Ethidium bromide changes the nuclease-sensitive DNA binding sites of the antitumor drug cis-diaminedichloroplatinum(II)

(intercalator/exonuclease III/DNA sequence determination/combination chemotherapy)

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Communicated by Nicholas J. Turro, March 8, 1982

ABSTRACT Exonuclease III has been shown previously to reveal the binding sites of the antitumor drug cis-diaminedichloroplatinum(II) on DNA. Pretreatment of the same DNA with the intercalator ethidium bromide causes new platinum binding sites to be detected by the exonuclease III method. In particular, a 5'-d(CpG-CpG)-3' sequence in a 165-base-pair restriction fragment of plasmid pBR322 becomes a preferred site for exonuclease III-detectable cis-diaminedichloroplatinum(II) binding. This switching of nuclease-sensitive platinum binding to new sites by the influence of another drug, ethidium bromide, offers an explanation at the molecular level for the phenomenon of synergism in combination chemotherapy.

cis-Diaminedichloroplatinum(II) (cis-DDP), a powerful antitumor agent, is generally used with other drugs in combination chemotherapy (1). There is much evidence that DNA is the biological target for cis-DDP (2) as well as for other anticancer drugs. We recently developed a method for determining the nuclease-sensitive positions of cis-DDP bound to a known sequence of DNA (3) by using exonuclease III (4) digestion along with DNA sequence determination techniques (5).

We now report that ethidium bromide, a compound known to bind to DNA by intercalation (6), can change the sites on a DNA molecule where the exonuclease III method detects covalent binding of cis-DDP. Intercalating drugs such as adriamycin are often used in chemotherapy with cis-DDP (1). Our results therefore offer a possible rationale at the molecular level for the synergism found for drugs used in combination with cis-DDP. We suggest that the DNA binding properties of the platinum drug might depend on the presence of another drug, in contrast to the more common explanation of combination chemotherapy which proposes independent action of two drugs—for example, at different phases in the cell cycle.

EXPERIMENTAL

Preparation of Labeled DNA. The singly end-labeled 165-base-pair (bp) DNA molecule (3) used in these experiments was prepared by digestion of plasmid pBR322 with the restriction endonuclease Hpa II (Bethesda Research Laboratories) and isolation of the second largest fragment, 527 bp, from a preparative agarose electrophoresis gel. Bacterial alkaline phosphatase (Bethesda Research Laboratories) was used to remove 5'-phosphates (5). The 5' ends were labeled with 32P by treatment with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-32P]ATP (Amersham; 3,000 Ci/mmol; 1 Ci = 3.7 × 1010 becquerels) (5). This doubly end-labeled molecule was digested with Hae III (Bethesda Research Laboratories), and the singly end-labeled 165-bp fragment was isolated by preparative polyacrylamide gel electrophoresis using the crush and soak technique of Maxam and Gilbert (5).

DNA Sequence Determination Reactions. The Maxam–Gilbert guanine-specific reaction (5) (dimethylsulfate/piperidine treatment) was performed on the singly end-labeled 165-bp DNA molecule. The reaction products were electrophoresed as described below in parallel with the products of exonuclease III digestion.

DNA–Drug Reactions. Samples were preincubated with ethidium bromide by addition of 1 μl of an aqueous solution of ethidium bromide at an appropriate concentration to 12 μl of labeled 165-bp DNA plus 1 μg of carrier salmon sperm DNA (Sigma) in 1 mM sodium phosphate/5 mM NaCl, pH 7.4. The concentration of ethidium bromide in stock solutions was determined by spectrophotometry. The reaction mixtures were incubated for 10 min at 37°C; then, 1-μl samples of aqueous cis-DDP solutions of the appropriate concentrations were added, and the mixtures were incubated for a further 3 hr. Drug-to-nucleotide ratios (D/N) for both ethidium bromide and cis-DDP were calculated relative to the amount of carrier DNA present in the reaction mixture. At the end of the incubation, NaCl was added to 100 mM and the mixtures were extracted with phenol. The aqueous layers were washed with ether and the DNA then was precipitated by addition of sodium acetate to 0.3 M and ethanol to 75% and chilling at −70°C. The suspensions were centrifuged at 12,000 × g for 5 min, and the supernatants were removed by aspiration. The pellets were redissolved in 0.3 M sodium acetate and again precipitated with ethanol. After washing with cold ethanol, the pellets were dried by lyophilization in a Speed Vac (Savant).

Enzyme Digestions. Exonuclease III (4) digestions were performed in 50 μl of pH 8.0 buffer consisting of 50 mM Tris-HCl, 5 mM MgCl2, 10 mM 2-mercaptoethanol. Escherichia coli exonuclease III (Bethesda Research Laboratories) was used at the ratio of 50 units per μg of carrier DNA in the reaction. Exonuclease III reactions were carried out for 1 hr at 37°C, and stopped by addition of EDTA to 20 mM and extraction with phenol. The aqueous layers were made 0.2 M in NaCN and incubated at 37°C for 3 hr to remove bound platinum prior to electrophoresis.

Electrophoresis. The DNA fragments were precipitated with ethanol, then redissolved in 80% (vol/vol) formamide/10 mM NaOH/1 mM EDTA/0.1% dyes, heated to 90°C for 3 min, and cooled in an ice bath. Aliquots (3 μl) were loaded onto prerun 8% polyacrylamide/7 M urea thin DNA sequencing gels (7). Electrophoresis was performed at 1,800 V. Gels were autoradiographed at −70°C on Kodak XAR-5 film with a Du Pont Lightning Plus intensifying screen. Densitometry of autoradiograms was performed with a Shimadzu CR-2000 microdensitometer.

The The Catalent Binding of cis-DDP to DNA was shown to be caused by the intercalation of the drug into the DNA helix. The binding of cis-DDP to DNA can be detected by the exonuclease III method, which reveals the sites of binding of the drug to DNA.

Abbreviations: cis-DDP, cis-diaminedichloroplatinum(II); bp, base pair; D/N, ratio of drug to nucleotide concentrations.

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RESULTS AND DISCUSSION

Our recent studies (3), as well as independent work by Haseltine and co-workers (8), used singly end-labeled DNA molecules of known sequence as substrates for platinum binding. Bound cis-DDP causes new products to appear in an exonuclease III digestion of the DNA. This enzyme has both 3' exonuclease and apurinic/apyrimidinic endonuclease activities. The sites of platination sensed by exonuclease III can be revealed by subjecting the digestion products to electrophoresis through a polyacrylamide/7 M urea DNA sequencing gel (5, 7). DNA fragments generated by the Maxam–Gilbert DNA sequence method (5) and electrophoresed in parallel are used as molecular "rulers" to identify the particular bases at which platination stops exonuclease III digestion. Both our experiments (3) and those of Haseltine and co-workers (8) showed that exonuclease III detects cis-DDP binding to oligo(dG) sites. This result is evident in lanes 1 and 8 of Fig. 1 where strong bands are seen corresponding to platinum binding at dG5, dG3, and dG2 sequences in a 165-bp DNA molecule. An anomaly in this experiment was apparent to us, however. There is a 5' d(G2-C-G2) 3' sequence 29 bases from the 3' end of this DNA molecule (9). Based on the postulated affinity of cis-DDP for oligo(dG) sequences (10–12) and the proximity of this site to the starting point of the 3'-exonucleolytic degradation of DNA by exonuclease III, we had expected a very strong band to be present corresponding to platination of the dG6 site. Yet only a relatively weak band was observed.

When the DNA molecule was incubated with ethidium bromide before the addition of cis-DDP, however, a dramatic change in the exonuclease III-digested platinum binding at the d(G6-C-G2) site was observed (lanes 2–5 and 9–12 in Fig. 1). The bands due to platination of the d(G6-C-G2) site became the

![Image of autoradiograph showing the effect of varying the ethidium bromide-to-nucleotide ratio on the position of cis-DDP binding to a 165-bp DNA molecule.](image-url)

**FIG. 1.** Autoradiograph of an 8% polyacrylamide/7 M urea electrophoresis gel showing the effect of varying the ethidium bromide-to-nucleotide ratio (ethidium bromide D/N) on the position of cis-DDP binding to a 165-bp DNA molecule. cis-DDP D/N: lanes 1–5, 0.01; lanes 6–12, 0.05. Ethidium bromide D/N: lanes 1 and 8, 0; lanes 2 and 9, 0.012; lanes 3 and 10, 0.057; lanes 4 and 11, 0.12; lanes 5 and 12, 0.23. Lanes 6 and 13 show Maxam–Gilbert G-specific reaction products (6). Lane 7 contained products of exonuclease III digestion of unplatinated 165-bp DNA. Electrophoresis was for 2 hr at 1,800 V.
major bands in the autoradiographic pattern not seen in control digestions (lane 7). Control experiments showed that treatment of unplatinated DNA with ethidium bromide and subsequent removal of the drug by phenol extraction and ethanol precipitation had no effect on the exonuclease III digestion pattern.

To determine in more detail the effect of ethidium bromide on the d(G6-C-G2) bands, we ran aliquots of these same samples on a gel giving higher resolution (Fig. 2). With no ethidium bromide present (lanes 1 and 8), either no bands or only weak bands appeared as a result of platination of the dG6 region. With approximately four ethidium bromide molecules bound to the 165-bp DNA molecule (ethidium bromide D/N = 0.012) (lanes 2 and 9), distinct bands due to platination of the dG2 site were evident. The dG6 site still showed little effect on exonuclease III digestion. With more ethidium bromide present in the platination reaction mixture (lanes 3–5 and 10–12), more cis-DDP binding to the dG6 region was detected and binding to the dG2 site appeared to decrease relatively. This result implies that the effect of ethidium bromide is not simply to increase the overall level of platinum binding. Independent studies on the quantitation of cis-DDP binding to plasmid pBR322 in the presence or absence of ethidium bromide have confirmed this conclusion (C. M. Merkel, personal communication).

A further characteristic of the ethidium bromide-promoted binding of cis-DDP to the d(G6-C-G2) sequence is clear in Fig. 3. This autoradiograph shows the results of an experiment in which the D/N of cis-DDP was varied from 0.005 to 0.01 to 0.05 at an ethidium bromide D/N of 0.17. The extremely high resolution of this gel reveals differences in intensity among the bands due to platination of the d(G6-C-G2) site. In particular, the peak of intensity is at the G band at the 3' end of the dG6 sequence. The intensities of the remaining bands fall off smoothly toward the 5' end.

What is the reason for this effect of ethidium bromide on the exonuclease III sensitive mode of binding of cis-DDP to DNA? Several recent experiments have shown that the structure of the DNA double helix is not uniform—it varies with base sequence. Rich and co-workers (13) have found that d(CpGpCpGp) crystallizes in a left-handed double-helical form, called Z-DNA, that is perhaps the most dramatic example of sequence-dependent DNA structure. The high-resolution x-ray crystallographic structure of the dodecanucleotide d(C-G-C-G-A-A-T-T-C-G-C-G) reported by Dickerson and Drew (14) shows another striking example of the variability of local DNA structure. Klug and co-workers have demonstrated that DNase I is sensitive to such structural variations (15). The rate of DNase I cutting at a given phosphodiester bond of the dodecanucleotide depends on the twist angle of the adjacent bases, as seen in the crystal structure (14). Other studies have reported sequence specificity in the digestion of DNA molecules by micrococcal nuclease (16, 17). In fact, such sequence specificity of enzymatic activity can be seen in our exonuclease III experiments. Control digestions (Fig. 1, lane 7) of unplatinated 165-bp DNA give rise to several products in a highly reproducible distribution. Clearly, DNA processing enzymes can be affected by the local structure of DNA, which depends on the sequence.

The present results can be explained by assuming that the DNA binding of the antitumor drug cis-DDP is sensitive to local DNA structure. Ethidium bromide, upon intercalation, is known to unwind the DNA helix by 26° per bound drug mol-

![Fig. 2. Autoradiograph of an electrophoresis gel showing the effect of ethidium bromide D/N on the position of cis-DDP binding to the 165-bp DNA molecule. Aliquots of the same samples used in the experiment of Fig. 1 were electrophoresed on an 8% polyacrylamide/7 M urea gel at 1,800 V for 4 hr. cis-DDP D/N: lanes 1–5, 0.01; lanes 8–12, 0.05. Ethidium bromide D/N: lanes 1 and 8, 0; lanes 2 and 9, 0.012; lanes 3 and 10, 0.057; lanes 4 and 11, 0.12; lanes 5 and 12, 0.23. Lane 6, Maxam–Gilbert G-specific reaction products (5). Lane 7 contained products of exonuclease III digestion of unplatinated 165-bp DNA.](image)
Ethidium bromide could bind selectively to certain regions of the DNA duplex (19), enhancing (or inhibiting) cis-DDP binding in the vicinity. Alternatively, bound ethidium bromide could change the mode of cis-DDP binding to DNA—for example, by switching the platinum from bidentate to monodentate coordination. Spectrophotometric experiments show that cis-DDP does not form a complex with ethidium bromide in solution (C. M. Merkel, personal communication).

The work we report here offers a rationale at the molecular level for the phenomenon of synergism in combination chemotherapy—namely, that one drug can switch the biochemically detectable binding site of another drug on its biological target. Further elaboration of this idea could lead to the rational design of new anticancer drugs.

We thank Engelhard Industries for a loan of K₂PtCl₄ used to prepare platinum complexes and the National Cancer Institute for National Research Service Postdoctoral Award CA-06406 to T.D.T. This work was supported by National Institutes of Health Research Grant CA-15826 from the National Cancer Institute.