Vol. 108, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1982 Pages 16-23

# CIS-DIAMMINEDICHLOROPLATINUM(II) MODIFICATION OF SV40 DNA OCCURS PREFERENTIALLY IN (G+C) RICH REGIONS: IMPLICATIONS INTO THE MECHANISM OF ACTION

William M. Scovell and Lee R. Kroos

Department of Chemistry
Bowling Green State University
Bowling Green, Ohio 43403

Received July 19, 1982

SUMMARY: The relative distribution of bound cis- and trans-$(NH_3)_2PtCl_2$ at specific sites in SV40 DNA is evaluated by monitoring the extent to which five restriction endonucleases, each of which cleave at a single, unique site, are inhibited as a result of the DNA modification. The order of cleavage inhibition is Bgl 1 ≳ Bam HI > Hpa II, Kpn I > Eco RI. Both isomers produce a comparable effect for any particular endonuclease. Inhibition correlates with the % (G+C) content within and about the recognition sequences. That modified sequences immediately adjacent to the recognition sequence influence cleavage is further supported by differential cleavage observed with the multicut Hind III endonuclease. The binding of cis-$(NH_3)_2PtCl_2$ at the hyper-reactive Bgl 1 site may well be directly responsible for inhibiting SV40 replication.

INTRODUCTION. Cis-diamminedichloroplatinum(II) (cis-DDP) has proven to be an effective anti-neoplastic agent, especialy when used in combination chemotherapy (1,2). The geometrical isomer, trans-DDP, is inactive. The critical cis-DDP interaction in mammalian cells is widely considered to be a direct modification of DNA, which in turn produces the selective inhibition of DNA synthesis both in vitro and in vivo (1). Recent findings reveal that the cis-DDP modification on DNA (1) produces a larger effective unwinding angle and also (2) stimulates far greater levels of S1 nuclease sensitivity than does the trans-DDP modification (3,4). Both these studies, and others (5,6,7), are consistent with the mode of binding for cis-DDP being different than that of trans-DDP and producing a more significant distortion in the DNA structure. Both isomers are expected to bind preferentially to guanines and (G+C) rich regions of DNA (5-9) and may form monodentate adducts or interstrand crosslinks (10). However, a mode of binding which is stereochemically unique to the cis-DDP isomer and may alter the double helix significantly involves an intrastrand crosslink to adjacent guanines (5).

0006-291X/82/170016-08$01.00/0

Kutinova et al. (11) have observed that cis-DDP treatment of SV40 virally infected permissive cells inhibits SV40 replication and V-antigen synthesis, while T-antigen production is unaltered. Because of the similarity of the effects of cis-DDP and ara-C, it was suggested that cis-DDP may inhibit the synthesis of SV40 DNA. These findings, coupled with the fact that the most (G+C) rich region in the SV40 genome is within the regulatory region (0.66-0.73 on the physical map) (12,13), prompted us to suggest that cis-DDP may bind preferentially in the regulatory region to inhibit metabolic processes, including SV40 viral DNA replication (3). In this communication, the relative distribution of bound DDP within and about specific sequences in the SV40 genome is evaluated by monitoring the relative cleavage inhibition of site specific restriction endonucleases (R.E.s). We find (1) preferential binding of both DDP isomers at the Bgl 1 and Bam HI sites and (2) that cleavage inhibition correlates with the (G+C) content in and about the R.E. sites. These findings are discussed in light of the effect of cis-DDP on SV40 replication in permissive cells.

## MATERIALS AND METHODS

SV40 DNA and the restriction endonucleases Bam HI, Bgl 1, Eco RI, Hpa II and Kpn I were purchased from Bethesda Research Laboratories. The cis- and trans-DDP were supplied by Mathey Bishop Co. and the National Cancer Institute, respectively. These solutions were prepared in DNA buffer solution (10 mM Tris-HCl, 5 mM NaCl, 1 mM EDTA, pH 7.6), filtered, adjusted to pH 7.6, stored in a foil wrapped container at 4°C and used within two weeks of preparation.

Reaction of SV40 DNA with cis- and trans-DDP: The reactions were carried out in DNA buffer for 3 hrs. at 25°C at 9 x $10^{-5}$ M nucleotide [DNA(N)] and 4.5 x $10^{-6}$ - 3.6 x $10^{-5}$ M DDP, yielding formal [DDP/DNA(N)] mole ratios of 0.05-0.2. Drop dialysis (14), using Millipore VM type membrane filters (0.05 um), was employed on all samples to remove unbound DDP prior to the cleavage reactions, including the DDP-free controls. Therefore, the $r_b$ levels are expected to be much lower than the formal mole ratios.

Restriction Endonuclease Cleavage of SV40 DNA: Restriction enzymes, Bam HI, Bgl 1, Eco RI, Hpa II and Kpn I, which produce cleavage at a single, unique sequence in SV40 DNA, were individually assayed for the number of units required to just completely cleave a mixture of nicked and supercoiled SV40 DNA under our reaction conditions (0.64 ug DNA in 20 ul for 120 minutes at 37°C). These same conditions were used in subsequent analyses of the DDP modified-DNA.

After cleavage reaction buffer was appropriately added to the dialyzed sample, the necessary amount of restriction enzyme was added to completely cleave the untreated DNA. Stop solution (25% glycerol, 5% SDS, 0.025% bromophenol blue) was added to the reaction mixture and aliquots containing 0.32 ug DNA were loaded on the gel.

Gel Electrophoresis: Agarose gel electrophoresis was carried out on vertical slabs containing 1.4% agarose prepared and run in TBE buffer (90 mM tris base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). The DNA samples were electrophoresed at 7 V/cm for about 6 hrs. at room temperature, after pre-electrophoresis of the gel for 30 min. at 7 V/cm. The gels were stained for 30

minutes in 10 ug/ml ethidium bromide, destained 30 minutes in electrophoresis buffer, UV illuminated and photographed on Polaroid P/N 55 film with a Polaroid MP-4 camera equipped with UV haze and red filters. The negative photographs of gels were scanned with a Joyce Loebl densitometer to quantitate band intensities and correlate the relative fluorescence in the gel to the amount of DNA applied. Samples with known amounts of DNA were employed to confirm that the peak areas in the densitometer traces were proportional to the amount of DNA.

## RESULTS AND DISCUSSION

SV40 DNA was reacted with DDP, spot dialyzed and then sufficient restriction endonuclease (R.E.) added to just completely cleave an equivalent amount of unmodified DNA. The recognition sequence for the R.E.s are located at 0/1.0 (Eco RI), 0.1432 (Bam HI), 0.6594 (Bgl 1), 0.7158 (Kpn I) and 0.7259 (Hpa II) on the SV40 DNA physical map. Previous studies carried out under these conditions suggest that there are comparable levels of bound cis- and trans-DDP (7). A comparison of the band intensities in each lane of the gel in Figure 1 indicates that cleavage by all R.E.s is inhibited, but that the extents of inhibition differ greatly for the 5 R.E.s. For any R.E., the extent of inhibition produced by cis- and trans-DDP is comparable. Similar results were obtained at incubated [DDP/DNA(N)] mole ratios of 0.2 and 0.05. The relative band intensities indicate that the relative order of cleavage inhibition for the R.E.s as a result of either cis- or trans-DDP binding to DNA is:

Bgl 1 $\gtrsim$ Bam HI > Hpa II, Kpn I > Eco RI

Assuming that a DDP modification in the immediate vicinity of the recognition site will significantly modulate the sequence specific protein-DNA interaction and thereby inhibit the R.E. cleavage process, these data reflect the relative distribution of bound DDP within or about specific sequences in the SV40 genome. The order of inhibition observed suggests the sequence at the Bgl 1 and Bam HI recognition sites are hyper-reactive to DDP binding in SV40 DNA. These two sites correspond to the site at or near the origin of replication (15) and a major site at which replicative intermediates accumulate during SV40 replication (16). Beard et al (17) have recently reported that N-acetoxy-acetylaminofluorene (AAAF), which binds almost exclusively to guanines also, reacts preferentially within the control region in intracellular SV40 minichromosome. Our findings with DDP, taken together with that of AAAF, suggest that

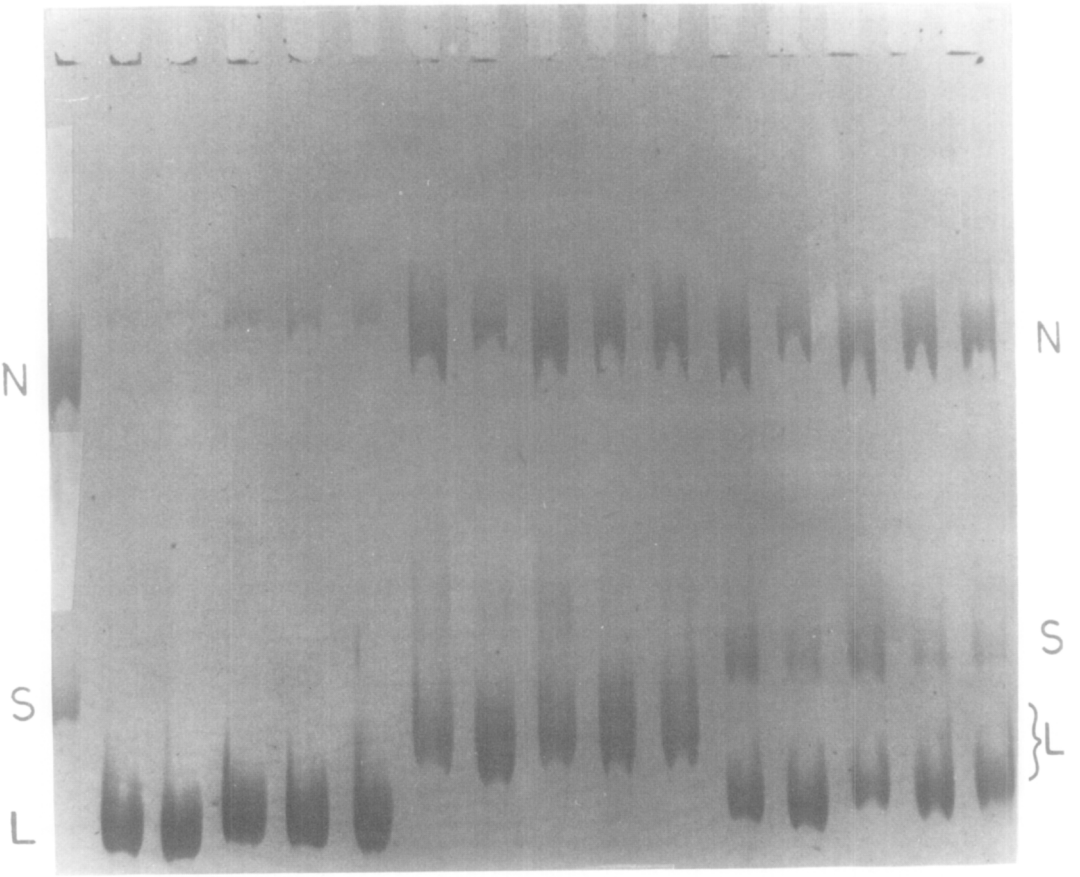


Figure 1: Cleavage of cis- and trans-DDP modified SV40 DNA by restriction endonucleases which cut at a single, unique site. Lane 1 shows the mobility of nicked (N) and supercoiled (S) forms of SV40 DNA. Lanes 2-6 contain SV40 DNA cleaved with Bam HI, Eco RI, Bgl 1, Hpa II and Kpn I, respectively. These controls demonstrate complete cleavage to the linear (L) form. Lanes 7-11 contain SV40 DNA [$9.0 \times 10^{-5}$ M DNA(N)], which was reacted with cis-DDP ($1.8 \times 10^{-5}$ M) and then cleaved with restriction enzymes. The order is the same as in the control series. Lanes 12-16 contain SV40 DNA reacted similarly with trans-DDP, followed by cleavage with restriction enzymes, again in the same order as controls.

agents which bind preferentially to guanines may exhibit a preference for binding to the regulatory region of SV40 DNA.

Previous studies have revealed that DDP binds preferentially to guanines and therefore to (G+C) rich regions in DNA (5,6,8,9). Stone et al (5), however,

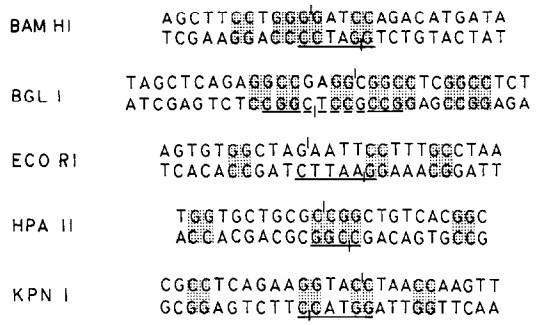


Figure 2: Cleavage sites for restriction endonucleases. The recognition sequence plus ten base pairs on each side is shown for Bam HI, Bgl 1, Eco RI, Hpa II and Kpn I. The base pairs that was essential in the recognition sequence are underlined with a solid line. Note that the central five base pair sequence for Bgl 1 underlined with a broken line is not an essential sequence. Sequences of two or more adjacent guanines are shown stippled. The vertical lines within the recognition sequence designate the cleavage site.

have shown a base sequence dependent difference in the binding of cis- and trans-DDP. In this regard we examined the recognition sequences and the sequences immediately adjacent to them, to discern whether the order of cleavage inhibition paralleled the % (G+C) content in the recognition sequences. As can be seen in Figure 2, the observed order of cleavage inhibition does not parallel the % (G+C) content within the recognition sequence. The most obvious discrepencies are with Hpa II and Bam HI, with 100% and 67% (G+C) contents, respectively. Consideration of the influence of an additional 5 or 10 nucleotides immediately adjacent to the recognition site seemed reasonable therefore, in that although the effect of DDP binding on DNA is not expected to be long range, its influence may extend well beyond the immediate site of binding and therefore modulate the R.E.-DNA interaction. Focusing on these larger base pair sequences, cleavage inhibition by the DDP binding parallels the number and relative position of the guanines in the sequence. Adjacent guanines enhance the inhibition more so than do non-adjacent guanines, with the inhibition being significantly greater if the number of adjacent guanines is more than two. This latter effect appears to be of particular importance in understanding the signi-

ficant inhibition of Bam HI. Although the % (G+C) content of the sequence shown in Figure 2 for Bam HI is lower (50%) than that of Bgl 1 (71%) and has fewer adjacent guanine clusters, it does have a nucleotide tract of four adjacent guanines, which is twice as large as any in the Bgl 1 sequence. Extending the analysis to more than 10 base pairs about the recognition sequences proved less justifiable (vide infra). Our findings are consistent with more limited studies of Kelman and Buchbinder (18) and Ushay et al. (19) to the extent that they found that cis-DDP bound to $\lambda$ and pBR322 DNA, respectively, inhibited Bam HI cleavage. However, no comparison with trans-DDP was reported.

The conclusions drawn from the analysis with the five single cut R.E.s are further supported by examination of Hind III cleavage of SV40 DNA, which occurs at 6 sites (data not shown). Differential inhibition is observed at the 6 equivalent recognition sequences, which can only be explained if DDP binding to nucleotides outside of the recognition sequence influences the cleavage process. The site which is inhibited to the greatest extent by far is at 0.3231 on the physical map and has a tract of 6 guanines immediately adjacent to the cleavage site. This is consistent with our conclusions noted above and similar to previous findings by Cohen et al. (20).

In addition, the observation that inhibition does not occur at the 0.9449 site, although it has a tract of 4 adjacent guanines which are 11-14 nucleotides from the recognition sequence, supports our assumption that although DDP binding can produce local distortions in DNA structure, its influence is reduced to undetectable levels if binding occurs ca 10 nucleotides or more from the recognition sequence.

Three final points should be noted. By this analysis, cis- and trans-DDP binding appears to exhibit some binding preference depending on the base pair sequence (at least with respect to guanines); however, we observe little or no sequence-dependent differences between cis- and trans-DDP. Secondly, this and other work highlight the fact that different probes used to monitor the influence of DDP binding on DNA structure may exhibit very different sensitivities to cis- and trans-DDP binding. Although studies using enzymatic or

physico-chemical probes have revealed differences in the binding of these isomers (5-8), the series of R.E.s used in this study appear relatively insensitive to the mode of binding and may be influenced predominantly by the local physical coverage of DDP at or adjacent to the recognition sequence. DNAase I, a general DNA nuclease, exhibits a similar character in that it also is inhibited comparably by either cis- or trans-DDP binding to DNA (21).

Finally, the observation that the Bgl 1 cleavage is most inhibited suggests that this site is hyper-reactive to DDP binding. T antigen, an SV40 viral protein, binds to three sequence-specific sites in SV40 DNA, one of which is at the Bgl 1 site (22,23). This binding interaction is essential for the autoregulation of T antigen production and stimulates SV40 viral DNA replication. Since T-antigen makes very close contact with guanines in this sequence-specific interaction (22), DDP binding to guanines may directly disrupt this regulatory binding interaction and possibly inhibit not only SV40 viral replication, but also other viral processes.

ACKNOWLEDGEMENTS

We thank Mathey Bishop Co. for the loan of cis-DDP and the National Cancer Institute for the gift of trans-DDP. This work was supported by the Ohio Division of the American Cancer Society, the Bowling Green State University Biomedical Research Support Grant, Alumni Association and Parents Club, Faculty Research Committee and the American Cancer Society Support Grant number IN-130.

REFERENCES

1. Roberts, J.J. and Thomson, A.J. (1978) Prog. Nucleic Acid Res. Mol. Biol. 22, 71-133.
2. Einhorn, L.H. (1981) Cancer Res. 41, 3275-3280.
3. Scovell, W.M. and Kroos, L.R. (1982) Biochem. Biophys. Res. Commun. 104, 1597-1603.
4. Scovell, W.M. and Capponi, V., in press.
5. Stone, P.J., Kelman, A.D., Sinex, F.M., Bhargava, M.M. and Halvorson, H.O. (1976) J. Mol. Biol. 104, 793-801.
6. Royer-Pokora, B., Gordon, L.K. and Haseltine, W.A. (1981) Nucleic Acids Res. 9, 4595-4609.
7. Tullius, T.D. and Lippard, S.J. (1981) J. Amer. Chem. Soc., 103, 4620-4622.
8. Scovell, W.M. and O'Connor, T. (1977) J. Amer. Chem. Soc. 99, 120-126.
9. Stone, P.J., Kelman, A.D. and Sinex, F.M. (1974) Nature 251, 736-737.
10. Deutsch, W.A., Spiering, A.L. and Newkome, G.R. (1980) Biochem. Biophys. Res. Commun. 97, 1220-1226.
11. Kutinova, L., Vonka, V., Zavadov'a, H. and Drobnik, J. (1972) Arch. ges. Virusforsch 39, 196-202.
12. Fiers, W., Contreras, R., Haegeman, G., Rogiers, R., van de Voode, A., Van Heuverswyn, H., Van Herreweghe, J., Volckaert, G. and Sebaert, M.Y. (1978) Nature 273, 113-120.
13. Reddy, V.D., Thunmaappaya, B., Dhar, R., Subramanian, K.N., Zain, B.S., Pan, J., Ghosh, P.K., Celma, M.L. and Weissman, S.M. (1978) Science 200, 494-502.

14. Murusyk, R. and Sergeant, A. (1980) Anal. Biochem. 105, 403-404.
15. Gutai, M.W. and Nathans, D. (1978) J. Mol. Biol 126, 259-274.
16. Tapper, D.P. and DePamphilis, M.L. (1980) Cell 22, 97-108.
17. Beard, P., Kaneko, M. and Cerutti, P. (1981) Nature 291, 84-85.
18. Kelman, A.D. and Buchbinder, M. (1978) Biochemie 60, 893-899.
19. Ushay, H.M., Tullius, T.D. and Lippard, S.J. (1981) Biochemistry 20, 3744-3748.
20. Cohen, G.L., Ledner, J.A., Bauer, W.R., Ushay, H.M., Carawanna, C. and Lippard, S.J. (1980) J. Amer. Chem. Soc. 102, 2487-2488.
21. Scovell, W.M. and Knezetic, J., unpublished studies.
22. Tjian, R. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 655-662.
23. Tooze, J. (1980) in DNA Tumor Viruses, Molecular Biology of Tumor Viruses, 2nd Ed., Part 2 (Tooze, J., Ed.) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.