[CANCER RESEARCH 44, 5100–5104, November 1984]

Inhibition of Growth of Human or Hamster Pancreatic Cancer Cell Lines by α-Difluoromethylornithine Alone and Combined with *cis*-Diamminedichloroplatinum(II)[1]

Barbara K. Chang,[2] Owen Black, Jr., and Robert Gutman

Department of Medicine, Section of Hematology/Oncology [B. K. C., R. G.], and Department of Cell and Molecular Biology [O. B.], Medical College of Georgia and Veterans Administration Medical Center, Augusta, Georgia 30910

ABSTRACT

A major problem in the therapy of pancreatic adenocarcinoma is its inherent resistance to most chemotherapeutic agents. Previously, we have reported that the four pancreatic cancer cell lines studied here have elevated levels of ornithine decarboxylase, a growth-regulating enzyme, and further that the degree of elevation tends to parallel the degree of chemoresistance. On the basis of these prior findings, we investigated the effects of α-difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase, alone and in combination with *cis*-diamminedichloroplatinum(II) (cisplatin), to which two of the four cell lines display relative resistance. The cell lines studied were: two of human origin, PANC-1 and COLO-357; and two of hamster origin, WD PaCa and PD PaCa. Colony formation (clonogenic) assays were used to evaluate drug effects. Cells were exposed continuously to DFMO in medium. For the combined treatments, cells were exposed to cisplatin for 1 hr, washed, and then plated in DFMO-containing medium. The inhibitory effects of DFMO were predominantly cytostatic, were reversible by putrescine, and were roughly additive when combined with cisplatin. Our panel of cell lines responded heterogeneously to DFMO, with PANC-1 and WD PaCa showing the most sensitivity. The combination of DFMO and cisplatin appears to be a promising experimental approach to overcoming drug resistance in pancreatic cancer.

INTRODUCTION

While the exact function of polyamines is still not understood at the molecular level, these ubiquitous organic cations have been shown to play an essential role in cellular growth and proliferation (19). Specific inhibitors of polyamine synthesis have been developed, and one of these, DFMO,[3] is the focus of the present study. DFMO is a specific, irreversible inhibitor of ODC (13, 15, 20), which is the rate-limiting enzyme for the biosynthesis of the polyamine putrescine and is known to be elevated in many malignantly transformed tissues (14, 17, 23). DFMO has been found to have minimal toxicity in animal studies (2) and predominantly gastrointestinal and platelet toxicity in Phase I clinical trials (1). It has been reported to enhance the effectiveness of 1,3-bis(2-chloroethyl)-1-nitrosourea against rat brain gliosarcoma cells (9), of 5-fluorouracil against a human colon adenocarcinoma cell line (10), of doxorubicin and vindesine against various animal tumor models (2), and of 1-β-D-arabinofuranosylcytosine against L1210 murine leukemia (21). In addition, Roizin-Towle (22) reported that polyamines added to the medium of cultures of hamster V79 cells diminished the cytotoxic effects of cisplatin, which indirectly suggests that blocking polyamine synthesis should enhance the effects of cisplatin. Oredsson *et al.* (18) have reported that a 48-hr pretreatment of rat brain gliosarcoma cells with 10 mM DFMO decreased the cytotoxic effects of cisplatin. They have also reported that a 72-hr pretreatment with 1 mM DFMO reduces the DNA cross-linking by cisplatin (25). However, the concentrations of DFMO that they used are outside the range of plasma concentrations, *i.e.*, 0.3 to 0.6 mM, seen in Phase I trials at the maximum tolerated dose under steady state conditions (1).

We have reported previously (3) that ODC is elevated, as compared to the normal pancreas, in the 4 pancreatic cancer cell lines used in the present study. Furthermore, as shown in Table 1, the levels roughly parallel the degree of differentiation and the chemotherapy resistance of the cell lines, with the more well-differentiated cell lines having higher levels of ODC and being more resistant to chemotherapeutic agents. Since primary drug resistance is a major problem in the treatment of advanced pancreatic adenocarcinoma, the present study was undertaken to determine whether DFMO could enhance the cytotoxicity of cisplatin against a panel of 4 pancreatic adenocarcinoma cell lines, 2 of which are resistant to cisplatin alone. In addition, the sensitivity to DFMO as a single agent was determined. Attention was focused upon the range of DFMO concentrations clinically achievable. DFMO was found to enhance the cisplatin-induced inhibition of the reproductive capacity of the pancreatic cancer cell lines in a roughly additive and cytostatic fashion, although differences in the degree of inhibition were found among the cell lines.

MATERIALS AND METHODS

Cell Lines. The characteristics of the cell lines used are summarized in Table 1. PANC-1 was obtained from the American Type Culture Collection, and it was originally described by Lieber *et al.* (11). COLO-357 was kindly supplied by G. E. Moore, University of Colorado (16). WD PaCa and PD PaCa, both of hamster origin, were adapted to tissue culture in our laboratory (7) from transplantable tumor models kindly supplied by D. G. Scarpelli and M. S. Rao, Northwestern University (24).

Culture Conditions. Cells are maintained in Roswell Park Memorial Institute Culture Medium 1640, supplemented with 10% heat-inactivated fetal bovine serum, glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Grand Island Biological Co., Grand Island, NY, except serum was from Flow Laboratories, McLean, VA). For the colony formation

[1] Supported by Grant CA34170, awarded by the National Cancer Institute, Department of Health and Human Services, and the Medical Research Service of the Veterans Administration. Presented in part at the annual meeting of the American Association for Cancer Research, San Diego, May 1983 (5).

[2] To whom requests for reprints should be addressed, at Hematology/Oncology (111E), Veterans Administration Medical Center, Augusta, GA 30910.

[3] The abbreviations used are: DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; cisplatin, *cis*-diamminedichloroplatinum(II).

Received March 8, 1984; accepted July 30, 1984.

Table 1

Characteristics of the pancreatic adenocarcinoma cell lines used in the present study

	Doubling time (hr)	Degree of differentiation	General chemosensitivity (4)	ODC activity (pmol/10^6 cells/30 min) (3)	
				Confluent	Log phase
PANC-1	20–28	Poorly differentiated	Moderately sensitive to many agents	1.1 ± 0.7[a]	6.59 ± 0.3
COLO-357	36–72	Well differentiated	Resistant to most agents	4.0 ± 0.3	21.0 ± 0.4
WD PaCa	20–28	Well differentiated	Resistant to most agents	4.4 ± 0.1	84.7 ± 1.7
PD PaCa	14–18	Poorly differentiated	Sensitive to many agents	1.6 ± 0.1	5.6 ± 1.0

[a] Mean ± S.E.

assay, the medium also includes 15% fetal bovine serum, 0.9% methylcellulose (4000 centipoise; Fisher Scientific Co., Fair Lawn, NJ), and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Grand Island Biological Co.). Cell lines are monitored periodically by transmission electron microscopy for mycoplasmal and viral contamination and by karyotyping (to rule out cross-contamination with other cell lines). Cell cultures are also *Mycoplasma* free by the Hoechst staining method (8).

Drugs and Chemicals. DFMO was supplied courtesy of Merrell Research Center, Merrell Dow Pharmaceuticals, Cincinnati, OH, and cisplatin was courtesy of Bristol Laboratories, Syracuse, NY. Putrescine was purchased from Sigma Chemical Co., St. Louis, MO. Drug dilutions were freshly prepared on the day of assay by dissolution in Hanks' balanced salt solution to a concentration of 20 mg/ml for DFMO, 20 mM for putrescine, and 1.0 mg/ml for cisplatin, followed by filter sterilization. An appropriate aliquot of the stock DFMO or stock putrescine or a dilution thereof was added directly to the methylcellulose assay medium. Cisplatin was serially diluted to concentrations of 20, 10, 5, 2, and 0.2 μg/ml. Mixtures (1:1) of cisplatin solutions and cell suspensions were used to give the desired final concentrations.

Dose-Response Assays. For dose-response determinations, cells were harvested in late-log- or early-stationary-phase growth by short exposure to 0.25% trypsin for the adherent cell lines (all except PD PaCa). Cells (1 to 2 × 10^6) were incubated at 37° in 5% CO_2 for 1 hr with cisplatin at 0, 0.1, 1.0, 2.5, 5.0, or 10 μg/ml. After incubation, cells were washed free of drug and plated at a density of 1 to 2 × 10^3 cells/ml in assay medium containing DFMO at concentrations of 0, 25, 50, 100, 500, or 1000 μg/ml (0.106 to 4.2 mM). Colony formation assays were carried out in quadruplicate in 35-mm culture dishes (Lux, Naperville, IL) with enumeration of colonies (>50 cells) using an inverted microscope after 7 to 14 days of incubation at 37° and 5% CO_2. Results for each assay were expressed as the T:C ratio (mean colonies treated:mean control colonies) for each drug concentration. Dose-response curves represent the mean of at least 3 separate experiments. Drug doses (μg/ml) at which there is a 50% inhibition or 90% inhibition of colony formation as compared to controls were determined from the semilog dose-response curves.

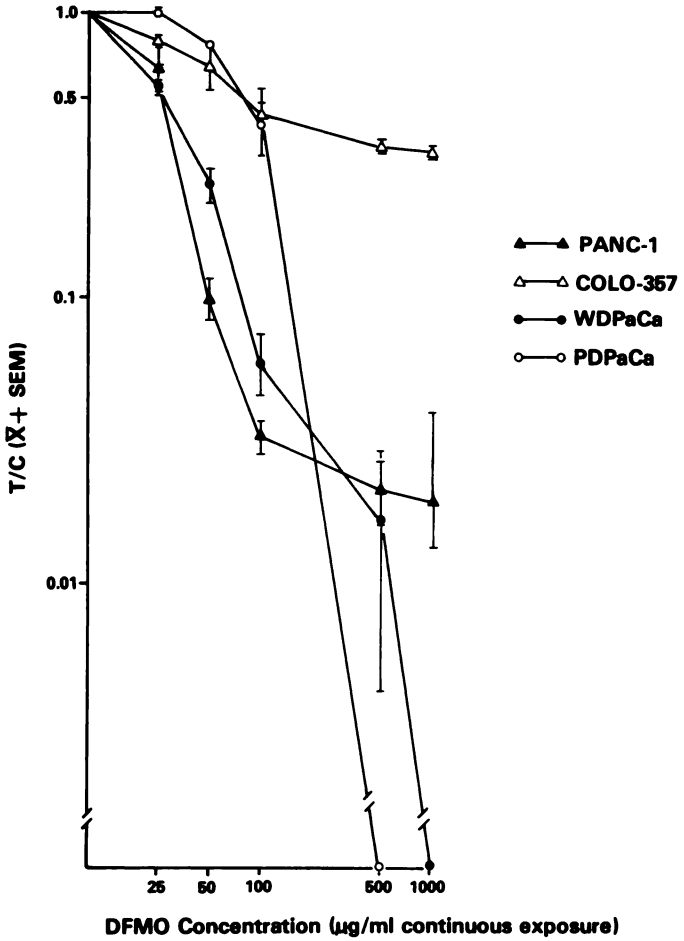

Chart 1. Dose-response curves of DFMO against pancreatic adenocarcinoma cell lines. Each dose-response curve is based upon 6 to 7 separate experiments. *T/C*, treated *versus* control; $\bar{X}$ + *SEM*, mean ± S.E.

RESULTS

The responses of the pancreatic adenocarcinoma cell lines to DFMO alone are summarized in Chart 1 and Table 2. It is important to note that, except at concentrations of ≥500 μg/ml, the effects of DFMO were cytostatic or antiproliferative; *i.e.*, culture dishes containing DFMO, 100 μg/ml (0.4 mM) or less, showed numerous viable cells and clusters of <50 cells, which is quite different from the absence of cells and clusters that we normally see when testing effective cytotoxic agents. Thus, a dose-dependent inhibition of colony formation was seen, reflecting effects on reproductive capacity without causing cell lethality at clinically achievable concentrations.

The specificity of the effects of DFMO was demonstrated by

Table 2

Sensitivity to DFMO alone as determined by the colony formation assay

Cell line	ID_{50}[a] (μg/ml DFMO)	ID_{90} (μg/ml DFMO)
PANC-1	22.6 (0.10)[b] ± 2.8[c]	50.6 (0.21) ± 4.1
WD PaCa	26.0 (0.11) ± 1.6	76.4 (0.32) ± 7.1
PD PaCa	71.1 (0.30) ± 10.6	138.4 (0.58) ± 8.5
COLO-357	84.2 (0.35) ± 11.9	>1000 (>4.2)

[a] ID_{50} or ID_{90}, drug concentration at which there is a 50% or 90% inhibition of colony formation compared to controls.
[b] Numbers in parentheses, mean concentrations in mM DFMO. See text for details.
[c] Mean ± S.E.

Table 3

Reversal of DFMO effects by putrescine

	T:C ratios (no. of colonies in treated groups:no. of colonies in untreated controls)				
	DFMO[a] alone	Putrescine alone (100 μM)	DFMO[a] + putrescine (100 μM)	Putrescine alone (10 μM)	DFMO[a] + putrescine (10 μM)
PANC-1	0	0.75 ± 0.08[b]	0.77 ± 0.07	1.015 ± 0.04	0.005 ± 0.002
COLO-357	0.31 ± 0.03	0.81 ± 0.09	1.08 ± 0.02	0.96 ± 0.03	0.70 ± 0.04
WD PaCa	0	0.92 ± 0.13	0.81 ± 0.10	1.05 ± 0.05	0.027 ± 0.01
PD PaCa	0	0.88 ± 0.08	0.83 ± 0.05	0.92 ± 0.03	0.017 ± 0.007

[a] The concentration of DFMO was 2.1 mM (500 μg/ml) for PANC-1, PD PaCa, and WD PaCa; for COLO-357, 4.2 mM (1000 μg/ml) was used.
[b] Mean ± S.D.

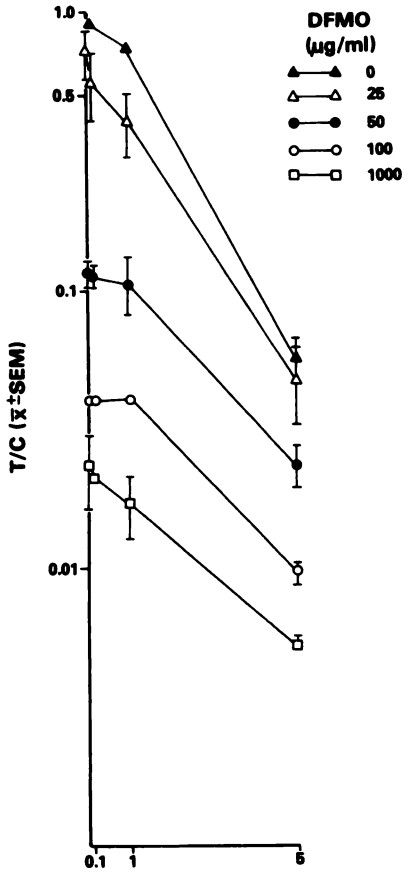

Chart 2. Dose-response curves of combined cisplatin (1 hr) followed by continuous exposure to DFMO in PANC-1. *T/C*, treated *versus* control; $\bar{X} \pm SEM$, mean ± S.E.

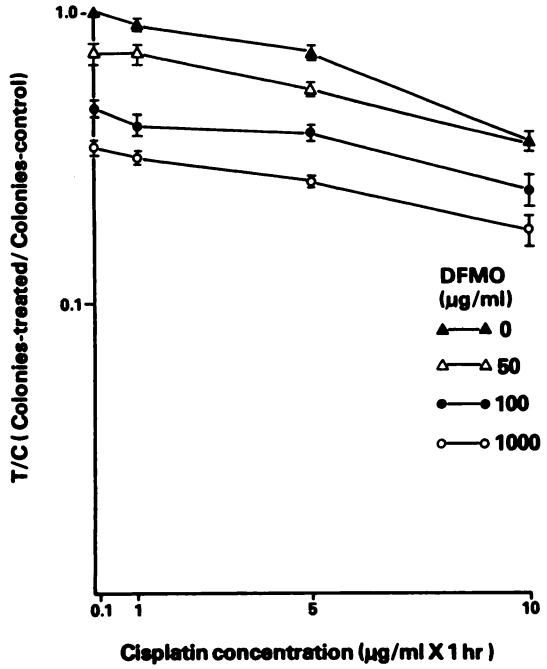

Chart 3. Dose-response curves of combined cisplatin (1 hr) followed by continuous exposure to DFMO in COLO-357. *T/C*, treated *versus* control; *bars*, S.E.

coculturing cells with DFMO and putrescine, as shown in Table 3. Putrescine (100 μM), while slightly toxic by itself, reversed 88 to 100% of the inhibition due to DFMO, whereas 10 μM putrescine could not reverse the antiproliferative effects of 2.1 to 4.2 mM DFMO.

Charts 2 to 5 show the effects on colony formation in each of the 4 cell lines of the combination of DFMO and cisplatin compared to those of cisplatin alone. The effects are roughly additive, with all dose-response curves showing basically the same slope as that seen with cisplatin alone. The magnitude of the combined effects also parallels the effects seen with DFMO alone, wherein PANC-1 and WD PaCa are the most sensitive cell lines. Continuous exposure to DFMO, 25 μg/ml, is capable of lowering the 50% inhibitory dose to cisplatin by a factor of 4 for PANC-1 (1.6 to 0.4 μg/ml) and by a factor of 7.5 for WD PaCa (4.5 to 0.6 μg/ml). In contrast, COLO-357 and PD PaCa are barely affected until DFMO concentrations of 100 μg/ml are reached.

DISCUSSION

We have shown that DFMO in combination with cisplatin is capable of enhancing the cisplatin-induced inhibition of reproductive capacity of pancreatic adenocarcinoma cell lines as assessed by the colony formation assay. The inhibition appears to be roughly additive and cytostatic or antiproliferative, rather than cytocidal. In this respect, our results are in agreement with findings of others, since DFMO appears to be cytostatic against most experimental models studied, with the exception of small cell lung cancer cell lines (12). PANC-1 and WD PaCa exhibit the most marked sensitivity to DFMO, alone and in combination with cisplatin. COLO-357 and PD PaCa are much less sensitive to DFMO, for reasons which are unclear at present. The differences in response to DFMO are not directly attributable to and are somewhat discordant with differences in the ODC activities of the cell lines (see Table 1). We are currently investigating the role that polyamine pool size and/or ODC half-lives may have in explaining the differences in sensitivity to DFMO.

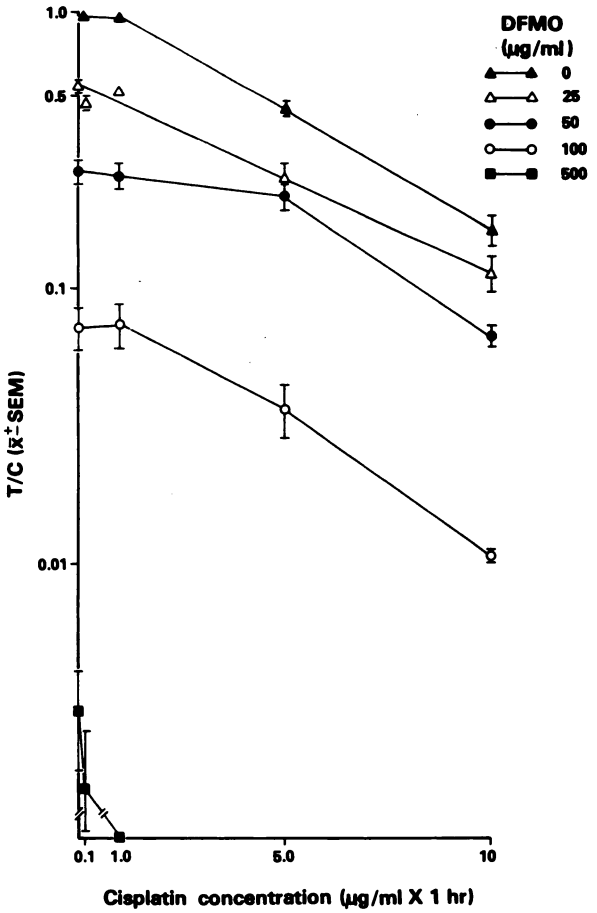

Chart 4. Dose-response curves of combined cisplatin (1 hr) followed by continuous exposure to DFMO in WD PaCa. *T/C*, treated *versus* control; $\bar{X} \pm SEM$, mean ± S.E.

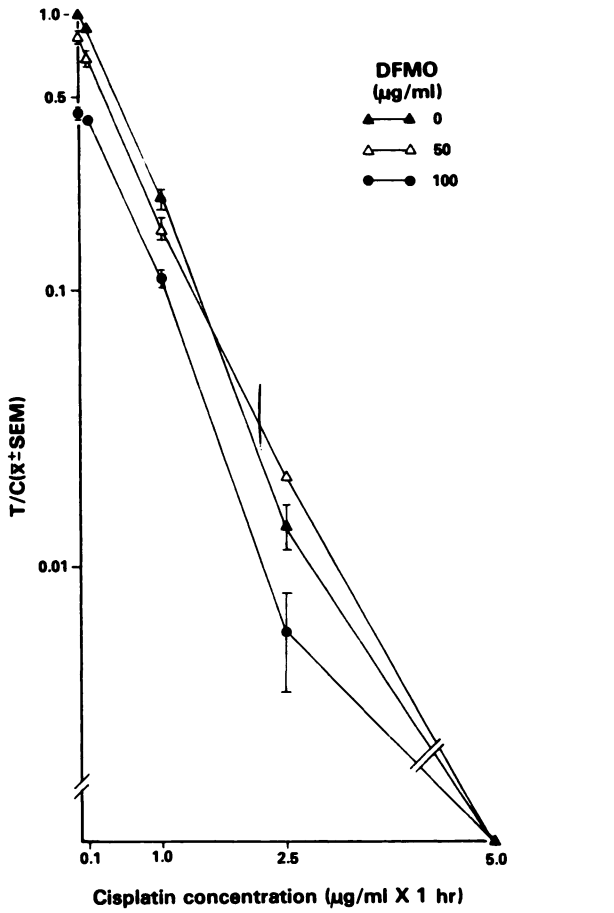

Chart 5. Dose-response curves of combined cisplatin (1 hr) followed by continuous exposure to DFMO in PD PaCa. *T/C*, treated *versus* control; $\bar{X} \pm SEM$, mean ± S.E.

It is important to point out that we did not see any inhibition of the cytotoxic activity of cisplatin as has been reported by Oredsson *et al.* (18) and Tolifon *et al.* (25). We think that the suppression of the effects of cisplatin that these workers observed may be schedule dependent, since in their study rat brain tumor cells were pretreated with DFMO prior to cisplatin exposure, whereas in our study cells were treated with cisplatin, followed by incubation with DFMO. We are presently exploring the question of schedule dependence further, in that cisplatin is an ideal agent to use in combination with DFMO because its toxicity (predominantly renal and neurological) does not overlap with that of DFMO (mainly myelosuppression and gastrointestinal). Our preliminary data do in fact show some inhibition of the effects of cisplatin when DFMO is given for only 2 or 5 to 6 days prior to cisplatin (6). The negative effect of DFMO pretreatment is, however, not seen when post-cisplatin treatment of the pancreatic adenocarcinoma cells with DFMO is given in addition to the pretreatment.

While there are many unanswered questions concerning the interaction of DFMO and other agents, the combination of the relatively low-toxicity agent DFMO with cytotoxic agents such as cisplatin appears to be a promising approach against pancreatic adenocarcinoma and perhaps other cancers exhibiting relative chemoresistance. Use of DFMO in combination with other agents may provide a means of overcoming primary drug resistance.

REFERENCES

1. Abeloff, M. D., Slavik, M., Luk, G., *et al.* Phase I trial and pharmacokinetic studies of α-difluoromethylornithine (DFMO), an inhibitor of polyamine biosynthesis. J. Clin. Oncol., *2:* 124–130, 1984.
2. Bartholeyns, J., and Koch-Weser, J. Effects of α-difluoromethylornithine alone and combined with Adriamycin or vindesine on L1210 leukemia in mice, EMT6 solid tumors in mice, and solid tumors induced by injection of hepatoma tissue culture cells in rats. Cancer Res., *41:* 5158–5161, 1981.
3. Black, O., Jr., and Chang, B. K. Ornithine decarboxylase enzyme activity in human and hamster pancreatic tumor cell lines. Cancer Lett., *17:* 87–93, 1982.
4. Chang, B. K. Differential sensitivity of pancreatic adenocarcinoma cell lines to chemotherapy agents in culture. Cancer Treat. Rep., *67:* 355–361, 1983.
5. Chang, B. K., and Black, O., Jr. Inhibition of the growth of pancreatic cancer cell lines by α-difluoromethylornithine (DFMO) alone and combined with cis-diamminedichloroplatinum (DDP). Proc. Am. Assoc. Cancer Res., *24:* 271, 1983.
6. Chang, B. K., Black, O., Jr., and Gutman, R. Schedule-dependent interaction of α-difluoromethylornithine (DFMO) and *cis*-diamminedichloroplatinum (DDP) against pancreatic cancer cell lines. Proc. Am. Assoc. Cancer Res., *25:* 316, 1984.
7. Chang, B. K., and Gutman, R. Chemotherapy of pancreatic adenocarcinoma: initial report on two transplantable models in the Syrian hamster. Cancer Res. *42:* 2666–2670, 1982.
8. Chen, T. R. *In situ* detection of *Mycoplasma* contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell Res., *104:* 255–262, 1977.
9. Hung, D. T., Deen, D. F., Seidenfeld, J., and Marton, L. J. Sensitization of 9L rat brain gliosarcoma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea by α-difluoromethylornithine, an ornithine decarboxylase inhibitor. Cancer Res., *41:* 2783–2785, 1981.
10. Kingsnorth, A. N., Russell, W. E., McCann, P. P., Diekema, K. A., and Malt, R. A. Effects of α-difluoromethylornithine and 5-fluorouracil on the proliferation of a human colon adenocarcinoma cell line. Cancer Res., *43:* 4035–4038, 1983.
11. Lieber, M., Mazzetta, J. A., Nelson-Rees, W., Kaplan, M., and Todaro, G. Establishment of a continuous tumor-cell line (PANC-1) from a human carci-

noma of the exocrine pancreas. Int. J. Cancer, *15:* 741–747, 1975.
12. Luk, G. D., Goodwin, G., Gazdar, A. F., and Baylin, S. B. Growth-inhibitory effects of DL-α-difluoromethylornithine in the spectrum of human lung carcinoma cells in culture. Cancer Res., *42:* 3070–3073, 1982.
13. Mamont, P., Duchesne, M-C., Grove, J., and Bey, P. Anti-proliferative properties of DL-α-difluoromethyl ornithine in cultured cells. A consequence of the irreversible inhibition of ornithine decarboxylase. Biochem. Biophys. Res. Commun., *81:* 58–66, 1978.
14. Matsushima, M., and Bryan, G. T. Early induction of mouse urinary bladder ornithine decarboxylase activity by rodent vesical carcinogens. Cancer Res., *40:* 1897–1901, 1980.
15. Metcalf, B. W., Bey, P., Danzin, C., *et al.* Catalytic irreversible inhibition of mammalian ornithine decarboxylase (E.C. 4.1.1.17) by substrate and product analogues. J. Am. Chem. Soc., *100:* 2551–2553, 1978.
16. Morgan, R. T., Woods, L. K., Moore, G. E., *et al.* Human cell line (COLO-357) of metastatic pancreatic adenocarcinoma. Int. J. Cancer, *25:* 591–598, 1980.
17. Olson, J. W., and Russell, D. H. Prolonged ornithine decarboxylase induction in regenerating carcinogen-treated liver. Cancer Res., *40:* 4373–4380, 1980.
18. Oredsson, S. M., Deen, D. F., and Marton, L. J. Decreased cytotoxicity of *cis*-diamminedichloroplatinum(II) by α-difluoromethylornithine depletion of polyamine in 9L rat brain tumor cells *in vitro*. Cancer Res., *42:* 1296–1299, 1982.
19. Pegg, A. E., and McGann, P. P. Polyamine metabolism and function. Am. J. Physiol., *243:* C212–C221, 1982.
20. Poso, H., and Pegg, A. E. Effect of α-difluoromethylornithine on polyamines and DNA synthesis in regenerating liver. Reversal of inhibition of DNA synthesis by putrescine. Biochim. Biophys. Acta, *696:* 179–186, 1982.
21. Prakash, N. J., and Sunkara, P. S. Combination chemotherapy involving α-difluoromethylornithine and 1-β-D-arabinofuranosylcytosine in murine L1210 leukemia. Cancer Res., *43:* 3192–3196, 1983.
22. Roizin-Towle, L. Studies with *cis*-diamminedichloro-platinum II and exogenous polyamines using mammalian cells in culture. Br. J. Cancer, *43:* 378–383, 1981.
23. Scalabrino, G., Poso, H., Holtta, E., Hannonen, P., Kallio, A., and Janne, J. Synthesis and accumulation of polyamines in rat liver during chemical carcinogenesis. Int. J. Cancer, *21:* 239–245, 1978.
24. Scarpelli, D. G., and Rao, M. S. Transplantable ductal adenocarcinoma of the Syrian hamster pancreas. Cancer Res., *39:* 452–458, 1979.
25. Tolifon, P. J., Deen, D. F., and Marton, L. J. α-Difluoromethylornithine-induced polyamine depletion of 9L tumor cells modifies drug-induced DNA cross-link formation. Science (Wash. DC), *222:* 1132–1135, 1983.