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Schedule-dependent Interaction of α-Difluoromethylornithine and *cis*-Diamminedichloroplatinum(II) against Human and Hamster Pancreatic Cancer Cell Lines[1]

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ABSTRACT

The interaction of *cis*-diamminedichloroplatinum (cisplatin) and α-difluoromethylornithine (DFMO) has been previously shown by us to be roughly additive in enhancing the growth-inhibitory effects of cisplatin and by another group of investigators to be antagonistic. Since two different schedules of administration were used, we sought to investigate systematically the role of schedule dependence in the interaction of cisplatin and DFMO in a panel of pancreatic adenocarcinoma cell lines (PANC-1, of human origin, and WD PaCa and PD PaCa, both of hamster origin). Dose-effect relationships of single drug alone and in combination were analyzed by the median-effect principle and by the combination indices for the quantitation of synergism or antagonism with the aid of a microcomputer. Pre-cisplatin administration of DFMO for 2 or 5 to 6 days at concentrations of 50 or 100 μg/ml (0.21 or 0.42 mM) was found to antagonize the effects of cisplatin to various degrees in the cell lines. In contrast, whenever post-cisplatin DFMO was administered, marked enhancement, which was synergistic in most instances, of cisplatin's inhibition of colony formation was found. Thus, the interaction of cisplatin and DFMO is felt to be schedule dependent with deleterious effects found only when DFMO is administered prior to and not following cisplatin. Furthermore, the combination shows promise as an approach to overcoming drug resistance in pancreatic cancer.

INTRODUCTION

DFMO[4] is a specific inhibitor of ODC (1–3), which is the rate-limiting enzyme in the biosynthesis of the polyamine putrescine and is known to be elevated in many malignantly transformed tissues (4–6). Polyamines, ubiquitous organic cations, play an essential role in cellular growth and proliferation (7), and one of their functions may be to stabilize DNA at various times in the cell cycle (8). Thus, DFMO, by virtue of its ability to induce polyamine depletion and its low toxicity in animal studies (9) and Phase I trials (10), is attractive as an antiproliferative agent to be used in combination with conventional cytotoxic agents.

DFMO has been combined to advantage with cytotoxic agents against a number of experimental tumor models and cell lines (9, 11–15). Nevertheless, previous reports did not consistently suggest benefits from the combination of DFMO with other agents, and in certain cases, antagonism has been documented (16–22). One of these instances of antagonism involved a 48-h pretreatment of brain tumor cells with 10 mM DFMO which decreased the cytotoxic effects of cisplatin (17).

Since our own work using DFMO as a post-cisplatin treatment of pancreatic adenocarcinoma cells showed enhancement of the cytotoxic effect (23), we hypothesized that the reported antagonism might be schedule dependent. Thus, the present study was undertaken to explore the schedule-dependent interaction of DFMO and cisplatin and to determine the optimal schedules for administering these agents in combination. Our data show that, under certain exposure conditions, less inhibition of colony formation was found after pre-cisplatin exposure to DFMO. However, no inhibition, but rather enhancement, of cisplatin's cytotoxic effects was seen when cisplatin administration was followed by DFMO or when DFMO was followed by cisplatin which was then followed by DFMO.

MATERIALS AND METHODS

Cell Lines. Characteristics of the cell lines used in this study have been previously described (23, 24). PANC-1, of human origin, was obtained from the American Type Culture Collection, Rockville, MD. WD PaCa and PD PaCa, both of hamster origin, were adapted to tissue culture in our laboratory (25) from transplantable tumor models (26) kindly supplied by D. G. Scarpelli and M. S. Rao, Northwestern University.

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 from Flow Laboratories, McLean, VA). For the colony formation assay, the medium also includes the following: 15% fetal bovine serum; 0.9% methylcellulose (4000 centipoise; Fisher Scientific Co., Fair Lawn, NJ); and 25 mM 4(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Grand Island Biological Co.).

Drugs and Chemicals. DFMO was supplied courtesy of Merrell Research Center, Merrell Dow Pharmaceuticals, Cincinnati, OH, and cisplatin courtesy of Bristol Laboratories, Syracuse, NY. 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 and 1.0 mg/ml for cisplatin, followed by filter sterilization. An appropriate aliquot of the stock DFMO or a dilution thereof was added directly to the culture medium or to the methylcellulose assay medium. Cisplatin was serially diluted to concentrations of 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-Effect Assays. The colony formation assay was used to assess drug effects. Cells were exposed to DFMO at concentrations of 0 and 50 μg/ml (0.21 mM) or 100 μg/ml (0.42 mM) continuously in tissue culture flasks for 2 or 5 to 6 days ("pre-cisplatin DFMO"), washed, and harvested by short exposure to 0.25% trypsin for the adherent cell lines (PANC-1 and WD PaCa). Cells (1 to 2 × 10^6) were incubated at 37°C, 5% CO_2, for 1 h with cisplatin at concentrations of 0, 0.1, 1.0, 2.5, or 5.0 μg/ml (0.33 to 16.7 μM). After incubation, cells were washed free of cisplatin and plated at a density of 1 to 2 × 10^3 cells/ml in assay medium containing DFMO at concentrations of 0 and 50 μg/ml (0.21 mM) or 100 μg/ml (0.42 mM) ("post-cisplatin DFMO"). Colony for-

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[4] The abbreviations and trivial terms used are; DFMO, α-difluoromethylornithine; ODC, ornithine decarboxylase; cisplatin, *cis*-diamminedichloroplatinum(II); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ID_{50}, drug dose at which there is a 50% inhibition of colony formation compared to untreated controls; C.I., combination index; DMF, dose-modifying factor.

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mation assays were carried out in quadruplicate in 35- x 10-mm suspension culture dishes (LUX, Naperville, IL) with enumeration of colonies (>50 cells) using an inverted microscope after 7 to 14 days of incubation at 37°C, 5% CO_2. Results for each assay were expressed as the treated:control ratio (mean colonies treated:mean colonies untreated control) for each drug concentration. Dose-effect curves represent the mean of at least 3 separate experiments. ID_{50}s (μg/ml) were determined from the median-effect equation (27). The C.I. for each dose/schedule exposure condition was calculated according to the method of analysis developed by Chou and Talalay (27) using computer software (28). According to this analysis a C.I. of 1.0 indicates summation or additive effects, <1.0 indicates synergism, and >1.0 indicates antagonism.

RESULTS

We have previously reported the responses of the pancreatic cancer cell lines to DFMO alone and have shown that the effects of DFMO are specific for ODC inhibition, since a reversal of these effects is seen when the cells are cocultured with exogenous putrescine and DFMO (23).

Tables 1 and 2 show the effects of scheduling and duration of DFMO exposure in relation to a standardized, 1-h exposure to cisplatin, in the 3 pancreatic adenocarcinoma cell lines. The concentrations of DFMO, 50 and 100 μg/ml (0.21 to 0.42 mM), were chosen to fall within the range of clinically achievable serum concentrations of DFMO (10). Table 1 summarizes the ID_{50}s for cisplatin and DFMO and their various combinations according to the schedule of administration; DMFs are shown to facilitate comparison of the various exposures. Table 2 shows the actual fractional inhibition of colony formation and the C.I.s for each exposure. As can be seen, pre-cisplatin DFMO interacted with cisplatin to produce antagonistic effects. In contrast, in most instances in which post-cisplatin DFMO was combined with cisplatin, synergistic effects were seen. The only exceptions to this were in the PD PaCa cell line in which weakly antagonistic (C.I.s of up to 1.4) or additive (C.I. = 1.0) effects were occasionally seen. These observations suggest that, while pre-cisplatin DFMO alone may be deleterious, as long as cisplatin is followed by DFMO largely synergistic (PANC-1 and WD PaCa) or additive (PD PaCa) effects were seen. Quantitative evaluation of the C.I. values indicates that deemphasizing the cisplatin doses and emphasizing the DFMO doses (*i.e.*, decreasing the cisplatin/DFMO ratio) maximize synergy.

DISCUSSION

Since either cisplatin or DFMO alone has inhibitory effects on colony-forming assays, it is necessary to define their additive effects before attempting to quantitate synergism or antagonism. The combined effects for additive interaction are not the sum of each drug's fractional inhibition (27). In addition, since the dose-effect curves for cisplatin or DFMO alone show a sigmoidal relationship (*i.e.*, $m > 1$), the combined effects for assumed additivism cannot be calculated from the fractional product method (27, 29). In the present studies, the dose-effect relationships are analyzed by the median-effect principle, the multiple-drug effect equation, and the recently developed concept of C.I. The C.I. allows for quantitative assessment of synergism and antagonism (27, 29). This method is compatible with the isobologram method. It has been shown in many recent studies involving cellular, subcellular, and animal systems (29) that the C.I. method is the method of choice because it is easy to use and because of its spectrum of applicability.

We have shown that, under certain exposure conditions, treatment of pancreatic cancer cell lines with DFMO prior to cisplatin produced antagonistic effects. Thus, in this sense, our results confirm the findings of Oredsson *et al.* (17), who reported that 48-h pre-cisplatin DFMO produced a diminution of cisplatin's cytotoxicity with dose-modifying factors of 2.0 to 2.1 at 10, 1, and 0.1% survival levels. Nevertheless, there are substantial differences between their study and our present one. Both the concentrations of DFMO (10 mM or 2366.5 μg/ml) and cisplatin (50 to 300 μM, or 15 to 90 μg/ml) that they used are well outside the range clinically achievable (10, 30). In addition, they did not specifically address the question of schedule dependence, whereas we have shown that the antagonistic effects of pre-cisplatin DFMO are avoided by the inclusion of post-cisplatin DFMO in the treatment protocol.

In related studies, Tofilon *et al.* reported that pre-cisplatin DFMO (10 mM for 72 h) followed by 0.5 μM (0.15 μg/ml) cisplatin for 1 h resulted in decreased sister chromatid exchanges compared to the effects of cisplatin alone, in contrast to enhanced sister chromatid exchanges induced by the same DFMO pretreatment of 9L cells followed by BCNU (19). Likewise, they found that preincubation of 9L cells with DFMO (1 mM or 236.7 μg/ml for 72 h) followed by either cisplatin (80 μM or 24 μg/ml) or BCNU (80 μM) caused a decrease in DNA

Table 1 *Effects of cisplatin and DFMO in pancreatic cancer cell lines*

Exposure	[DFMO] (μg/ml)	PANC-1 ID_{50} (μg/ml)[a]	PANC-1 DMF[b]	WD PaCa ID_{50} (μg/ml)	WD PaCa DMF	PD PaCa ID_{50} (μg/ml)	PD PaCa DMF
Cisplatin alone		0.42		2.13		0.24	
DFMO alone	25–1000	16.16		28.48		91.25	
2-Day pre-cisplatin DFMO	50	2.22	5.3	2.99	7.2	0.41	1.7
	100	1.22	2.9	4.18	9.9	0.25	1.0
5–6-Day[c] pre-cisplatin DFMO	50	2.17	5.2	2.05	4.9	0.23	1.0
	100	2.09	5	1.27	3.0	0.11	0.5
2-Day pre- and post-cisplatin DFMO	50	0.0004	0.0001	<0.000		0.04	0.2
	100	<0.000		<0.000		0.002	0.01
5–6-Day pre- and post-cisplatin DFMO	50	0.0009	0.002	0.0006	0.001	0.2	0.4
	100	<0.000		<0.000		0.0043	0.02
Post-cisplatin DFMO	50	0.005	0.01	0.0002	0.0005	0.13	0.5
	100	0.0001	0.0002	0.0004	0.001	0.04	0.2

[a] ID_{50}, dose of cisplatin in μg/ml that results in 50% inhibition of colony formation compared to untreated controls.
[b] DMF, dose-modifying factor, or ID_{50} (cisplatin + DFMO):ID_{50} (cisplatin alone).
[c] Pre-cisplatin incubation with DFMO was 6 days for PANC-1 and 5 days for WD PaCa and PD PaCa.

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Table 2 *Fractional inhibitions and C.I.s of cisplatin and DFMO in three pancreatic cancer cell lines*

Exposure	D1[a] [cisplatin][b]	D2 [DFMO]	PANC-1		WD PaCa		PD PaCa	
			fa	C.I.	fa	C.I.	fa	C.I.
2-Day pre-cisplatin DFMO	0.1	50	−0.156		−0.084		0.097	2.24
	1.0	50	0.091	37.39	0.084	12.78	0.740	1.19
	5.0	50	0.912	3.15	0.769	1.67	0.966	1.88
	0.1	100	0.237	19.29	−0.005		0.240	2.13
	1.0	100	0.561	6.98	0.068	22.00	0.804	2.71
	5.0	100	0.978		0.581	4.61	0.980	1.73
5–6-Day pre-cisplatin DFMO[c]	0.1	50	−0.125		−0.014		0.274	1.36
	1.0	50	0.105	32.70	0.293	3.99	0.811	2.24
	5.0	50	0.910	3.21	0.749	1.71	0.970	1.77
	0.1	100	−0.118		0.047	16.82	0.457	1.56
	1.0	100	0.140	42.14	0.334	0.596	0.950	1.44
	5.0	100	0.897	3.94	0.767	2.56	0.987	1.43
2-Day pre- and post-cisplatin DFMO	0.1	50	0.946	0.25	0.928	0.50	0.769	0.65
	1.0	50	0.949	0.58	0.930	0.52	0.948	1.13
	5.0	50	0.992	0.63	0.969	0.37	0.994	0.80
	0.1	100	0.993	0.71	0.979	0.53	0.970	0.69
	1.0	100	0.994	0.15	0.975	0.58	0.991	0.76
	5.0	100	0.999	0.20	0.989	0.39	0.998	0.63
5–6-Day pre- and post-cisplatin DFMO	0.1	50	0.954	0.22	0.861	0.73	0.539	0.91
	1.0	50	0.959	0.49	0.891	0.67	0.918	1.42
	5.0	50	0.995	0.45	0.960	0.43	0.990	1.03
	0.1	100	0.987	0.11	0.988	0.40	0.983	0.58
	1.0	100	0.986	0.28	0.991	0.35	0.9993	0.27
	5.0	100	0.998	0.31	0.996	0.23	0.9997	0.36
Post-cisplatin	0.1	50	0.915	0.38	0.864	0.71	0.442	1.04
	1.0	50	0.960	0.48	0.882	0.70	0.921	1.39
	5.0	50	0.995	0.49	0.953	0.48	0.996	0.70
	0.1	100	0.978	0.19	0.954	0.79	0.807	1.03
	1.0	100	0.989	0.23	0.984	0.46	0.989	0.82
	5.0	100	0.998	0.30	0.994	0.28	0.998	0.62
Cisplatin alone	0.1		0.128		0.091		0.198	
	1.0		0.625		0.194		0.859	
	5.0		0.989		0.644		0.991	
DFMO alone		25.0	0.271		0.457		0.0002	
		50.0	0.883		0.734		0.178	
		100.0	0.960		0.928		0.564	
		500.0	0.974		0.997		1.00	

[a] D1, Dose 1; D2, Dose 2; fa, fraction affected, or [1 − (treated/control)]; C.I., $(D1/Dx1) + (D2/Dx2)$, where $Dx = Dm[fa/(1 - fa)]^{1/m}$ or the dose required to affect x%; Dm = the median effect dose or ID_{50}, and m = the slope of the median-effect plot (27) which signifies the sigmoidicity of the dose-effect curve. [C.I. = 1.0, additive; <1.0, synergism; >1.0, antagonism.] For a conservative calculation of C.I., a third term $(D1/Dx1)(D2/Dx2)$ should be added for mutually nonexclusive drugs. This will result in slightly higher C.I. values (27–29).

[b] For PD PaCa, the highest [cisplatin] was 2.5 μg/ml (instead of 5.0 μg/ml).

[c] Pre-cisplatin incubation with DFMO was 6 days for PANC-1 and 5 days for WD PaCa and PD PaCa.

interstrand cross-linking with cisplatin and an increase with BCNU (18). Based upon these studies, they have concluded that the interaction of DFMO and cytotoxic agents depends upon a given drug's mechanism of action. They also point out the need for preclinical studies of DFMO whenever it is used in combination with a given agent.

While concurring with their conclusions, we would also emphasize the need for examination of the role of schedule dependence in combining DFMO with other agents. Schedule of administration would seem to be a critical factor, since DFMO induces a lower rate of cellular proliferation, which theoretically may protect cells from the lethal effects of cytotoxic, particularly cycle (or phase)-specific, agents which are most effective against rapidly dividing cells. In fact, some evidence to support the preceding supposition is derived from studies using the 9L rat brain tumor model showing that DFMO induces a block in the G_1- to S-phase transition (20, 21, 31). Although cisplatin is generally considered to be a cycle-nonspecific agent, it is quite possible that the DFMO-induced lower rate of cell division may influence its cytotoxicity since cell cycle-nonspecific agents may display a cell cycle age response. In addition, based upon our finding of an enhanced recovery of cells pretreated with DFMO and not exposed to cisplatin, we hypothesize that release from DFMO inhibition followed by polyamine repletion may result in an enhanced growth fraction of cells that were temporarily protected, in part, from the cytotoxic effects of cisplatin. We plan to explore this question further using cell cycle analysis by flow cytometry and sequential determination of polyamine levels.

Nevertheless, regardless of the mechanism of DFMOs antagonism of cisplatin when administered as a pretreatment, it should be stressed that no antagonism, but rather enhancement of cisplatin's effects was seen when DFMO followed cisplatin in our panel of pancreatic cancer cell lines. These results suggest that the optimal scheduling would be to administer DFMO between cycles of chemotherapy to prevent cancer cell recovery. Such an approach should be feasible due to the low toxicity of DFMO, particularly at doses which give serum levels similar

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to those used in the present study, and due to the higher levels of ODC in malignant as compared to normal tissues. The approach is especially attractive in cancers such as pancreatic adenocarcinoma which are relatively resistant to conventional chemotherapy.

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