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### Authors

BREGMAN, MARVIN D  
BUCKMEIER, JULIE  
FUNK, CAROLE  
[et al.](#)

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# Median Effect and Long Term Recovery Analysis of Biological Modifier Interactions With Difluoromethylornithine on the Proliferation of Human Melanoma Cells

MARVIN D. BREGMAN, JULIE BUCKMEIER, CAROLE FUNK, AND  
FRANK L. MEYSKENS, JR.

Arizona Cancer Center, University of Arizona, Tucson, Arizona 85724

The ability of difluoromethylornithine (DFMO), a specific polyamine synthesis inhibitor, to interact with various biological modifiers to inhibit the colony-forming growth of human melanoma cells was determined by using the median effect principle to computer model the strength of two agent interactions. Either alpha- or gamma-IFN (interferon) in combination with DFMO resulted in a synergistic inhibition on human melanoma colony formation. For human melanoma cells which were not resistant to 13-cis RA (retinoic acid), an additive suppression on colony formation was obtained with the retinoid-DFMO combination. Dexamethasone (DEX) interacted with DFMO to yield a synergistic reduction in melanoma colony number on glucocorticoid sensitive cells and no growth enhancement with DFMO on glucocorticoid resistant melanoma lines.

Human melanoma cells displayed differential long-term growth sensitivity to DFMO treatment. C8146C human melanoma cells were terminally growth-inhibited by a 96 h exposure to DFMO, in a manner which was concentration and time dependent. The proliferation of C82-7A melanoma cells was inhibited by 95% in presence of DFMO, but upon removal of DFMO the cells regained their ability to proliferate and form colonies. The simultaneous addition of DEX plus alpha-IFN plus 13-cis-RA with DFMO caused most of the human melanoma cells in these lines to become permanently growth arrested. Pre-treatment with DEX plus alpha-IFN plus 13-cis RA, but without DFMO, did not have any long term effect on the ability of melanoma cells to recover and proliferate in soft agar. Since human melanoma cells are heterogeneous with respect to sensitivity to these hormones, it may be advantageous to use DFMO with several of these agents in combination.

**Key words:** Interferon, Dexamethasone, Retinoic acid

## INTRODUCTION

The proliferation of various human melanoma cell lines can be inhibited by exposure to either DEX, DFMO,  $\alpha$ -IFN, or RA (Bojar and Fabry, 1985; Bregman et al., 1983a; Bregman and Meyskens, 1983b; Disorbo et al., 1983; Lotan, 1979; Meyskens and Salmon, 1979; Salmon et al., 1983). However, cell populations resistant to pharmacologically relevant concentrations have been observed (Bregman and Meyskens, 1986). In this paper, we utilized the median effect principle (Chou and Talalay, 1984) to measure the strength of the cooperative interaction between (biological modifier) BMF and DFMO in suppressing the proliferation of human melanoma cells. The median effect principle has recently been advanced to quantitate the effects of multiple inhibitors on cellular systems and to provide a measure-

ment of summation effects over the entire dose-effect curve (Chang et al., 1985; Chou and Talalay, 1984). In this paper, we show that human melanoma cells treated with combinations of hormones, 13-cis RA, DEX, and alpha-IFN, and DFMO become irreversibly growth inhibited.

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Abbreviations: BMF, biological modifier; DEX, dexamethasone; DFMO, difluoromethylornithine; FA, fraction affected; FU, fraction unaffected; IFN, human recombinant-interferon; RA, retinoic acid; and 13-cis RA, 13-cis retinoic acid.

Address reprint requests to Marvin D. Bregman, Hipple Cancer Research Center, 4100 S. Kettering Boulevard, Dayton, OH.  
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## MATERIALS AND METHODS

### Materials

13-cis RA was purchased from Eastman Kodak Chemical Company (Rochester, NY), and standards and experimental concentrations were verified by high pressure liquid chromatography. RPMI 1640, fetal calf serum, and gentamicin were purchased from GIBCO (Santa Clara, CA). DEX was obtained from Sigma Chemical Co. (St. Louis, MO) and concentrations measured by UV spectrophotometry (239 nm,  $\epsilon$  14, 900). The other BMFs were generous gifts: alpha-IFN from Dr. P.F. Sorter, Hoffman-La Roche (Nutley, NJ), and DFMO from Dr. P. McCann, Merrell Dow Pharmaceuticals, Inc. (Cincinnati, OH). Priority of these agents was determined by the respective companies and provided to us on a weight basis.

### Establishment and Culture of Melanoma Cell Strains and Line

Human melanoma cell strains were developed from human biopsies as previously described (Bregman and Meyskens, 1983b). Early subcultures were frozen in liquid nitrogen for repetitive experiments. The cells were maintained in monolayer in RPMI 1640 medium supplemented with 10% FBS, glutamine (0.8  $\mu\text{g}/\text{ml}$ ), and gentamicin 10  $\mu\text{g}/\text{ml}$ . All experiments that assessed colony formation with short term human melanoma cell strains were done on cells subcultured less than seven times.

### Soft Agar Assay

Human melanoma cells were plated with the upper 0.3% soft agar layer which contained RPMI 1640 medium supplemented with 10% FBS, glutamine (0.8  $\mu\text{g}/\text{ml}$ ), and gentamicin (10  $\mu\text{g}/\text{ml}$ ). Optimal cell concentrations were chosen for plating and were those in the mid-range of concentrations showing a linear relationship between number of cells plated and number of colonies formed (Meyskens et al., 1983). Exponentially growing monolayer cultures of human melanoma were harvested using 0.25% trypsin. The melanoma cell strains C8146C, C8161, C82-7A, and C83-2CY were plated in the overlayer at a cell concentration of 10,000 cells/plate. In the experiments, the single and two agent combinations were added with the cells to the overlayer. The plates were incubated in a sealed, humidified modular incubator chamber (Phillips-Rothenberg, Inc., Del Mar, CA) gassed with a mixture of 95% air and 5%  $\text{CO}_2$  and placed in a 37°C incubator. The colonies (growth units) from short term melanoma cell strains were counted on day 14. Dose effect growth inhibition curves were generated using growth units of 42 micron diameter as a minimum size cut-off.

### Median Effect Analysis of Dose-Effect Curves

The colony-forming assay data was analyzed by the median effect equation as defined by Chou and Talalay (1984), (fraction affected/fraction unaffected = dose  $D$ /  $D_m^m$ ).  $D_m$  is the  $\text{ID}_{50}$  dose. The dose-effect data was fitted by linear regression analysis of log (fraction affected/

fraction unaffected) versus log (dose). The doses for the combination were chosen from the single agent dose response curves. If a strong interaction occurred, then the experiment had to be re-run using sub-threshold single agent doses in the combination mixture. Each concentration was done in triplicate and each individual determination was used as a data point, instead of the mean. A linear regression coefficient  $> .899$  indicates that the data is explained by the median effect equation. All dose-effect curves fitted by the linear regression method in this study had linear regression coefficients larger than 0.9.

The combination index profile was computer generated following the equations of Chou and Talalay (1984). The average combination index was determined by averaging the combination index values obtained at 0.1, 0.2, 0.4, 0.6, 0.8, and 0.9 fraction affected points.

### Pre-treatment and Ability of Melanoma Cells to Recover Growth in Soft Agar

Five hundred thousand human melanoma cells were seeded in 75  $\text{cm}^2$  flasks (Costar, Cambridge, MA). The various BMFs were added with the media. On day four, the cells, including cells which were floating in the media, were harvested. The cells were washed 3 $\times$  in media, counted, and viability determined by trypan blue. The treated cells were then tested for their ability to continue proliferation in soft agar. Also on day four, the eight day exposure experiments received new media with BMFs. The untreated control flasks had to be split 1:2 into a new flask. The cells were harvested on day eight and plated in soft agar as previously described. The size and number of colonies in the pretreated plates were compared to the control.

## RESULTS

### Combination Index: Strength of Two Agent Interactions Over the Entire Dose-Effect Curve

The increase in growth inhibition seen when the individual hormones were combined with DFMO was measured utilizing the median effect principle. Single agent dose-response curves were generated, along with combination dose-response curves in which the agents were maintained at a constant molar ratio. Only melanoma cells which were responsive to the particular hormone used were tested. This is important for retinoids and glucocorticoids because many human melanoma cells are either partially or completely resistant (Bojar and Fabry, 1985; Meyskens and Salmon, 1979). Most of our melanoma cell lines responded to either  $\alpha$ -IFN or  $\gamma$ -IFN. The combination of  $\alpha$ -IFN and DFMO was tested on three human melanoma cell lines. As shown in Table 1, the average combination index ranged from 0.34 to 0.79. These values are indicative of synergism. The lower the value, the stronger the synergism.  $\gamma$ -IFN + DFMO were tested in combination on the human melanoma line, C83-2cy. The average combination index value for  $\gamma$ -IFN + DFMO was 0.62, which is indicative of synergism. The growth in-

**TABLE 1. Median Effect Analysis of the Melanoma Growth Inhibition Produced by DFMO in Combination with Various Growth Regulators**

Cell	Combination	Average combination index
C8146C	13-Cis RA + DFMO	0.95
C8161	a-IFN + DFMO	0.49
C82-7A	DEX + DFMO	0.75
	a-IFN + DFMO	0.34
C83-2CY	13-Cis RA + DFMO	No Interaction
	a-IFN + DFMO	0.79
	g-IFN + DFMO	0.62
M1RW5	13-Cis RA + DFMO	No Interaction
	DEX + DFMO	No Interaction

hibitory effect on this melanoma line with the gamma-IFN + DFMO combination was stronger than that obtained with alpha-IFN + DFMO.

The DEX-DFMO combination was tested on the weakly glucocorticoid responsive human melanoma cell line C82-7A. The ID<sub>50</sub> for DEX inhibition of colony formation was 1.75  $\mu$ M. The average combination index obtained for the DEX-DFMO combination was 0.75, which shows a stronger than additive interaction. This combination was also tested on a glucocorticoid resistant human melanoma line, M1RW5, and, as expected, the addition of DEX had no effect on the DFMO dose-response curve.

The 13-cis RA-DFMO combination was tested on a retinoid sensitive human melanoma line, C8146C. The ID<sub>50</sub> for colony formation inhibition by 13-cis RA was 5 nM. The average combination index obtained for the RA-DFMO combination was 0.95, which is close to that of true additivity. The 13-cis RA-DFMO combination was tested on two retinoid resistant lines, C83-2cy and M1RW5, and the addition of 13-cis RA did not improve the DFMO dose-response curve.

#### The Ability of Combination DFMO-BMF Treatment to Produce Long-Term Growth Suppression

C8146C cells were pre-treated for four or eight days with individual BMF, combination BMF, DFMO, and DFMO with combination BMF (Table 2). The mono-

layer cells were harvested, washed, and plated in soft agar to measure their ability to express colony-forming growth. C8146C human melanoma cells which were pre-treated with a-IFN at a concentration of 800 units/ml, immediately regained the ability to clone in agar and proliferate. The size of the colonies was almost the same as those obtained from the untreated cells for both the four and eight day pre-exposures. When a-IFN was added simultaneously with the cells to soft agar this concentration caused a 90% reduction in colony formation. Thus these results demonstrate that a-IFN elicited a suppression of melanoma proliferation which is dependent on constant exposure and is rapidly reversible.

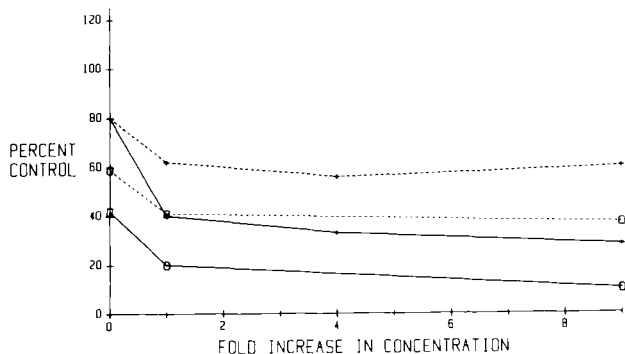
Similar results were obtained with DEX and 13-cis RA. Removal of the BMF before plating in soft agar allowed the C8146C cells to regain their ability to clone in agar and proliferate (Table 2). The addition of DEX, 0.1  $\mu$ M, or 13-cis RA, 0.1  $\mu$ M, with C8146C cells in a soft agar assay caused a 65% and 95% reduction in colony formation. Pre-treatment with DEX, a-IFN and 13-cis RA in combination for four days did not prevent the melanoma cells from regaining their proliferative capacity. The a-IFN-DEX-13-cis RA combination resulted in a 90% reduction in monolayer cell growth. Subsequent plating in soft agar after the treatment showed that the ability of the mixture to control cellular proliferation was transient (Table 2). Adding the a-IFN-DEX-13-cis RA mixture to the cells in soft agar caused a

**TABLE 2. Long-Term Effect of Biological Regulators and DFMO on the Subsequent Growth of C8146C Melanoma Cells in Soft Agar**

Condition	Concentration	Percent control colony size, $\mu$ m		
		> 42	> 60	> 104
<b>Four Days Pre-treatment</b>				
a-IFN	800 units/ml	105	102	103
DEX	0.1 $\mu$ M	84	84	88
13-Cis RA	0.1 $\mu$ M	105	105	105
DFMO	0.1 mM	54	48	32
DEX + a-IFN + 13-Cis RA		110	108	105
DFMO + DEX + a-IFN + 13 Cis RA		14	14	13
<b>Eight Days Pre-treatment</b>				
a-IFN	800 units/ml	89	91	94
DEX	0.1 $\mu$ M	77	77	76
RA	0.1 $\mu$ M	105	105	105
DFMO	0.1 mM	22	19	11
DFMO + DEX + a-IFN + 13 Cis RA		5	4	2

100% inhibition on C8146C colony forming units. Alone or in combination, DEX, a-IFN, and 13-cis RA exerted a contact-dependent cytostatic control of human melanoma cell growth.

Pre-treatment with DFMO resulted in long term suppression on the ability of the C8146C cells to regain their proliferative capacity and clone in agar. The DFMO effect was time dependent. Exposure to a DFMO concentration of 0.1 mM for four days resulted in a 50% decrease in the number of C8146C colonies formed (Table 2). Pre-treatment for eight days resulted in a 78% reduction in the number of C8146 cells that recovered and proliferated in agar. Adding the combined BMF simultaneously with DFMO increased the number of C8146C cells brought under permanent growth control. Exposing C8146C cells to the combination of DEX, a-IFN, 13-cis RA, and DFMO for four days and then plating the washed cells in soft agar resulted in an 86% reduction in the number of colonies formed. The combined four day pre-treatment was more effective than an eight day exposure to DFMO alone. An eight day pre-treatment with DFMO-hormone combination resulted in an almost completely permanent arrest in the ability of the C8146C to recover and demonstrate colony-forming growth. Thus DEX, a-IFN, and 13-cis RA interacted with DFMO to promote the permanent growth inhibition of human melanoma cells. The ability of the BMF + DFMO combination to irreversibly inhibit the growth of C8146C cells was concentration dependent (Fig. 1).



**Fig. 1.** The ability of increasing concentrations of DFMO and DFMO in combination with the hormones to permanently inhibit the colony-forming growth of C8146C human melanoma cells. A dose-effect series was set up by exposing exponentially growing C8146C cells in tissue culture flasks to increasing incremental concentration of DFMO (+ · · +), DEX + a-IFN + 13-cis RA (· · · ·), and DFMO + DEX + a-IFN + 13-cis RA (O—O). The starting concentrations were 0.1 mM for DFMO, 10 nM for DEX, 100 units/ml for a-IFN and 10 nM for 13-cis RA. The combination and single agent concentrations were increased in increments up to 9-fold. The cells were treated for four days and the washed cells plated in soft agar. The viability of the cells was at least 92% at the time of plating in agar. Before plating putrescine (50  $\mu$ M) was added to an aliquot of C8146C cells obtained from the DFMO (+ · · +) and DFMO + DEX + a-IFN + 13-cis RA (O · · · O) treated flasks. The resultant colonies were measured and sized on day 14. 85% of the control colonies grew to at least 86 microns in diameter. All colonies reaching at least 42 microns in diameter were counted.

The irreversibility of DFMO pre-treatment was further verified by plating the pre-treated cells with putrescine, 50  $\mu$ M. The colony-forming growth of C8146C cells was not affected when DFMO and putrescine were added simultaneously (Bregman and Meyskens, 1986). The addition of putrescine to the soft agar media rescued a large number of the pre-treated DFMO cells from permanent growth inhibition (Fig. 1). But even with putrescine rescue, 40% of the C8146C cells pre-treated with DFMO did not regain their ability to clone in agar. BMF + DFMO pre-treated C8146C cells were even less susceptible to putrescine rescue. The removal of BMF + DFMO combination followed by the subsequent addition of putrescine to the soft agar did not rescue over 60% of the colony-forming melanoma cells from permanent growth inhibition.

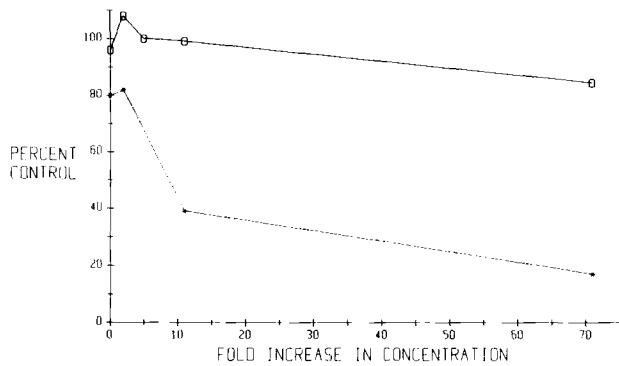
Not all human melanoma cells were susceptible to single agent DFMO pre-treatment. The recovery of growth of C82-7A cells in soft agar was not affected by a four day exposure to either the combined BMF or DFMO, 0.07 to 0.7 mM (Fig. 2). However, the addition of increasing concentration of DEX, a-IFN, and 13-cis RA with DFMO impaired the recovery of growth of C82-7A in a dose dependent manner. Whereas none of the C82-7A colony-forming cells were affected by either DFMO or BMF pre-treatment alone, up to 80% of the C82-7A cells were unable to recover and grow in soft agar assay following the combined DFMO + BMF pre-treatment.

## DISCUSSION

DFMO is a potent inhibitor of murine melanoma growth in vitro and in vivo (Kapyaho, 1985; Sunkara et al., 1983; Sunkara et al., 1985). DEX, a-IFN, and RA in combination synergistically enhanced human melanoma cell sensitivity to growth inhibition by continuous DFMO exposure. In the current study, we analyzed the interaction of each BMF component with DFMO and quantified the strength of their interaction on melanoma cell growth in soft agar.

Synergistic combination indexes were obtained with a-IFN + DFMO, g-IFN + DFMO, and DEX + DFMO. Kovach and Svingen (1985) have reported that a-IFN and DFMO synergistically inhibited the colony-forming growth of human melanoma. There is also a recent report describing only an additive to sub-additive interaction between DFMO and a-IFN on the proliferation of HM7 melanoma cells (Wolf et al., 1985). The degree of interaction may reflect the sensitivity of melanoma cells to a-IFN. In our study, we used interferon sensitive human melanoma cells strains, which have only gone through a limited number of subcultures after isolation from the original biopsy cells, and were able to consistently identify synergism between a-IFN and DFMO.

The cellular kinetic data suggest that these hormones may be modulating one or more enzymes on the polyamine pathway or some other pathway which has a common end-point. We are currently investigating the ability of these BMF to modulate various polyamine-associated metabolite levels.



**Fig. 2.** The ability of increasing concentrations of DFMO and DFMO in combination with DEX + a-IFN + 13-cis RA to permanently inhibit the colony-forming growth of C82-7A human melanoma cells. A dose-effect series was set up. Different groups were exposed to increasing incremental concentrations of DFMO (o-o), and DFMO + a-IFN + DEX + 13-cis RA (★-★). The starting concentrations were 0.01 mM for DFMO, 10 mM for DEX, 35 units for a-IFN and 10 nM for 13-cis RA. The combination and single agent concentrations were increased in increments up to 71-fold. The cells were treated for four days, washed and plated in soft agar. The viability of the cells was at least 95%. Pre-treatment with the three BMFs in combination did not inhibit the subsequent colony-forming ability of C82-7A cells.

The question remained whether melanoma cells treated by exposure to BMF and DFMO in combination were irreversibly growth-inhibited. This was answered by the DFMO pre-treatment experiments in the current study which demonstrated that some human melanoma cells cannot recover from a four day exposure to this polyamine synthesis inhibitor. Some, but not all of these growth-arrested melanoma cells, could be rescued by the subsequent addition of putrescine, which was produced by the DFMO target enzyme ornithine decarboxylase. Together DEX, a-IFN, and 13-cis RA in a dose-dependent manner dramatically increased the percentage of human melanoma cells that were irreversibly growth-inhibited by DFMO. That the permanent cellular damage was due to DFMO and not the combined BMF was shown by the inability of DEX, a-IFN, or 13-cis RA to suppress human melanoma cell growth following their removal from the media. The combination of the three BMFs synergistically enhanced the ability of DFMO to promote the permanent growth-arrest of human melanoma cells. Several groups have recently reported that the pre-treatment of murine melanoma cells with high levels of DFMO (2–2.5 mM) resulted in a marked long term reduction in growth and an induction of the melanogenesis (Kapyaho et al., 1985; Sunkara et al., 1985). In this study we demonstrated that human melanoma cells treated with DEX, a-IFN, and 13-cis RA become permanently growth-arrested in the presence of pharmacologically achievable levels of DFMO.

This study yields further information supporting the use of multiple hormones in combination for the treatment of human melanoma. Since the experimental conditions may not represent long-term and continuous exposure of the melanoma cells to the BMF, these results should be carefully interpreted. On the one hand, true continuous exposure may lead to even better inhi-

bition of cellular growth; however, the exposure of tumor cells in vivo is unlikely to ever be truly optimal. DEX, 13-cis RA, a-IFN, and gamma-interferon each interacted in an additive or better manner with DFMO. Since human melanoma cells are heterogeneous with respect to sensitivity to these hormones (Bregman and Meyskens, 1986; Meyskens and Salmon, 1979), it may be advantageous to use DFMO with several of these agents in combination.

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