Kinetics of Formation and Disappearance of a DNA Cross-linking Effect in Mouse Leukemia L1210 Cells Treated with cis- and trans-Diammine-dichloroplatinum(II)


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ABSTRACT

cis- and trans-diamminedichloroplatinum(II) (PDD) produced a DNA cross-linking effect in mouse leukemia L1210 cells, which was demonstrable after subtoxic treatments with the DNA alkaline elution technique. Cross-linking effects developed following treatments with concentrations as low as 1 μM for cis-PDD and 5 μM for trans-PDD, which permitted over 80% survival of colony-forming ability. The maximum cross-linking effect by cis-PDD required about 12 hr of posttreatment incubation before it was fully developed, whereas the cross-linking effect of trans-PDD was fully developed at the end of the 1-hr drug exposure. The cross-linking effects of both agents were reversed upon further incubation of the cells.

INTRODUCTION

The formation of covalent cross-links involving DNA has long been suspected to be responsible for the potent cytotoxic and antitumor actions of bifunctional alkylating agents (9, 15, 46). Covalent cross-linking of the paired strands of the DNA helix has been studied extensively with bifunctional nitrogen mustards (5, 21, 30-32, 49), and it is clear that such lesions, if unrepaired, would interfere with DNA replication. Nitrogen mustard has also been reported to cross-link DNA to protein (16, 18, 25). Both types of cross-links have recently been observed with the potent antitumor compounds of the 1-chloroethyl-1-nitrosourea group, and a mechanism has been proposed whereby cross-links can result from a single chloroethylation reaction (10, 26).

Interstrand DNA cross-linking has also been demonstrated with Pt(II) complexes (8, 19, 22, 37, 38) presumably produced by nucleophilic displacement reactions analogous to those of alkylating agents (2). The Pt(II) complexes have 4 stable coordination sites oriented in a plane, so that there are distinct cis and trans isomers for complexes such as PDD1 (Chart 1). cis-PDD is highly active against several animal (40, 43, 45) and human (43) cancers, whereas trans-PDD had much less antitumor activity (6, 7). Although both isomers produce interstrand cross-links when reacted with purified DNA (19, 37, 44), the cis isomer is much more effective than is the trans isomer in producing interstrand cross-links in intact cells (37). Interstrand cross-linking has been proposed as the major cytotoxic lesion produced by cis-PDD.

For exploration of the possible role of DNA cross-linking in cytotoxic actions produced by various agents, it is necessary to measure the effects on DNA under the dosage conditions at which the major biological effects are observed. The possibility for DNA measurements of the required sensitivity has recently been enhanced by the development of the DNA alkaline elution technique (10, 28, 29). This technique permits the detection of DNA interstrand cross-linking and/or DNA protein cross-linking in cells treated at pharmacological dosage with nitrogen mustards and chloroethylnitrosoureas (10, 12, 14, 41, 42). Using this technique, we have investigated the effects of cis- and trans-PDD. It was of interest to determine whether the higher potency of the cis isomer for the production of interstrand cross-links, previously observed by other techniques, would also be exhibited as a difference between the 2 isomers in alkaline elution assays. Since the repair of cross-links is likely to be a crucial factor in cell survival and has been reported to occur following treatment of cells with cis-PDD (3, 47, 48), DNA cross-linking was studied as a function of time after treatment.

MATERIALS AND METHODS

Cells and Radioactive Labeling. L1210 mouse leukemia cells were grown in suspension culture in Roswell Park Memorial Institute Medium 1630 supplemented with 20% fetal calf serum plus penicillin and streptomycin. Stock cultures were maintained in static bottles without antibiotics and were used to initiate suspension cultures. Cultures utilized to assess drug effects were in exponential growth phase with a doubling time of 12 hr.

DNA was labeled in exponentially growing cultures by exposure to [2-14C]thymidine (0.01 to 0.02 μCi/ml) or to [methyl-3H]thymidine (0.1 to 0.2 μCi/ml; 10-8 M unlabeled thymidine added) for 20 hr at 37°. Radioactive label was removed 1 hr prior to drug treatment by centrifuging the cells at 900 rpm at 37° and resuspending them in fresh medium.

Drugs. cis-PDD (NSC 119875) and trans-PDD (NSC 131558) were obtained from the Drug Synthesis and Chemistry Branch of the Division of Cancer Treatment, National Cancer Institute. These drugs were dissolved in sterile 0.85% NaCl solution. All drug treatments were for 1 hr at 37°. After treatment, drug was removed by centrifuging the cells at 900 rpm for 3 min, and the cells were resuspended in fresh medium.

Alkaline Elution Analysis. Alkaline elution was carried out as previously described (10, 13, 28). For the introduction of a known frequency of DNA single-strand breaks.
prior to analysis, cells were irradiated with 600 R with a 200-kV X-ray source as previously described (28). During and after irradiation, cells were kept at ice temperature to prevent DNA repair. Cells were then diluted in cold phosphate-buffered saline [150 mM NaCl-4.28 mM KH2PO4-0.71 mM KH2PO4 (pH 7.4)] and deposited on a polyvinyl chloride filter (Millipore Corp., Bedford, Mass.). The cells were lysed on the filter with 5 ml of 2 M NaCl-0.2% Sarkosyl (Ciba-Geigy, Ardsley, N.Y.)-0.04 M EDTA, pH 10.0. This lysis solution was allowed to flow through the filter by gravity, and it removed most of the cell protein and RNA. The remaining DNA (more than 97% of that applied to the filter) was analyzed by elution with tetrapropylammonium hydroxide-EDTA, pH 12.1 (RSA Corp., Ardsley, N.Y.), at a constant flow rate of 0.03 to 0.04 ml/min. Fractions were collected every 90 min, mixed with 3.3 volumes of Aquasol (New England Nuclear, Boston, Mass.) plus 0.3% acetic acid, and counted in a liquid scintillation spectrometer.

Alkaline elution assays of drug-treated cells ([3H]thymidine label) were normalized relative to the elution of an internal standard ([3H]thymidine-labeled L1210 cells exposed to 150 R) as previously described (13). The 14C- and 3H-labeled cells were mixed immediately prior to assay. The 14C-labeled DNA retained on the filter was plotted against the 3H-labeled DNA retained on the filter, which can be considered to be a corrected time scale because the 3H-labeled DNA from cells irradiated with 150 R elutes linearly with time in the semilogarithmic plot (28). Cross-linking was quantitated in terms of "relative retention," arbitrarily defined as the fraction of the [3H]DNA retained on the filter with 60% of the [3H]DNA was retained. The essential findings were unaffected by the choice of [3H]DNA retention end points between 50 and 85%.

Survival Curves. Cells treated with drug for 1 hr at 37° were washed twice in fresh medium and assayed for colony survival in soft agar by the method of Chu and Fisher (4). Colonies were counted after 10 days of incubation at 37°. Cloning efficiency of control cells was 85 to 95%.

RESULTS

The effects of cis- and trans-PDD on survival of L1210 cells are shown in Chart 2. The D50 doses (i.e., dose increment that reduces survival by a factor of 1/e in the exponential portion of the survival curve) were 7 μM for cis-PDD and approximately 56 μM for trans-PDD. D50 values (the intercept of the extrapolation of the exponential portion of the curve with the abscissa) were 6 and 80 μM, respectively. Similar values for these 2 agents were found in HeLa cells by Pascoe and Roberts (37). These authors described a shoulder in the trans-PDD curve comparable to that seen in Chart 2.

In alkaline elution analysis, drug-induced DNA cross-linking is measured as a reduction in elution of DNA from drug-treated cells compared to that of DNA from untreated cells (Chart 3). For improvement of the sensitivity of the assay, a known frequency of single-strand breaks has been introduced into the DNA by X-irradiation after drug treatment and removal. Although we do not in this work attempt to quantitate cross-link frequencies or to distinguish between different possible types of cross-links, it is possible to assign relative magnitudes to the elution results. We do this by means of an empirical relation that has previously been found to be proportional to cross-link frequency, as judged from its observed linear dependence on nitrogen mustard concentrations in 30-min treatments (14, 41, 42). The linear measure of cross-link frequency used is log r/log r0, where r is the relative retention of DNA from irradiated, untreated control cells and r0 is the relative retention of DNA from irradiated and treated cells (14, 41, 42) (Chart 4). This relationship applies only in the absence of a significant amount of drug-induced single-strand breaks. Such single-strand breaks would be detected by an increase in elution of DNA from drug-treated, unirradiated cells (10).

Chart 3 displays typical alkaline elution curves following 1-hr treatments with cis- (Chart 3a) and trans-PDD (Chart 3b) at several concentrations. Results are shown for the time of maximum cross-link development, which occurred 12 hr after treatment with cis-PDD and immediately after the 1-hr treatment with trans-PDD. As described in "Materials and Methods," [3H]DNA retained on the filter is plotted against [3H]DNA retained (internal standard cell DNA). As can be seen, the DNA from drug-treated cells irradiated with 600 R elutes more slowly than did DNA from cells that were irradiated but not drug treated. The retention increases as drug dose increases.

An increase in DNA retention due to drug treatment was
Chart 3. The effect of cis-PDD (a) and trans-PDD (b) on DNA alkaline elution kinetics of L1210. Cells were exposed to the indicated concentrations of drug for 1 hr at 37°C. In the case of cis-PDD (a), the cells were then incubated for 12 hr in the absence of drug; whereas in the case of trans-PDD (b), the cells were assayed immediately after drug removal. At these times, the cross-linking effects produced by the 2 agents were maximal. Alkaline elution assays were performed either without the use of X-ray (○, □, ◦) or after exposure of the cells to 600 R at ice temperature (●, ■, ●,*). Arrows, point at which 60% of 3H-labeled internal standard DMA remains on the filter; the fraction of the [14C]DNA retained on the filter at this point is called relative retention.

also seen in assays that did not use X-ray (Chart 3, open symbols). Thus the small effect of the DNA strand breaks accumulating during alkaline elution (28) was masked by the action of drug in the same way that X-ray-induced DNA breaks are masked. This result also argues against the production of extensive DNA strand breakage (10) by the Pt(II) complexes.

The concentration dependence of the DNA cross-linking effect in cells treated with cis- and trans-PDD for 1 hr is shown in Chart 4. Each curve represents a different time period of incubation after drug removal. In Chart 5, the same data are plotted against time of incubation after drug removal and show the formation and disappearance of the cross-linking effect for cis- and trans-PDD. For cis-PDD the effect increased with time up to 12 hr and then decreased. With trans-PDD, the maximum effect was seen immediately after drug removal and decreased thereafter.

The cross-linking effects of equitoxic doses of these 2 agents are compared in Chart 6 where relative retention is plotted against time after drug removal for 10 µM cis-PDD and 200 µM trans-PDD, the doses that yielded approximately 10% colony-forming ability. At 0 and 6 hr after drug removal, trans-PDD displayed a much greater cross-linking effect than did cis-PDD. However the cross-linking effects were approximately equal at 12 to 18 hr after drug removal.

The question may be raised as to whether the observed reductions in elution may be due to drug-induced reductions in X-ray sensitivity of intracellular DNA. The drug effects would then be attributable to a reduction in X-ray-induced strand breaks rather than to the production of cross-links. We therefore used another method to introduce the strand breaks required for the assay. It has previously been reported that single-strand breakage occurs during the course of alkaline elution (28). At the usual elution pH of 12.1, this alkaline hydrolysis effect is slight, but at pH 12.8 the effect is substantial. Chart 7 demonstrates that the treatment of cells with cis-PDD reduces elution when strand breaks are introduced by alkaline hydrolysis during elution at pH 12.8. The drug effect is thus of the same nature in this assay, which does not use X-ray, as it is in the assays in which X-ray is used to generate DNA breaks.

**DISCUSSION**

DNA interstrand cross-linking by Pt(II) complexes has previously been demonstrated both in purified DNA and in
cells (8, 19, 22, 37, 38). The sensitivity of the previous methods of assay, however, is inadequate for routine pharmacological application. Our results with the technique of alkaline elution show an effect of Pt(II) complexes on DNA in L1210 mouse leukemia cells treated with doses that permitted up to 80% survival of colony-forming ability. We believe that the observed effects represent 1 or more forms of DNA cross-linking on the basis of mechanistic considerations and observations with other agents that produce similar effects. The mechanistic considerations are that the effect of the drug is to reduce the alkaline elution rate as if the drug treatment had increased the size of the DNA strands. Interstrand cross-linking would produce such an increase in size. Elution rate could, however, be reduced by linkage of DNA to substances such as protein, which tend to stick to filters, so that the observed effect could be due to cross-links between DNA and other molecules (10–14). The question of the type of cross-link responsible for our results is still under study. Support for the idea that some kind of cross-linking is involved, however, stems from previous observations of similar effects on alkaline elution by agents known from other types of evidence to be capable of forming cross-links (10–12, 26, 30, 41, 42).

As an alternative to cross-linking, it could be proposed that the drug treatment reduced the X-ray sensitivity of the DNA. Irradiation of the cells to generate the strand breaks required for the cross-linking assays would then have produced fewer breaks and given a spurious result. The magnitude of the reduction in X-ray sensitivity that would have to be assumed, however, is so large as to be unprecedented. We have carried out experiments in which strand breaks were introduced by alkaline hydrolysis at pH 12.8 instead of by X-ray (Chart 7). The results showed that at least a major part of the effect of cis-PDD is independent of the use of X-ray in the assay. A similar effect can be seen at pH 12.1 because drug-treated, unirradiated cells elute more slowly than untreated, unirradiated ones (Chart 3, open symbols). The possibility cannot be excluded, however, that changes in X-ray sensitivity might have small effects on the magnitudes of observed changes in elution.

The cross-linking effect produced by cis- and trans-PDD was observed to change with time after drug treatment and removal. cis-PDD produced a maximum cross-linking effect 12 hr after drug removal, decreasing thereafter. In contrast, trans-PDD produced its maximum effect immediately, and the effect then decreased. In the alkaline elution system, disappearance of cross-linking effect with time after drug removal may result from either drug-induced single-strand breaks or cellular repair of drug-induced cross-links (10, 12–14). Since we did not detect single-strand breaks in drug-treated, unirradiated cells, it is likely that cross-links were removed. Effects that are consistent with removal of DNA cross-linking induced by cis-PDD have been described (47).

The cross-linking effect produced by cis-PDD increased for many hr after removal of drug, suggesting a 2-step process in which platinum first binds to DNA and then forms cross-links through a slow second reaction. This type of delayed cross-linking has been observed with chloroethylnitrosoureas (26). trans-PDD, however, did not exhibit any delay in cross-link formation. The cross-linking effect that was seen with trans-PDD by alkaline elution was maximal immediately after a 1-hr drug treatment and diminished over a 12-hr period thereafter. This suggests that cis- and trans-PDD may differ in the types of cross-links they produce.

For the comparison of the kinetics with cytotoxicity, we examined the cross-linking effect of these drugs at equitoxic doses (~10% survival). Although the trans isomer displayed the greater cross-linking effect from 0 to 6 hr, at 12 and 18 hr the effects produced by the 2 isomers were approximately equal. The types of cross-links present at these later times, however, remain to be determined.

DNA interstrand cross-linking has been demonstrated in HeLa cells by the buoyant density labeling method (37); cis-PDD produced these cross-links with 10-fold greater potency than did trans-PDD. The limited sensitivity of this method, however, required the use of supralethal drug doses. Although this and other studies (36, 37, 44) detected interstrand cross-linking as a rare event, with estimates for cis-PDD between 1/30 and 1/400 cross-links per bound platinum, the possible existence of slowly forming cross-links was not examined. Delayed effects upon cellular DNA have been reported to occur after cis-PDD treatment (23, 47), and it is interesting to compare our results with the findings of these studies. Van Den Berg and Roberts (47) investigated the effects of cis-PDD on Chinese hamster cells using a DNA alkaline sedimentation method. The cis-PDD dosage, 30 μM for 2 hr, used by these authors yielded 10% survival of colony-forming ability, which is within the range of our experiments. This treatment increased the fraction of the DNA sedimenting rapidly (>650S), and it was...
suggested that this may reflect DNA cross-linking. This result is consistent with our findings if there is a common factor between such rapidly sedimenting DNA and DNA retention on filters in alkaline elution, as has been previously suggested (27). After a 6-hr incubation following drug removal, the DNA sedimentation was reduced to a narrow band of about 350S, suggesting the presence of DNA strand breaks. Although our experiments showed no signs of DNA strand breaks, DNA cross-links may more effectively mask DNA strand breakage in alkaline elution than in alkaline sedimentation assays (27). Both studies suggest that some repair of DNA lesions has occurred by 20 to 24 hr after treatment and that this repair is likely to involve the formation of DNA strand breaks.

Howie and Gale (23), using incorporation of [methyl-3H]thymidine into the acid-insoluble fraction of Ehrlich ascites tumor cells, demonstrated inhibition of DNA synthesis in cells treated in vitro and in vivo with cis-PDD. Maximum inhibition of DNA synthesis was observed at 4 to 6 hr, illustrating another example of a delayed pharmacological effect of cis-PDD.

DNA cross-linking by cis- and trans-PDD has also been examined by treatment of isolated nucleosides, binucleotides, polynucleotides, and DNA with Pt(II) compounds in an attempt to correlate biochemical effects with drug activity. Several studies did show that only cis-PDD could produce DNA interstrand cross-links (8, 19, 20, 22, 34, 39). The most frequently proposed site for this interstrand cross-link was between 6-NH2 groups of adenines within A-T pairs on opposite strands of DNA. These lie directly above one another within the DNA helix with an interplane distance that approximates the distance between the leaving groups of cis-PDD (1, 20, 39). It is proposed that steric considerations preclude the trans isomer from producing such a cross-link, thus explaining the differential activity.

Other investigators postulate cis-PDD binding to N-7 and O-6 of guanine on a single strand that can cause strand separation and disrupt hydrogen bonding, thus preventing accurate DNA replication (17, 33, 35). Such disruption could eventually allow subsequent interstrand cross-linking as well (24). However, the actual form of binding of the Pt(II) compounds to DNA and the lethal lesions of these agents remain unknown. The exact reason for the activity of cis-PDD and the relative inactivity of trans-PDD is, likewise, unexplained.

We have demonstrated a cross-linking effect by these 2 drugs. The kinetics of formation and disappearance of this effect with time after drug treatment and removal were distinctive and different for each agent. Further, comparing equitoxic doses of cis- and trans-PDD revealed greater early cross-linking effect with the less active agent (trans-PDD), while only at later times (12 to 18 hr) were the effects of the
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