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α-Difluoromethylornithine-Induced Polyamine Depletion of 9L Tumor Cells Modifies Drug-Induced DNA Cross-Link Formation

Abstract. *Depletion of intracellular levels of polyamines, which are believed to have a role in the intranuclear stabilization of DNA, alters the cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea and* cis*-diamminedichloroplatinum II in 9L rat brain tumor cells. Alkaline elution techniques were used to show that polyamine depletion alters the number of DNA cross-links formed by these cytotoxic agents.*

The polyamines putrescine, spermidine, and spermine have various functions in cells, including an apparent role in the stabilization of DNA (*1*). Polyamines stabilize cell-free DNA, thus inhibiting enzymatic degradation (*2*) and denaturation by x-rays (*3*) and heat (*4*). α-Difluoromethylornithine (DFMO), an enzyme-activated, irreversible inhibitor of ornithine decarboxylase, the first enzyme in the polyamine biosynthetic pathway (*5*), depletes polyamine in 9L rat brain tumor cells (*6*). Polyamine depletion appears to cause an alteration in the conformation of 9L cell DNA (*7*). These effects of polyamines on the structural integrity of DNA, the probable target for the cytotoxic action of many anticancer drugs, suggest a possible role for polyamine depletion in cancer chemotherapy. We have shown that depletion of polyamines in 9L cells modifies the cytotoxic effects of several antineoplastic drugs both in vitro and in vivo (*8*). However, the mechanism by which polyamine depletion alters the reaction between active drug species and DNA has not been definitively established. We now report that polyamine depletion changes the number of cross-links formed by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and *cis*-diamminedichloroplatinum II (*cis*-platinum).

The cytotoxicity of chloroethylnitrosoureas is thought to be the result of a sequence of reactions in which an initial alkylation of DNA by a chloroalkyl carbonium ion intermediate is followed by nucleophilic displacement of a primary chlorine group to form DNA interstrand cross-links (*9*). We have shown in 9L cells that polyamine depletion caused by DFMO does not affect the cytotoxicity of nitrosoureas that do not cross-link DNA, does not change the number of DNA monoadducts in cells treated with ^{14}C-labeled 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU), and does not alter the activity of several enzymes that repair alkylation damage (*8*). However, DFMO-induced polyamine depletion increases the cytotoxicity of the chlorethylnitrosoureas BCNU, MeCCNU, and chlorozotocin (*8*), all of which cross-link DNA.

cis-Platinum is an anticancer agent that is thought to kill cells by forming DNA cross-links (*10*). However, in contrast to results obtained with chloroethylnitrosoureas, the cytotoxicity of *cis*-platinum was decreased by DFMO-induced polyamine depletion (*11*). The two chlorine leaving groups of *cis*-platinum are separated by 3.3 Å (*12*), a distance similar to the interplanar separation of DNA bases (3.4 Å) (*13*). A number of nucleophiles in native DNA are separated by the same distance (*14*). Presumably, after the initial formation of a chloroplatinate-base covalent bond in native DNA, other base nucleophiles displace the second chlorine and form either intra- or interstrand cross-links. An alteration in DNA structure caused by polyamine depletion may change the spatial configuration of these nucleophiles, which could make the cross-linking reaction with *cis*-platinum mechanistically unfavorable.

Our data for the experiments with chloroethylnitrosoureas and *cis*-platinum were obtained with a colony-forming efficiency (cell survival) assay, which reflects the combined effects of many factors that lead to cell death. With this method it was not possible to determine directly the mechanism by which polyamine depletion affected the cytotoxicity of chloroethylnitrosoureas and *cis*-platinum. The sister chromatid exchange assay, which measures effects on the chromosomal level, is an established method used to measure damage to DNA (*15*). Polyamine depletion increased BCNU-induced and decreased *cis*-platinum-induced sister chromatid exchanges, the same relative changes observed in the cytotoxicity experiments (*16*), which suggests that polyamine deficiency alters drug-induced DNA damage.

Our data suggested that the modification of cytotoxicity is the result of a change in the number of DNA cross-links formed by these anticancer drugs. We therefore used the alkaline elution assay to measure changes in the number of DNA cross-links formed by treatment with BCNU and *cis*-platinum in polyamine-depleted 9L cells. In the alkaline elution assay, cells are lysed on a filter, and the rate at which single-stranded DNA passes through the filter is determined (*17*). The presence of strand breaks increases the rate of elution. For the determination of cross-links, cells are irradiated before lysis to induce a fixed number of random single-strand breaks. Cross-linking between DNA strands or between DNA and protein is the source of the larger molecular species that remain after lysis. The presence of these larger species reduces the effects of irradiation and decreases the amount of DNA eluted from the filter. In the presence of proteinase K, the number of DNA-protein cross-links is greatly reduced; thus, if the enzyme is present in the lysing solution, the number of DNA interstrand cross-links is measured (*17*).

The 9L cells were seeded and cultured as described for our cytotoxicity and sister chromatid exchange experiments (*11, 16*). Cells were incubated with 1 m*M* DFMO for 72 hours and then treated with BCNU or *cis*-platinum for 1 hour. This procedure was also followed in experiments in which putrescine was added to the culture medium to replenish intracellular polyamine levels; after 48 hours of incubation with DFMO, putrescine was added to achieve a final concentration of 1 m*M*. After drug treatment, cells were incubated for 6 hours, the approximate length of time required for the maximum number of cross-links to form for 9L and other cell lines (*18*). The alkaline elution assay and the fluorometric quantitation of DNA were performed as described by Murray and Meyn (*19*).

Treatment of 9L cells with DFMO alone had no effect on the elution profiles

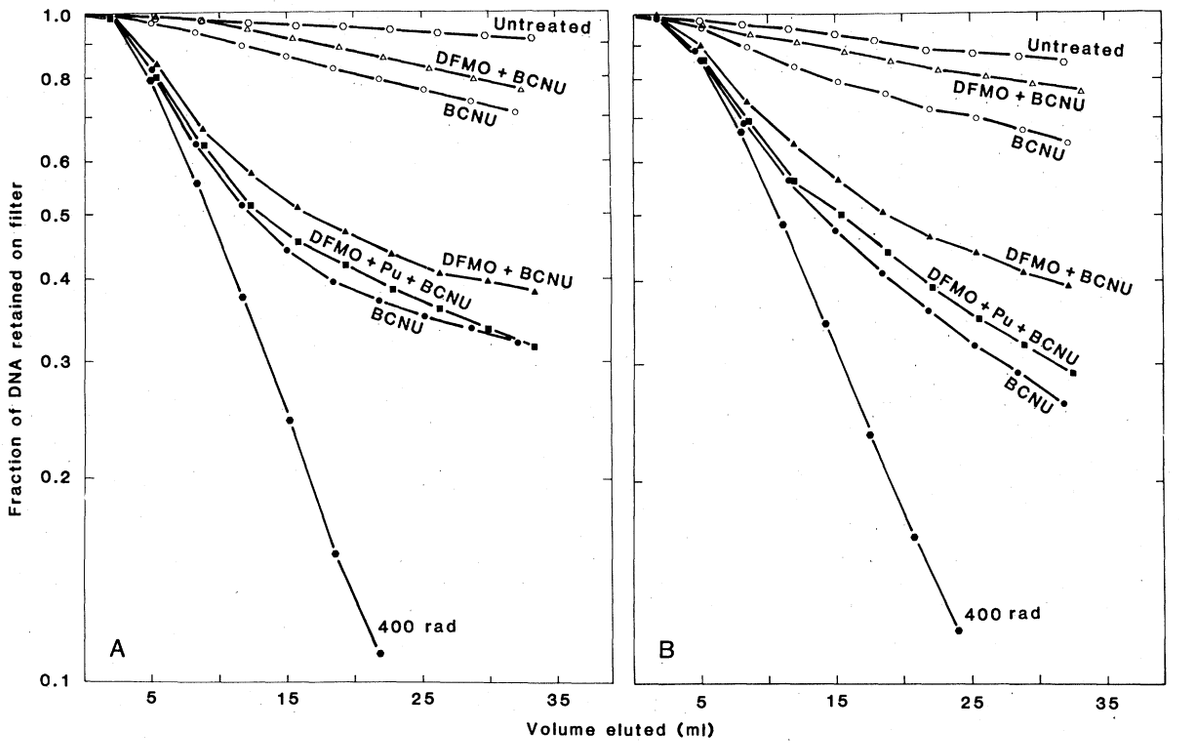

Fig. 1. Representative alkaline elution profiles obtained after treatment of 9L cells with BCNU (80 μ*M*) alone, with DFMO (1 m*M*) and BCNU, or with DFMO, putrescine (*Pu*) (1 m*M*), and BCNU. After trypsinization, 9L cell suspensions to be assayed were irradiated with 400 rad of ^{137}Cs gamma radiation at 4°C (closed symbols). Cell suspensions assayed for DNA strand breaks were not irradiated (open symbols). Cells (3.6×10^6 to 4.1×10^6) were deposited on polycarbonate filters (pore size, 2 μm) and lysed with a solution containing 2*M* NaCl, 0.04*M* Na_2EDTA, and 0.2 percent Sarkosyl (*p*H 10.0), for measuring total cross-linking (A) without proteinase K, or for measuring DNA interstrand cross-linking (B) with proteinase K (0.5 mg/ml). The filter was washed with 0.02*M* Na_2EDTA (*p*H 10.0), and the DNA was eluted in the dark with tetrapropylammonium hydroxide 0.02*M* H_4EDTA (*p*H 12.2) at a flow rate of 0.036 to 0.038 ml/min. Fractions were collected every 90 minutes for 18 hours. After the elution procedure, DNA was removed from the filters by heating at 65°C for 20 minutes in 5 ml of the eluting solution, followed by extensive vortexing. DNA remaining in the filter holder or barrel was removed by flushing with 5 ml of eluting buffer (*19*). The amount of DNA in each fraction, including the filter and wash solutions, was determined with the assay described by Murray and Meyn (*19*) that detects DNA by measuring fluorescence from bound Hoechst 33258 dye (*20*).

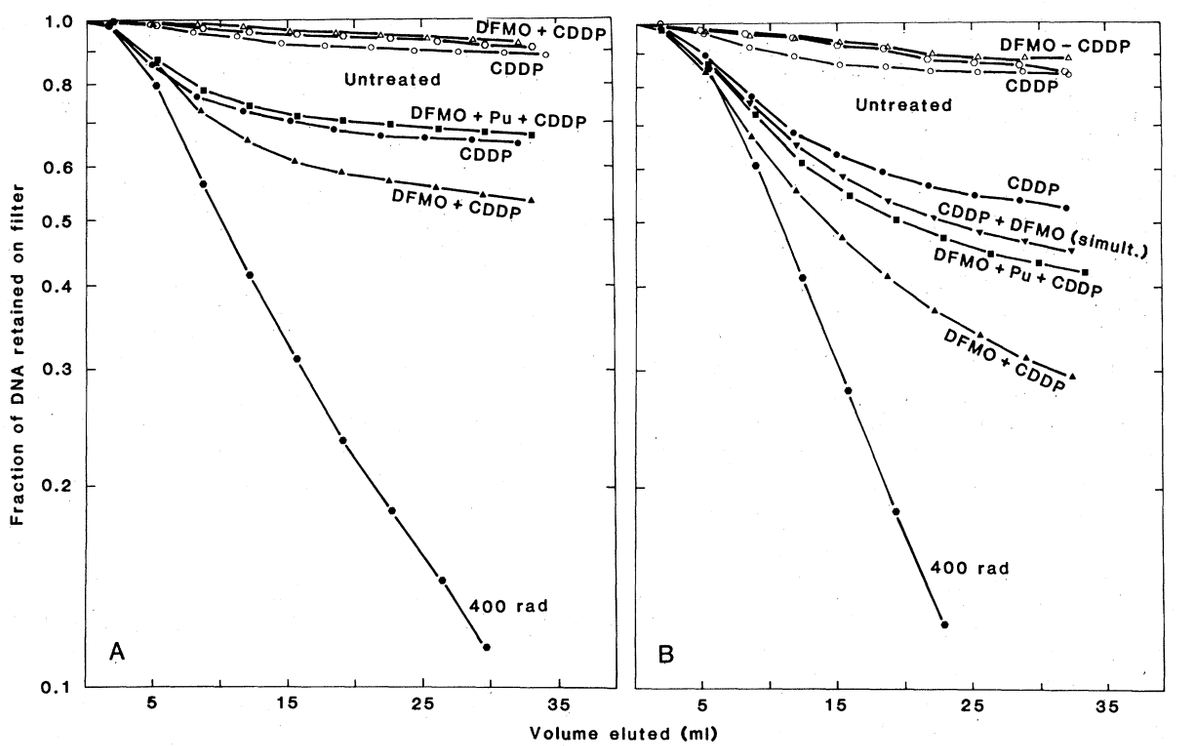

Fig. 2. Typical alkaline elution profiles after treatment of cells with *cis*-platinum (*CDDP*) (80 μ*M*) alone, with DFMO (1 m*M*) and *cis*-platinum, with DFMO, putrescine (*Pu*) (1 m*M*), and *cis*-platinum, or with *cis*-platinum and DFMO simultaneously. The alkaline elution assay was performed as described in the legend to Fig. 1. (A) Total cross-linking; (B) DNA interstrand cross-linking.

Table 1. The effects of DFMO on DNA cross-linking by BCNU (80 μM) and *cis*-platinum (80 μM). The cross-linking factor (CLF) was defined as $[(1 - R_o)/(1 - R_1)]^{1/2} - 1$, where R_o and R_1 are the fractions of the DNA remaining on the filter after 30 ml of elution for control irradiated cells and drug-treated irradiated cells, respectively (*17*). Calculations were based on data from Figs. 1A and 2A; Pu, putrescine.

Total cross-linking (no proteinase K) (CLF × 10^3)			
BCNU		*cis*-Platinum	
BCNU	184	*cis*-Platinum	613
DFMO	246	DFMO + *cis*-platinum	387
DFMO + Pu + BCNU	189	DFMO + Pu + *cis*-platinum	663

Table 2. The effects of DFMO on DNA interstrand cross-linking by BCNU (80 μM) and *cis*-platinum (80 μM). The CLF was defined in Table 1. Alkaline elution was performed with proteinase K in the lysis solution. Values represent the mean of two experiments (values from the independent experiments are given in parentheses).

DNA interstrand cross-linking (with proteinase K) (CLF × 10^3)					
BCNU			*cis*-Platinum		
BCNU	146	(139, 152)	*cis*-Platinum	378	(407, 349)
DFMO + BCNU	282	(257, 306)	DFMO + *cis*-platinum	155	(161, 148)
DFMO + Pu + BCNU	185	(167, 203)	DFMO + Pu + *cis*-platinum	286	(283, 289)
			cis-Platinum + DFMO (simultaneous)	287	(318, 256)

of unirradiated or irradiated cells (data not shown). Treatment of 9L cells with 80 μM BCNU alone reduced the amount of DNA eluted, an indication that BCNU cross-links DNA (Fig. 1A). When cells were treated with DFMO for 72 hours and then with 80 μM BCNU (Fig. 1A), the cross-linking factor was approximately 34 percent greater than that obtained with BCNU alone (Table 1). Administration of putrescine to DFMO-treated cells 24 hours before BCNU treatment prevented this increase, an indication that the DFMO-mediated increase in BCNU cross-linking is secondary to polyamine depletion. A lower concentration of BCNU (35 μM) produced similar results (data not shown); prior treatment with DFMO caused an increase in the BCNU-induced cross-linking, which could be prevented by the addition of putrescine to the culture medium.

Typical alkaline elution profiles illustrating the effects of DFMO on BCNU-induced DNA interstrand cross-links are shown in Fig. 1B. Use of proteinase K in these experiments eliminated DNA-protein cross-links, which reduced by approximately 20 percent the total cross-linking factor for treatment with 80 μM BCNU (Tables 1 and 2); therefore, even when proteinase K is not used in the assay, most of the cross-links detected after BCNU treatment are DNA interstrand cross-links. Prior treatment with DFMO produced a 93 percent increase in BCNU interstrand cross-linking (Table 2); the addition of putrescine prevented the major portion of this increase.

As shown by the increase in DNA eluted from unirradiated cells, BCNU both cross-links and induces strand breaks in DNA (Fig. 1, A and B); this has been observed also in other cell lines (*17*). The concurrent induction of strand breaks and cross-links complicates quantification of the alkaline elution data because the number of occurrences of each change can be underestimated when they occur together (*17*). We attempted to minimize this possible source of error in the determination of cross-linking by using a relatively large radiation dose to induce many more strand breaks than are induced by BCNU treatment alone (*17*). Because DFMO alone does not induce strand breaks, all strand breaks found in cells treated with both DFMO and BCNU can be attributed to BCNU alone; the error in the determination of the cross-linking factor that results from strand breaks should be the same for experiments with BCNU alone and BCNU plus DFMO. Prior treatment with DFMO appears to reduce the number of BCNU-induced strand breaks (Fig. 1, A and B); it is probably the DFMO-mediated increase in BCNU cross-link formation that is responsible for the apparent decrease in induction of strand breaks.

The effects of 80 μM *cis*-platinum on DNA eluted from irradiated and unirradiated cells are shown in Fig. 2. While *cis*-platinum alone reduces the elution rate for irradiated cells, it has no effect on the elution rate for unirradiated cells; thus *cis*-platinum cross-links but does not induce strand breaks in DNA. Similar results have been reported by others (*18*). The total cross-linking assay was performed for a *cis*-platinum concentration of 80 μM; DFMO treatment reduced the total cross-linking factor for *cis*-platinum by 37 percent (Table 1). Similar results were obtained for treatment with 17 μM *cis*-platinum (data not shown). For both concentrations of *cis*-platinum, administration of putrescine prevented this decrease, which suggests that DFMO-mediated polyamine depletion caused the decrease in total *cis*-platinum cross-linking.

Prior treatment with DFMO reduced the total cross-linking factor for *cis*-platinum by 60 percent (Fig. 2B and Table 2); addition of putrescine prevented a substantial portion of the decrease. In experiments in which cell survival was used as an end point (*11*), simultaneous treatment of 9L cells for 1 hour with *cis*-platinum and DFMO, a protocol that does not deplete polyamines, reduced *cis*-platinum cytotoxicity slightly. When cells were treated simultaneously for 1 hour with 80 μM *cis*-platinum and 1 mM DFMO, the interstrand cross-linking factor for *cis*-platinum was reduced similarly by 24 percent. Because this effect is apparently not polyamine-mediated, it is not surprising that this decrease in cross-linking persists in cells to which putrescine had been added.

Our data provide direct evidence that the DFMO-mediated modification of BCNU and *cis*-platinum cytotoxicity previously observed (*8*) was the result of a change in the number of DNA cross-links formed. Currently, DFMO is undergoing phase I and II clinical trials. Because the effects of polyamine depletion on the action of cancer chemotherapeutic agents can be either beneficial or detrimental, as shown for BCNU and *cis*-platinum, respectively, it is important that the effects of DFMO treatment for a given agent be studied in the laboratory before any combination is used in a clinical setting.

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Human Trophoblast-Lymphocyte Cross-Reactive (TLX) Antigens Define a New Alloantigen System

Abstract. *Antisera to human syncytiotrophoblast microvillous cell surface membranes from different placentas are cytotoxic for lymphocytes from some people but not others, demonstrating the presence of allotypic trophoblast-lymphocyte cross-reactive (TLX) antigens. Exploratory principal components factor analysis, performed on limited data consisting of 300 cytotoxic reactions produced by ten separate trophoblast antisera on a panel of lymphocytes from 30 random donors, suggested the presence of three distinct TLX antigen groupings. It is proposed that such TLX alloantigens are central in establishing maternal recognition and protection of the blastocyst, and that lack of recognition results in implantation failure and spontaneous abortion. These findings are compatible with contemporary results of immunotherapy to prevent recurrent spontaneous abortions, and their implications extend to other conditions of allogeneic coexistence, such as organ transplantation and the tumor-host relationship.*

During human pregnancy, the principal anatomical interfaces between maternal and extraembryonic tissues are formed by the trophoblast (*1, 2*). Immunohistological studies of these human placental villous trophoblasts show none of the major histocompatibility complex (HLA) (*3, 4*) or ABO blood group (*5*) antigens; the membranes do, however, express two unique groups of species-specific cell-surface trophoblast antigens (TA) that have been designated TA1 and TA2 (*6*). Antisera defining the TA1 group are trophoblast-specific, yet they can functionally inhibit allogeneic recognition as shown by their ability to block the mixed lymphocyte culture (MLC) reaction (*7*). Antisera of TA2 specificity are cytotoxic for lymphocytes (*8*), indicating the presence of trophoblast-lymphocyte cross-reactive (TLX) antigens. We have proposed that these TA groups act in a hapten-carrier model of pregnancy (*6*), TA1 as the carrier and TA2 the hapten. TA1 can modulate cell-mediated immunity that would theoretically result within placentas from such a hapten-carrier system (*9*), and the need for stimulating maternal recognition reactions has been met by data showing the TA2 group of TLX antigens to be allotypic (*10*). We now report findings suggestive of a new alloantigen system for TLX as revealed by principal components factor analysis (*11, 12*) of the differential lymphocytotoxicity produced by trophoblast antisera with human peripheral blood lymphocytes (PBL). The results show that trophoblast antisera produce cytotoxic reaction patterns with PBL which are suggestive of three TLX antigen groups. Groups 1 and 3 were found to represent two independent factors, and group 2 was a combination of the reaction patterns produced by groups 1 and 3.

A principal components factor analysis with varimax rotations (*13*) was used in an exploratory manner for the purpose of providing a statistical organization of the cytotoxicity reactions of ten trophoblast antisera (A through J) with a panel of PBL from 30 donors. This analysis showed that the reaction patterns could be attributable to two factors (Fig. 1). The similarity of the reactions of five of the antisera (D, F, G, H, and I) with the 30 PBL donors is indicated by sizable factor loadings of these sera on a common factor (factor 1). Likewise, the similarity of the reactions of three sera (B, C, and E) with the 30 PBL donors is indicated by loadings on a second factor (factor 2). Sera D, F, G, H, and I had loadings of 0.68 or greater on factor 1 and 0.36 or less on factor 2 (Table 1), whereas sera B, C, and E had loadings of 0.60 or greater on factor 2 and 0.22 or less on factor 1. Sera A and J had approximately equal loadings on factors 1 and 2 (Table 1). These findings suggest the existence of three distinct TLX antigen groups in terms of their reactions with lymphocytes. The three reaction groupings are depicted in Fig. 2 in which the reactions

Table 1. Factor structure matrix showing the loadings on factors 1 and 2 for the lymphocytotoxic reaction patterns produced by ten antisera to trophoblasts.

Sera	Factor 1	Factor 2
A	0.62	0.62
B	0.18	0.68
C	0.22	0.86
D	0.81	0.36
E	−0.08	0.60
F	0.76	0.05
G	0.79	0.12
H	0.84	0.06
I	0.68	0.30
J	0.38	0.69

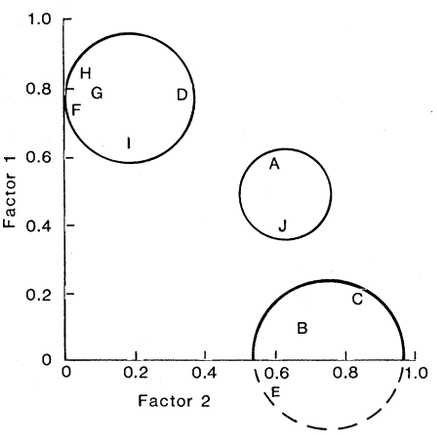

Fig. 1. Principal components factor analysis with varimax rotation of the cytotoxicity reaction patterns of ten antisera to trophoblasts provides evidence for three groups of TLX antigens. A similar factor structure with the same TLX antigen grouping was obtained with principal axis factor analysis with varimax rotation.