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Modification of the Recognition of Restriction Sites of Plasmid DNA by the Antitumor Drug Pt-Pentamidine

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ABSTRACT. The rate of binding of the antineoplastic drugs Pt-pentamidine $[(cis\text{-}PtCl_2)_3(pentamidine)_3][PtCl_4]_2$ and cis-DDP [cis-diamminedichloroplatimum(II)] to pUC8 DNA, as well as the effect of the binding of these platinum compounds on the cutting effectiveness of Bam HI, Hind III, and Sal I restriction endonucleases, were determined by flameless atomic absorption spectroscopy and gel electrophoresis, respectively. The results show that covalent DNA platination is 12% to 22% lower in DNA:Pt-pentamidine complexes than in DNA:cis-DDP at the same molar rate of platinum/nucleotide, and the number of Pt-pentamidine molecules bound to DNA is significantly lower in Pt-pentamidine:DNA complexes than in cis-DDP:DNA complexes. Although both compounds inhibit Bam HI cleavage of pUC8 DNA, Pt-pentamidine does not prevent the cutting activity of Hind III, in contrast with cis-DDP. Neither cis-DDP nor Pt-pentamidine inhibits the cutting activity of Sal I, whose recognition sequence neighbors the Bam HI and Hind III sites. BIOCHEM PHARMACOL 52;6: 851–856, 1996.

KEY WORDS. DNA interactions; Pt-pentamidine; cis-Pt(II)

The interaction of cis-DDP† and its analogs with DNA has been extensively studied as a consequence of the discovery of the DNA double helix as the main intracellular target of these drugs. This binding may explain the antitumor properties of the platinum-based drugs [1, 2, 3]. The most common adducts formed by cis-DDP on DNA are d(GpG) and d(ApG) intrastrand dimers, together with a low percentage of interstrand cross-links [4]. Because the DNA modification induced by the adducts inhibits the formation of particular restriction fragments [5], the analysis of the activity of specific endonucleases has been a very useful tool for the mapping of drug-DNA binding sites [6, 7, 8]. That the adducts formed do not inhibit the activity of the restriction enzymes and the absence of DNA cutting are only apparent due to the maintaining together of the resulting DNA restriction fragments through covalent drug-DNA intrastrand cross-links [9]; whether or not the local DNA distortion provoked by the adducts inhibits or slows down the cutting activity of the enzyme [10] is still an open question.

To determine the DNA binding sites of the cis-Pt(II) centers of the antineoplastic drug Pt-pentamidine [11, 12, 13], we have carried out restriction enzyme inhibition studies using the Bam HI and Hind III sites of pUC8 DNA. The Bam HI and Hind III enzymes were chosen because pUC8 DNA presents only one restriction site for each of the enzymes containing target sites for the cis-Pt(II) centers [4], such as GG or AG base pairs, respectively.

MATERIALS AND METHODS

Isolation of pUC8 Plasmid and Drug Solutions

pUC8 plasmid was isolated from the *Escherichia coli* strain JM83 according to the alkaline lysis method [14]. Cis-DDP was supplied by Bristol-Myers (Spain). Pt-pentamidine was synthesized as described previously [11].

The platinum drugs were dissolved in 2.5% DMSO in water. Pentamidine isethionate (Aldrich, Spain) was prepared in water. The stock solutions (1 mg/mL) were stored in the dark at room temperature until use.

Quantification of Platinum Binding to DNA

pUC8 DNA samples (4 μg/mL) were incubated with the platinum drugs in TE buffer (10 mM Tris · HCl, pH 7.4, EDTA 0.1 mM) at several r_i. The incubations were carried out in the dark for 24 hr at 37°C. DNA samples were

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† *Abbreviations:* cis-DDP, cis-diamminedichloroplatimum(II); Pt-pentamidine, $[(cis\text{-}PtCl_2)_3(pentamidine)_3][PtCl_4]_2$; K_2PtCl_4, potassium tetrachloroplatinate; FAAS, flameless atomic absorption spectroscopy; oc, open circular plasmid DNA form; ccc, covalently closed circular plasmid DNA form; r_i, input molar ratio of platinum to nucleotide; d_i, input molar ratio of drug to nucleotide; r_b, molar ratio of platinum bound to nucleotide; d_b, molar ratio of drug to nucleotide.

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TABLE 1. Molar ratio values of drug bound to nucleotide obtained in pUC 8 DNA modified by cis-DPP and Pt-pentamidine at several input molar ratios of drug to nucleotide

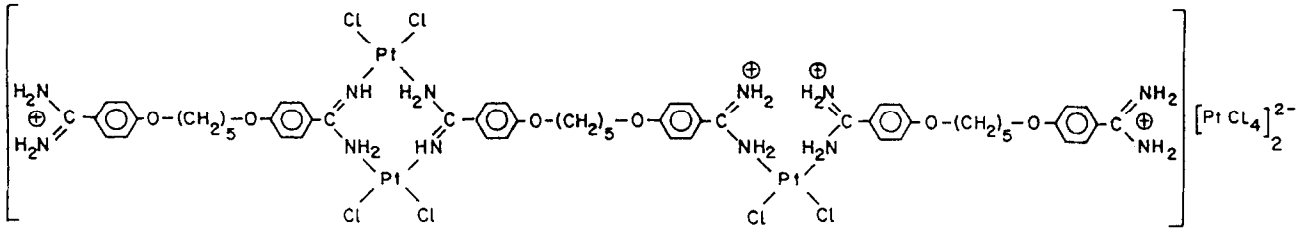
Compound	r_i	r_b	r_b/r_i (%)	d_i	d_b	d_b/d_i (%)
cis-DDP	0.05	0.049 ± 0.002	98	0.05	0.049 ± 0.002	98
	0.15	0.135 ± 0.006	90	0.15	0.135 ± 0.006	90
	0.25	0.200 ± 0.010	80	0.25	0.200 ± 0.010	80
	0.50	0.400 ± 0.017	80	0.50	0.400 ± 0.017	80
Pt-pent.	0.05	0.038 ± 0.003	76	0.016	0.013 ± 0.001	81
	0.15	0.105 ± 0.005	70	0.050	0.035 ± 0.002	70
	0.25	0.170 ± 0.008	68	0.083	0.057 ± 0.003	71
	0.50	0.360 ± 0.015	68	0.166	0.113 ± 0.005	71

pUC8 DNA samples were incubated for 24 hr at 37°C with the platinum drugs in TE buffer at several r_i. DNA samples were concentrated by evaporation and subsequently digested in HCl + HNO_3. Then, the samples were evaporated again and, finally, resuspended in 10% HCl in water. The platinum content of the drug:DNA complexes was measured at 265.9 nm by FAAS. The data represent the mean values from 3 independent experiments. r_i = input molar ratio of platinum to nucleotide; d_i = input molar ratio of drug to nucleotide; r_b = molar ratio of platinum bound to nucleotide; d_b = molar ratio of drug to nucleotide.

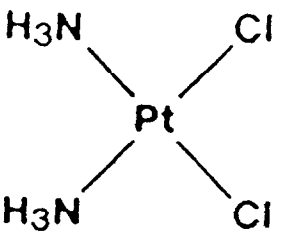
concentrated by evaporation in a speed vacuum concentrator (SAVANT SVH 100H) and, subsequently, digested in 3 volumes of HCl + 1 volume of HNO_3. The samples were evaporated again and finally resuspended in 10 mL of 10% HCl in water. The platinum content of the drug:DNA complexes was measured at 265.9 nm by FAAS (Perkin Elmer 372 AAS and HGA programmer). Calibration was carried out with standard solutions of K_2PtCl_4. The results were expressed as platinum atoms bound per nucleotide (r_b), with the mean molecular weight taken as 330 per nucleotide. Each value represents the mean obtained from 3 independent experiments.

Digestion of pUC8 with Bam HI, Hind III, and Sal I Restriction Endonucleases

Aliquots containing 1 μg of DNA were incubated with the compounds in TE buffer (pH 7.4) at different r_i values for 24 hr at 37°C. Drug:DNA complexes were ethanol-precipitated and resuspended in 25 μL of restriction buffer



Pt-pentamidine complex



cis-DDP

FIG. 1. Molecular structure of Pt-pentamidine complex and cis-DDP.

(10 mM Tris · HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM 2-mercaptoethanol). The drug:DNA complexes were digested with Bam HI, Hind III, or Sal I until completion. The DNA was extracted by phenol-chloroform, precipitated with ethanol, resuspended in 20 μL of TE buffer and subjected to 1.5% agarose gel electrophoresis for 16 hr at 25 V in TAE buffer (40 mM Tris-acetate and 2 mM EDTA, pH 8.0). DNA was stained with ethidium bromide (0.5 μg/mL) and the gels photographed under UV light. Densitometry of cut vs uncut DNA fractions was performed to estimate enzymatic activity using a Molecular Dynamics 300 A densitometer. All experiments were repeated 3 times.

RESULTS

Pt-pentamidine and cis-DDP Binding to pUC8 DNA

Because the inhibition of restriction cutting is determined by the amount of DNA:drug adducts formed, we calculated the r_b after incubation of pUC8 DNA with cis-DDP and Pt-pentamidine (Table 1). An almost linear correlation between the r_i and r_b was observed for both drugs. Although the amount of platinum bound to DNA was 12–22% lower in Pt-pentamidine:DNA than in cis-DDP:DNA complexes, the number of drug molecules bound was significantly lower in Pt-pentamidine:DNA complexes than in cis-DDP:DNA complexes, because there were 3 cis-Pt(II) centers in Pt-pentamidine in contrast to 1 in cis-DDP (Fig. 1). Thus, it would be expected that the number of bound Pt-pentamidine molecules needed to inhibit the activity of a particular restriction enzyme would be approximately 4-fold higher than that of cis-DDP (Table 1).

Inhibition of Bam HI Cleavage in Drug:pUC8 DNA Complexes

Figure 2 shows the results of the agarose gel electrophoresis of native and drug-modified DNA after Bam HI digestion (A) and the percentage of inhibition of enzyme cleavage versus the r_i (B). It was observed that binding of cis-DDP at an r_i of 0.05 only produced partial inhibition of the enzyme cleavage because oc and ccc DNA bands were detected, in addition to the pUC8 linear form (Fig. 2A, lane 3). A densitometric evaluation of the different DNA forms revealed that the cleavage was inhibited by 50% (Fig. 2B). At an r_i of 0.15, the inhibitory effect reached 89% because only a faint band corresponding to the linear form was observed (Fig. 2A, lane 4). At a cis-DDP r_i of 0.25 and higher, the

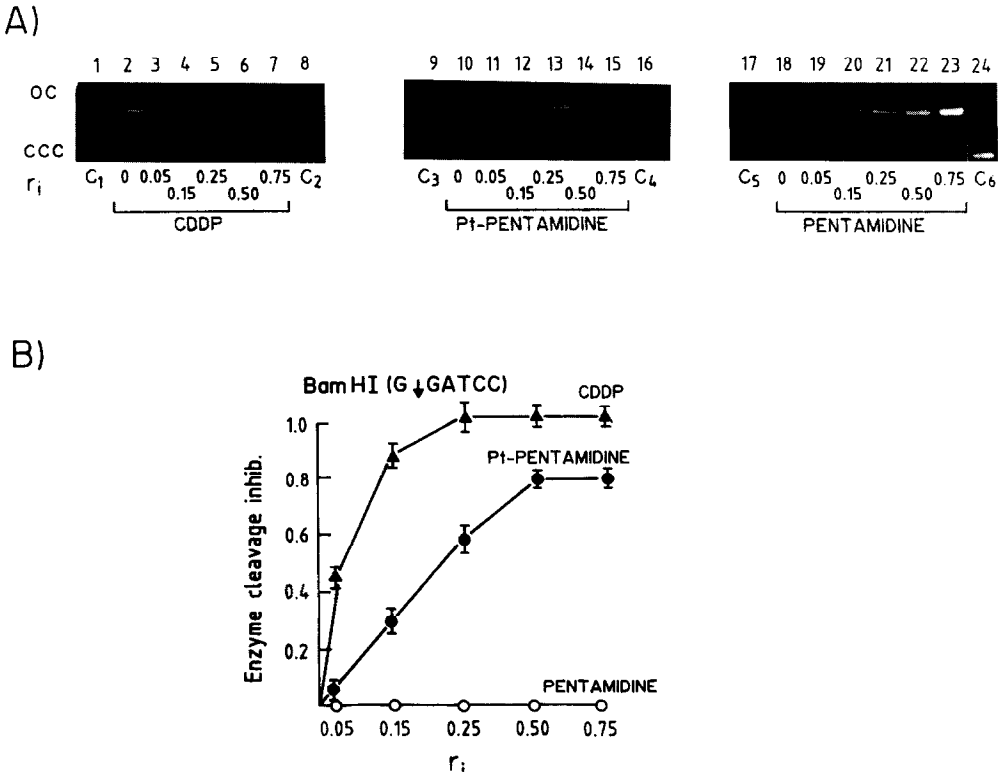


FIG. 2. (A) Electrophoresis in agarose gels following digestion with Bam HI of pUC8 DNA modified by cis-DDP (lanes 3 to 7), Pt-pentamidine (lanes 11 to 15), and pentamidine (lanes 19 to 23); r_i = 0.05, 0.15, 0.25, 0.50, and 0.75. Lanes 1, 9, and 17: native pUC8 DNA; lanes 2, 10, and 18: native pUC8 DNA after digestion with Bam HI; lanes 8, 16, and 24: pUC8 DNA incubated with the drugs at r_i = 0.75. (B) Inhibition of Bam HI cleavage of pUC8 DNA modified by cis-DDP (▲); Pt-pentamidine (●); and pentamidine (○) vs r_i. Inhibition was determined densitometrically by comparing the fractions of the uncut versus the cut plasmid. The symbols represent the mean value obtained from 3 independent experiments. The vertical bars indicate the SD.

inhibitory effect was 100% because only a single band, corresponding to both the oc form (with an increased mobility) and the ccc form (with a decreased mobility), slightly slower than that of the linear form, was detected (Fig. 2A, lanes 5 to 8). We observed that Pt-pentamidine at a r_i = 0.05 did not inhibit the cutting capacity of the enzyme (Fig. 2A, lane 11). Partial inhibition was detected at a Pt-pentamidine r_i of 0.15 and 0.25 (35% and 55%, respectively) (Fig. 2A, lanes 12 and 13). At a Pt-pentamidine r_i = 0.5, the inhibition of Bam HI cleavage reached a plateau of 80% (Fig. 2A, lanes 14 and 15 and Fig. 2B). The inhibition capacity of the drug was not due to binding of the ligand because pentamidine did not interfere with the cutting capacity of Bam HI at any of the r_i tested (Fig. 2A, lanes 19 to 23).

Inhibition of Hind III Cleavage in Drugs:pUC8 Complexes

Figure 3A shows the results of agarose gel electrophoresis after Hind III digestion of native or drug-modified pUC8 DNA. There was a gradual increase in the percentage of inhibition of Hind III cleavage with growing amounts of platinum bound to DNA after cis-DDP binding (lanes 3 to 7). From the data presented in Fig. 3B, it may be calculated that 50% of the inhibition was reached at a cis-DDP r_i of 0.1, increasing to 100% at the r_i of 0.25. Neither Pt-pentamidine nor pentamidine, however, produced inhibition of Hind III cleavage at any of the r_i tested (Fig. 3A, lanes 11 to 15 and 19 to 23, respectively). It is likely, then, that in contrast to the cis-Pt(II) centers of cis-DDP, the cis-Pt(II) centers of Pt-pentamidine do not form adducts within or near the Hind III restriction-site sequences.

Inhibition of Sal I Cleavage in Drug:DNA Complexes

To assess whether the inhibition of Bam HI or Hind III activity on platinated pUC8 DNA is restricted to sequences with which the drug may form adducts or if the inhibition also extends to neighboring DNA sequences, we carried out experiments with the Sal I restriction enzyme, whose cutting site is located 6 base pairs downstream of Bam HI and 12 base pairs upstream of Hind III. Because the Sal I target-site sequences do not contain GG or AG base pairs, it is not expected to form Pt-DNA bifunctional adducts with the cis-Pt(II) centers. It was observed that the binding of either cis-DDP (Fig. 4, lanes 3 to 7) or Pt-pentamidine (lanes 9 to 13) to pUC8 did not inhibit Sal I cleavage at any of the r_i

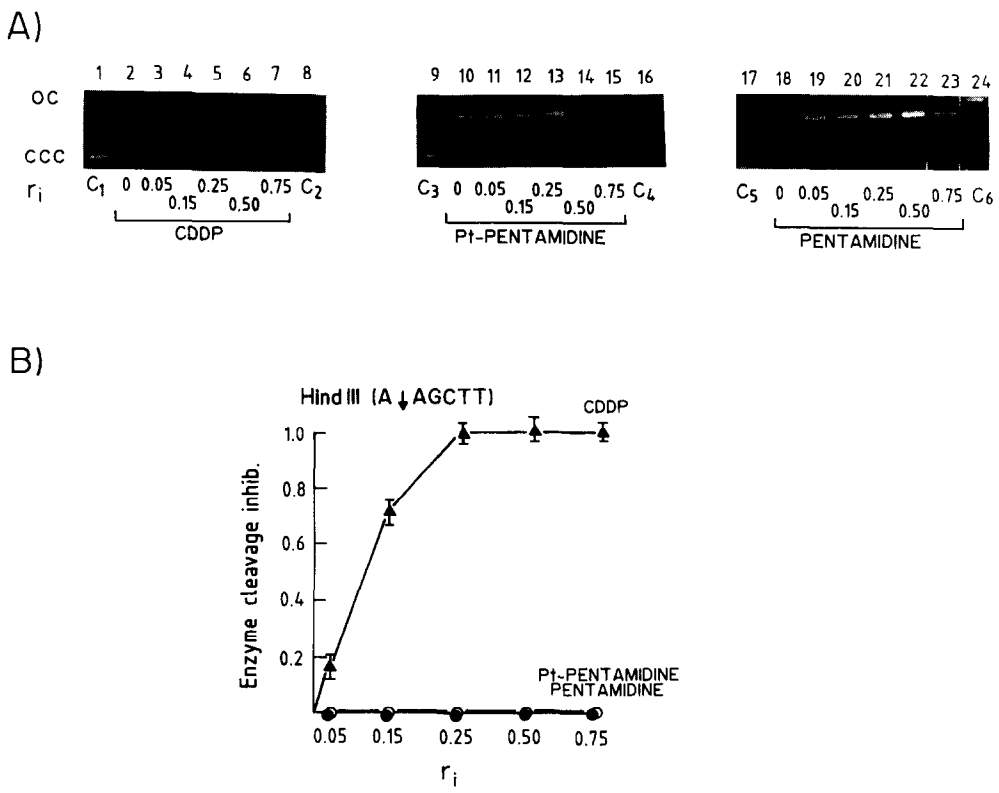


FIG. 3. (A) Electrophoresis in agarose gel following digestion with Hind III of pUC8 DNA modified by cis-DDP (lanes 3 to 7), Pt-pentamidine (lanes 11 to 15), and pentamidine (lanes 19 to 23); r_i = 0.05, 0.15, 0.25, 0.50, and 0.75. Lanes 1, 9, and 17: native pUC8 DNA; lanes 2, 10, and 18: native pUC8 DNA after digestion with Hind III; lanes 8, 16, and 24: pUC8 DNA incubated with the drugs at r_i = 0.75. (B) Inhibition of Hind III cleavage of pUC8 DNA modified by cis-DDP (▲), Pt-pentamidine (●), and pentamidine (○) vs r_i. Inhibition was determined densitometrically by comparing the fractions of the uncut versus the cut plasmid DNA. The symbols represent the mean value obtained from 3 independent experiments. The vertical bars indicate the SD.

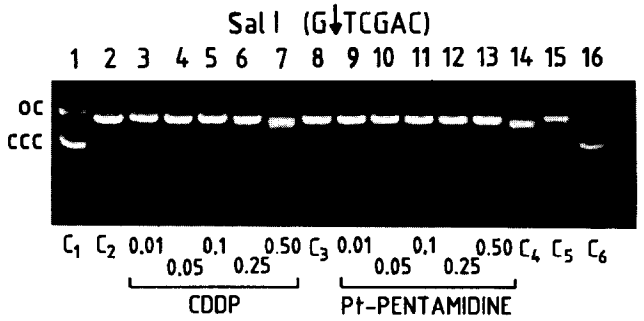


FIG. 4. Electrophoresis in agarose gel following digestion with Sal I of pUC8 DNA modified by cis-DDP (lanes 3 to 7) and Pt-pentamidine (lanes 9 to 13); r_i = 0.01, 0.05, 0.1, 0.25, and 0.50. Lane 1: native pUC8 DNA; lane 2: native pUC8 DNA after digestion with Sal I; lane 8: digestion by Sal I of pUC8 DNA modified by pentamidine (r_i = 0.50); lanes 14, 15, and 16: pUC8 DNA modified by cis-DDP, Pt-pentamidine and pentamidine at r_i = 0.50.

tested. These data suggest that the platinum adducts are formed at or near to the Bam HI and Hind III restriction sites, because they do not inhibit the cutting of neighboring restriction sites.

DISCUSSION

Although the data obtained by FAAS show that the amount of platinum bound to pUC8 DNA due to the cis-Pt(II) centers of Pt-pentamidine was only 12–22% lower than that due to cis-DDP, the number of molecules bound to the DNA was much lower, because Pt-pentamidine is an oligomer containing 3 cis-Pt(II) centers per molecule [11]. The results obtained herein, showing that increasing binding of cis-DDP and Pt-pentamidine to pUC8 DNA gradually prevents the formation of restriction fragments by Bam HI, suggest that both drugs may form intrastrand cross-links of the adjacent guanines located at the cutting site of the enzyme. Because the pUC8 plasmid also contains a $d(G)_3.d(C)_3$ stretch at 5′ in the Bam HI restriction site [15], a bifunctional binding of the cis-Pt(II) centers of Pt-pentamidine and cis-DDP to neighboring guanines may occur, inducing a conformational change capable of preventing localization of the recognition sequence by the enzyme [9]. However, it cannot be ruled out that monofunctional binding to guanine may also occur within or close to the restriction site because, as has previously been reported platination with $[Pt(Eten)_2(NH_3)_3Cl]Cl$, which cannot bind bifunctionally, inhibits DNA cleavage by Bam HI [7]. The data show, moreover, that Pt-pentamidine does not inhibit the cleavage of pUC8 DNA by Hind III, suggesting that the cis-Pt(II) centers of Pt-pentamidine do not bind either to the 5′A-A3′ base pair at the cutting site or to the 5′A-G3′ adjacent sequence. Because monofunctional binding of Pt-pentamidine to the guanine of the 5′A-G3′ base pair that flanks the restriction site of Hind III inhibits, to some extent, the enzyme activity [7], it seems that the cis-Pt(II) centers of Pt-pentamidine need the presence of adjacent guanines to bind to DNA. However, cis-DDP inhibits the cleavage of pUC8 DNA by Hind III, so the drug must bind to the AG pair located near the restriction sequence of Hind III. In fact, it has previously been reported that, although cis-DDP mainly forms intrastrand cross-links of adjacent guanines, the drug can also form this kind of adduct on AG pairs in which adenine is located at 5′ [16]. However, our data show that the concentration of cis-DDP needed to inhibit 50% of the cutting activity of Hind III is 2-fold higher than that needed to inhibit 50% of cutting activity of Bam HI (r_i = 0.10 vs r_i = 0.05) and that the inhibitory effect of the drugs must be due to binding within or near the restriction sites, because the Sal I restriction site was not affected. Thus, we believe that the pentamidine residues alter the potential DNA binding sites of the cis-Pt(II) centers of Pt-pentamidine relative to the cis-Pt(II) center of cis-DDP. These results are in agreement with Pt-pentamidine:DNA melting data, which indicated that 30% of total DNA in Pt-pentamidine:DNA complexes was not melted at 79°C, in contrast with that observed in cis-DDP:DNA complexes in which the melting temperature value decreased relative to native DNA [17]. Thus, we believe that novel platinum drugs may be designed in which the ligand restricts or confers base-specificity to the interaction of the cis-Pt(II) centers with DNA.

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References

1. Umapathy P, The chemical and biochemical consequences of the binding of the antitumour drug cisplatin and other platinum group metal complexes to DNA. *Coord Chem Rev* **95:** 129–181, 1989.
2. Newman PC, Williams DM, Cosstick R, Seela F and Conolly BA, Interaction of the EcoRV restriction endonuclease with the deoxyadenosine and thymidine bases in its recognition hexamer d(GATATC). *Biochemistry* **29:** 9902–9910, 1990.
3. Lippert B, From cisplatin to artificial nucleases—the role of metal ion-nucleic acid interactions in biology. *Biometals* **5:** 195–208, 1992.
4. Pinto AL, Lippard SJ, Binding of the antitumor drug cis-diamminedichloroplatinum (II) (cisplatin) to DNA. *Biochem Biophys Acta* **780:** 167–180, 1985.
5. Ushay HM, Tullius TD and Lippard SJ, Inhibition of the Bam HI cleavage and unwinding of pBR3222 deoxyribonucleic acid by the antitumour drug cis-dichlorodiammineplatinum (II). *Biochemistry* **21:** 3744–3748, 1981.
6. Cohen GL, Bauer WR, Barton JK and Lippard SJ, Binding of cis- and trans-dichlorodiammine platinum (II) to DNA: Evidence for unwinding and shortening of the double helix. *Science* **203:** 1014–1015, 1979.
7. Herman TS, Teicher BA, Chan V, Collins LS, Kaufman ME and Loh C, Effect of hyperthermia on the action of cis-diammine dichloroplatinum (II), (rhodamine 123)$_2$ tetrachloroplatinum (II) and potassium tetrachloroplati-

nate "in vitro" and "in vivo." *Cancer Res* **48:** 2335–2341, 1988.
8. Brabec V and Balcarova Z, Restriction-enzyme cleavage of DNA modified by platinum(II) complexes. *Eur J Biochem* **216:** 183–187, 1993.
9. Kelman AD and Buchbinder M, Platinum-DNA crosslinking: platinum antitumor drug interactions with native lambda bacteriophage DNA studies using a restriction endonuclease. *Biochimie* **60:** 893–899, 1978.
10. Cohen GL, Ledner JA, Bauer WR, Ushay HM, Caravana C and Lippard SJ, Sequence dependent binding of cis-diammine dichloroplatinum (II) to DNA. *J Am Chem Soc* **102:** 2487–2488, 1980.
11. Pérez JM, Navarro-Ranninger MC, Requena JM, Jiménez-Ruíz A, Craciunescu D, Parrondo E, López MC and Alonso C, DNA binding properties and antileukemic activity of a Pt-pentamidine complex. *Chem-Biol Interact* **77:** 341–355, 1991.
12. Pérez JM, Requena JM, Craciunescu D, López MC and Alonso C, The anti Z-DNA reactivity of Z-DNA forming sequences is affected by platinum antitumor drugs. *J Biol Chem* **268:** 24774–24778, 1993.
13. Pérez JM, Requena JM, Craciunescu D, Doadrio JC and Alonso C, Binding of Pt-pentamidine to nucleosomal DNA. Studies of the antiproliferative activity of the drug against human cancer cells. *Chem-Biol Interact* **89:** 61–72, 1993.
14. Maniatis T, Fritsch EF and Sambrook J, *Molecular Cloning, a laboratory manual*. Cold Spring Harbor Laboratory Press, New York, 1989.
15. Viera J and Messing J, The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19:** 259–268, 1982.
16. Eastman A, Reevaluation of interaction of cis-dichloro (ethylenediamine) platinum (II) with DNA. *Biochemistry* **25:** 3912–3915, 1986.
17. López MC, Ruíz LM, Craciunescu D, Doadrio A, Osuna A and Alonso C, Studies on the interaction between antitrypanosome cis-DDP analogs with DNA. *Chem-Biol Interact* **59:** 99–111, 1986.