Alteration and activation of sequence-specific cleavage of DNA by bleomycin in the presence of the antitumor drug *cis*-diamminedichloroplatinum(II)

[intercalator/combo chemotherapy/*trans*-diamminedichloroplatinum(II)/DNA sequence determination/antibiotic]

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ABSTRACT The antitumor drug *cis*-diamminedichloroplatinum(II) dramatically alters the sequence-specific cleavage of the bleomycin A₂-iron(II)-O₂ system. Preferred bleomycin cleavage sites adjacent to oligo(dG) regions on two restriction fragments of plasmid pBR322 DNA were masked by pretreatment with *cis*-diamminedichloroplatinum(II). *trans*-Diamminedichloroplatinum(II), which is inactive as an antitumor drug, showed similar but not identical behavior. The DNA-cleaving activity of bleomycin was substantially modified by *cis*-diamminedichloroplatinum(II), and a number of specific new cutting sites in guanine-rich parts of the sequence were activated by both isomers of the platinum complex. The results further emphasize the possibility that the synergism found when *cis*-diamminedichloroplatinum(II) and bleomycin are used in combination chemotherapy may be due to interactions at the level of DNA-drug binding.

Bleomycin and *cis*-diamminedichloroplatinum(II) (*cis*-DDP) (Fig. 1) are used in combination chemotherapy to treat malignant melanoma (2), head-neck carcinoma (3), and testicular carcinoma (4). The two drugs exhibit synergism in the clinical management of head and neck cancer (5). The biological target for both *cis*-DDP (6) and bleomycin (7) is believed to be DNA. Bleomycin cleaves double-stranded DNA specifically at 5'→3' G-C and G-T sequences in the presence of ferrous ion and molecular oxygen (1, 8-12), and the anticancer activity of this glycopeptide antibiotic is related to DNA strand scission. *cis*-DDP, on the other hand, binds covalently to DNA. Attachment of *cis*-DDP to poly(dG) regions through intrastrand crosslinking of guanosine N7 atoms is thought to be responsible for its various biological activities (13-17). Previously we showed that the intercalating drug ethidium bromide changes the nuclease-sensitive DNA-binding sites of *cis*-DDP (15) and that ethidium bromide, distamycin A, and actinomycin D modify the sequence-specific cleavage mode of bleomycin (18). Here we address the related question of whether prior covalent binding of *cis*-DDP to DNA might alter the specific nucleotide cleavage pattern of bleomycin.

EXPERIMENTAL

Preparation of DNA Restriction Fragments. Restriction fragments of 396 and 527 base pairs (bp), respectively, were obtained by digestion of purified plasmid pBR322 DNA (19) with the restriction endonucleases *Hinf*I and *Hpa* II. After incubation with bacterial alkaline phosphatase the 5' ends were labeled with ³²P by treatment with bacteriophage T4 polynucleotide kinase and [γ -³²P]ATP. The doubly end-labeled fragments were then digested with *Hae* III and the resulting singly end-labeled 327- and 165-bp fragments were isolated by preparative 6% polyacrylamide gel electrophoresis (14, 15).

DNA-Drug Reactions and Analysis. Reaction samples containing the 165-bp ³²P-labeled DNA piece and sonicated calf thymus carrier DNA (1 mg/ml) were incubated with *cis*- or *trans*-DDP at a drug-to-nucleotide (D/N) mole ratio of 0.33, 0.165, 0.083, or 0.041 in 1 mM sodium phosphate/5 mM sodium chloride buffer, pH 7.4, at 37°C for 3 hr. In studies with the 327-bp fragment, the carrier DNA and *cis*-DDP concentrations were 20 μ g/ml and 40 μ M, respectively, corresponding to D/N = 0.67. Sodium chloride was added to 0.2 M final concentration to stop the reaction and the platinated DNA was precipitated by addition of sodium acetate (0.3 M) and ethanol. The pellets were rinsed with cold 75% (vol/vol) ethanol and dried (15). The ratio of bound platinum per nucleotide for both *cis*- and *trans*-DDP was determined by flameless atomic absorption spectroscopic analysis of carrier DNA platinated under identical conditions. Next, bleomycin A₂ samples containing bound Fe(II) (2 and 4 μ M in two different experiments) and 2-mercaptoethanol (20 mM) in deaerated water were added to the platinated DNA dissolved in 20 mM Tris-HCl buffer, pH 8.3, and the mixtures were incubated at 37°C for 10 min (327 bp) or 4 min (165 bp). A 10 μ M solution of Na₂EDTA was added to stop the reaction and the DNA was precipitated by addition of cold ethanol. The pellets were treated with 0.2 M sodium cyanide (150 μ l) at 37°C for 4 hr to remove bound platinum before electrophoresis (14). The DNA samples were collected once again by precipitation, washed three times with cold 75% ethanol, and subjected to electrophoresis together with the Maxam-Gilbert sequencing fragments (20) on a 10% or 15% polyacrylamide/7 M urea slab gel.

RESULTS AND DISCUSSION

The two restriction fragments used in this work start from different 5' end points on pBR322 DNA but terminate at the same 3' end. Their sequences in the regions of interest are given in Fig. 2. The 165-bp fragment was allowed to react with various concentrations of *cis*- and *trans*-DDP and the bleomycin concentration was also varied. For the 327-bp piece, only *cis*-DDP was used to check whether similar interactions occur farther up the DNA nucleotide chain.

Abbreviations: *cis*- and *trans*-DDP, *cis*- and *trans*-diamminedichloroplatinum(II); D/N, mole ratio of drug to nucleotide concentrations; bp, base pair(s).

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FIG. 1. Structures of compounds used in this study: bleomycin A₂, *cis*-diamminedichloroplatinum(II), and *trans*-diamminedichloroplatinum(II). The absolute configuration of bleomycin A₂ is drawn in ref. 1.

Figs. 3 and 4 display the gel electrophoresis results obtained with the 165- and 327-bp DNA molecules, respectively. The products of four Maxam–Gilbert base-specific chemical reactions were run in parallel lanes (lanes 1–4) to identify the bleomycin cleavage fragments. Cleavage of 5'-end-labeled DNA with bleomycin is known to produce a major fragment that runs slightly faster than fragments of equal base length created by the Maxam–Gilbert sequencing reactions and a minor fragment with mobility indistinguishable from that of the Maxam–Gilbert generated pieces (1, 8). We confirm this observation, which is especially apparent for short, rapidly migrating fragments (Fig. 3). Control digestions, performed in the absence of any added

platinum reagent (Fig. 3, lanes 5 and 14; Fig. 4, lane 6), reveal the known propensity of bleomycin in the presence of Fe(II) and oxygen to cleave double-stranded DNA preferentially at 5'→3' G-C and G-T sequences. Strong bands appear at positions T₁₆, C₃₀, and C₄₂ in the 165-bp DNA and at positions C₃₃, T₃₆, C₄₁, T₅₃, T₅₇, and C₅₉ in the 327-bp DNA control lanes (Figs. 2–4). Interestingly, strong bands also appear at selected purine bases adjacent to the 3' side of guanosine: A₂₆ and G₄₉ for the 165-bp fragment and G₄₈ for the 327-bp fragment. Although 5'→3' G-A- and G-G-specific cleavages have been previously identified for bleomycin A₂-Fe(II) (10), they usually constitute only a small minority of the cutting sites. In the present case

FIG. 2. Partial sequences of restriction fragments studied here. Numbers in parentheses refer to the base positions in the entire sequence map of pBR322 (19). Solid arrows indicate strong cleavage sites and broken arrows depict weaker sites for bleomycin (BLM) in the absence and presence of *cis*- or *trans*-DDP.

FIG. 3. Autoradiogram of a 15% polyacrylamide/7 M urea electrophoresis gel showing the effect of addition of various quantities of *cis*- and *trans*-DDP on the bleomycin-Fe(II)-O₂ cleavage of a 165-bp DNA molecule. Lanes 1-4 show the Maxam-Gilbert sequencing reactions for G, G+A (also in lane 15), C+T, and C, respectively (20). Lanes 5 and 14 are control bleomycin digestions of DNA that had not been pretreated with platinum. Lanes 6, 8, 10, and 12 were bleomycin digests of DNA incubated with *cis*-DDP at D/N = 0.33 (bound D/N = 0.080), 0.165 (0.052 bound), 0.083 (0.024 bound), and 0.041 (0.014 bound), respectively, while lanes 7, 9, 11, and 13 are the corresponding products of bleomycin treatment of DNA allowed to react with the same amounts of *trans*-DDP (D/N bound = 0.054, 0.027, 0.019, and 0.013, respectively). On the right is shown the sequence with numbers referred to the ³²P-labeled 5' end (*Hpa* II site). Electrophoresis was carried out for 3 hr at 55 W, 1,800 V.

the adenosine and guanosine cleavage sites are nested in oligo(dG)-oligo(dC) runs of duplex. Recent x-ray crystal structure studies of oligonucleotides containing (dG)_n sequences revealed A-DNA conformations (21-23), which might possibly be suited to bind bleomycin A₂-Fe(II). Previous studies of the sequence dependence of bleomycin cutting were carried out with DNA molecules that lacked extensive oligo(dG) sequences.

When the DNA molecules were incubated with *cis*- or *trans*-

FIG. 4. Autoradiogram of a 10% polyacrylamide/7 M urea electrophoresis gel showing results for bleomycin digestion of a 327-bp pBR322 DNA restriction fragment. Lanes 1-4 have G, A>C, C+T, and C Maxam-Gilbert base-specific chemical reaction products (20), lane 5 has results for bleomycin digestion of *cis*-DDP/DNA, and lane 6 is the control bleomycin reaction. The numbers on the left of the figure represent length of the DNA fragment from the ³²P-labeled *Hinf*I site. Sequences on the right show preferential cleavage sites (solid underlines indicate strong cleavage sites and broken underlines indicate weaker sites; see also Fig. 2).

DDP prior to the addition of bleomycin, dramatic changes were observed in the sequence-specific cleavage patterns (Figs. 2-4). Many more short cleavage products appeared in lanes containing bleomycin-digested DNA that had previously been allowed to react with either *cis*- or *trans*-DDP at D/N = 0.041, corresponding to ≈ 0.013 bound platinum atom per nucleotide. Specifically, strong bands appeared at positions C₈, G₁₀, C₁₁, A₂₈, and C₃₂ of the 165-bp DNA that were either weak or absent from the control lanes. Cleavage at C₁₇ along the 165-bp fragment was enhanced by *cis*- but not *trans*-DDP. Pretreatment of DNA with low D/N of either *cis*- or *trans*-DDP also masked the preferred bleomycin cleavage at G₄₉, the second nucleotide of the (dG)₅ sequence in the 165-bp DNA, and a higher D/N of *cis*-DDP produced the same effect at the G-G-

G-G-G-G-T sequence in the 327-bp piece.

Increasing the formal D/N resulted in many fewer bleomycin cleavage products appearing in lanes corresponding to DNA pretreated with *cis*-DDP (Fig. 3). We attribute this effect to enhanced cleaving of DNA by bleomycin between the point of platinum attachment and the 5' end label. Moreover, the bands that did appear on the gel were smeared, indicating that some platinum remained on the DNA despite treatment with cyanide. This cyanide-resistant fraction of *cis*-DDP bound to DNA, especially at high D/N ratios, has been observed and discussed previously (14). Similar behavior was not exhibited in experiments with the *trans* isomer.

Recent NMR studies indicate that *cis*-DDP forms intrastrand crosslinks between N7 atoms of adjacent deoxyguanosine residues in the sequences [d(A-G-G-C-C-T)]₂ (24) and [d(T-G-G-C-C-A)]₂ (25). It is likely that *cis*-DDP binding to (dG)_n sequences in the present experiments is responsible for masking preferred bleomycin cleavage sites within or in the vicinity of oligo(dG) regions in both the 165- and 327-bp restriction fragments. Platinum binding not only inhibits bleomycin cleavage but also stimulates it elsewhere, at sites that normally would not be cut by the antibiotic. This stimulation could arise either from long-range duplex structural changes induced by the binding of platinum or simply because destruction of the primary cleavage sites permits more bleomycin to bind and cut DNA at secondary targets.

The pronounced alteration of the bleomycin cleavage patterns upon *cis*-DDP binding demonstrates that *cis*-DDP alters the bleomycin interaction with DNA, their acknowledged common biological target. Binding of *cis*-DDP to DNA has a definite role in directing the cleavage mode of DNA by bleomycin. The *trans* isomer, because of its different geometry, is less effective at inhibiting bleomycin cutting near oligo(dG) sequences but does activate several new sites, especially at d(GNG)_n sequences. Further work is required to elucidate the detailed structural changes responsible for alteration of bleomycin cutting activity by both platinum isomers.

CONCLUSION

cis-DDP is generally used with other drugs such as bleomycin in combination chemotherapy for cancer treatment. The present study demonstrates that *cis*-DDP alters the sequence specific cleavage of DNA by bleomycin, masking cutting sites near oligo(dG) sequences and stimulating cutting elsewhere. These results support the general notion that drugs used in combination chemotherapy might exhibit their synergism through binding to a common biological target, in this case DNA.

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1. Kross, J., Henner, W. D., Hecht, S. M. & Haseltine, W. A. (1982) *Biochemistry* **21**, 4310-4318.
2. Bajetta, E., Rovej, R., Buzzoni, R., Vaglini, M. & Bonadonna, G. (1982) *Cancer Treat. Rep.* **66**, 1299-1302.
3. Perry, D. J., Weltz, M. D., Brown, A. W., Jr., Henderson, R. L., Neglia, W. J. & Berenberg, J. L. (1982) *Cancer* **50**, 2257-2260.
4. Davis, H. L., Jr., von Hoff, D. D., Henney, J. E. & Rozenzweig, M. (1978) *Cancer Chemother. Pharmacol.* **1**, 83-90.
5. Wittes, R. E., Brescia, F. & Young, C. W. (1975) *Oncology* **32**, 202-207.
6. Roberts, J. J. & Thomson, A. J. (1979) *Prog. Nucleic Acid Res. Mol. Biol.* **22**, 71-133.
7. Umezawa, H. (1978) in *Bleomycin: Current Status and New Developments*, eds. Carter, S. K., Crooke, S. T. & Umezawa, H. (Academic, New York), pp. 15-19.
8. D'Andrea, A. D. & Haseltine, W. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3608-3612.
9. Takeshita, M., Grollman, A. P., Ohtsubo, E. & Ohtsubo, H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5983-5987.
10. Takeshita, M., Kappen, L. S., Grollman, A. P., Eisenberg, M. & Goldberg, I. H. (1981) *Biochemistry* **20**, 7599-7606.
11. Sugiura, Y., Suzuki, T., Otsuka, M., Kobayashi, S., Ohno, M., Takita, T. & Umezawa, H. (1983) *J. Biol. Chem.* **258**, 1328-1336.
12. Mirabelli, C. K., Beattie, W. G., Huang, C.-H., Prestayko, A. W. & Crooke, S. T. (1982) *Cancer Res.* **42**, 1399-1403.
13. Cohen, G. L., Ledner, J. A., Bauer, W. R., Ushay, H. M., Caravana, C. & Lippard, S. J. (1980) *J. Am. Chem. Soc.* **102**, 2487-2488.
14. Tullius, T. D. & Lippard, S. J. (1981) *J. Am. Chem. Soc.* **103**, 4620-4622.
15. Tullius, T. D. & Lippard, S. J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3489-3492.
16. Fichtinger-Schepman, A. M. J., Lohman, P. H. M. & Reedijk, J. (1982) *Nucleic Acids Res.* **10**, 5345-5356.
17. Brouwer, J., Fichtinger-Schepman, A. M. J., van de Putte, P. & Reedijk, J. (1982) *Cancer Res.* **42**, 2416-2419.
18. Sugiura, Y. & Suzuki, T. (1982) *J. Biol. Chem.* **257**, 10544-10546.
19. Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 77-90.
20. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65**, 499-560.
21. Connor, B. N., Takano, T., Tanaku, S., Itakura, K. & Dickerson, R. E. (1982) *Nature (London)* **295**, 294-299.
22. Shakked, Z., Rabinovich, D., Cruse, W. B. T., Egert, E., Kennard, O., Sala, G., Salisbury, S. A. & Viswamitra, M. A. (1981) *Proc. R. Soc. London Ser. B* **213**, 479-487.
23. Wang, A. H.-J., Fujii, S., van Boom, J. H. & Rich, A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3968-3972.
24. Caradonna, J. P., Lippard, S. J., Gait, M. J. & Singh, M. (1982) *J. Am. Chem. Soc.* **104**, 5793-5795.
25. Girault, J.-P., Chottard, J.-C., Guittet, E. R., Lallemand, J.-Y., Huynh-Dinh, T. & Igolen, J. (1982) *Biochem. Biophys. Res. Commun.* **109**, 1157-1163.