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The growth-inhibitory effect of 4-hydroperoxycyclophosphamide against human non-small cell lung carcinoma is enhanced by low-dose difluoromethylornithine*

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Summary. Surgically unresectable human non-small cell lung carcinoma (NSCC) is highly resistant to present chemotherpy and radiation therapy regimens. Cyclophosphamide, a potent alkylating agent, has shown some efficacy, especially in combination chemotherapy. Difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ornithine decarboxylase (ODC) which produces minimal toxicity in animals and humans, has shown antiproliferative effect against human SCC in culture but a much smaller effect (cytostatic) against NSCC. We therefore investigated 4-hydroperoxycyclophosphamide (4HC) and DFMO alone and in combination against a human NSCC line (NCI-H157). Cells were treated with DFMO at graded concentrations of 0 to 800 μM from day 0 to day 7. On day 3, cells were exposed for 1 h to 4HC at graded concentrations of 0 to 80 μM, washed, and refed with media containing DFMO at initial concentrations. On day 7, cells were counted by hemacytometer. Cells treated with DFMO or 4HC alone exhibited dose-dependent growth inhibition. Growth inhibition by 4HC was enhanced through combination with DFMO. On day 7, 50 μM (5×10^{-5} M) DFMO effected a 37% inhibition, 8 μM 4HC 47% inhibition, and the combination of 50 μM DFMO and 8 μM 4HC yielded an elevated 71% inhibition. The growth inhibitory effect and potentiating effect of DFMO were reversible upon addition of putrescine (PU) to the culture medium. The combination of DFMO and 4HC, two agents with different toxicity spectra, may represent an effective chemotherapeutic regimen for the treatment of lung cancer.

Introduction

Human lung cancer is the leading cause of cancer death in the USA today. NSCC constitutes 70%–80% all lung cancers [25]. Curative surgical resection in lung disease has generally been unsuccessful; consequently, the need for development of effective adjuvant chemotherapy is urgent in view of the debilitating systemic effects of lung cancer [25]. Unfortunately, management of surgically unresectable NSCC remains one of the most challenging clinical problems [25]. Single-agent chemotherapy, combination chemotherapy, or chemotherapy in combination with radiotherapy against NSCC has thus far manifested only minimal benefits in the most advanced cases. There has been some limited success with cyclophosphamide. A cisplatin-adriamycin-cyclophosphamide combination treatment proved to be superior to cyclophosphamide alone in several studies [5, 9, 26]. Therefore, investigation of other potent combinations may be valuable. Large cell lung carcinoma, the lung tumor subtype which is the subject of our present investigation, has been suggested to be the least responsive histological subtype of NSCC [25].

In general, all eucaryotic cells contain the polyamines PU, spermidine, and spermine [20]. Although the physiological role that these polyamines play still awaits clarification, it is now known that these ubiquitous low-molecular-weight organic cations are highly regulated and are necessary for normal cellular growth and proliferation [20]. ODC is an enzyme that catalyzes the first and rate-limiting step in polyamine formation, the conversion of ornithine to PU [20]. Dramatic rises in ODC activity have been observed in rapidly growing tissues [10, 20]; elevation of polyamine levels has been documented in body fluids of patients with certain neoplasms [10] and in tissues transformed by carcinogens [15, 17].

Aside from studying ODC inhibitors as a means of pinpointing the role of polyamines in the cell, the above association of polyamines with rapid cellular turnover has brought hopes that these polyamine inhibitors could prove useful in the therapy of proliferative diseases and neoplasms. DFMO, one of the two agents of focus in the present study, is a potent, specific, enzyme-activated irreversible inhibitor of ODC and polyamine biosynthesis [16, 20]. Human SCC in vitro has shown a striking sensitivity to DFMO inhibition of polyamine biosynthesis, responding with a decrease in cell growth as well as cell viability [12, 13].

However, against NSCC, the tumor type forming the subject of our present study, DFMO has shown a less dramatic antiproliferative activity [13]. DFMO has been well tolerated in murine studies [3] and has shown only moder-

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Abbreviations: DFMO, difluoromethylornithine; FBS, fetal bovine serum; 4HC, 4-hydroperoxycyclophosphamide; NSCC, non-small cell lung carcinoma; ODC, ornithine decarboxylase; PBS, phosphate-buffered saline; PU, putrescine; RPMI 1640, Roswell Park Memorial Institute 1640 medium; SCC, small cell lung carcinoma

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ate and reversible gastrointestinal upset, thrombocytopenia, and decreased auditory acuity in phase I clinical trials [1]. Because of the minimal side effects produced by DFMO, the addition of the drug might permit the administration of the maximal dosage of other anticancer agents [26]. Indeed, DFMO has been documented to enhance the efficacy of several types of chemotherapeutic agents in vitro [2, 6, 11, 18, 19, 22, 23] and in vivo [3, 14, 21].

Since NSCC is so highly resistant to present chemotherapy regimens, and because DFMO has exhibited minimal toxicity and potent sensitization of several cytotoxic agents, we sought to examine whether DFMO could enhance the growth-inhibitory effect of 4HC, an analogue of cyclophosphamide, against an NSCC cell line in vitro. The goals of the study were threefold: (a) to study NSCC sensitivity to DFMO and to 4HC singly at clinically applicable doses; (b) to examine whether DFMO could enhance the growth-inhibitory effect of 4HC against this tumor cell line; and (c) to confirm that the enhancement effect is due to polyamine depletion by DFMO.

Materials and methods

Cell culture. We used the NCI-H157 cell, a large cell carcinoma of NSCC lineage, for this study [13]. The cell line was maintained in RPMI 1640 (GIBCO, Grand Island, NY) containing 9% heat-inactivated FBS (Sterile Systems, Inc., Logan, Utah) and 1% L-glutamine (GIBCO) at 37°C with 5% CO_2 and 95% humidified air. The H157 cells grew in monolayers and were maintained in logarithmic growth at a cell concentration varying from 0.5 to 5.0 × 10^6 cells/ml. This large cell carcinoma NSCC cell line, a slow-growing cell line (doubling time 96 h), was monitored for and found to be free from mycoplasmal, bacterial and viral contamination, as previously described [13].

Drugs and chemicals. DFMO was a gift from the Merrel-Dow Research Institute, Cincinatti, Ohio. 4HC was a gift from Dr. M. Colvin, Johns Hopkins Oncology Center, Baltimore, Md. [24]. PU was purchased in the form of the dihydrochloride salt from Sigma Chemical Company, St. Louis, Mo. DFMO as a 500 m*M* stock solution was kept at 4°C, and 4HC as a 5 m*M* stock solution in PBS (GIBCO), without calcium or magnesium was kept at −20°C. PU was dissolved in 0.1 *N* HCl and frozen in 1 m*M* stock aliquots. 4HC solutions were made on the day of drug treatment by dilution with PBS without calcium or magnesium. Similarly, DFMO was prepared by dilution with PBS immediately before studies. Appropriate aliquots of the stock PU were added directly to the culture medium.

Drug studies. Single-cell seeding was done in 25-cm^2 tissue culture flasks containing 3 ml medium with graded concentrations of DFMO from 0 to 800 μ*M* present continuously from day 0 to day 7 (and up until day 14 for the time-course studies). The culture medium was changed twice weekly. On day 3, the cells were incubated for 1 h at 37°C with 4HC at graded concentrations of 0 to 80 μ*M*, washed twice with PBS, and refed with media containing DFMO at the initial concentrations. Continuous exposure to DFMO has previously been shown to inhibit polyamine biosynthesis in these cells [13]. Endogenous polyamines in the cells were depleted after 72 h DFMO exposure, and there ensued a reversible decrease in cell proliferative activity [13]. PU was added at 10 μ*M* on day 0 in studies designed to test for reversal of the growth-inhibitory and potentiating effect of DFMO. In clinical trials DFMO is given orally every 6–8 h for up to 21 days, or by continuous i.v. infusion [1]. On the other hand, 4HC (and cyclophosphamide) is an alkylating agent which affects preformed DNA, RNA, and protein in vitro [7]. Cyclophosphamide is administered in humans in short intravenous boluses [8].

At time of cell harvest, the cell monolayers were detached with 0.25% trypsin and 0.02% EDTA in PBS, and resuspended in 0.5 ml PBS buffer containing 0.75 m*M* dithiothreitol. Cell counts were determined microscopically with a hemacytometer using trypan blue. Growth curves were derived from the means of at least four separate experiments. Results are expressed as number of viable cells (excluding trypan blue) in the flask.

Biochemical assays. ODC activity was assayed in supernatants of cell sonicates by measuring $^{14}CO_2$ liberated from DL-[1-^{14}C] ornithine as previously described [4, 12, 13]. The reaction mixture contained 10 μl ^{14}C-ornithine (Amersham, Arlington Heights, Ohio), 10 μl pyridoxal-5-phosphate (Sigma), 60 μl dithiothreitol-PBS buffer and 80 μl of the cell sonicate, and was incubated at 37°C for 2 h, exactly as previously described [4, 12, 13].

Polyamines in cell sonicates were quantitated fluorometrically after separation on a cation exchange column and derivatization with *O*-phthaldehyde, as previously described [12].

Results

We found a dose-dependent growth-inhibitory effect on the H157 cells by DFMO at doses ranging from 50 to 800 μ*M*; and by 4HC at doses ranging from 1 to 80 μ*M* (data not shown). These concentrations are in the range of plasma levels achievable in humans [1, 9]. Subsequent studies were therefore done at the low end of these dose ranges, with DFMO at 50 μ*M* and 4HC at 8 μ*M*. These doses about 1 log lower than the achievable steady state concentration at the maximal tolerated oral dose when the drugs are given alone [1, 8].

Untreated cells showed an increase in ODC activity with the onset of cell growth (Fig. 1). Cells treated with 50 μ*M* DFMO exhibited a marked suppression of the ODC activity, consistent with results obtained by our laboratory [12] and by other investigators [2, 6, 11, 18, 19, 22].

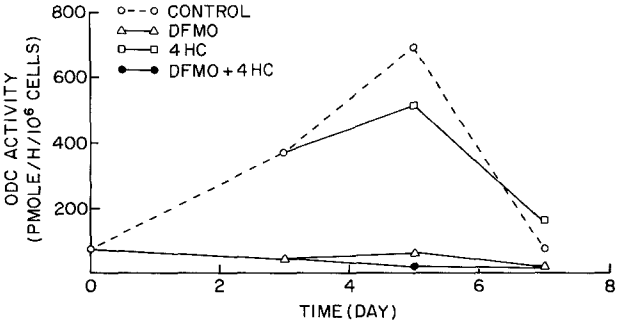

Fig. 1. ODC activity in NSCC cells treated with 50 μ*M* DFMO and 8 μ*M* 4HC, singly and in combination. Each point represents the mean from three to six studies. SEM not shown (height of bar would have been less than height of symbol)

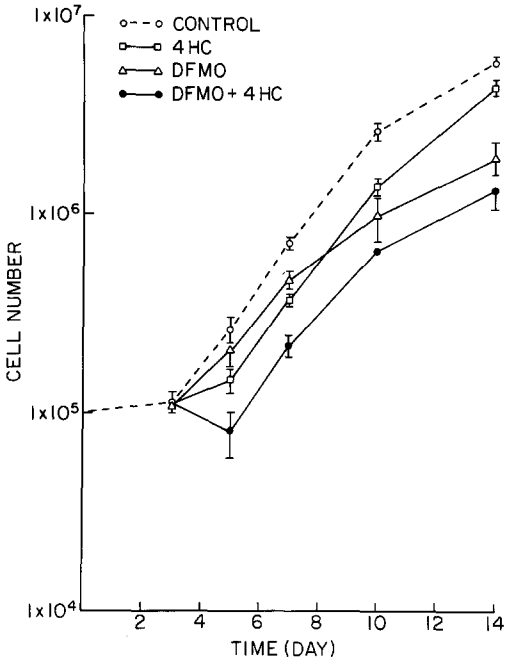

Fig. 2. Effect of 50 μ*M* DFMO and 8 μ*M* 4HC, singly and in combination, on the growth of NSCC cells in vitro. Each point represents the mean from three to six separate experiments. *Bars* represent SEM (not shown when height of bar less than height of symbol)

Treatment with 8 μ*M* 4HC, either by itself or in combination with 50 μ*M* DFMO, showed no significant changes in ODC activity as compared to cells not given 4HC. This observation confirms that 4HC does not affect cells via ODC suppression (Fig. 1).

Untreated cells entered exponential growth after the typical initial lag period of approximately 2 days (Fig. 2). DFMO alone produced continued growth suppression of the H157 cells (Fig. 2). 4HC-treated cells exhibited an initial marked growth suppression, with this inhibition gradually decreasing in magnitude as time progressed. This cell response is consistent with 4HC's mechanism of action as an alkylating agent and the subsequent recovery of cells from alkylating injury. However, the addition of DFMO to 4HC produced a marked enhancement of the growth-inhibitory effect, with a consistent increase in growth inhibition that surpassed the inhibition of either agent given alone throughout the entire 14-day course of the study (Fig. 2).

Table 1. Effect of DFMO and 4HC, singly and in combination, on the cellular polyamine contents of H157 cells

Day 7	Polyamines (pmol/10^6 cells)		
	PU	Spermidine	Spermine
Control	1450±136	2830±237	3190±228
DFMO	230± 83*	540±141*	1880±192*
4HC	1420±174	2780±245	3340±188
DFMO + 4HC	250± 91*	620± 97*	1750±212*

DFMO 50 μ*M* was added continuously beginning with seeding, and 4HC 8 μ*M* was added for 1 h on day 3

* Significantly different from control, $P < 0.05$

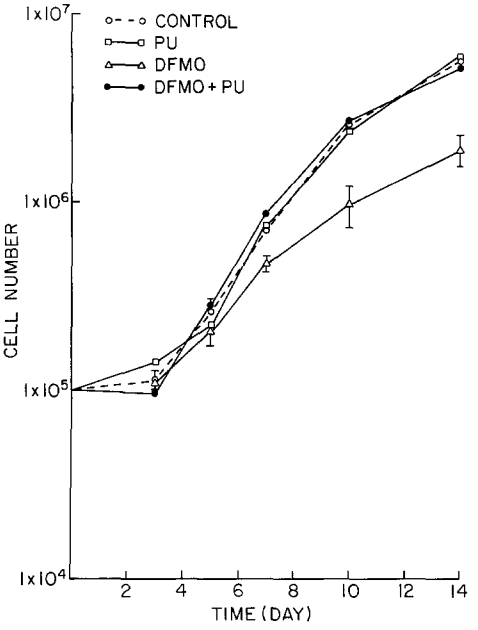

Fig. 3. Reversal of the growth-inhibitory effect of 50 μ*M* DFMO by addition of 10 μ*M* PU in NSCC cells. Each point represents the mean from three to six experiments. *Bars* represent SEM (not shown when height of bar less than height of symbol)

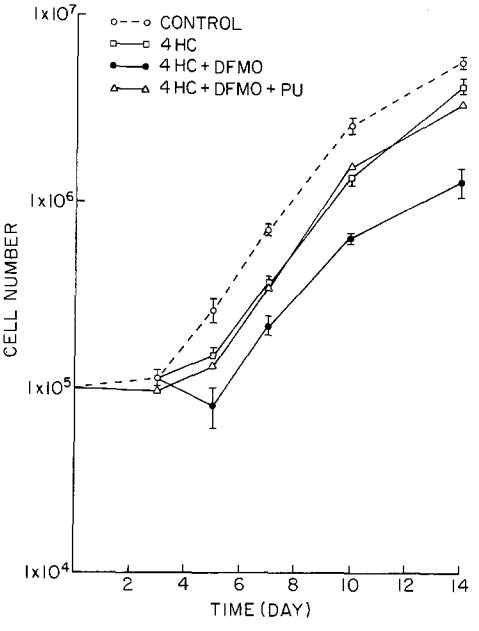

Fig. 4. Reversal of the growth-inhibitory enhancement effect of 50 μ*M* DFMO on 8 μ*M* 4HC by addition of 10 μ*M* PU in NSCC cells. Each point represents the mean from three to six studies. *Bars* represent SEM (not shown when height of bar less than height of symbol)

The specificity of the effects of DFMO is confirmed by polyamine depletion in treated cells and by the abrogation of DFMO effects with the addition of exogenous PU. DFMO produced increasing growth inhibition, with a 66% inhibition by day 14. The growth inhibition was associated with inhibition of ODC activity (Fig. 1) and depletion of intracellular polyamine levels (Table 1). PU alone had no effect on cell growth (Fig. 3). However, the simultaneous

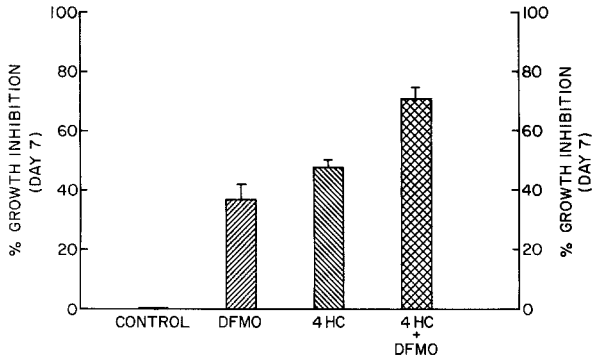

Fig. 5. Growth inhibition of NSCC cells by 50 μ*M* DFMO and 8 μ*M* 4HC, singly and in combination. Each histogram represents the mean from three to six studies. *Bars* represent SEM

addition of 10 μ*M* PU to the DFMO in culture medium on day 0 completely abrogated the growth-inhibitory effect of DFMO (Fig. 3). This observation confirms that the observed DFMO effects are due to the resultant polyamine depletion, which is in agreement with previous results obtained by our laboratory [12] and by other investigators [2, 6, 11, 18, 19, 22, 23].

Addition of PU to the combination of 4HC and DFMO promptly reversed the DFMO enhancement effect: the enhancement of growth inhibition appears to be completely abolished, showing a return to the growth curve resulting from 4HC administered alone (Fig. 4). These results indicate that the growth-inhibitory enhancement of 4HC by DFMO is likely due to the depletion of polyamines.

The growth-inhibition enhancement effect of DFMO on 4HC is summarized in Fig. 5. The growth inhibition patterns confirm our initial hypothesis that administration of DFMO can enhance the antiproliferative effect of 4HC against NSCC cells. 50 μ*M* DFMO showed 33%, 8 μ*M* 4HC 43% and the combination of DFMO and 4HC 74% growth inhibition.

Discussion

The results of the present study show that both 4HC and DFMO inhibited NSCC cell growth, and that very low doses (5×10^{-5} *M*) of DFMO potentiated the 4HC antiproliferative effect. This dose of DFMO is one of the lowest effective in vitro doses of DFMO used to date. The growth-inhibitory effect and 4HC-potentiating effect of DFMO are both abrogated by the simultaneous addition of PU, confirming that the observed effects of DFMO are related to depletion of polyamines.

Furthermore, the low concentrations of the agents used in the study are about 1 log below those clinically achievable and produce minimal toxicities when used alone. It appears promising that significant effects were observed at low drug dosages in large cell lung carcinoma, suggested to be the least responsive histological subtype of NSCC.

These results are in agreement with those of other investigators that report DFMO enhancement of the antiproliferative effects of antineoplastic agents, including those of cis-diamminedichloroplatinum against human and hamster pancreatic cell lines [6], of 1,3-bis(2-chloroethyl)-1-nitrosourea and other chloroethylnitrosoureas against 9L rat brain tumor cells and multicellular spheroids [2, 14, 18, 19, 22] and human tumor cells [23], of 1-β-D-arabinofuranosylcytosine against L1210 murine leukemia cells [21], of doxorubicin and vindesine against several animal tumor models [3] and of 5-fluorouracil against a human colon adenocarcinoma cell line [11].

In addition, the cytostatic effect of DFMO shown in the present study is comparable to that found in most other studies. DFMO in vitro, either alone or in combination with other chemotherapeutic agents, often shows growth inhibition but not complete growth cessation or frank cytotoxicity with cell loss from culture [11, 14, 18, 19, 21, 22]. Nevertheless, when DFMO is employed in vivo using the same tumor cell lines, growth inhibition is effected [2, 3, 21].

Thus, DFMO in combination with cyclophosphamide may be effective in the therapy of human NSCC. The specific combination may be valuable because of the low doses required and because the relatively low toxicity of DFMO (predominantly gastrointestinal toxicity and ototoxicity) does not overlap with that of cyclophosphamide (myelosuppression and cystitis) [8]. The demonstration of the sensitization effects of DFMO in this study and in other combination protocols should stimulate further investigations of this biochemical modulation that may lead to the development of effective, nontoxic chemotherapeutic treatment of lung cancer.

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