells. In previous work, we showed that Jurkat cells exposed to 65 $\mu$M cisplatin in the presence of carbonate-containing culture medium took up Pt at a rate of 125 amol of Pt h$^{-1}$ cell$^{-1}$ (*45*). Thus, of the 0.060 $\mu$mol of Pt present in 920 $\mu$L of the cisplatin solution used, the fraction that was taken up by a single cell is $2.1 \times 10^{-9}$, showing that the ratio of uptake rates for cisplatin and carboplatin is $(21 \times 10^{-10})/(1.4 \times 10^{-10}) = 15$. This is close to the 13.3 ratio seen with the L1210 murine lymphomasrcoma cell line for the two drugs (*54*). Ghezzi et al. (*55*) found the ratio of uptake rates, cisplatin to carboplatin, to be 3.1 for MCF-7 breast cancer cells. Pereira-Maia and Garnier-Suillerot (*56*) found ratios of 15 and 8 for susceptible and resistant small lung-cancer cells. They reported Pt uptake rates of ~0.2 mM h$^{-1}$ for lung-cancer cells exposed to 100 $\mu$M cisplatin (average over four cell types); with an assumed cell volume of $10^{-12}$ L, this corresponds to 200 amol of Pt h$^{-1}$ cell$^{-1}$, about the same as we found for Jurkat cells. Other values of uptake rates and a discussion of the mechanisms by which the platinum drugs enter the cell have been previously published (*7*, *57*).

With a binding rate of ~15 amol of Pt h$^{-1}$ cell$^{-1}$, about 9 million platinum ions in some chemical form strongly bind to the cell in 1 h. Earlier studies by us (*58*) and by Ghezzi et al. (*55*) show that only about 1% of the ions that bind to the cell are actually bound to DNA, so that ~90 000 Pt ions would be attached to genomic DNA in 1 h. Since cells exposed to conditions similar to those used here would enter into drug-induced apoptosis (*58*), reaction of the remaining 99% of the Pt bound to the cell with molecules other than DNA could be responsible for the initiation of death. As pointed out by Bose (*6*), there are many potential non-DNA targets, the platination of which could jeopardize the survival of the cell. Clearly, additional study to resolve this important issue appears warranted.

As is evident from Figure 5 and Table 1, RJ cells have reduced capacity to bind platinum when in contact with solutions containing significant concentrations of carbonato species. This is probably because the RJ cells were selected for their ability to survive higher concentrations of cisplatin and their survival is associated with mechanisms to prevent platinum from entering cells, by chemically modifying it in solution (*45*), and/or rapidly removing platinum from cells by an efflux mechanism. Since substitution reactions occur at much greater rates with cisplatin than with carboplatin, the products shown on the right side of Scheme 1 would be present in significant concentrations in the

solutions to which cells are exposed when selecting cells for their resistance to cisplatin. Thus it is not surprising that the RJ cells exhibit reduced uptake (reduced amount of platinum strongly bound to the cell) from solutions containing these species. This implies that carbonato species, regardless of the drug from which they are derived (i.e., cisplatin or carboplatin), are highly toxic to the cells and cell survival depends on finding mechanisms to minimize exposure to these agents.

During intravenous infusion of carboplatin, the concentration of the drug in blood is ∼130 $\mu$M (*53*). While it is widely believed that the drug is entirely in the chelated form **1** (Scheme 1), this is clearly not the case: infusion solutions contain small amounts (a few percent) of the hydrolyzed form (*39*). The $pK_a$ values for deprotonation of the cisplatin aquo complexes are near physiological pH (*13*), and it is likely that a carboplatin aquo species would have a $pK_a$ for deprotonation in the same range. Since hydroxo compounds can react rapidly with aquated $CO_2$ to form carbonato complexes, injection of the infusion solution of carboplatin into blood could lead to the rapid formation of small amounts of **2** and other species shown in Scheme 1. As we earlier showed (*9*), carbonate ion $CO_3^{2-}$, small concentrations of which are present in blood, can displace the CBDCA chelate ring of **1**, and the ion appears to be an important nucleophile for attacking carboplatin in the aggressive nucleophilic environment of culture medium (*9*, *43*). Our findings show that carbonato species are significantly more toxic to neuroblastoma, proximal tubule, and BL cells than **1,** so even small concentrations of these species in therapy may be important. Although carbonato complexes have not been detected in therapy, free CBDCA has been found in the urine of cancer patients who have received carboplatin (*17*), indicating that some nucleophile present in the body, perhaps but not necessarily carbonate, can completely displace the chelate ring of carboplatin.

As is shown in Scheme 1, the reaction of carboplatin in carbonate medium produces a number of complexes that are anions which, as the cytotoxicity studies show, are toxic to the cell. While the mechanism used by these compounds for entering the cell is unknown, anionic drugs, toxins, and xenobiotics having molecular weights similar to those shown in Scheme 1 enter the cell by use of organic anion-transporting polypeptides (OAT) (*59*). This transporter, which has many variations, can be found in multiple tissues including the blood−brain barrier, kidney, heart, lung, placenta, and testis. For example the anticancer drug methotrexate, which is a dianion and has a molecular mass of ∼450 Da, is believed to be taken into mouse proximal tubule cells by an OAT (*60*). These transporters have also been implicated in resistance in that they appear to facilitate the removal of drugs from the cell (*61*). Recently, Zhang et al. (*62*) studied the uptake of cisplatin, carboplatin, and oxaliplatin by cells having overexpressed organic *cation* transporters, OCT. The study concluded that oxaliplatin, but not cisplatin or carboplatin, is an excellent substrate for OCT, suggesting that a cationic forms of this platinum drug may be present in the medium and taken up by the cell.

In summary, entry of a carbonate ligand into the primary coordination sphere of a platinum anticancer drug creates a variety of different reaction and transport options for the drug under biological conditions. As this study shows, carbonato complexes of carboplatin are capable of killing cells, which indicates that they are taken up by the cell and can interact with critical cellular components to induce apoptosis. Clearly, additional work will be needed to further characterize the structures of these interesting compounds, the factors that control their rates and mechanism of formation, and most importantly, the molecular mechanism by which they kill cells.

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