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POTENTIATION BY α-DIFLUOROMETHYLORNITHINE OF THE ACTIVITY OF 3,4-DIHYDROXYBENZYLAMINE, A TYROSINASE-DEPENDENT MELANOLYTIC AGENT, AGAINST B16 MELANOMA

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Abstract—Continuous exposure for 96 hr of B16 melanoma cells in culture to 2.5 mM α-difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ornithine decarboxylase, resulted in a marked increase in the activity of the enzyme tyrosinase, and also 20% cell kill as assessed by clonogenic assay. A 4-hr exposure to 0.4 mM 3,4-dihydroxybenzylamine (DHBA), a compound which is melanolytic due to its conversion to a cytotoxic quinone by the tumor specific enzyme tyrosinase, was found to be approximately equitoxic to 2.5 mM DMFO. However, a combination of DFMO (2.5 mM) and DHBA (0.4 mM) produced greater than 95% cell kill. This observed cytotoxicity with the combination suggests that induction of tyrosinase by DFMO sensitizes B16 melanoma cells to the melanolytic activity of DHBA. Oral administration of DFMO to mice bearing subcutaneous B16 melanomas also resulted in marked increases in the activity of tyrosinase in the tumor tissue. In mice inoculated intraperitoneally with 10^5 B16 melanoma cells, administration of DFMO via the drinking water (2%) increased the survival time by 8.5 days, whereas intraperitoneal administration of 300 mg/kg of DHBA for 14 days resulted in an increase in life span of 4.5 days compared to untreated controls. A combination of DFMO and DHBA prolonged the survival time by 14.6 days. These results indicate that DFMO in combination with an appropriate tyrosinase-dependent melanolytic agent might be useful in the chemotherapy of malignant melanomas.

Several catechol compounds, including the naturally occurring levodopa and dopamine, have been shown to possess significant antitumor activity against a variety of experimental tumor cells both *in vitro* and *in vivo* [1–6]. These compounds have received considerable attention in recent years as specific melanolytic agents because of their ability to be accepted as substrates for the enzyme tyrosinase, the activity of which is relatively high in melanoma cells. The preferential cytotoxicity of some of the catechols to melanoma cells has been attributed as being due to the biotransformation of these compounds to their corresponding quinones by the enzyme tyrosinase [7, 8]. Recently, Wick [6] reported that 3,4-dihydroxybenzylamine, a catecholamine, is a tyrosinase-dependent melanolytic agent with a therapeutic effect superior to that of other catechols against B16 melanoma in mice.

Recently, Kapyaho and Janne [9] reported that, in Cloudman S91 melanoma cells grown in culture, depletion of intracellular polyamines by α-difluoromethylornithine (DFMO) is accompanied by increases in the activity of tyrosinase and also the concentration of melanin in these cells. Similar results were reported by Sunkara *et al.* [10] with B16 melanoma cells in culture. These findings suggested the possibility of preferential sensitization of B16 tumor cells to DHBA by DFMO pretreatment to raise the levels of tyrosinase activity in the cells.

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MATERIALS AND METHODS

Chemicals

DFMO was synthesized at the Merrell Dow Research Institute according to published procedures [11]. DHBA was purchased as the hydrobromide salt from the Sigma Chemical Co., St. Louis, MO. All other chemicals were of reagent grade, obtained from MCB Reagents, Gibbstown, NJ.

Cells

B16-F_1 melanoma cells were supplied by Dr. I. J. Fidler, National Cancer Institute, Frederick Cancer Research Center, Frederick, MD. The cells were cultured as monolayers at 37° in Eagle's minimum essential medium (MEM) supplemented with heat-inactivated fetal calf serum (10%), non-essential amino acids, sodium pyruvate, glutamine, and penicillin–streptomycin mixture, in an atmosphere containing 5% CO_2.

In vitro *cytotoxicity*

Cytotoxicity of test compounds and their combinations to B16 melanoma cells in culture was assessed by determining their effects on growth rate and cloning efficiency of the cells. Growth rate was estimated from periodic cell count data. Cell enumeration was done in a Coulter Counter after trypsinization of the cells. Cloning efficiency was evaluated according to the procedure described in detail earlier [12].

In vivo *assays*

Tumor maintenance. B16 melanoma was maintained as a solid tumor in C57/BL mice by subcutaneous inoculation of 10^5 B16 cells into the intrascapular region. On selected days following tumor cell inoculation, the animals were divided into two groups. One group was offered 2% aqueous DFMO as the sole drinking fluid while the control group received only tap water to drink. Animals were killed subsequently at regular intervals, and the tumors were extirpated, weighed, and frozen at −70° for subsequent polyamine and tyrosinase assays.

Antitumor activity. Male C57/BL mice, initial body weight 18–20 g, obtained from Jackson Laboratories, Bar Harbor, ME, were employed for antitumor studies. Animals were inoculated intraperitoneally with 10^5 B16 melanoma cells on day 0, and treatment with test compounds was started on day 1. DFMO was administered orally as a 2% aqueous solution as the sole drinking fluid. DHBA was injected intraperitoneally as an aqueous solution in a volume of 1 ml/100 g body weight. Therapeutic effect was evaluated by comparing the life span of treated groups with the untreated controls. Statistical evaluations were done by the Newman–Keuls multiple range test [13].

Biochemical assays

Tyrosinase assay. The frozen tumor tissue was homogenized in 4 vol. (w/v) of 25 mM Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The homogenates were centrifuged at 25,000 *g* for 30 min. The supernatant fraction was employed for the determination of tyrosinase activity according to the procedure of Pomerantz [14] as modified by Korner and Pawelek [15]. Protein was determined by the method of Bradford [16].

Polyamine assay. The tumor tissues were homogenized in 0.4 M perchloric acid, and the supernatant fractions obtained after centrifugation were used for polyamine determination by dansylation and subsequent thin-layer chromatography as described earlier [12].

RESULTS

Effects of DFMO, DHBA, and DFMO + DHBA on the growth of B16 melanoma cells in culture

Melanoma cells seeded in culture medium containing 2.5 mM DFMO grew at a substantially slower rate than did those cells seeded in drug-free medium (Fig. 1). A significant growth inhibitory effect was discernible after 96 hr of continuous exposure to the drug. Thereafter, the increase in cell number was minimal compared to untreated cells. The effect of 0.4 mM DHBA on cell growth was qualitatively similar to that of DFMO, although complete growth arrest was observed up to day 4 following exposure to the compound. Complete growth arrest followed by a decrease in cell number was observed when the cells were treated with a combination of DFMA and DHBA, indicating actual cell death.

Moderate cytotoxicity (<20% cell kill) could be demonstrated for DFMO (2.5 mM) or DHBA (0.4 mM) alone by the clonogenic assay (Fig. 2).

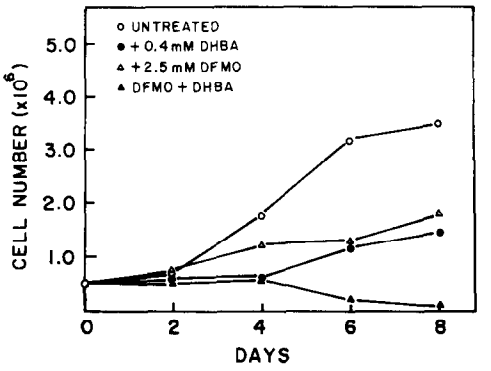

Fig. 1. Effects of DFMO, DHBA and a combination of DFMO and DHBA on the growth of B16 melanoma cells in culture. The cells were grown as monolayers in the presence of the test compounds at the indicated concentrations. Each data point represents the average of two separate experiments. Key: (○) untreated controls; (●) DHBA-treated cells; (△) DFMO-treated cells; and (▲) DFMO + DHBA-treated cells.

However, a combination of DFMO and DHBA was found to be considerably more cytotoxic to these cells. Thus, although 0.1 mM DHBA was only able to bring about a slight reduction in viable cell number, a combination of DFMO and 0.1 mM DHBA produced almost 40% cell kill. Greater than 95% cell kill was observed with a combination of DFMO and 0.4 mM DHBA, indicating substantial potentiation of DHBA cytotoxicity by DFMO.

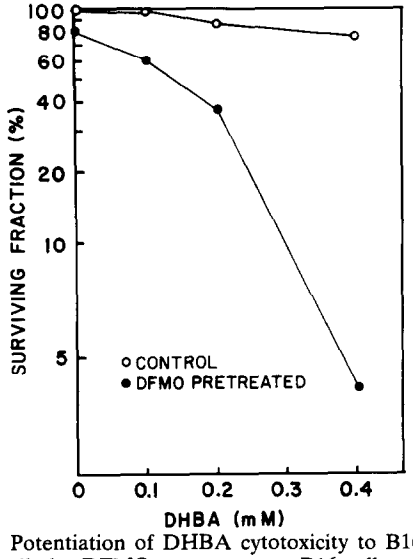

Fig. 2. Potentiation of DHBA cytotoxicity to B16 melanoma cells by DFMO pretreatment. B16 cells grown as monolayers were pretreated with DFMO (2.5 mM) for 96 hr. Both untreated controls and DFMO-treated cells were then exposed to indicated concentrations of DHBA for 4 hr. Thereafter, the cells were washed free of the test compounds. Approximately 200 cells were then plated in 35 mm dishes in regular medium for 10 days, and thereafter the colonies were fixed and counted. The number of colonies observed in the untreated controls was taken to represent 100%. Each data point represents the mean of three separate experiments. Key: (○) surviving fractions of control cells treated with DHBA; and (●) surviving fractions of DFMO + DHBA-treated cells.

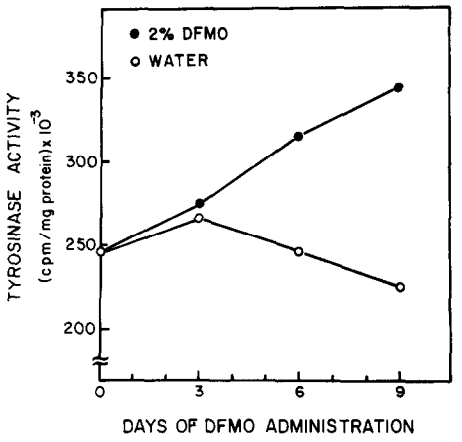

Fig. 3. Tyrosinase activity of subcutaneous solid tumors in mice. Tumor-bearing animals (10^5 cells s.c.) were offered 2% DFMO with drinking fluid beginning on day 14 (day 0 on figure) post tumor inoculation. Control animals were on tap water. Tumors were removed on indicated days, and enzyme activity was determined. Each data point represents the average of two determinations on tumors from two animals. Key: (○) enzyme activity of control tumors; and (●) enzyme activity of tumors from DFMO-treated animals.

Effect of DFMO on solid tumor tyrosinase activity

Tyrosinase activity of solid tumors from control animals remained fairly constant with a trend towards declining activities with increasing age of the tumors (Fig. 3). However, a progressive increase in the activity of tyrosinase was observed with increasing duration of DFMO treatment. The induction of tyrosinase following DFMO treatment was more pronounced if the treatment was started on day 5 after tumor inoculation when the tumor burden was low (Table 1). DFMO treatment also resulted in the expected decrease in the concentrations of putrescine and spermidine with little change in the concentration of spermine in the tumor tissue [17]. A similar decrease in putrescine and spermidine was observed in tumor tissues from animals that received DFMO beginning on day 14 post tumor inoculation (data not presented).

In vivo *antitumor activity*. Animals inoculated i.p. with 10^5 melanoma cells had a mean survival time of 13.6 days (Table 2). Administration of DFMO, beginning on day 1 post tumor inoculation until death, increased the survival time to 22.1 days. Animals that received DHBA at a dose of 300 mg/kg, administered i.p. daily from day 1 through 14, had a mean survival time of 17.6 days. A combination of DHBA and DFMO prolonged survival time to 28.2 days. DFMO or DHBA alone, as well as the combination, was well tolerated by the animals.

DISCUSSION

Results of the present investigation indicate that tyrosinase activity is increased following polyamine depletion in mice bearing solid B16 melanoma tumors, in agreement with earlier findings in melanoma cell cultures [9, 10]. The degree of polyamine depletion in the tumors was comparable to that reported in an earlier study [17]. The tumors from animals treated with DFMO were more highly pigmented on visual inspection than those from controls, indicating enhanced melanogenesis. A similar expression of differentiated function by B16 melanoma cells has been reported following colchicine treatment [18] and exposure to anthracycline antitumor agents [19]. These experimental observations and also the results of several other investigations [20] are strongly indicative of a role for these polycations in the expression of differentiated function. On the other hand, since DFMO has been reported to exert striking inhibitory effects on the growth of subcutaneous B16 melanomas [17], the expression of differentiated biochemical traits in these cells might merely be an effect secondary to growth inhibition.

It seems probable that enhancement of antitumor activity of DHBA by DFMO is due to the increased tyrosinase activity induced by the latter drug. Speci-

Table 1. Effects of DFMO on the tyrosinase activity and polyamine concentrations of subcutaneous solid B16 melanomas in C57/BL mice

Days post inoculation	Treatment	Tyrosinase activity* (cpm/mg protein)	Tumor polyamine concentrations* (nmoles/g wet weight)		
			Putrescine	Spermidine	Spermine
9	Control	277.0 ± 88.0	127.0 ± 28.0	1429.0 ± 324.0	594.0 ± 130.0
	DFMO†	1123.5‡	3.16‡	183.2	487.6
11	Control	307.0 ± 46.0	184.0 ± 43.0	1659 ± 164.0	686.0 ± 105.0
	DFMO	779.0 ± 135.0	1.7 ± 0.6	127.0 ± 25.0	575.0 ± 157.0
15	Control	118.0 ± 61.0	43.0 ± 35.0	1070.0 ± 339.0	554.0 ± 23.0
	DFMO	882.0 ± 162.0	1.1 ± 0.2	82.4 ± 29.0	459.0 ± 68.0
19	DFMO§	666.0 ± 53.0	0.6 ± 0.1	71.5 ± 8.4	472.0 ± 49.0

* Values represent mean ± S.E.M. of determinations on four tumors except for the DFMO group on day 9.
† DFMO was offered as a 2% solution in drinking water beginning on day 5 post tumor inoculum.
‡ Average of two determinations on tumor tissue sample pooled from two animals; only 2/18 animals in the DFMO-treated group had palpable tumors on day 9.
§ Mortality of animals in control group precluded tumor sampling on day 19 for this group.

Table 2. Effects of DHBA, DFMO, and DHBA + DFMO on the survival time of mice inoculated intraperitoneally with 10^5 B16 melanoma cells

Group	Treatment	Survival time in days [mean ± S.E.M. (N)]	% Increase in life span
1	Control	13.6 ± 0.2 (23)	
2	DHBA*	17.6 ± 0.5† (16)	29
3	DFMO‡	22.1 ± 0.4† (23)	63
4	DFMO + DHBA§	28.2 ± 0.5† (16)	107

* Three hundred mg/kg i.p. daily beginning on day 1 post inoculation for 14 days.
† Significantly different from control ($P < 0.001$); groups 2, 3 and 4 are also significantly different from one another ($P < 0.001$).
‡ Two percent in drinking water beginning on day 1 post inoculation.
§ Doses of DHBA and DFMO, respectively, are the same as those for groups 2 and 3.

ficity of this effect of DFMO is suggested by the fact that exogenous putrescine reverses the increase in tyrosinase activity in Cloudman S91 melanoma cells [9]. Thus, it is likely that exogenous polyamines would also inhibit the potentiation of cytotoxicity of DHBA by DFMO in B16 melanoma. Preliminary studies (unpublished data) with B16 amelanotic melanoma, a tumor with negligible tyrosinase activity, indicated no chemotherapeutic advantage with a combination of DFMO and DHBA, indirectly confirming a role for tyrosinase in the increased DHBA cytotoxicity.

Whatever the precise mechanism by which DFMO induces the tyrosinase in B16 melanoma cells, results of the present investigation indicate that the phenomenon can be exploited to achieve enhanced killing of these cells both *in vitro* and *in vivo* in conjunction with a melanolytic agent. Elegant studies by Wick [8] have shown that DHBA *in vitro* is inhibitory to DNA polymerase only when activated by the enzyme tyrosinase. The present *in vitro* cytotoxicity data of DHBA on control and DFMO-treated B16 cells *in vitro* are also in agreement with these earlier experimental observations. The *in vivo* therapeutic effect observed with a combination of DFMO and DHBA reported here was marginally greater than the expected additive effect with respect to these two agents administered alone. However, in view of the low host toxicities of these agents at the doses employed, the therapeutic effect observed *in vivo* with the combination warrants further investigation into the design and search for more potent melanolytic agents capable of providing improved antitumor effects.

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REFERENCES

1. M. M. Wick, L. Byers and E. Frei III, *Science* **197**, 468 (1977).
2. F. W. Knock, R. M. Galt, Y. T. Oester and R. Sylvester, *Oncology* **26**, 515 (1972).
3. W. Chavin, E. J. Jelonek, Jr., A. H. Reed and L. R. Binder, *Science* **208**, 408 (1980).
4. M. M. Wick, *Science* **199**, 775 (1978).
5. M. M. Wick, *J. invest. Derm.* **71**, 163 (1978).
6. M. M. Wick, *J. natn. Cancer Inst.* **63**, 1465 (1972).
7. H. Sugano, I. Sugano, K. Turnbow and T. B. Fitzpatrick, *Cancer Res.* **35**, 3126 (1975).
8. M. M. Wick, *Cancer Res.* **40**, 1414 (1980).
9. K. Kapyaho and J. Janne, *Biochem. biophys. Res. Commun.* **113**, 18 (1983).
10. P. S. Sunkara, C. C. Chang, N. J. Prakash and P. S. Lachmann, *J. Cell. Biol.* **97**, 59a (1983).
11. B. W. Metcalf, P. Bey, C. Danzin, M. J. Jung, P. Casara and J. P. Vevert, *J. Am. chem. Soc.* **100**, 2551 (1978).
12. P. S. Sunkara, N. J. Prakash, C. C. Chang and A. Sjoerdsma, *J. natn. Cancer Inst.* **70**, 505 (1983).
13. B. J. Winer, *Statistical Principles in Experimental Design*, p. 208. McGraw-Hill, New York (1971).
14. S. H. Pomerantz, *Science* **164**, 838 (1973).
15. A. Korner and J. Pawelek, *Nature, Lond.* **267**, 444 (1977).
16. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
17. P. S. Sunkara, N. J. Prakash, G. D. Mayer and A. Sjoerdsma, *Science* **219**, 851 (1983).
18. S. S. Bleehan, *Pigment Cell* **1**, 202 (1973).
19. A. Raz, *J. natn. Cancer Inst.* **68**, 629 (1982).
20. O. Heby, *Differentiation* **19**, 1 (1981).