DNA Hairpin Formation in Adducts with Platinum Anticancer Drugs: Gel Electrophoresis Provides New Information and a Caveat

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Abstract: The duplex form of the self-complementary oligonucleotide, 5′d(A(1)T(2)G(3)G(4)G(5)T(6)A(7)C(8)C-(9)C(10)A(11)T(12)), was treated with *cis*-$Pt(A_2)Cl_2$ anticancer drugs ($A_2 = (NH_3)_2$ or en (ethylenediamine)). Previous NMR reports of the Pt(en) reaction revealed *one* hairpin-like product with platination at two of G(4), G(5), or A(7) but *not* at G(3). To resolve these issues, 3′-^{32}P-end-labeling of reaction mixtures was examined. Gel electrophoresis gave *one* pronounced product band (autoradiographic detection) with hairpin-like mobility for both drugs. However, in conflict with the NMR studies, this product has a G(3),G(4) intrastrand crosslink (DNA sequencing methods). Furthermore, with each drug, 5′-^{32}P-end-labeling and gel electrophoresis gave *two* significant comparable hairpinlike product bands: one for the G(3),G(4) crosslinked adduct identified as the exclusive product by 3′-^{32}P-end-labeling and the second for a G(4),G(5) crosslinked adduct consistent with NMR studies. To resolve these issues, the Pt(en) reaction was subjected to exhaustive 5′-end-labeling with nonradioactive ATP. The G(3),G(4) adduct identified as the exclusive product by 3′-^{32}P-end-labeling was found by UV-visualization to constitute only 4% of the product. The major product (96%) was the G(4),G(5) crosslinked adduct. From gel electrophoresis under denaturing conditions, the G(3),G(4) adduct exists in the denatured state to a much greater extent than the G(4),G(5) adduct. Clearly, during both types of enzymatic labeling, the minor product (probably as the denatured form) was labeled at much higher efficiency, suggesting that caution should be exercised in interpreting the increasingly widely used ^{32}P/gel electrophoresis methods. In a GGG sequence, the N7 of the central residue, G(4), is the most nucleophilic site, and we found that the monofunctional complex, [chloro(diethylenetriamine)platinum(II)] chloride, preferentially attacks this site. A(7) binding was shown to be insignificant both by the diethyl pyrocarbonate reaction and by enzymatic digestion, which reveals only G,G crosslinking. These results suggest that an initial G(4) monoadduct forms a 1,2 crosslink to the 3′-end G(5) much more favorably than to the 5′-end G(3) and that 1,2 G,G crosslinking is much more favorable than 1,4 G,A crosslinking in either direction. Relatively stable hairpins such as the G(4),G(5) adduct described here could explain features of the ^{31}P NMR spectrum observed on treating polymeric DNA with Pt anticancer drugs and could stabilize cruciforms in palindromic regions. The latter possibility is discussed in the light of the recent discoveries on the structure-specific recognition protein and its partial sequence homology with the high mobility group protein-1, a species known to recognize cruciforms [Bruhn, S. L.; Pil, P. M.; Essigman, J. M.; Housman, D. E.; Lippard, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 2307. Pil, P. M.; Lippard, S. J. *Science* **1992**, *256*, 234].

Considerable evidence exists that the anticancer activity of *cis*-diamminedichloroplatinum(II) (*cis*-DDP or cisplatin) resides in its reaction with cellular DNA.[1-11] Chromatographic analysis of enzyme digests of adducts of anticancer platinum drugs and DNA demonstrates that an N7,N7 ~GpG~ crosslink is the major lesion.[12,13] Such crosslinks have been identified in vivo.[14,15] Furthermore, in a ^{31}P NMR survey of various oligonucleotides treated with cisplatin or related compounds, a significant downfield shift was noted in the ^{31}P NMR signal of the ~GpG~ crosslink.[16-18] A downfield signal with a similar shift in DNA spectra is consistent with this lesion and suggests a similar structural distortion.[18-20] However, the area of the downfield ^{31}P peak appears to be too large to be limited to the ~GpG~ crosslink.[16]

Recently, we used modern NMR techniques to elucidate many of the solution structural features of Pt(en){d(ATGGGTACC-CAT)} (en = ethylenediamine).[21,22] The ApC ^{31}P signal is downfield shifted; thus, such species could also contribute to the downfield signal observed in polymeric DNA. Together with electrophoretic and UV and CD spectroscopic studies, the NMR evidence suggests a hairpinlike structure for the Pt adduct under

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all conditions. The unplatinated counterpart, however, exists mainly as a duplex at all but the lowest oligomer concentrations.[23]

Since the N7 of purines is exposed in the major groove, this is the principal reaction site for platination. Despite the alternative binding sites (six purine N7 sites, two GpG, one GpNpG, one ApNpG, one GpNpA, and one GpNpNpA) in the 12-mer, 5′d-(A(1)T(2)G(3)G(4)G(5)T(6)A(7)C(8)C(9)C(10)A(11)T-(12)), remarkably, only one major species is obtained from the reaction mixture of equimolar $Pt(en)Cl_2$ and 12-mer, as evidenced by NMR (^1H, ^{31}P) and gel electrophoresis experiments.[21,22]

With NMR methods, we were unable to determine the precise sites of platination.[21,22] Sequential assignment methods for the aromatic H8 signals were unsuccessful in the loop region due to the absence of one signal in the aromatic shift range. Although the reason for the absence of this signal was not known, most other signals could be assigned. Since the NMR results clearly showed that A(1), G(3), and A(11) were not platinated, the possibilities that seemed most reasonable were crosslinking involving G(4) and G(5), G(4) and A(7), or G(5) and A(7).[21] The limitation of the NMR procedures prompted us to explore other procedures in order to define the 12-mer Pt binding sites for $Pt(en)Cl_2$ and, for the first time, for the widely used anticancer drug, cisplatin. We employed a strategy utilizing base-specific reactions, DNA sequencing methodologies, and enzyme digest experiments. Essentially identical results were observed for the two platinum complexes. However, initial studies using standard, widely applied methodologies[24] were contrary to some of our NMR studies. These findings and their resolution, together with new insights into the reaction of the 12-mer and the nature of the adducts, are the basis of this report. In addition to meeting our objectives, our results provide an important caveat, since often the ^{32}P tracer methods we have found wanting are the only procedures used to gain insight into the chemistry and biochemistry of nucleic acids in a broad range of studies. The results led us to further verify the conclusions using well defined enzymatic digestion procedures described by Lippard and co-workers.[25]

Experimental Procedures

Materials. The oligodeoxyribonucleotide, 5′-d(ATGGGTACCCAT), was prepared by the phosphoramidite method.[26-28] The crude dimethoxytrityl-protected material was dissolved in triethylamine and purified as described by Kline.[22] After deprotection, the 12-mer was found to contain only one NMR detectable species. The properties of this 12-mer have been investigated, and it has been shown to exist as an equilibrium mixture of duplex and hairpin forms. These have been characterized by NMR and electrophoresis.[23] $Pt(en)Cl_2$ was prepared by the method of Dhara.[29] *cis*-$Pt(NH_3)_2Cl_2$ was purchased from Aldrich. [Chloro(diethylenetriamine)platinum(II)] chloride ([Pt(dien)Cl]Cl) was kindly supplied by Prof. B. Lippert of the University of Dortmund, Germany.

Pt Reactions. The Pt reactions with the 12-mer were performed by the method of Kline.[22] In a typical reaction, an aqueous solution of the 12-mer (8–12 mM in base) was mixed with 1 equiv per strand of the desired Pt complex. (A value of ϵ_{260} (25 °C) = 6300 M^{-1} cm^{-1} was used to calculate concentrations.) The solution was kept in the dark at 14 °C for 5 days. Progress of the reaction was monitored by (nondenaturing) gel electrophoresis (ethidium bromide staining) and ^1H NMR. In all cases, only one major product was observed. Samples stored at −20 °C were good for several months.

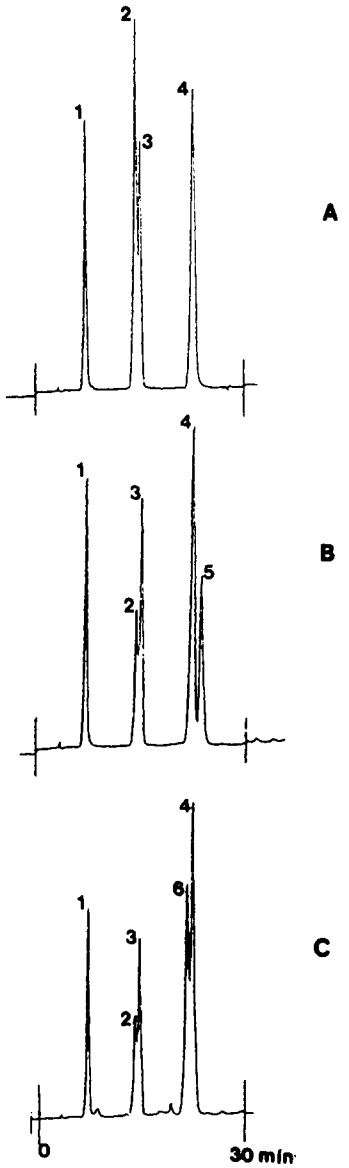

Figure 1. Reversed-phase HPLC analysis of the enzyme digest of (A) 12-mer, (B) Pt(en)/12-mer, and (C) *cis*-$Pt(NH_3)_2$/12-mer. Peaks 1, 2, 3, 4, 5, and 6 are dC, dG, dT, dA, $[Pt(en)\{d(GpG)\}]^+$, and *cis*-$[Pt(NH_3)_2\{d(GpG)\}]^+$, respectively. Gradient 5-22.5% eluate B over 30 min, eluate A is 0.10 M NH_4OAc (pH 6.0), and eluate B is a 1:1 A/CH_3-CN solution; detection at UV 260 nm; flow rate of 1 mL/min.

Enzymatic Digestion of the Pt Adducts. The enzymatic digestion experiments were performed as described previously.[25] In a typical reaction, a lyophilized Pt adduct (20 nmol) or control 12-mer was digested with DNase I (200 units) (Boehringer Mannheim, from bovine pancreas) and nuclease P1 (1 unit) (Boehringer Mannheim, from *Penicillium citrinum*) in the presence of $MgCl_2$ (10 mM) and sodium acetate (50 mM, pH 5.5). The reaction mixture (total volume, 200 μL) was incubated at 37 °C for 16 h. The pH was raised to ~9 by adding 24.2 μL TRIS-HCl (1 M), pH 9. Alkaline phosphatase (5 units) (Boehringer Mannheim, from calf intestine) was then added, and incubation continued at 37 °C for another 4 h. Products of the digest were analyzed by reversed-phase HPLC on a C-18 column using a gradient described in the caption for Figure 1. Peaks were identified by comparison with authentic 2′-deoxynucleosides (Sigma), $[Pt(en)\{d(GpG)\}]^+$, and *cis*-$[Pt(NH_3)_2\{d(GpG)\}]^+$. The latter two were prepared by mixing equivalent amounts of each of the Pt complexes with d(GpG) (1 mM in each, 100 μL) at 14 °C for 5 days. This platination reaction was monitored by HPLC under the conditions described above.

Modified Maxam–Gilbert Sequencing Experiments. The platinum binding sites were determined with base-specific chemical reactions after

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5′- and 3′-^{32}P-end-labeling of platinated and unplatinated 12-mers. A sample (~1 nmol in oligonucleotide strand) withdrawn from the platination reaction mixture was 5′-^{32}P-labeled with [γ-^{32}P] ATP (17 pmol) (Amersham, specific activity 3000 mCi/mMol) using T4 polynucleotide kinase (10–20 units, New England Biolabs) or 3′-^{32}P labeled with dideoxyadenosine [α-^{32}P] triphosphate (ddATP, 28 pmol, Amersham, specific activity 3000 mCi/mMol) using terminal deoxynucleotidyl transferase (10 units, Bethesda Research Lab).[30] The ^{32}P-labeled 12-mers were purified on a preparative nondenaturing 20% polyacrylamide gel (1.5 mm) at 4 °C (~20 h at 500 V). The Maxam–Gilbert[31] G+A and C+T sequencing reactions were performed on the ^{32}P-labeled 12-mer. The G-specific dimethyl sulfate (DMS) reaction was performed for a longer time (10–15 min) than described by Maxam and Gilbert.[31] $KMnO_4$ was used for T-specific reactions.[32] Diethylpyrocarbonate (DEPC) was used for purine-specific reactions (A more reactive than G) as described by Herr[33] with minor modifications. (Reaction time was 30 min at 90 °C DEPC, 2 μL, was added at 0, 10, and 20 min, with vortexing.)-

Products of the base-specific chemical reactions were treated with 0.1 M KCN (50 μL) overnight, precipitated with ethanol, cleaved with piperidine (2 M), subjected to 20% polyacrylamide/7 M urea gel (0.5 mm) electrophoresis (~7 h at 1000 V), and analyzed by autoradiography. Samples were not heated before loading on the gels.

Exhaustive 5′-End-Labeling. In a typical reaction (50 μL), the platinated 12-mer mixtures (~1 nmol) were exhaustively 5′-end-labeled with 85 nmol ATP using T4 polynucleotide kinase (10–20 units) in 25 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, and 10 mM dithiothreitol at 37 °C for 60 min. The reaction was stopped by adding 50 μL of 0.6 M NaOAc, pH 7.5, followed by extraction of the enzyme with 25:24:1 phenol/chloroform/isoamyl alcohol (see ref 30 for more detail). Reaction products were precipitated using ethanol and analyzed on 20% denaturing polyacrylamide gels.

Electrophoresis. Polyacrylamide gel electrophoresis was performed by using a published procedure[30] with gels containing Tris-borate-EDTA (TBE) buffer (0.1 M Tris-borate, pH 8.3, and 0.2 mM EDTA) or TBE buffer with 7 M urea (denaturing gel). To prepare the gels, 600 μL of a 10% ammonium persulfate solution was added to 60 mL (analytical gel) or 150 mL (preparative gel) of 20% acrylamide followed by 20 μL of *N,N,N′,N′*-tetramethylethylenediamine. Electrophoresis was carried out at room temperature. Preparative gel electrophoresis was carried out at ~4 °C.

Densitometric Measurements. The intensities of the bands from negatives of the UV-illuminated gels were determined using a Biorad densitometer, Model Video 620.

Results

In order to determine the Pt binding sites, the Pt(en)/12-mer and *cis*-Pt$(NH_3)_2$/12-mer products were digested as described by Lippard[25] and Eastman[34] with DNase I and nuclease P1 (to hydrolyze the phosphodiester bond (3′ to 5′) to generate 5′ phosphate nucleotides) and then phosphatase (to generate nucleosides). The mixture was analyzed by HPLC (Figure 1). Peaks that coeluted with authentic samples of [Pt(en){d(GpG)}]$^+$ and *cis*-[Pt$(NH_3)_2${d(GpG)}]$^+$ were observed for the Pt(en)/12-mer and *cis*-Pt$(NH_3)_2$/12-mer solutions, respectively. The expected decrease in the dG peak was observed with no decrease in the dA, dC, or dT peaks. Furthermore, after treatment of the digest mixture with 0.1 M KCN, HPLC traces in both cases lacked the peak for the d(GpG) adduct but, instead, gave an additional peak with a retention time indentical to that of d(GpG). Both 5′- and 3′-dGMP have short retention times with this method. Thus, this experiment establishes that the adducts are GpG crosslinked species and not GA adducts. GA adducts were not ruled out by reported NMR studies.[21]

The results in the previous paragraph agree with ^{195}Pt NMR data for the Pt(en)-12-mer, suggesting that the Pt(en) moiety is bound to two N7's of purines.[21] It eliminates all monofunctional adducts and restricts the major adduct to an intrastrand crosslinked adduct, G(3),G(4) or G(4),G(5). Since our NMR results clearly show that G(3) is not likely to be platinated, these results suggest that the major product is a G(4),G(5) crosslink.

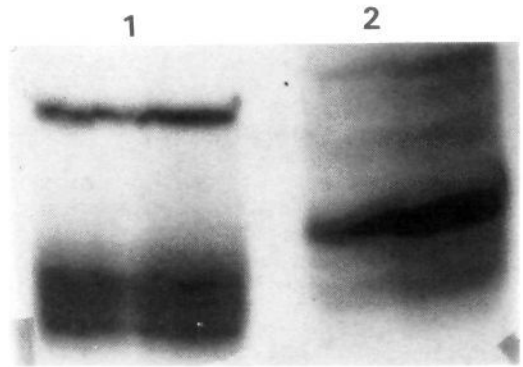

Figure 2. Autoradiogram of nondenaturing polyacrylamide gel of 3′-end-labeled species (lane 1) 12-mer and (lane 2) Pt(en)/12-mer reaction product. The bands in lane 1 are the duplex (slower) and hairpin (faster) forms of the 12-mer. The multiple bands for the 12-mer hairpin form are an artifact typically found for this 12-mer. Only one band was found in similar experiments, and when the multiple hairpin bands were eluted and analyzed with denaturing gels, only one band was observed in every case, see lane 7, Figure 3.

Samples of the 12-mer and of the Pt(en)/12-mer (Figure 2) and *cis*-Pt$(NH_3)_2$/12-mer reaction mixtures were 3′-end-^{32}P labeled with [α-^{32}P] ddATP using terminal transferase.[30] This procedure adds dideoxyadenosine [α-^{32}P] monophosphate (ddAMP) to the 3′-end. The three samples were analyzed by gel electrophoresis under nondenaturing conditions (20% polyacrylamide). For *both* Pt complexes, the reaction mixtures appeared by this method of analysis to contain mainly one significant oligonucleotide product. These appear to be hairpin-type species from the relative mobilities of these products, which are only slightly slower than the less abundant hairpin form of the 12-mer. The abundant duplex form of the 12-mer has a much slower mobility (Figure 2).

To determine the specific G's bound to Pt, base-specific chemical cleavage DNA sequencing methodologies were employed as described by Leng[24] and Lippard[25] on the three 3′-end-^{32}P-labeled 12-mers. After elution from the gel with 0.3 M sodium acetate (pH 7) and ethanol precipitation, the three samples were dried and dissolved in water. The DMS reaction was used to methylate specifically the N7 of G, making each modified site labile to piperidine cleavage. However, if these sites are effectively protected from alkylation through coordination to Pt, cleavage by piperidine should not occur.[24,25,35,36] The cleavage products were electrophoresed on denaturing DNA sequencing gel (20% polyacrylamide, 7 M urea). Prior to piperidine cleavage the samples were treated with 0.1 M KCN in order to remove the Pt,[36] which otherwise would alter electrophoretic mobility.[24,25,35,36] Results of such an experiment are shown in Figure 3; lanes 1 and 2 are DMS reactions on 12-mer and Pt(en)/12-mer products, respectively. As expected, the 12-mer (lane 1) exhibits cleavage at all G's. However, the Pt(en)/12-mer product (lane 2) has missing fragments (bands) at G(3) and G(4) (from the 5′-end), indicating platination at N7 of these G's. This result is *contrary* to our previous studies by 2D-NMR,[21,22] which suggested no coordination of Pt to G(3).

Even more striking, when the same 12-mer reaction mixtures were 5′-end-^{32}P-labeled with [γ-^{32}P] ATP using polynucleotide kinase and analyzed on nondenaturing gel, *two* Pt adducts (X and Y), each in comparable amount, were observed in both cases (Figure 4). Essentially identical results were obtained when the reaction mixture was heated to 90 °C for 30 min prior to labeling. In our previous NMR studies, we found only *one* Pt(en)-12-mer adduct. For each of the solutions of the Pt complexes, the two adducts X and Y have slower and faster electrophoretic mobilities,

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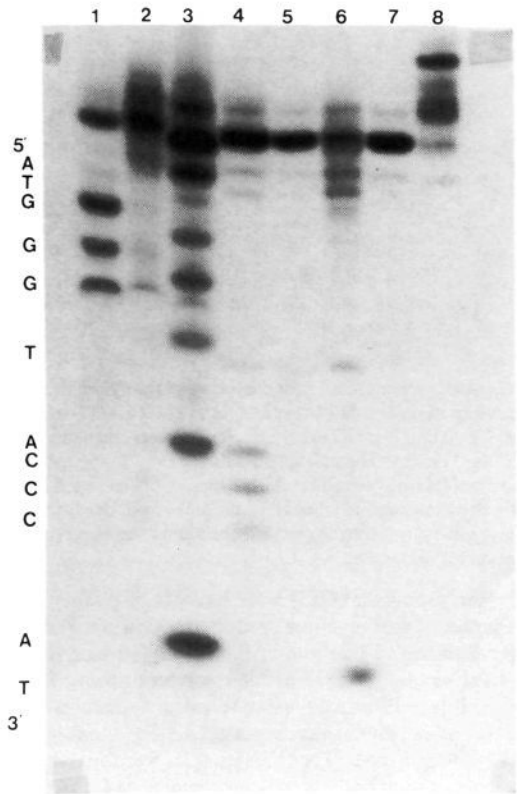

Figure 3. Autoradiogram of denaturing gels of Maxam–Gilbert sequencing reactions of 12-mer and Pt(en)/12-mer reaction mixture 3′-^{32}P-end labeled with [α-^{32}P]-ddATP using terminal transferase: lane 1, G-specific reactions on 12-mer; lane 2, G-specific reaction on Pt(en)-12-mer; lane 3, Purine-specific reaction on 12-mer; lane 4, pyrimidine-specific reaction on 12-mer; lane 5, T-specific reaction on 12-mer; lane 6, T-specific reaction on Pt(en)-12-mer; lane 7, 12-mer; lane 8, Pt(en)-12-mer. There is an apparent increase in electrophoretic mobility in all G and A fragments in the purine-specific sequencing reactions (lane 3). A similar mobility increase is apparent in results published by Clarke et al.[37] but was not discussed. This increase may be explained if the grafted 3′-end A is labile to depurination in acidic or basic media. Subsequent δ-elimination would leave a 3′-terminal phosphate group. Such fragments, because of charge and size, are known to have higher electrophoretic mobility than their counterparts with a terminal sugar moiety.[38]

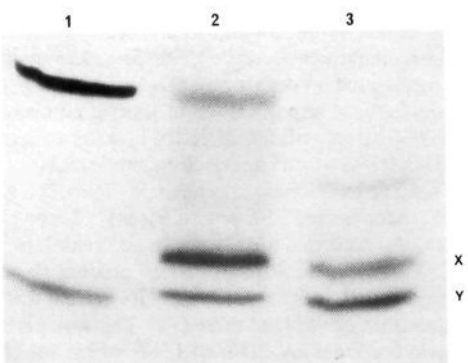

Figure 4. Autoradiograms of nondenaturing polyacrylamide gel of 5′-^{32}P-end-labeled: (lane 1) 12-mer; (lane 2) *cis*-Pt(NH$_3$)$_2$/12-mer reaction products; (lane 3) Pt(en)/12-mer reaction products. Some unplatinated 12-mer duplex form is still present in lane 2. The two bands in lane 1 are the duplex (slower) and hairpin (faster) forms of the 12-mer.

respectively. From base-specific reactions, adduct X corresponds to the only species detected during the 3′-end-^{32}P-labeling experiment. Although adduct Y has an electrophoretic mobility in a nondenaturing gel almost identical to that of the hairpin form of the parent 12-mer, it is shown below that Y is *not* unplatinated 12-mer.

The 5′-end-^{32}P-labeled X and Y adducts were separately eluted from the gel. The Pt binding sites for both drugs were mapped by performing base-specific chemical reactions[24,25] on 5′-end-^{32}P-labeled 12-mer and on the two products (X and Y) of the Pt(en)/12-mer and *cis*-Pt(NH$_3$)$_2$/12-mer reactions. For both platination reactions, the binding sites observed for X and Y with the DMS cleavage procedure discussed above were G(3),G(4) and G(4),G(5), respectively. (Figure 5, lanes 1, 2, and 3 reflect products of G-specific reactions on 12-mer, Pt(en)-12-mer adduct X, and Pt(en)-12-mer adduct Y, respectively. The missing fragments for adduct X are G(3) and G(4), while those of adduct Y are G(4) and G(5).)

In addition, the DEPC reaction was used to determine if A's were involved in coordination to Pt. DEPC is known to carbethoxylate N7 of purines (reactivity of A is greater than G), making each modified A labile to piperidine cleavage.[33] The reactions were performed in denaturing conditions (90 °C) in order to avoid any secondary structure that could possibly hinder the purines from reacting with DEPC. If the N7 of A is protected through coordination to Pt, cleavage by piperidine should not occur. The DEPC control (Figure 5, lane 8) indicates cleavage at A(7). The DEPC-modified X and Y samples were treated with 0.1 M KCN prior to piperidine cleavage. Analysis of the samples by denaturing gel electrophoresis indicates that no A's are coordinated to Pt (lanes 9 and 10). Together with the enzymatic digestion studies, the DMS and DEPC reactions indicate that the two species are [Pt(en){d(ATGGGTACCCAT)-N7-G(3), N7-G(4)}] (minor adduct X) and [Pt(en){d-(ATGGGTACCCAT)-N7-G(4), N7-G(5)}] (major adduct Y) (Figure 6). Both adducts revert to the parent 12-mer upon treatment with KCN (Figure 5, lanes 11 and 12).

In a complementary type of experiment, one can assess G-specific reactions without removal of the Pt. In this type of experiment, after cleavage with piperidine, only fragments with G's that are situated 5′ from the binding site can be observed at the gel mobilities usual for a fragment of a given size. If the G's, however, are situated 3′ from the binding site, only the Pt bound fragment would be observed, but at a slower gel mobility. Lanes 4 and 5 (Figure 5) contain DMS-treated samples of X and Y, respectively, that were *not* treated with KCN to remove the Pt. Lane 4 for X shows missing fragments at G(3) and G(4) and a Pt-bound fragment with slower gel mobility. This result suggests that the binding site for X is G(3),G(4), in agreement with results obtained with the corresponding KCN-treated X sample (lane 2). Lane 5 for Y shows mainly a fragment at G(3), suggesting that Pt is bound to G(4),G(5), in agreement with the results obtained with the corresponding KCN-treated Y sample (lane 3). (The relatively much lighter bands at G(4) and G(5) probably result from residual unplatinated 12-mer hairpin form.)

Recently, Lippard and co-workers[25] reported a novel linkage isomerization of a *trans*-diammineplatinum(II)-dodecanucleotide 1,3-crosslink adduct to a 1,4-crosslink adduct. To test if adducts X and Y would isomerize, we separately incubated both species (5′-^{32}P-end-labeled) at 90 °C for 30 min and analyzed the solutions by denaturing gel electrophoresis. For both species, the control and heated lanes were identical. Therefore, neither species isomerizes to the other. Similarly, *both* X and Y were stable to piperidine (2 M) cleavage at 90 °C for 30 min.

The observation of X with *both* 3′ and 5′ labeling and the presence of *two* products is in clear contradiction to our NMR studies.[21] In order to resolve such anomalies, the unplatinated 12-mer and Pt(en)/12-mer mixture were phosphorylated at the 5′-end with [γ-^{32}P]ATP or an excess of *nonradioactive* ATP using polynucleotide kinase, and each reaction mixture was ethanol precipitated, lyophilized, and electrophoresed on a denaturing gel. UV-visualization analysis of the gel is shown in Figure 7. The result clearly indicates that the Pt(en)Cl$_2$-treated 12-mer (*lane 6 or 8*) *contains one major product* Y (*~96% by* densitometric measurements) having a slightly faster electrophoretic mobility than the unplatinated 12-mer (lane 3). The

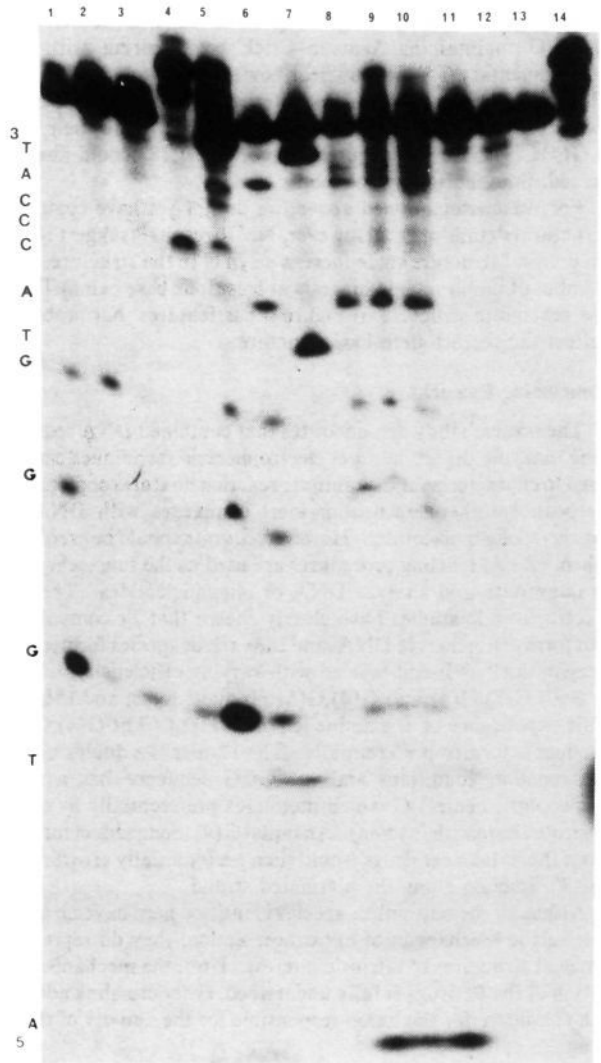

Figure 5. Base specific strand cleavage on the Pt(en)-12-mer X and Y adducts and the 12-mer (5′-^{32}P-end-labeling) in lanes 1 to 10 and some uncleaved controls in lanes 11–14: lanes 1, 6, 7, and 8, products of Maxam–Gilbert and DEPC base-specific reactions on the 12-mer; lanes 2, 4, and 9, products of Maxam–Gilbert and DEPC base-specific reactions on the minor adduct (X); lanes 3, 5, and 10, products of Maxam–Gilbert and DEPC base-specific reactions on the major adduct (Y); lanes 1–5, G-specific sequencing reactions; lane 6, purine-specific sequencing reaction; lane 7, T-specific reaction; lanes 8–10, DEPC reaction; lanes 2, 3, 9, 10, platinum removed by KCN prior to piperidine cleavage; lanes 4 and 5, platinum was not removed; lanes 11 and 12, major (Y) and minor (X) species, respectively; platinum removed by treatment with KCN prior to electrophoresis; lane 13, 12-mer; lane 14, minor adduct (X).

greater electrophoretic mobility of the major adduct Y is even more apparent in the UV experiment with the phosphorylated species, lanes 4 and 5 (or 7). These increases in electrophoretic mobility for the major products may be attributed to the distorted structure induced by Pt.[21,22] A minor product X (~4%) (lane 6 or 8) has a slower electrophoretic mobility than the parent 12-mer (lane 3); cf. also the phosphorylated species (platinated lanes 5 or 7 and unplatinated lane 4). The positive charge of the Pt moiety may explain the slow electrophoretic mobility of the minor species, which probably is denatured. This experiment is in agreement with the NMR studies since products with <5% abundance would be difficult to detect and the major adduct, Y, does not show platination at G(3). It is also in agreement with previous gel electrophoresis experiments, which were limited to nondenaturing gels. The minor component X is also a hairpin under these conditions. In previous experiments,[21,22] UV-visualization of unphosphorylated product was used, and the minor

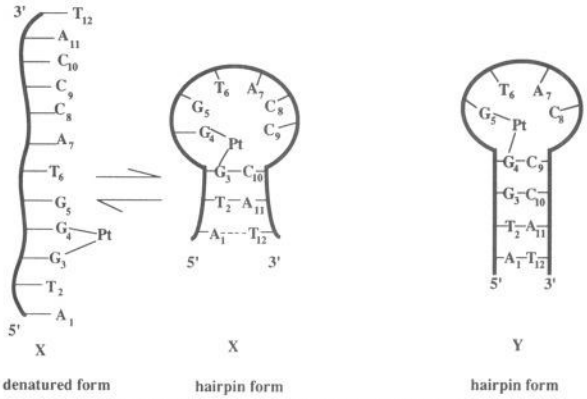

Figure 6. Schematic representation of adducts X and Y. These are not meant to be precise. NMR data on adduct Y suggest this general structure,[21] but since X is found in trace amounts, no NMR data are available for it.

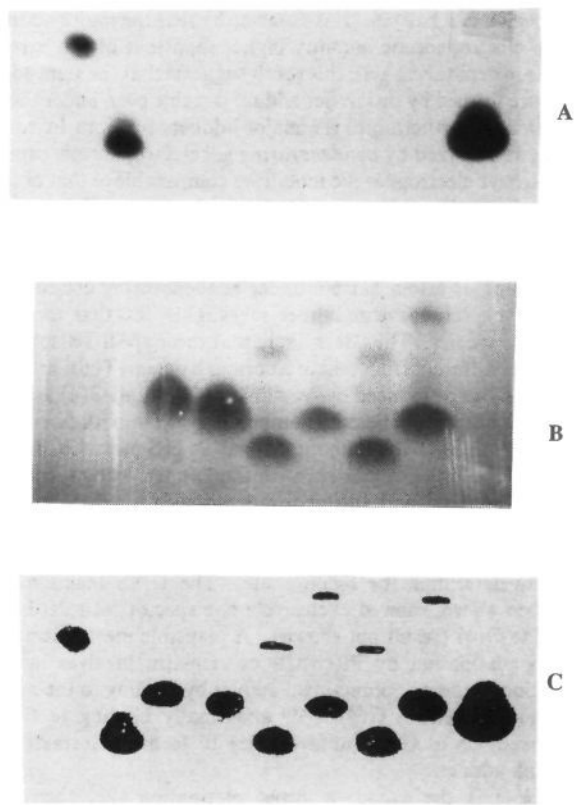

Figure 7. Detection on the *same* denaturing gel of the 12-mer and the Pt(en)Cl_2/12-mer reaction products by (A) ^{32}P-labeling methods and by (B) UV methods. Phosphorylation followed standard protocols for 5′ end-labeling procedures.[30] The "cold" labeling procedure involved an excess of ATP. Radioactively labeled species (lanes 1, 2, and 9) were detected from X-ray autoradiograms, while the rest were detected by illuminating with UV light at 254 nm and Polaroid photography. The part C composite shows spots traced manually from the gel and is not quantitative. For quantitation, negatives of similar experiments were used: lanes 1 and 2, minor (X) and major (Y) 5′-^{32}P-end-labeled Pt-(en)-12-mer adducts isolated for use as controls; lane 3, 12-mer (unphosphorylated); lane 4, 5′-end-phosphorylated 12-mer using "cold" ATP; lane 5, 5′-end-phosphorylated Pt(en)/12-mer reaction product mixture using "cold" ATP; lane 6, Pt(en)/12-mer reaction product mixture (unphosphorylated); lanes 7 and 8, repeat of lanes 5 and 6, respectively, but of a second preparation; lane 9, 5′-end-^{32}P labeled 12-mer.

hairpin component would have been lost in the tail of the major hairpin component.

Apparently, the minor product, X, is more efficiently 5′-end-^{32}P labeled than the major species, Y. It is known that single-stranded

DNAs are phosphorylated more efficiently than those with blunt ends.[30] The major Pt(en)-12-mer adduct as evidenced by NMR studies[21,22] has base pairing in the stem region and has a blunt end, which is thus more difficult to phosphorylate. However, when a greater than 5000-fold excess of nonradioactive ATP was used during phosphorylation of the products of the $Pt(en)Cl_2$/12-mer reaction, it appears that all of the major Pt adduct (Y) in the mixture was eventually phosphorylated (Figure 7, lane 5 or 7).

According to the denaturing gel electrophoresis experiments performed in this study, the minor adducts obtained from both Pt complexes have slower electrophoretic mobility than the 12-mer, suggesting that in the minor Pt adducts the equilibrium lies more toward the denatured state (i.e., nonhairpin) structure than in the major Pt adducts. We anticipate slower mobility for a single strand because the Pt moiety adds two positive charges. The bound Pt in the minor (X) species, as demonstrated by base-specific chemical cleavage experiments, is closer to the 5'-end than the major adduct, disrupting possible base pairing at the stem region of a hairpin. It is noteworthy that the major adduct has an electrophoretic mobility higher than that of the parent 12-mer in denaturing gels; this result suggests that the stem-loop structure formed by this major adduct is stable even under these conditions. Furthermore, the major adducts for both Pt complexes, as analyzed by *nondenaturing* gel electrophoresis experiments, have electrophoretic mobilities comparable to that of the hairpin form of the 12-mer[23] and of a single-stranded 9-mer (not shown), suggesting a possible hairpin structure.[21,22]

By these criteria, the minor species (X), readily detected by ^{32}P labeling, is also a hairpin under nondenaturing conditions. The minor species have mobilities only slightly less than those of the major species. Thus, it is likely that these species also have structures different from that of a normal hairpin. Thus, hairpin formation can be induced by crosslinking G(3) and G(4) as well as G(4) and G(5). The common binding site in both X and Y is the N7 of G(4). A monofunctional Pt complex, [Pt(dien)Cl]Cl, was allowed to interact with the 12-mer, using the same protocol described for the drugs, in order to find the most favorable site of coordination. The reaction mixture was 5'-end-^{32}P-labeled, and base-specific chemical cleavage reactions were employed in order to determine the binding site. The DMS reaction, as described above, showed exclusively one species with Pt(dien) bound to G(4) (result not shown). A plausible mechanism for 12-mer platination by $Pt(en)Cl_2$ or cisplatin involves initial formation of the monofunctional adduct by binding to the more electron-rich central G(4)[23,39,40] and finally binding to G(5) (preferred) or to G(3) (unfavorable) to form an intrastrand crosslink adduct.

If G(4) is the exclusive initial platination site, then 1,2 crosslinking is ~20 times more favorable in the 3' direction than in the 5' direction. This factor would be even larger, if any initial platination occurs at G(3), the next most nucleophilic site.[23] The apparently favorable 1,2 crosslinking in the 3' direction would account for the small amount of minor adduct. This factor would be smaller if any platination occurs at G(5), but this site is not a likely metalation site.[23] Clearly, 1,3 and 1,4 crosslinking are not favored.

The structures in Figure 6 are meant to be schematic. We have no information on the H-bonding for species X, which was identified only in electrophoretic studies. Duplexes with PtGpG adducts appear to have base pairing modification at the 5'G, with the 3'G maintaining Watson–Crick base pairing with the complementary C.[41] It is unclear how the loop would affect this H-bonding. Thus, a reasonable alternative stem could have the two AT base pairs, a disrupted or modified G(3)C(10) pair, and a G(4)C(9) base pair. Furthermore, G(5)C(8) could also be paired, thus shortening the loop.

For reasons mentioned above, we do not yet have complete structural details on Y. However, NMR results[21] suggest it has an unusual structure since there is a syn G in the structure. The number of imino signals suggests at least four base pairs. Thus, the schematic structure in Figure 6 has features that probably reflect the correct stem-loop structure.

Concluding Remarks

The present study demonstrates that combined DNA sequencing, enzyme digest, and gel electrophoresis techniques can be used to characterize and quantitate reaction mixtures of platinum or possibly other substitution-inert complexes with DNA or synthetic oligonucleotides. However, *caution* should be exercised when ^{32}P-end labeling procedures are used as the sole technique to quantitate and analyze DNA or oligonucleotides. The gel electrophoretic studies have clearly shown that Pt compounds can form distortions in DNA, and the various species formed are enzymatically ^{32}P-end-labeled with varying efficiencies.

Both G(3),G(4) and G(4),G(5) products form, and there is little dependence on the amine {en or $(NH_3)_2$}. The G(4),G(5) product is formed preferentially. The 12-mer is a duplex under the reaction conditions and the GGG sequence has a very nucleophilic central G which metalates preferentially as demonstrated here with Pt(dien). An initial G(4) monoadduct formed from the anticancer drugs would then preferentially crosslink in the 3' direction along the platinated strand.

Although the hairpinlike species identified here have no clear role in the mechanism of anticancer action, they do represent unusual structures of intrinsic interest. Until the mechanism of action of the Pt drugs is fully understood, every crosslink adduct is a candidate for the lesion responsible for the activity of these drugs.

In our previous studies, we suggested that the larger than expected ^{31}P downfield signal in DNA[16,20] could be due to unusual structures caused by GG crosslinking and that hairpins can form in palindromic regions.[21] In an extended duplex, it is possible that a cruciform may be formed by GG intrastrand crosslinking if platination occurs in a palindromic region. Recently, studies by Lippard and colleagues have led to the identification of a structure-specific recognition protein (SSRP1).[42] The SSRP1 protein has been sequenced and a 75-residue segment exhibits extensive areas of homology (47%) with the high mobility group protein, HMG1.[43] In turn, HMG1 is known to recognize cruciform structures.[44] Thus, the SSRP1 may recognize a variety of lesions in addition to the kinked structure that was used to identify it, including the hairpin structures from the present work. Further work on the recognition protein will undoubtedly determine if Pt-induced cruciforms are also recognized.

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