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Effects of DFMO-induced polyamine depletion on human tumor cell sensitivity to antineoplastic DNA-crosslinking drugs*

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Summary. We investigated the effect of pretreatment with difluoromethylornithine (DFMO), an ornithine decarboxylase inhibitor, on the cytocidal responses of four human adenocarcinoma cell lines to two alkylating and crosslinking agents: chlorambucil and *N,N',N''*-triethylenethiophosphoramide (thiotepa). The cell lines studied included HuTu-80 (duodenum), HT-29 (colon), ME-180 (cervix), and A-427 (lung). A 48- to 72-h pretreatment with DFMO reduced intracellular putrescine and spermidine contents to $<10\%$ and $<1\%$ of control levels. This treatment also caused a 30%–70% decline in spermine content. Survival of control and DFMO-pretreated cells after treatment with chlorambucil or thiotepa was measured by a plating efficiency assay. For three of the four lines studied, the DFMO-induced partial polyamine depletion significantly protected cells from the lethal effects of chlorambucil. In ME-180 cultures alone, DFMO pretreatment did not alter the cytocidal efficacy of chlorambucil. Addition of exogenous putrescine to cultures of HuTu-80, HT-29, or A-427 24 h after DFMO addition but 24 h before treatment with chlorambucil reversed the polyamine depletion and its protective effects on chlorambucil-induced cell kill. In contrast to the above observations, DFMO and partial polyamine depletion had no effect on cell survival after thiotepa treatment for any of the cell lines investigated.

Introduction

The enzyme-activated irreversible ODC inhibitor, DFMO [12], is currently under investigation as a potential modulator of cellular responses to other antineoplastic drugs. A number of laboratories have demonstrated enhanced *in vitro* cytocidal efficacy for BCNU, a DNA alkylating and crosslinking drug [10], in human [20] and rodent [3, 8, 15] tumor cell lines partially depleted of intracellular polyamines by pretreatment with DFMO. Similar enhancement of cell kill was reported in polyamine-deficient 9L cells for other CENU drugs that crosslink DNA [14, 16]. DFMO pretreatment also increases the production of DNA interstrand crosslinks in 9L cells subsequently treated with BCNU [25]. Based on these observations, it was hypothesized that alterations in the structure or conformation of intracellular DNA resulting from partial polyamine depletion may increase accessibility of the DNA to BCNU. This may, in turn, be responsible for potentiation of both BCNU-induced cell kill and crosslink production. In support of this hypothesis, they have used viscoelastometry to demonstrate altered structure or stability of DNA isolated from DFMO-treated 9L cells [9]. However, measurements of BCNU-induced crosslinks in DFMO-pretreated and control human adenocarcinoma cells failed to detect increased crosslink production analogous to that observed with 9L (J. Seidenfeld and L. C. Erickson, unpublished observations). Nevertheless, BCNU-induced cell kill is enhanced by DFMO pretreatment to the same degree in the human tumor cell lines as in 9L, or to a greater extent [20]. Thus, the molecular mechanisms underlying enhanced cytocidal efficacy for CENUs in polyamine-deficient cells may not be the same in rodent as in human tumor cells.

The effect of polyamine depletion on cytocidal responses to alkylating and crosslinking agents with chemical structures and mechanisms of action that differ from those of CENUs has been studied for very few drugs. The report that *cis*-PT(II) is a less effective cytocidal agent in DFMO-pretreated 9L cells than in controls [13] suggests that synergism between polyamine depletion and crosslinking agents may be unique to the CENUs. However, *cis*-Pt(II) may owe its antitumor efficacy more to DNA intrastrand crosslinks than to interstrand crosslinks [11]. Thus, the differing effects of DFMO-induced polyamine depletion on cytocidal responses to BCNU and *cis*-Pt(II) do not rule out potentiation for other interstrand crosslinking agents. In fact, DFMO increased the cytotoxicity and DNA interstrand crosslinking produced by treatment of human lymphoma cells with *L*-phenylalanine mustard [5].

Chlorambucil is a nitrogen mustard-type alkylating and crosslinking agent currently used for treatment of a variety of human neoplasms [2]. As with other nitrogen mustard-type drugs, alkylation and crosslinking of DNA by chlorambucil occurs via an aziridine intermediate [4].

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Abbreviations. BCNU, 1,3-*bis* (2-chloroethyl)-l-nitrosourea; CENU, chloroethylnitrosourea; *cis*-Pt (II), *cis*-diamminedichloroplatinum (II); DFMO, difluoromethylornithine; ODC, ornithine decarboxylase; PU, putrescine; SD, spermidine; SP, spermine; thiotepa, *N,N',N''*-triethylenethiophosphoramide

Thiotepa is an antineoplastic alkylating and crosslinking agent with three aziridinyl moieties already present in the drug molecule [17]. We examined the effects of DFMO-induced partial polyamine depletion on the cytocidal responses of four human adenocarcinoma cell lines to these two agents. In contrast to the results reported for the combination of DFMO with *L*-phenylalanine mustard in human lymphoma cells [5], the data we present here show that polyamine deficiency protects three of four human adenocarcinoma cells from chlorambucil-induced cell kill. Also, DFMO pretreatment failed to produce a similar protective effect for thiotepa in the same cell lines.

Materials and methods

Chemicals and drugs. PU dihydrochloride, 1,7-diaminoheptane, and chlorambucil were obtained from Sigma Chemical Co. (St. Louis, Mo). SD trihydrochloride and SP tetrahydrochloride were purchased from Calbiochem-Behring (La Jolla, Calif). Thiotepa was provided by the Division of Cancer Treatment, National Cancer Institute. DFMO was a generous gift of the Merrell-Dow Research Center (Cincinnati, Ohio). Stock solutions of 30 and 150 m*M* DFMO and of 10 m*M* PU were prepared in Dulbecco's modification of Eagle's medium, adjusted to pH 7.2–7.4, sterilized by membrane filtration (0.2 μm pores) and stored at −20 °C. Chlorambucil solutions in dimethylsulfoxide and thiotepa solutions in deionized sterile water were prepared immediately before use.

Cell culture and drug treatments. The human adenocarcinoma cell lines used for these investigations included HuTu-80, duodenum [18]; HT-29, colon [6]; ME-180, cervix [24]; and A-427, lung [7]. Cell culture and treatment with DFMO and/or PU were carried out as previously described [19, 20]. After an initial 24-h incubation, cells were treated at a final concentration of 1 or 5 m*M* DFMO. In experiments testing the ability of exogenous PU to reverse the effects of DFMO, a final concentration of 0.1 m*M* PU was added 24 or 48 h after DFMO treatment. The 60-min treatments with chlorambucil or thiotepa were carried out either 48 or 72 h after DFMO, or 24 h after PU addition. Controls were treated with an equal volume of solvent (dimethylsulfoxide or deionized sterile water) at the same time. Cells were then harvested and survival measured by a plating efficiency assay, as previously described [20].

Polyamine analysis. Extraction of washed and pelleted cells for measurement of polyamine content was done as previously described [19, 20]. Polyamine concentrations in aliquots of the cell extracts were determined using a minor modification of the reverse-phase paired-ion high-pressure liquid chromatography method of Seiler and Knodgen [23]. Some determinations were also carried out using a newly developed method involving: pre-column derivatization with dansyl chloride; preliminary clean-up of derivatized samples by adsorption on Bond-Elut C_{18} columns (Analytichem International, Harbor City, Calif), and elution with methanol; separation on octadecylsilyl columns (15 cm × 4.6 mm; 5 μm particles) eluted with a gradient of 45%–90% acetonitrile in 10 m*M* phosphate buffer, pH 4.4; and fluorometric detection and quantitation (P. M. Kabra et al., 1985, paper submitted for publication).

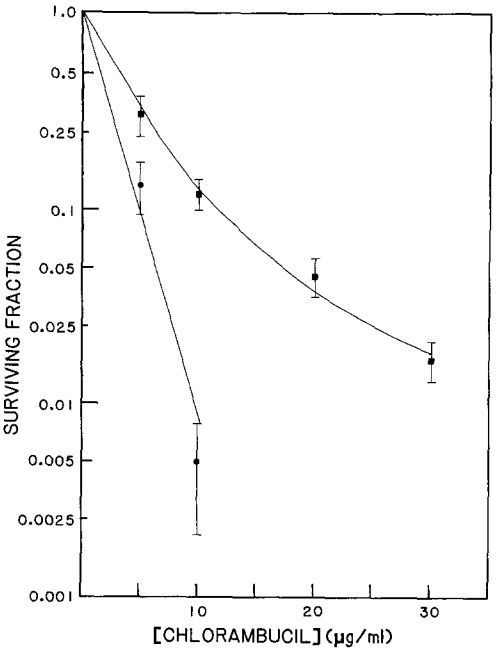

Fig. 1. DFMO-induced protection of HT-29 cells from chlorambucil. Cultures were treated with 5 m*M* DFMO or with vehicle 1 day after seeding of 5×10^5 cells per 75-cm^2 flask. After 3 days at 37 °C, one control flask (●) and one DFMO flask (■) were treated with chlorambucil for 60 min at each of the indicated concentrations. Cells were then harvested and assayed for survival by plating efficiency assay. *Points* are the means (±SD) of 8–12 replicate dishes

Results

Figure 1 shows survival curves for control cultures of HT-29 cells and for cultures treated with 5 m*M* DFMO on day 1 that were treated with chlorambucil on day 4. The data show that the 72-h preincubation with DFMO resulted in a marked increase in the fraction surviving chlorambucil treatment. Similar experiments were carried out using cultures treated with chlorambucil only 48 h after DFMO addition (i.e., 3 days after seeding). In control cultures, the surviving fractions were 5- to 10-fold higher when treatment with chlorambucil was on day 3 rather than on day 4 (data not shown). DFMO-pretreated cultures showed much smaller increases in chlorambucil sensitivity from day 3 to day 4. The degree of protection afforded by a 48-h DFMO treatment was accordingly smaller than that seen 72 h after DFMO addition, yet remained statistically significant (data not shown).

Equivalent results were obtained with control and DFMO-pretreated cultures of HuTu-80 and A-427 cells treated with chlorambucil on either day 3 or day 4 after seeding (data not shown). Lower concentrations of DFMO (1.0 m*M*) also protected all three cell lines from chlorambucil cytotoxicity (data not shown). In contrast, DFMO pretreatment had no effect on the cytocidal response of ME-180 cells to chlorambucil regardless of DFMO concentration or time after seeding at which chlorambucil treatment took place (Fig. 2). Comparison of Figs. 1 and 2 shows that ME-180 controls were markedly less sensitive to chlorambucil than the HT-29 cells. Control HuTu-80 and A-427 cells were equally sensitive to chlorambucil, as were the HT-29 cells (data not shown).

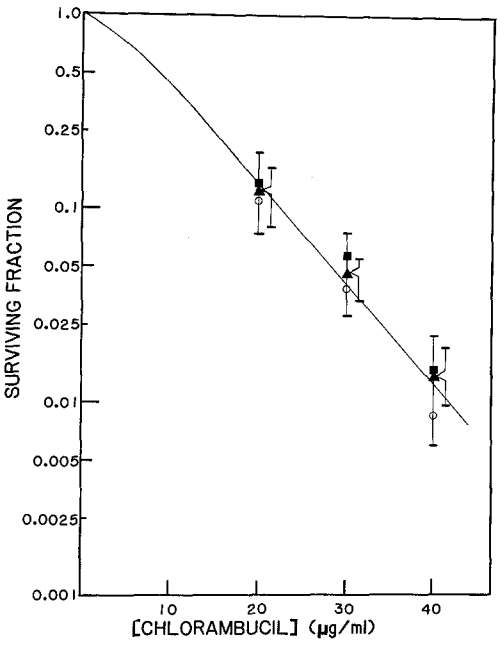

Fig. 2. Absence of DFMO-induced protection from chlorambucil in ME-180 cells. Cultures were treated with 5 m*M* DFMO or with vehicle 1 day after seeding of 5×10^5 cells per 75-cm^2 flask. After 2 days at 37 °C, half the DFMO flasks received 0.1 m*M* PU and the flasks were incubated for a further 24 h. One flask each of controls (○), DFMO-treated cultures (■), and DFMO/PU-treated cultures (▲) were then incubated for 60 min with the indicated concentrations of chlorambucil. Cells were harvested and assayed for survival by plating efficiency assay. *Points* are the means (±SD) of 8–12 replicate dishes

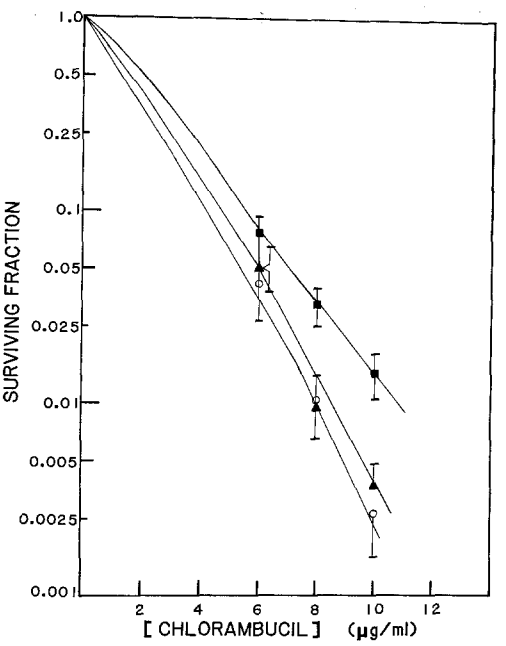

Fig. 3. Reversal of the DFMO-induced protection of A-427 cell from chlorambucil by exogenous PU. Cultures were treated with 5 m*M* DFMO or with vehicle 1 day after seeding of 5×10^5 cells per 75-cm^2 flask. After 24 h at 37 °C half the DFMO flasks had 0.1 m*M* PU added and were then incubated for a further 24 h. One flask each of controls (o), DFMO-treated cells (■), and DFMO/PU-treated cells (▲) were then incubated for 60 min with the indicated concentrations of chlorambucil. Cells were harvested and assayed for survival by plating efficiency assay. *Points* are the means (±SD) of 8–12 replicate dishes

Figure 3 shows survival curves obtained when 0.1 m*M* PU was added to DFMO-pretreated cultures of A-427 cells 24 h before treatment with chlorambucil. The data show that exogenous PU reversed the DFMO-induced protection from the cytocidal effects of chlorambucil. Similar experiments demonstrated that PU reversed the effects of DFMO for HuTu-80 and HT-29 cells (data not shown). We also verified for all three cell lines that addition of PU to cultures not treated with DFMO did not alter cytocidal responses to chlorambucil (data not shown). Furthermore, the data of Fig. 2 show that addition of PU to DFMO-treated ME-180 cells also did not alter the fraction of cells surviving after chlorambucil treatment.

Measurements of intracellular polyamine contents in extracts of cells from all of the experiments described above confirmed that DFMO reduced intracellular PU and SD contents to < 10% and < 1% of control levels (data not shown). SP content was 30%–70% of controls. The degree of polyamine depletion for ME-180 cells at the time of chlorambucil treatment did not differ from that seen for the other three cell lines. We also found that 72 h in the presence of DFMO did not substantially decrease the polyamine content below that observed 48 h after DFMO addition in any of the four cell lines. The addition of 0.1 m*M* PU to DFMO-treated cells restored the intracellular polyamine content to control levels in all cases (data not shown). Our observations in the present studies thus confirm those previously reported from this laboratory for these cell lines [19, 20].

Table 1 lists the fractions of cells surviving treatment with thiotepa for control and DFMO-pretreated cultures of the same four cell lines as in our experiments with chlorambucil. The data show that DFMO and the resulting polyamine depletion neither potentiated the cytocidal effects of thiotepa nor protected cells of any of these lines

Table 1. Survival of DFMO-pretreated and control cells after thiotepa[a]

Cell line	Thiotepa (μg/ml)	Surviving fraction	
		Control cultures	DFMO cultures
HuTu-80	20	0.388±.101	0.469±.128
	30	0.023±.007	0.027±.008
HT-29	40	0.031±.001	0.040±.009
	60	0.020±.005	0.023±.005
ME-180	40	0.181±.042	0.196±.046
	80	0.013±.003	0.020±.004
A-427	20	0.173±.030	0.172±.038
	50	0.006±.002	0.004±.001

[a] DFMO (5 mM) was added to cultures 1 day after seeding 5×10^5 cells per 75 cm^2 flask. Thiotepa treatment (60 min at the indicated concentrations) was on day 4. Data are the means (±SD) of 8–12 replicate dishes

from them. Similar results were obtained whether thiotepa treatment was on day 3 or day 4 after seeding (i.e., 48 or 72 h after DFMO addition). Measurements of polyamine contents in these studies verified that DFMO produced the same degree of polyamine depletion as in the experiments with chlorambucil (data not shown).

Discussion

The data obtained in the present investigation show that DFMO reduces the cytocidal efficacy of chlorambucil in three of four human adenocarcinoma cell lines (Fig. 1). Exogenous PU restored intracellular polyamine contents to control levels [19, 20] and also reversed the protective effects of DFMO in these three lines (Fig. 3). These observations support our contention that polyamine deficiency is causally related to the increased survival after chlorambucil treatment. Since chlorambucil and other nitrogen mustards are known to alkylate and crosslink DNA by way of an aziridine intermediate [4], it was quite surprising that DFMO pretreatment did not also protect the same cell lines from the lethal effects of thiotepa (Table 1). The reason behind these divergent effects of polyamine depletion on the efficacy of two agents with such similar mechanisms remains obscure.

The ME-180 cell line differed from the others in that DFMO did not protect it from chlorambucil. Two possible explanations for this dissimilarity were considered. The first is the differing effects of DFMO on cell cycle phase distributions between ME-180 and the other lines studied. All but ME-180 respond to DFMO treatment with a marked increase in the G_1 phase fraction [22]. However, Bhuyan et al. reported that the cell cycle age response of DON cells for chlorambucil showed greatest sensitivity for cells in G_1 with a progressive decrease as cells progressed through S and G_2 [1]. DFMO increases the G_1 fraction in cultures of the three lines protected from chlorambucil by polyamine depletion [22]. Unless the age response of human adenocarcinoma cells differs from that reported for DON cells [1], our observations [22] would thus predict enhanced sensitivity to chlorambucil in DFMO-pretreated cultures of HT-29, HuTu-80, and A-427 cells, which accumulate in G_1. However, this was not the case. Perturbations of cell cycle phase distributions are thus most likely not responsible for the protection we observed and for its absence in ME-180 cells. The second possible explanation is that the relative insensitivity of control cultures of ME-180 to chlorambucil (compare Figs. 1 and 2) may be responsible for the lack of a protective effect resulting from polyamine depletion.

It also seems rather difficult to reconcile the observations we report here with those of Ducore, showing enhanced efficacy of *L*-phenylalanine mustard in DFMO-treated cultures of a human lymphoma cell line [5]. Both chlorambucil and *L*-phenylalanine mustard are aromatic nitrogen mustard alkylators that crosslink DNA [2, 4]. Unless there is a systematic difference between epithelial and lymphoma cell lines in the consequences of polyamine depletion for nitrogen mustard efficacy, we see no rational explanation for the opposing directions of the effects observed. Investigation of the effect of DFMO treatment on sensitivity to *L*-phenylalanine mustard in our cell lines would be of interest. These divergent responses, both across differing cell types and across similar but slightly different drugs, point to the dangers of drawing generalized conclusions from such in vitro studies with a small number of cell lines.

The hypothesis upon which the present work was based postulated an alteration of the structure or conformation of intracellular DNA in polyamine-deficient cells [21], which would enhance the accessibility of the DNA to alkylating and crosslinking agents. Observations of increased cytocidal efficacy for CENUs in DFMO-treated cells [3, 8, 14–16, 20] provided evidence to support that hypothesis. While the observations we report here do not rule out this hypothesis as an explanation for the potentiation of CENU cytotoxicity, they clearly do establish that the phenomenon is not general in all antineoplastic agents whose mode of action depends on interstrand crosslinking of intracellular DNA.

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