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ACCELERATED COMMUNICATION

Difluoromethylornithine Prevents the Down-regulation of Type I Interferon Receptors: A Possible Mechanism for a Synergistic Antiproliferative Effect

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SUMMARY

Type I interferon and difluoromethylornithine have been shown to exert an antiproliferative effect, both alone and in combination, in several tumor cell lines. Using B16 melanoma cells, we have shown that these two drugs inhibit growth over 72 hr *in vitro*. The estimated ED_{50} values for difluoromethylornithine and type I interferon were 31.1 ± 1.1 μM and 22.3 ± 2.7 IU/ml, respectively. When used in combination, a marked synergism was observed, as detected by isobologram analysis. Type I interferon, at concentrations that exhibited synergistic activity with difluoromethylornithine, did not affect ornithine decarboxylase activity or intracellular polyamine concentrations. These data suggest that the synergistic antiproliferative effect of murine type I interferon in combination with difluoromethylornithine is not mediated via polyamine depletion. When we examined the type I interferon receptor numbers on the B16 cells exposed to 5 IU/ml murine type I interferon for 72 hr, a 40% decrease was observed, compared with that seen in control cells. Difluoromethylornithine, at 10 μM, did not affect type I interferon receptor numbers. However, when added to the cells in the presence of murine type I interferon, difluoromethylornithine completely inhibited down-regulation, suggesting that down-regulation of the type I interferon receptor is a polyamine-dependent process. These observations may provide a basis for enhancing the therapeutic efficacy of interferon treatment through control of interferon receptor down-regulation.

Type 1 IFNs are a large family of soluble proteins consisting of α and β subtypes. All members of the family exhibit antiproliferative activity both *in vitro* and *in vivo* (1). Clinical studies have shown varying therapeutic responses to IFN in a number of neoplasms including hairy cell leukemia, malignant melanoma, multiple myeloma, acquired immunodeficiency syndrome-related Kaposi's sarcoma, and cutaneous T cell lymphoma (2). Several mechanisms have been proposed to explain the antiproliferative activity of IFN. These include a direct effect upon the growth of the target cell, an enhancement of the host's immune system, differentiation of the target cell, phenotype reversion, and antiviral effects (3).

The cellular responses to IFN are mediated through the interaction of the protein with cell surface receptors, resulting in a number of biochemical effects including a marked alteration in gene expression. As is the case for most other biological response modifiers and polypeptide hormones, IFN-receptor complexes are localized in clathrin-coated pits on the cell surface (4). The complexes are internalized by endocytosis and then degraded (4).

The ODC inhibitor difluoromethylornithine inhibits the growth of tumor cells both *in vitro* and *in vivo* by depleting intracellular polyamine levels (5). Recently, several studies have demonstrated an enhanced antitumor effect when difluoromethylornithine was administered along with IFN (6–9) or IFN inducers (10). *In vitro*, this combination has been shown to exert a synergistic antiproliferative effect against human Daudi cells (11) and human renal carcinoma cells (8). A phase 1 clinical study using difluoromethylornithine and human IFN-α has been reported (12).

Currently, it is not known why these two agents exert a synergistic antiproliferative effect upon tumor cells. However, several mechanisms have been proposed. IFN has been shown to suppress ODC activity *in vitro* (13) and, therefore, may enhance the inhibition of polyamine synthesis caused by difluoromethylornithine. Although Gohji *et al.* (8) have reported that IFN can reduce intracellular polyamine levels *in vitro*, Sunkara *et al.* (6) did not observe such an effect *in vivo*. Difluoromethylornithine reportedly stabilizes 2′,5′-isoadenylate oligomers, whereas IFN is known to induce 2′,5′-oligo-

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ABBREVIATIONS: IFN, interferon; ODC, ornithine decarboxylase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

isoadenylate synthetase (11). It has also been suggested that polyamine depletion may enhance the phosphorylation of eukaryotic initiation factor by IFN-induced kinases, leading to inhibition of protein synthesis and possibly cell death (6).

In the present study, we demonstrate that difluoromethylornithine and murine type I IFN exhibit a synergistic antiproliferative effect against murine B16 melanoma cells. We have investigated the effects that the combination of these two agents have upon intracellular polyamine levels, ODC activity, and type I IFN membrane receptor populations. The data obtained suggest that the synergy seen may be a direct result of difluoromethylornithine-mediated inhibition of the normal down-regulation of type 1 IFN receptors.

Materials and Methods

Cell growth curves. B16 melanoma cells were seeded at a density of 1.75×10^4 cells/cm^2 in 24-well plates and were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 50 μg/ml gentamicin, 2.5 μg/ml amphotericin B, and 50 μM 2-mercaptoethanol. The cells were cultured for 24 hr at 37° in a humidified incubator, with a 5% CO_2 in air atmosphere. The cells were then washed and treated with difluoromethylornithine (Merrell Dow), IFN (murine type I IFN, 1.7×10^6 IU/ml, 5.0×10^6 IU/mg; Lee Biomolecular Research, San Diego, CA), or a combination of each at concentrations shown in Results. Cells were treated with new media containing the drugs every 24 hr up to 72 hr. The cells were then washed and DNA content was determined as previously described (14). Preliminary studies established that none of the treatments affected cell adhesion. Growth curves were constructed over a difluoromethylornithine concentration range of 0–1 mM and an IFN concentrate range of 0–1000 IU/ml.

Data have been expressed as the relative change in DNA content/well (Δ) with respect to the maximum possible change, that is:

$$\Delta = (DNA_c - DNA_{obs})/(DNA_c - DNA_{max})$$

where DNA_{obs} = DNA content in each well treated with either difluoromethylornithine or IFN, DNA_c = DNA for untreated cells, and DNA_{max} = DNA in each well treated with the maximum concentration of each drug. The resulting data were modelled to a sigmoidal curve by nonlinear least squares regression analysis and the ED_{50} was determined as the point of inflection on the curve. Curves were constructed from at least four independent experiments. The estimated ED_{50} values are presented as mean ± standard error, with the degrees of freedom determined as the number of observations minus the number of model parameters. All other results have been presented as mean ± standard error, with the number of observations (n) from independent experiments given in parentheses.

ODC activity and intracellular polyamine concentrations. ODC activity was measured in control and treated cells by the method of $^{14}CO_2$ release from [^{14}C]ornithine, as described elsewhere (15). The supernatant from 5×10^5 cells, in 1 ml of buffer containing 0.1 mM pyridoxyl-5-phosphate, 0.1 mM EDTA, 25 mM Tris, and 5 mM dithiothreitol, pH 7.6, was incubated with 1 μCi of [^{14}C]ornithine for 20 min. Liberation of $^{14}CO_2$ was linear with time for at least 50 min. The intracellular concentration of each polyamine was quantified by high pressure liquid chromatography, as described elsewhere (15).

Type 1 IFN receptor binding. Recombinant ^{35}S-labeled murine IFN-α1 was produced by *in vitro* transcription and translation. The detailed methods for *in vitro* transcription of linearized pSP64T-based templates containing human IFN-α and direct *in vitro* translation of the synthetic mRNA have been described elsewhere (16). The murine IFN-α1 gene was modified by site-directed *in vitro* mutagenesis, such that expression of the mature protein could be achieved *in vitro*. pSP64T vectors containing the modified murine IFN-α1-coding region were linearized with *Xba*1 at a point 3′ to the IFN gene. Transcription was carried out using SP6 RNA polymerase (Promega Biotech, Madison, WI), which initiates mRNA synthesis at the SP6 promoter sequence of pSP64T. Following transcription, the reaction mixture was directly translated using rabbit reticulocyte lysate (Promega Biotech) in the presence of [^{35}S]methionine (>1000 Ci/mmol; Amersham, Bucks, UK). The final incubation mixture was chromatographed twice on a Bio-Rad 10DG column equilibrated with RPMI 1640. The resulting protein fraction contained a single labeled protein with a molecular weight of 17,500, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To quantify type I IFN receptors, 30,000 dpm of ^{35}S-labeled murine IFN-α1 (25 IU/μl; 10,000 dpm/μl) were added to a final concentration of 150 IU/ml to control cells or cells treated previously with 10 μM difluoromethylornithine, 5 IU/ml IFN, or their combination. After 150 min at 37°, the cells were washed twice with RPMI and once with HEPES-buffered saline. The monolayer was digested in 1 M NaOH and radioactivity was quantified by liquid scintillation counting. Nonspecific binding was measured by the addition of an excess (4.1×10^5 IU/ml) of unlabeled IFN. Under these conditions, nonspecific binding was not affected by drug treatment and accounted for 5650 ± 280 dpm/mg of DNA.

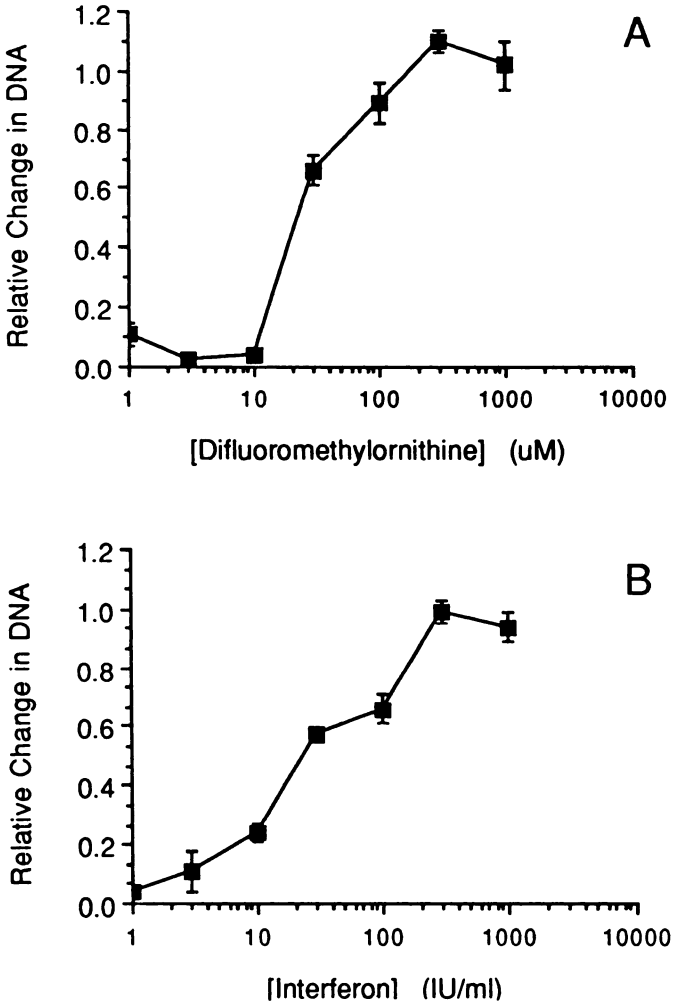

Fig. 1. Dose-response curves for the antiproliferative effects of difluoromethylornithine (A) and IFN (B) in B16 melanoma cells. Cells were grown for 72 hr in the presence of increasing concentrations of each drug. Growth inhibition was determined as the relative change in DNA content relative to control cells. Each *point* is the mean ± standard error (n = 4).

Results and Discussion

Fig. 1 illustrates the antiproliferative effects of both difluoromethylornithine and type 1 IFN against B16 cells. At maxi-

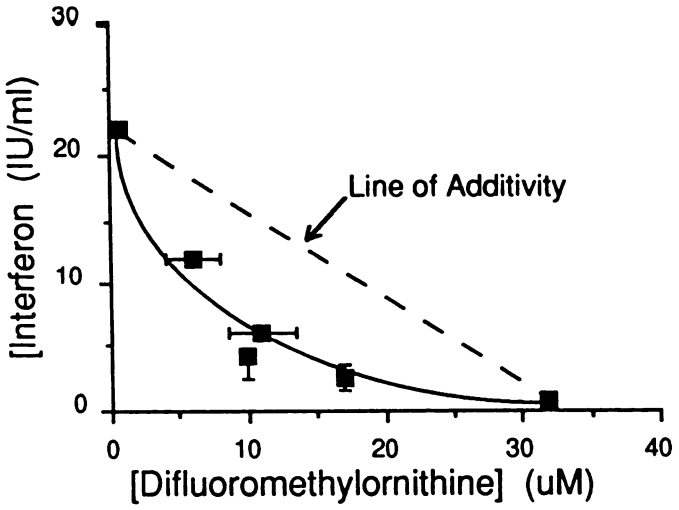

Fig. 2. Isobologram for the interaction of difluoromethylornithine and IFN on the growth of B16 melanoma cells. *Points with horizontal error bars*, ED_{50} values for difluoromethylornithine determined in the presence of a fixed IFN concentration. *Points with vertical error bars*, ED_{50} values for IFN determined in the presence of a fixed difluoromethylornithine concentration. – – –, predicted ED_{50} values if the antiproliferative effects of both agents were additive. Each *point* represents the mean ± standard error (degrees of freedom = 8).

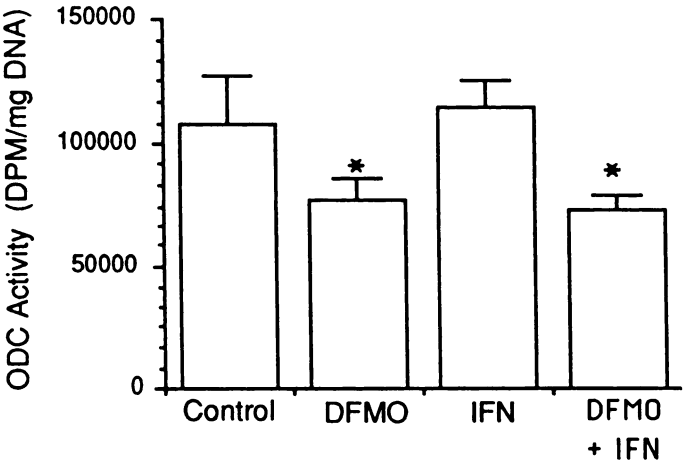

Fig. 3. Effect of difluoromethylornithine and IFN on ODC activity in B16 melanoma cells. ODC activity was determined as described in Materials and Methods after exposure for 3 days to difluoromethylornithine (10 μM) (*DFMO*), IFN (5 IU/ml), or the combination. Data are presented as mean ± standard error (n = 4). *, Treatment significantly different from controls.

mal concentrations, each agent decreased cell growth after 72 hr by 50–60%. This is similar to the effects of each agent on B16 cell growth reported previously (6). The estimated ED_{50} values were 31.3 ± 1.1 μM (degrees of freedom = 8) and 22.3 ± 2.7 IU/ml (degrees of freedom = 8) for difluoromethylornithine and IFN, respectively. The effect of the combination of both drugs was assessed by isobologram analysis (11). For these studies, the ED_{50} for IFN was determined in the presence of 10 and 17 μM difluoromethylornithine. Conversely, the ED_{50} for difluoromethylornithine was determined in the presence of 6 and 12 IU/ml IFN. The results are shown in Fig. 2. The resulting curve fell below the line of additivity, which joins the ED_{50} values determined in the absence of the second drug (Fig. 2). The data indicate that difluoromethylornithine and IFN exert a synergistic antiproliferative effect on B16 melanoma

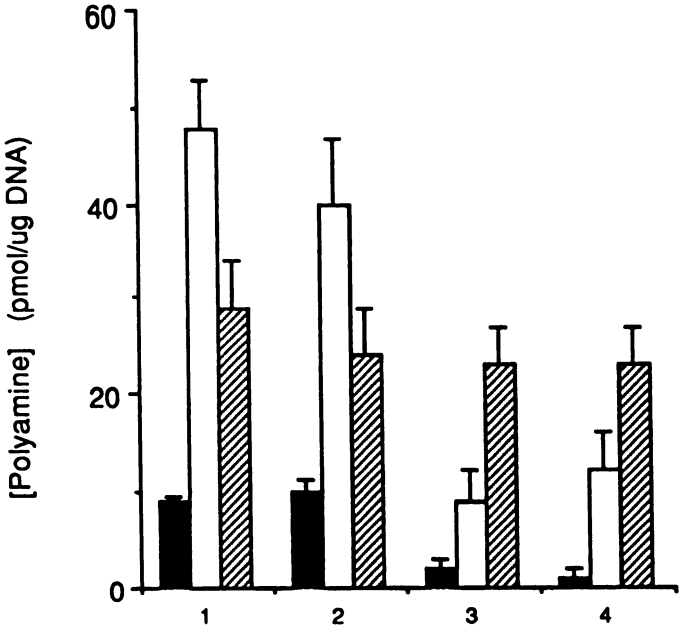

Fig. 4. Effect of difluoromethylornithine and IFN on intracellular polyamine concentrations in B16 melanoma cells. Following treatment with each drug for 3 days, the cells were washed and dissolved in 1 M NaOH. Polyamine content was determined by high pressure liquid chromatography (15). *1*, Control cells; *2*, cells treated with IFN (5 IU/ml); *3*, cells treated with difluoromethylornithine (10 μM); and *4*, cells treated with both drugs. Putrescine (■), spermidine (□), and spermine (▨) concentrations are shown as mean ± standard error (n = 6–9).

TABLE 1

Effects of difluoromethylornithine and IFN on type I IFN receptors on B16 melanoma cells

B16 cells were treated for 3 days with IFN (5 IU/ml), difluoromethylornithine (10 μM), or the combination. The cells were then washed and IFN receptors were assayed as described in Materials and Methods. Control cells exhibited a binding of 8430 ± 1720 dpm/mg of DNA. Nonspecific binding was determined in the presence of 4.1×10^5 IU/ml unlabeled IFN.

Treatment	Receptor number
	% of control
IFN (5 IU/ml)	59.3 ± 14.5[a]
Difluoromethylornithine (10 μM)	103 ± 7.8
Difluoromethylornithine + IFN	123 ± 9.2

[a] Significantly different from control ($p < 0.05$), as determined by one-way analysis of variance.

cells. This observation is in agreement with data obtained using other cell lines (8, 11).

Sunkara *et al.* (6) have previously reported that difluoromethylornithine, in the presence of IFN, exerted a greater antiproliferative effect in B16 cells than would be expected from a purely additive effect. In their studies, 2.5 mM difluoromethylornithine and 500 IU/ml IFN were used. They also presented data showing that IFN (100 IU/ml) depleted intracellular putrescine and spermidine. In combination with difluoromethylornithine, it caused a decrease in intracellular polyamines similar to that seen with difluoromethylornithine alone. In contrast, Gohji and associates (8), who observed synergism between difluoromethylornithine and IFN in renal carcinoma cells, reported a marked effect of interferon (1000 IU/ml) on polyamine levels, particularly in the presence of difluoromethylornithine (5 mM). We attempted to confirm whether this observation was also seen at the much lower drug concentrations employed in the present study. Fig. 3 illustrates

the effect of each drug on cellular ODC activity. IFN, at 5 IU/ml, did not affect ODC activity. Moreover, when IFN and difluoromethylornithine (10 μM) were administered together, ODC activity was the same as that seen in cells treated with difluoromethylornithine alone. IFN, either alone or in combination with difluoromethylornithine, also did not affect the level of intracellular putrescine, spermidine, or spermine (Fig. 4). We conclude from these data that, at the concentrations of drugs used in the present study, the synergistic antitumor activity of IFN and difluoromethylornithine against B16 cells is not mediated through changes in the polyamine content.

We also investigated the cell surface type I IFN receptor population following treatment with each agent and their combination. Receptor down-regulation for type I IFN has been reported following prolonged exposure to the drug (17). Moreover, it has been suggested that the effect of IFN *in vivo* may be limited by the rate of receptor down-regulation (18). When B16 cells were exposed to 5 IU/ml IFN for 72 hr, the number of cell surface receptors decreased by 41% (Table 1). Difluoromethylornithine at 10 μM did not affect Type I IFN receptor numbers. When cells were treated with difluoromethylornithine and IFN together, no loss of receptors was seen. These data indicate that difluoromethylornithine prevents the normal down-regulation of IFN receptors that is observed during prolonged exposure. Because the antiproliferative effect of IFN is related to the number of cell surface receptors (19), this effect of difluoromethylornithine may explain the synergistic growth-inhibitory activity of the two drugs. In patients receiving IFN, receptor numbers on peripheral blood lymphocytes decrease by up to 60% following 3–5 days of treatment (18). Prevention of receptor down-regulation may not only be important in optimizing IFN therapy but may also have clinical implications for other biological response modifiers, because receptor down-regulation appears to be a general phenomenon, the mechanism of which may be regulated by a common biochemical pathway (20).

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