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Pt(IV) complexes as prodrugs for cisplatin

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ABSTRACT

The antitumor effects of platinum(IV) complexes, considered prodrugs for cisplatin, are believed to be due to biological reduction of Pt(IV) to Pt(II), with the reduction products binding to DNA and other cellular targets. In this work we used pBR322 DNA to capture the products of reduction of oxoplatin, *c,t,c*-$[PtCl_2(OH)_2(NH_3)_2]$, **3**, and a carboxylate-modified analog, *c,t,c*-$[PtCl_2(OH)(O_2CCH_2CH_2CO_2H)(NH_3)_2]$, **4,** by ascorbic acid (AsA) or glutathione (GSH). Since carbonate plays a significant role in the speciation of platinum complexes in solution, we also investigated the effects of carbonate on the reduction/DNA-binding process. In pH 7.4 buffer in the absence of carbonate, both **3** and **4** are reduced by AsA to cisplatin (confirmed using ^{195}Pt NMR), which binds to and unwinds closed circular DNA in a manner consistent with the formation of the well-known 1, 2 intrastrand DNA crosslink. However, when GSH is used as the reducing agent for **3** and **4**, ^{195}Pt NMR shows that cisplatin is not produced in the reaction medium. Although the Pt(II) products bind to closed circular DNA, their effect on the mobility of Form I DNA is different from that produced by cisplatin. When physiological carbonate is present in the reduction medium, ^{13}C NMR shows that Pt(II) carbonato complexes form which block or impede platinum binding to DNA. The results of the study vis-à-vis the ability of the Pt(IV) complexes to act as prodrugs for cisplatin are discussed.

1. Introduction

In the earliest reports of the anticancer effects of cisplatin, *cis*-$[PtCl_2(NH_3)_2]$, **1**, Fig. 1, Rosenberg and coworkers noted that certain complexes containing Pt(IV) exhibit potent antitumor activity [1–5]. Since complexes of Pt(IV) are slow to exchange their bound ligands, a central question concerning the mechanism of action of these compounds is how they may be activated *in vivo* to produce their antitumor effects. Tobe and Khokhar [6], and later Cleare et al. [7], suggested that Pt(IV) complexes may be reduced to Pt(II) complexes *in vivo* and the latter exert their cytotoxic effects in a manner analogous to cisplatin and its analogs. The reduction hypothesis was reinforced when it was observed that patients receiving the Pt(IV) complex, iproplatin, *cis*, *trans*, *cis*-$[PtCl_2(OH)_2((CH_3)_2CHNH_2)_2]$, **2**, Fig. 1, in clinical trials had, *cis*-$[PtCl_2((CH_3)_2CHNH_2)_2]$, in their plasma and urine indicating that **2** was reduced in the body to a Pt(II) complex [8,9].

Ascorbic acid, which is present in blood plasma (50–150 μM) and the cytosol (~1 mM) [10,11], is a two-electron reducing agent capable of reducing Pt(IV) to Pt(II). In the case of iproplatin and the related complex oxoplatin, *cis*, *trans*, *cis*-$[PtCl_2(OH)_2(NH_3)_2]$, **3**, Blatter et al. [12] used ^{195}Pt NMR and other techniques to show that ascorbate reduces these Pt(IV) complexes to *cis*-$[PtCl_2((CH_3)_2CHNH_2)_2]$, and cisplatin, respectively, and that the reduction products can bind to and unwind closed circular PM2 DNA. Green and Evans [13] and Choi et al. [14] measured the second order rate constant for the ascorbate reduction of iproplatin at 40 °C obtaining $k = 0.33$ and 0.103 $M^{-1}\,s^{-1}$, respectively, while Weaver and Bose [15] investigated the mechanism of reduction of both **2** and **3** by ascorbate. The later study concluded that reduction of the Pt(IV) compounds by ascorbate proceeds by an outer sphere mechanism with a rate constant similar to those earlier reported but [13,14], after some Pt(II) is produced in the reaction mixture, the reduction is greatly accelerated by the ability of Pt(II) species to serve as catalysts for the further rapid reduction of Pt(IV) to Pt(II).

The typical concentration of the tripeptide, glutathione (GSH), γ-glutamylcysteinylglycine, which contains a cysteine thiol residue, is ~2 mM in the cytosol and ~850 μM in blood [2,16]. Reduced GSH is a potent one-electron reducing agent and, while it is capable of reducing Pt(IV) to Pt(II), the nature of the GSH-produced reduction products has been the subject of inquiry [3]. Recently, Volckova, et al. [17] used a variety of physical techniques to show that GSH rapidly reduces iproplatin, **2**, to produce *cis*-di(isopropylamine) chloro-glutathionatoplatinum(II) which has the thiolate of a GSH molecule bound to Pt(II). Since this type of complex could not form a 1, 2 intrastrand crosslink with DNA [18], it was suggested that GSH-reduced iproplatin may exhibit its cytotoxic effects by modifying biological targets other than DNA in the cell. As the work described in this manuscript was being finalized, Nakai, et al. [19], reported that the reduction by GSH of the Pt(IV) complex *cis*-$[PtCl_4(NH_3)_2]$, which

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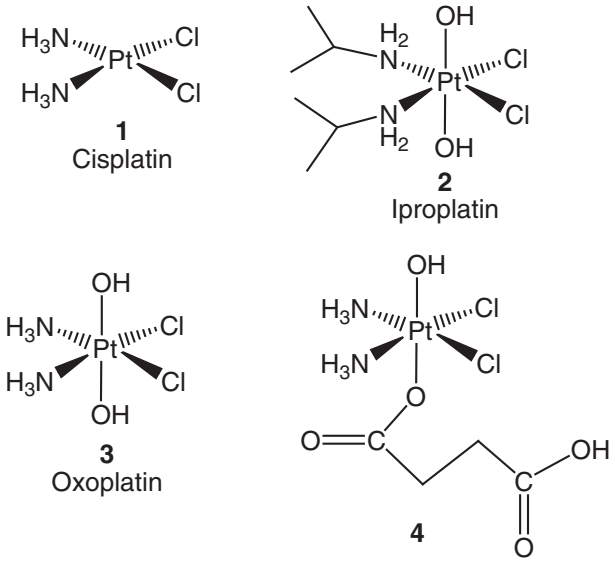

Fig. 1. Structures of the platinum compounds in the study.

is considered a prodrug of cisplatin, actually does not produce cisplatin. The study also showed that if calf thymus DNA is present in the reduction medium, the Pt(II) product produced in the reaction which is captured by DNA does not form the 1, 2 intrastrand DNA crosslink.

While both ascorbic acid and GSH are small molecule reducing agents often cited in connection with the reductive activation of Pt(IV) complexes, Gibson and coworkers [20,21] monitored the rate of reduction of Pt(IV) complexes in extracts obtained from whole cells. These investigators found that reductive elimination of *cis*-diam(m)inedichloridoplatinum(IV) complexes with axial acetato ligands by whole cell extracts does exclusively produce a Pt(II) product with the expected, *cis*-$PtCl_2N_2$ geometry.

In considering the activation of Pt(IV) complexes, Platts et al. [22], correlated the electrochemical peak potentials and octanol-water partition coefficients of 31 Pt(IV) complexes having the potential to produce cisplatin or cisplatin-type complexes through reductive elimination to Pt(II) compounds. These investigators found a good correlation between the aforementioned properties and the calculated surface area, orbital energies, dipole moments and atomic partial charges of the compounds and suggested that the models developed in the study have the potential to predict the antiproliferative properties of the compounds from calculated data.

When compared to Pt(II) compounds, complexes containing Pt(IV) have relatively slow ligand exchange rates, a feature that allows chemical modification of the bound ligand without breaking the metal-ligand bond. For example, in the synthesis of the drug candidate, satraplatin, hydroxide ion bound to Pt(IV) serves as a nucleophile for reaction with acetic anhydride, in the process converting the hydroxo ligand to an acetato ligand [2,23]. The exchange inertness of ligands bound to Pt(IV) is also playing an increasingly important role in new strategies for delivering platinum drugs to cancer cells involving nanotechnology [24–28]. An approach often employed is to chemically modify one of the ligands attached to a Pt(IV) complex and to tether many copies of the modified complex to a nanoparticle. Since a nanoparticle has numerous attachment sites, some of which can be equipped with a vector capable of targeting a specific cell type, the nanoparticle becomes an efficient vehicle for transporting many copies of a platinum complex inside the cell. If the Pt(IV) compound attached to the nanoparticle is designed to release cisplatin upon reduction, the nanoparticle becomes a "super prodrug" for cisplatin capable of delivering many copies of the potent anticancer drug to the interior of the cell.

In this report we examine the ability of oxoplatin, **3**, and one of its carboxylate modified analogs, **4**, Fig. 1, to produce cisplatin in the presence of the biologically common reducing agents, ascorbic acid, AsA, and glutathione, GSH. Oxoplatin exhibits potent oral anticancer activity [29,30] and **4** and its analogs have been used in a host of drug delivery applications involving nanotechnology [24–28]. Since Pt(II) complexes bind to DNA, we use pBR322 DNA as a capture agent for the reduction products of **3** and **4** and measure the mobility of the platinated DNA forms in an agarose gel. Since it is known that physiological carbonate plays a significant role in the speciation of cisplatin in solution which affects the kinetics of the platinum–DNA interaction and possibly the nature of the DNA adduct formed [31–36], we also investigated platinum binding to pBR322 DNA in the presence and absence of carbonate buffer. In addition to using a closed circular DNA and gel electrophoresis to detect the effects of the reduction products on DNA mobility, we also used ^{195}Pt NMR to identify the nature of the platinum species formed in the reduction and ^{13}C NMR to detect Pt(II) carbonato complexes when the reduction is carried out in carbonate media.

2. Materials and methods

2.1. Synthesis of oxoplatin (3)

This compound was synthesized in a manner analogous to that earlier reported by Brandon and Dabrowiak [37]. Hydrogen peroxide (30 wt.%, 20 mL) was added dropwise to a suspension of cisplatin, **1**, (0.4 g, 1.33 mmol) in H_2O (12 mL) at 60 °C. After 4 h, the bright yellow solution was cooled at room temperature overnight to afford yellow crystals. The crystals were recovered by filtration and washed with ice cold water. Yield: 65.6% (0.291 g, 0.872 mmol). IR 3458 (s, OH stretch), 1072 (m, Pt-OH bend), 536 (m, Pt-N(O) stretch). ^{195}Pt NMR (500 MHz, D_2O), δ (ppm) = 853.

2.2. Synthesis of c,t,c-[$PtCl_2(OH)(O_2CCH_2CH_2CO_2H)(NH_3)_2$], (4)

This compound was synthesized in a manner analogous to that described by Dhar et al. [25]. To a suspension of compound **3** (0.2 g, 0.6 mmol) in DMSO (16 mL) was added succinic anhydride (0.06 g, 0.6 mmol) and the mixture was stirred overnight at RT to afford a bright yellow solution. After reducing the volume of the solution in *vacuo* to ~0.5 mL, addition of ~10 mL of ice cold acetone caused precipitation of a pale yellow solid, which was collected via filtration, washed with acetone and dried in vacuo. Yield: 97.2% (0.253 g, 0.583 mmol). ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm) = 5.92 m, 6H (NH_3); 2.39 m, 4H (CH_2). $J(^1H\text{-}^{195}Pt) = 103.2$ Hz, $J(^1H\text{-}^{14}N) = 25.5$ Hz. ^{13}C NMR (75 MHz, DMSO-d_6): δ = 179.73 (C=O), 174.19 (C=O), 30.21 (CH_2), 31.24(CH_2) ppm. MS (1:1 THF:MeOH with NaCl) calculated m/e of $(C_4H_{12}N_2O_5Cl_2Pt)Na^+ = 456.0$, observed m/e = 456.0. ^{195}Pt NMR, δ (ppm) = 943.

2.3. DNA binding studies

The DNA binding/capture studies were done in a total volume of 20 μL containing 38.5 μM (bp) pBR322 DNA (New England BioLabs Inc.), in 12 mM HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonicacid (pH 7.4) buffer or, when sodium bicarbonate was present, in 12 mM HEPES buffer plus 12 mM sodium bicarbonate (pH 7.4). The two reducing agents used were glutathione (GSH, 2 mM) and ascorbic acid (AsA, 50 μM). Cisplatin, **1**, was used as purchased (Sigma-Aldrich, St. Louis, MO). Stock solutions of the platinum compounds in distilled water, **1** and **3**, or DPBS (Dulbecco's phosphate buffered saline) buffer, **4**, were in the range of 40–650 μM. Appropriate volumes of the stock solutions were added to solutions containing the buffer and DNA to give the final concentrations given earlier and values of *r*, where *r* = [platinum compound]/[DNA-bp], given in the

captions to figures. The samples were incubated at 37 °C for 24 h in sealed Eppendorf tubes after which time 2.5 μL of a loading dye containing 50% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol was added. An 8 μL volume of each sample containing the loading dye was loaded in the wells of a 1% agarose gel. Electrophoresis was carried out for a period of ~4 h, at 100 V. After electrophoresis, the gel was immersed in 300 mL of deionized water containing 300 μL of a 0.5 mg/mL solution of ethidium bromide for 30 min to stain the DNA, and then soaked in water alone for 15 min to de-stain the background of the gel. A digital image of the stained gel was captured using a Kodak Gel Logic 100 imaging system equipped with Fisher Biotech IT-88A transilluminator.

2.4. Instrumentation

One dimensional ^{1}H NMR was obtained using a Bruker Avance 300 MHz spectrometer with chemical shifts determined by setting the hydrogen impurity in DMSO-D_6 to 2.50 ppm. One dimensional ^{195}Pt NMR spectra were recorded on a Bruker Avance 500 MHz NMR instrument utilizing either a Nalorac 5 mm indirect detection gradient probe or a Nalorac 10 mm broadband observe probe. A solution of 10 mM potassium hexachloroplatinate in 95% H_2O/5% D_2O was used as an external standard (0 ppm). All experiments were done at 25 °C using a 2 s relaxation delay time and a spectral width of 600 ppm with 32 K points. One dimensional carbon-13 NMR spectra were recorded at 25 °C on a Bruker Avance 500 MHz NMR instrument equipped with a 5 mm Bruker QXI gradient probe. The standard ^{13}C pulse sequence was used with proton broad-band decoupling and a spectral window of 250 ppm. The relaxation delay was set to 2 s, the total number of points to 16 K, and the total number of scans set to 6000. The internal chemical shift standard, set at 126.1 ppm, was $^{13}CO_{2(aq)}$. Infrared spectra were obtained on nujol mulls using a Nicolet 200 FT-IR spectrometer and the mass spectral data were obtained using positive electrospray ionization with a Bruker 12 Tesla APEX-Qe FTICR-MS with an Apollo II ion source (COSMIC Lab, Norfolk, VA).

2.5. ^{195}Pt NMR

Compound **3** (10 mM) was incubated at 37 °C for 24 h in the presence of 10 mM ascorbic acid or 20 mM GSH in 12 mM HEPES buffer with or without 12 mM $NaHCO_3$, pH 7.4. Compound **4** (10 mM) was incubated at 37 °C for 24 h in the presence of 10 mM ascorbic acid or 20 mM GSH in DPBS buffer with or without 12 mM $NaHCO_3$, pH 7.4. The solvent of the aforementioned samples was H_2O/D_2O (95/5) and the concentrations given are the final concentrations.

2.6. ^{13}C NMR

Compound **3** (10 mM) was incubated at 37 °C for 24 h in the presence of 10 mM ascorbic acid in 12 mM HEPES buffer with 0.5 M $NaH^{13}CO_3$, pH 7.4. Compound **4** (10 mM) was incubated at 37 °C for 24 h in the presence of 10 mM ascorbic acid in DPBS buffer with 0.5 mM $NaH^{13}CO_3$, pH 7.4. The solvent of the aforementioned samples was H_2O/D_2O (90/10); the final concentrations in the reaction mixture are given.

3. Results

3.1. DNA capture–gel electrophoresis experiments

Fig. 2(a)–(e)shows images of agarose gels of pBR322 DNA in the presence of reducing agents and either **3** or **4** in the presence and absence of carbonate while Fig. 2(f) shows the binding of cisplatin, **1**, to pBR322 DNA in the presence and absence of GSH. Fig. 3(a)–(f) shows mobility plots, in which the distance of migration of the DNA form from the loading well in arbitrary units is plotted versus *r*, where $r = [Pt]/[DNA\text{-}bp]$, with linear fits to the data. Since the concentrations of DNA, the reducing agent, carbonate (when present) and time are constant in the experiments, the plots show changes in DNA mobility as a function of the total concentration of Pt(IV) complex initially present in the reaction medium. In these experiments the closed and nicked circular forms of pBR322 DNA were simply used as capture agents for the reduction products, in this case Pt(II) compounds, which bind to the DNA forms and affect their mobilities in the gel. Since electrophoresis conditions varied slightly from experiment to experiment which changed the distance of migration in the gel, the linear fits to the data in Fig. 3 were divided by the y-intercept to give relative slopes. The relative slopes, with statistical errors, are given in Table 1.

The agarose gel of pBR322 DNA with oxoplatin, **3**, reduced by ascorbic acid (AsA) is shown in Figs. 2(a) and 3(a) and Table 1 give the mobility plots and their relative slopes. When carbonate is present, the relative slopes of Form I and II are zero indicating that mobility is independent on *r* suggesting that the Pt(II) species produced in the reduction are not binding to DNA.

When carbonate is absent from the medium Form I is difficult to observe, Form II runs slightly faster than its counterpart without carbonate and its relative slope is positive (the mobility of DNA increases with *r*), Table 1. While the increase in mobility and positive slope of Form II is consistent with the formation of the well-known cisplatin 1, 2 intrastrand crosslink, the difficulty in detecting Form I (a weak band for this form is observed in lane 12) suggests that Form I is co-migrating with Form II at some values of *r* and/or the crosslink blocks ethidium bromide binding to DNA leading to reduced staining [34]. ^{195}Pt NMR shows that the reduction of **3** with AsA produces cisplatin and its aquated/substituted products and ^{13}C NMR indicates that in carbonate media Pt(II) carbonato complexes are present (*vide infra*).

Figs. 2(b) and 3(b) and Table 1 give the results of the DNA capture experiment when the oxoplatin analog, **4**, is reduced by AsA in the presence and absence of carbonate. In the absence of carbonate, Figs. 2(b) and 3(b) show that the mobilities of both DNA forms are much different than the controls indicating that binding of Pt(II) to DNA is taking place. However, as is evident in Table 1, the slope for Form II is zero while the slope for Form I is likely positive. In this DNA capture experiment, the stock solution of **4** was in DPBS, which contains chloride ion. Since the chloride ion suppressed the aquation of cisplatin and [Cl^-] is proportional to *r* in the reaction medium, the crosslink appears to be formed but there is not much change in the mobility of Form I with *r*. When carbonate is present in the reduction reaction, mobilities are like those of the controls with the slope of Form I zero and the slope of Form II small and positive with a substantial uncertainty. ^{195}Pt NMR shows that the reduction of **4** with AsA produces cisplatin (not its aquated/substituted products) and ^{13}C NMR indicates that in carbonate media Pt(II) carbonato complexes are present (*vide infra*).

Figs. 2(c) and 3(c) and Table 1 show the effects on pBR322 DNA mobility when **3** is reduced with GSH. In the presence of carbonate, the mobilities of both forms are like controls with the relative slope of Form I zero and the slope of Form II weakly positive (with a significant error) showing that 12 mM carbonate blocks or impedes the interaction of Pt(II) products with DNA. However, when carbonate is absent from the reaction mixture, the mobility of Form I is slower than the control and its relative slope is negative (mobility decreases with *r*) while the slope of Form II is positive (mobility increases with *r*). ^{195}Pt NMR analysis shows that reduction of **3** with GSH does not produce cisplatin (*vide infra*).

Figs. 2(d) and 3(d) show the gel and mobility plots when oxoplatin analog, **4**, is reduced by GSH. When carbonate is present, the mobilities of both forms are similar to those of the controls but the slopes of both forms are only weakly positive consistent with little

platinum binding to DNA, Table 1. When carbonate is absent, the mobilities of both forms appear shifted from controls and the relative slopes of both forms are significant with Form I having a positive slope (mobility increases with r) and Form II a negative slope, opposite to what is observed for **3** being reduced by GSH Figs. 2(c) and 3(c), Table 1.

In an effort to better understand the DNA capture experiment involving the reduction of **4** by GSH in the absence of carbonate, a larger number of values of r were investigated, (see Figs. 2(e) and 3(e) and Table 1). It is evident that both forms are shifted from the controls and their mobilities change systematically with r. From linear fits of the mobilities to r, Form I has a positive slope while Form II has a negative slope with signs and magnitudes comparable to those observed in Figs. 2(d) and 3(d) and Table 1. However closer inspection reveals that the mobility is weakly bimodal in nature with much better fits to the data being obtained by two lines, one for $r<3.3$ and a second for $r>3.3$ (see Table 1). While the mobility of Form I moves closer to that of Form II for higher r, the two forms never co-migrate. ^{195}Pt NMR analysis shows that reduction of **4** with GSH does not produce cisplatin (*vide infra*).

Figs. 2(f) and 3(f) show cisplatin binding to pBR322 DNA in the presence and absence of GSH; the relative slopes of the mobility plots are given in Table 1. In the absence of GSH, the mobility of Form I is much lower than the control and Forms I and II nearly co-migrate at $r=0.9$. Table 1 also shows that the sizes of the relative slopes of both forms are much larger than any other relative slope showing that the effect of the drug on the mobility of the form is very large. This is due to the formation of the 1, 2 intrastrand cross-link. Since the slope of Form I is positive, the writhe is positive which indicates that there is a high loading of platinum on DNA [38,39]. The negative slope for Form II is also consistent with a high loading of platinum on DNA.

Figs. 2(f) and 3(f), show the effects of cisplatin, **1**, in the presence of GSH on Forms I and II and Table 1 gives the slopes of the mobilities. Both Forms I and II have large negative and positive slopes, respectively, which is the same slope pattern observed for the reduction of **3** with GSH in the absence of carbonate. This suggest that some cisplatin may also be produced in the reduction of **3** with GSH and that possibly a cisplatin-GSH complex is interacting with DNA.

3.2. ^{195}Pt NMR

^{195}Pt NMR was used to identify the nature of the platinum species in solution when **3** and **4** are reduced by either AsA or GSH. The incubation time of the reduction solution was 24 h, following which time NMR spectra were obtained; the solutions contained no DNA. Also, the ratio of reducing agent to platinum complex was 1:1 for AsA reducing **3** and **4** and 2:1 for GSH reducing **3** and **4**, much different than the ratios employed in the DNA capture-gel studies. The choice of ratios of reducing agent to platinum was largely influenced by the relatively low sensitivity of ^{195}Pt NMR which required millimolar concentrations of the platinum complex in the NMR tube. The concentration of the reducing agent, if the ratios used in the gel studies were to be maintained, would be unrealistically large. In these

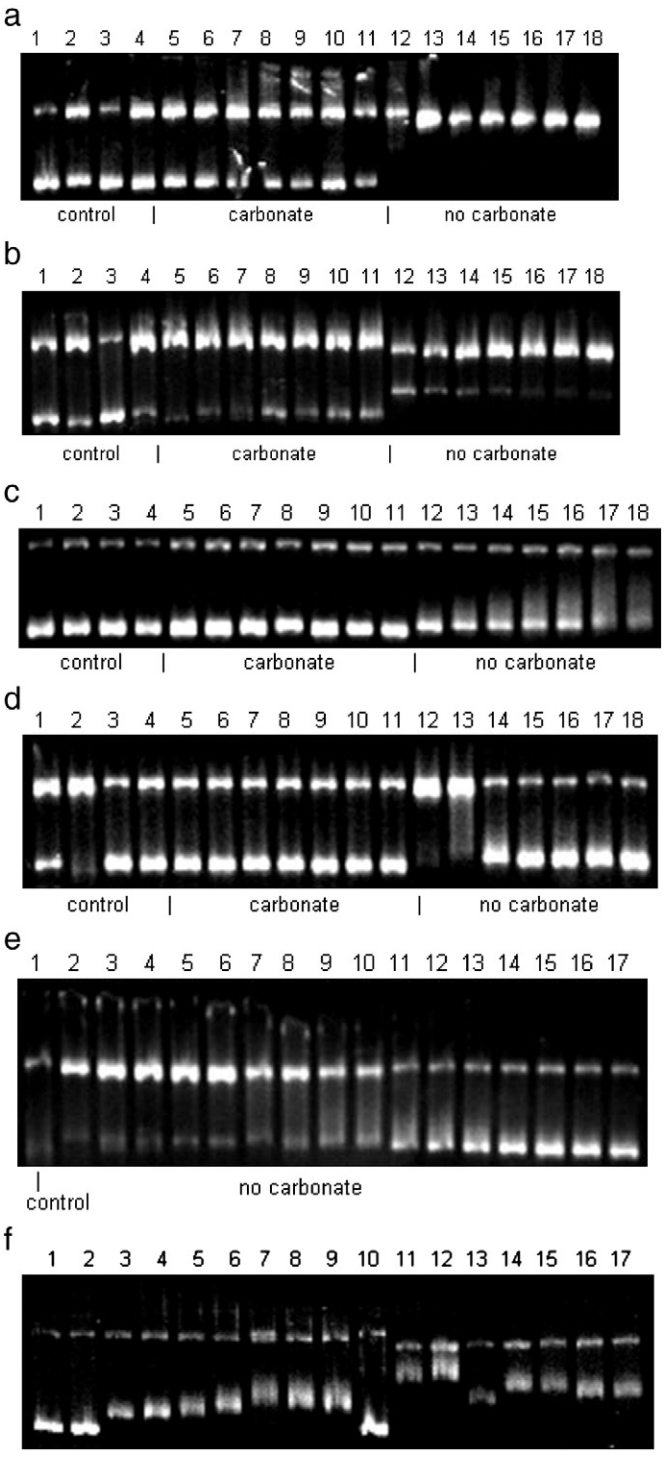

Fig. 2. (a)–(f) Ethidium bromide stained agarose gels of pBR322 DNA (38.5 μM). (a)–(e), pBR322 DNA in the presence of either **3**, or **4**, in the absence or presence of 12 mM sodium bicarbonate with either ascorbic acid, AsA (50 μM) or glutathione, GSH (2 mM) as the reducing agent. The fastest migrating band (bottom band) is covalently closed circular form I DNA while the slowest migrating band (top band) is nicked circular form II DNA. (a) Compound **3**, AsA, sodium bicarbonate, lanes 5–11; no bicarbonate, lanes 12–18. For this and the subsequent gels, lane, r, i.e., the lane number followed by r where, $r=$ [compound]/[DNA-bp], are given. Lane, r: 5, 0.26; 6, 0.52; 7, 1.04; 8, 1.17; 9, 1.30; 10, 1.43; 11, 1.56; 1, 0 (DNA); 2, 0 (DNA + AsA); 3, 0 (DNA + bicarbonate); 4, 0 (DNA + AsA + bicarbonate). The values of r for lanes 12–18 are the same as for lanes 5–11. (b) Compound **4**, AsA, sodium bicarbonate, lanes 5–11; no bicarbonate, lanes 12–18. Lane, r: 5, 0.26; 6, 0.52; 7, 1.04; 8, 1.17; 9, 1.30; 10, 1.43; 11, 1.56; 1, 0 (DNA); 2, 0 (DNA + ascorbic acid); 3, 0 (DNA + bicarbonate); 4, 0 (DNA + ascorbic acid + bicarbonate). The values of r for lanes 12–18 are the same as for lanes 5–11. (c) Compound **3**, GSH, sodium bicarbonate, lanes 5–11; no bicarbonate, lanes 12–18. Lane, r: 5, 1.69; 6, 3.38; 7, 5.06; 8, 6.75; 9, 7.60; 10, 8.44; 11, 10.13; 1, 0 (DNA); 2, 0 (DNA + GSH); 3, 0 (DNA + bicarbonate); 4, 0 (DNA + GSH + bicarbonate). The values of r for lanes 12–18 are the same as for lanes 5–11. (d). Compound **4**, GSH, sodium bicarbonate, lanes 5–9; no bicarbonate, lanes 12–18. Lane, r: 5, 1.30; 6, 2.60; 7, 3.90; 8, 5.19; 9, 5.84; 10, 6.49; 11, 7.79; 1, 0 (DNA); 2, 0 (DNA + GSH); 3, 0 (DNA + bicarbonate); 4, 0 (DNA + GSH + bicarbonate). The values of r for lanes 12–18 are the same as for lanes 5–11. (e). Compound **4**, GSH. Lane, r: 3, 1.30; 4, 1.49; 5, 1.69; 6, 1.88; 7, 2.08; 8, 2.27; 9, 2.60; 10, 2.92; 11, 3.25; 12, 3.57; 13, 3.90; 14, 4.22; 15, 4.55; 16, 4.87; 17, 5.19; 18, 5.52; 1, 0 (DNA); 2, 0 (DNA + GSH). (f) Compound **1**, cisplatin, 2 mM GSH in 12 mM HEPES buffer, pH 7.4, lanes 3–9; no GSH, lanes 11–17, the values of r for lanes 3–9 and 11–17 are the same (condition error, lane 13). Lane, r: 3 (11), 0.08; 4 (12), 0.09; 5 (13), 0.10; 6 (14), 0.11; 7 (15), 0.12; 8 (16), 0.13; 9 (17), 0.14; 1 (10), 0 (DNA), 2, 0 (DNA + GSH).

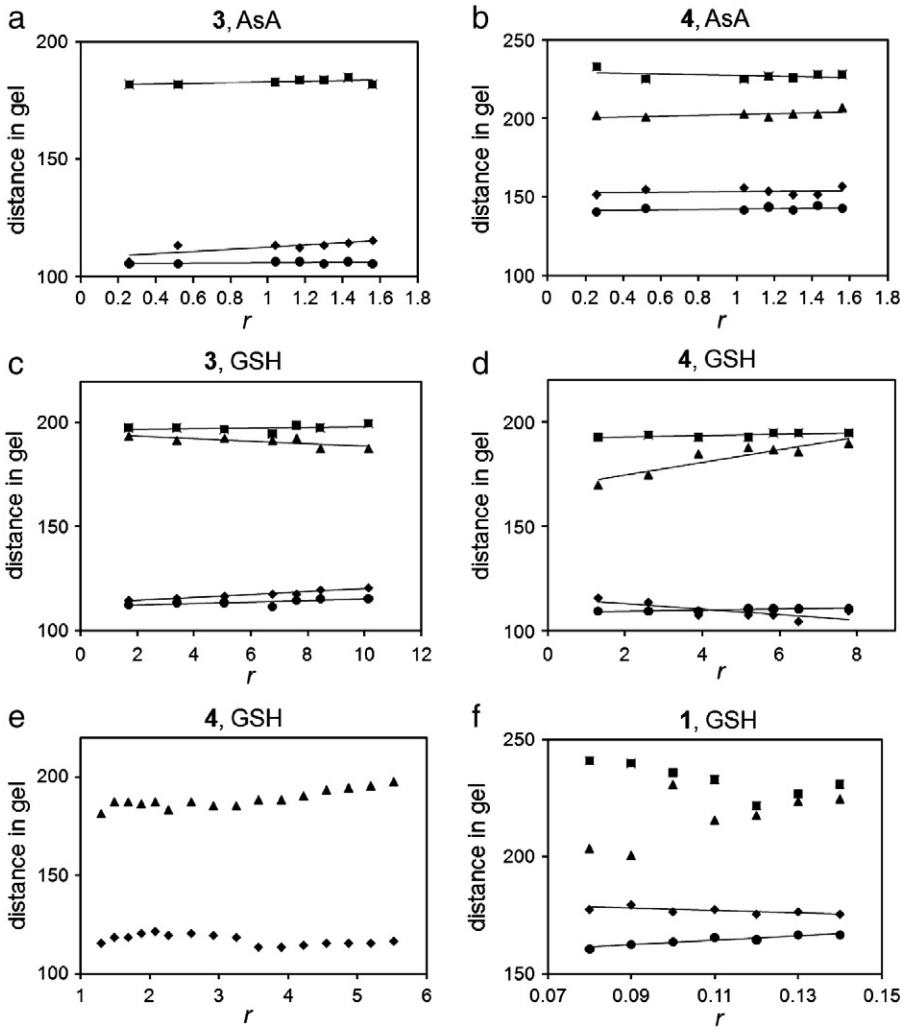

Fig. 3. Distance of migration of the band from the loading well in the gel in arbitray units as a function of r, where $r = [Pt]/[DNA\text{-}bp]$, referred to as mobility plots, for the agarose gels shown in Fig. 2. The lines shown are linear fits to the data. Square is closed circular Form I pBR322 DNA in the presence of carbonate; triangle is Form I DNA without carbonate; circle is nicked circular Form II DNA in the presence of carbonate; diamond is Form II DNA in the absence of carbonate.

Table 1
Relative slopes (slope divided by the y-intercept) from the mobility plots shown in Fig. 3, with statistical errors.

System[a]	Form I[b]	Form II	Form I + HCO_3^-	Form II + HCO_3^-
AsA and **3** (a)	No data	45 ± 15 (+)	7.8 ± 5.2 (0)	3.6 ± 4.4 (0)
AsA and **4** (b)	13.2 ± 7.5 (+)	6.1 ± 12.4 (0)	−9.4 ± 10.4 (0)	11.4 ± 7.3 (+)
GSH and **3** (c)	−3.3 ± 1.2 (−)	6.2 ± 0.6 (+)	0.9 ± 1.2 (0)	3.0 ± 1.5 (+)
GSH and **4** (d)	18.0 ± 3.5 (+)	−11.1 ± 4.0 (−)	1.6 ± 0.7 (+)	1.9 ± 0.5 (+)
GSH and **4** (e)	15.5 ± 2.4[c](+)	−9.2 ± 3.4[c](−)	no data	no data
Cisplatin (f)	Form I + GSH (1.92 ± 0.96) × 10^3(+)	Form II + GSH −260 ± 110 (−)	−960 ± 340 (−)	630 ± 100 (+)

[a]Refers to the reducing agent and the Pt(IV) compound involved in the reduction reaction; the letter in parenthesis refers to the gel image in Fig. 2 and the mobility plot in Fig. 3. The values given in the table are relative slopes of the linear fits to the mobility plots shown in Fig. 3 in units of r^{-1} multiplied by 10^3, followed by the sign of the slope in parentheses, $r = [Pt]/[DNA\text{-}bp]$. Positive and negative slopes indicate that the DNA form migrates faster and slower, respectively, with increasing r, than the same form in the absence of added platinum compound. [b]Form I and Form II are closed and nicked circular pBR322 DNA, respectively. [c]For (e), a much better fit (sum of square deviations = 60 instead of 377) is obtained by using one line for $r < 3.3$ and a second for $r > 3.3$).

reactions, ascorbic acid acts as a two electron reducing agent to convert one molecule of a Pt(IV) complex to a Pt(II) product and the AsA is oxidized to dehydroascorbic acid [15]. In the case of the reduction of the Pt(IV) complex with iproplatin, **2**, *three* molecules of GSH are required to completely reduce one molecule of **2**. Two GSH molecules supply the two electrons necessary to convert Pt(IV) to Pt(II) (producing the disulfide, GSSG), and one molecule of GSH, in its deprotonated form, becomes bound to Pt(II) to give as the final product, *cis*-di(isopropylamine)chloro-glutathionatoplatinum(II), *cis*-[PtCl(GS)($NH_2CH(CH_3)_2$)$_2$], [17].

The ^{195}Pt NMR spectra of the platinum species observed in the reduction reactions are shown in Fig. 4. Fig. 4(a) shows that the reaction of **3** with AsA in 12 mM HEPES buffer produces a strong signal for cisplatin at −2140 ppm, a number of weak signals for Pt(II) species in the region −1670 to −1830 ppm having a PtN_2O_2 coordination environment [40], and a weak signal for unreacted **3**, at 853 ppm.

Interestingly, when two equivalents of GSH are used as the reducing agent for oxoplatin, only a signal of reduced intensity for **3** is observed in the ^{195}Pt NMR spectrum at 853 ppm, i.e., no Pt(II) products are observed, Fig. 4(d). Although an exhaustive search in the chemical shift range −4000 to 1500 ppm failed to find additional ^{195}Pt NMR resonances, the electrophoresis results clearly show that some Pt(II) complexes are produced in the reduction and that they bind to DNA, Figs. 2(c) and 3(c) and Table 1.

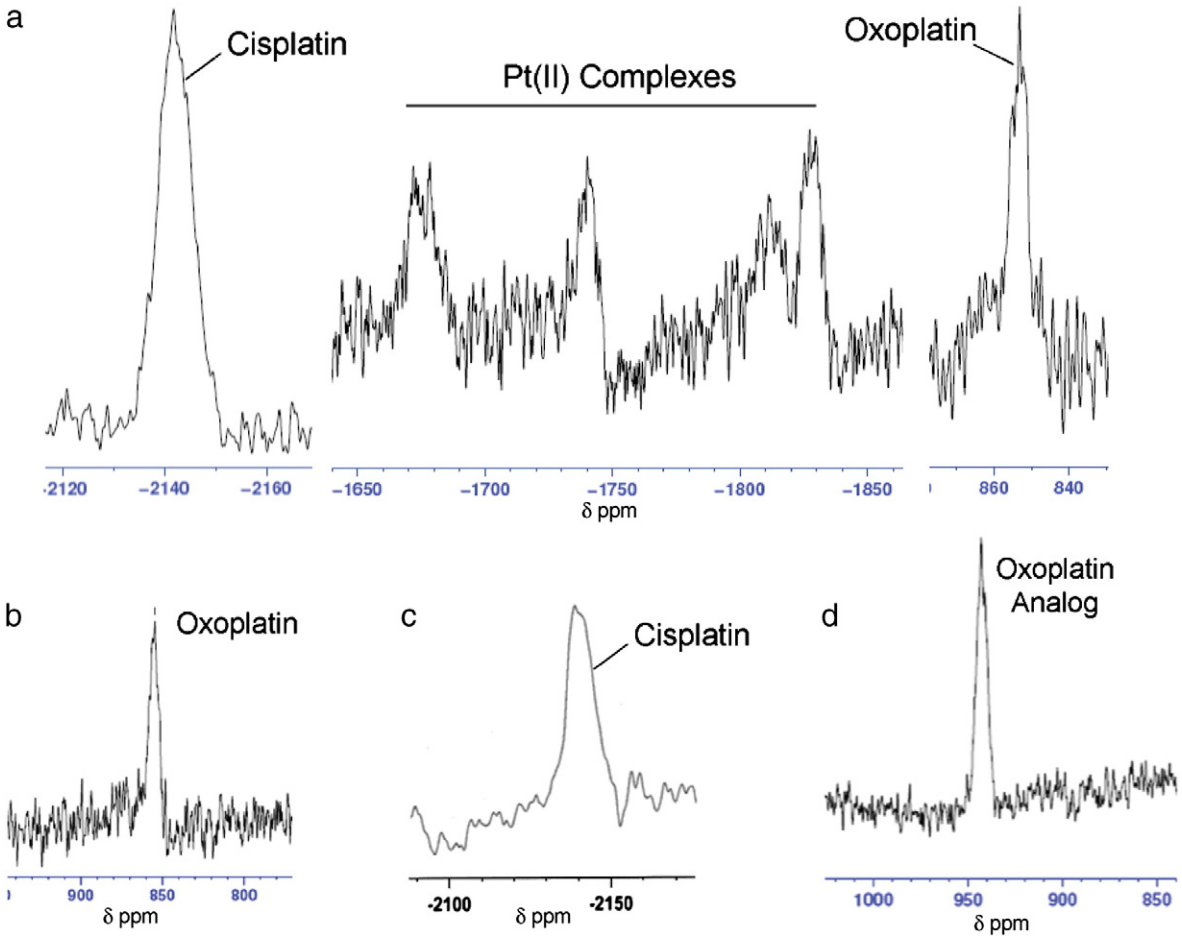

Fig. 4. ^{195}Pt NMR spectra. (a) Reduction of oxoplatin, **3**, with AsA. (b) Reduction of oxoplatin, **3**, with GSH. (c) Reduction of the oxoplatin analog, **4**, with AsA. (d) Reduction of the oxoplatin analog, **4**, with GSH.

Fig. 4(c) shows the ^{195}Pt spectrum of the oxoplatin analog, **4**, 24 h after exposure to one equivalent of AsA in DPBS buffer. As is evident from the figure, the only detectable ^{195}Pt NMR signal is the strong peak at −2140 ppm which due to cisplatin. The absence of the Pt(IV) starting material at 943 ppm shows that the reaction of **4** with AsA went to completion. In addition, the lack of Pt(II) aqua species and/or cisplatin reaction products with ascorbate or dehydroascorbic acid, as in Fig. 4(a), shows that the presence of a high concentration of chloride ion, ~140 mM, in DPBS, suppresses the reaction of cisplatin with components in solution, keeping the drug in its dichloro form.

The ^{195}Pt NMR spectrum of the reaction of **4** with two equivalents of GSH for 24 h in DPBS buffer only exhibits a resonance (of reduced intensity) for the unreacted complex, **4,** at 943 ppm, i.e. no resonances for cisplatin/other Pt(II) complexes are observed, Fig. 4(d).

3.2. ^{13}C NMR

In addition to ^{195}Pt NMR, the reduction of **3** and **4** with AsA, which produces cisplatin, was monitored with ^{13}C NMR in the presence of $H^{13}CO_3^-$. Fig. 5 shows that the reduction of either compound with AsA produces a number of Pt(II) carbonato complexes which have resonances on the low field side of free bicarbonate ion at 162.0 ppm [31,33]. Reduction of **3** in carbonate produces carbonato resonances at, 168.1–168.7 ppm, while reduction of **4** with AsA gives a group of carbonato resonances in the region 167.2–169.7 ppm. Since carbonato complexes reduce the binding of cisplatin to DNA [34–36], the ^{13}C NMR results explain the observed decrease in binding of Pt(II) compound to DNA when carbonate is present in the reduction medium, Figs. 2 and 3 and Table 1.

4. Discussion

In this report we show that the biologically common reducing agent ascorbic acid reduces oxoplatin, **3**, and one of its analogs having an attached carboxylate ligand, **4**, to cisplatin but that treatment of the Pt(IV) compounds with glutathione does not produce cisplatin. While the Pt(II) product(s) produced by GSH bind to closed circular pBR322 DNA, the gel analyses show that they do not form the well-known 1, 2 intrastrand DNA crosslink that is characteristic of cisplatin binding to DNA. We also show that reduction of **3** and **4** in the presence of physiological carbonate prevents binding of the Pt(II) products to pBR322 DNA. Collectively, these observations limit the conditions under which oxoplatin and one of its analogs that is useful in nanotechnology can serve as prodrugs of cisplatin.

Bose and coworkers [15] studied the kinetics and mechanism of reduction of oxoplatin, **3**, with ascorbic acid and iproplatin, **2**, with glutathione. These researchers showed that AsA reduces **3** with a second order rate constant of ~2 M^{-1} s^{-1} at pH 7.3 at 22 °C and that cisplatin is produced by the reduction. Fig. 5 shows that cisplatin is indeed produced in the reduction and ^{195}Pt NMR further indicates that the drug reacts during the 24 h reaction period to produce Pt(II)N_2O_2 type products which appear to be aquated and/or carboxyl substituted products of the drug, the carboxylate ligand most likely being supplied by dehydroascorbic acid [40]. Interestingly, not all **3** has been reduced to Pt(II) products as evidenced by the relatively weak resonance at 853 ppm, Fig. 5, although this reduction reaction would be expected to have an approximate half-life of ~1 min under the conditions of the ^{195}Pt NMR experiment ([AsA], [**3**] = 10 mM, 37 °C). It is possible that either the stoichiometry of the reduction is

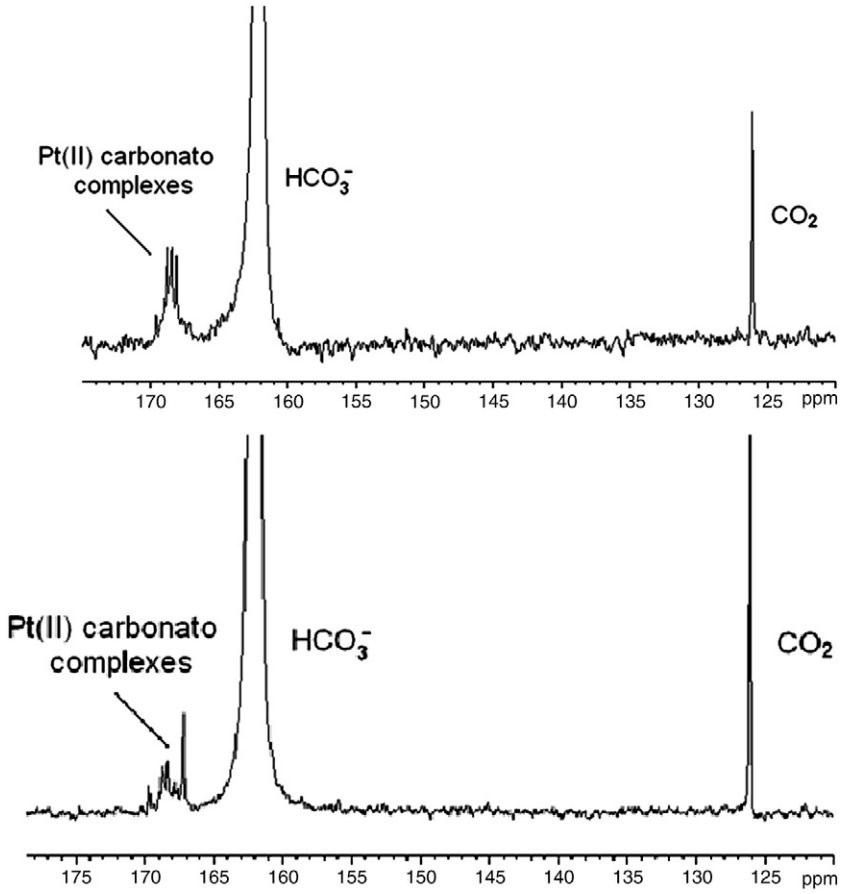

Fig. 5. ^{13}C NMR spectra of the products of reduction of **3** (top spectrum) and **4** (bottom spectrum) with ascorbic acid in the presence of $NaH^{13}CO_3$.

not 1:1 or there is a stoichiometric imbalance in the amounts of **3** and AsA in the reaction mixture. Since aquated and/or carboxyl substituted products of cisplatin should react with DNA to form the 1, 2 intrastrand crosslink, the ^{195}Pt NMR results reinforce the interpretation of the gel DNA mobility studies that suggest that the reduction product of **3** with AsA produces the crosslink, Figs. 2(a) and 3(a) and Table 1.

Fig. 4(b) shows that when oxoplatin is reduced by GSH using a 1:2 ratio of reactants, only a weakened signal for **3** at 853 ppm is observed in the ^{195}Pt NMR spectrum. While it is evident that a reaction has occurred, no Pt(II) products were observed in the ^{195}Pt NMR spectrum. In the case of the related complex, iproplatin, **2**, reduction with GSH occurs with a second order rate constant of 0.49 M^{-1} s^{-1} at 22 °C at pH 7.0 with three molecules of GSH being required to reduce one equivalent of **2** to produce, *cis*-di(isopropylamine)chloro-glutathionatoplatinum(II) [17]. Since iproplatin, **2**, and oxoplatin, **3**, have similar structures, it is reasonable to assume that **3** would also react with GSH in a 1:3 stoichiometry which explains the peak at 853 ppm as due to unreacted oxoplatin in the ^{195}Pt NMR experiment. Furthermore if the rates of reaction for **2** and **3** with GSH are similar, the $t_{1/2}$ of the reaction of **3** with GSH under the conditions of the ^{195}Pt NMR experiment ([GSH], [3] = 10 mM, 37 °C) is ~3 h, so that the reaction is complete. Although Volckova et al. [17] show that reduction of **2** with GSH produces *cis*-di(isopropylamine)chloro-glutathionatoplatinum(II), the corresponding Pt(II) complex for the reduction of **3**, *cis*-diamminechloro-glutathionatoplatinum(II), *cis*-[PtCl(GS)$(NH_3)_2$], is not observed in Fig. 4(b) (its resonance would be expected at ~−3000 ppm [40]). Failure to observe the Pt(II) product (s) in the ^{195}Pt NMR spectrum most likely means that although the Pt(II)-GSH adduct is formed, due to the polydentate nature of GSH and the long reaction period, 24 h, it converts to other species that have too low intensity to be detected in the NMR experiment. The DNA capture experiments clearly show that a Pt(II) complex is produced in the reaction, and is captured by DNA, but its effect on the supercoiling of DNA is not like that of cisplatin, i.e., the 1, 2 intrastrand crosslink is not formed, Fig. 2(f), Table 1[2,34–36]. If the initial reduction product in the reaction is *cis*-[PtCl(GS)$(NH_3)_2$], it could react with DNA through the loss of the chloride ligand to produce a monofunctional adduct, which because of the presence of the thiolate ligand, would be unlikely to yield an intrastrand crosslink.

The reduction of **4** by AsA mirrors that of oxoplatin except that only cisplatin is observed in the ^{195}Pt NMR spectrum, Fig. 5(c), showing that the reaction is complete. The absence of hydrolysis products and/or reaction products of cisplatin is explained by the high chloride concentration in the DPBS buffer (~140 mM) in the ^{195}Pt NMR sample. Since the reduction of **4** with AsA in the presence of DNA (no carbonate) was carried out in a HEPES buffer with some DPBS added with **4**, some reactive Pt(II) species should be present which explains the shift of Form I DNA relative to its control, Figs. 2(b) and 3(b). However, in this case mobilities are essentially independent of *r*. This is because, in the DNA reaction mixture, [Cl^-] is proportional to *r*, and since chloride suppresses DNA binding, mobilities are not very sensitive to *r*, Table 1.

The ^{195}Pt NMR spectrum of the reduction of **4** with GSH is similar to that of **3** with the tripeptide in that only some of the Pt(IV) complex remains after 24 h in the NMR solution. While this agrees with the reaction stoichiometry of **2** with GSH, Volckova et al. [17], i.e., some Pt(IV) should remain unreacted, Pt(II) products are not observed in the NMR spectrum. However, when the reduction is carried out in the presence of DNA without carbonate, DNA captures a Pt(II) complex, but the bound platinum does not affect mobility in the same way as the intrastrand crosslink, i.e. Forms I and II never co-migrate

at any value of *r*, Figs. 2(d,e) and 3(d,e), Table 1. By analogy with the reaction of **3** with GSH, the Pt(II) product is likely *cis*-[PtCl(GS)$(NH_3)_2$] which would be expected to bind to DNA in a monofunctional manner (loss of Cl^-) and not produce the intrastrand crosslink.

Fig. 5 shows ^{13}C NMR spectra of reductions of **3** and **4** with AsA in the presence of ^{13}C-labeled carbonate. As is evident from the spectra, the reduction results in the formation of Pt(II) carbonato complexes which exhibit characteristic ^{13}C NMR resonances in the region 167.2–169.7 ppm, on the low field side of $H^{13}CO_3^-$ [31,32]. It is well known that carbonato complexes can form by either a conventional ligand substitution reaction at the metal center, in which carbonate is exchanged with a ligand bound to the metal ion, or by a unique and rapid route in which the oxygen atom of a metal-bound hydroxo ligand attacks the carbon atom of molecular carbon dioxide, to produce a bound carbonate (hydrogencarbonate) ligand, Fig. 6 [41]. Since the latter reaction does not involve a metal–ligand bond breaking step, it generally occurs with much larger rates than conventional ligand exchange reactions, which, depending on the metal ion, are in the range, 50–600 M^{-1} s^{-1} [41]. The ^{13}C NMR spectra in Fig. 5 show that there are a greater number of distinct carbonato species formed in the reduction of **4** with AsA than in the corresponding reduction of **3**. The additional carbonato species observed for the former could be due to the liberated dicarboxylate ligand which could act as a ligand toward Pt(II) thereby increasing the number of carbonato products observed in the reaction.

In the period following the initial report of the anticancer activity of cisplatin, Rosenberg proposed that the anticancer properties of the drug are due to its ability to form an intrastrand crosslink at two adjacent purine bases of DNA [1]. Subsequent work by many research groups over the intervening years confirmed the existence of this type of lesion, finding that an adduct at the sequence GG is most common [2]. The mechanism for cisplatin binding to DNA starts with the mono-aqua complex, *cis*-$[PtCl(H_2O)(NH_3)_2]^+$, which reacts with N-7 of guanine through the loss of a the water molecule to form the monofunctional adduct *cis*-$[PtCl(G)(NH_3)_2]^+$ [42]. This DNA adduct then converts to the 1, 2 intrastrand crosslink, *cis*-$[Pt(GG)(NH_3)_2]^{+2}$, directly through the loss of the chloride ligand from Pt(II) or through an intermediate step involving the aqua complex. If carbonate is present, the bound water molecule of *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ may be partially or fully converted into a carbonate form, through the reaction in Fig. 6, and since carbonate/hydrogencarbonate is a poorer leaving ligand than water [43], binding of the complex to DNA would be reduced. This explains the gel studies that show that binding is slight when carbonate is present [34–36].

Todd et al. [36] argued that carbonate in the medium does not change the type of adduct formed on DNA, i.e. the 1, 2 intrastrand crosslink is formed, but that carbonate only reduces the amount of the crosslink that forms. However, characterization of the DNA adduct in these studies required techniques (HPLC, mass spectrometry, etc.) that would allow CO_2 gas to escape from the system. This could shift the equilibria given in Fig. 6 to the left and, the cisplatin-DNA adduct that initially forms in carbonate could have been inadvertently destroyed in the workup thus producing the 1, 2 intrastrand crosslink. Binter et al. [34], on the other hand, measured mobilities and band intensities of platinated DNA in gel electrophoresis experiments, concluding that the presence of carbonate causes cisplatin to form a monofunctional adduct on DNA. While these studies had none of the structural characterization rigor of the study by Todd et al. [36] they were much less prone to shifts in the chemical equilibrium in Fig. 6. In a following study, Sorokanich et al. [35] were unable to reproduce the band intensity changes observed by Binter et al. [34] but the investigators found that at large values of *r*, Forms I and II never co-migrate in the gel, again suggesting that the adduct in carbonate is not the same as the 1, 2 intrastrand crosslink. The living cell maintains a cytosolic concentration of ~12 mM carbonate, of which dissolved CO_2 is an important component, so cisplatin adducts that form on genomic DNA are exposed to a "carbonate pressure" that may be difficult to maintain in laboratory experiments. Since carbonate can change the speciation of cisplatin and carboplatin, additional study on how carbonate influences the rate and mechanism of the formation of the DNA adduct and its structure is warranted.

Presently, science is racing to discover new ways of delivering proven anticancer drugs to tumor sites using nanotechnology [2,24–28,44]. A reoccurring theme in the case of the platinum drugs is tethering a Pt(IV) complex of the type exemplified by **4** to a nano-sized delivery vehicle capable of targeting a cancer cell. While this strategy promises to uncover exciting new ways for treating cancer, these sophisticated delivery vehicles may not survive passage through the blood to the tumor site. To date, most studies have been done using cells growing in culture and since culture medium contains only a small amount of GSH (~3 μM) and no ascorbic acid, the reducing environment in a cell culture study is very different than that encountered in chemotherapy.

Using the published rate constants for the reduction of **3** with ascorbic acid and reduction of iproplatin, **2**, with GSH [15,17], it is interesting to speculate on the nature of the Pt(II) products formed if **3** and **4** are present in blood. Although k_{AsA}/k_{GSH}~4, the ratio [AsA]/[GSH] in blood (plasma) is 100 mM/850 mM = ~0.12 [2,10,11,16] which suggests that only half (4 × 0.12) of the reduction product would be cisplatin, i.e., reduction by AsA, with the remaining half being a non-cisplatin complex, formed by GSH reduction. An additional aspect is that the reduction potentials of **3** and **4** are −0.82 and −0.46 V (NHE) [45,25] respectively, which shows that **4** is more easily reduced than **3**. The reduction potentials for AsA and GSH are −0.065 V and −0.16 V respectively, so that GSH is a slightly better reducing agent than AsA [46,47]. Since the circulation time of a particle in the blood is 1–10 h, there would be ample opportunity for a compound such as **4** to be reduced on the way to the tumor site [15,17–19] with much of the reduction product being a thiol adduct of cisplatin. The platinum "cargo" released from the carrier before the vehicle reaches its intended target could also reach and kill tumor cells, which could impart characteristics to the delivery system that are a combination of both the nano-carrier and the free drug.

In the environment in the cytosol, where [AsA]/[GSH]~1 mM/2 mM~0.5, the situation found in blood is that the formation of cisplatin through reduction by AsA is favored by 2 to 1 over the formation of non-cisplatin product through reduction by GSH. If the nano-carrier with its attached load of Pt(IV) enters the cell intact and is reduced by intracellular GSH, the commonly cited mechanism, the expected product would be a cisplatin-GSH adduct and not, as is often suggested, cisplatin. The likelihood of an intracellular Pt(II)-glutathione adduct being formed under these conditions is supported by the work of Raynaud et al. [48] who showed that the Pt(IV) complex, satraplatin, is reduced inside the cell and that the amount of intracellular Pt(II)-adduct formed is dependent on the amount of

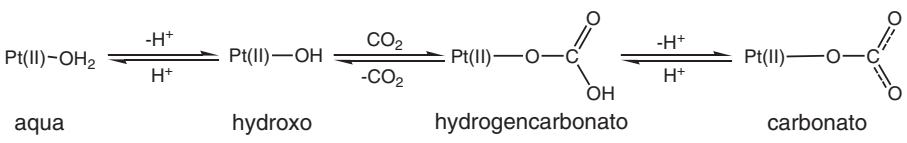

Fig. 6. Scheme showing the reaction of a Pt(II) aqua species with molecular carbon dioxide and associated equilibria.

GSH in the cell. While the reaction of GSH with cisplatin is widely cited as an inactivation mechanism for the drug, implying that the GSH adduct is not toxic to the cell, such an adduct has one remaining coordination site (loss of the chloride ligand) for interaction with biological targets in the cell. Given the uncertainty of just how cisplatin kills cells, formation of a cisplatin-GSH adduct inside the cell could be sufficient to modify proteins and DNA, impairing their function, thus causing the cell to enter into apoptosis and die. Professor Dan Gibson appropriately summed up the progress in the area in a recent paper titled, "The mechanism of action of the platinum anticancer agents—What do we really know about it?" [21]. Despite more than 40 years of research on the mechanism of action of cisplatin and its analogs we really do need to learn a lot more about how these important drugs work. This is almost certain to challenge long standing paradigms and, most importantly, open new avenues for finding more effective platinum drugs for treating cancer.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2011.10.012.

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