Biophysical Chemistry, 35 (1990) 129–141
Elsevier

BIOCHE 01418

Biophysical studies of the modification of DNA by antitumour platinum coordination complexes

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Accepted 25 May 1989

cis-Diamminedichloroplatinum(II); *trans*-Diamminedichloroplatinum(II); Diethylenetriaminechloroplatinum(II) chloride; Antitumor activity; DNA-drug interaction

Cisplatin (*cis*-diamminedichloroplatinum(II)) is widely used in the treatment of various human tumours. A large body of experimental evidence indicates that the reaction of cisplatin with DNA is responsible for the cytostatic action of this drug. Several platinum-DNA adducts have been identified and their effect on the conformation of DNA has been investigated. Structural studies of platinum-DNA adducts now permit a reasonably good explanation of the biophysical properties of platinated DNA. Antitumour-active platinum compounds induce in DNA, at low levels of binding, local conformational alterations which have the character of non-denaturing distortions. It is likely that these changes occur in DNA due to the formation of intrastrand cross-links between two adjacent purine residues. On the other hand, the modification of DNA by antitumour-inactive complexes results in the formation of more severe local denaturation changes. Conformational alterations induced in DNA by antitumour-active platinum compounds may be reparable with greater difficulty than those induced by the inactive complexes. Alternatively, non-denaturation change induced in DNA by antitumour platinum drugs could represent more significant steric hindrance against DNA replication as compared with inactive complexes.

1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II), *cis*-DDP) is one of the most widely used anticancer drugs [1,2]. It is a very simple inorganic molecule consisting of only 11 atoms, six of which are hydrogens (fig. 1). Although cisplatin has so far been successfully applied in the treatment of various human tumours, the mechanism of its antitumour activity is not yet fully understood.

There is a large body of experimental evidence that DNA is the critical target for the cytostatic activity of cisplatin [3–5]. As a result, numerous investigators have studied its reactions with DNA. A necessary step in this investigation is to determine the platinum-DNA adducts. The research in this area is well advanced, at least with regard to the modification of DNA in vitro and this article will summarize the results.

Nevertheless, it is still unclear as to how the platinum-DNA adducts inhibit DNA replication which is assumed to be the main biochemical

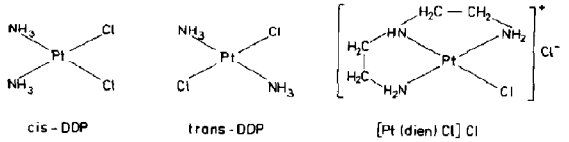

Fig. 1. Structures of three bivalent platinum complexes: *cis*-diamminedichloroplatinum(II) (*cis*-DDP), its *trans* isomer (*trans*-DDP) and diethylenetriaminechloroplatinum(II) chloride {[Pt(dien)Cl]Cl} used in studies of pharmacological structure-activity relations. Only *cis*-DDP is an active antitumour agent.

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event responsible for antitumour activity of cisplatin. Adducts alter the local conformation around the site of platination and replication is likely to be inhibited via a specific conformational change. It is, therefore, evident that structural alterations induced in DNA by platinum binding are of great interest. Various physical and physico-chemical techniques have been employed in elucidating the conformational changes induced in DNA by platinum complexes and this article will review the principal results.

2. Platinum-DNA adducts

Cisplatin reacts with DNA in the cell nucleus, where the concentration of chlorides is markedly lower than in extranuclear or extracellular fluids. The drug loses its chloride ligands in media containing low concentrations of chloride to form positively charged monoaqua and diaqua species [6,7].

The respective rate constants for the loss of the first and second chloride at 25°C have been evaluated as 2.5×10^{-5} and 3.3×10^{-5} s^{-1} [8]. The aqua ligand is in equilibrium with its deprotonated hydroxo form. The pK_a values are 5.6 for the diaqua species and 7.3 for the monoaquamonohydroxo and monoaquamonochloride species [9–11]. The hydroxo moiety forms a stable bond with platinum while water is a good leaving group. The aquated forms bind covalently to DNA. If a solution of freshly dissolved cisplatin is added to DNA, formation of monoaqua species is the rate-limiting step [12,13]. The half-life for this reaction in water amounts to 4 h at 37°C.

2.1. Cisplatin-DNA adducts in vitro

Various experimental approaches have been used to identify cisplatin-DNA adducts. The most successful of these involve digestion of cisplatin-modified DNA by enzymes (deoxyribonuclease I, nuclease P_1 and alkaline phosphatase [14,15]) or by acid hydrolysis (depurination by hot concentrated formic acid [16]). The products are separated chromatographically and the adducts quantitated by a suitable method, such as atomic absorption spectroscopy or determination of radioactivity when radiolabelled equivalent of cisplatin is used. Adducts are identified by NMR or through comparison with standard compounds prepared with deoxyribonucleotides or dinucleotides and cisplatin.

A typical experiment [14,15], in which the adducts of cisplatin-DNA were analyzed after 16 h reaction at $r_b = 0.002$,* gave the following results. The major adducts are cross-links with dinucleotides containing two deoxyguanosines (63%) or deoxyguanosine and deoxyadenosine (22%). It is interesting that in the latter adduct, deoxyadenosine is always the 5′ nucleotide. The minor adduct (7%) is a cross-link between two deoxyguanosines without a linking phosphate group. Investigations of these adducts by NMR spectroscopy reveal that cisplatin is bound to the purine base at the N(7) position.

The high frequency of modifications at GG sequences cannot be explained by a random initial reaction at any guanine and subsequent cross-linking to a neighbouring purine. Thus, cisplatin may react preferentially with DNA at GG sequences. Platinum forms a stable coordinate covalent bond at the N(7) position of guanine and as a result the selectivity of this reaction is under kinetic rather than thermodynamic control. Rate constants for the reaction of aquated cisplatin are 3-times greater for the reaction with GpG than ApG. It has been proposed [17] on the basis of molecular mechanics calculations that this selectivity is the result of hydrogen bonding of a pentacoordinated intermediate to the O(6) position of 5′-G which significantly stabilizes the transition state for GpG but not for ApG or GpA.

The minor adduct is a cross-link between two non-adjacent deoxyguanosines. This adduct could be formed between two guanines either in different strands or in one strand but separated by one or more bases. It has been shown [18,19] that the amount of interstrand cross-links in double-stranded DNA modified by cisplatin does not exceed 1%. The adducts which can be formed in

* r_b is defined as the number of platinum atoms fixed per nucleotide.

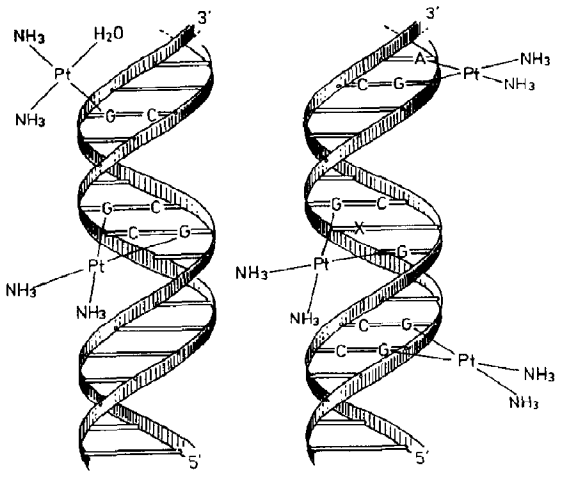

Fig. 2. Schematic representation of modes of *cis*-DDP binding to DNA. Distortions of the double helix which occurred due to platination are not shown. G, A, C and T denote guanine, adenine, cytosine and thymine residues, respectively, X being any of the four bases.

DNA by cisplatin in vitro are schematically shown in fig. 2.

Various pieces of experimental evidence suggest that the reaction of cisplatin with DNA is a two-step process [14–16]. A monofunctional adduct is formed in the initial step. The monofunctional adducts can be trapped with ammonium bicarbonate, cyanide, radioactive guanine, or thiourea [14,15,20,21]. For example, thiourea is rapidly exchanged with the remaining leaving ligand of cisplatin which has been monofunctionally bound to DNA [15]. If this approach is employed, monofunctional adducts account for 42% of cisplatin after 15 min reaction. It has been shown that the monoadducts are tranformed to bifunctional adducts in a biphasic reaction [16,21]. The first phase involves the majority of the lesions and reaches completion during 2 h reaction with cisplatin. The second reaction concerns 5–10% of the lesions which disappear at 37 °C with a half-life of about 20 h.

2.2. *Cisplatin-DNA adducts in vivo*

Early experiments quantitated DNA interstrand cross-links and the cross-links between DNA and proteins in cultured cells treated with platinum compounds [22]. Interstrand cross-links are relatively minor lesions involving about 1% of the platinum [19]. Although the frequency of these minor adducts is correlated with the cytotoxicity of cisplatin in some biological systems, conflicting results have been reported [23,24].

The major adducts – intrastrand cross-links between adjacent purine bases – have been quantitated in DNA isolated from blood cells of human patients treated with cisplatin. These cross-links can be detected by polyclonal antibodies elicited against platinum-DNA adducts in vitro [25,26]. This approach has been used to quantitate platinum lesions in DNA isolated from Chinese hamster ovary cells or murine L1210 leukemia cells [25,26]. A correlation between the level of DNA adducts in leukocytes of patients treated with cisplatin and the effectiveness of chemotherapy via this drug has been reported [27].

2.3. *The adducts of DNA wih platinum complexes exhibiting no antitumour activity*

Structure-activity studies often employ inactive compounds such as *trans*-DDP and [Pt(dien)Cl]Cl (fig. 1). These compounds have been widely used to investigate the mechanism of action of cisplatin. In this approach, one searches for differences between active and inactive compounds which may be responsible for the pharmacological effect.

The leaving ligands in *trans*-DDP are sterically arranged in such a way that this isomer cannot form an adduct between the N(7) positions of adjacent purine residues in double-helical DNA. On the other hand, the adducts of *trans*-DDP with guanine residues in one strand separated by one or more bases have been described [28]. After 2 h incubation, *trans*-DDP forms about 85% monofunctional adducts in double-helical DNA in vitro, which are transformed into bifunctional adducts very slowly (50% of the monofunctional adducts remain after 24 h) [21,29]. Bifunctional lesions formed by *trans*-DDP occur between deoxyguanosine and either deoxycytosine, deoxyadenosine, or another deoxyguanosine [30].

The adducts formed between DNA and [Pt(dien)Cl]Cl in vitro have been isolated by acid

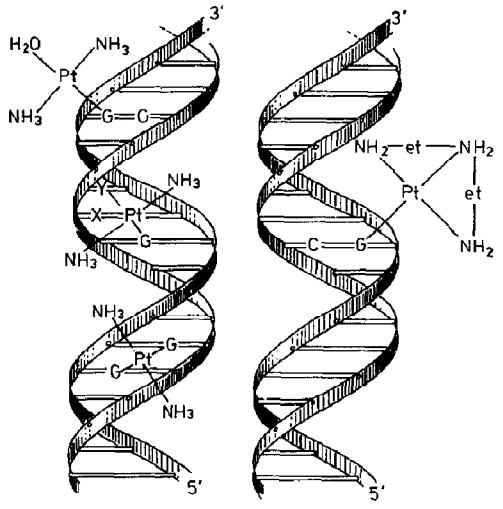

Fig. 3. Schematic representation of the binding modes of antitumour-inactive platinum complexes *trans*-DDP (left) and [Pt(dien)Cl]Cl (right) to DNA. Y denotes any one of the three bases guanine, adenine and cytosine. Other details as in fig. 2.

depurination and characterized [31]. At levels of binding below $r_b = 0.1$, this platinum complex is bound exclusively to guanine residues at the N(7) position.

Lesions formed in double-helical DNA modified in vitro by the inactive antitumour compounds *trans*-DDP and [Pt(dien)Cl]Cl are summarized in fig. 3. Interstrand and protein-DNA cross-links have been observed in cells treated with *trans*-DDP [22]. Intrastrand adducts in cells treated with these complexes have not been investigated.

3. Physico-chemical studies of the conformation of platinum-DNA complexes

3.1. Circular dichroism spectroscopy and pulse polarography

The first detailed information about the changes in DNA conformation induced by platinum binding was obtained by CD spectroscopy. Tamburro et al. [32] observed that the complexation of DNA with both *cis*- and *trans*-DDP at high r_b values led to a decrease in the positive CD band of DNA at about 280 nm. These CD spectral changes were interpreted as being indicative of a B → C transformation with increased winding of the DNA helix.

A subsequent extensive study involved CD spectroscopy, ultraviolet absorbance, denaturation measurements, viscometry and electron microscopy of a variety of platinum-DNA complexes with a wide range of r_b values (table 1).

On the basis of these investigations, three classes of platinum-DNA binding were identified [34,35]: (i) *cis*-bifunctional binding, which increased the positive DNA band at 280 nm at low r_b values; (ii) *trans*-bifunctional binding, which decreased this band; (iii) monofunctional platinum-DNA binding which was not accompanied by any changes in the positive CD band at low r_b values. CD spectral changes were more pronounced in complexes of DNAs with a higher content of guanine residues [35]. Platinum compounds characteristic of the three binding types are *cis*-DDP, *trans*-DDP and [Pt(dien)Cl]Cl, respectively.

In addition to the changes in CD spectra, a hyperchromic effect in ultraviolet absorption is observed after complexation with bifunctional, but not monofunctional platinum compounds. These spectroscopic changes reflect the disruption of electronic interactions between adjacent nucleobases. Furthermore, binding of a single bifunctional platinum compound prevents the intercala-

Table 1

Changes in conformation and stability of DNA after fixation of Pt(II) chloroamines in vitro ($r_b = 0.01$; data taken from the work of Macquet et al. (ref. 33 and references cited therein))

	cis-DDP	*trans*-DDP	[Pt(dien)Cl]Cl
CD 280 nm	increase	decrease	no change
Ultraviolet hyperchromism	+	+	0
Exclusion of intercalating agents	+	+	0
Renaturation	+	+	0
Viscosity	decrease	decrease	no change
Electron microscopy	shortening	shortening	no change
Change in melting temperature (°C)	−2.4	+1.3	+3.3

tion of one ethidium bromide molecule into the DNA double helix [36]. On the basis of these results, it was suggested [34] that base stacking is disrupted at the site of platination by the formation of intrastrand cross-links between adjacent nucleotides. In addition, bidentate, but not monodentate platinum compounds enhance the renaturation of DNA after thermal or alkaline denaturation; these results indicate that the formation of interstrand cross-links maintains the two DNA strands in register during denaturation. Finally, measurements based on electron microscopy and viscometry show that complexation of DNA with bifunctional platinum compounds shortens the DNA molecule.

Such experiments readily distinguish between monodentate and bidentate platinum complexes by their capacity to form intra- and interstrand cross-links. The most remarkable difference between the two bidentate compounds is that the *cis* isomer destabilizes DNA at low r_b values whereas the *trans* isomer stabilizes the polynucleotide (table 1).

More recently, conformational changes induced in DNA on binding a wide variety of platinum complexes showing different antitumour activities have been investigated via pulse-polarographic analysis (fig. 4D and E), CD spectroscopy (fig. 5A–C) and denaturation-renaturation experiments [37–41]. The results indicate the existence of two binding types. The first (type I) increases both the positive CD band of DNA and a peak in the pulse-polarographic curve at −1.38 V (the so-called peak II); this type of binding is characteristic of *cis*-DDP and other antitumour-active platinum compounds at low r_b values. In contrast, type II binding decreases the positive CD band at low levels of binding and causes the appearance and increase of a new, more negative pulse-polarographic peak at −1.43 V (designated as peak III); the latter binding type is observed for *trans*-DDP and the monofunctionally interacting [Pt(dien)Cl]Cl, which exhibit no antitumour activity.

Pulse-polarographic analysis sheds considerable light on the conformational basis for both types of binding. It has been shown [42] in carefully isolated preparations of double-helical DNA that

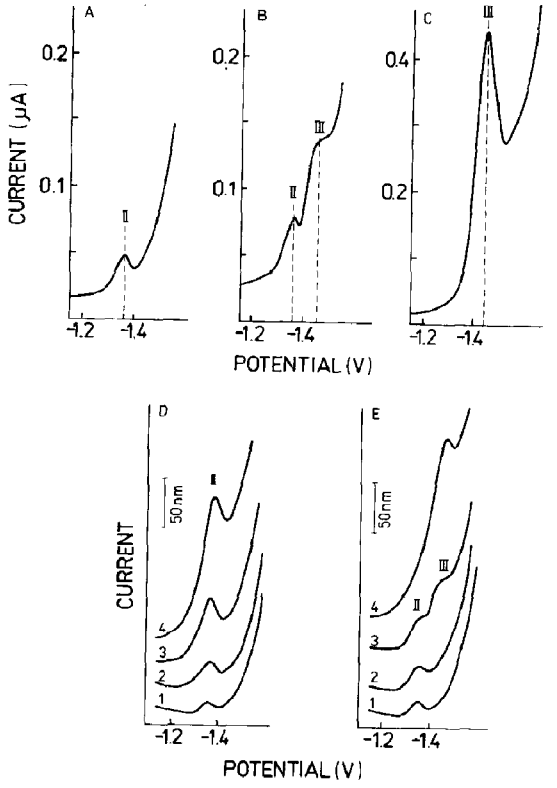

Fig. 4. Differential pulse polarograms of calf thymus DNA in 0.3 M ammonium chloride and 0.01 M Tris-HCl buffer (pH 7.0) recorded at 25°C. (A) Native DNA at 0.4 mg/ml; (B) native DNA at 0.4 mg/ml containing 1.2% thermally denatured DNA; (C) thermally denatured DNA at 0.05 mg/ml; (D) *cis*-DDP-DNA complexes: curve 1, $r_b = 0$; curve 2, $r_b = 0.001$; curve 3, $r_b = 0.005$; curve 4, $r_b = 0.02$; (E) *trans*-DDP-DNA complexes: curve 1, $r_b = 0$; curve 2, $r_b = 0.001$; curve 3, $r_b = 0.01$; curve 4, $r_b = 0.02$. Interaction of platinum compounds with DNA was allowed to proceed in 0.01 M sodium perchlorate at 28°C in the dark until completion was reached. Reference: saturated calomel electrode.

pulse-polarographic peak II (Fig. 4A) is very small. Intact double-helical DNA is polarographically inactive. The reduction sites are involved in hydrogen bonds and are unable to make contact with the working mercury electrode in a manner suitable for electron transfer. Electroreduction of adenine and cytosine residues present in distorted but still double-stranded (non-denatured) regions of DNA is responsible for the appearance of peak II. For example, this peak is increased after intro-

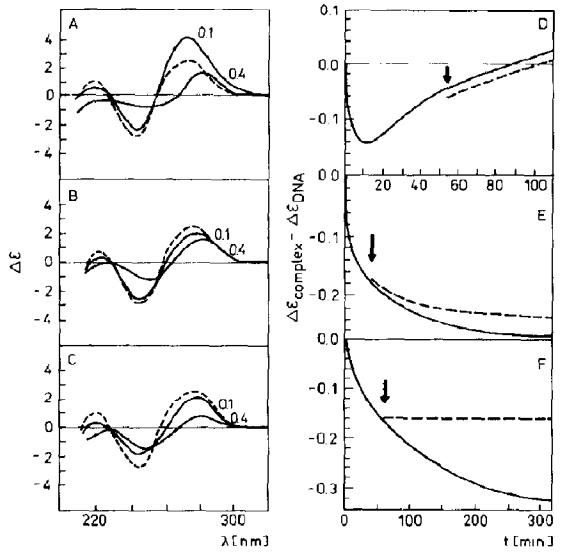

Fig. 5. Changes in CD spectra of DNA (A–C) induced by reaction with *cis*-DDP (A), *trans*-DDP (B), and [Pt(dien)Cl]Cl (C) measured in equilibrated reaction mixtures for the r_b values beside the respective curves; (- - -) free DNA. Kinetics of CD spectral changes at 280 nm during the reaction of DNA with *cis*-DDP (D), *trans*-DDP (E), and [Pt(dien)Cl]Cl (F). Input r was 0.08. (———) Kinetic curves of unseparated mixtures; (- - -) kinetic curves after separation of the unreacted platinum at the times denoted by arrows (see text). Reactions were performed in 0.01 M sodium perchlorate at 25 °C.

ducing local distortions of a non-denaturational character, such as single- and double-strand breaks or the introduction of thymine dimers into the DNA helix [42]. Such distortions may induce local base unstacking but not necessarily disruption of interbase hydrogen bonds. Nevertheless, some bases in these distorted regions become more accessible for electroreduction at the mercury electrode and can yield a small polarographic current.

On the other hand, the appearance of peak III (fig. 4B and C) on pulse polarograms of DNA indicates the presence of single-stranded, denatured regions in the DNA molecule in which hydrogen bonds between complementary bases have been broken. Peak III increases in height during thermal denaturation of DNA and becomes several orders of magnitude more intense than peak II of double-helical DNA. This dramatic enhancement of the polarographic activity of DNA probably reflects the large number of bases in denatured DNA which are readily accessible to the electrode. Differences in the absorption properties of double-helical and denatured DNA at the mercury electrode [43] may give rise to the different reduction potentials which are observed for the two DNA conformations.

Thus, qualitative differences in the polarographic behaviour of the two binding types and the dependence of the heights of peaks II and III on r_b support the view that type I binding induces only small distortions of a non-denaturational nature in the DNA helix. On the other hand, type II binding leads to the formation of short single-stranded segments containing unpaired bases (denatured regions) [37,41].

The above results indicate that even monofunctional attachment of platinum complexes such as [Pt(dien)Cl]Cl can result in conformational changes in DNA. This view is supported by the observation that the binding of [Pt(dien)Cl]Cl facilitates the transition of poly(dG-dC) · poly(dG-dC) from the B conformation to the Z form [44]. The ability to stabilize the left-handed Z structure is much stronger for [Pt(dien)Cl]Cl than for bifunctional *cis*- and *trans*-DDP [45]. Nevertheless, results obtained by Raman spectroscopy of DNA modified by *cis*-DDP and its *trans* isomer confirm that both complexes may also facilitate the transition of DNA from the B to Z form [46,47]. On the other hand, CD analysis clearly indicates that all three compounds inhibit the transition of DNA from the B to A conformation [48] although *cis*-DDP and *trans*-DDP are more effective than [Pt(dien)Cl]Cl.

Binding types I and II have also been demonstrated to occur during formation of liquid crystalline microphases of DNA. DNA preparations modified with platinum compounds characterized by CD spectroscopy as type I are unable to form a liquid crystalline phase of the cholesteric type in the presence of polyethylene glycol. In contrast, platinum compounds attached by type II binding do not interfere significantly with this transformation [49–51].

It has been proposed that both binding types distinguished by CD spectroscopy and pulse polarography might serve as a simple tool for

selecting platinum compounds as potential anti-cancer drugs. In fact, all anti-cancer or cytostatic platinum compounds tested to date exhibit the characteristics of type I binding [37,39,41]. However, compounds which modify DNA in this manner do not require the reactive leaving groups to be in a *cis* configuration. For example, a *trans*-chloronitro analogue of *trans*-DDP apparently binds to DNA according to a type I process but the binding kinetics is rather slow (ref. 50 and unpublished results).

3.2. NMR spectroscopy and X-ray diffraction

In section 3.1, two types of conformational change were described, namely, those induced in DNA by *cis*-DDP, on the one hand, and *trans*-DDP and [Pt(dien)Cl]Cl on the other. We now attempt to establish correlations between the two different lesions in DNA and more detailed data from structural analysis of platinum adducts with oligonucleotides.

NMR spectroscopy and X-ray diffraction analysis provide information on the conformation of DNA at a resolution on the atomic level. Known structures of platinum complexes with DNA bases and oligonucleotides have been reviewed recently by Reedijk et al. [7]. A description of the NMR techniques employed in such studies can be found in the latter article.

NMR studies of complexes between *cis*-DDP and trinucleotides differing in base sequences confirm the well-established fact that platinum reacts at the N(7) atom of guanine. In the trinucleotide d(CGG) *cis*-DDP chelates the two neighbouring guanines and C(1) is stacked above G(2) [52]. This structure has been confirmed by X-ray diffraction analysis [53] demonstrating that the stacked structure is only slightly distorted by chelation of two neighbouring bases.

If two guanines in a trinucleotide are separated by another base [d(GBG)], *cis*-DDP chelates the guanines G(1) and G(3) [54,55]. In d(pGGG) chelation occurs mainly between G(1) and G(2) [56]. However, in d(GAG), chelation to adenine is also possible [54] and about 20% AG chelate is formed. Initial monofunctional binding of *cis*-DDP takes place mainly at G(3) which indicates a preference for AG chelation in the 5′ direction. Platinum binding to bases other than guanine and adenine has not been observed in trinucleotides of the type d(GBG), even though chelation to cytosine residues has been reported in dinucleotide complexes [57]. Surprisingly, *trans*-DDP also binds bifunctionally to d(GTG) [55].

Platination of self-complementary tetra- or hexanucleotides disrupts the helical structure [58–60] while platinated decanucleotides or larger oligonucleotides remain double-stranded. Hence, conformational studies of platinated oligonucleotides are performed in the following way. A single-stranded oligonucleotide containing one target sequence, -GG- or -GBG-, is reacted with *cis*-DDP and then annealed with the complementary strand. The short duplexes formed are then analyzed by ^{1}H- or ^{31}P-NMR [61–64]. The following conclusions have been drawn from analysis of the NMR spectra:

(1) The platinated duplex is destabilized as compared with the unplatinated form.

(2) Platinated guanine residues may form hydrogen bonds of a particular type with the complementary cytosines.

(3) A small degree of distortion of the duplex occurs at the platination site which has been described as a kink of about 40–70° wihout a larger change in helix winding. This kink has also been detected by electron microscopy measurements [65] and gel retardation experiments [66].

Such NMR investigations cannot provide information concerning the structural effects of interstrand cross-links.

NMR analysis of the undecamer, d(TCTCGTGTCTC), treated with *cis*-DDP revealed the two central guanines surrounding a thymine residue to be platinated [67]. Upon addition of a complementary oligonucleotide duplex formation is observed. However, NMR spectra indicate the absence of hydrogen bonds between the central thymine and adenine residues, apparently due to distortion of the platinated -GTG- segment. Thermal destabilization of this duplex is greater than in the case of -GG- platination.

A significant difference has been observed between changes in the CD spectra of oligonucleotides with sequences -GG- and -GTG- platinated

with *cis*-DDP. In the former case, platination leads to an increase in the long-wavelength positive CD band, whereas a diminution is observed in the latter [67]. These changes strongly resemble type I and II binding of platinum compounds to polymeric DNA, respectively (vide supra) and indicate that the intrastrand cross-linking between neighbouring bases is the most frequent event during the reaction of *cis*-DDP with DNA.

In light of the spectroscopic and polarographic changes induced by type II platination described in section 3.1, it would be interesting to investigate the effect of monofunctional platinum binding on the duplex conformation. The only data available are those on the X-ray structure of the self-complementary dodecamer, d(CGCGAATTCGCG), containing monofunctionally bound *cis*-DDP which was obtained by soaking dodecamer crystals in a solution of cisplatin [68]. Platination occurs at N(7) of three guanines of the eight potential binding sites and induces their shift toward the major groove. It has been proposed that this shift may weaken the glycosidic bond and lead to depurination. However, unlike alkylation, platination of the N(7) position of guanosine stabilizes the glycosyl linkage [31]. It is not clear as to whether the displacement of platinated guanines might be responsible for the effects induced in the CD spectrum of DNA by monofunctional platination.

The experiments with oligonucleotides can be summarized in the following points:

(1) The primary binding site of *cis*-DDP is the N(7) position of guanine residue.

(2) *cis*-DDP can chelate either the neighbouring bases, -GG- or with lower probability -AG-, or two guanines separated by another base, -GBG-.

(3) Platination of neighbouring bases by *cis*-DDP induces only a small degree of non-denaturing distortion in the DNA double helix, which has been characterized as a kink. Hydrogen bonds between paired bases are not disrupted.

(4) Platination of a -GBG- sequence by *cis*-DDP leads to the disruption of hydrogen bonds involving the central base.

(5) Data on binding of *trans*-DDP to the trinucleotide d(GTG) indicate that this antitumour-inactive compound can form intrastrand cross-links between two guanine residues separated by a third base.

(6) Changes in the CD spectra of oligonucleotides induced by *cis*-DDP chelation of neighbouring base residues (-GG-, -AG-) and the -GBG- sequence are similar to those observed upon platination of DNA. Both of these types of intrastrand cross-links are characteristic of binding types I and II of bifunctional platinum compounds, respectively.

3.3. Other chemical and biochemical methods

Among techniques suitable for the analysis of local conformational alterations in DNA are those utilizing chemical probes. The characteristic property of such probes is their ability to react only with a small but altered part of the biomacromolecule.

Terbium is a sensitive fluorescent probe for guanine bases present in single-stranded denatured sequences of DNA; it also detects local, non-denatured distortions of double-helical DNA in which the vertical stacking of base-pairs has been altered. Modification of DNA by *cis*-DDP or its analogues with leaving groups in the *cis* configuration enhances terbium fluorescence [69–72]. In contrast, DNA modification by *trans*-DDP does not increase terbium fluorescence. Apparently, this probe can also differentiate between type I and II binding of platinum complexes to DNA.

Other chemical probes such as chloroacetaldehyde, diethyl pyrocarbonate, and osmium tetroxide have been used to analyze the perturbations induced by binding of *cis*-DDP to DNA. Such studies indicate that while only a few complementary bases are unpaired, marked distortion of the double helix occurs [73].

Similarly, single-strand-specific nucleases have been used to study local conformational alterations in DNA. It has been shown that double-stranded DNAs modified by both *cis* and *trans*-DDP are sensitive to digestion with S_1 nuclease [18,74]; *cis*-DDP results in a far greater concentration of S_1 nuclease-sensitive sites than in the case of the *trans* isomer. This finding, however, cannot be interpreted as being due to *cis*-DDP binding

producing single-stranded regions, which would contradict the results of CD, pulse-polarographic, NMR, and X-ray measurments. It has been suggested [41] that S_1 nuclease may recognize and excise not only single-stranded segments but also regions in DNA which are double-stranded, yet in some way distorted by the lesions.

Moreover, intrastrand cross-linking between two neighbouring guanine residues induced in double-stranded oligodeoxynucleotides by cisplatin represents a hindrance to the digestion of the oligomer by deoxyribonuclease I, snake venom and calf spleen phosphodiesterases [75]; parallel data on the effect of platination of the oligomer by *trans*-DDP are, however, lacking. This comparison was carried out in the case of the study of cleavage of platinum-modified DNA by restriction nucleases.

Various restriction endonucleases have been used to monitor the influence of platination on DNA structure [76–80]. The extent to which restriction nucleases are inhibited as a result of DNA modification by *cis*-DDP, *trans*-DDP, and [Pt(dien)Cl]Cl is comparable for all enzymes employed so far. Platination by these complexes alters enzyme-substrate interactions at sequences beyond the immediate site of binding. Antitumour *cis*-DDP produces more extensive effects for any particular restriction nuclease in comparison with *trans*-DDP and [Pt(dien)Cl]Cl. Thus, restriction nucleases appear to be sensitive to the differences between binding types I and II of platinum complexes to DNA as classified according to data from CD spectroscopy and pulse polarography. Hence, such enzymatic assays may be sensitive to conformational changes which have not yet been studied by physico-chemical methods.

Polyclonal and monoclonal antibodies have been employed to probe the alterations induced in DNA by modification with platinum complexes [81–86]. It has been shown that the antibodies elicited against DNA modified by *cis*-DDP exhibit high specificity for DNA modified by all antitumour bivalent platinum complexes having *cis* stereochemistry. Poor immunoreactivity of these antibodies is observed toward DNA modified by *trans*-DDP. Similarly, antibodies elicited against DNA modified by *trans*-DDP exhibit poor immunoreactivity toward complexes of DNA with *cis*-DDP [87]. It has been proposed [45,88,89] that the antibodies recognize a conformational change in DNA. If this conclusion is correct, immunochemical methods might be used to investigate the different types of platinum binding to DNA which have been established on the basis of CD and pulse-polarographic investigations.

3.4. Kinetic aspects

Adducts of bifunctional platinum compounds with DNA are formed in two steps. In the first, platinum is attached monofunctionally to guanine residues at the N(7) position in either strand of the DNA duplex. In the following step the other labile ligand is substituted by a second purine base. As already discussed, bifunctional binding may result in the formation of intrastrand or interstrand cross-links; two types of intrastrand cross-links have been observed which join either neighbouring purine bases or two guanines separated by at least one other nucleotide. It has also been shown that monofunctional binding and both of these types of intrastrand cross-links can produce different local conformational changes in the DNA duplex. It is thus of interest to use techniques capable of quantitating these changes to investigate the kinetics of their formation.

There exist relatively few reports on the kinetics of DNA platination. The kinetics of initial attachment of *cis*-DDP, *trans*-DDP, and their aqua derivatives to DNA have been determined using compounds labelled with radioactive platinum [90]. For the diaqua species only one reaction was detected.

More recently, Schaller et al. [20] investigated the kinetics of DNA platination by the same compounds. Several parameters were measured; some, such as the CD spectral data, are directly correlated with conformational changes and others indirectly (inhibition of ethidium bromide intercalation [36] and inhibition of DNA polymerase activity). In addition, the kinetics of disappearance of the monofunctional lesions were measured via their reaction with radiolabelled cyanide. The results obtained with the different techniques were interpreted within the framework of a two-step binding

mechanism where both the monofunctional and bifunctional reaction are followed by a conformational rearrangement. However, none of the procedures employed is capable of detecting all four steps and the various methods monitor different DNA properties. Therefore, this complex reaction scheme remains to be verified.

The experiments reveal that the reaction kinetics are strongly dependent on the extent of *cis*-DDP aquation. For the diaqua species, the rate constant for the initial binding step is one order of magnitude greater as compared to the monoaquamonochloro analogue; maximum monofunctional binding is observed within approx. 2 min after the onset of the reaction in the former case and within 20–40 min in the latter. The monoaquamonochloro analogue of *trans*-DDP is bound mainly monofunctionally under the conditions of this experiment [20].

In a subsequent kinetic study carried out in our laboratory [91,92], CD spectrophotometry was used to investigate the kinetics of DNA conformational changes during platination and a polarographic method was employed to determine the concentration of unreacted platinum complex in the reaction mixture. The polarographically determined kinetics, which measure the initial monofunctional binding, demonstrated that this reaction has two phases for both *cis*-DDP equilibrated in 0.01 M sodium perchlorate and its diaqua analogue [92]. The results suggest that initial binding of these compounds with leaving groups in the *cis* configuration may occur at DNA sites with different degrees of accessibility for platinum compounds.

CD measurements of DNA mixed with *cis*- or *trans*-DDP in 10 mM sodium perchlorate showed two-phase kinetics, characterized by parameters similar to those reported previously [20]. However, an important qualitative difference was observed for the *cis*-DDP reaction. In the initial rapid phase, a decrease in the positive CD band at about 280 nm was observed, similar to that found during the reaction of *trans*-DDP or [Pt(dien)Cl]Cl (fig. 5D) [91,92]. Only in the second phase of the reaction does the CD band begin to increase, resulting in a spectrum characteristic of the final *cis*-DDP-DNA complex [29–31].

In order to understand these results, it was necessary to separate the kinetics corresponding to the monofunctional attachment and bifunctional rearrangement. For this purpose, unreacted platinum was removed from the platinated DNA after a brief period of treatment by centrifugation through a Sephadex column [91,92]. Monophasic kinetics of CD spectral changes corresponding only to bifunctional rearrangement were then recorded (fig. 5D–E). An increase occurs during post-treatment incubation after separation of the unreacted *cis*-DDP. On the other hand, the bifunctional rearrangement of *trans*-DDP induces a further decrease of the CD band (fig. 5E). No changes are observed, as expected, for the monofunctionally bound [Pt(dien)Cl]Cl, which cannot be transformed to bifunctional adducts (fig. 5F) [91,92].

The results of the above kinetic investigations support our previous conclusions based on CD spectroscopic and pulse-polarographic measurements of platinum-DNA complexes on termination of the reaction. Apparently, the initial monofunctional attachment of any of the platinum compounds investigated gives rise to distortions in the DNA double helix in which a number of hydrogen bonds between bases in opposite strands are broken. The distortions prevail in *trans*-DDP-DNA complexes even after longer periods of incubation when the bifunctional rearrangement takes place. In contrast, the bifunctional reaction of *cis*-DDP also induces a change in the DNA conformation at the platination site which results in a different type of local distortion of the DNA duplex, apparently non-denaturational in character without disruption of hydrogen bonds. This type of reaction results in an increase in the positive CD band of DNA and polarographic peak II.

The initial decrease in intensity of the CD band, observed during the early stages of the reaction of *cis*-DDP and its analogues, is most clearly evident if the CD experiment is performed with a platinum compound which reacts slowly with DNA. This is probably the reason why the initial decrease in the CD band was not detected by Schaller et al. [20], who performed the experiments with the highly reactive diaqua derivative of *cis*-DDP.

4. Discussion

Cisplatin forms several types of adducts upon interaction with DNA (fig. 2). The particular adduct that is responsible for the specific cytostatic effect of the drug remains to be established conclusively. In an initial study [4], evidence was adduced that the ability of cisplatin to form DNA interstrand cross-links is causally related to the cytostatic effect of the drug. On the other hand, if structure-pharmacological activity relationships are taken into consideration, the most likely candidate for a critical lesion resulting in the inhibition of cell division appears to be an intrastrand cross-link between two neighbouring purine bases. Only this type of adduct is formed exclusively by antitumour-active cisplatin and is in fact the major adduct formed by this drug on DNA. *trans*-DDP cannot yield this adduct due to steric constraints.

Resolving the problem as to which lesion is critical for the cytotoxic activity of platinum drugs might be simplified if the critical lesion were to be a specific conformational change induced in DNA by the binding of these compounds. Antitumour-active platinum compounds induce local conformational alterations in DNA which have the character of non-denaturational distortions at low levels of binding. A large body of experimental evidence [7,92,93] supports the view that such non-denaturational alterations occur in DNA due to the formation of intrastrand cross-links. On the other hand, antitumour-inactive platinum compounds induce more severe denaturational changes in DNA under the same conditions. Therefore, the question as to why a relatively subtle local change in DNA conformation is able to trigger a system of regulation or other physiological processes in the tumour cell leading finally to the cytostatic effect while this effect cannot be initiated by more severe denaturational change remains to be resolved.

One possibility is the hypothesis that conformational alterations induced in DNA by *cis*-DDP are reparable with greater difficulty as compared to those induced by *trans*-DDP. It assumes that the relatively subtle conformational distortion induced in DNA by *cis*-DDP would be less readily recognized by the intracellular repair system than denaturational distortions induced in DNA by antitumour-inactive *trans*-DDP. This proposal, however, lacks sufficient experimental support; papers dealing with this problem have provided rather conflicting results [94–98].

Alternatively, non-denaturational changes induced in DNA by cisplatin could represent more significant steric hindrance arising from protein-DNA interactions during the process of DNA replication. There is some evidence available to support this contention. DNA damaged by *cis*-DDP inhibits thymidine incorporation in *Escherichia coli* an order of magnitude more efficiently than the *trans* isomer while [Pt(dien)Cl]Cl has little effect [99]. In this experiment platinum-DNA adducts formed by *cis*-DDP underwent less excision repair [99] and less SOS repair [100] than those formed by *trans*-DDP or [Pt(dien)Cl]Cl. Similarly, platinum-DNA lesions formed by *cis*-DDP also inhibited replication of phage T_7 DNA by a crude bacterial extract 5-times more efficiently than *trans*-DDP [6]. Finally, *cis*-DDP blocks the replication of single-stranded M13 DNA by *E. coli* polymerase I and mammalian polymerase-α at well-defined sites dG_n ($n \geq 2$). *trans*-DDP arrests polymerization at more remote locations while monofunctional adducts have little effect [101,102]. Such differences may reflect a decreased binding constant for the polymerase at platinated nucleotide sequences [20]. The precise structural features of the platinum-DNA adduct responsible for these different template activities are unknown but probably reflect the conformational changes observed with biophysical techniques.

In order to determine the nature of the critical lesion responsible for the antitumour effect of platinum compounds and the specific role played its characteristic features, elucidation of the structure-pharmacological activity relationships of the platinum complexes must be performed at the molecular level. Studies aimed at clarifying the effects of platinum lesions on single gene regulation or other physiological events, whose error-free course is a prerequisite for correct completion of the complex process of DNA replication, are envisaged in the near future.

References

1 B. Rosenberg, Cancer 55 (1985) 2303.
2 A.R. Prestayko, S.T. Crooke and S.K. Carter, Cisplatin, current status and development (Academic Press, New York, 1980).
3 V. Kleinwachter, Stud. Biophys. 73 (1978) 1.
4 J.J. Roberts and A.J. Thomson, Prog. Nucleic Acid Res. Mol. Biol. 22, (1979) 71.
5 J. Drobnik, Cancer Chemother. Pharmacol. 10 (1983) 145.
6 N.P. Johnson, J.D. Hoeschele, N.B. Kuemmerle, W.E. Masker and R.O. Rahn, Chem.-Biol. Interact. 23 (1978) 267.
7 J. Reedijk, A.M.J. Fichtinger-Schepman, A.T. van Oosterom and J. van de Putte, Structure and Bonding (Springer, Berlin, 1987).
8 J.W. Reishus and D.S. Martin, Jr, J. Am. Chem. Soc. 83 (1961) 2457.
9 M.C. Lim and R.B. Martin, J. Inorg. Nucl. Chem. 38 (1976) 1911.
10 J.R. Perumareddi and A.W. Adamson, J. Phys. Chem. 72 (1968) 414.
11 K.A. Jensen, Z. Anorg. Allg. Chem. 242 (1939) 87.
12 P. Horáček and J. Drobnik, Biochim. Biophys. Acta 254 (1971) 341.
13 M. Green, Transition Metal Chem. 12 (1987) 186.
14 A.M.J. Fichtinger-Schepman, J.L. van der Veer, J.H.J. den Hartog, P.H.M. Lohman and J. Reedijk, Biochemistry 24 (1985) 707.
15 A. Eastman, Biochemistry 25 (1986) 3912.
16 N.P. Johnson, A.M. Mazard, J. Escalier and J.P. Macquet, J. Am. Chem. Soc. 107 (1985) 6376.
17 A. Laoui, J. Kozelka and J.C. Chottard, Inorg. Chem. 27 (1988) 2751.
18 A. Eastman, Biochem. Biophys. Res. Commun. 105 (1982) 869.
19 J.J. Roberts and F. Friedlos, Biochim. Biophys. Acta 655 (1981) 146.
20 W. Schaller, H. Reisner and E. Holler, Biochemistry 26 (1987) 943.
21 J.L. Butour, N.P. Johnson, Biochemistry 25 (1986) 4534.
22 L.A. Zwelling, T. Anderson and K.W. Kohn, Cancer Res. 39 (1979) 365.
23 A.L. Pinto and S.J. Lippard, Biochim. Biophys. Acta 780 (1985) 167.
24 A.C.M. Plooy, M. van Dijk, F. Berends and P.H.M. Lohman, Cancer Res. 45 (1985) 4178.
25 M.C. Poirier, S.J. Lippard, L.A. Zwelling, H.M. Ushay, D. Kerrigan, C.C. Thill, R.M. Santella, D. Grunberger and S.H. Yuspa, Proc. Natl. Acad. Sci. U.S.A. 79 (1982) 6443.
26 A.C.M. Plooy, A.M.J. Fichtinger-Schepman, H.H. Shutte, M. van Dijk and P.H.M. Lohman, Carcinogenesis 6 (1985) 561.
27 E. Reed, R.F. Ozols, R. Tarone, S.H. Yuspa and M.C. Poirier, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 5024.
28 A. Eastman, Pharmacol. Ther. 34 (1987) 155.
29 A. Eastman and M.A. Barry, Biochemistry 26 (1987) 3303.
30 A. Eastman, M.M. Jennerwein and D.L. Nagel, Chem.-Biol. Interact. 67 (1988) 71.
31 N.P. Johnson, J.P. Macquet, J.L. Wiebers and B. Monsarrat, Nucleic Acids Res. 10 (1982) 5255.
32 A.M. Tamburro, L. Celotti, D. Furlan and V. Guantieri, Chem.-Biol. Interact. 16 (1977) 1.
33 J.P. Macquet, J.L. Butour and N.P. Johnson, in: Platinum, gold and other metal chemotherapeutic agents, ed. S.J. Lippard (American Chemical Society, 1983) p. 75.
34 J.-P. Macquet and J.-L. Butour, Biochimie 60 (1978) 901.
35 J.-P. Macquet and J.-L. Butour, Eur. J. Biochem. 83 (1978) 375.
36 J.-L. Butour and J.-P. Macquet, Eur. J. Biochem. 78 (1977) 455.
37 V. Brabec, O. Vrána, V. Kleinwachter and F. Kiss, Stud. Biophys. 101 (1984) 135.
38 V. Kleinwachter and H. Rau, Stud. Biophys. 103 (1984) 5.
39 V. Kleinwachter, V. Brabec, O. Vrána, F. Kiss and I. Závodná, in: CMEA Chemotherapy Symposium 1984, eds. S. Eckhard and S. Kerpel-Fronius (Akadémiai Kiadó, Budapest, 1985) p. 207.
40 V. Kleinwachter and H. Rau, Drugs Exp. Clin. Res. 12 (1986) 523.
41 O. Vrána, V. Brabec and V. Kleinwachter, Anti-Cancer Drug Design 1 (1986) 95.
42 E. Paleček, in: Topics in bioelectrochemistry and bioenergetics, vol. 5, ed. G. Milazzo (Wiley, New York, 1983) p. 65.
43 V. Brabec and E. Paleček, Biopolymers 11 (1972) 2577.
44 B. Malfoy, B. Hartmann and M. Leng, Nucleic Acids Res. 9 (1981) 5659.
45 J.-M. Malinge and M. Leng, EMBO J. 3 (1984) 1273.
46 W.L. Peticolas and G.A. Thomas, in: Structure and motion: Membranes, nucleic acids and proteins, eds. E. Clementi, G. Corongiu, M.H. Sarma and R.H. Sarma (Adenine Press, New York, 1985) p. 497.
47 W.L. Peticolas, W.L. Kubasek, G.A. Thomas and M. Tsuboi, in: Biological applications of Raman spectroscopy: vol. 1– Raman spectra and the conformations of biological macromolecules, ed. T.G. Spiro (Wiley, New York, 1987) p. 81.
48 Z. Balcarová and V. Brabec, Biochim. Biophys. Acta 867 (1986) 31.
49 N. Akimenko, V. Kleinwachter and Yu. Yevdokimov, FEBS Lett. 156 (1983) 58.
50 N. Akimenko, P. Cheltsov, Z. Balcarová, V. Kleinwachter and Yu. Yevdokimov, Gen. Physiol. Biophys. 4 (1985) 597.
51 V.I. Salyanov, P.M. Chel'tsov, V. Kleinwachter and Yu.M. Yevdokimov, Dokl. Akad. Nauk S.S.S.R. 301 (1988) 1241 (in Russian).
52 J.H.J. den Hartog, C. Altona, G.A. van der Marel and J. Reedijk, Eur. J. Biochem. 147 (1985) 371.
53 G. Admiraal, J.L. van der Veer, R.A.G. de Graaf, J.H.J. den Hartog and J. Reedijk, J. Am. Chem. Soc. 109 (1987) 592.

54 J.L. van der Veer, H. van der Elst, J.H.J. den Hartog and J. Reedijk, Inorg. Chem. 25 (1986) 4657.
55 J.L. van der Veer, G.J. Ligvoet, H. van der Elst and J. Reedijk, J. Am. Chem. Soc. 108 (1986) 3860.
56 J.L. van der Veer, Ph.D. Thesis, State University Leiden, (1986).
57 J.P. Girault, G. Chottard, J.Y. Lallemand, F. Huguenin and J.C. Chottard, J. Am. Chem. Soc. 106 (1984) 7227.
58 J.C. Chottard, J.P. Girault, G. Chottard, J.Y. Lallemand and D. Mansuy, J. Am. Chem. Soc. 102 (1980) 5565.
59 A.T.M. Marcelis, G.W. Canters and J. Reedijk, Recueil Trav. Chim. Pays-Bas 100 (1981) 391.
60 J.P. Caradonna, S.J. Lippard, M.J. Gait and M. Singh, J. Am. Chem. Soc. 104 (1982) 5793.
61 J.H.J. den Hartog, C. Altona, J.H. van Boom and J. Reedijk, FEBS Lett. 176 (1984) 393.
62 J.H.J. den Hartog, C. Altona, J.H. van Boom, G.A. van der Marel, C.A.G. Haasnoot and J. Reedijk, J. Am. Chem. Soc. 106 (1984) 1528.
63 B. van Hemelryck, E. Guittet, G. Chottard, J.P. Girault, T. Huynh-Dinh, J.Y. Lallemand, J. Igolen and J.C. Chottard, J. Am. Chem. Soc. 106 (1984) 3037.
64 J.H.J. den Hartog, C. Altona, J.H. van Boom, G.A. van der Marel, C.A.G. Haasnoot and J. Reedijk, J. Biomol. Struct. Dyn. 2 (1985) 1137.
65 B. Revet, J.M. Malinge, E. Delain, M. Le Bret and M. Leng, Nucleic Acids Res. 12 (1984) 8349.
66 J.A. Rice, D.M. Crothers, A.L. Pinto and S.J. Lippard, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 4158.
67 J.H.J. den Hartog, C. Altona, H. van den Elst, G.A. van der Marel and J. Reedijk, Inorg. Chem. 24 (1985) 986.
68 R.M. Wing, P. Pjura, H.R. Drew and R.E. Dickerson, EMBO J. 3 (1984) 1201.
69 M. Arquilla, L.M. Thomson, L.F. Pearlman and H. Simpkins, Cancer Res. 43 (1983) 1211.
70 C. Houssier, M.C. DePauw-Gillet, R. Hacha and E. Fredericq, Biochim. Biophys. Acta 739 (1983) 317.
71 C. Houssier, M.N. Maquet and E. Fredericq, Biochim. Biophys. Acta 739 (1983) 312.
72 Z. Balcarová and V. Brabec, Biophys. Chem. 33 (1989) 55.
73 L. Marrot and M. Leng, Biochemistry 28 (1989) 1451.
74 W.M. Scovell and V.J. Capponi, Biochem. Biophys. Res. Commun. 124 (1982) 1138.
75 K. Inagaki and Y. Kidani, Inorg. Chim. Acta 106 (1985) 187.
76 A.D. Kelman and M. Buchbinder, Biochimie 60 (1978) 893.
77 H.M. Ushay, T.D. Tullius and S.J. Lippard, Biochemistry 20 (1981) 3744.
78 W.M. Scovell and L.R. Kroos, Biochem. Biophys. Res. Commun. 108 (1982) 16.
79 Z. Balcarová and V. Brabec, Stud. Biophys. 130 (1989) 59.
80 Z. Balcarová and V. Brabec, Gen. Physiol. Biophys., submitted.
81 S.J. Lippard, H.M. Ushay, C.M. Merkel and M.C. Poirier, Biochemistry 22 (1983) 5165.
82 M.C. Poirier, E. Reed, L.A. Zwelling, R.F. Ozols, C.L. Litterst and S.H. Yuspa, Environ. Health Perspect. 62 (1985) 89.
83 A. Rahmouni, J.-M. Malinge, A. Schwartz and M. Leng, J. Biomol. Struct. Dyn. 3 (1985) 363.
84 W.I. Sundquist, S.J. Lippard and B.D. Stollar, Biochemistry 25 (1986) 1520.
85 W.I. Sundquist, S.J. Lippard and B.D. Stollar, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 8225.
86 B. Malfoy, B. Hartmann, J.P. Macquet and M. Leng, Cancer Res. 41 (1981) 4127.
87 V.I. Kiselova, O. Vrána, V. Brabec and A.M. Poverennyi, Gen. Physiol. Biophys., submitted.
88 B. Malfoy, N. Rousseau and M. Leng, Biochemistry 21 (1982) 5463.
89 A. Eastman and V.M. Richon, in: Biochemical mechanisms of platinum antitumour drugs, eds. D.C.H. McBrien and T.F. Slater (IRL Press, Oxford, 1985) p. 91.
90 N.P. Johnson, J.D. Hoeschele and R.O. Rahn, Chem.-Biol. Interact. 30 (1980) 151.
91 V. Kleinwachter, O. Vrána, V. Brabec and N.P. Johnson, in: Platinum and other metal coordination compounds in cancer chemotherapy, ed. M. Nicolini (Martinus Nijhoff, Dordrecht, 1988) p. 102.
92 V. Kleinwachter, O. Vrána, V. Brabec and N.P. Johnson, Stud. Biophys. 123 (1988) 85.
93 O. Vrána and V. Brabec, Bioelectrochem. Bioenerg. 19 (1988) 145.
94 R.B. Ciccarelli, M.J. Solomon, A. Varshavsky and S.J. Lippard, Biochemistry 24 (1985) 7533.
95 J.J. Roberts and F. Friedlos, Cancer. Res. 47 (1987) 31.
96 E. Janovská and V. Kleinwachter, Stud. Biophys. 103 (1984) 13.
97 D.J. Beck, S. Popoff, A. Sancar and W.D. Rupp, Nucleic Acids Res. 13 (1985) 7395.
98 S.C. Popoff, D.J. Beck and W.D. Rupp, Mutat. Res. 183 (1987) 129.
99 R. Alazard, M. Germanier and N.P. Johnson, Mutat. Res. 93 (1982) 327.
100 B. Salles and C. Lesca, Biochem. Biophys. Res. Commun. 105 (1982) 202.
101 A.L. Pinto and S.J. Lippard, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 4616.
102 G. Villani, U. Hubscher and J.L. Butour, Nucleic Acids Res. 16 (1988) 4407.