Use of Antibodies To Probe the Stereochemistry of Antitumor Platinum Drug Binding to Deoxyribonucleic Acid†

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ABSTRACT: A previously prepared antiserum elicited against DNA modified with *cis*-diamminedichloroplatinum(II) (*cis*-DDP) (Poirier et al., 1982) was found by a competitive enzyme-linked immunosorbent assay (ELISA) to show high specificity for *cis*-DDP bound to poly(dG)·poly(dC) but not to the alternating heterocopolymer poly[d(GC)]·poly[d(GC)]. Poor immunoreactivity was shown toward calf thymus DNA modified by a variety of antitumor-inactive platinum analogues including *trans*-DDP, *trans*-[$Pt(NH_2CH_3)_2Cl_2$], and [Pt(dien)Cl]Cl whereas the antiserum exhibited good specificity for DNA modified by analogues having cis stereochemistry. Platinum complexes included in the latter category are [Pt(en)Cl_2], *cis*-[$Pt(NH_2CH_3)_2Cl_2$], [Pt(CP)(DACH)], and *cis*-diammineplatinum α-pyridone blue. The ELISA immunoreactivity of *cis*-DDP-DNA treated with cyanide ion or thiourea after modification or with ethidium bromide during modification was also monitored. Taken together, the results of this study suggest that a primary three-dimensional structure recognized by the antibody is a bidentate adduct of *cis*-DDP in which two chloride ions have been replaced by two adjacent deoxyguanosines on the same DNA strand.

cis-Diamminedichloroplatinum(II), *cis*-DDP,[1] is an antitumor drug whereas the trans isomer is inactive [Lippard (1982) and references cited therein]. This simple relationship between drug activity and stereochemistry has evoked a variety of explanations (Barton & Lippard, 1980) on the basis of the assignment (Roberts & Pera, 1983) of DNA as the principal target for anticancer coordination compounds. Recent studies of *cis*-DDP binding to DNA in vitro (Tullius et al., 1983) and to short oligonucleotides of defined sequence (Marcelis et al., 1981; Caradonna et al., 1982; Chottard et al., 1983) support a model (Kelman et al., 1977) in which the two chloride ions are replaced by two N7 atoms of adjacent deoxyguanosine residues in the d(GpG) sequence. To date, there has been no evidence that such an intrastrand d(GpG) cross-link is induced by *cis*-DDP in vivo although base-pair substitution mutagenesis experiments have suggested the occurrence of closely related d(GAG) and d(GCG) intrastrand cross-linking by *cis*-DDP (Brouwer et al., 1981). The alkaline-elution method (Kohn, 1979) has been employed to demonstrate that DNA–protein and DNA interstrand cross-linking is caused by *cis*-DDP in vivo (Zwelling et al., 1979), but these cross-links were shown to be only a small percentage of the total drug–DNA product distribution (Roberts & Pera, 1983).

Recently, we reported the preparation of an antiserum elicited against *cis*-DDP-modified calf thymus DNA that is specific for adducts of *cis*- (but not *trans*-) DDP-DNA formed in cultured cells and intact animals (Poirier et al., 1982). This antiserum, which fails to recognize *cis*-DDP alone, DNA alone, and DNA interstrand cross-linked by *cis*-DDP, has the potential for determining femtomole quantities of *cis*-DDP-DNA adducts through a highly sensitive competitive enzyme-linked immunosorbent assay (ELISA). In the present study, we used this antiserum to investigate by ELISA specificity for DNA samples modified by a variety of platinum complexes (Figure 1). We also investigated DNA to which *cis*-DDP was bound in the presence of EtdBr, which is known to alter the mode of platinum binding (Merkel & Lippard, 1983). The results yielded new information concerning the nature of the immunogen adduct and other cis-DDP-DNA adducts formed in vitro and in vivo.

Materials and Methods

Source of Platinum Compounds, Preparation of the Immunogen, Immunization, and ELISA. All procedures for preparing *cis*- and *trans*-DDP, calf thymus DNA, and platinum-modified DNAs, for immunization, and for ELISA were as previously described (Poirier et al., 1982). [Pt(en)Cl_2] was made by a literature procedure (Johnson, 1966); [Pt(dien)Cl]Cl was prepared from the recrystallized hydrochloride salt of diethylenetriamine and K_2PtCl_4; *cis*-[$Pt(NH_2CH_3)_2Cl_2$] was obtained from methylamine and K_2PtCl_4 by a route analogous to the preparation of *cis*-DDP; *cis*-diammineplatinum α-pyridone blue and *cis*-[$Pt(NH_3)_2(OH)]_2(NO_3)_2$ were provided by L. S. Hollis; [Pt(CP)(DACH)] was a gift from J. H. Burchenal; *trans*-[$Pt(NH_2CH_3)_2Cl_2$] was provided by C. Caravana; and *cis*-[$Pt(NH_2$-*i*-$C_3H_7)_2Cl_2$] was a gift from M. Cleare. Unless otherwise specified, "buffer" refers to 1 mM sodium phosphate–3 mM sodium chloride, pH 7.4.

Preparation of cis-DDP-Modified Homo- and Heterocopolymers. Poly(dG)·poly(dC) (P-L Biochemicals, Inc.) and poly[d(GC)]·poly[d(GC)] (Collaborative Research Inc.) were purchased as lyophilized solids. The polymers were dissolved in 25 mM Tris-HCl–1 mM EDTA, pH 7.8, to a final concentration of 2 A_{260} units/mL by gentle stirring at room

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); EtdBr, ethidium bromide; PPB, *cis*-diammineplatinum α-pyridone blue; TU, thiourea; dien, diethylenetriamine; [Pt(CP)(DACH)], (4-carboxyphthalato)(1,2-diaminocyclohexane)platinum(II); en, ethylenediamine; ELISA, enzyme-linked immunosorbent assay; D/N, drug-to-nucleotide ratio, equivalent to percent modification; *cis*-DDP-DNA, calf thymus DNA containing bound *cis*-DDP at a specified D/N value; EDTA, sodium salt of ethylenediaminetetraacetate; AAS, carbon-rod atomic absorption spectroscopy; Tris, tris(hydroxymethyl)aminomethane.

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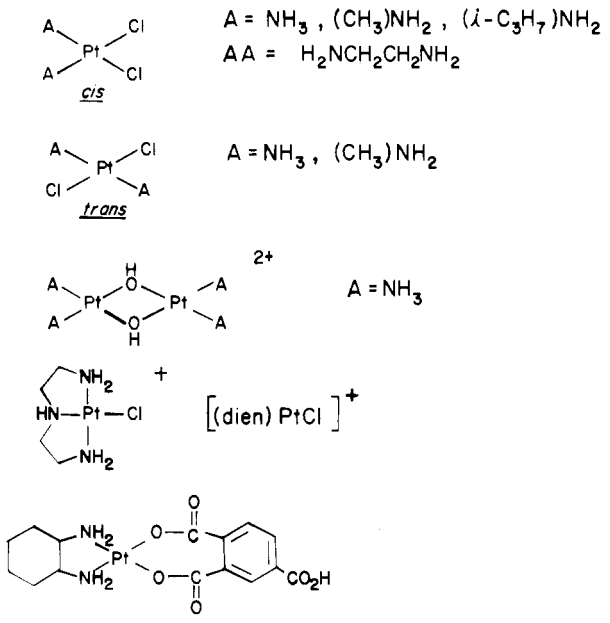

FIGURE 1: Platinum complexes studied by competitive ELISA.

temperature for 2 days. *cis*-DDP at a final concentration (by AAS) of 2.66×10^{-5} M was incubated for 2 days at 37 °C with 3 A_{260} units of each polymer. The *cis*-DDP-DNA complexes were then dialyzed at room temperature against four changes of buffer. In these and all subsequent experiments, the final platinum binding level was determined by AAS and the polymer concentrations from the published extinction coefficients (Inman & Baldwin, 1964; Wells et al., 1970).

cis-DDP-Modified DNA Prepared or Treated with Other Agents. In these experiments, the final calf thymus DNA concentration was 2.3 mM. To *cis*-DDP-DNA (D/N = 0.058) was added a sufficient quantity of 1 M NaCN at pH 9 to yield a cyanide ion concentration of 0.2 M (Bauer et al., 1978; Lippard & Hoeschele, 1979). Following incubation for 3 h at 37 °C and dialysis against buffer, the final platinum modification was D/N = 0.003. Calf thymus DNA modified to D/N = 0.072 with *cis*-DDP was incubated for 3 h at 37 °C in the presence of 1 M thiourea, dialyzed against buffer, and found to have D/N = 0.02.

cis-DDP (0.15 mM) was incubated with calf thymus DNA in the presence of saturating amounts (0.2 EtdBr/nucleotide) of ethidium bromide at 37 °C for 12 h. The sample was then dialyzed against buffer, phenol extracted, and exhaustively dialyzed, and the D/N was determined.

Preparation of DNA Modified with Various cis-DDP Analogues. Calf thymus DNA (0.5 mg in 0.5 mL final volume of buffer) was allowed to react with the various platinum compounds (Figure 1) in buffer at 37 °C for 24 h and dialyzed as reported previously (Poirier et al., 1982). In most cases, the ratio of added platinum per nucleotide was 0.05 except for the α-pyridone blue and *cis*-DDP samples where it was 0.10. The final D/N ratio was calculated from the platinum and DNA phosphate concentrations as determined by AAS and optical spectroscopy, respectively.

Results

Immunoreactivity of Anti-cis-DDP-DNA toward cis-DDP Coordinated to Homo- and Heterocopolymers of Deoxyguanosine and Deoxycytosine. By use of the competitive ELISA for calf thymus DNA modified with *cis*-DDP described previously (Poirier et al., 1982), standard curves were obtained in assays in which the DNA coated on microtiter wells and those used as competitors were both modified to the same extent (D/N ~ 0.06–0.08; Figures 2–4). The quantity of platinum giving 50% inhibition was 66.0 ± 7.0 fmol (mean ± range) for several assays at a serum dilution of 1:16 000 and 25.5 ± 4.5 fmol at a serum dilution of 1:32 000.

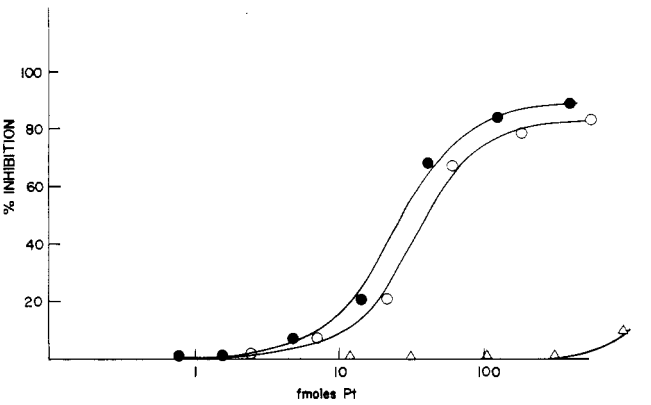

FIGURE 2: Competitive ELISA of homo- and heterocopolymers of deoxyguanosine and deoxycytidine. 0.4 ng of *cis*-DDP-DNA (D/N = 0.083) was coated on microtiter wells; antiserum (rabbit no. 48, Platina) was used at a dilution of 1:32 000, and 10 μg of unmodified calf thymus DNA as carrier was present [see Poirier et al. (1982) for more details of the procedures for ELISA]. On the abscissa are plotted increasing concentrations of platinum as *cis*-DDP-modified calf thymus DNA [(●) D/N = 0.062, control], poly(dG)·poly(dC) [(○) D/N = 0.042], and poly[d(GC)]·poly[d(GC)] [(△) D/N = 0.049].

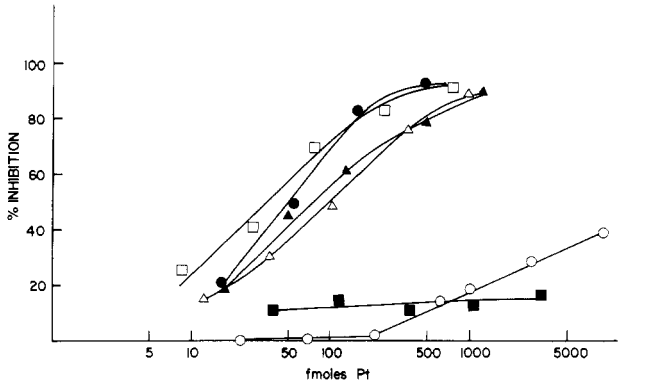

FIGURE 3: Competitive ELISA under the conditions described in the legend to Figure 2 except that a 1:16 000 dilution was used, which accounts for the decreased sensitivity of the standard curve. On the abscissa are plotted increasing concentrations of platinum on calf thymus DNA modified with *cis*-DDP (●), *cis*- (□) or *trans*-[Pt-$(NH_2CH_3)_2Cl_2$] (■), *cis*-DDP-DNA treated with TU (▲) or cyanide (○), and *cis*-DDP-DNA prepared in the presence of EtdBr (△).

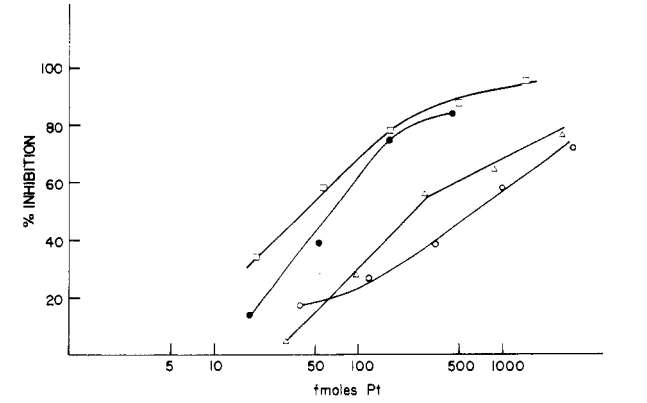

FIGURE 4: Competitive ELISA under the conditions described in the legend to Figure 3. On the abscissa are plotted increasing concentrations of platinum on DNAs modified with *cis*-DDP (●), *cis*-[Pt-$(NH_2$-*i*-$C_3H_7)_2Cl_2$] (△), [Pt(CP)(DACH)] (□), and *cis*-[Pt-$(NH_3)_2(OH)]_2(NO_3)_2$ (○).

Table I: ELISA[a] Immunoreactivity of *cis*-DDP-DNA Treated with Cyanide or Thiourea or with Ethidium Bromide during Modification

sample[b]	platinum modification (D/N × 100)	fmol of Pt at 50% inhibition
cis-DDP-DNA before cyanide treatment	5.8	62
cis-DPP-DNA after cyanide treatment	0.3	20 000
cis-DDP-DNA before TU treatment	7.2	52
cis-DDP-DNA after TU treatment	2.0	70
cis-DDP-DNA modified in the presence of EtdBr	2.8	105

[a] ELISA was performed on plates coated with 0.4 ng of *cis*-DDP-DNA (D/N = 0.084), anti-*cis*-DDP-DNA diluted 1:16 000, and 10 μg/well of calf thymus DNA as carrier. Samples were assayed in triplicate for each point and on two to four different occasions. [b] See text.

As shown in Figure 2, poly(dG)·poly(dC) modified to D/N = 0.042 with *cis*-DDP exhibited 50% inhibition at 42 fmol of added platinum at a serum dilution of 1:32 000. This result is nearly the same as found for the control *cis*-DDP-DNA sample under the same conditions. By contrast, the *cis*-DDP-modified poly[d(GC)]·poly[d(GC)] was not recognized at all by the antibody. The immunological data clearly demonstrate the profound effect of base sequence upon the stereochemistry of the *cis*-DDP adducts of DNA.

Immunoreactivity of Anti-cis-DDP-DNA toward cis-DDP-DNAs Treated with Cyanide or Thiourea or Modified in the Presence of Ethidium Bromide. Cyanide ion (Bauer et al., 1978; Lippard & Hoeschele, 1979) and thiourea (Filipski et al., 1979) are commonly used to reverse the covalent linkage of platinum to DNA. Not all the platinum can be removed by these reagents, however, and it was of interest to learn whether the CN^-- or TU-resistant fraction of *cis*-DDP-DNA would be immunoreactive. When a portion of *cis*-DDP-DNA modified to D/N = 0.058 was incubated with cyanide ion (see Materials and Methods), D/N was reduced to 0.003, and the platinum species remaining on the DNA was virtually unreactive toward the antibody (Table I). By ELISA, 2×10^4 fmol of platinum on cyanide-treated *cis*-DDP-DNA was required to yield 50% inhibition (○, Figure 3). Treatment with TU yielded a smaller reduction in the platinum-modification level, from D/N = 0.072 to 0.02, and the DNA was significantly although not substantially less reactive (Table I) toward the antibody by competitive ELISA (△, Figure 3).

EtdBr has been shown to reduce long-range cross-linking (Merkel & Lippard, 1983) and to alter the nuclease-sensitive site binding preferences (Tullius & Lippard, 1982) when present during the reaction of *cis*-DDP with DNA. *cis*-DDP was allowed to react with calf thymus DNA in the presence of EtdBr (two experiments); the DNAs were modified to D/N = 0.028 and 0.04, respectively. The inhibition profiles were 1.5–2-fold less sensitive than the *cis*-DDP-DNA standard curves by competitive ELISA (Table I and Figure 3, ▲).

Immunoreactivity of Anti-cis-DDP-DNA toward Calf Thymus DNAs Modified with Other Platinum Compounds. In order to elucidate the nature of the adduct(s) on the original immunogen DNA, the specificity of the anti-*cis*-DDP-DNA for a series of calf thymus DNAs incubated with various platinum compounds was monitored by competitive ELISA. Table II and Figure 3 show that virtually no antibody specificity was directed toward DNAs modified with *trans*-DDP, *trans*-$[Pt(NH_2CH_3)_2Cl_2]$, or [Pt(dien)Cl]Cl.

Three compounds giving modified DNAs with specificity similar to that of the original immunogen, $[Pt(en)Cl_2]$ (Table II), *cis*-$[Pt(NH_2CH_3)_2Cl_2]$ (Figure 3, □), and *cis*-$[Pt(NH_2\text{-}i\text{-}C_3H_7)_2Cl_2]$ (Figure 4, △), contained *cis*-$PtCl_2$ units capable of forming a bidentate adduct. The bulky isopropylamine platinum adducts were less well recognized by the antibody than the smaller methylamine and ethylenediamine platinum adducts (Table II). By ELISA, DNA modified with the methylamine platinum complex appeared to be more sensitive than the standard *cis*-DDP-DNA (Table II and Figure 3, □). Although the two curves fell in the same range, the slope of the former was decreased substantially as compared to the standard DNA. This phenomenon was also observed with the [Pt(CP)(DACH)] complex (Figure 4, □) and *cis*-diammineplatinum α-pyridone blue modified DNAs (Table II). DNA modified with the hydroxide-bridged dimer *cis*-$[Pt(NH_3)_2(OH)]_2(NO_3)_2$ was an order of magnitude less immunoreactive by competitive ELISA than *cis*-DDP-DNA (Table II and Figure 4, ○).

Discussion

The antiserum elicited against *cis*-DDP-modified calf thymus DNA exhibits excellent specificity for *cis*-DDP bound to poly(dG)·poly(dC) but virtually none for the platinated copolymer poly[d(GC)]·poly[d(GC)]. These results demonstrate that the *cis*-$Pt(NH_3)_2^{2+}$ moiety bound to adjacent deoxyguanosine residues on the same strand forms a structure constituting a major antigenic determinant for this antiserum. It is likely that this or a stereochemically similar adduct occurs in vivo since DNAs from cultured cells (Poirier et al., 1982), mouse ascites fluid (Poirier et al., 1982), and human blood mononucleated cells (M. Poirier, E. Reed, L. Zwelling, and S. Yuspa, unpublished results) treated with *cis*-DDP all inhibit antigen–antibody binding in the competitive *cis*-DDP-DNA ELISA. Since the ELISA profiles of these biological DNA samples indicate less efficient antibody binding than for the

Table II: ELISA[a] Immunoreactivity of Calf Thymus DNAs Modified with Various Platinum Compounds

compound[b]	platinum modification (D/N × 100)	fmol of Pt giving no inhibition	fmol of Pt giving 50% inhibition
cis-DDP	8.4		66 ± 7
cis-$[Pt(en)Cl_2]$	4.9		62
cis-$[Pt(NH_2CH_3)_2Cl_2]$	4.2		35[c]
[Pt(CP)(DACH)]	2.2		45
PPB	5.8		20
cis-$[Pt(NH_2\text{-}i\text{-}C_3H_7)_2Cl_2]$	3.5		210
cis-$[Pt(NH_3)_2(OH)]_2(NO_3)_2$	3.6		750
trans-DDP	3.8	5000	
[Pt(dien)Cl]Cl	3.8		15 000
trans-$[Pt(NH_2CH_3)_2Cl_2]$	4.6	3000	

[a] ELISA conditions are the same as for Table I, footnote *a*. [b] See Figure 1. [c] The apparent increase in sensitivity is the result of a shallow slope; see Figure 3.

immunogen DNA, as judged by a decrease in the slope of the competition curve, a more heterogeneous population of *cis*-DDP-DNA adducts probably exists in vivo.

Previously, Malfoy et al. (1981) reported that *cis*-DDP-modified poly(dG)·poly(dC) but not poly[d(GC)]·poly[d(GC)] was recognized by an antiserum to *cis*-DDP-modified calf thymus DNA. These investigators used a competitive radioimmunoassay ~4 orders of magnitude less sensitive than ours, however, and their modified homopolymers, including poly(dA)·poly(dT) and poly[d(AC)]·poly[d(TG)], were at least 100 times less efficiently recognized by their antibodies than platinated control DNA. Moreover, they found no inhibition by rat liver DNA modified by *cis*-DDP in vivo. The much lower sensitivity of the assay employed in this work makes it difficult to assess its relevance to our own results.

The competitive ELISA profiles for DNAs modified with various *cis*-DDP analogues (Figure 1) support the conclusion that a primary three-dimensional structure recognized by the antiserum is a bidentate adduct of *cis*-DDP linking two adjacent deoxyguanosines on the same DNA strand. Only compounds that can generate a *cis*-PtA_2-d(GpG) fragment (A = amine, see Figure 1) were capable of forming DNA adducts recognized by the antibody. In contrast, the trans isomers and [Pt(dien)Cl]Cl, which cannot yield this fragment, formed adducts that were not immunoreactive. DNA adducts with platinum complexes in which the amine ligands had organic substituents (CH_3–, $-CH_2CH_2-$, *i*-C_3H_7–) showed reduced immunoreactivity. This result demonstrates that it is the chloride ion and not the amine ligands that are displaced from platinum upon DNA binding, a conclusion that is consistent with previous studies using radiolabeled amine ligands (Tullius et al., 1983; Macquet et al., 1983). None of these results, however, rule out the possibility that there may be specificity directed toward a monodentate *cis*-DDP adduct, and experiments to identify immunogenically such a species are currently in progress. Evidence for the formation of another class of adduct comes from the cyanide-treated samples, which contained only 5% of the original D/N and which were virtually unrecognized by the antibody. Although the chemical nature of this adduct is currently unknown, it is probable that the immunogen DNA also contained a small proportion of cyanide-resistant bound platinum but not enough to elicit antibodies.

cis-DDP-DNA modified in the presence of ethidium was immunoreactive only to a slightly lesser degree than the control DNA modified in the absence of EtdBr. Ethidium is known to alter the mode of binding of *cis*-DDP to DNA, presumably by blocking the formation of long-range inter- or intrastrand cross-links that produce DNA shortening (Tullius et al., 1983; Merkel & Lippard, 1983). The present immunological results show that *cis*-DDP binding to DNA in the presence of EtdBr does not affect recognition by the antiserum to a substantial degree. Thus, the chemical structure responsible for DNA shortening is probably not one that is recognized by the antibodies.

It is significant that all of the *cis*-bis(amine)platinum(II) compounds exhibiting good recognition by the antibody are also effective antitumor agents. The high immunoreactivity of the [Pt(CP)(DACH)] and platinum α-pyridone blue platinum adducts, in particular, is interesting since both of these compounds are under study as new-generation antitumor drugs.

In summary, the present immunological study supports the hypothesis that a *cis*-DDP-induced intrastrand d(GpG) cross-link forms as an important chemical and stereochemical determinant when the antitumor platinum drug binds to its supposed biological target. Is such an adduct primarily responsible for tumorocidal activity? The answer to this question requires further work.

Acknowledgments

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Registry No. *cis*-DDP, 15663-27-1; poly(dG)·poly(dC), 25512-84-9; poly[d(GC)], 36786-90-0; *trans*-DDP, 14913-33-8; *trans*-[Pt(NH_2CH_3)$_2Cl_2$], 15319-09-2; [Pt(dien)Cl]Cl, 14215-58-8; *cis*-[Pt(en)Cl_2], 14096-51-6; *cis*-[Pt(NH_2CH_3)$_2Cl_2$], 15273-32-2; [Pt(CP)(DACH)], 65296-81-3; *cis*-diammineplatinum α-pyridone blue, 62782-86-9.

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