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# AN IN VITRO CHARACTERIZATION OF INTERSTRAND CROSS-LINKS IN DNA EXPOSED TO THE ANTITUMOR DRUG CIS-DICHLORODIAMMINEPLATINUM(II)

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Summary. The cis-isomer of the antitumor drug dichlorodiammineplatinum(II) [cis-Pt(II)] was tested for its abilty to introduce nicks (single-strand breaks) into supercoiled PM2 DNA. Whereas incubations up to 24 h show no indication of cis-Pt(II)-treated DNA having single-strand breaks, DNA interstrand cross-links were detected in the first 15 min of incubation. Furthermore, the formation of DNA interstrand cross-links was both inhibited and fully reversed after incubation with 2 mM thiourea.

Introduction. The cis-isomer of dichlorodiammineplatinum(II) [cis-Pt(II)] has been shown to be an effective antitumor drug towards a broad spectrum of animal tumors (for review see ref. 1). It is generally regarded that cis-Pt(II) exerts its antitumor properties by acting on DNA, resulting in an eventual interruption of DNA replication (1). The exact sequence of events leading to this impairment in DNA synthesis is unclear, but presumably involves certain DNA lesions which are refractive to DNA synthesis. A number of DNA lesions produced by cis-Pt(II) has been identified, one of which is the creation of single-stranded nicks in the DNA phosphodiester backbone (2). In an attempt to identify such lesions created by organometallic reagents presently under study in our laboratory (3), we have taken advantage of an assay system which measures the selective retention on nitrocellulose filters of denatured DNA or DNA containing single-stranded regions (4). This in vitro system has been successfully used to detect DNA modifications produced by carcinogenic agents (4). As well, the properties of nitrocellulose have been exploited in the purification of DNA binding proteins (5,6). Utilizing the nitrocellulose filter assay system, we find that the first detectable lesion produced by cis-Pt(II) is DNA

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interstrand cross-links, and that these DNA cross-links can be inhibited as well as reversed by thiourea.

## MATERIALS AND METHODS

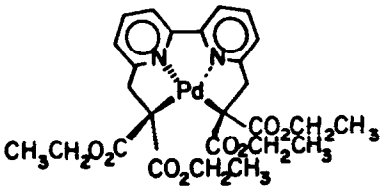
Analysis of single-strand breaks: Reaction mixtures (0.05 ml) contained 1.2 nmol of PM2 DNA (60 fmol of PM2 DNA molecules with a specific activity of 197 cmp/fmol circle), 25 mM Tris, pH 7.5, 10 mM $MgCl_2$, and transition metal reagent. Incubations were at 37°C for the indicated time. Measurement of single-strand breaks has been previously described (4). Values have been corrected according to a Poisson distribution of target sites.

Analysis of DNA interstrand cross-links: Reaction mixtures were the same as for the analysis of single-strand breaks. The DNA was denatured with 0.4 ml of 0.15 M $K_2HPO_4$, 2.5 N KOH, pH 13.2. After 10 min at room temperature, the reaction mixtures were neutralized with 0.2 ml of 1 M $KH_2PO_4$-HCl (pH 4.0); 0.2 ml of 5 M NaCl and 4 ml of 1 M NaCl/50 mM Tris-HCl (pH 8.1) were then added. Filtration was through a Schleicher and Schuell type BA-85 nitrocellulose filter.

Other Methods: The preparation of phage PM2 DNA has been described (4), except that infection was at an MOI of 2.

## Results and Discussion

During the course of examinations on transition metal reagents and their ability to produce lesions in DNA, we found that one such complex, cis-Pd(dedp), synthesized in our laboratory could produce nicks in DNA (3).




As a logical extension of the above studies, it was of interest to learn if a similar type of DNA lesion could be detected by a known antitumor drug. We chose to examine cis-dichlorodiammineplatinum(II) [cis-Pt(II)] since recent reports had implied that it caused single-strand breaks in DNA (2). Values of DNA strand-scission obtained for the cis-Pt(II) complex in the present study, however, were consistently less, even after 24 h, than values obtained in control reactions incubated in the absence of any transition metal reagent (Table 1). This suggested that perhaps the two DNA strands were kept in register by interstrand cross-links, thus making the DNA molecule stable to alkali. To test this, the cis-Pt(II)-treated DNA was incubated for various periods of time, and then exposed to a pH which irreversibly denatures DNA. As

Table 1
Strand breaks introduced into DNA.

| Time | Nicks introduced (fmol) | | | |
|---|---|---|---|---|
| | cis-Pd (dedp) (17μM) | cis-Pt(II) (84 μM) | no addition (Blk) | total strand breaks[a] |
| 5 min | 74 | — | 18 | 56 |
| 120 min | -- | 16 | 30 | -(14) |

[a] Nicks introduced with added agents less blank.

can be seen in Fig. 1A, after incubations of only 15 min, DNA molecules contained cross-links. The amount retained decreased from an original 85% of the DNA bound to the filter to about 37% in 2 h (acid-soluble counts were not being liberated that could account for the decrease in DNA bound to the filter). As a control, nitrous acid, a known cross-linking agent, was also tested on DNA (Fig. 1B). The average number of cross-links per DNA molecule treated with cis-Pt(II) and nitrous acid can be calculated by assuming a Poisson distribution according to the equation $C = \ln P/N$, where P and N are the fractions of cross-linked and untreated DNA, respectively. The number of cross-links per DNA molecule calculated with this equation is about 0.4 after 1 h in the presence of cis-Pt(II) and about 2.1 in the presence of nitrous acid. The latter value for nitrous acid is in excellent agreement with that found by Legerski, et al. (7).

To determine if the DNA cross-linking produced by cis-Pt(II) could be reversed or nullified, thiourea was utilized. Reports on the effects of thiourea on cross-linked DNA are inconsistent. Zwelling, et al. (8) observed that DNA cross-link formation by cis-Pt(II) in mouse leukemia cells could be prevented by thiourea, but not reversed. On the other hand, Filipski, et al.

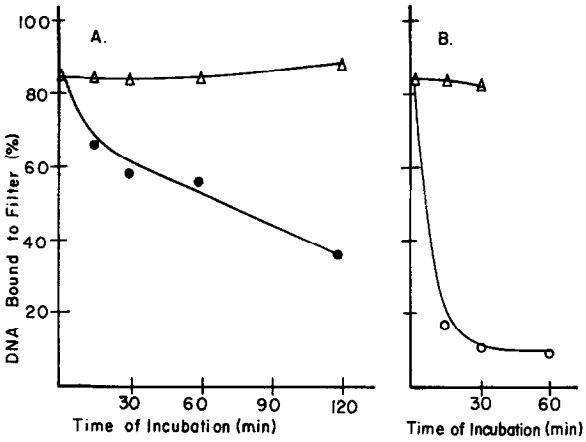



Fig. 1. The production of DNA cross-links by cis-Pt(II) and nitrous acid. (A) Reaction mixtures (0.3 ml) were described in Materials and Methods, with 7.2 nmol of DNA nucleotide and 87 μM cis-Pt(II). At the indicated times, 0.05 ml was removed and 0.15 ml of SE buffer (0.01% sodium dodecyl sulphate, 2.5 mM EDTA-NaOH, pH 7.0) was added. Denaturation of DNA was described in Materials and Methods. Δ-Δ-Δ, reaction tubes in the absence of cis-Pt(II); ●-●-●, reaction mixtures in the presence of 87 μM cis-Pt(II).

(B) Reacton mixtures (0.2 ml) contained freshly prepared 0.9 M $NaNO_2$ in BE buffer (0.1 M NaCl, 20 mM Tris-HCl, and 1 mm EDTA, pH 8.1) and 4.8 nmol of DNA nucleotide. After equilibration at 25°C, the pH was adjusted to 4.4 by addition of 8 μl of 2 M acetic acid. Volumes of 0.05 ml were removed at the indicated times and the reaction stopped with the addition of 10 μl of 2 M Tris-HCl, pH 8.2. SE buffer (0.15) ml was then added, and the DNA denatured as described in Materials and Methods. Δ-Δ-Δ, no acetic acid added; O-O-O, 2 M acetic acid added.

(9) showed that the predominant effect of thiourea is to reverse the lethal lesions in transfecting λ DNA, and . . . "not merely to prevent their formation." We tested whether thiourea could prevent the formation of DNA cross-links by initially incubating thiourea and cis-Pt(II) together in the same reaction tube. No difference was found between this and incubations in the absence of cis-Pt(II), indicating that thiourea was preventing the formation of DNA cross-links (data not shown). The ability of thiourea to subsequently effect cross-links in DNA first exposed to the cis-Pt(II) agent was also tested. As can be seen in Table 2, a 1 hour incubation in the presence of cis-Pt(II) produced approximately 0.5 cross-links per DNA molecule (46% of the DNA bound to the filter). After addition of 2 mM thiourea, about 50% of the

TABLE 2

Cross-links introduced by cis-Pt(II) treatment and reversal by thiourea.

| Incubation in the presence of cis-Pt(II) (time) | | | |
|---|---|---|---|
| 1 h | | 2 h | |
| min after thiourea addition | % DNA bound to filter | min after thiourea addition | % DNA bound to filter |
| 0 | 46 | 0 | 33 |
| 15 | 58 | 15 | 36 |
| 30 | 60 | 30 | 44 |
| 60 | 72 | 60 | 66 |

Reaction mixtures (0.25 ml) were described in Materials and Methods, with 6 nmol of DNA nucleotide. Incubations were for 1 h or 2 h at 37°C in the presence of cis-Pt(II) (87 μM). After 1 h or 2 h, 0.05 ml was removed to determine the amount of PM2 DNA molecules sensitive to alkali denaturation (0 time). Then thiourea was added to the 1 h or 2 h incubation tubes to give a final concentration of 2 mM thiourea; 0.05 ml was removed at the above times (15, 30 and 60 min) and tested for sensitivity to alkali denaturation. For reaction tubes incubated in the absence of cis-Pt(II), 75% of the DNA was denatured by alkali.

cross-links were removed (0.25 cross-links per DNA molecule, 58% of the DNA bound to the filter) in the first 15 min. Virtually all of the DNA cross-links produced by à 1 h exposure to cis-Pt(II) were removed in a subsequent 1 h incubation in the presence of 2 mM thiourea. If DNA was first exposed for 2 h to cis-Pt(II), thiourea was still capable of efficiently reversing the DNA cross- links from an original 0.8 cross-link (33% of DNA bound to the filter) to 0.1 cross-link per DNA molecule after 1 h in the presence of 2 mM thiourea. Also evident from Table 2 is that the number of DNA cross-links generated by cis-Pt(II) almost double between 1 and 2 h in the absence of thiourea; almost all of the DNA cross-links produced in the 1 h incubation, however, were

subsequently reversed by thiourea. Thus the kinetics of cross-link formation suggest that thiourea acts not only to reverse cross-links but also to prevent their formation.

Although we were unable to detect DNA single-strand breaks in the current study, it is not unlikely that nicks in DNA could eventually occur as a result of DNA interstrand cross-links. For example, it is known that cross-linking agents, such as nitrous acid, do eventually cause single-strand breaks (4). It is still unclear from other studies whether either of these lesions or a combination of many DNA lesions leads to the cytotoxic effects of cis-Pt(II). What is noteworthy from the present study is the rapid rate by which DNA cross-links do occur in vitro. Since other DNA lesions were not detected within 24 h, it seems reasonable that a primary impact in vivo by cis-Pt(II) would be the initial creation of DNA cross-links, although such lesions as DNA intrastrand cross- links could conceivably be produced as rapidly.

Our results also show that the formation of DNA cross-links can be prevented as well as reversed in the presence of 2 mM thiourea, whereas results previous to this indicate that either prevention (8) or reversal (9) occur. Furthermore, the concentration of thiourea required for the reversal of DNA cross-links in the present study is much less than that documented for the reversal of cross-links in the phage λ transfectivity test (9), but agrees well with the concentration necessary to prevent cross-link formation by cis-Pt(II). In conclusion, we find that the concentration of thiourea necessary for blocking DNA cross-link formation is adequate for cross-link reversal.

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