Volume 17 Number 23 1989 Nucleic Acids Research

Interaction of novel bis(platinum) complexes with DNA

John D.Roberts[1], Bennett Van Houten[2]*, Yun Qu[3] and Nicholas P.Farrell[3]*

Vermont Regional Cancer Center and the Departments of [1]Medicine, [2]Pathology and [3]Chemistry, University of Vermont, Burlington, VT 05405, USA

Received August 16, 1989; Revised and Accepted October 20, 1989

ABSTRACT

Bis(platinum) complexes [{*cis*-$PtCl_2(NH_3)$}$_2H_2N(CH_2)_nNH_2$] are a novel series of potential anticancer agents in which two *cis*-diamine(platinum) groups are linked by an alkyldiamine of variable length. These complexes are potentially tetrafunctional, a unique feature in comparison with known anticancer agents. Studies of DNA interactions of bis(platinum) complexes in comparison with cisplatin demonstrate significant differences. Investigations of interstrand crosslink formation in which crosslinking of a short DNA fragment is detected by gel electrophoresis under denaturing conditions demonstrate that interstrand crosslinks are 250 fold more frequent among bis(platinum) adducts than among cisplatin-derived adducts under the conditions examined. These investigations indicate that bis(platinum) adducts contain a high frequency of structurally novel interstrand crosslinks formed through binding of the two platinum centers to opposite DNA strands. Unlike cisplatin, bis(platinum) complex binding does not unwind supercoiled DNA. Studies with the *E. coli* UvrABC nuclease complex demonstrate that both linear and supercoiled DNA containing bis(platinum) adducts are subject to incision by the repair enzyme complex. Initial studies using UvrABC nuclease as a probe to define the base and sequence specificity for bis(platinum) complex binding suggest that the specificity of the bis(platinum)s is similar, but not identical, to that of cisplatin.

INTRODUCTION

The cytotoxic effects of cisplatin ([*cis*-diamminedichloroplatinum(II), *cis*-DDP, *cis*-$[PtCl_2(NH_3)_2]$), an important anticancer agent, are thought to result from binding of the bifunctional *cis*-diammine(Pt) group to DNA (reviewed in 1,2,3). The final adducts which result are crosslinks of three types: intrastrand, interstrand, and DNA-protein. The most common adduct is an intrastrand crosslink bridging adjacent guanines bound at the N(7) position (4,5,6,7). Interstrand crosslinks represent 2% or less of total adducts (8,9). The relative contribution of intrastrand and interstrand crosslinks toward cytotoxic and therapeutic effects of cisplatin is a topic of some controversy and the subject of ongoing investigation. The presence of DNA-protein crosslinks correlates poorly with cytotoxic effects (10). Each of these adduct types is subject to removal by DNA repair enzymes (11,12).

Two significant drawbacks of cisplatin are that it has limited activity against many common human cancers (13) and that it is susceptible to the phenomenon of acquired drug resistance. Preclinical models of the acquired resistance phenomenon suggest it to be multifactorial, with one factor being enhanced repair activity (12,14).

Considerable effort has been expended to discover new platinum complexes which may be complementary or superior to cisplatin in the clinic (15). Nearly all the products of this effort represent variations upon the structure of cisplatin in which identical amines

(or a complex diamine) are coordinated to platinum in a *cis* configuration. Much emphasis has been placed upon complexes containing a 1,2-diaminocyclohexane ligand as such complexes are active *in vitro* and *in vivo* against cell lines rendered resistant to cisplatin (16). It is likely, however, that the adducts formed by all *cis*-diamine(Pt) analogs are similar to those of cisplatin (17), and it remains to be demonstrated that such analogs are complementary or superior to cisplatin in the clinic.

One of us has previously reported the synthesis of a novel series of bis(platinum) complexes [[{*cis*-$PtCl_2(NH_3)$}$_2H_2N(CH_2)_nNH_2$], bis(Pt)s] in which two *cis*-diammine(Pt) groups are linked by an alkyldiamine of variable length (Figure 1)(18). This series represents a significant departure from previous platinum chemistry efforts. Bis(platinum) complexes are potentially tetrafunctional and so are unique as compared with all known anticancer agents. Bis(platinum) complexes are remarkable as they are potent cytotoxic agents against wild type L1210 cells as well as against L1210 rendered resistant to either cisplatin or 1,2-diaminocyclohexanesulfato(platinum)II (19). Further, the complexes are therapeutically active against both wild type and cisplatin-resistant L1210 and P388 in murine tumor model(20). Such activity *in vitro* and *in vivo* is unprecedented for platinum complexes.

In this report we demonstrate that the products of bis(platinum)-DNA interaction differ significantly from those of cisplatin with regard to 1) structure and frequency of interstrand crosslink formation, and 2) effects upon supercoiled plasmid tertiary structure. Further, it is shown that, like those of cisplatin, bis(Pt) adducts are subject to incision by *E. coli* UvrABC nuclease complex (ABC), and that this complex may be used as a probe for the characterization of bis(Pt) adduct structures. As chloride salts of the bis(platinum)s are very sparingly water soluble, these investigations were undertaken with bis(malonate) substituted bis(Pts) [{$Pt(mal)(NH_3)$}$_2H_2N(CH_2)_nNH_2$]. Variations in anionic ligands (e.g., the chlorides of cisplatin) affect chemical and pharmacokinetic properties, which, in turn, may affect organ directed toxicities and therapeutic index (21), but there is no evidence which suggests that these variations affect the structure of stable adducts (22) or the spectrum of susceptible cancers (23).

MATERIALS AND METHODS

Platinum complexes, plasmids, and enzymes. Bis(Pt)s as chloride salts were synthesized as previously reported (18). The complexes [{$Pt(mal)(NH_3)$}$_2H_2N(CH_2)_nNH_2$] were prepared by reaction of the parent dichlorides as a suspension in H_2O with two equivalents of silver malonate dissolved in H_2O. The solution, which becomes clear upon stirring overnight, is filtered and evaporated to dryness. The off-white solid is recrystallised by redissolving in H_2O, addition of ethanol, and cooling at 3°C. Elemental analysis and spectroscopic data (IR, ^1H, and ^{195}Pt NMR) were fully consistent with the formulation. Cisplatin was purchased from Sigma. Platinum complexes were stored as 1mM stock solutions in Tris-HCl 10mM (pH 8.0)/ EDTA 0.1mM at 4°C.

pBR322, purified in the absence of cesium chloride or ethidium bromide in order to minimize DNA damage, was purchased from Applied Genetics, Inc. pUC12 was obtained from N. Heintz (University of Vermont), propagated under standard conditions, and the supercoiled form purified in cesium chloride gradients (24). This plasmid is a dimer of 5.3Kb which upon digestion with EcoR I migrates as 2.6Kb during electrophoresis in agarose gels.

The UvrA, UvrB, and UvrC subunits were purified from the *E. coli* overproducer strain CH296 containing the plasmids pUNC45, pUNC211, or pDR3274, respectively (obtained

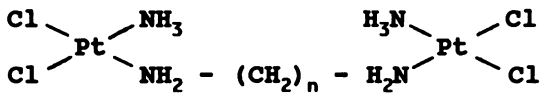

Figure 1. Structure of bis(platinum) complexes in which two *cis*-diamine(platinum) groups are linked by an alkyldiamine of variable length (n = 4, 5, and 6). Malonate complexes are derived by substitution of each pair of *cis* chlorides with a bidentate CH_2 $(COO)_2$ group.

from A. Sancar, University of North Carolina) essentially as described by Thomas (25).

Platinum complex binding to DNA. Each experiment began with reactions involving platinum complexes and DNA performed in the following standard fashion. Platinum complexes and DNA were incubated in Tris-HCl 10mM (pH 7.5)/ EDTA 0.1mM (TE) for one hour at 37°C. Further reaction was inhibited by addition of NaCl (final concentration 50mM). Unbound platinum complexes were removed by dialysis against TE performed in a microdialysis block chilled in an ice-water slurry (volumes less than 30μl) or against water at 4°C (volumes greater than 30μl). Platinated DNA was stored at −20°C pending further analysis.

Platinum complex reactivity with DNA. Platinum complexes were bound to calf thymus DNA (4μg/ml) (Worthington) as described above (with the exception that final NaCl concentration for reaction inhibition was 200mM) in a reaction volume of 1.2ml. Final DNA concentrations were measured by fluorescence after reaction with Hoescht 33258 dye (26). Final (bound) Pt concentrations were measured by flameless atomic absorption spectroscopy (FAAS; Perkin Elmer 560 AAS, HGA 500 programmer and AS40 autosampler); samples requiring concentration prior to FAAS were partially digested with 2% nitric acid, concentrated by evaporation, and resuspended in nitric acid. Results for platinum complex binding are expressed as platinum complexes per nucleotide (r_b) assuming an average molecular weight for nucleotides in DNA of 330.

Interstrand crosslink assay. The interstrand crosslink assay was performed with the (Dde I-EcoR $I)_{65}$ restriction fragment of pBR322 uniquely 5′ labelled at the EcoR I restriction site with [*gamma*-^{32}P]-ATP using standard methods (24). The fragment (approximately 1−5ng per reaction) was mixed with calf thymus DNA (4μg/ml) prior to platination performed as described above in a reaction volume of 20μl. The DNA was concentrated by evaporation, mixed in formamide with tracking dyes, denatured by heating, and subjected to electrophoresis in Tris-borate-EDTA buffer through an 8% polyacrylamide sequencing gel containing 8M urea (24). The dried gel was exposed to x-ray film (Kodak XAR) for 1 to 7 days. Selected autoradiograms were scanned with a densitometer (Zeineh Soft Laser Model SL-TRFF, Biomed Instruments). From the r_b estimates as previously described, the fragment size, and the non-crosslinked fragment fraction as determined by scanning densitometry, the frequency of interstrand crosslinks as a function of total platinum complex bound was calculated using the Poisson distribution.

Interhelical crosslink assay. A approximately equimolar mixture of linearized pUC12 12μg/ml and the (EcoR I−Hae $III)_{174}$ restriction fragment of pBR322 uniquely 5′labelled at the EcoR I restriction site was platinated as described above in a reaction volume of 20μl. Aliquots were either analyzed by electrophoresis under denaturing conditions as described above or mixed in glycerol with tracking dyes and subjected to electrophoresis in an 0.8% agarose gel in Tris-borate-EDTA buffer (24). Location of the pUC12 plasmid in gels was visualized by fluorescence under UV irradiation after staining with ethidium

bromide; subsequently, the gel was dried and subjected to autoradiography, revealing the location of the labelled fragment.

UvrABC nuclease plasmid incision assay. pUC12 plasmid 16μg/ml was platinated as described above in a reaction volume of 80μl containing platinum complexes at a concentration of 25μM. Plasmid subsequently was incubated with UvrABC nuclease subunits (UvrA 1.0 pmol, UvrB 7.4 pmol, and UvrC 2.6 pmol) in KCl 50mM/ Tris-HCl 50mM (pH 7.5)/ $MgCl_2$ 10mM/ ATP 2mM/ dithiothreitol 5mM/ bovine serum albumin 50μg/ml (ABC buffer) in a final volume of 80μl for 5 to 20 minutes. At various times portions were withdrawn and further reaction inhibited by the addition of tracking dyes in glyercol containing 0.1% SDS and 25μM EDTA; control (nondamaged) plasmid was incubated in ABC buffer with UvrABC subunits for 20 minutes. The products of reaction were subjected to electrophoresis through an 0.8% agarose gel, and blotted onto a nylon membrane by the method of Southern (27). The membrance was probed with radiolabelled pUC12 and subjected to autoradiography.

UvrABC nuclease fragment incision assay. The (EcoR I−Hae III)$_{174}$ restriction fragment (15−50ng) of pBR322 uniquely labelled at the EcoR I restriction site was platinated as described above or UV irradiated to 3000 J/m^2 at 254nm. The fragment was then incubated with UvrABC nuclease (UvrA 1.0 pmol, UvrB 7.4 pmol, and UvrC 2.6 pmol) in a reaction volume of 56μl for 30 minutes. The reaction products were rapidly frozen, lyophilized to dryness, and resuspended in formamide with tracking dyes. Aliquots were subjected to electrophoresis in 8.0% polyacrylamide sequencing gels containing 8.0M urea. In order to have markers for incision sites, the fragment was sequenced using the Maxam-Gilbert chemical degradation reactions (A+G, G, T+C, and C) (28) and subjected to electrophoresis with the products of incision reactions. The gels were dried and imaged by autoradiography. The relative amount of each incision in incision clusters specific to platination damage (see Figure 7) was measured by scanning densitometry.

RESULTS

From structural considerations it is reasonable to propose that bis(platinum) complexes form novel interstrand crosslinks in which the two platinum centers are bound to opposite strands of DNA. An experiment was undertaken to compare the frequency of interstrand crosslink formation by bis(Pt)s and cisplatin following a short incubation with DNA. The (Dde I−EcoR I)$_{65}$ fragment of pBR322 was incubated with bis(Pt)s of n = 4, 5, and 6 or cisplatin (each at 25μM), or exposed to UV irradiation (3000 J/m^2 at 254nm), and subjected to electrophoresis under denaturing conditions (Figure 2). Under these conditions, non-crosslinked 5'labelled fragments migrate as 65b single strands whereas crosslinked fragments migrate as a higher (approximately 2×) molecular weight species. Crosslinked fragments are apparent after reaction with each of the bis(Pt)s under these conditions (lanes 4−9), whereas crosslinking is not apparent after reaction with cisplatin (lanes 2 and 3) or in the UV-irradiated (lane 1) fragments. In order to demonstrate that this reflects a difference in the frequency of interstrand crosslink formation rather than differences in reactivity of the complexes, it was necessary to quantitate total adduct formation. The bis4(Pt) complex was selected for further study.

Binding of Bis4(Pt) to DNA.

The detection limit of FAAS for Pt (approximately 0.5ng) renders direct measurement of platinum complex binding to a labelled DNA fragment impractical. In order to compare reactivity of bis4(Pt) and cisplatin with DNA, calf thymus DNA (4μg/ml) was incubated

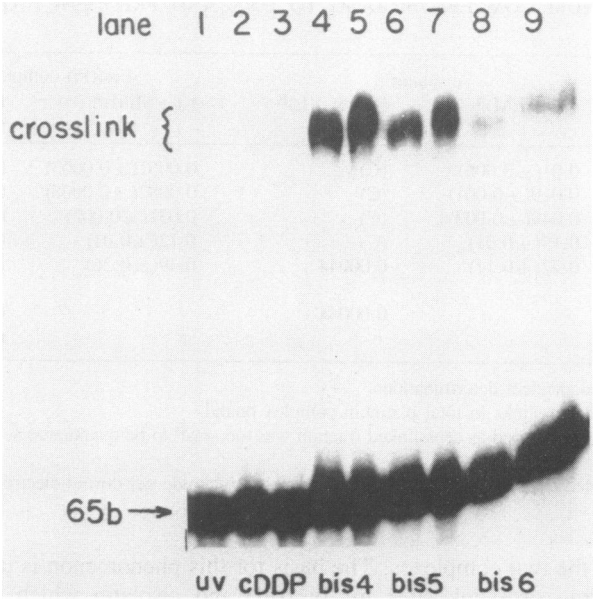

Figure 2. Crosslinking of the (Dde I−EcoR I)$_{65}$ fragment. The fragment was uniquely 5′ labelled at the EcoR I restriction site and exposed to UV irradiation (3000 J/m^2 at 254nm) (control, lane 1) or reacted with a platinum complex at 25μM (cisplatin [cDDP], lanes 2 & 3; bis4(Pt), lanes 4 & 5; bis5(Pt), lanes 6 & 7; bis6(Pt), lanes 8 & 9) prior to electrophoresis upon a polyacrylamide sequencing gel. Crosslinked fragments, apparent as a higher molecular weight species, are present after incubation with each of the bis(Pt)s, but not after cisplatin or UV irradiation.

with platinum complexes for 1 hour at 37°C, unbound complexes were removed by dialysis, and platinum complex binding was determined by measurement of the final concentrations of DNA (by fluorescence after binding of Hoescht 33258) and platinum (by FAAS). Inspection of results for DNA quantitation indicated inhibition of fluorescence after addition of Hoescht dye especially for bis4(Pt) at $r_b > 2\times10^{-2}$; accordingly, final DNA concentrations for samples of lower r_b were averaged and assumed to apply to other samples handled identically in each group. Results are expressed as platinum complexes bound per nucleotide in DNA (r_b) (Table 1). Binding of bis4(Pt) was approximately 40−80% that of cisplatin.

Interstrand Crosslink Formation by Bis4(Pt).

In order to compare the frequency of interstrand crosslink formation of bis4(Pt) and cisplatin, the interstrand crosslink assay was repeated with a mixture of calf thymus DNA and labelled fragment so that platinum complex binding values as previously determined could be used to calculate the number of adducts per labelled fragment. The (Dde I−EcoR I)$_{65}$ fragment (1 to 5ng) mixed with calf thymus DNA (4μg/ml) was incubated with platinum complexes in concentrations ranging from 1μM to 256μM, and the interstrand crosslink assay performed upon the reaction products as described above (Figure 3). Inspection of the autoradiogram reveals an apparent difference in crosslinking throughout the range of concentrations tested. It is notable that the crosslinked fragments have migrated as a broad smear within which there are two discernible bands. Distribution of crosslinked fragments within these two

TABLE 1. PLATINUM COMPLEX BINDING TO DNA AND FREQUENCY OF INTERSTRAND CROSSLINKS.

concentration (μM)	cisplatin r_b(±SEM)[A]	cisplatin F(inter XL)[B]	bis4(Pt) complex r_b(±SEM)[A]	bis4(Pt) complex F(inter XL)[B]
1	0.01(±0.006)	(C)	0.0051(±0.0003)	0.046
4	0.019(±0.001)	(C)	0.0082(±0.0008)	0.21
16	0.049(±0.002)	(C)	0.031(±0.002)	0.135
64	0.15(±0.01)	(C)	0.12(±0.01)	0.054
256	0.72(±0.11)	0.00044	0.49(±0.26)	(D)
mean F(inter XL) (±SEM)		0.00044		0.11 (±0.04)

[A] Results of three independent determinations.
[B] Ratio of interstrand crosslinks to total platinum complex bound.
[C] Value could not be determined as crosslinked fraction was too small to be quantitated accurately by scanning densitometry.
[D] Value not determined as DNA sample did not enter polyacrylamide gel during electrophoresis.

bands differs for the two complexes. The basis for this phenomenon is uncertain. It may be due to differing crosslink sites for bis4(Pt) and cisplatin which would result in conformational heterogeneity among the crosslinked fragments. In addition, the tetrafunctional bis4(Pt) would carry a positive charge for each unbound coordination site which would result in reduced electrophoretic mobility. The autoradiogram was scanned densitometrically to obtain estimates of the fraction of non-crosslinked fragment under each condition. From the fraction of non-crosslinked fragment, r_b values, and the fragment size, the frequency of interstrand crosslinks as a function of total complex bound was calculated using the Poisson distribution (Table 1). From these calculations the frequency of interstrand crosslinks after cisplatin is 0.00044, whereas the average frequency of crosslinks after bis4(Pt) is 0.11. Thus, bis4(Pt) creates interstrand crosslinks 250 times more frequently than cisplatin under identical conditions of these experiments.

Assay for Interhelical Crosslink Formation.

An unexplained finding of the fragment crosslinking assay was the failure of highly bis4(Pt)-modified fragment to enter the gel during electrophoresis (see Figure 3, lanes marked bis4(Pt) 64μM and 256μM). Such a phenomenon might be observed if a bis(Pt) adduct may form an interhelical crosslink, that is, a crosslink between two DNA duplexes which are separate fragments or remote regions of the same molecule. Extensive interhelical crosslinking of mixture of DNA fragments would result in a DNA matrix of high molecular weight. Such structures have been hypothesized for cisplatin(29), but never demonstrated (1). In order to test this possibility, the labelled $(\text{EcoR I}-\text{Hae III})_{174}$ fragment and linearized pUC12 were incubated with platinum complexes and the reaction products analyzed by gel electrophoresis. Autoradiography of samples run on a polyacrylamide sequencing gel (not shown) confirmed that bis4(Pt) incubation had created interstrand crosslinks and that a fraction of the fragment had failed to enter the gel at the highest concentration tested as previously observed. Migration of linearized pUC12 through a native agarose gel was detected by flouresence under UV light after staining of the gel with ethidium bromide; subsequently, autoradiography of the dried gel was performed to localize

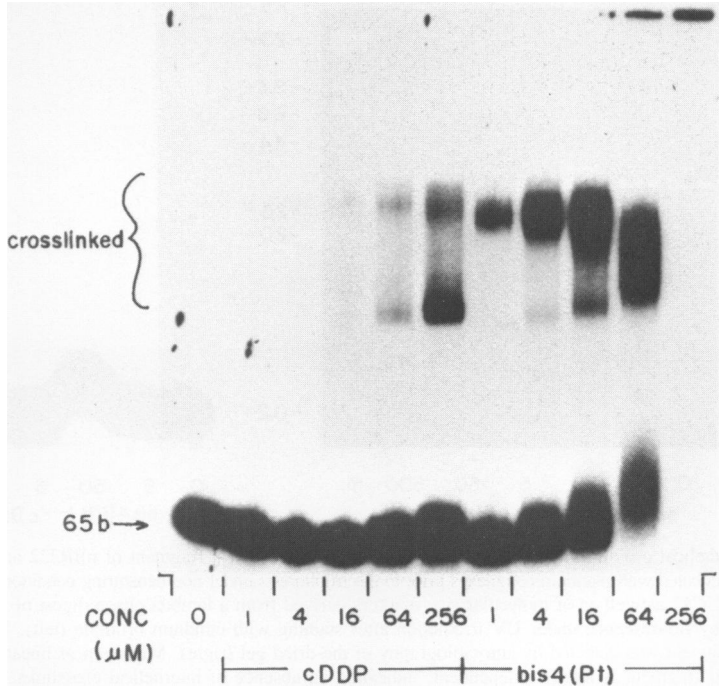

Figure 3. Quantitation of interstrand crosslinks induced by bis4(Pt) and cisplatin. A trace amount (1−/ng) of labelled (Dde I−EcoR I)$_{65}$ fragment in the presence of calf thymus DNA (4μg/ml) was incubated with the indicated concentration of bis4(Pt) or cisplatin prior to electrophoresis through a polycarylamide sequencing gel.

the labelled fragment (Figure 4). No discernible fraction of the labelled fragment was found to migrate with the linearized plasmid, and so, under these conditions, interhelical crosslink formation was not observed. It is notable that high bis4(Pt)-modification (see lane marked bis4(Pt) 50μM) slows migration of both fragment and linearized plasmid DNA during electrophoresis under native conditions. A reasonable explanation for this is that positive charges present when bis(Pt) complexes are bound to DNA at less than four sites alter electrophoretic mobility.

UvrABC Nuclease Incision of Bis4(Pt)-damaged Plasmid.

The UvrABC nuclease is an enzyme complex isolated from *E. coli* which is capable of recognizing diverse types of DNA damage and initiating DNA repair by producing single strand incisions flanking a damage site (reviewed in 30). Incision usually occurs at the 8th phosphodiester bond 5′ and the 4th or 5th phosphodiester bond 3′ from the site of a damaged base, although variant incisions sites have been noted (30,31). *In vivo* ABC damage recognition and single strand incision is followed by processes mediated by helicase II, pol I and ligase which release a single strand oligo fragment containing the damage, replace the excised nucleotides by template-directed synthesis, and reseal the repaired region (30). Monoadducts, bifunctional intrastrand adducts, and interstrand adducts are recognized by ABC (30). In the case of asymmetric interstrand adducts, such as a psoralen diadduct, ABC incisions may be limited to one strand of DNA (32,33,34). It has been proposed

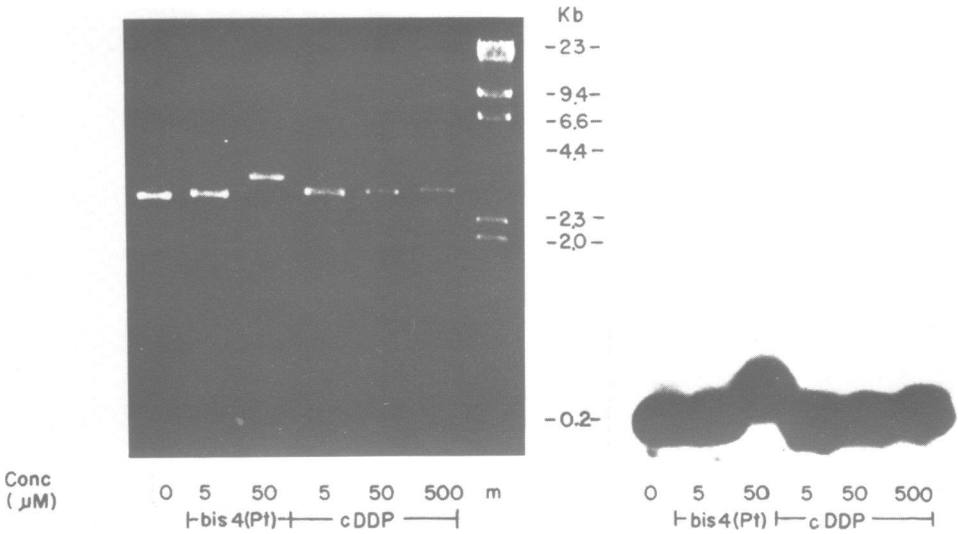

Figure 4. Interhelical crosslink assay. The labelled (EcoR I− Hae III)$_{174}$ fragment of pBR322 and linearized pUC12 were incubated with platinum complexes prior to electrophoresis under non-denaturing conditions. Migration of linearized pUC12 as well as of molecular size markers derived from a lambda phage digest of Hind III (m) were detected by flourescence under UV irradiation after staining with ethidium bromide (left). Migration of the labelled fragment was detected by autoradiography of the dried gel (right). Migration of linearized pUC12 and the labelled fragment is totally independent, indicating an absence of interhelical crosslinks.

that ABC recognition of DNA damage is mediated by binding, not to the adduct *per se*, but rather to distortions in DNA tertiary structure induced by an adduct (30,32). ABC has been used as a probe to map the position of specific DNA adducts by monitoring either 5′ or 3′ incision sites (35,36,37,38,39). Previous investigations have demonstrated that ABC is capable of recognizing cisplatin-induced damage in both circular plasmid and linear DNA (40,41). Of interest, it has been reported that ABC is capable of recognizing damage induced by *trans*-DDP in circular, but not linear, DNA (40).

The basis of the plasmid incision assay is that single strand incisions produced in a damaged plasmid by UvrABC nuclease will convert supercoiled plasmid (form I) to open circle (form II). In order to determine whether ABC could recognize damage induced by bis(Pt)s in supercoiled plasmid, pUC12 was platinated as previously described with platinum complex concentrations of 25μM. Assuming that platinum binding observes first order kinetics as a function of platinum complex concentration for r_b values < 0.05 and extrapolating from platinum binding observed under the previously described conditions, the calculated number of complexes bound per plasmid following this incubation is 17 for bis4(Pt) and 28 for cisplatin. Damaged plasmid was incubated with ABC for 5 to 20 minutes. The products of reaction were subjected to electrophoresis through a 0.8% agarose gel and blotted to a nylon membrane. The membrane was probed with radiolabelled pUC12 and imaged by autoradiography (Figure 5). Inspection of the autoradiogram reveals ABC incision of both bis4(Pt)- and cisplatin-damaged plasmid with similar efficiency and kinetics. Note that treatment of plasmid with platinum complexes alone did not cause strand breaks (see Figure 6).

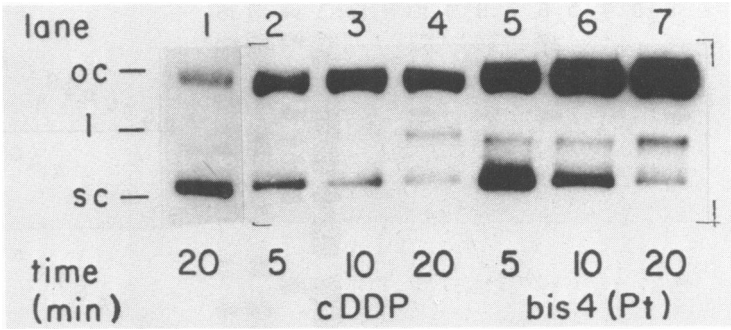

Figure 5. UvrABC nuclease plasmid incision assay. pUC12 plasmid, 70% supercoiled (sc) and 30% relaxed circular form (oc), was incubated with bis4(Pt) or cisplatin (25μM). Damaged plasmid was then incubated for 5, 10, or 20 minutes, and control (nondamaged) plasmid for 20 minutes the UvrABC nuclease. Conversion from supercoiled to open circle form occurs as ABC creates single strand incisions flanking sites of platinum complex damage; formation of a small amount of linear (1) form may be due to overlapping ABC incisions on opposite strands yielding a double strand break. Reaction products were electrophoresed through an 0.8% agarose gel and blotted by the method of Southern to a nylon membrane; the membrane was probed with radiolabelled pUC12 and subjected to autoradiography.

During the course of these studies differences in the effects of bis(Pt) and cisplatin binding upon the tertiary structure of supercoiled plasmids were observed. Binding of both cisplatin and *trans*-DDP to supercoiled plasmid induces unwinding of the double helix resulting in the loss of negative supercoils and, consequently, decreased electrophoretic mobility in agarose gels approaching as a limit the mobility of relaxed circular DNA (42). It is of interest, then, that bis4(Pt) binding induces no discernible change in the electrophoretic mobility of supercoiled plasmid (Figure 6), suggesting that the structure of bis4(Pt) adducts may stabilize, rather than unwind, supercoiled plasmid.

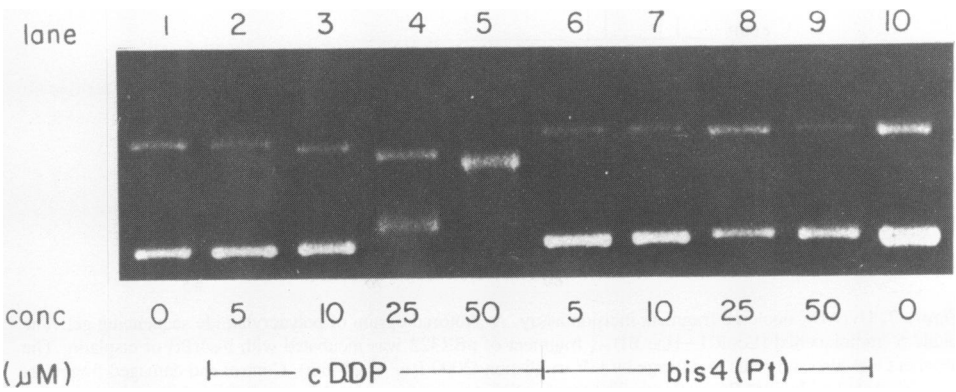

Figure 6. Electrophoresis of supercoiled plasmid after reaction with bis4(Pt) and cisplatin. pUC12 plasmid, 70% supercoiled and 30% relaxed circular form, was incubated with bis4(Pt) or cisplatin, and the reaction products were subjected to electrophoresis through an 0.8% agarose gel. The gel was stained with ethidium bromide and photographed under UV irradiation.

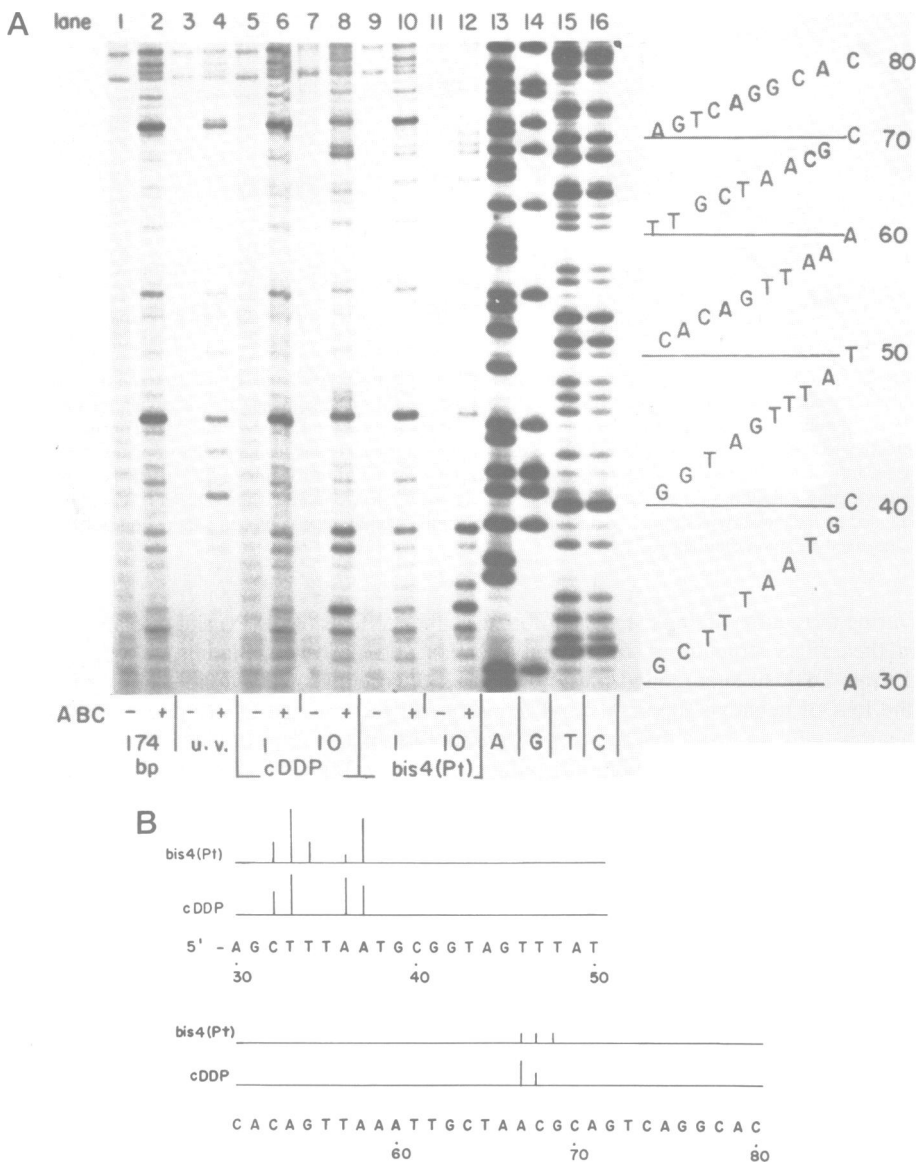

Figure 7. UvrABC nuclease fragment incision assay. A: Autoradiogram of polyacrylamide sequencing gel. The single 5′-radiolabelled (EcoR I−Hae III)$_{174}$ fragment of pBR322 was incubated with bis4(Pt) or cisplatin. The fragment was also subjected to damage by UV irradiation (3000 J/m^2 at 254nm). Control and damaged fragments were incubated with UvrABC nuclease. The fragment also was subjected to Maxam-Gilbert chemical degradation reactions (A, G = A + G, T, C = T + C). The digestion products were electrophoresed under denaturing conditions. B: Histogram of densitometric scanning of the autoradiogram (lanes marked cDDP 10uM and bis4(Pt) 10μM). Only ABC cutting sites specific to bis4(Pt) and cisplatin are shown; marker height represents relative frequency for each incision site within each of the two incision clusters (bp 32−37 and 67−69) which were scanned independently.

UvrABC Incision of Bis4(Pt)-damaged Fragment and Base and Sequence Specificity of Bis4(Pt) Binding to DNA.

Information concerning base and sequence specificity of cisplatin binding to DNA has been developed from two sorts of experimental techniques: stop site analysis of exonuclease digestion of platinated fragments (43); and separation and identification of platinum-base, platinum-nucleoside, or platinum-nucleotide complexes derived from digests of platinated DNA (5,6,44,45). We were interested in approaching this problem with UvrABC nuclease and comparing specificities of the bis(Pt)s with cisplatin.

The (EcoR I-Hae III)$_{174}$ restriction fragment of pBR322 uniquely 5′ labelled at the EcoR I restriction site was damaged with platinum complexes. The fragment also was damaged by irradiation with UV light (3000 J/m^2 at 254nm). Control and damaged fragments were incubated with UvrABC nuclease. The fragment also was subjected to Maxam-Gilbert sequencing reactions. The reaction products were electrophoresed through a sequencing gel which was dried and subjected to autoradiography (Figure 7A). The ABC incision sites and relative incision frequencies specific for damage by each platinum complex are indicated in the histogram (Figure 7B) derived from a densitometric scan of the autoradiogram. The two clusters of specific incision sites (bases 32−37 and 67−69) were scanned independently. Inspection of the autoradiogram reveals that during the preparation and handling of these fragments some unintended DNA damage occurred as indicated by the background ABC incision pattern apparent in all fragment preparations. These incisions are not due to a protein contaminant as they occurred only upon the addition of all three subunits (Figure 7A, lanes 1 and 2) and only in the presence of ATP (data not shown). ABC has been found to recognize certain types of oxidative DNA damage, including thymine glycols (46), which could have arisen upon reaction of thymines with the decay products of the ^{32}P isotopic label. The pattern of damage recognition by ABC after UV irradiation in this experiment (analyzed for bases 21 through 80) is identical to that previously reported (35).

From the UvrABC nuclease incision pattern several observations can be made: First, ABC incises linear DNA containing bis4(Pt) damage. Second, the presence of six common incision sites suggests that the base and sequence specificity of bis4(Pt) binding is quite similar to that of cisplatin. Third, two unique incision sites occur after bis4(Pt) binding. These may represent modification of additional bases by the tetrafunctional complex, or an identical pattern of base damage by bis4(Pt) may lead to multiple ABC incision sites. It is apparent that complete characterization of adducts derived from a bis(Pt) will be a complex task. Future experiments with ABC using both 5′ and 3′ labelled fragments, perhaps in conjunction with exonuclease digestion experiments, will contribute to characterization of the adduct structures of these interesting complexes.

DISCUSSION

The most common adduct structure resulting from interaction of the anticancer agent cisplatin with DNA is an intrastrand diadduct bridging adjacent guanines through the N(7) positions. Binding of *cis*-diamine(Pt) to the dinucleotide d(GpG) results in major conformational changes (47). This lesion appears to induce in the DNA helix an approximate 40° bend oriented toward the major groove (48). Current evidence suggests that this adduct constitutes a block to both replication and transcription (49,50).

Synthesis of the bis(platinum) complexes was motivated by the premise that the conformational distortion induced in DNA by two Pt atoms bound in close proximity might

be a more cytotoxic lesion as compared with that derived from cisplatin. This molecular approach to modification and enhancement of Pt-DNA interactions may result in new complexes of clinical use and in new insights toward further rational drug development. Studies of the interaction of the bis(Pt)s with DNA were initiated in order to gain insights concerning mechanisms of biological activity of these complexes. Several important conclusions can be made on the basis of investigations reported here.

Studies of platinum complex binding to DNA after 1 hour at 37°C indicate that binding of bis(malonate)bis4(Pt) is 40% to 80% that of cisplatin. This result may be compared with previously published investigations of binding, as measured by inhibition of restriction enzyme activity, in which the bis4(Pt) complex was more active than cisplatin and, within the bis(Pt) series, activity was in the order $n = 4 > 5 > 6$ (18). In the previously reported experiments chloride salts of bis(Pt)s were the subject of investigation rather than bis(malonate)s. Chloride complexes are significantly more reactive than analogous malonate-substituted complexes, and further comparison will require quantitation of binding of chloride complexes.

Results from the interstrand crosslinking assay indicate that interstrand crosslinks represent approximately 10% of total adduct structures created by bis4(Pt); this value is 250 fold greater than the frequency of interstrand crosslinks among cisplatin-derived adducts. The estimate for frequency of cisplatin interstrand crosslinks in this report is much lower than previously reported estimates ranging from 0.007 to 0.02 (3,51). Thus, the interstrand crosslink frequency for bis4(Pt) after a 1 hour incubation exceeds the highest reported frequency for cisplatin interstrand crosslink formation by a factor of 5. It follows that in bis(Pt) adducts most interstrand crosslinks form through binding of the two platinum centers to opposite strands of DNA. Molecular models confirm the ease of formation of this novel bis(Pt) crosslink which is depicted schematically (Figure 8). In a 'fully-extended' form the Pt-Pt distance of the bis(Pt)s varies from approximately 9.5Å ($n = 4$) to 12.0Å ($n = 6$) and, allowing for the Pt-N(7)-guanine bond distance of 2.0−2.1Å, it can be easily appreciated that a bis(Pt) lesion can span up to a four-base pair segment of DNA. We emphasize that Figure 8 is a schematic representation of a feasible interaction, and that interstrand crosslinking should not be considered to be limited to a four base pair segment.

Consideration of the mode and frequency of interstrand crosslink formation by the bis4(Pt)s in conjunction with their biological activities suggests that these complexes may be more similar to the alkylating agents and nitrosoureas, clinically important classes of anticancer agents which form predominantly interstrand crosslinks (52), than to monomeric platinum complexes. This comparison is relevant to future chemical and pharmacological studies of these complexes.

We observed in the course of these investigations several unusual phenomena after bis(Pt) binding to DNA. DNA fragments highly modified with bis4(Pt) failed to migrate from the origin when subjected to electrophoresis in a polyacrylamide sequencing gel (Figure 3, lane 11). Such an effect might occur if bis4(Pt) produced interhelical as well as interstrand crosslinks resulting in the formation of a DNA matrix of high molecular weight. While specific evidence for interhelical crosslink formation was not observed in the experiment reported here (Figure 4), the possibility remains that such structures may be formed by bis(Pts). Absence of an unwinding effect (Figure 6) such as cisplatin induces upon binding to supercoiled DNA could be due to interhelical crosslinks located at twists of the supercoiled duplex after bis(Pt) binding.

Another interesting observation made in the course of these investigations is that binding

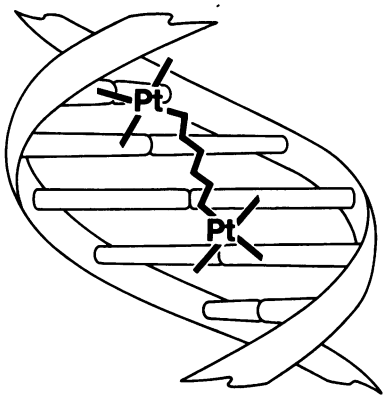

Figure 8. Schematic representation of a possible mode of bis(Pt) binding to a DNA segment. An interstrand crosslink is shown in which each of the platinum centers is bound in a bidentate manner to a single strand. The linkage depicted is the $NH_2(CH_2)_4NH_2$ unit.

of Hoescht 33258, which binds to the minor groove of DNA, is inhibited by bis4(Pt) to a much greater extent than by cisplatin. This may reflect distortion of the dye binding site, competition for binding sites on DNA, or an interaction between the dye and the platinum complex exclusive of DNA. Formation of ternary complexes between cisplatin-derived adducts and intercalating agents has been described (53,54,55) and, from chemical considerations, would appear quite likely with the tetrafunctional bis(Pt)s as well.

As repair of cisplatin-derived damage is a mechanism of acquired resistance to cisplatin demonstrable in preclinical systems (12,14), it is important to consider whether new platinum complexes are subject to DNA repair processes. The UvrABC nuclease complex derived from *E. coli* represents an enzyme system with which such questions may be addressed. Further, ABC may be used as a probe for the characterization of the base and sequence specificity of platinum complex binding. The present investigations demonstrate that ABC recognizes bis(Pt)-derived damage in both supercoiled and linear DNA. These studies also reveal common and unique incision sites for DNA damaged by bis4(Pt) as compared with cisplatin. These data suggest that, although the base and sequence specificity for DNA binding of these two agents is similar, bis(Pt) adducts either involve additional bases or otherwise produce other alterations in DNA tertiary structure which alter ABC incision patterns.

In conclusion, these studies demonstrate that there are significant differences in the products of interaction of the bis(platinum) complexes with DNA as compared with those of the clinically important agent cisplatin. Specifically, the frequency of interstrand crosslink formation as well as the presumed structure of interstrand crosslinks differs between the two complexes. Differential effects upon tertiary DNA structure are apparent in studies of supercoiled plasmids and suggested by effects upon the DNA binding agent Hoescht 33258. Nevertheless, as is the case with cisplatin, bis(Pt) adducts are recognized by the DNA repair enzyme complex UvrABC nuclease, and the feasibility of using this complex as a probe for investigation of the base and sequence specificity of bis(Pt) binding has been demonstrated. The relationship between these novel chemical features and their promising biological activities will be the subject of further investigations.

ACKNOWLEDGEMENTS.

We thank Mr. David Gaines for his technical assistance. This work was supported by NIH Grants CA01143 (JDR) and CA22435 (VRCC) and by a grant from the Vermont chapter of the American Cancer Society (BVH).

*To whom correspondence should be addressed.

Studies of DNA interactions were performed in the laboratory of BVH.

REFERENCES

1. Sherman, S.E., and Lippard, S.J. (1987) Chem. Rev. 87, 1153–1181.
2. Eastman, A. (1987) Pharmac. Ther. 34, 155–166.
3. Roberts, J.J., and Friedlos, F. (1987) Pharmac. Ther. 34, 215–246.
4. Pinto, A.L. and Lippard, S.J. (1984) Biochim. Biophys. Acta 780, 167–180.
5. Eastman, A. (1983) Biochemistry 22, 3927–3933.
6. Eastman, A. (1986) Biochemistry 25, 3912–3915.
7. Fichtinger-Shepman, A.M.J., van der Veer, J.L., den Hartog, J.H.J., Lohman, P.H.M., and Reedijk, J. (1985) Biochemistry, 24, 707–713.
8. Rahmouni, A. and Leng, M. (1987) Biochemistry 26, 7229–7234.
9. Roberts, J.J. and Friedlos, F.F. (1982) Chem.-Biol. Interactions 39, 181–189.
10. Zwelling, L.A., Anderson, T., Kohn, K.W. (1979) Cancer Res. 39, 365–369.
11. Pera, Jr., M.F., Rawlings, C.J., and Roberts, J.J. (1981) Chem.-Biol. Interactions 37, 245–261.
12. Eastman, A. and Schulte, N. (1988) Biochemistry 27, 4730–4734.
13. Loehrer, P.J., and Einhorn, L.H. (1984) Ann. Int. Med. 100, 704–713.
14. Masuda, H., Ozols, R.F., Lai, G.-M., Fojo, A., Rothenberg, M., and Hamilton, T. (1988) Cancer Res. 48, 5713–5721.
15. Farrell, N. (1989 in press) In, James, B.R. and Ugo, R. (eds) Catalysis by Metal Complexes, Reidel-Kluwer, Dordrecht, Holland, Chapter 2.
16. Burchenal, J.H., Kalaher, K., O'Toole, T., and Chisholm, J. (1977) Cancer Res. 37, 3455–3457.
17. Eastman, A., Biochemistry 25, 3912–3915.
18. Farrell, N.P., de Almeida, S.F., and Skov, K.A. (1988) J. Am Chem. Soc. 110, 5018–5019.
19. Farrell, N., Qu, Y., and Hacker, M.P. (1989) Proc. AACR 30, 462.
20. Kraker, A., Elliott, W., Van Houten, B., Farrell, N., Hoeschele, J., Roberts, J. (1989) J. Inorg. Biochem. 36, (3–4) 160.
21. Harrap, K.R. (1985) Cancer Treatment Reviews 12 (Supplement A), 21–33.
22. Knox, R.J., Friedlos, F., Lydall, D.A., and Roberts, J.J. (1986) Cancer Res. 46, 1972–1979.
23. Canetta, R., Bragman, K., Smaldone, L., and Rozencweig, M. (1988) Cancer Treatment Reviews 15 (Supplement B) 17–32.
24. Maniatis, R., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
25. Thomas, D.C., Levy, M. and Sancar, A. (1985) J. Biol. Chem. 200, 9875–9883.
26. Labarca, C., and Paigen, L. (1980) Anal. Biochemistry 102, 344–352.
27. Southern, E. (1975) J. Mol. Biol. 98, 503.
28. Maxam, A.M. and Gilbert, W. (1980) Methods in Enzymology 65, 499–560.
29. Macquet, J.-P. and Butour, L.-L (1978) Biochimie 60, 901–914.
30. Sancar, A., and Sancar, G.B. (1988) Ann. Rev. Biochem. 57, 29–68.
31. Myles, G.M., Van Houten, B., and Sancar, A. (1987) Nucl. Acids Res. 15, 1227–1244.
32. Van Houten, B., Gamper, H., Holbrook, S.R., Hearst, J.E., and Sancar, A. (1986) Proc. Natl. Acad. Sci. USA 83, 8077–8081.
33. Sladek, F., Munn, M.M., Rupp, W.D., and Howard-Flanders, P. (1989) J. Biol. Chem. 264, 6755–6765.
34. Jones, B.K. and Yeung, A.T. (1988) Proc. Natl. Acad. Sci. USA 85, 8410–8414.
35. Sancar, A., Franklin, K.A., Sancar, G.B., and Tang, M.S. (1985) J. Molec. Biol. 184, 725–734.
36. Selby, C.P. and Sancar, A. (1988) Biochemistry 27, 7184–7188.
37. Tang, M-S., Lee, C-S., Doisy, R., Ross, L., Needham-Vandevanter, D.R., and Jurley, L.H. (1988) Biochemistry 27, 893–901.
38. Yeung, A.T., Jones, B.K., Vapraro, M. and Chu, T. (1987) Nucl. Acids Res. 15, 4957–4971.

39. Walter, R.B., Pierce, J., Case, R., and Tang, M-S. (1988) J. Mol. Biol. 203, 939–947.
40. Beck, D.J., Popoff, S., Sancar, A. and Rupp, W.D. (1985) Nucl. Acids Res. 13, 7395–7412.
41. Popoff, S.C., Back, D.J., and Rupp, W.D. (1987) Mutation Research 183, 129–137.
42. Scovell, W.M. and Collart, F. (1985) Nucl. Acids Res. 13, 2881–2895.
43. Royer-Pokara, B., Gordon, L.K., and Haseltine, W.A. (1981) Nucl. Acids Res. 9, 4595–4609.
44. Fichtinger-Shepman, A.M., vander Veer, J.L., den Hartog, J.H.J., Lohman, P.H.M., and Reedijk, J. (1985) Biochemistry 24, 707–713.
45. Johnson, N.P., Mazard, A.M., Escalier, J., and Macquet, J.P. (1985) J. Am. Chem. Soc. 107, 6376–6380.
46. Kow, W., Wallace, S., and Van Houten, B. unpublished observations.
47. den Hartog, J.H.J., Altona, C., van Boom, J.H., Marcelis, A.T.M., van der Marel, G.A., Rinkel, L.J., Wille-Hazeleger, G., and Reedijk, J. (1983) Eur. J. Biochem. 143, 485–495.
48. Rice, J.A., Crothers, D.M., Pinto, A.L., and Lippard, S.J. (1988) Proc. Natl. Acad. Sci. USA 85, 4158–4161.
49. Pinto, A.L. and Lippard, S.J. (1985) Proc. Natl. Acad. Sci. USA 82, 4616–4619.
50. Eastman, A., Schulte, N., Sheibani, N., and Sorenson, C.M. (1988) In Nicolini, M. (ed). Platinum and Other Metal Coordination Complexes in Cancer Chemotherapy, Martinus Nijhoff Publishing, Boston, p. 178–196.
51. Rahmouni, A. and Leng, M. (1987) Biochemistry 26, 7229–7234.
52. Calabresi, P. and Parks, Jr., R.E. (1985) In Gilman, A.G., Goodman, L.S., Rall, T.W., and Murad, F. (eds). The Pharmacological Basis of Therapeutics, 7th edn, p. 1252.
53. Malinge, J.-M., Leng, M. (1986) Proc. Natl. Acad. Sci. USA 83, 6317–7321.
54. Malinge, J.-M., Schwartz, A., Leng, M. (1987) Nucl. Acids Res. 15, 1779–1797.
55. Sundquist, W.I., Bancroft, D.T., Chassot, L., Lippard, S.J. (1989) J. Am. Chem. Soc. 110, 8559–8560.

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.