Cleavage by Restriction Enzymes of DNA Modified with the Antitumour Drug *cis*-diamminedichloroplatinum(II)

Z. BALCAROVÁ, J. MRÁZEK, V. KLEINWÄCHTER and V. BRABEC*

Institute of Biophysics, Czechoslovak Academy of Sciences, Královopolská 135, 612 65 Brno, Czechoslovakia

Abstract. The effect of binding of an antitumour drug *cis*-diamminedichloroplatinum(II) (*cis*-$[Pt(NH_3)_2Cl_2]$) to DNA on cutting effectiveness of BamHI, EcoRI, and SalI restriction endonucleases was quantitatively determined. The platinum complex inhibits the cleavage of plasmid pHC624 DNA linearized by BglI restrictase. From the present results we conclude that the yield of restriction endonuclease cleavage is also lowered if the platinum complex is bound outside the recognition DNA sequence of these enzymes. We propose that the origin of platinum adducts on DNA outside the recognition sequence can decrease the yield of restriction enzyme cleavage via inducing a conformational perturbation in the recognition DNA sequence of these enzymes and also via inhibition of the linear diffusion of these enzymes on DNA.

Key words: Platinum complexes — Plasmid DNA — Restriction endonucleases — Gel electrophoresis

Introduction

The anticancer activity of platinum complexes is generally accepted to involve binding to DNA, a process about which there is substantial chemical and structural information (Eastman 1987; Reedijk 1987; Sherman and Lippard 1987; Johnson et al. 1989; Brabec et al. 1990; Lepre and Lippard 1990; Bellon et al. 1991). It has been shown that the processing of platinum - DNA adducts by cellular proteins is an important aspect of the molecular mechanism of antitumour activity of platinum coordination complexes (Bellon et al. 1991; Bruhn et al. 1992). It is therefore of considerable importance to learn how platinum - DNA adducts modulate their interaction with these proteins. The model system to investigate these interactions can also comprise restriction endonucleases and platinated DNA fragments containing the recognition sequence of these enzymes. In addition, the

* To whom correspodence should be addressed

inhibitory effect of physical and chemical agents on the cutting effectiveness of restriction endonucleases has been intensively investigated (e.g. Moore et al. 1987; Newman et al. 1990; Olsen et al. 1990; Williams et al. 1991; Sip et al. 1992). Thus, knowledge of the effect of platinum binding to DNA on its cleavage by restriction enzymes can contribute to a further understanding of how DNA can be protected from the enzyme-catalyzed hydrolysis.

DNA adducts of both antitumour active and inactive platinum complexes have been shown to inhibit the cleavage of DNA (Balcarová and Brabec 1992). These findings were interpreted to mean that no clear connection exists between the ability of a specific platinum – DNA adduct to inhibit restriction enzyme cleavage of DNA and antitumour effectiveness of platinum compounds.

In the present investigation we used the electrophoretic gel mobility shift methodology to directly measure the yield of cleavage of plasmid DNA modified by antitumour *cis*-$[Pt(NH_3)_2Cl_2]$ by three restriction enzymes. Statistical evaluation of these experiments reveals that platinum complexes can also inhibit cutting of DNA by restriction endonucleases if the complexes are bound outside the recognition DNA sequence of these enzymes.

Materials and Methods

Plasmid pHC624 DNA (2016 base pairs [bp]) (Fig. 1) was isolated as described in the literature (Boros et al. 1984). The platinum complexes were allowed to react with DNA after its previous linearization with BglI restrictase (which cleaves pHC624 DNA at a single site located 932, 942, and 926 bp from single and unique BamHI, EcoRI, and SalI recognition sequences in pHC624 DNA, respectively).

cis-$[Pt(NH_3)_2Cl_2]$ was kindly supplied by the Research Institute of Pure Chemicals, Lachema, Brno, restriction nucleases BamHI, BglI, EcoRI, and SalI were purchased from the Institute of Sera and Vaccines, Prague. The location of the recognition sequences of these enzymes on pHC624 DNA and the composition of BamHI, EcoRI, and SalI restriction sequences are shown in Fig. 1.

In a typical binding experiment the reaction between the platinum complex and pHC624 DNA was carried out by mixing equal volumes of linearized pHC624 DNA in 0.01 mol/l sodium perchlorate with a freshly prepared solution of the platinum compound in the same medium; the reaction mixture was incubated at 37 °C till equilibrium (typically for 48 hours). The concentration of pHC624 DNA in the reaction mixture was always 54 μg/ml while the concentration of *cis*-$[Pt(NH_3)_2Cl_2]$ varied to yield, after equilibration, a required r_{bp} value (r_{bp} is defined as the number of platinum atoms covalently bound per one base pair). Under these conditions *cis*-$[Pt(NH_3)_2Cl_2]$ was bound to DNA quantitatively so that no fraction of the platinum compound remained free in the solution, as revealed by a pulse polarographic assay (Kim et al. 1990).

Digestions with BamHI, BglI, EcoRI, and SalI were performed in the same way as described earlier (Ushay et al. 1981). The amount of enzyme used was that necessary to cut 2 μg of nonplatinated pHC624 DNA (linearized by BglI) in 1 hour at 37 °C. The DNA fragments were subjected to electrophoresis in agarose (1.0% gel) in 40 mmol/l Tris – acetate (pH 8.0) and 1 mmol/l EDTA medium at 25 °C for 16 hours with a potential drop

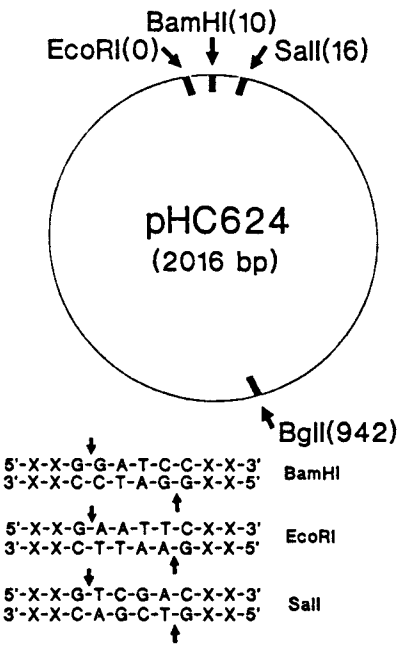

Figure 1. Map of plasmid pHC624 DNA showing cutting sites by BamHI, BglI, EcoRI, and SalI restriction endonucleases. The sequence of bases at each of the cutting sites is also shown.

of 0.8 V/cm. The gel was stained with ethidium bromide and samples were visualized and photographed in UV light. After development the film was scanned on a Vitatron MPS Type 940.800 scanning densitometer.

Results

BamHI, EcoRI, and SalI cleavage of DNA modified by cis-*diamminedichloroplatinum(II)*

The results of electrophoresis after BamHI, EcoRI, and SalI digestions of linearized pHC624 DNA modified by *cis*-$[Pt(NH_3)_2Cl_2]$ at different r_{bp} are presented in Fig. 2. At very low levels of *cis*-$[Pt(NH_3)_2Cl_2]$ binding ($r_{bp} < 0.001$) the cutting was complete with all DNA migrating as bands corresponding to two pHC624 DNA fragments. As the amount of *cis*-$[Pt(NH_3)_2Cl_2]$ bound to DNA increased, the yield of these fragments gradually decreased. At a level of *cis*-$[Pt(NH_3)_2Cl_2]$ binding corresponding to $r_{bp} = 0.2$, the two restriction fragments could no more be detected. The mobilities of the two linear fragments produced by BamHI, EcoRI, and SalI digestions were not changed with the increasing *cis*-$[Pt(NH_3)_2Cl_2]$ binding. On the other hand, the mobility of linearized pHC624 DNA increased slightly with the growing level of binding though only at $r_{bp} > 0.1$ (Fig. $2A - C$, channels 8 and 9). These results support the view that the binding of *cis*-$[Pt(NH_3)_2Cl_2]$ resulted in a shortening of linear DNA.

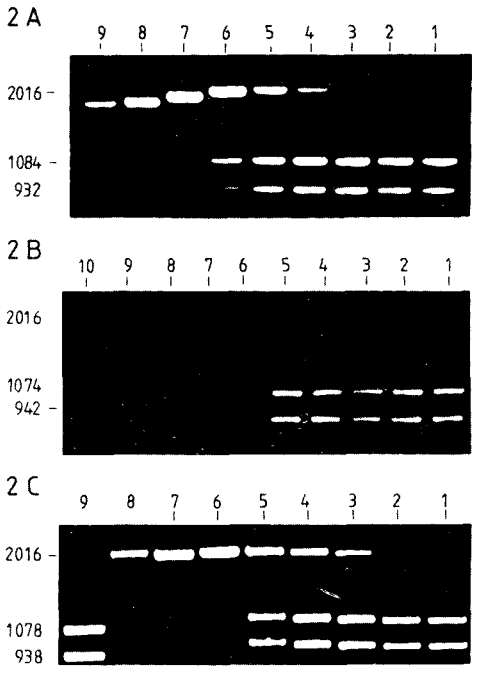

Figure 2. Electrophoresis in agarose gel following digestion with BamHI (*A*), EcoRI (*B*), and SalI (*C*) of pHC624 DNA (linearized by BglI) modified by *cis*-$[Pt(NH_3)_2Cl_2]$. (A) channel – r_{bp}: 1 – 0.0 (control), 2 – 0.001, 3 – 0.002, 4 – 0.005, 5 – 0.01, 6 – 0.02, 7 – 0.05, 8 – 0.1, 9 – 0.2. (B) channel – r_{bp}: 1 – 0.0 (control), 2 – 0.001, 3 – 0.002, 4 – 0.005, 5 – 0.01, 6 – 0.02, 7 – 0.05, 8 – 0.1, 9 – 0.2, 10 – 0.0 (control) (C) channel – r_{bp}: 1 – 0.001, 2 – 0.002, 3 – 0.005, 4 – 0.01, 5 – 0.02, 6 – 0.05, 7 – 0.1, 8 – 0.2, 9 – 0.0 (control)

We could demonstrate (Balcarová and Brabec 1992) that the inhibition of the enzymatic digestion is not due to platination of restriction endonucleases or a platinum-induced DNA – restrictase cross-link (Ushay et al. 1981).

Statistical analysis

Restriction endonucleases have stringent structural requirements on DNA recognition elements. In addition, platinum complexes can induce distortions of the DNA double helix not only in the base pairs to which a platinum complex is bound but also in some distance from this site (den Hartog et al 1984a,b, Reedijk 1987; Sherman and Lippard 1987; Marrot and Leng 1989; Brabec et al 1990; Lepre and Lippard 1990). In this way, even platinations outside the recognition sequence may be expected to induce a conformational perturbation in this sequence and thus inhibit the cleavage of DNA by the restriction enzyme. In other words, we assume that there exists a critical distance D of the nearest single platination site from the restriction site; all DNA molecules with at least one platinum adduct formed within this distance D from the restriction site are protected from cleavage, whereas all others undergo digestion.

In order to determine the distance D we evaluated the results of agarose electrophoresis in the following way: Let us define N_0 as the total number of pHC624

DNA molecules which enter the cleavage experiment, and N as the number of these molecules, which after the platination at r_{bp}, remain unmodified within the distance D from the restriction DNA site. Then, the ratio N/N_0 is determined from the sum of integrated densities of the lower two bands corresponding to the restriction fragments (Fig. $2A-C$) divided by the sum of the densities of all bands, and equals 1 at $r_{bp} = 0$.

Each potential platinum-binding site on a DNA molecule is defined by a unique serial number n according to its distance from the restriction cleavage site ($n = 1$ for the potential platinum-binding site nearest to the cleavage site). The probability k of a platinum complex being attached to a particular binding site on DNA is given by the expression

$$k = r_{bp}L/P$$

where L is the length of the DNA molecule ($L = 2016$ bp for pHC624 DNA) and P is the number of binding sites for the particular platinum compound borne by this DNA molecule. A simple consideration leads to the deduction of an expression for the probability w determining that at a particular r_{bp} there is no platinum bound to any of the binding sites within a segment containing binding sites with serial numbers lower than or equal to n,

$$w = (1-k)^n.$$

N/N_0 has been defined as a fraction of binding sites that remain unplatinated within a distance D from the cleavage site. This fraction is, however, determined by the probability w so that in this case the ratio N/N_0 equals the probability w. The expression for n defining the distance D is thus given by

$$n = \log(N/N_0)/\log(1-k).$$

It is apparent that the critical distance D equals the distance of the platinum-binding site of the serial number n.

Table 1 summarizes the results of these calculations for which the input data were taken from our electrophoretic measurements (Fig. $2A-C$). d(GG) dinucleotide units were taken as the binding sites for *cis*-$[Pt(NH_3)_2Cl_2]$ (Table 2) (Fichtinger-Schepman et al. 1985; Eastman 1986). The probability of a platinum complex being attached to its particular binding site on pHC624 DNA is assumed to be the same for all these sites. Thus, P of 241 was taken for *cis*-$[Pt(NH_3)_2Cl_2]$ (there are 241 d(GG) sites in both strands of pHC624 DNA). If BamHI cleavage of pHC624 DNA is investigated the modification of DNA by *cis*-$[Pt(NH_3)_2Cl_2]$ results in a systematic reduction of distance D with the growing r_{bp} value. The same is also observed for DNA modified with *cis*-$[Pt(NH_3)_2Cl_2]$ and digested by EcoRI and SalI restriction endonucleases (Table 1).

Table 1. Inhibition of cleavage by restriction endonucleases of pHC624 DNA modified with *cis*-$[Pt(NH_3)_2Cl_2]$ at various r_{bp}^a

A: BamHI

r_{bp}	N/N_0	$\frac{\log(N/N_0)}{\log(1-k)}$	n	D^b
0.002	0.88	7.55	8	26
0.004	0.84	5.30	5	3
0.010	0.74	3.52	4	1
0.020	0.58	2.93	3	0[c]
0.040	0.47	1.85	2	0[c]
0.100	0.16	1.03	1	0[c]

B: Summary of D^b values for three restriction endonucleases

r_{bp}	BamHI	EcoRI	SalI
0.002	26	16	10
0.004	3	5	7
0.010	1	3	5
0.020	0[c]	1	1
0.040	0[c]	1	1
0.100	0[c]	0[c]	0[c]

[a] See text for abbreviations and acronyms.

[b] The distance from the outer base pair of the restriction enzyme recognition sequence of the platinum-binding site in base pairs corresponding to the value of n.

[c] Zero in this column indicates that the corresponding platinum-binding site is within the restriction enzyme recognition sequence.

Table 2. Potential binding sites for *cis*-$[Pt(NH_3)_2Cl_2]$ in the vicinity of the recognition sequences in pHC624 DNA of the restriction endonucleases used in this work. The recognition sequences are underlined. The platinum-binding sites are indicated by asterisks

BamHI

```
          * *   * * *       *
A A G A A T T C C C G G G G A T C C G T C G A C C T G C A G
```

EcoRI

```
                                * *   * * *       *
C G T C T T C A A G A A G A A T T C C C G G G G A T C C G T
```

SalI

```
    * *   * * *       *               *
T C C C G G G G A T C C G T C G A C C T G C A G A T C T C T
```

Table 3. Inhibition of the cleavage by BamHI restriction endonuclease of pHC624 DNA modified by *cis*-$[Pt(NH_3)_2Cl_2]$ at various r_{bp}. *cis*-$[Pt(NH_3)_2Cl_2]$ is assumed to bind with an equal probability to all base pairs in pHC624 DNA

r_{bp}	N/N_0	$\frac{\log(N/N_0)}{\log(1-k)}$	n	D^b
0.002	0.88	63.63	64	13
0.004	0.84	44.99	45	8
0.010	0.74	30.63	31	5
0.020	0.58	26.58	27	4
0.040	0.47	18.48	18	2
0.100	0.16	17.63	18	2

[a] See text for abbreviations and acronyms.

[b] The meaning of D is the same as in the Table 1.

Also, we performed calculations assuming that *cis*-$[Pt(NH_3)_2Cl_2]$ is bound to all base pairs in pHC624 DNA with an equal probability ($P = 2016$). This situation, of course, cannot occur in reality but represents an extreme case if further binding sites in DNA are to be taken into account. This attempt was undertaken to show that *cis*-$[Pt(NH_3)_2Cl_2]$ being able to also form minor adducts at sequences different from d(GG) has no effect on the qualitative conclusion that distance D decreases with the growing r_{bp}. Distance D is calculated using the relation $D = n/4-3$ (D is measured from the edge of the recognition sequence but n is calculated from its center). The values of distance D calculated for this extreme case are shown in Table 3. They differ from the values of D given in Table 1, but again, the decrease of D with the growing r_{bp} is evident.

Comparison with previous results

It has already been attempted to estimate D for DNA modified by *cis*-$[Pt(NH_3)_2Cl_2]$ and cleaved by BamHI restriction enzyme (Ushay et al. 1981). To substantiate our approach to the analysis of experimental data based on statistical calculations, the following facts should be considered. The approach used previously (Ushay et al. 1981) was based on the estimation of the minimum level of platination that induced complete restriction enzyme blockage. This minimum level of platination was determined from the disappearance of the electrophoretic bands corresponding to the restriction fragments. However, various distributions were obtained for platination of binding sites on identical molecules of plasmid DNA sample, and they obeyed statistical rules. Thus, even at very high (close to infinitely high) levels of platination, a fraction of DNA molecules remained unmodified. It is, therefore, evident that the determination of a platination level that would induce complete blockage of restriction enzyme action in all DNA molecules present in the sample is

principally impossible In addition, a circular DNA modified to different r_{bp} values in a kinetic experiment was used in the previous work (Ushay et al 1981) Thus, not only r_{bp} values but also the character of DNA adducts of *cis*-$[Pt(NH_3)_2Cl_2]$, their relative amounts, and superhelical density of DNA have been changed All these factors could have influenced the cutting effectiveness of BamHI restriction enzyme, and no attempt was undertaken to separate the effects of these single variables

In order to circumvent these problems in the present experiments, we used linearized pHC624 DNA modified by the platinum complex to different r_{bp} values using different concentrations of *cis*-$[Pt(NH_3)_2Cl_2]$ in reaction mixture with DNA This mixture had always been incubated until binding equilibrium was attained In addition, we used statistical analysis for the determination of D, this made it possible on one hand to overcome the problem of inadequateness of the estimation of D from a minimum level of platination that induces complete restriction enzyme blockage (Ushay et al 1981), and on the other hand to estimate D as a function of r_{bp}

Discussion

A statistical evaluation of our experimental data (Fig 2, Table 1) reveals that the distance from the restriction site that defines an area around this site in which platinum binding completely inhibits restriction enzyme cleavage, becomes reduced with growing r_{bp} This result is somewhat surprising since one would expect this distance to be independent of the level of DNA platination In addition, rather high values of D were obtained for low levels of DNA platination ($D = 10$ at $r_{bp} = 0\ 002$), which means that platinum adducts formed on DNA in the distance of one helix turn or perhaps a little more from the restriction site still block the cleavage Structural studies of lesions induced in DNA by a single platinum adduct have revealed that the distortions extend over only few base pairs from the platination site (den Hartog et al 1984a,b, Reedijk 1987, Sherman and Lippard 1987, Marrot and Leng 1989, Brabec et al 1990, Lepre and Lippard 1990) Hence, the high values of D obtained at low r_{bp} can hardly be explained on the basis of a mechanism that would only involve inhibition of cleavage due to a structural perturbation affecting the recognition sequence

Under conditions of our cleavage experiments (Table 1) a linear diffusion of restriction enzymes on DNA was involved in localizing the recognition sites (Balcarová and Brabec 1992) A linear diffusion model (Ehbrecht et al 1985, von Hippel and Berg 1989) assumes that a restriction endonuclease, after its nonspecific association with the DNA molecule, may slide along (or hop around) the DNA in a random fashion As it has been demonstrated in our recent paper (Balcarová and Brabec 1992) this process is inhibited at low levels of DNA platination

(r_{bp} = 0 01) Thus, platinum compounds bound to DNA could constitute a barrier across which the linear diffusion cannot occur

Let us first consider the case when the platinum adduct is formed on DNA at a distance from the restriction site equal to or smaller than the length of the DNA segment over which the conformational distortion induced by this single adduct extends The probability that this occurs increases with the growing r_{bp} Then, the inhibition is caused by the distortion in the recognition sequence so that a contribution from blocking the linear diffusion to the overall inhibition of cleavage need not be considered On the other hand, this contribution becomes significant if the platinum adduct is formed on DNA at a greater distance than in the previous case (it becomes the more significant the larger this distance) The probability of DNA platination occurring at a site more distant from the restriction site than the length of the DNA segment distorted by the formation of this single adduct increases with the decreasing r_{bp}

It is, therefore, reasonable to conclude that the inhibition of DNA cleavage by the binding of platinum complexes may presumably include inhibition of the linear diffusion of restriction enzymes along DNA in addition to the mechanism associated with a structural distortion of the recognition sequence The significance of this mechanism in the cleavage process becomes more apparent with the decreasing r_{bp}, which may explain the increase of distance D observed with the decreasing r_{bp} (Table 1)

References

Balcarová Z , Brabec V (1992) Inhibition of restriction enzyme cleavage of DNA modified by platinum complexes Eur J Biochem , (submitted)

Bellon S F , Coleman J H , Lippard S J (1991) DNA unwinding produced by site-specific intrastrand cross-links of the antitumor drug *cis*-diammineдichloroplatinum(II) Biochemistry USA **30,** 8026—8035

Boros I , Postfai G , Venetianer P (1984) High-copy-number derivatives of the plasmid cloning vector pBR322 Gene **30,** 257—260

Brabec V , Kleinwachter V , Butour J -L , Johnson N P (1990) Biophysical studies of the modification of DNA by antitumour platinum coordination complexes Biophys Chem **35,** 129—141

Bruhn S L , Pil P M , Essigmann J M , Housman D E , Lippard S J (1992) Isolation and characterization of human cDNA clones encoding a high mobility group box protein that recognizes structural distortions to DNA caused by binding of the anticancer agent cisplatin Proc Nat Acad Sci USA **89,** 2307—2311

den Hartog J H J , Altona C , van Boom J H , van der Marel G A , Haasnot C A G , Reedijk J (1984a) *cis*-Diammineдichloroplatinum(II) induced distortion in a double-helical DNA fragment J Amer Chem Soc **106,** 1528—1530

den Hartog J H J , Altona C , van Boom J H , Reedijk J (1984b) A phosphorus NMR study of the reaction of *cis* diammineдichloroplatinum(II) with a double-helical oligonucleotide and with DNA FEBS Lett **176,** 393—397

Eastman A. (1986): Reevaluation of interaction of *cis*-dichloro(ethylenediamine)platinum-(II) with DNA. Biochemistry USA **25**, 3912—3915

Eastman A. (1987): The formation, isolation, and characterization of DNA adducts produced by anticancer platinum complexes. Pharmacol. Ther. **34**, 155—166

Ehbrecht H.-J., Pingoud A., Urbanke C., Maass G., Gualerzi C. (1985): Linear diffusion of restriction endonucleases on DNA. J. Biol. Chem. **260**, 6160—6166

Fichtinger-Schepman A. M. J., van der Veer J. L., den Hartog J. H. J., Lohman P. H. M., Reedijk J. (1985): Adducts of the antitumor drug *cis*-diamminedichloroplatinum-(II) with DNA: Formation, identification and quantitation. Biochemistry USA **24**, 707—713

Johnson N. P., Butour J.-L., Villani G., Wimmer F. L., Defais M., Pierson V., Brabec V. (1989): Metal antitumor compounds: The mechanism of action of platinum complexes. Prog. Clin. Biochem. Med. **10**, 1—24

Kim S. D., Vrána O., Kleinwächter V., Niki K., Brabec V. (1990): Polarographic determination of subnanogram quantities of free platinum in reaction mixture with DNA. Anal. Lett. **23**, 1505—1518

Lepre C. A., Lippard S. J. (1990): Interaction of platinum antitumor compounds with DNA. In: Nucleic Acids and Molecular Biology 4 (Eds. F. Eckstein and D. M. J. Lilley) pp. 9—38, Springer Verlag, Berlin

Marrot L., Leng M. (1989): Chemical probes of the conformation of DNA modified by *cis*-diamminedichloroplatinum(II). Biochemistry USA **28**, 1454—1461

Moore S. P., McAleer M. A., Moss S. H. (1987): Restriction enzyme cleavage of UV-irradiated DNA: A comparison of far-UV and mid-UV wavelengths. Photochem. Photobiol. **45**, 253—263

Newman P. C., Williams R. Cosstick R., Seela F., Connolly B. A. (1990): Interaction of the EcoRV restriction endonuclease with the deoxyadenosine and thymidine bases in its recognition hexamer d(GATATC). Biochemistry USA **29**, 9902—9910

Olsen D. B., Kotzorek G., Eckstein F. (1990): Investigation of the inhibitory role of phosphorothioate internucleotidic linkages on the catalytic activity of the restriction endonuclease EcoRV. Biochemistry USA **29**, 9546—9551

Reedijk J. (1987): The mechanism of action of platinum antitumor drugs. Pure Appl. Chem. **59**, 181—192

Sherman S.E., Lippard S.J.(1987): Structural aspects of platinum anticancer drug interactions with DNA. Chem. Rev. **87**, 1153—1181

Sip M., Schwartz A., Vovelle F., Ptak M., Leng M. (1992): Distortions induced in DNA by cis-platinum interstrand adducts. Biochemistry USA **31**, 2508—2513

Ushay H. M., Tullius T. D., Lippard S. J. (1981): Inhibition of the BamHI cleavage and unwinding of pBR322 deoxyribonucleic acid by the antitumor drug *cis*-dichlorodiammineplatinum (II). Biochemistry USA **20**, 3744—3748

von Hippel P. H., Berg O. G. (1989): Facilitated target location in biological sytems. J. Biol. Chem. **264**, 675—678

Williams D. M., Benseler F., Eckstein F. (1991): Properties of 2′-fluorothymidine-containing oligonucleotides: Interaction with restriction endonuclease EcoRV. Biochemistry USA **30**, 4001—4009

Final version accepted October 26, 1992