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cis-DIAMMINEDICHLOROPLATINUM(II) (DDP)-INDUCED CROSSLINKING AND CROSSLINK REMOVAL IN L1210 CELLS *IN VITRO* AFTER THEOPHYLLINE CO-TREATMENT*

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Abstract—The present study investigated the mechanism by which theophylline decreases *cis*-diamminedichloroplatinum(II) (DDP)-induced DNA crosslinking in L1210 cells. Alkaline elution of DNA from L1210 cells treated with DDP in the presence and absence of 1 mM theophylline showed that theophylline decreased interstrand crosslinking by 20%. DNA-protein crosslinking (PXL) immediately following platinum removal (used as a measure of active drug delivered to the cell nucleus) was not altered by theophylline nor were the kinetics of either DNA interstrand (ISC) or DNA-protein crosslink formation after DDP treatment changed by the presence of theophylline. Peak protein crosslinking occurred 6 hr and peak interstrand crosslinking occurred 12 hr after DDP removal. We measured crosslink removal by using thiourea to block the conversion of platinum monoadducts to crosslinks. DNA-protein crosslinks were removed more rapidly in the presence of theophylline. There was no change in interstrand crosslink removal rate when theophylline was present. We conclude that the addition of theophylline to DDP treatment results in decreased amounts of DNA interstrand crosslinking most likely by increasing the removal of DPP-DNA monoadducts. This latter process may be reflected by the increased removal rates for DNA-protein crosslinks.

Although methylxanthines can enhance the cytotoxicity of alkylating agents, ionizing radiation, and ultraviolet irradiation [1–5], this does not appear to be mediated by increased DNA damage. There is usually a reversal of the damage-induced DNA synthesis inhibition [2, 3, 5–7]. We have, however, noted decreased DNA interstrand crosslinking following bifunctional alkylating agent treatment in the presence of theophylline. This has occurred whether cells are sensitive or resistant to the cytotoxicity-enhancing effects of the methylxanthine [5, 7]. We decided to study this effect in greater detail, and chose the drug *cis*-diamminedichloroplatinum(II) (DDP‡) because of our ability to separate certain aspects of crosslink formation and removal as outlined below.

MATERIALS AND METHODS

Cell culture. Murine L1210 leukemia cells were grown in log phase in suspension culture in Roswell Park Memorial Institute (RPMI) medium 1630 with 15% heat-inactivated fetal bovine serum (Dutchland Laboratories, Denver, PA) with 1 mM L-glutamine and penicillin and streptomycin. Cultures were maintained in a forced draft incubator with 7% carbon dioxide and 90% humidity.

Drug treatment. DDP was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The drug was dissolved in distilled water at a concentration of 1 mM immediately prior to each drug treatment. Theophylline (1,3-dimethylxanthine, Eastman Kodak Co., Rochester, NY) was dissolved directly in the tissue culture medium at the concentration desired (1 mM). For drug treatments, cells were removed from the growth medium and resuspended in RPMI 1630 with 1% heat-inactivated fetal bovine serum plus glutamine, penicillin, and streptomycin either with or without theophylline. After 15 min, the DDP was added and the cells were reincubated for 1 hr. The drug was removed by centrifugation, and the cells were washed with RPMI 1630 and 15% heat-inactivated fetal bovine serum with or without theophylline as appropriate. The cells were then counted, put into flasks, and reincubated at a concentration of 5×10^5 cells/ml. For thiourea treatments, thiourea (Fisher Scientific Co.) was dissolved in RPMI 1630 with 15% fetal bovine serum at a concentration of 0.1 M. The solution was sterilized by filtration. Six hours following DDP removal, cells were suspended in the thiourea containing medium for 1 hr. They were washed with RPMI 1630 with 15% fetal calf serum, put back into flasks, and reincubated.

Radionuclide labeling. Cells were grown for 24–36 hr in log phase in medium containing [2-^{14}C]-thymidine at 0.02 μCi/ml (58 mCi/mmole, New England Nuclear, Boston, MA). The medium was removed 12 hr prior to drug treatment, and the cells were resuspended in fresh RPMI 1630 with 15% serum without label. Cells used as internal standards were grown for 24 hr in medium containing [*methyl*-^{3}H]thymidine at 0.05 μCi/ml (20 Ci/mmole, New England Nuclear).

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‡ Abbreviations: DDP, *cis*-diamminedichloroplatinum(II); RPMI, Roswell Park Memorial Institute; ISC, DNA-DNA interstrand crosslinking; and PXL, DNA-protein crosslinking.

Alkaline elution. The alkaline elution of DNA from membrane filters was performed as described previously [8, 9]. Briefly, at intervals after drug treatment, cells were removed from culture and placed in plastic tubes on ice. For crosslinking assays, the cells underwent [^{137}Cs] gamma irradiation (Gammacell 40, Atomic Energy of Canada, Ltd.). Cells were then mixed with tritium-labeled cells, and the mixtures were applied by gravity to 3 μm pore-sized polycarbonate filters (Nucleopore Corp., Pleasanton, CA) and lysed *in situ* with 2% sodium dodecyl sulfate (SDS, 99% purity, BDH Biochemicals, Poole, England), 0.02 M EDTA and 0.1 M glycine, pH 10. Following the lysis solution, 2 ml of a deproteinizing solution (proteinase K, Fisher Scientific, 0.5 mg/ml in the lysis solution) was layered onto the filters and this was followed by the elution solution of tetrapropyl ammonium hydroxide (RSA Corp., Ardsley, NY), 0.02 M EDTA (acid form), and 0.1% SDS at pH 12.1. Pump rates were 0.035 to 0.045 ml/min and fractions were collected at 3-hr intervals. Following sample processing as described previously [9], samples were counted in Budgetsolve (RPI, Elk Grove Village, IL) in a Packard Tricarb liquid scintillation counter. For DNA interstrand crosslink assays, experimental cells received 300 rads of irradiation. All internal standard (tritium labeled cells) received 300 rads of irradiation. For protein crosslink (PXL) assays, the alkaline elution technique as modified by Kohn and Ewig [10] was performed. Such assays utilized 2 μm polyvinyl chloride filters (Millipore Corp., Bedford, MA). Following the lysis step, 5 ml of 0.02 M EDTA (pH 10) wash was dripped through the filter to remove any excess SDS. Proteinase K was not used, and the eluting solution of tetrapropyl ammonium hydroxide was made without SDS. All cells for protein crosslink assays received 1000 rads of irradiation.

Calculations. DNA interstrand crosslinking was quantitated according to the formula

ISC (in rad equivalents)

$$= \left[\left(\frac{1 - \frac{R_0}{r_0}}{1 - \frac{R}{r}} \right)^{1/2} - 1 \right] (P_{br} + P_{bd})$$

where ISC is the relative crosslinking effect, R_0 is the fraction of DNA retained in irradiated control cells after 15 hr of elution, R is the fraction of DNA retained on the filter in irradiated drug-treated cells after 15 hr of elution, r and r_0 are the fractions of DNA remaining after the lysis in drug-treated and control cells, respectively, and P_{bd} and P_{br} are frequencies of single-strand breaks by drug and irradiation respectively [11]. DNA-protein crosslinking was quantiated according to the method of Kohn and Ewig [10] as refined by Ross *et al.* [12] according to the formula:

$$\text{PXL} = \left[(1 - R)^{-1/2} - (1 - R_0)^{-1/2} \right) P_{br}$$

where PXL is the frequency of drug-induced DNA protein crosslinks, P_{br} is the frequency of X-ray-induced single-strand breaks, and R and R_0 are the fractions of the DNA eluting in the slow component in the presence and absence of drug respectively.

RESULTS

Figure 1 shows a representative interstrand crosslink alkaline elution profile of DNA from L1210 cells treated with DDP at different doses in the presence and absence of theophylline measured 6 hr after DDP treatment. While increasing the dose of DDP resulted in increasing numbers of interstrand crosslinks (decreased elution of the DNA) at each dose, cells pretreated with theophylline showed slightly decreased interstrand crosslinking from cells treated without theophylline. While it appears from Fig. 1 that theophylline decreased the X-ray-induced strand breakage in control cells, this was not the case overall. The [^{14}C] retention at 30% of internal standard was 031.57% ($\pm$ 0.0404) and 032.65% ($\pm$ 0.0411) for cells treated without and with theophylline respectively ($P < 0.4$, paired t-test, 16 degrees of freedom). A change in X-ray responsiveness cannot explain the crosslinking changes.

Figure 2 shows the least squares linear regression dose–response curves for interstrand crosslinking with and without theophylline measured 6 hr after DDP treatment. While a clear dose-response was seen in either case, the slope of the curve with theophylline was slightly but significantly decreased from that obtained without theophylline ($P < 0.02$, t-test for equality of regression, slopes, 3 degrees of freedom). The ratio of the slopes obtained from the regression lines is 0.8.

We next tested whether this decrease in DNA interstrand crosslinking could be the result of decreased platinum binding to DNA. It is known that interstrand crosslink formation is a time-dependent phenomenon. The initial step of the reaction is the formation of a drug-DNA monoadduct and the second event (which forms the crosslink) takes some time to form [13, 14]. The kinetics of protein crosslink formation are much more rapid, however [13], and we and others have used DNA-protein crosslinking immediately following drug treatment as a measure of active drug species reaching the cell nucleus [13]. Figure 3 shows a representative DNA-protein crosslink alkaline elution profile of L1210 DNA immediately following DDP treatment. With this method the level at which the slow eluting portion of the elution curve flattens out becomes a direct measurement of the number of DNA-protein crosslinks formed. At both doses there appear to be slightly fewer DNA-protein crosslinks in theophylline-treated cells. Figure 4 shows the relationship of protein crosslink formation to DDP dose immediately following drug treatment from several experiments. These data do not substantiate a difference in protein crosslinking in cells treated with or without theophylline ($P < 0.5$, Chi squared for combined tests of significance [15], 4 degrees of freedom).

Because crosslink formation is time dependent, we investigated whether the addition of theophylline resulted in changes in the time dependency of crosslink formation. Figure 5 shows this tendency. Crosslinks are plotted as percent of peak crosslinking. No

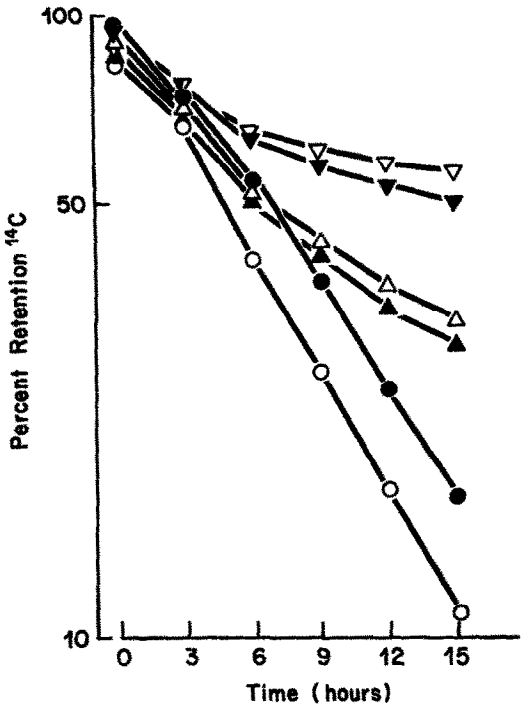

Fig. 1. DNA-DNA interstrand crosslinking alkaline elution profile from L1210 cells treated with DDP in the presence or absence of theophylline. Open symbols, no theophylline; solid symbols, 1 mM theophylline. Key: (○, ●) no drug; (△, ▲) 30 μM DPP; and (▽, ▼) 60 μM DDP. All cells received 300 rads of irradiation.

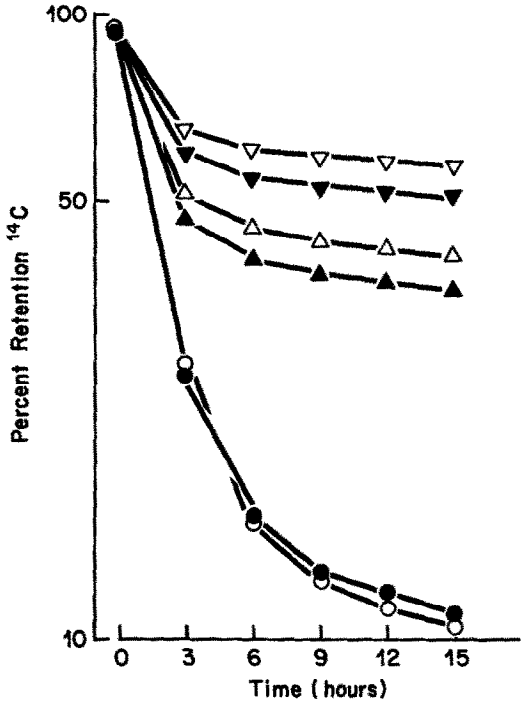

Fig. 3. DNA-protein crosslinking alkaline elution profile from L1210 cells treated with DDP in the presence or absence of theophylline. Open symbols, no theophylline; solid symbols, 1 mM theophylline. Key: (○, ●) no drug, (△, ▲) 30 μM DDP and (▽, ▼) 60 μM DDP. All cells received 1000 rads of irradiation.

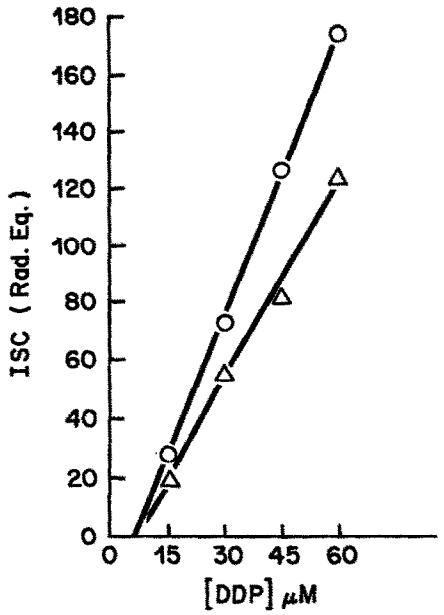

Fig. 2. DNA-DNA interstrand crosslinking in L1210 cells treated with DDP in the presence or absence of theophylline. Crosslinking measurements were performed 6 hr after DDP treatment. Symbols represent the means from four experiments, solid lines are the least squares linear regression lines from the individual data points. Key: (○) no theophylline, and (△) 1 mM theophylline.

alterations in crosslink formation versus time were noted with theophylline. Peak DNA-protein crosslinking was seen 6 hr after DDP treatment and peak DNA-DNA interstrand crosslinking was seen 12 hr after DDP treatment, whether or not theophylline was present.

As Micetich *et al.* [13] have clearly elucidated, crosslinking as measured at any one point in time is a function of crosslink formation and removal.

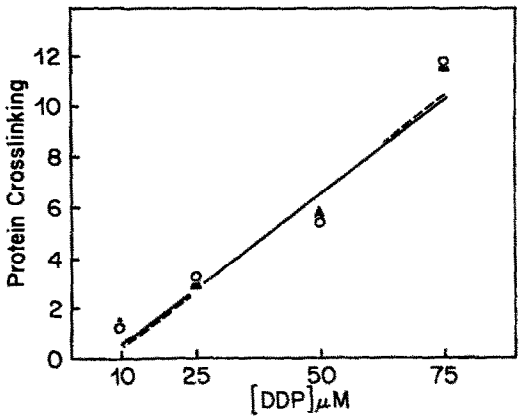

Fig. 4. DNA-protein crosslinking in L1210 cells treated with DDP in the presence or absence of theophylline. Symbols represent the means from four experiments. Lines are the least squares linear regression lines from the original data points. All assays were performed immediately following DDP treatment. Key: (○) no theophylline, and (▲) 1 mM theophylline.

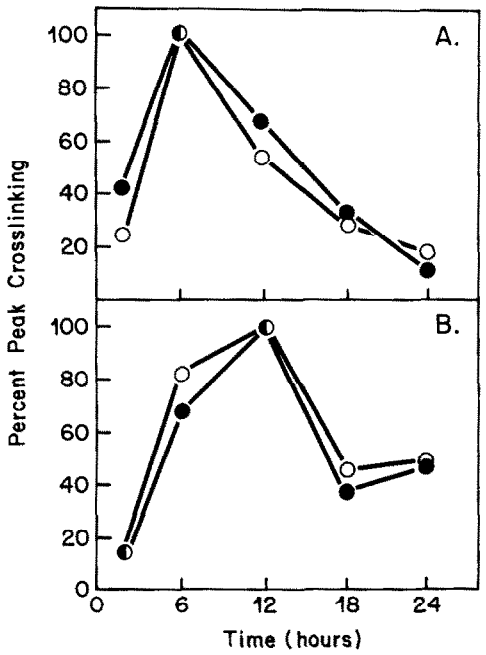

Fig. 5. DNA crosslinking (as percent of peak crosslinking) as a function of time after DDP removal in L1210 cells treated in the presence or absence of theophylline. Open symbols, no theophylline; solid symbols, 1 mM theophylline. (A) DNA-protein crosslinking, and (B) DNA-DNA interstrand crosslinking. One hundred percent (peak) values: PXL 29.04 (0 mM), 17.01 (1 mM) – PXL/10^7 nucleotides; ISC 554 (0 mM), 563 (1 mM) – Rad Eq.

Because thiourea binds to platinum DNA monoadducts thereby preventing subsequent crosslink formation [16], by treating cells with thiourea after DDP treatment, fewer crosslinks reflect crosslink removal since no new crosslinks are being formed [13]. We utilized this technique to investigate whether theophylline also altered the rate of crosslink removal in L1210 cells. In these experiments cells were treated with DDP in the presence or absence of theophylline and reincubated for 6 hr to allow crosslinks to form. Cells were then incubated with thiourea for 1 hr to quench any remaining DNA platinum monoadducts, and interstrand and protein crosslinking was measured immediately and following an additional incubation (24 hr following DDP removal). Table 1 summarizes these results. Protein crosslinking was not different 6 hr after DDP removal (immediately after thiourea) with mean crosslinking levels of 4.13 and 3.16 crosslinks/10^7 nucleotides for cells treated in the absence and presence of theophylline respectively. A significant difference was seen 24 hr after DDP treatment (18 hr after thiourea) with mean values of 2.50 crosslinks/10^7 nucleotides in control cells and 1.55 crosslinks/10^7 nucleotides in cells co-treated with theophylline. If it is assumed that a simple enzymatically mediated crosslink removal system is present, the rate constant for crosslink removal without theophylline is −0.028/hr. Removal in the presence of theophylline is −0.040/hr. From this it would appear that protein crosslink removal is more rapid in the presence of theophylline than in its absence.

Table 1. Removal of DNA-protein crosslinks after thiourea block of further crosslink formation

Theophylline (mM)	PXL/10^7 nucleotide: Time after DDP 6 hr	PXL/10^7 nucleotide: Time after DDP 24 hr	Removal rate constant* (per hr)
0	4.13 ± 2.57	2.50 ± 0.63	−0.028
1	3.16 ± 2.02	1.55 ± 0.38	−0.040
P value†	<0.2	<0.02	

See text for complete discussion of timing of DDP and thiourea treatments. All measurements were performed after thiourea removal. Values are means ± S.D.

* Assumes a simple enzyme mediated removal system.

† Paired *t*-test, comparing values at each time point, N = 6 (5 degrees of freedom) at each time point.

Interstrand crosslinking showed the expected 20% reduction in cells co-treated with theophylline (37 and 29 rad equivalents) and measured immediately after thiourea. There was no difference in ISC measured 12 or 18 hr after thiourea in cells treated in the presence or absence of theophylline. The rate constants for ISC removal were −0.07/hr and −0.12/hr for cells treated without and with theophylline respectively ($P < 0.25$).

DISCUSSION

We have shown that the combination of theophylline and DDP results in a 20% decrease in DNA interstrand crosslinking in L1210 cells. We have demonstrated this previously in L1210 cells treated with theophylline and a nitrosourea [5] and in a human Burkitt's lymphoma cell line treated with theophylline and L-phenylalanine mustard [7]. Measurements of DNA protein crosslinking immediately following drug removal did not show a difference for cells treated in the presence or absence of theophylline. We and others have used this measurement as an indication of the amount of active alkylating species reaching the cell nucleus [13]. These data therefore suggest that the decrease in interstrand crosslinking is not due to less platinum in the cell nucleus.

Experiments utilizing thiourea to quench monoadduct conversions to crosslinks allow a direct measurement of crosslink removal [13] since in the normal situation crosslinking at any one period of time is a function of crosslink formation and removal. Because crosslink formation is blocked following thiourea treatment [16], measurement of crosslink diminution over time gives a true indication of the rate of crosslink removal. With this technique we demonstrated that, although interstrand crosslink removal was not affected by the presence of theophylline, DNA protein crosslink removal occurred more rapidly in the presence of theophylline.

We hypothesize that the addition of theophylline to our cells results in a pattern of events similar to that described by Micetich *et al.* comparing DDP sensitive and resistant L1210 lines [13]. In their comparison, there was no difference in protein crosslinking immediately following DDP treatment. ISC was decreased in the platinum resistant line but ISC removal rates were similar between the two lines.

They postulated that the primary difference must be related to monoadduct removal with the DDP resistant line showing more rapid rates of monoadduct removal. This more rapid removal would result in fewer platinum monoadducts being available to form interstrand crosslinks. They did not measure DNA protein crosslinking except immediately following drug removal. We would infer a similar situation in our cells following theophylline treatment and suggest that the increased rate of DNA-protein crosslink removal mirrors the hypothesized increase in platinum monoadduct removal as well.

With the exception of the O^6-methylguanine-methyltransferase enzyme system, the mechanisms by which cells remove monoadducts, protein and interstrand crosslinks are not well understood. Some researchers have suggested that methylxanthines may interact with DNA resulting in altered conformation of the chromatin [3]. Such changes could increase the accessibility of repair enzymes to damaged bases, thereby facilitating adduct removal. Whether such a conformational change results in increased rates of removal of both DNA-protein crosslinks and monoadducts, or whether the two lesions are truly excised by a similar mechanism, remains to be elucidated.

Finally, it is evident that the major effect of theophylline on the cellular response to alkylating agent treatment is not that of increased damage repair. While in the system described by Micetich *et al.* increased repair which lowered the levels of interstrand crosslinking resulted in increased cell survival, such is not the case in our system. We have published previously the survival characteristics of L1210 cells treated with a number of bifunctional alkylating agents including DDP in the presence and absence of theophylline. While such theophylline co-treatment increases the sensitivity of L1210 cells to nitrosoureas and *L*-phenylalanine mustard [5, 6], such co-treatment does not change survival with DDP [6]. This was despite the fact that the decrease in replicon initiation seen with DDP was reversed by theophylline co-treatment in L1210 cells, a result which usually correlates with increased cytotoxicity [5–7]. In addition, while theophylline decreased ISC in a human Burkitt's lymphoma cell line treated with L-phenylalanine mustard, there was also no alteration in drug (L-PAM) induced cytotoxicity [7]. The relationship between the induction of DNA damage by alkylating agents, the repair of such damage, and the functional consequences of such lesions is complex, and the precise mechanism by which the physical damage of DNA results in the functional consequence of cytotoxicity remains to be determined. However, it is clear that, regardless of its effect on the cytotoxic potential of alkylators and DDP, theophylline does alter the pattern of DNA damage induced by these agents.

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