

DNA Interactions of New Antitumor Aminophosphine Platinum(II) Complexes

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ABSTRACT

Mechanistic studies are presented of a novel class of aminophosphine platinum(II) complexes as potential anticancer agents. These new agents, which have demonstrated activity against murine and human tumor cells including those resistant to cisplatin are *cis*-[PtCl₂(Me₂N(CH₂)₃PPh₂-P)₂] (Com1) and *cis*-[PtCl(C₆H₁₁NH(CH₂)₂PPh₂-N,P)(C₆H₁₁NH(CH₂)₂PPh₂-P)] (Com2). We studied modifications of natural and synthetic DNAs in cell-free media by Com1 and Com2 by various biochemical and biophysical methods and compared the results with those obtained when DNA was modified by cisplatin. The results indicated that Com1 and Com2 coordinated to DNA faster than cisplatin. Bifunctional Com1 formed DNA adducts coordinating to single adenine or guanine residues or by forming cross-links between these residues. In comparison with cisplatin, Com1 formed the adducts more frequently at adenine

residues and also formed fewer bidentate lesions. The monofunctional Com2 only formed DNA monodentate adducts at guanine residues. In addition, Com1 terminated DNA synthesis *in vitro* more efficiently than cisplatin whereas Com2 blocked DNA synthesis only slightly. DNA unwinding studies, measurements of circular dichroism spectra, immunochemical analysis, and studies of the B-Z transition in DNA revealed conformational alterations induced by the adducts of Com1, which were distinctly different from those induced by cisplatin. Com2 had little influence on DNA conformation. It is suggested that the activity profile of aminophosphine platinum(II) complexes, which is different from that of cisplatin and related analogs, might be associated with the specific DNA binding properties of this new class of platinum(II) compounds.

Current platinum-based drugs are limited in their use by their severe side effects, narrow spectrum of anticancer activity, and problems due to drug resistance. We are investigating the design of novel platinum(II) aminophosphine complexes as potential anticancer agents.

The aminophosphine complexes of platinum(II), *cis*-[PtCl₂(Me₂N(CH₂)₃PPh₂-P)₂] (Com1) and *cis*-[PtCl(C₆H₁₁NH(CH₂)₂PPh₂-N,P)(C₆H₁₁NH(CH₂)₂PPh₂-P)] (Com2; Fig. 1) contain the possibility of *cis* amine ligands, which are a feature found in many active platinum anticancer agents

(Reedijk, 1996), together with *cis* phosphine ligands. Certain diphosphines have also been shown to exhibit anticancer activity, especially 1,2-diphenylphosphine (dppe) complexes of Cu(I), Ag(I), and Au(I) (Berners-Price and Sadler, 1988). Furthermore, lipophilic cations such as [Au(dppe)]⁺ can disrupt the mitochondrial membrane potentials (Berners-Price et al., 1997), and thereby act via a different mode of action to platinum am(m)ine antitumor complexes for which DNA is the target (Johnson et al., 1989).

Metal aminophosphine complexes have been shown to be cytotoxic toward cancer cells with a potency approaching that of *cis*-diamminedichloroplatinum(II) [*cis*-[PtCl₂(NH₃)₂]] (cisplatin) and, moreover, are active against some cisplatin-resistant cell lines (Habtemariam and Sadler, 1996; Papathanasiou et al., 1997). These complexes can exist in chelate ring-opened and ring-closed forms in aqueous solution (Fig. 2). The equilibrium can be controlled under conditions of

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ABBREVIATIONS: Com1, *cis*-[PtCl₂(Me₂N(CH₂)₃PPh₂-P)₂]; Com2, *cis*-[PtCl(C₆H₁₁NH(CH₂)₂PPh₂-N,P)(C₆H₁₁NH(CH₂)₂PPh₂-P)]; Ab_{cis}, polyclonal antibodies that bind selectively to adducts formed on linear double-helical DNA by cisplatin at r_b = 0.08; bp, base pair(s); CD, circular dichroism; cisplatin, *cis*-diamminedichloroplatinum(II) [*cis*-[PtCl₂(NH₃)₂]]; [PtCl(dien)]Cl, chlorodiethylenetriamineplatinum(II) chloride {[PtCl(H₂NCH₂CH₂NHCH₂CH₂NH₂)]Cl}; DPP, differential pulse polarography; EtBr, ethidium bromide; FAAS, flameless atomic absorption spectrophotometry; RP, reversed phase; ICL, interstrand cross-link; r_b, the number of the molecules of the platinum complex fixed per nucleotide residue; r_i, the molar ratio of free platinum complex to nucleotide-phosphates at the onset of incubation with DNA; CT, calf thymus.

biological relevance by variation of pH and chloride concentration (Habtemariam and Sadler, 1996). The ring-closed form (Fig. 2A), being a lipophilic cation, could thus act as an antimitochondrial agent and the ring-opened forms (Fig. 2, B and C), which contain labile chloride ligands, offer potential binding sites to DNA.

Recently we have shown that chelate ring-opening platinum(II) aminophosphine complexes can bind rapidly and reversibly to the DNA bases guanine (Habtemariam and Sadler, 1996) and thymine as well as to the RNA base uracil (Margiotto et al., 1997), under physiologically relevant conditions, in contrast to platinum am(m)ine anticancer complexes (Reedijk, 1996). The binding was observed to the bases contained in monomeric nucleotides or in a short oligonucleotide duplex [8 base pairs (bp)]. In this work the modification of natural DNA and synthetic single- or double-stranded polydeoxyribonucleotides in cell-free media was studied by using various biomedical and biophysical methods. Com1 was chosen for these studies as a representative of the bi-functional aminophosphine complexes with leaving chloride ligands in *cis* position to compare its effects on double-helical DNA with those of cisplatin, whereas Com2, which has one chloride-leaving group, was used as a model.

Experimental Procedures

Materials. Cisplatin and chlorodiethylenetriamineplatinum(II) chloride $\{[PtCl(H_2NCH_2CH_2NHCH_2CH_2NH_2)]Cl\}$ ($[PtCl(dien)]Cl$) were synthesized and characterized in Lachema (Brno, Czech Republic). Com1 and Com2 (Fig. 1) were prepared and characterized as reported elsewhere (A.H., R. Palmer, P. Potter, and P.J.S., submitted). Calf thymus (CT) DNA (42% guanine + cytosine, mean molecular mass ca. 2×10^7) was prepared and characterized as described previously (Brabec and Paleček, 1970). Denatured CT DNA was prepared by heating at 100°C for 10 min and subsequent rapid cooling on an ice bath. Plasmids pSP73 (2464 bp) or pSP73KB (2455

bp) were isolated according to standard procedures and banded twice in CsCl/ethidium bromide (EtBr) equilibrium density gradients. Synthetic single-stranded homopolydeoxyribonucleotides poly(dA), poly(dC), poly(dG), poly(dT), and double-helical alternating polydeoxyribonucleotides poly(dA-dT) and poly(dG-dC) were purchased from Boehringer-Mannheim Biochemica (Mannheim, Germany) [all references in the text to poly(dA-dT) and poly(dG-dC) refer to duplex molecules] (the concentrations of synthetic polynucleotides are related to their phosphorus content). The oligodeoxyribonucleotide duplex containing 40 bp, 5'-CCCGATTATACGGCTTAAACCAAATTGCTTAAATTGGCC/5'-GGCCAATTTAAGCAATTTGGTTTAAGCCGTATAATCCGGG was obtained from East Port (Prague, Czech Republic); the single-stranded oligonucleotides constituting this duplex were purified by strong anion exchange chromatography (Pharmacia MonoQ column) on a Pharmacia fast protein liquid chromatography system with 10 mM NaOH, 0.2 to 0.8 M NaCl gradient. The duplex was formed by heating the mixture of the complementary single-stranded oligonucleotides at equal concentrations (related to the mononucleotide content) at 90°C for 5 min followed by incubation at 25°C for 4 h. Restriction endonucleases and Thermal Cycle Dideoxy DNA Sequencing Kit with Vent_R(exo⁻) or Vent_R(exo⁺) DNA polymerases were purchased from New England Biolabs (Beverly, MA). A primer 5'-d(GATTTAGGTGACACTATAG) was obtained from BioVendor (Brno, Czech Republic). T4 polynucleotide kinase and Klenow fragment of DNA polymerase I were also obtained from Boehringer-Mannheim Biochemica (Mannheim, Germany). DNase I from bovine pancreas, nuclease P1 from *Penicillium citrinum*, and alkaline phosphatase from calf intestine were purchased from Sigma-Aldrich (Prague, Czech Republic). EtBr, acrylamide, (bis)acrylamide, urea, and agarose were obtained from Merck KgaA (Darmstadt, Germany). The radioactive products were purchased from Amersham (Arlington Heights, IL).

Platination Reactions. DNAs were modified by platinum complexes in 10 mM NaClO₄ (pH 7.0) at 37°C in the dark for 48 h unless stated otherwise. In these samples, the number of the molecules of the platinum complex fixed per nucleotide residue (r_b) was determined by flameless atomic absorption spectrophotometry (FAAS) or by differential pulse polarography (DPP; Kim et al., 1990).

HPLC Analyses. These analyses were performed using a Hitachi Series 4 liquid chromatograph equipped with a LCI-100 computing integrator and a Waters μ Bondapak C18 column. If not stated otherwise, the products were separated by reversed phase (RP)-HPLC (isocratic elution with 0.1 M ammonium acetate, pH 5.0 in 4% CH₃CN at 1 ml/min flow rate). The following enzymatic digestion protocol was used to characterize the platinated deoxyribooligonucleotides. The samples (50 μ g of the oligonucleotide) were incubated with 72 U DNase I at 37°C. After 4 h nuclease P1 (40 μ g) was added, and the reaction was allowed to continue at 37°C for 18 h. Finally, alkaline phosphatase (39 U) was added and the incubation continued for additional 4 h at 37°C. The digested samples containing constituent nucleosides were then heated for 2 min at 80°C, centrifuged, and the supernatant was analyzed by RP-HPLC. Each analysis was performed four times and the data varied on average $\pm 1\%$ from their mean.

Sequence Specificity of DNA Adducts. The (*Hpa*I/*Nde*I) restriction fragment of pSP73KB DNA (212 bp) was obtained as described previously (Brabec and Leng, 1993). Ten micrograms of pSP73KB were treated with *Nde*I to obtain linear plasmid followed by treatment with alkaline phosphatase. The linear fragment was then 5'-end labeled by treatment with T4 polynucleotide kinase in the presence of [³²P] γ -ATP. The 212-bp fragment was obtained by subsequent treatment with *Hpa*I and isolated by electrophoresis through a preparative 1% agarose gel. The modification of this fragment by cisplatin, Com1, or Com2 was carried out in 10 mM NaClO₄ (pH 7) for 48 h at 37°C to obtain $r_b = 0.01$. Circumvent Thermal Cycle Dideoxy DNA Sequencing Kit with Vent_R(exo⁻) or (exo⁺) DNA polymerases was used with the protocol for thermal cycle DNA

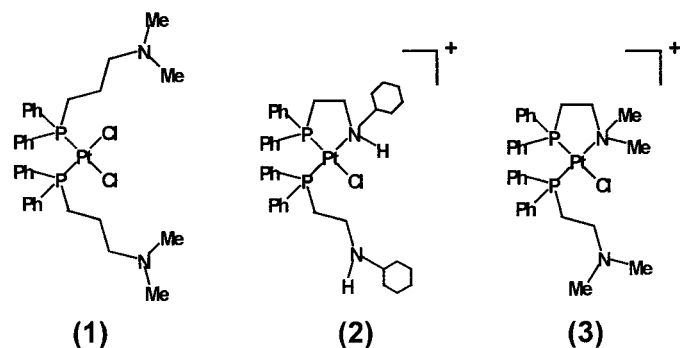


Fig. 1. Structures of bis(aminophosphine) platinum(II) complexes: 1, *cis*- $[PtCl_2(Me_2N(CH_2)_3PPh_2)_2]$, 2, *cis*- $[PtCl(C_6H_{11}NH(CH_2)_2PPh_2-N, P)(C_6H_{11}NH(CH_2)_2PPh_2-P)]$, 3, *cis*- $[PtCl(Me_2N(CH_2)_2PPh_2-N, P)(Me_2N(CH_2)_2PPh_2-P)]$.

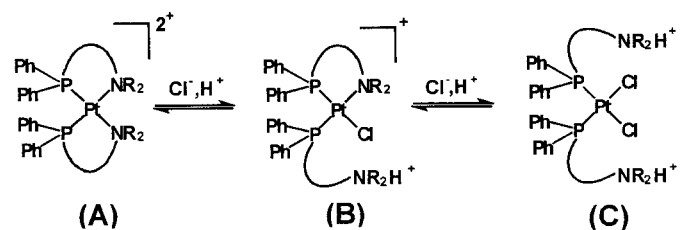


Fig. 2. Schematic representation of chelate ring opening in bis(aminophosphine) platinum(II) complexes.

sequencing with 5' end-labeled primer recommended by the manufacturer with small modifications (Nováková et al., 1995).

EtBr Fluorescence. These measurements were performed with the aid of a Shimadzu RF 40 spectrofluorophotometer using a 1-cm quartz cell. Fluorescence measurements of DNA modified by platinum in the presence of EtBr were performed using the excitation wavelength of 546 nm and the emitted fluorescence was measured at 590 nm. The fluorescence was measured at 25°C in 0.4 M NaCl to avoid the second fixation site of EtBr to DNA (Butour and Macquet, 1977). The concentrations were 0.01 mg/ml for DNA and 0.04 mg/ml for EtBr, which corresponded to the saturation of all intercalation sites of EtBr in DNA (Butour and Macquet, 1977).

Unwinding of Negatively Supercoiled DNA. Unwinding of closed circular supercoiled pSP73 plasmid DNA was assayed by an agarose gel mobility shift assay (Keck and Lippard, 1992). The unwinding angle Φ , induced per platinum-DNA adduct was calculated upon the determination of the r_b value at which the complete transformation of the supercoiled to relaxed form of the plasmid was attained. Samples of pSP73 plasmid were incubated with Com1 or Com2 in 10 mM NaClO₄, pH 7.0 at 37°C in the dark for 48 h. All samples were precipitated by ethanol and redissolved in TAE or the TBE buffer (0.04 M Tris-acetate + 1 mM EDTA, pH 7.0 or 0.09 M Tris-borate + 1 mM EDTA, pH 9.0, respectively). An aliquot of the precipitated sample was subjected to electrophoresis on 1% agarose gels running at 25°C in the dark with TAE or TBE buffer with voltage set at 30 V. The gels were then stained with EtBr, followed by photography on Polaroid 667 film with transilluminator. The other aliquot was used for the determination of r_b values by FAAS.

Interstrand Cross-Link (ICL) Assay. If not stated otherwise, Com1 or Com2 at varying concentrations were incubated with 2 μ g of pSP73 DNA linearized by *Eco*RI. The platinated samples were precipitated by ethanol and analyzed for DNA ICLs in the same way as described in several recent papers (Farrell et al., 1990; Brabec and Leng, 1993). The linear duplexes were first 3'-end labeled by means of Klenow fragment of DNA polymerase I and [α -³²P]dATP. The samples were deproteinized by phenol, precipitated by ethanol, and the pellet was dissolved in 18 μ l of a solution containing 30 mM NaOH, 1 mM EDTA, 6.6% sucrose, and 0.04% bromophenol blue. The number of ICLs was analyzed by electrophoresis under denaturing conditions on alkaline agarose gel (1%). After the electrophoresis was completed, the intensities of the bands corresponding to single strands of DNA and ICL duplex were quantified by means of a Molecular Dynamics PhosphorImager (Storm 860 System with ImageQuant software; Sunnyvale, CA). As shown below, some platinated DNA samples were also analyzed for the ICLs using milder conditions, when the DNA samples were treated with formamide or dimethyl sulfoxide at 40 or 55°C and then analyzed in native agarose gel. The details of these milder assays can be found in the papers previously published (Konopa, 1983; Cullinane and Phillips, 1994).

Circular Dichroism (CD). If not stated otherwise, CD spectra of DNA modified by the platinum complexes were recorded at 25°C in 10 mM NaClO₄ (pH 7.0) on a JASCO spectropolarimeter, Model J720 (Tokyo, Japan).

Immunochemical Analysis. Polyclonal antibodies that bind selectively to adducts formed on linear double-helical DNA by cisplatin at $r_b = 0.08$ (Ab_{cis}) were elicited against double-helical calf-thymus DNA modified by cisplatin at $r_b = 0.08$ in 10 mM NaClO₄ for 48 h at 37°C. They were purified and characterized as described in previously published papers (Sundquist et al., 1987; Vrána et al., 1992). The procedures for their immunoenzymatic analysis and enzyme-linked immunosorbent assay have been also described (Sundquist et al., 1987; Vrána et al., 1992).

Results and Discussion

DNA Binding. Solutions of double-helical CT DNA at a concentration of 32 μ g/ml were incubated with Com1 or Com2 at the molar ratio of free platinum complex to nucle-

otide-phosphates at the onset of incubation with DNA (r_i) values of 0.08 in 10 mM NaClO₄ (pH 7.0) at 37°C. At various time intervals an aliquot of the reaction mixture was withdrawn and assayed by DPP for the amount of platinum bound to DNA (r_b) (Kim et al., 1990). Figure 3 shows a plot of r_b against the time of DNA incubation with Com1 or Com2. The amount of platinum coordinated to DNA increased with time. After 48 h, approximately 100% or 80% of the complex Com1 or Com2, respectively, present in the reaction mixtures was coordinated to DNA [exhaustive dialysis of the samples of DNA treated with Com1 or Com2 against platinum-free background solution (10 mM NaClO₄) did not affect the amount of the platinum bound to DNA]. In these binding reactions, the time at which the binding reached 50% ($T_{50\%}$) was ca. 3 h for both Com1 and Com2. The value of $T_{50\%}$ for the reaction of cisplatin or monofunctional [PtCl(dien)]Cl with DNA under conditions identical with those specified in Fig. 3 were ~4 h, respectively (Bancroft et al., 1990). This comparison indicates that the presence of the aminophosphine groups as nonleaving ligands enhances the rate of the coordination of monofunctional chloro or bifunctional dichloro platinum(II) complexes to natural double-helical DNA. When the same binding experiment was carried out with thermally denatured CT DNA, the binding of Com2 remained unchanged whereas the binding of Com1 was somewhat faster ($T_{50\%}$ was ~2 h; Table 1).

We also studied the binding of Com1 and Com2 to several synthetic single-stranded homopolydeoxyribonucleotides and double-helical synthetic alternating polydeoxyribonucleotide complexes (Table 1). The polynucleotides at a concentration of 1×10^{-4} M (the concentration is the mononucleotide content) were incubated with Com1 or Com2 at $r_i = 0.08$ in 10 mM NaClO₄ (pH 7.0) at 37°C. The binding of the platinum compounds was quantified in the same way as described above for the reaction of Com1 and Com2 with CT DNA. Both complexes bound readily to single-stranded poly(dA), poly(dC), and poly(dG) (the values of $T_{50\%}$ were about 2 h for Com1 and 6–7 h for Com2; Table 1). Importantly, at pH 7 Com1 did not bind to single-stranded poly(dT) whereas Com2

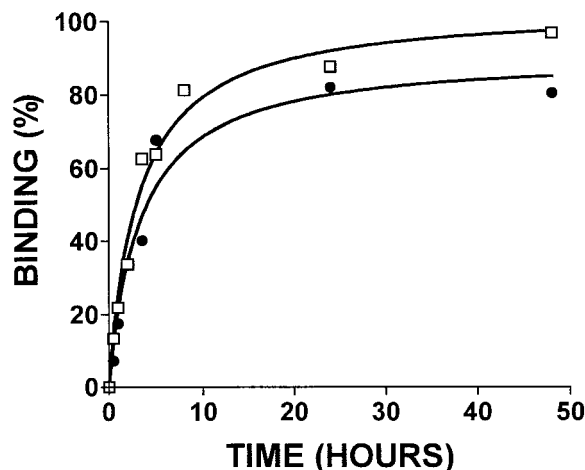


Fig. 3. Formation of DNA adducts by bis(aminophosphine) platinum(II) complexes as a function of incubation time. CT DNA at the concentration of 32 μ g/ml was mixed with Com1 (\square) or Com2 (\bullet) at $r_i = 0.08$ in 10 mM NaClO₄ and at 37°C in the dark. At various time intervals the aliquots were withdrawn and platinum coordinated to DNA was estimated by DPP assay. Data points measured in triplicate varied on average $\pm 2\%$ from their mean.

was bound to poly(dT), but considerably more slowly than to other single-stranded polynucleotides (Table 1). Also importantly, both platinum complexes bound to double-stranded poly(dA-dT) or poly(dG-dC) with markedly higher rates than to single-stranded polynucleotides (Table 1). The binding of Com1 and Com2 to double-helical CT DNA and poly(dT) was also quantified in the following way. Aliquots of the reaction withdrawn at various time intervals were quickly cooled on an ice bath and then exhaustively dialyzed against 10 mM NaClO₄ at 4°C to remove free (unbound) platinum compound. The content of platinum in these samples was determined by FAAS. Results identical with those obtained using the DPP assay were obtained. Taken together, the results of these binding studies suggest that at pH 7 Com1 binds to adenine, cytosine, and guanine residues in synthetic polynucleotides with approximately the same rate, and it does not bind to thymine residues. On the other hand, the monofunctional complex Com2 seems to bind to all base residues in synthetic polynucleotides including thymine residues. The binding to the latter residues is, however, noticeably slower than to other DNA base residues, and in general the DNA binding of Com2 is slower than that of Com1. Thus, these results suggest that Com1 and Com2 bind to different base residues in DNA and have different base sequence preferences than their simpler analogs cisplatin or [Pt-Cl(dien)]Cl, which both bind preferentially to guanine residues, and cisplatin to a less extent to adenine residues (Johnson et al., 1982; Fichtinger-Schepman et al., 1985; Eastman, 1987).

It has been shown by NMR studies that under physiological conditions the monofunctional complex 3 (Fig. 1) binds to the mononucleotide dTMP or thymine residues at the ends of a short (8-bp) oligodeoxyribonucleotide duplex (Margiotta et al., 1997). It was found that these reactions were fast (completed within several minutes) and that the amount of binding increased with increasing pH. Similar results have been obtained for Com1, with binding to dTMP observed over a large pH range (4–12). For this reason we repeated studies on the binding of Com1 and Com2 to poly(dT) at pH 8 (the pH was adjusted by adding a small amount of 2 M NaOH or 1

mM Tris/HCl buffer, pH 8, to 10 mM NaClO₄). The binding of Com2 was affected only negligibly whereas 30% of Com1 was now bound after 48 h (Table 1). However, when the binding of Com1 and Com2 to CT DNA was also investigated at pH 9 (the pH was again adjusted by adding either a small amount of 2 M NaOH or 1 mM Tris/HCl buffer, pH 9, to 10 mM NaClO₄) no binding of either complex was observed.

The amount of binding of the complexes to poly(dT) is lower than might have been expected from the NMR work on the monomeric nucleotides. The reason for this is unclear but it may imply that N3 atoms in poly(dT) under these conditions are not accessible to platinum.

In addition, it was previously shown by NMR studies (Habtariam and Sadler, 1996) that the addition of 0.5 M KCl led to the displacement of coordinated 5' dGMP from *cis*-[PtCl{Me₂N(CH₂)₂PPh₂-N,P}{Me₂N(CH₂)₂PPh₂-P}(5'-dGMP-N7)]³⁺. Therefore, we prepared a sample of CT DNA modified by Com1 or Com2 at *r*_b = 0.08 (at 37°C in 10 mM NaClO₄ for 48 h). The concentration of KCl in these samples was adjusted by 5 M KCl to give a final concentration of 0.5 M. The samples were incubated at 37°C for an additional 48 h, and the content of unbound platinum was determined by DPP. No free platinum compound was found, indicating that the presence of chloride ions did not result in the displacement of Com1 or Com2 from high molecular mass DNA. Therefore, it appears that the duplex structure of DNA increases the kinetic stability of platinum aminophosphine adducts perhaps by shielding platinum from attack by chloride ions. Such a shielding is thought to increase the stability of 5'-G monofunctional adducts with cisplatin (Reeder et al., 1997).

Characterization of DNA Adducts by HPLC Analysis of Enzymatically Digested DNA. To further characterize the coordination mode of the two aminophosphine platinum(II) complexes, a 40-bp deoxyribooligonucleotide duplex (with a random nucleotide sequence, see *Experimental Procedures*) modified by Com1 or Com2 (in 0.1 M NaClO₄, pH 7) was enzymatically digested to mononucleosides and analyzed by RP-HPLC. RP-HPLC analysis of enzymatic digests of Com1- or Com2-modified oligonucleotide duplex was performed by recording optical density at 260 nm. The profile in Fig. 4 (curve 1) shows well resolved mononucleoside peaks that reflect the proper proportions of the single mononucleosides in unplatinated duplex when integrated and normalized by their extinction coefficients. Digestion of the platinated samples (Fig. 4, curves 2 and 3) resulted in the decrease of the integrated area of the deoxyriboguanosine peak and, in the case of the digestion of the duplex modified by Com1, also in the decrease of deoxyriboadenosine peak. The peaks of deoxyribocytidine and thymidine were not affected. It was verified by FAAS that no product containing platinum coeluted with the peaks corresponding to unplatinated deoxyribonucleosides. The platinated products were not retained by the column under the conditions used, so they could not be identified and quantified.

The integrated area of the deoxyriboguanosine peak decreased by ~24% as a consequence of the platination of the duplex by the monofunctional compound Com2 at *r*_b = 0.05 (Fig. 4, curve 3). The 40-bp duplex used in these analyses contained 17 guanine residues. Thus, this decrease corresponds to the loss of four guanine residues due to the modification by Com2. The peaks corresponding to other nucleosides were unchanged, which implies that only guanine

TABLE 1

The binding of the Com1 and Com2 to CT DNA and synthetic polydeoxyribonucleotides determined by DPP^a

Nucleic Acid	<i>T</i> _{50%} ^b		Binding after 48 h	
	1	2	1	2
	<i>h</i>		%	
ds CT DNA ^c	3.0	3.3	99	78
Denatured CT DNA ^d	1.7	3.3	99	85
Poly(dA)	2.2	5.7	89	89
Poly(dC)	1.9	7.6	79	75
Poly(dG)	2.0	6.2	83	79
Poly(dT), pH 7.0		18.2	0	68
Poly(dT), pH 8.0 ^e		17.6	30	70
Poly(dA-dT)	0.3	4.0	90	80
Poly(dG-dC)	0.7	1.5	96	91

^a CT DNA (double-stranded or denatured) at the concentration of 32 μg/ml or synthetic single- or double-stranded polynucleotides at the concentration of 1 × 10⁻⁴ M were mixed with the platinum complexes at *r*_i = 0.08 in 10 mM NaClO₄ (pH 7) and at 37°C in the dark. Data measured in triplicate varied on average ±2% from their mean.

^b The time of the reaction at which the binding reached 50% (*r*_b = 0.04).

^c Double-helical DNA.

^d DNA was thermally denatured before the platinum complex was added.

^e pH was adjusted by NaOH.

residues were platinated by Com2 in a monofunctional manner. Thus, the DNA binding mode of Com2 appears to be similar to that of the other monofunctional platinum(II) complex, [PtCl(dien)]Cl, which also binds preferentially to guanine residues in DNA (Johnson et al., 1982). In other words, the presence of aminophosphine groups in the coordination sphere of platinum in monofunctional triamineplatinum(II) complexes has no effect on the preference of these complexes to bind to guanine residues in double-helical DNA.

The modification of the duplex by bifunctional Com1 at $r_b = 0.09$ resulted in the decrease of the integrated area of the deoxyriboguanosine and deoxyriboadenosine peaks by ~28% and ~21%, respectively (Fig. 4, curve 2; the duplex contained 17 guanine and 23 adenine residues). This decrease corresponds to the loss of five guanine and five adenine residues. The duplex was, however, modified at $r_b = 0.09$, which implies that each duplex only contained 7.2 adducts on the average. The loss of ten unmodified bases due to the platination by Com1 suggests that ca. three adducts, i.e., ca. 40% of all platinum adducts, were bifunctional cross-links. The results of RP-HPLC analyses are consistent with the idea that cisplatin analog Com1 preferentially forms DNA adducts by coordinating to both a single purine or to two purine residues.

For comparative purposes, the modification of the 40-bp duplex used in the present study by cisplatin at $r_b = 0.09$ was also investigated. This modification also resulted in the decrease of the integrated areas of the deoxyriboguanosine and deoxyriboadenosine peaks (not shown). However, in contrast to the modification by Com1, the areas of these peaks decreased by 70% and 5%, respectively. These decreases correspond to the loss of ~12 guanine and ~1 adenine residues, which suggests that ca. 80% of all cisplatin adducts were

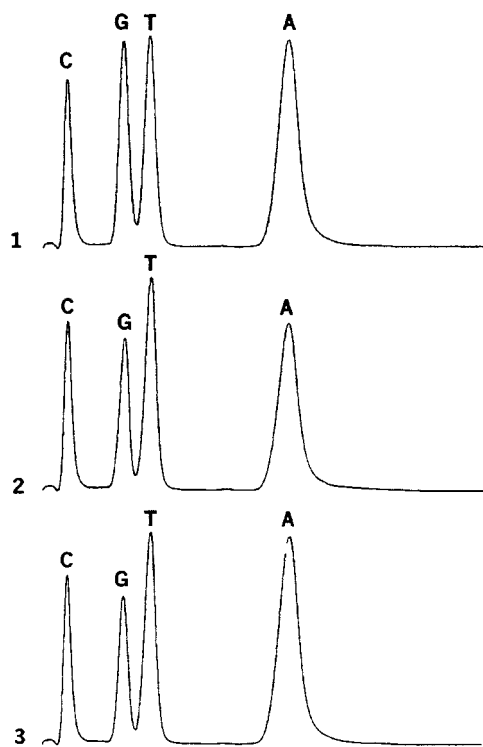


Fig. 4. RP C18 HPLC separation of the products of enzymatic digest of the 40-bp oligonucleotide duplex nonmodified (curve 1) or modified by Com1 at $r_b = 0.09$ (curve 2) or by Com2 at $r_b = 0.05$ (curve 3). For other details, see the text.

bifunctional cross-links. This is in good agreement with the previous analyses (Fichtinger-Schepman et al., 1985; Eastman, 1987) indicating that cisplatin forms mainly bifunctional adducts (intrastrand cross-links and ICLs) and that the preferential sites involved in the adducts of cisplatin are guanine residues and, in a considerably smaller extent, also adenine residues. Thus, the results of the present work based on RP-HPLC analysis of enzymatically digested platinated DNA strongly support the view that in comparison with cisplatin, Com1 forms DNA adducts more frequently at adenine residues and also forms fewer bifunctional lesions.

Mapping of DNA Adducts. Recent work has shown that the in vitro DNA synthesis by DNA polymerases on DNA templates containing several types of bidentate adducts of platinum complexes can be prematurely terminated at the level or in the proximity of adducts (Comess et al., 1992; Murray et al., 1992; Vrána et al., 1996). Importantly, the efficiency of monofunctional DNA adducts of several platinum(II) complexes to terminate DNA synthesis is considerably smaller (Comess et al., 1992).

DNA synthesis by Vent_R DNA polymerase using *HpaI*/*NdeI* fragment of pSP73KB modified by Com1 at $r_b = 0.01$ in 10 mM NaClO₄ (pH 7) yielded fragments corresponding to the DNA synthesis that was prematurely terminated at the level of the platinum adducts (Fig. 5A, lane 1). Thus, the adducts formed by Com1 formed on the DNA template were capable of terminating DNA synthesis in vitro. Several termination sites were identical with those yielded by the adducts of cisplatin, but several termination sites were different. Com1 terminated DNA synthesis preferentially at guanine and adenine residues whereas cisplatin terminated DNA synthesis preferentially at guanine residues in d(GG) and 5'-d(AG)-3' sites (Comess et al., 1992; Murray et al., 1992; Vrána et al., 1996), the termination sites produced by Com1 were mainly at guanine and adenine residues flanked by various base residues [CGA, GGT, TGA, GGG, TAA, TAT, AAC, CAG (bold and italic letters represent the termination site)] (Fig. 5B). These results are consistent with the results of RP-HPLC analysis of enzymatically digested DNA modified by Com1 (Fig. 4), which suggest that the preferential sites in DNA at which Com1 is coordinated are guanine and adenine residues. In addition, the results of the present replication mapping studies indicate that Com1 binds to DNA with a less regular sequence preference, i.e., with a considerably different nucleotide sequence specificity than cisplatin.

The monofunctional compound Com2 terminated DNA synthesis only slightly. Very faint bands were observed if the DNA template was modified at r_b values as high as 0.05 (Fig. 5A, lane 2). This result indicates that the efficiency of DNA adducts of Com2 to inhibit DNA synthesis in vitro is low, and similar to that of DNA adducts of the monofunctional platinum(II) complexes such as [PtCl(dien)]Cl and [PtCl(NH₃)₃]Cl.

Characterization of DNA Adducts by EtBr Fluorescence. EtBr as a fluorescent probe can be used to distinguish between perturbations induced in DNA by monofunctional and bifunctional adducts of platinum(II) compounds (Butour and Macquet, 1977; Žaludová et al., 1997b). Binding of EtBr to DNA by intercalation is blocked in a stoichiometric manner by formation of the bifunctional adducts of a series of platinum complexes including cisplatin and transplatin,

which results in a loss of fluorescence intensity (Butour and Macquet, 1977; Žáková et al., 1998). On the other hand, modification of DNA by monodentate platinum(II) complexes (having only one leaving ligand) results in only a slight decrease of EtBr fluorescence intensity as compared with nonplatinated DNA-EtBr complex.

Double-helical DNA was modified by Com1 or Com2 and for comparative purposes also by cisplatin or [PtCl(dien)]Cl for 48 h. The levels of the modification corresponded to the values of r_b in the range between 0 and 0.15. Modification of DNA by all platinum complexes resulted in a decrease of EtBr fluorescence (Fig. 6). In accordance with the results published earlier (Butour and Macquet, 1977; Žaludová et al., 1997b; Žáková et al., 1998), cisplatin considerably decreased the fluorescence. The binding of Com1 to DNA also considerably decreased EtBr fluorescence although less than DNA adducts of cisplatin. On the other hand, the decrease of the fluorescence intensity by the adducts of Com2 was only very small and similar to that induced by the adducts of [PtCl(dien)]Cl. This result suggests that Com2 forms the DNA adducts, which resemble, from the viewpoint of their

capability to inhibit EtBr fluorescence, those formed by monofunctional platinum complexes. Taken together, the fluorescence analysis is consistent with the idea and supports the postulation that the major DNA adducts of Com2 are monofunctional lesions even after long incubations of DNA with this platinum complex (48 h). On the other hand, under comparable conditions Com1 forms monofunctional adducts and also bifunctional cross-links on DNA that are capable of inhibiting EtBr fluorescence. The amount of the cross-links is, however, smaller in comparison with DNA modification by cisplatin.

CD. CD spectroscopy has already been used to obtain information about the global changes in DNA conformation induced by platinum complexes. It has been shown (Vrána et al., 1986; Brabec et al., 1990) that the intensity of the positive CD band yielded by B-DNA at ~ 275 nm is increased as a consequence of DNA modification by the complexes containing the *cis*-[PtCl₂(amine)₂] unit (Fig. 7, B and C); at higher levels of the modification ($r_b > \sim 0.5$) the intensity of this CD band begins to decrease (Fig. 7, B and C). On the other hand, the modification of DNA by clinically ineffective transplatin or dienPt only slightly decreases this positive band (Vrána et al., 1986; Brabec et al., 1990). It has been suggested (Vrána et al., 1986; Brabec et al., 1990) that the enhancement of the CD band at ~ 275 nm due to the modification by the complexes containing *cis*-[PtCl₂(amine)₂] unit reflects distortions in DNA of a non-denaturational nature. The slight reduction of this CD band induced by the binding of platinum complexes is consistent with the occurrence of short segments containing unpaired bases (denatured regions).

The modification of CT DNA by Com2 had only a negligible effect on the CD spectrum (not shown). In this respect, the monofunctional Com2 resembled other platinum(II) complexes that bind to DNA monodentately [e.g., [PtCl(dien)]Cl or [PtCl(NH₃)]Cl]. In contrast, the bifunctional compound Com1 (having leaving ligands in *cis* positions) radically affected the CD spectrum of DNA at pH 7 (Fig. 7A), but in a distinctly different way than the binding of cisplatin (Fig. 7B). As a linear function of r_b , there was a marked loss in the intensity of the positive band at around 280 nm already at low r_b values (< 0.05 ; Fig. 7, A and C) accompanied by a loss in the intensity of the negative band at around 245 nm. The changes observed in the positive band are reminiscent of

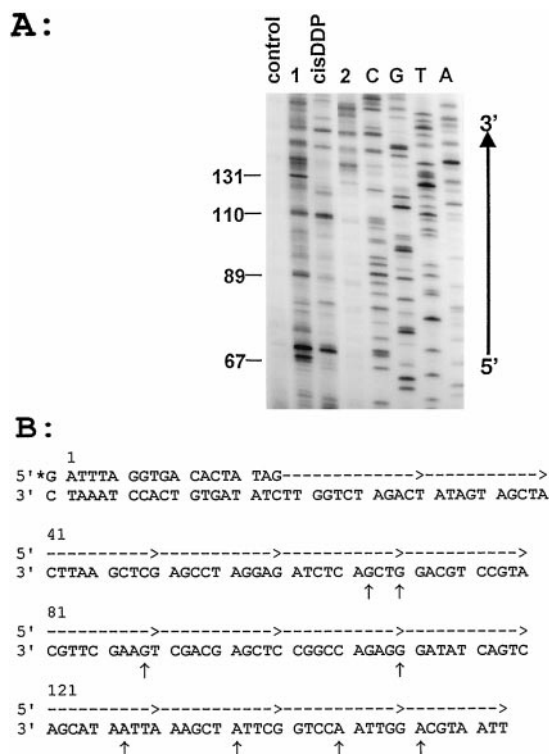


Fig. 5. A, autoradiogram of 6% polyacrylamide/8 M urea sequencing gel showing inhibition of DNA synthesis by Vent_R DNA polymerase on the *HpaI/NdeI* restriction fragment of pSP73KB plasmid DNA modified by platinum complexes. The gel contained the linear amplification products of DNA treated with Com1, Com2, and cisplatin. Lanes: control, unmodified template; cisDDP, DNA modified by cisplatin at $r_b = 0.01$; 1, DNA modified by Com1 at $r_b = 0.01$; 2, DNA modified by Com2 at $r_b = 0.05$; C, G, T, A, chain-terminated marker DNAs (note that these dideoxy-sequencing lanes give the sequence complementary to the template strand). The numbers correspond to the nucleotide sequence numbering of 5B. B, schematic diagram showing a portion of the sequence used to monitor inhibition of DNA synthesis on the template containing adducts of the platinum complexes. * indicates the 5'-end labeling of the primer. The dashed arrow indicates the start site of the DNA polymerase and the direction of the synthesis. ↑, stop signals from A, lane 1. Nucleotides 1 and 18 correspond to 2539 and 1 on the pSP73KB nucleotide sequence map.

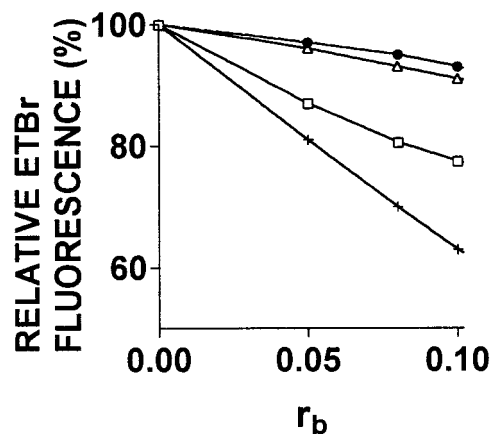


Fig. 6. Dependences of the EtBr fluorescence on r_b for DNA modified by various platinum complexes in 10 mM NaClO₄ at 37°C for 48 h. (+), cisplatin; (Δ), [PtCl(dien)]Cl; (□), Com1; and (●), Com2. Data points measured in triplicate varied on average $\pm 2\%$ from their mean.

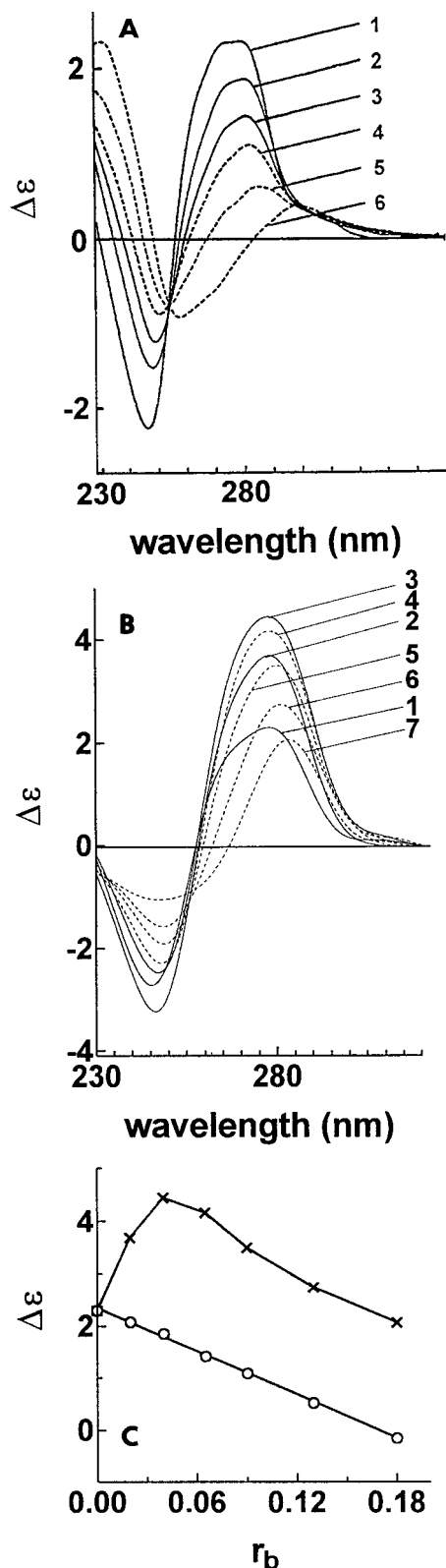


Fig. 7. CD spectroscopy of CT DNA modified by Com1 and cisplatin. The spectra were recorded for DNA in 10 mM NaClO₄, pH 7.0. A, CD spectra of DNA modified by Com1. Curves: 1, control (nonmodified) DNA; 2, $r_b = 0.04$; 3, $r_b = 0.065$; 4, $r_b = 0.09$; 5, $r_b = 0.13$; 6, $r_b = 0.18$. B, CD spectra of DNA modified by cisplatin. Curves: 1, control (nonmodified) DNA; 2, $r_b = 0.02$; 3, $r_b = 0.04$; 4, $r_b = 0.065$; 5, $r_b = 0.09$; 6, $r_b = 0.13$; 7, $r_b = 0.18$. C, dependence of the maximum ellipticity of the positive CD band at around 280 nm on r_b . (x), cisplatin; (o), Com1. Data points measured in duplicate varied on average $\pm 1\%$ from their mean.

DNA transformations seen in the presence of high concentrations of electrolyte or by the reaction with a variety of amines. For instance, at the level of DNA binding corresponding to $r_b = 0.13$, the reduction of the rotational strength of the positive band above 260 nm in the CD spectrum of DNA in 10 mM NaClO₄, pH 7.0 (Fig. 7A) was similar to that obtained for underivatized DNA in ca. 6 M LiCl or at ca. 0.1 mol of *n*-butylamine covalently fixed (via CH₂O)/mol of nucleotide (Chen et al., 1981; Gray, 1996). This transformation has been demonstrated to be roughly correlated with the reduction of the electrostatic repulsive interactions in the DNA molecule. These interactions are reduced by high concentrations of electrolytes in the solution or by simple amines (retaining their positive charge) covalently attached to DNA. At pH 7, binding of Com1 to DNA could position two positively charged amino groups in the dangling arms of this complex close to DNA phosphate groups. Such salt bridges could result in an additional stabilization for DNA adducts of Com1 compared to those of Com2 (which contains only one dangling arm with amino group) or cisplatin. This interpretation is also corroborated by the observation that the marked loss in the intensity of the positive band at around 280 nm due to the binding of Com1 became considerably less pronounced if the sample of DNA modified by Com1 at pH 7 for 48 h was transferred into the medium of pH 9 (not shown). The increase of pH could decrease the number of positively charged amino groups of dangling arms due to their deprotonization. It was verified by DPP that due to the increase of pH to 9, no platinum compound dissociated from the DNA modified by Com1 at pH 7.

The changes induced in double-helical DNA by Com1 do not have the character of extensive denaturational distortions. The cooperative character of the melting profile and the hyperchromic increase upon melting are the same as for the unplatinated control, which contains intact Watson-Crick hydrogen bonds (not shown). It has been shown (Chen et al., 1981; Gray, 1996; Johnson, 1996) that the conformation of DNA in concentrated electrolyte solutions or when modified by some amines, which gives rise to a CD spectrum with a markedly decreased rotational strength above 260 nm, is a variant of the B structure. This variant has a higher winding angle than that present in the nonmodified DNA in more modest concentrations of electrolyte. Thus, we suggest that double-helical DNA modified by Com1 adopts some features of the conformation of DNA in concentrated electrolyte solutions or DNA to which positively charged amines are covalently bound. Also importantly, this conformational alteration is apparently unique for bifunctional DNA binding of bifunctional bis(aminophosphine) compounds (no change such as that seen for Com1 was observed for monofunctional Com2).

Unwinding Induced in DNA by Platinum Coordination. The results described above suggest that Com1 forms bifunctional adducts on DNA although to a smaller extent than its bifunctional analog, cisplatin. It has been shown that DNA adducts of cisplatin (and its direct analogs) unwind DNA (e.g., Bellon et al., 1991; Keck and Lippard, 1992; Huang et al., 1995; Gelasco and Lippard, 1998). CD spectra of DNA modified by Com1 (Fig. 7A) suggest that this modification also results in an overall overwinding of the DNA molecule as a whole if the platinated DNA is dissolved in neutral media, whereas no changes in CD spectra indicating over-

winding occur if DNA is modified under identical conditions by cisplatin or if the pH of the sample of DNA modified by Com1 is adjusted to 9.

The unwinding induced by a random modification of DNA by various platinum(II) complexes including cisplatin can be determined by electrophoresis in native agarose gels by monitoring the degree of supercoiling in plasmid DNA (Keck and Lippard, 1992). A compound that unwinds the DNA duplex reduces the number of supercoils in closed circular, negatively supercoiled DNA. This decrease upon binding of unwinding agents causes a decrease in the rate of migration through agarose gels, which makes it possible that the unwinding can be observed and quantified. We used this assay to determine the unwinding induced in pSP73 plasmid by Com1 (Fig. 8). Figure 8A shows an electrophoresis gel run at pH 9 of samples in which an increasing amount of Com1 was bound to a mixture of nicked and supercoiled pSP73 DNA. The unwinding angle is given by $\Phi = 18 \sigma / r_b(c)$ where σ is the superhelical density and $r_b(c)$ is the value of r_b at which the supercoiled and nicked forms comigrate (Keck and Lippard, 1992). Under the present experimental conditions, σ was calculated to be -0.063 on the basis of the data for cisplatin for which the $r_b(c)$ was determined in this study and $\Phi = 13^\circ$ was assumed (Keck and Lippard, 1992). The $r_b(c)$ for Com1 was determined to be 0.08 (Fig. 8A; this value represents the mean from three measurements and varied on average $\pm 3\%$ from this mean) so that the unwinding angle for Com1 at pH 9 is $14 \pm 1^\circ$. This value for the average unwinding angle caused by DNA adducts of Com1 is very similar to that found for DNA adducts of cisplatin and its direct analogs using the same experimental approach (13° ; Keck and Lippard, 1992). Thus, unwinding of DNA modified by Com1 and transferred

into the medium of pH 9 is similar to that of DNA modified by cisplatin and its direct, simple analogs.

CD spectra of DNA modified by Com1 and dissolved in neutral media (Fig. 7A) made it possible to suggest that at pH 7 DNA unwinding induced by the binding of Com1 is more complicated than at pH 9. It is reasonable to suggest that DNA adducts of Com1 unwind DNA similar to cisplatin or Com1 at pH 9 [i.e., that they increase the number of bp per turn of B-DNA by ca. 0.38 or 0.41 (these values correspond to the unwinding angle of 13° found for cisplatin or 14° found for Com1)]. In addition, CD spectra of DNA modified by Com1 and dissolved in neutral media (Fig. 7A) suggest that Com1, upon binding to DNA and at pH 7, is also capable of overwinding DNA (i.e., to decrease the number of bp per turn of DNA). It has been shown (Gray, 1996; Johnson, 1996) that the dramatic reduction of the intensity of DNA-positive CD band at around 280 nm (Fig. 7, A and C) corresponds to a decrease in the number of bp per turn in B-DNA by approximately 0.2 , which corresponds to the winding angle of $\sim 7^\circ$. Thus, the resulting DNA unwinding by Com1 at pH 7 could be the sum of the two antagonistic effects. The $r_b(c)$ value for the sample of plasmid pSP73 determined by means of gel electrophoresis at pH 7 was 0.2 (Fig. 8B) so that the total unwinding angle for Com1 at pH 7 was only $6 \pm 1^\circ$. A plausible explanation of this observation is that in neutral media DNA unwinding caused by the adducts of Com1 ($\sim 14^\circ$) is partially compensated by overall overwinding of DNA molecules ($\sim 7^\circ$) induced by the binding of Com1 and deduced from CD spectra (Fig. 7, A and C).

No comigration of the relaxed and supercoiled forms of pSP73 DNA at pH 7 or 9 was reached even at a value of r_b as high as 0.2 if the sample of the plasmid was modified by monofunctional Com2 (not shown). This result indicates that

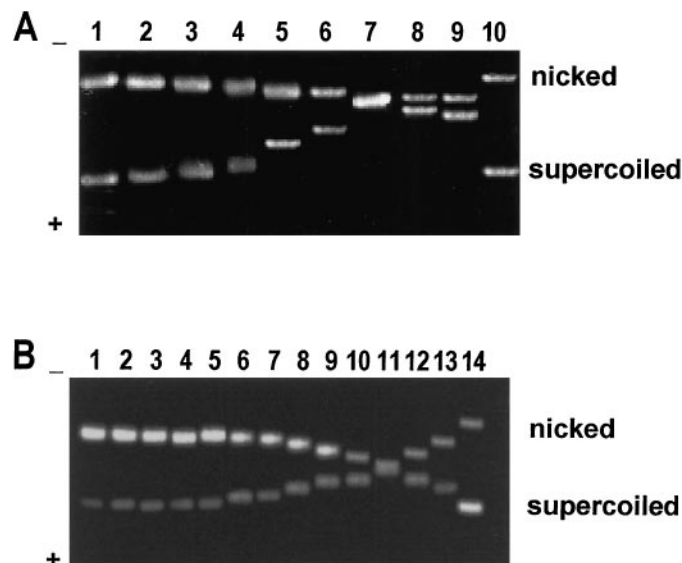


Fig. 8. Unwinding of supercoiled pSP73 plasmid DNA by Com1. DNA was modified in 10 mM NaClO_4 , pH 7, precipitated by ethanol, dissolved in TBE buffer, pH 9.0 (A) or TAE buffer, pH 7.0 (B), and analyzed by gel electrophoresis using the same buffer in which DNA was dissolved. The top bands correspond to the form of nicked plasmid and the bottom bands to the closed, negatively supercoiled plasmid. A, lanes: 1 and 10, $r_b = 0$; 2, $r_b = 0.008$; 3, $r_b = 0.01$; 4, $r_b = 0.02$; 5, $r_b = 0.04$; 6, $r_b = 0.06$; 7, $r_b = 0.08$; 8, $r_b = 0.09$; 9, $r_b = 0.11$. B, lanes: 1 and 14, $r_b = 0$; 2, $r_b = 0.0008$; 3, $r_b = 0.002$; 4, $r_b = 0.004$; 5, $r_b = 0.0064$; 6, $r_b = 0.008$; 7, $r_b = 0.02$; 8, $r_b = 0.04$; 9, $r_b = 0.064$; 10, $r_b = 0.08$; 11, $r_b = 0.2$; 12, $r_b = 0.4$; 13, $r_b = 0.64$.

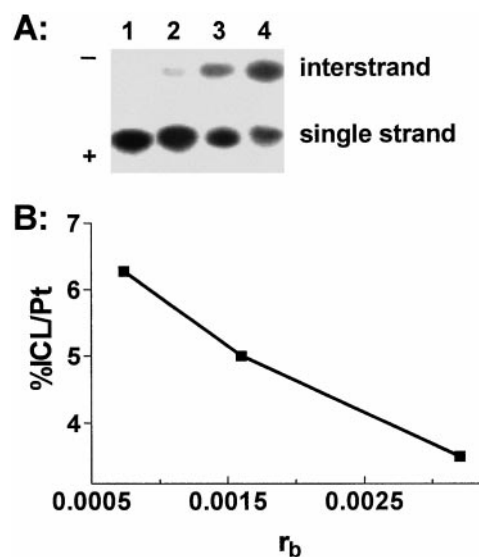


Fig. 9. The formation of ICLs by Com1 in the pSP73 plasmid linearized by *EcoRI*. A, autoradiogram of a denaturing 1% agarose gel of DNA which was $3'$ -end labeled. The interstrand cross-linked DNA appears as the top bands migrating on the gel more slowly than the single-stranded DNA (contained in the bottom bands). DNA was incubated with Com1 for 48 h at 37°C . r_b values: lane 1, 0 ; lane 2, 0.0007 ; lane 3, 0.0016 ; lane 4, 0.0032 . B, dependence on r_b of the number of ICLs per adduct ($\% \text{ICL/Pt}$). The ratio of ICLs to total platinum bound was calculated as described previously (Farrell et al., 1990; Brabec and Leng, 1993); $\% \text{ICL/Pt}$ was then calculated by multiplying this ratio by 100 . Data points measured in triplicate varied on average $\pm 3\%$ from their mean.

Com2 induces a very small DNA unwinding ($\ll 6^\circ$), which is consistent with a low DNA unwinding efficiency of monofunctional adducts of several platinum(II) compounds observed earlier (Keck and Lippard, 1992; Žaludová et al., 1997b; Balcarová et al., 1998).

Interstrand Cross-Linking. The amounts of ICLs formed by Com1 or Com2 in linear DNA were measured in pSP73 plasmid (2464 bp) that was first linearized by *EcoRI* (*EcoRI* cuts only once within pSP73 plasmid) and subsequently modified by Com1 or Com2 at various r_b . The samples were analyzed for the ICLs by agarose gel electrophoresis under denaturing conditions.

An electrophoretic method for precise and quantitative determination of interstrand cross-linking by platinum complexes in DNA was described previously (Farrell et al., 1990; Brabec and Leng, 1993). Upon electrophoresis under denaturing conditions, 3'-end labeled strands of linearized pSP73 plasmid containing no ICLs migrated as a 2464-base single strand, whereas the interstrand cross-linked strands migrated more slowly as a higher molecular mass species. The bands corresponding to more slowly migrating interstrand-cross-linked fragments were observed if Com1 was used to modify linearized DNA at r_b as low as 7×10^{-4} (shown in Fig. 9A, lane 2). The intensity of the more slowly migrating band increased with the growing level of the modification. The radioactivity associated with the individual bands in each lane was measured to obtain estimates of the fraction of noncross-linked or cross-linked DNA under each condition. The frequency of ICLs (the amount of ICLs per one molecule of the platinum complex bound to DNA) was calculated using the Poisson distribution from the fraction of noncross-linked DNA in combination with the r_b values and the fragment size (Farrell et al., 1990). Com2 showed no interstrand cross-linking efficiency even at a high r_b such as 0.1 (not shown).

On the other hand, Com1 formed in DNA ICLs with a similar efficiency as cisplatin (frequency of ICLs formed in DNA was small, 4–6%; Fig. 9B).

It has been demonstrated that the ICLs formed in DNA by some nonmetal-based anticancer drugs are destroyed at alkaline pH and/or elevated temperatures. It is, therefore, possible that the lability of the ICLs of Com1 or Com2 in alkaline medium used in gel electrophoresis under denaturing conditions (Fig. 9) could account for the negligible frequency of DNA ICLs of Com2 or could result in underestimation of the frequency of ICLs formed in DNA by Com1. Therefore, we have also used a milder procedure that has allowed determination of alkali-unstable DNA ICLs formed by several anthracyclines (Konopa, 1983; Cullinane and Phillips, 1994). These techniques are based on the denaturation of modified DNAs at significantly lower temperatures, in the presence of either formamide or dimethyl sulfoxide and subsequent analysis in native agarose gel. The milder DNA interstrand cross-linking assays gave, however, frequencies of the ICLs of Com1 or Com2 that are similar to those determined by means of the agarose gel electrophoresis in an alkaline medium. Thus, it appears unlikely that Com1 or Com2 form alkali-unstable ICLs in DNA.

Immunochemical Analysis. We prepared Ab_{cis} , which recognizes two neighboring purine residues of the same strand of DNA *cis* coordinated to the platinum atom of *cis*-[Pt(amine)₂]²⁺ (Sundquist et al., 1987; Vrána et al., 1992). They do not recognize monofunctional platinum adducts and the adducts of transplatin and its analogs.

Using competitive enzyme-linked immunosorbent assay, the inhibition of the binding of Ab_{cis} to their immunogens (double-stranded CT DNA modified by cisplatin at $r_b = 0.08$ for 48 h) by double-stranded DNA modified by Com1 or Com2 at various r_b values in the range of 0.005 to 0.1 was mea-

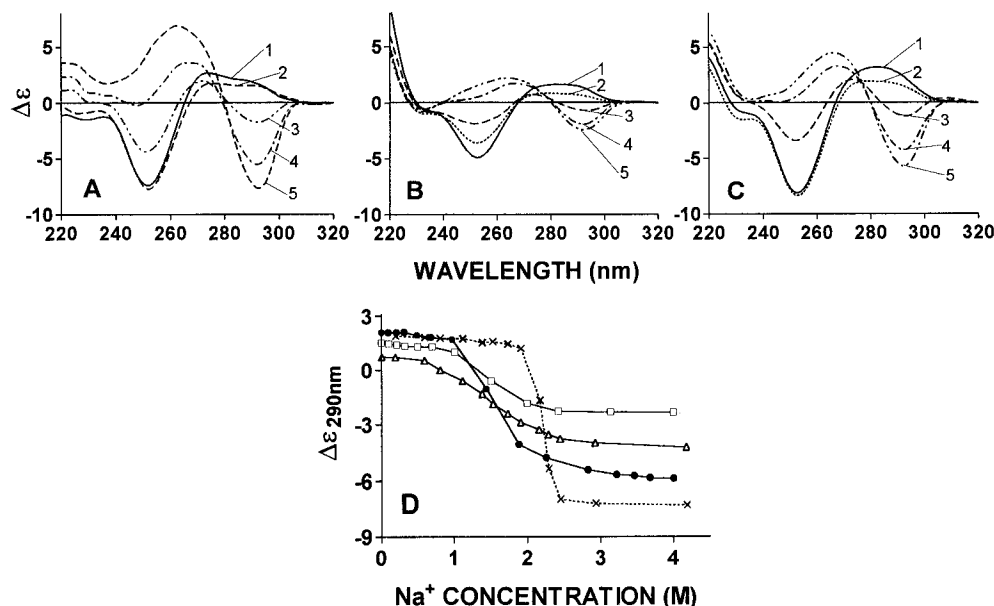


Fig. 10. CD spectroscopy of poly(dG-dC) nonmodified (A) or treated with Com1 (B) or Com2 (C) at $r_b = 0.1$. The polymer was modified when it was in B-form (in 10 mM $NaClO_4$) and was subsequently transferred into the media containing 10 mM $NaClO_4$ with 1 mM phosphate buffer, pH 7.5, plus 10 mM EDTA and various concentrations of NaCl. A, control, nonmodified polymer; curves: 1, 0.19 M NaCl; 2, 1.53 M NaCl; 3, 2.17 M NaCl; 4, 2.29 M NaCl; 5, 4.18 M NaCl. B, polymer modified by Com1; curves: 1, no NaCl added; 2, 1.01 M NaCl; 3, 1.51 M NaCl; 4, 2.00 M NaCl; 5, 3.14 M NaCl. C, polymer modified by Com2; curves: 1, no NaCl added; 2, 0.97 M NaCl; 3, 1.44 M NaCl; 4, 1.89 M NaCl; 5, 2.82 M NaCl. D, plot of the molar ellipticity at 290 nm ($\Delta\epsilon_{290\text{nm}}$) as a function of Na^+ concentration for poly(dG-dC) nonmodified (\square), Com1 (\bullet), and cisplatin at $r_b = 0.1$ (Δ). Data points measured in duplicate varied on average $\pm 1\%$ from their mean. Other details are described in the text.

sured. It was found (not shown) that double-stranded DNA modified by Com1 or Com2 did not inhibit the binding of the Ab_{cis}.*

Ab_{cis} exhibits an equally good specificity for DNA modified by several analogs of cisplatin having varied nonleaving amine groups (Sundquist et al., 1987; Vrána et al., 1992). The observation that Ab_{cis} did not recognize DNA modified by bifunctional Com1 is very likely related to distinct conformational alterations induced in DNA by Com1 in comparison with cisplatin.

B→Z Transition. The effect of various platinum compounds on the salt-induced B→Z transition in poly(dG-dC) has already been described in several papers (for instance, Ushay et al., 1982; Pérez-Martin et al., 1993; Žaludová et al., 1997a). In the present work, DNA modifications by Com1 or Com2 are compared with those by cisplatin or [PtCl(dien)]Cl, respectively. Cisplatin was found to facilitate the B→Z transition, but the resulting Z form was distorted and the transition cooperativity was considerably reduced. [PtCl(dien)]Cl has been shown to stabilize the Z-form of DNA; for [PtCl(dien)]Cl-treated poly(dG-dC), the Z conformation is observed at a considerably lower salt concentration than for the nontreated polymer, and the transition cooperativity is considerably reduced.

The effect of Com1 and Com2 binding on the B→Z transition in DNA was investigated in poly(dG-dC) during salt-induced transition from the right- to left-handed double helix. The transition was monitored by CD spectroscopy at a series of NaCl concentrations between 0 and 4 M added to the medium containing 10 mM NaClO₄, 1 mM phosphate buffer, pH 7.5 and 0.1 mM EDTA. All of our experiments were done at r_b of 0 or 0.1. The r_b value of 0.1 was chosen as a compromise between a low value, which would have some relevance to the therapeutic effects of the platinum compounds, and a larger value, which would cause more pronounced changes in the spectra.

The CD spectra of nonplatinated poly(dG-dC) at various concentrations of NaCl are shown in Fig. 10A. The figure shows the characteristic inversion in the CD spectrum on going from the right-handed B form to the left-handed Z form after addition of at least 2.0 M NaCl, and is used as the standard with which to compare the platinum-treated duplexes. The CD spectra at different NaCl concentrations of poly(dG-dC) pretreated with either Com1 or Com2 in 10 mM sodium perchlorate (i.e., the polymer was treated when it was in the B conformation) are shown in Fig. 10, B and C, respectively.

A plot of the molar ellipticity at 290 nm as a function of the concentration of NaCl added to 10 mM NaClO₄ with 0.1 mM EDTA plus 1 mM phosphate buffer, pH 7.5 (Fig. 10D) can be used to monitor the B→Z transition in poly(dG-dC) (Žaludová et al., 1997a). From an examination of these plots presented in Fig. 10D it is evident that the maximum variation in the ellipticity at 290 nm for the nonmodified (control) polymer and the polymers treated with Com1 or Com2 occurs over a more or less narrow range in salt concentration. Thus, the transitions appear to be cooperative. The efficiency of platinum complexes to inhibit or facilitate the B→Z transition in DNA can be evaluated by means of the comparison of the midpoints in salt-induced transitions of control and modified polymers. The transition midpoint for control poly(dG-dC) occurs at 2.3 M whereas the transition midpoints of poly(dG-

dC) modified by Com1 or Com2 were at ~1.6 M NaCl (Fig. 10D).

The results of the present work indicate that Com1 and Com2 facilitate the B→Z transition in DNA, but the resulting Z forms are distorted. Com1 distorts the Z-DNA more extensively than Com2. Com1 and Com2 affect B→Z transition differently, which implicates a different mode of their binding to DNA. In addition, the different effects of Com1 or Com2 on the B→Z transition in comparison with the effects of cisplatin (Fig. 10D) and [PtCl(dien)]Cl are consistent with unique DNA binding modes of the new aminophosphine platinum(II) complexes, as potent drugs with antitumor efficacy different from that of cisplatin.

Conclusions. In broad terms, we have demonstrated that the DNA binding mode, and very likely also the mechanism of antitumor activity of the new class of platinum(II) aminophosphine complexes, is different from that of cisplatin. This difference reflects the distinct nature of nonleaving ligands. The replacement of amine ligands in *cis*-dichloroplatinum(II) complexes by phosphine groups has been shown to influence considerably the selectivity for the adducts containing adenine and guanine residues and for monodentate and bidentate lesions. In addition, conformational alterations induced in DNA by Com1 are different from those induced by cisplatin. The different conformational changes and the increased bulk of the lesions are consistent with a role of these factors in the processing of DNA platinated by Com1. In particular, Com1 is more efficient than cisplatin in blocking DNA synthesis. A specific role of aminophosphine ligands in the modification of DNA by Com1 is also evident from the observation that DNA platinated by Com1 is not recognized by the antibodies elicited against DNA modified by cisplatin despite an equally good specificity of these antibodies for DNA modified by several analogs of cisplatin having varied nonleaving amine groups. A further processing of platinum(II) adducts by cellular components has been suggested to play an important role in the mechanism underlying antitumor activity of platinum compounds. The different cellular processing of DNA modified by *cis*-dichloroplatinum(II) complexes containing on the one hand nonleaving amine groups and aminophosphine ligands on the other might be relevant to the different biological activity of these two classes of platinum compounds. Structural studies of site-specific DNA adducts with aminophosphine platinum(II) complexes should provide a basis for analyzing and re-evaluating the structure-pharmacological activity relationships of platinum compounds. Studies toward this end are in progress, from which may ultimately arise a rational basis for design of a novel class of platinum antitumor drugs.

References

- Balcarová Z, Kašpárková J, Žáková A, Nováková O, Sivo MF, Natile G and Brabec V (1998) DNA interactions of a novel platinum drug, *cis*-[PtCl(NH₃)₂(N7-acyclovir)]⁺. *Mol Pharmacol* **53**:846–855.
- Bancroft DP, Lepre CA and Lippard SJ (1990) Pt-195 NMR kinetic and mechanistic studies of *cis*-diamminedichloroplatinum and *trans*-diamminedichloroplatinum(II) binding to DNA. *J Am Chem Soc* **112**:6860–6871.
- Bellon SF, Coleman JH and Lippard SJ (1991) DNA unwinding produced by site-specific intrastrand cross-links of the antitumor drug *cis*-diamminedichloroplatinum(II). *Biochemistry* **30**:8026–8035.
- Berners-Price SJ, Bowen J, McKeage MJ, Galetti P, Ding L, Baguley C and Brower W (1997) Selective antitumor activity of metal complexes of bidentate pyridylphosphines. *J Inorg Biochem* **67**:154.
- Berners-Price SJ and Sadler PJ (1988) Phosphines and metal phosphine complexes: Relationship of chemistry to anticancer and other biological activity. *Struct Bonding (Berlin)* **70**:27–102.
- Brabec V, Kleinwächter V, Butour JL and Johnson NP (1990) Biophysical studies of

- the modification of DNA by antitumour platinum coordination complexes. *Biophys Chem* **35**:129–141.
- Brabec V and Leng M (1993) DNA interstrand cross-links of *trans*-diamminedichloroplatinum(II) are preferentially formed between guanine and complementary cytosine residues. *Proc Natl Acad Sci USA* **90**:5345–5349.
- Brabec V and Paleček E (1970) The influence of salts and pH on polarographic currents produced by denatured DNA. *Biophysik* **6**:290–300.
- Butour JL and Macquet JP (1977) Differentiation of DNA-platinum complexes by fluorescence. The use of an intercalating dye as a probe. *Eur J Biochem* **78**:455–463.
- Chen C, Kilkuskie R and Hanlon S (1981) Circular dichroism spectral properties of covalent complexes of deoxyribonucleic acid and *n*-butylamine. *Biochemistry* **20**:4987–4995.
- Comess KM, Burstyn JN, Essigmann JM and Lippard SJ (1992) Replication inhibition and translation synthesis on templates containing site-specifically placed *cis*-diamminedichloroplatinum(II) DNA adducts. *Biochemistry* **31**:3975–3990.
- Cullinane C and Phillips DR (1994) Sequence specificity of (cyanomorpholino)adriamycin adducts in human cells. *Biochemistry* **33**:6207–6212.
- Eastman A (1987) The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol Ther* **34**:155–166.
- Farrell N, Qu Y, Feng L and Van Houten B (1990) Comparison of chemical reactivity, cytotoxicity, interstrand cross-linking and DNA sequence specificity of bis(platinum) complexes containing monodentate or bidentate coordination spheres with their monomeric analogues. *Biochemistry* **29**:9522–9531.
- Fichtinger-Schepman AMJ, Van der Veer JL, Den Hartog JHJ, Lohman PHM and Reedijk J (1985) Adducts of the antitumor drug *cis*-diamminedichloroplatinum(II) with DNA: Formation, identification, and quantitation. *Biochemistry* **24**:707–713.
- Gelasco A and Lippard SJ (1998) NMR solution structure of a DNA dodecamer duplex containing a *cis*-diammineplatinum(II) d(GpG) intrastrand cross-link, the major adduct of the anticancer drug cisplatin. *Biochemistry* **37**:9230–9239.
- Gray DM (1996) Circular dichroism of protein-nucleic acid interactions, in *Circular Dichroism and The Conformational Analysis of Biomolecules* (Fasman GD ed) pp 469–500, Plenum Press, New York.
- Habtemariam A and Sadler P (1996) Design of chelate ring-opening platinum anticancer complexes: Reversible binding to guanine. *Chem Commun* 1785–1786.
- Huang HF, Zhu LM, Reid BR, Drobny GP and Hopkins PB (1995) Solution structure of a cisplatin-induced DNA interstrand cross-link. *Science (Wash DC)* **270**:1842–1845.
- Johnson JW (1996) Determination of the conformation of nucleic acids by electronic CD, in *Circular Dichroism and The Conformational Analysis of Biomolecules* (Fasman GD ed) pp 433–468, Plenum Press, New York.
- Johnson NP, Butour J-L, Villani G, Wimmer FL, Defais M, Pierson V and Brabec V (1989) Metal antitumor compounds: The mechanism of action of platinum complexes. *Prog Clin Biochem Med* **10**:1–24.
- Johnson NP, Macquet JP, Wiebers JL and Monsarrat B (1982) Structures of adducts formed between [Pt(dien)Cl]Cl and DNA in vitro. *Nucleic Acids Res* **10**:5255–5271.
- Keck MV and Lippard SJ (1992) Unwinding of supercoiled DNA by platinum ethidium and related complexes. *J Am Chem Soc* **114**:3386–3390.
- Kim SD, Vrána O, Kleinwächter V, Niki K and Brabec V (1990) Polarographic determination of subnanogram quantities of free platinum in reaction mixture with DNA. *Anal Lett* **23**:1505–1518.
- Konopa J (1983) Adriamycin and daunomycin induce interstrand DNA cross-links in HeLa S3 cells. *Biochem Biophys Res Commun* **110**:819–826.
- Margiotta N, Habtemariam A and Sadler PJ (1997) Strong, rapid binding of a platinum complex to thymine and uracil under physiological conditions. *Angew Chem Int Ed Engl* **36**:1185–1187.
- Murray V, Motyka H, England PR, Wickham G, Lee HH, Denny WA and McFadyen WD (1992) The use of Taq DNA polymerase to determine the sequence specificity of DNA damage caused by *cis*-diamminedichloroplatinum(II), acridine-tethered platinum(II) diammine complexes or 2 analogues. *J Biol Chem* **267**:18805–18809.
- Nováková O, Kašpárková J, Vrána O, vanVliet PM, Reedijk J and Brabec V (1995) Correlation between cytotoxicity and DNA binding of polypyridyl ruthenium complexes. *Biochemistry* **34**:12369–12378.
- Papathanasiou P, Salem G, Waring P and Willis AC (1997) Synthesis of gold(I), silver(I) and copper(I) complexes containing substituted (2-aminophenyl)phosphines. Molecular structure of [AuI(2-H₂NC₆H₄PPhe)], [AuI((±)-2H₂NC₆H₄PMePh)] and (±)-[Cu(2-H₂NC₆H₄PPh₂)₂]PF₆. *J Chem Soc Dalton Trans* 3435–3443.
- Pérez-Martin JM, Requena JM, Craciunescu D, López MC and Alonso C (1993) The anti Z-DNA reactivity of Z-DNA forming sequences is affected by platinum antitumor drugs. *J Biol Chem* **268**:24774–24779.
- Reeder F, Guo ZJ, Murdoch PD, Corazza A, Hambley TW, BernersPrice SJ, Chottard JC and Sadler PJ (1997) Platination of a GG site on single-stranded and double-stranded forms of a 14-base oligonucleotide with diaqua cisplatin followed by NMR and HPLC—Influence of the platinum ligands and base sequence on 5'-G versus 3'-G platination selectivity. *Eur J Biochem* **249**:370–382.
- Reedijk J (1996) Improved understanding in platinum antitumor chemistry. *Chem Commun* 801–806.
- Sundquist WI, Lippard SJ and Stollar BD (1987) Monoclonal antibodies to DNA modified with *cis*- or *trans*-diamminedichloroplatinum(II). *Proc Natl Acad Sci USA* **84**:8225–8229.
- Ushay HM, Santella RM, Caradonna JP, Grunberger D and Lippard SJ (1982) Binding of [(dien)PtCl]Cl to poly(dG-dC).poly(dG-dC) facilitates the B→Z conformational transition. *Nucleic Acids Res* **10**:3573–3588.
- Vrána O, Boudný V and Brabec V (1996) Superhelical torsion controls DNA interstrand cross-linking by antitumor *cis*-diamminedichloroplatinum(II). *Nucleic Acids Res* **24**:3918–3925.
- Vrána O, Brabec V and Kleinwächter V (1986) Polarographic studies on the conformation of some platinum complexes: Relations to antitumor activity. *Anti-Cancer Drug Des* **1**:95–109.
- Vrána O, Kiseleva VI, Poverenny AM and Brabec V (1992) Conversion of DNA adducts of antitumor *cis*-diamminedichloroplatinum(II)-immunochemical analysis. *Eur J Pharmacol* **226**:5–13.
- Žáková A, Nováková O, Balcarová Z, Bierbach U, Farrell N and Brabec V (1998) DNA interactions of antitumor *trans*-[PtCl₂(NH₃)(quinoline)]. *Eur J Biochem* **254**:547–557.
- Žaludová R, Natile G and Brabec V (1997a) The effect of antitumor *trans*-[PtCl₂(E-iminoether)₂] on B→Z transition in DNA. *Anticancer Drug Des* **12**:295–309.
- Žaludová R, Žáková A, Kašpárková J, Balcarová Z, Vrána O, Coluccia M, Natile G and Brabec V (1997b) DNA modifications by antitumor *trans*-[PtCl₂(E-iminoether)₂]. *Mol Pharmacol* **52**:354–361.

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