HPLC Determination of Binding of Cisplatin to DNA in the Presence of Biological Thiols: Implications of Dominant Platinum-Thiol Binding to Its Anticancer Action

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Purpose. The purpose of this work is to evaluate the extent of the binding of cisplatin (cis-diamminedichloroplatinum(II)) to DNA in the presence and absence of biological thiols, glutathione, and cysteine, and to test the hypothesis whether the platinum-thiol complexes can serve as a drug reservoir for subsequent binding to DNA.
Methods. Reactions of cisplatin (50 μM to 1.0 mM) with calf thymus DNA (870 μM to 6.75 mM) in the presence and absence of glutathione and cysteine (0 to 10mM) were carried out at pH 4.4, 7.0, and 7.3. Following the reactions, the DNA was enzymatically digested with nucleases, separated by RP HPLC, and analyzed to determine the extent of DNA binding. The method was independently verified by proton NMR measurements.
Results. At neutral pH, and equimolar concentrations of DNA and thiols, only a very small amount of platinum (<5%) was coordinated to DNA, and most of the platinum was coordinated to the thiols. At pH 4.4, binding to DNA was dominant over the binding to thiols. No conversion of platinum-thiol to platinum-DNA complexes was observed up to 7 days of incubation.
Conclusion. At physiological pH, the cisplatin was exclusively coordinated to biological thiols and platinum-DNA was a minor adduct. Data presented in this paper does not support the "drug reservoir" hypothesis.

KEY WORDS: cis-diamminedichloroplatinum(II) (cisplatin); platinum-glutathione; platinum-DNA binding.

INTRODUCTION

Cis-diamminedichloroplatinum(II) (cisplatin) is widely used for the treatment of testicular, ovarian, and other forms of cancer (1–3). The platinum compound arrests the cell cycle at the G2 phase by a mechanism commonly known as apoptosis (4–6). At the molecular level, it is generally believed that the platinum compound binds the cellular DNA primarily though an intra-strand binding mode (7). *In vitro* experiments indicate that this intra-strand binding is mainly due to coordination through the N7 sites of the purine ring of two adjacent guanine and guanine-adenine bases. Other modes of binding including inter-strand DNA, protein, and DNA-protein crosslinks have been reported. Eastman and coworkers (8) have estimated various DNA binding modes by separating the platinated DNA lesions by HPLC and measuring the platinum content in the major fragments by atomic absorption. An estimation of DNA binding under cellular environment is important for designing effective drug delivery mechanisms to the target biomolecules. In the cellular milieu, thiol containing ligands such as glutathione and cysteine are present in substantial concentration. Platinum(II) has tremendous affinity for binding to sulfur donors, especially thiols, compared to nitrogen donor ligands such as DNA bases (9–12). The intra-cellular concentrations of sulfhydryl groups including cysteine and glutathione could be as high as 10 mM. Because nitrogen donor sites in DNA cannot compete with thiols and thio-ether donors in peptides and proteins, it is not well understood how DNA competes for platinum with biological thiols and thio-ethers in cellular milieu. A conventional hypothesis is that sulfur containing nucleophiles initially bind to the platinum atom and then convert to platinum-DNA complexes, thermodynamically more stable products (13). It is therefore desirable to estimate the extent of platinum-DNA binding in the presence of thiol containing small amino acids and peptides and follow the fate of platinum-thiol complexes. In this article, we describe the extent of DNA binding in the presence and absence of biological thiols as monitored by HPLC method by measuring the concentration of unbound bases. The concentrations of the thiols used in our experiments are similar to those found in the cellular milieu. Single stranded DNA was selected as the model substrate due its ease of hydrolysis by nucleases. A longer hydrolysis time, as much as 16h (33), is required for the double stranded DNA hydrolysis and therefore, a precise determination of concentration-time profile would be difficult using our method of analysis.

MATERIALS AND METHODS

Materials

L-cysteine, glutathione, 2′-deoxyadenosine (dA), 2′-deoxycytosine (dC), 2′-deoxythymidine (dT), and 2′-deoxyguanosine (dG) are purchased from Sigma. Other reagents, ammonium formate, formic acid, and HPLC grade methanol and acetonitrile were also received from Sigma. *Cis*-diamminedichloroplatinum(II) was prepared by the literature method (14). Deuterium oxide (99% atom, Sigma) was used as a solvent for the NMR experiment. Water was purified via an Ion Pure Plus 150 filtration system with 0.2 μm filter. Single stranded calf thymus DNA (CT ssDNA) was purchased from Sigma as a lyophilized powder. Nuclease P1 (400 u/μg) and alkaline phosphatase (20 u/μL) were obtained from US Biochemicals. Other reagents such as tris-HCl, acetic acid, MgCl2 etc. were of highest purity grade materials.

Methods

Chromatographic Separation

High performance liquid chromatography experiments were performed on a Waters HPLC system equipped with an ISCO ternary gradient programmer and a photodiode array detector (Waters). The separations were performed on a C18 column (Waters, Novapak) by using a gradient method utilizing a mobile phase consisting of 50 mM ammonium formate

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buffer (pH, 4.0) (solvent A) and methanol (solvent B). The gradient was set up such that for the first 15 min the solvent B was linearly increased from 0% to 15% followed by a steeper gradient, 15 to 100% of B in the next 15 min. Finally, an additional 8 min of separation was extended with 100% B. In some separations, 15 mM phosphoric acid (pH = 2.2) with 7% methanol was used as the mobile phase. These isocratic separations were performed on a Lichrosorb R18 column.

The chromatograms were detected at 260 nm. However, UV spectra in the range 350 to 200 nm for all eluates were recorded by a diode array detector. These spectra and retention times were used to establish the identity of analytes. A 20 μL aliquot of sample was injected for each separation. Each separation was repeated three times. Each experiment was then duplicated by using fresh starting materials. Therefore, the concentrations derived from chromatograms are in most cases the average of six runs. Calibration curves for four bases, dA, dC, dG, and dT were established from nucleoside standards.

Nuclear Magnetic Resonance Measurements

Spectra were recorded on a 500 MHz (Varian, Unity 500) instrument using a deuterium lock. The proton signals are referenced to H-O-D resonance at 4.68 ppm. Typically, spectral width (sw), 10 kHz was selected with 32k data points. Usually, 16 scans were sufficient to collect spectra with S/N > 50. A presaturation pulse exactly at water resonance was applied to suppress the peak for water. A line broadening factor of 2 Hz was introduced before the Fourier Transformation.

Reactions of Cisplatin with DNA in the Presence and Absence of Biological Thiols

The stock solution of DNA was prepared by denaturing the nucleic acid at 70°C for 15 min and immediately storing on an ice to prevent annealing. In a typical measurement, CT-ssDNA (870 μM to 4.0 mM) was reacted with cisplatin (0 to 1.0 mM) in the absence and presence of cysteine or glutathione in 10 mM phosphate buffer of pH = 7 and 7.3. The reaction mixture was incubated at 37°C for variable length of time, 18 h to 7 days. Aliquots of cisplatin-DNA reaction mixtures were treated with nucleases at desired time interval. Nuclease P1 (15 units) was added in 100 μL sample in two steps followed by the addition of alkaline phosphatase (10 units total) twice at thirty minute intervals. The mixture was stored at –20°C until the HPLC separation was performed.

Reaction in acidic solution was carried out in acetate buffer (10 mM, pH = 4.4) by using platinum and DNA concentrations stated above. The same cleavage protocol was applied except that the pH of the reaction mixture was raised to 7.2 before the enzymatic digestion. When the experiments were conducted in unbuffered solution at pH 3, irreproducible results were obtained most probably due to precipitation of platinum-DNA complex; although the precipitation was not visible to naked eyes.

The DNA displacement reaction was initiated by adding glutathione to cisplatin-DNA complex which was prepared as described above by incubating the platinum complex with DNA for 24 h at 37°C. Aliquots of the reaction mixture were then subjected to enzymatic digestion at different time intervals up to seven days, and followed by chromatographic separation.

RESULTS

Figure 1 shows the chromatographic separations of cisplatin-DNA reactions, carried out either in the presence or absence of glutathione, and subjected to the enzyme digest. The concentration of four deoxynucleosides were determined from the standard calibration curves established from pure nucleosides. These standard curves were linear from 20 μM up to 5.0 mM for each nucleoside and the value of r^2 for each of the standard curve was calculated to be >0.999. For the

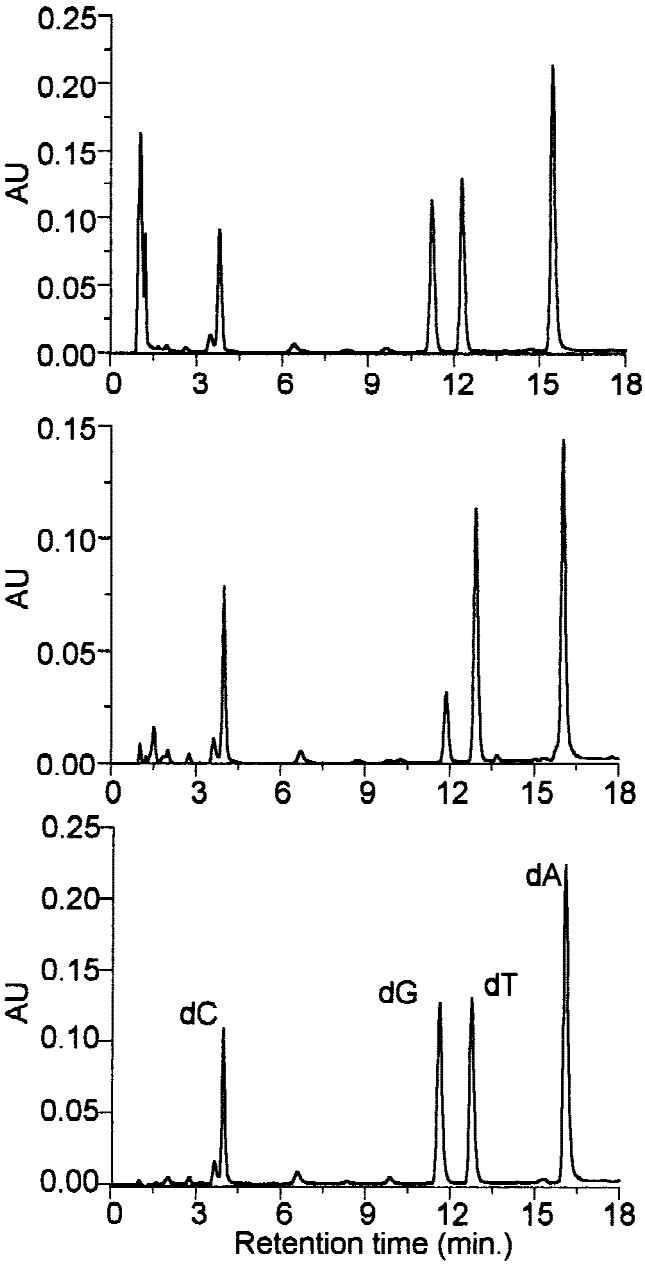

Fig. 1. Chromatograms of DNA (2.25 mM) after digestion with nucleases: Bottom, DNA alone; middle, DNA plus cisplatin (1.0 mM); top, DNA, cisplatin (1.0 mM) and glutathione (1.0 mM). The released nucleosides are marked in the bottom chromatogram.

platinated DNA, the nucleases do not cleave the nucleic acid at the sites where platinum(II) is coordinated (8). Therefore, the difference between the concentration of each of the free bases obtained from the control experiments and that evaluated after incubating platinum with DNA provides the estimation of bound nucleosides. In addition to four bases, several other peaks appear in the chromatograms which are for platinum complexes bound to DNA bases and biological thiols.

Table I lists the concentrations of unbound nucleosides in the presence and absence of cisplatin and glutathione. The selection of initial DNA concentration is based on the fact that the platinum compound favors the formation of bis-(nucleoside) complexes upon the release of two chlorides from its coordination sphere (15–16). The lowest nucleoside (dA+dC+dG+T) concentrations, 2.25 mM was above two-fold of platinum concentrations (1.0 mM) to ensure complete reaction with the DNA. Under these conditions, the reaction of DNA with the platinum compound in the absence of glutathione accounts for the loss of total nucleoside concentration of 1.2 mM. When the concentration of DNA was doubled, a loss of 1.6 mM nucleoside due to the coordination to platinum was observed. When the reaction was carried out in 1.0 mM glutathione with 1.0 mM cisplatin, the amount of uncoordinated nucleosides were found to be almost the same as in the control experiment indicating that insignificant amount of DNA was bound to platinum. By doubling the DNA concentration, only 0.10 mM nucleobase, exclusively dG, was found to be coordinated to the metal ion. Figure 2 shows the chromatograms of cisplatin-DNA reactions in the presence and absence of cysteine. Table II lists the concentrations of unbound nucleosides in the presence and absence of cysteine. In the presence of 1.0 mM cysteine, only a loss of 0.25 mM nucleoside compared to the control experiment was observed. By doubling the DNA concentration from 2.25 mM to 4.50 mM and keeping the cysteine concentration invariant, no significant increase in the nucleoside binding was observed. Assuming that bis(nucleoside) complexes are predominantly formed, less than 15% of the original cisplatin was bound to DNA when both platinum and cysteine were present in equimolar concentrations. When cysteine concentration was less than that of cisplatin, a significant amount of platinum reacted with the nucleic acid. Kinetic and Pt-195 NMR data for the cisplatin-cysteine reaction indicate a favorable formation of a bis(cysteine) complex.(9) Since the reaction is much faster than that of DNA (10), it is imperative that the platinum compound reacts with the DNA when the amino acid is almost completely depleted from the reaction mixture. In fact, no traces of unreacted cysteine can be detected in the chromatogram. Furthermore, no significant increase in DNA binding was observed even when the concentration of the nucleic acid was doubled.

Table I. Estimation of Unbound Nucleoside Concentrations (mM) for the Reaction of Cisplatin (1.0 mM) with DNA in the Presence of Glutathione at pH 7.0. Concentrations for Control Experiments Were Measured in the Absence of Cisplatin

[GSH]	[dA]	[dC]	[dG]	[dT]
		[DNA] = 2.45 mM		
0.0	0.404 ± .004	0.402 ± .003	0.084 ± .001	0.384 ± 0.003
0.20	0.608 ± .006	0.530 ± .006	0.172 ± .002	0.481 ± 0.005
0.50	0.725 ± .008	0.567 ± .007	0.398 ± .004	0.510 ± .006
1.00	0.759 ± .009	0.629 ± .007	0.477 ± .005	0.577 ± .012
Control	0.787 ± .012	0.630 ± .010	0.497 ± .012	0.567 ± .009
		[DNA] = 4.85 mM		
0.0	1.114 ± .021	0.959 ± .016	0.309 ± .006	0.860 ± .012
0.20	1.432 ± .021	1.117 ± .019	0.530 ± .008	1.030 ± .015
0.50	1.513 ± .025	1.165 ± .020	0.871 ± .009	1.052 ± .015
1.00	1.589 ± .035	1.220 ± .030	0.872 ± .012	1.098 ± .021
Control	1.570 ± .029	1.207 ± .021	0.976 ± .011	1.082 ± .014

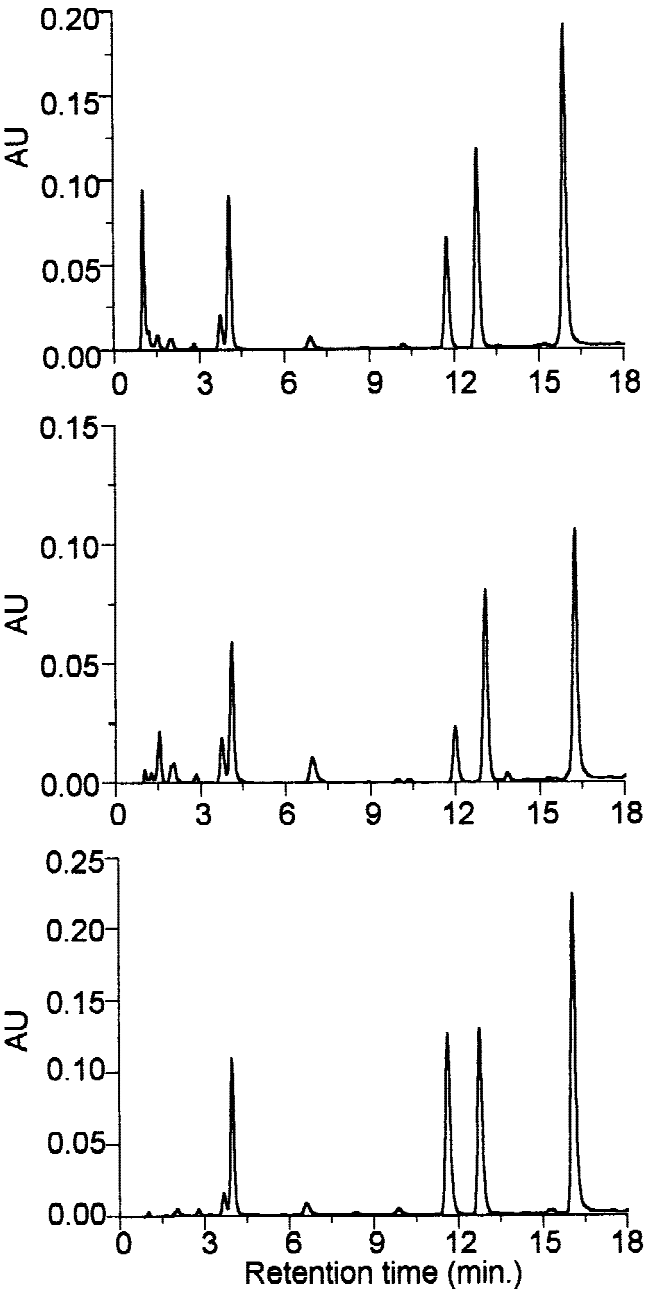

Fig. 2. Chromatograms of DNA (2.25 mM) after digestions with nucleases: Bottom, DNA alone; middle, DNA plus cisplatin (1.0 mM); top, DNA, cisplatin (1.0 mM) and cysteine (1.0 Mm). The released nucleosides are marked in the bottom chromatogram.

Table II. Estimation of Unbound Nucleoside Concentrations (mM) for the Reaction of Cisplatin (1.0 mM) and DNA in the Presence of Cysteine at pH 7.0. Concentrations for Control Experiments were Measured in the Absence of Cisplatin

[cysteine]	[dA]	[dC]	[dG]	[dT]
		[DNA] = 2.45 mM		
0.0	0.404 ± .004	0.402 ± .003	0.084 ± .001	0.384 ± .003
0.20	0.547 ± .005	0.494 ± .004	0.169 ± .002	0.449 ± .005
0.50	0.638 ± .006	0.520 ± .005	0.217 ± .002	0.463 ± .004
1.0	0.724 ± .006	0.568 ± .006	0.388 ± .004	0.506 ± .006
Control	0.787 ± .012	0.630 ± .010	0.497 ± .012	0.567 ± .009
		[DNA] = 4.85 mM		
0.0	1.114 ± .021	0.959 ± .011	0.309 ± .006	0.860 ± .010
0.50	1.336 ± .020	1.069 ± .012	0.515 ± .012	0.937 ± .011
1.00	1.523 ± .031	1.119 ± .020	0.818 ± .011	1.052 ± .012
10.0	1.58 ± .03	1.21 ± .02	0.92 ± .02	1.06 ± .02
Control	1.570 ± .029	1.207 ± .021	0.976 ± .011	1.082 ± .014

The platinum-thiol complexes exhibit very short retention times on a C18 column with our mobile phase. The intensity of the peaks representing these complexes did not change within a few days, after the primary reactions were over. Likewise, no decrease in unbound nucleoside concentrations was apparent during the same time indicating that no further transformation of platinum-thiol complexes to platinum-DNA complexes took place during our experiments.

The reactions between the platinum complex and DNA were also examined by using therapeutically relevant cisplatin and physiologically appropriate glutathione concentrations. Figure 3 (Table III) displays the concentration of unbound DNA bases for a reactions that used 50.0 μM cisplatin and 870 μM DNA in the presence and absence of 2.0 mM glutathione. As can be seen from Table III, in the absence of glutathione, the total bound base concentration is close to 100 μM, twice the concentration of cisplatin. These data indicate that the formation of bis(nucleoside) complex, $Pt(NH_3)_2(Base)_2$ is virtually complete. In the presence of glutathione, however, concentrations of the unbound bases were almost identical to those found in control experiments indicating that DNA cannot effectively compete with the thiol to bind platinum. Since the chromatographic data bear uncertainties up to ±2.0%, we conclude that the DNA bound species must be below the error limit.

The replacement of coordinated glutathione by DNA was also monitored for up to seven days. The variations of the concentrations of unbound bases are shown in Table III (Fig. 3). The changes in base concentrations fall within the experimental uncertainty indicating that DNA did not replace coordinated glutathione. We have also examined the replacement of coordinated DNA by glutathione. Here again, the Platinum-DNA complexes were not substituted by the thiol ligand. It appears then that both Pt-glutathione and -DNA complexes are quite robust toward substitution.

When the reactions were carried out in acidic solution (pH 4.4), significant coordination to DNA was observed.

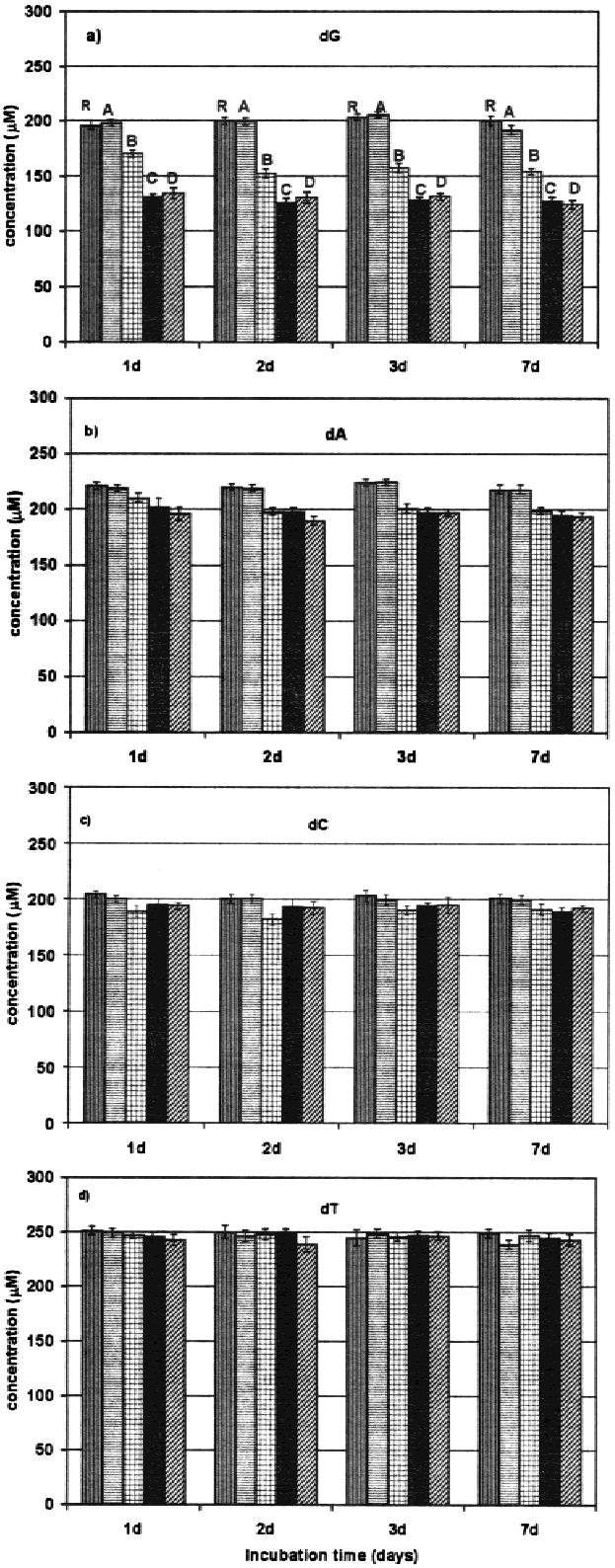

Fig. 3. Evaluations of concentrations of unbound nucleosides a = dG, b = dA, c = dC, d = dT for the reaction of cisplatin(50 μM) and DNA (870 μM) under various experimental conditions: Reaction A contained cisplatin, DNA and 2mM glutathione at pH = 7.3, B: cisplatin, DNA and 2 mM glutathione at pH = 4.4, C: cisplatin and DNA at pH = 7.3, D: cisplatin, DNA at pH = 7.3 with 2 mM glutathione added 24 h after the initiation of the reaction, Control (R): DNA alone. Column labels for b, c, d are identical to those in a.

Table III. Determination of Concentrations of Unbound Nucleosides for the Reaction of Cisplatin (50 μM) and DNA (870 μM) under Various Experimental Conditions

	Incubation time (days)	Base Concentration (μM)				
		Control (R)	A	B	C	D
dG	1	196 ± 4	198 ± 3	170 ± 3	131 ± 3	135 ± 5
	2	200 ± 3	200 ± 3	152 ± 4	126 ± 4	131 ± 5
	3	203 ± 3	206 ± 4	157 ± 4	128 ± 3	132 ± 3
	7	201 ± 4	192 ± 5	154 ± 3	127 ± 4	124 ± 4
dA	1	221 ± 3	219 ± 4	210 ± 4	202 ± 8	196 ± 6
	2	220 ± 3	219 ± 4	198 ± 3	198 ± 3	190 ± 4
	3	224 ± 3	225 ± 6	201 ± 4	197 ± 4	197 ± 3
	7	218 ± 4	218 ± 4	199 ± 3	195 ± 4	194 ± 3
dC	1	204 ± 3	200 ± 5	189 ± 4	195 ± 5	194 ± 3
	2	200 ± 4	201 ± 3	182 ± 4	193 ± 7	192 ± 6
	3	203 ± 5	200 ± 4	190 ± 3	194 ± 3	195 ± 7
	7	201 ± 4	200 ± 3	191 ± 4	191 ± 4	192 ± 3
dT	1	251 ± 4	249 ± 4	243 ± 3	247 ± 3	246 ± 5
	2	250 ± 6	247 ± 5	239 ± 5	248 ± 5	248 ± 7
	3	245 ± 7	249 ± 4	247 ± 4	246 ± 4	247 ± 4
	7	249 ± 4	239 ± 4	243 ± 5	247 ± 4	245 ± 5

Reaction A contained cisplatin, DNA and 2 mM glutathione at pH = 7.3, B: cisplatin, DNA and 2 mM glutathione at pH = 4.4, C: cisplatin and DNA at pH = 7.3, D: cisplatin, DNA at pH = 7.3 with 2 mM glutathione added 24 hours after the initiation of the reaction, Control (R): DNA alone.

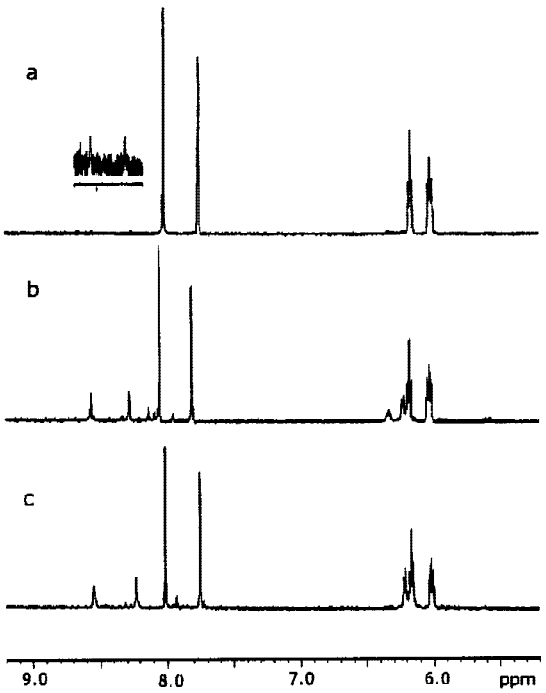

Fig. 4. Selected regions from the1D H-NMR spectra of the reactions of 1mM cisplatin with 2 mM GpG a in the presence of 2mM glutathione, pH = 7.0, b in the presence of 2mM glutathione, pH = 4.4, and c in the absence of glutathione, pH = 7.0.

These observations were made from both the chromatographic measurements and from the NMR experiments. When the thiol and DNA concentrations were equal, the DNA binding is dominant. Figure 3 also shows the concentrations of unbound bases from cisplatin (50 μM)-DNA (870 μM) reaction carried out at pH 4.4 in the presence of 2.0 mM glutathione. Taking bis(nucleoside) is the dominant complex in the reaction mixture, the data indicate that about 80% of platinum is bound to DNA. Even when the thiol concentrations were exceeded five fold over the DNA, 20% of the nucleotide was bound to cisplatin.

To further validate the method that in the presence of equimolar cysteine or glutathione, DNA cannot compete effectively with the biological thiols, reactions of dGpG with cisplatin in the presence of glutathione was monitored. This dinucleotide has been identified as the main bifunctional platinum adduct in the DNA lesion. The free di-nucleotide exhibits two H8 resonances at 7.75 and 8.01 ppm assigned to 5′ and 3’ guanosines as they relate to the bonding with their respective sugar moieties through the 3’ and 5′ positions to the phosphate diester. The reaction of the dinucleotide with cisplatin shifted these two signals down field to 8.54 and 8.23 ppm indicating the coordination through the N7 sites of the nucleotide. In the presence of equimolar concentration of glutathione, almost no detectable signals for coordinated dinucleotide were observed (Fig. 4). In contrast, the signals for the methylene protons next to the thiol group of glutathione exhibit significant broadening and downfield shift compared to the corresponding signal of the free tripeptide. These data clearly support the chromatographic results that platinum does not significantly bind to the DNA in the presence of thiol containing small amino acids and peptides.

DISCUSSION

As can be seen from Tables I–III, in the absence of biological thiols, cisplatin did not completely bind to all available guanine bases. In fact, significant binding to other bases was observed. Substitution reactions of platinum(II) complexes are primarily kinetically rather than thermodynamically controlled processes. Furthermore, substitution reactions of cisplatin usually follow consecutive kinetics due to the release of two coordinated chlorides in a stepwise fashion (10,12,15–17). Therefore, once a platinum center is bound to a specific guanine base, it is forced to react either with the adjacent base or remains as a monofunctional adduct. The latter adduct either could be a mono(chloro)(base) or mono(aqua)(base) complex. However, the aqua- species is highly reactive and known to bind even weak ligands such as phosphate and carboxylate (18). Because we have selected a calf thymus DNA in which 20% bases are guanine and that only a fraction of those guanine bases are adjacent to each other, other bases were forced to react once a platinum atom is anchored to a specific guanine sites. We therefore believe that the coordination to cytosine and thymine was not due to the inherent preference toward these bases, but rather a situation in which the platinum(II) center was forced to react due to the initial anchoring to either guanine or adenine bases. As the concentration of the DNA was increased, disproportionately higher percentage of G base was coordinated. Nonetheless, other bases were also bound, although to a lesser extent. The observed distributions of the bound nucleosides are consistent with established reactivity of DNA bases, dG>dA>dC>dT toward the platinum complex (19).

In the presence of glutathione, the amount of base coor-

dination was systematically reduced as the concentration of thiol was increased. In fact, when equimolar concentrations of cisplatin and glutathione were used, only a small amount of G base coordination was observed. For the cysteine reaction however, relatively more base coordination, exclusively at G base was observed. From a kinetic point of view, these observations might represent a paradox in that more base coordination would be expected for the glutathione reaction since the latter is a much bigger molecule and that platinum substitution is controlled mainly by an associative mechanism. The observed anomaly can be explained as follows. Both cysteine and glutathione reactions with cisplatin are much faster than that of the DNA reaction. When the thiols are completely depleted, DNA begins to react with the remaining cisplatin. However, since cysteine is a much smaller ligand, bis(cysteine) coordination is more favorable over the corresponding bis(glutathione) complexation. In fact, we have shown that the second step of the cysteine reaction leading to the formation of bis(cysteine) complex is kinetically more favorable than the initial coordination of the thiol moiety (10). Therefore, more platinum is expected to be exposed to DNA reaction due the favorable 1:2 platinum:cysteine stoichiometry. Moreover, the formation of cysteine-DNA mixed ligand complex would also be more favorable over the glutathione-DNA counterpart due to the same steric reasons cited above.

The preference of DNA coordination over thiol in acidic solutions needs to be addressed. At lower pH values, all thiols, but not the N7 sites of purines, are exclusively protonated. The sluggish reactions of protonated thiols over the N7 purine sites led to a significant base coordination. In fact, similar observations were made by other workers in describing the reactivity of other platinum(II) complexes (20). These authors extrapolated their observations in acidic solution in justifying cellular DNA coordination in the presence of biological thiols. At or near neutral pH, although less than 10% of thiols are deprotonated, the bases cannot compete with the thiol containing amino acids and peptides, due to the enhanced reactivity of deprotonated thiols by several orders of magnitude (9). Therefore, the binding primarily takes place through the sulfur donor sites.

Results of the present investigation need to be discussed in the cellular context. Firstly, the reactivity of double stranded DNA toward cisplatin must be compared with single stranded DNA. Cisplatin reacts with both single- and double stranded-DNA primarily through rate limiting aquation steps. Thiol binding, on the other hand, proceeds through the direct reaction with the dichloro species as well through the aquated pathway. The direct bimolecular reaction of the dichloro species with the tripeptide is expected to dominate in the intracellular environment due to substantial intracellular glutathione concentration. Since the reactive platinum species for DNA binding is $Pt(NH_3)_2(Cl)(H2O)+$, rates of single and double stranded DNA reaction with the aqua-chloro-species need to be compared. It has been shown that the rate of initial reaction of a duplex DNA with $Pt(NH_3)_2(Cl)(H2O)+$ is doubled compared to that of a single stranded counterpart (32). The rate of chelation, however, was slightly less for the double stranded substrate. Although the rate of double stranded DNA reaction is higher than that for the single stranded analog, the combined concentration of intracellular thiols needs to be considered for appropriate comparisons. Glutathione alone has been reported to be as high as 10 mM. Moreover, many cytosolic enzymes and proteins including replication enzymes and transcription factors contain many cysteine residues. Furthermore, these concentrations of thiols are many orders of magnitude higher than the anticipated platinum concentration in the cell. For example, only a fraction of administered drug is transported inside the cell. Taking all the rates and concentration profiles into account, we conclude that majority of platinum is expected to bind thiol nucleophiles. We concur with the analysis by Eastman and co-workers (4) that only about 1% of the administered platinum drug is bound to genomic DNA.

The issue of transformation of platinum-thiol species to platinum-DNA complexes also needs to be addressed. To address the kinetic vs. thermodynamic preference for platinum substitution reactions, several model systems with a monofunctional platinum(II) complex and several ligands that contain both nitrogen and sulfur donor sites have been examined. For example, Pt(dien)Cl+ was reacted with SAH (S-adenosyl-L-homocysteine), and SGH (S-guanosyl-L-homocysteine). For the SAH reaction, the coordination exclusively took place through the sulfur sites and through the amine of the homocysteine moiety, but not through the N1 or N7 sites of the purine base (21). However, for the SGH system, rapid coordination to the sulfur atom followed by migration to the N7 site of the purine was observed (22). Similar observations were made from a methionine anchored trinucleotide, Met-d(TpGpG) (23). An intermolecular competition reaction of Pt(dien)Cl+ with a mixture of methionine and 5′-GMP revealed that Pt(dien)(methionine) complex can be slowly (t1/2 ~ 7 days at 310 K) replaced by the nucleotide (24). Similar competition experiments of the bifunctional platinum complex, cis-dichloro(ehylenediamine)platinum(II) and its hydrolyzed products with a mixture of 5′-GMP or dGpG and thio-ether containing di- and tri-peptides, also afforded sulfur bound intermediates followed by N7-coordinated guanine products (25–27). Here again, the transformation from the sulfur ligation of the peptide to the nitrogen coordination to DNA was slow. Although such migration of thioether to guanine ligation seems to be common, we did not observe any measurable transition from platinum-thiol to platinum-DNA coordination within seven days. Since platinum-glutathione complex is transported out of the cells after 12 h through an ATP-dependent transport mechanism (28), an extremely sluggish kinetic process for converting Pt-glutathione to Pt-DNA complex may be mechanistically irrelevant.

Because cisplatin readily reacts with glutathione and that as much as 67% of administered platinum has been found to coordinate to the tripeptide (28), the role of glutathione toward the cytotoxicity of cisplatin needs to be addressed. The role of glutathione appears to be dual; the tripeptide both deactivates and activates cisplatin. An enhancement of glutathione concentration in the cell induced by the platinum complex has been reported (29–30). Because the tripeptide is major detoxifying agent for both exogenous and endogenous toxins, the cellular response to increase the level of glutathione is certainly understandable. In one study, the ineffectiveness of cisplatin to several different ovarian cells has been correlated with the increased level of glutathione (31). In another study, the platinum glutathione complex has shown to arrest the protein synthesis by blocking the translational

activity (28). The higher effectiveness of cisplatin has also been demonstrated by co-administering cisplatin and glutathione in patients. It is not clear whether this increase in effectiveness is due to the reduced toxicity or due to the modification of the platinum drug by covalent binding to the metal center.

In closing, considering the breakthrough work of Eastman and co-workers (4–6) that apoptosis takes place at the G2 phase, cellular and molecular events including the signal transduction pathways other than Pt-DNA binding events, must be involved in the cisplatin induced anti-carcinogenesis. In fact, involvement of some transcription factors has already been demonstrated. Since several sulfur donor ligands are usually co-administered with cisplatin to reduce the toxicity (32–33), further work is needed to unveil whether modified platinum complexes coordinated to 'rescue ligands' trigger apoptosis as well.

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