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Combined Use of α-Difluoromethylornithine and an Inhibitor of S-Adenosylmethionine Decarboxylase in Mice Bearing P388 Leukemia or Lewis Lung Carcinoma

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The antitumor and antimetastatic effects of α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, combined with an inhibitor of S-adenosylmethionine decarboxylase, either methylglyoxal bis(guanylhydrazone) (MGBG) or ethylglyoxal bis(guanylhydrazone) (EGBG), were studied in mice bearing P388 leukemia or Lewis lung carcinoma. Although EGBG is a more specific inhibitor of polyamine biosynthesis than the widely used MGBG, the antitumor effect of the DFMO-EGBG combination on P388 leukemia-bearing mice was less than that of the DFMO-MGBG combination. The prolongation of survival time by the DFMO(1000 mg/kg)-MGBG(25 mg/kg) combination was 2.65-fold, while that of the DFMO(1000 mg/kg)-EGBG(50 mg/kg) combination was 1.34-fold. When mice were fed a polyamine-deficient diet, stronger antitumor effects were exerted; the prolongation of survival time by the DFMO-MGBG and the DFMO-EGBG combinations was 2.89-fold and 2.03-fold, respectively. The antitumor effect of combined use of the two polyamine antimetabolites with mice on normal and polyamine-deficient diets correlated with a decrease of polyamine charge contents in the tumor cells. The above *in vivo* results were confirmed clearly in the KB cell culture system. The antimetastatic activity of DFMO on Lewis lung carcinoma-bearing mice was strengthened by the addition of MGBG or EGBG. The antimetastatic activity of the DFMO-MGBG or DFMO-EGBG combination did not parallel the polyamine charge contents in the primary tumor and blood.

Key words: Polyamine antimetabolites — Antitumor effect — Antimetastatic effect — Polyamine-free diet

Since polyamine biosynthesis is related closely to cell growth, the possibility that inhibitors of polyamine biosynthesis may function as anticancer agents has been examined.[1] Two chemicals, used widely as inhibitors in polyamine biosynthesis, are α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase[2] and methylglyoxal bis(guanylhydrazone) (MGBG), an inhibitor of S-adenosylmethionine decarboxylase.[3] These inhibitors of two different segments of polyamine biosynthesis have exhibited antitumor activity in experimental tumor studies,[4, 5] and the antitumor effect of DFMO has been studied extensively.[1] In addition, the combination of DFMO-MGBG has been shown to be more effective therapeutically than either drug alone in a number of *in vivo* model systems, which include Ehrlich ascites carcinoma,[6] murine L1210 leukemia,[7, 8] murine renal adenocarcinoma,[9] and rat prostate cancer.[10] We have also shown recently that the DFMO-MGBG combination is more effective than either inhibitor alone on human stomach cancer cells xenotransplanted into nude mice.[11] This is probably due to the greater decrease of polyamine charge contents resulting from the combined use of inhibitors of two different segments of polyamine biosynthesis as compared with either inhibitor alone. In this paper, we have compared the effects of the DFMO-MGBG and DFMO-EGBG [ethylglyoxal bis(guanylhydrazone)] combinations on P388 leukemia in mice. We expected that the antitumor effect of the DFMO-EGBG combination would be greater than that of the DFMO-MGBG combination

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since EGBG is known to be a more specific inhibitor of S-adenosylmethionine decarboxylase than MGBG.[12, 13] Furthermore, we have examined how dietary polyamines influence the antitumor effect of the polyamine antimetabolites. Finally, the antimetastatic effect of combined use of the two polyamine antimetabolites has been examined, since it has been reported that DFMO has antimetastatic activities.[14]

Materials and Methods

Determination of Antileukemic Activity of DFMO, MGBG and/or EGBG against P388 Leukemia in Mice P388 murine leukemia cells (1×10^6) were inoculated ip into female CDF_1 mice (7 weeks old) on day 0. The mice were maintained on laboratory chow (MF as normal diet and B as polyamine-deficient diet, Oriental Yeast Co.) and tap water *ad libitum*. The contents of putrescine, spermidine and spermine in normal diet were 125, 325 and 305 nmol/g, respectively, and those in polyamine-deficient diet were 0.5, 12.4 and 9.6 nmol/g, respectively. The inhibitors, DFMO, MGBG and EGBG, were suspended in saline and given daily (ip) in 2 divided doses at about 9 AM and 4 PM from day 1 until death at the following doses: DFMO, 1000 mg/kg/day; MGBG, 25 mg/kg/day; EGBG, 50 mg/kg/day. In some experiments, the MGBG and EGBG doses were changed, as shown in the tables. The DFMO was a gift from Merrell Dow Pharmaceuticals, Inc., MGBG was purchased from Aldrich Chemical, and EGBG was synthesized according to the method of Podorebarac *et al.*[15] The antitumor effect was estimated according to the NCI tumor panel screening.[16]

Determination of Antimetastatic Activity of DFMO, MGBG, and/or EGBG against Lewis Lung Carcinoma in Mice Lewis lung carcinoma cells (5×10^5) were inoculated sc into the footpads of female BDF_1 mice (10 weeks old) on day 0. Inhibitors of polyamine biosynthesis were given daily as described above, and the tumor-bearing foot was amputated on day 12 after tumor inoculation. Administration of DFMO, MGBG and/or EGBG was continued until day 20, and the pulmonary metastasis was determined on day 21 according to the method of Wexler.[17]

Culture of KB Cells KB cells (1×10^4 cells/ml), supplied by the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, were cultured in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co.) with 10% heat-inactivated fetal calf serum (Gibco Oriental) containing gentamycin (80 μg/ml, Schering Co.) in a 5% CO_2 humidified incubator at 37°. The cells were grown with a doubling time of about 19 hr at 37° under the standard conditions. The polyamine antimetabolites were added to the medium at 24 hr after the start of incubation. The concentrations of DFMO, MGBG, and EGBG used were 5m*M*, 8 μM, and 100μM, respectively. After 96 hr, the cells were harvested, washed with phosphate-buffered saline, treated with 0.25% trypsin-0.5m*M* EDTA at 37° for 5 min and collected for the determination of cell number and polyamine contents. Viable cell number was counted in the presence of 0.25% trypan blue.

Measurement of Polyamine Contents The tumor tissues were homogenized in 0.2*N* perchloric acid, and the supernatants obtained after centrifugation were used for polyamine determination. Polyamine levels were determined by high-performance liquid chromatography as described previously.[18] The amounts of polyamines in tumors were expressed as nmol/mg protein. Protein in tumors was determined by the method of Lowry *et al.*[19] on precipitates obtained from the 0.2*N* perchloric acid homogenates. Polyamine charge contents [2 × putrescine (nmol/mg protein) + 3 × spermidine (nmol/mg protein) + 4 × spermine (nmol/mg protein)][20] were used as the index of growth-stimulating activity of polyamines.

Statistical Analysis The statistical significance of differences in tumor weight, polyamine levels and number of pulmonary metastases was assessed by applying Student's *t*-test.

Results

Effect of Polyamine Antimetabolites on P388 Leukemia Cells in Mice As shown in Table I, mice inoculated with 1×10^6 P388 cells survived 10.3 days. Treatment with DFMO (1000 mg/kg) and MGBG (25 mg/kg) extended survival by 1.5 and 5.5 days, respectively, while EGBG (50 mg/kg) shortened the survival time slightly. The combination of DFMO-MGBG or DFMO-EGBG extended the life span by 17.0 and 3.5 days, respectively. This extension is longer than expected for an additive drug interaction.

Although EGBG is a more specific inhibitor of polyamine biosynthesis than the widely used MGBG, the antileukemic activity of the DFMO-EGBG combination was much less than that of the DFMO-MGBG combination. The prolongation of survival time nearly paralleled the decrease of P388 leukemia cells in mice, and there was some correlation between these factors and a decrease of polyamine charge contents in P388 leukemia cells (Table II). The level of spermidine and

Table I. Effect of Combination Therapies with Two Polyamine Antimetabolites in CDF_1 Mice on a Normal or Polyamine-deficient Diet and Inoculated with 1×10^6 P388 Cells

Diet	Treatment	Dose (mg/kg)	Number of mice	Survival time (day)	T/C (%)	Weight gain /4 days (g)
Normal	Control		8	10.3	100	0.5
	DFMO[a)]	1000	8	11.8	115	0.7
	MGBG	25	8	15.8	153	0.6
	EGBG	50	8	9.9	96	0.9
	DFMO	1000	8	19	184	0.2
	MGBG	12.5				
	DFMO	1000	8	27.3	265	0.5
	MGBG	25				
	DFMO	1000	8	12.4	120	0.3
	EGBG	25				
	DFMO	1000	8	13.8	134	0
	EGBG	50				
Polyamine-deficient	Control		8	9.0	100	−0.5
	DFMO	1000	8	12.0	133	0
	MGBG	25	8	17.7	197	−0.3
	EGBG	50	8	10.0	111	0
	DFMO	1000	8	21.3	237	−0.3
	MGBG	12.5				
	DFMO	1000	8	26.0	289	−1.3
	MGBG	25				
	DFMO	1000	8	14.6	162	−0.6
	EGBG	25				
	DFMO	1000	8	18.3	203	−0.9
	EGBG	50				

a) Polyamine antimetabolites were injected intraperitoneally.

Table II. Polyamine Contents in Ascitic P388 Leukemia Cells on the 7th Day after Tumor Inoculation of CDF_1 Mice on a Normal or Polyamine-deficient Diet

Diet	Treatment	Dose (mg/kg)	Wet weight of leukemia cells[a)] (mg)	Polyamines (nmol/mg protein)			Polyamine charge contents
				Putrescine	Spermidine	Spermine	
Normal	Control		652±81[b)]	1.72±0.10[b)]	10.63±0.76	11.08±0.94	79.7
	DFMO	1000	77±9**	1.33±0.04	6.82±0.32**	6.05±0.42*	47.3
	MGBG	25					
	DFMO	1000	343±83**	12.69±0.42**	7.88±0.33**	5.14±0.10**	69.6
	EGBG	50					
Polyamine-deficient	Control		556±81	1.69±0.19	9.65±0.43	8.28±0.23	65.5
	DFMO	1000	52±10**	0.65±0.07**	4.40±0.52**	4.22±0.42**	32.7
	MGBG	25					
	DFMO	1000	117±12**	7.83±0.11**	5.96±0.24**	3.05±0.13**	45.7
	EGBG	50					

a) More than 97% of the ascitic cells were leukemia cells judging from their morphology.
b) Mean±SE; *, $P<0.05$; **, $P<0.01$.

Table III. Effect of Polyamine Antimetabolites on the Growth and Polyamine Contents of KB Cells

Polyamine antimetabolite in medium	Cell number at 96 hr ($\times 10^6$/ml)	Polyamines (nmol/mg protein)			Polyamine charge contents
		Putrescine	Spermidine	Spermine	
None	1.83±0.09[a]	1.71±0.21	6.47±0.21	8.56±0.21	57.1
DFMO (5m*M*)	1.23±0.11	0.01±0.01	0.99±0.05	8.33±0.13	36.3
MGBG (8μM)	1.12±0.10	2.62±0.19	3.37±0.14	5.12±0.13	35.8
EGBG (100μM)	1.36±0.10	3.30±0.10	3.25±0.09	5.83±0.14	39.7
DFMO (5m*M*) MGBG (8μM)	0.16±0.02	0.08±0.01	2.13±0.17	4.46±0.12	24.4
DFMO (5m*M*) EGBG (100μM)	0.23±0.04	0.18±0.03	2.93±0.10	4.88±0.11	28.7

a) The values are means±standard deviations.

spermine in cells from mice treated with the DFMO-MGBG combination was less than that of control mice, and the level of putrescine did not increase in the DFMO-MGBG treated mice. In contrast, the level of putrescine in cells from mice treated with the DFMO-EGBG combination was about 7-fold greater than the level observed in the control mice, even though the level of spermidine and spermine was less than that of control mice. The increase of putrescine may contribute to the reduced effectiveness of the DFMO-EGBG combination in comparison with the DFMO-MGBG combination.

Similar experiments were performed with mice on a polyamine-deficient diet. When mice were fed a polyamine-deficient diet, body weight decreased slightly (Table I). However, the antileukemic acitvity of the DFMO-MGBG and DFMO-EGBG combinations was superior to that in mice on a normal diet. The indices of antileukemic activity of the DFMO-MGBG (1000–12.5 mg/kg) and the DFMO-EGBG (1000–50 mg/kg) combinations increased from 184 and 134 to 237 and 203, respectively, and that of the DFMO-MGBG (1000–25 mg/kg) combination increased slightly from 265 to 289 (Table I). The level of polyamines in P388 leukemia cells from mice on a polyamine-deficient diet was lower than that of mice on a normal diet. It was also observed that the prolongation of survival time nearly paralleled the decrease of P388 leukemia cells in mice and of polyamine charge contents in P388 leukemia cells among this group.

Effect of Polyamine Antimetabolites on the Growth of KB Cells in Culture *In vitro* studies with KB cells have been performed to clarify the situation. As shown in Table III, the combination of two polyamine antimetabolites was more effective than either drug alone, and the DFMO-MGBG combination was more effective than the DFMO-EGBG combination. The DFMO-MGBG combination inhibited the growth of cancer cells by 90%. In addition, inhibition of the growth of KB cells nearly paralleled the decrease of polyamine charge contents.

Effect of Polyamine Antimetabolites on the Pulmonary Metastasis of Lewis Lung Carcinoma Cells Inoculated into the Footpads of Mice As shown in Tables IV and V, DFMO inhibited the growth of Lewis lung carcinoma in the footpad and the spontaneous metastasis into the lung. The level of putrescine and spermidine in Lewis carcinoma cells from mice treated with DFMO was less than that of control mice. The results confirmed the previous report by Sunkara *et al.*[14] When combined therapy was tried, the antimetastatic activity of DFMO increased in proportion to the increase of MGBG (or EGBG), but the amounts of primary tumor and of putrescine and spermidine in the primary tumors increased when larger doses of MGBG or EGBG were used in combination with DFMO. The growth of Lewis carcinoma cells nearly paralleled the increase of polyamine charge contents. No pulmonary metastasis was observed in mice treated with the DFMO-MGBG (1000–50 mg/kg) combination. The

Table IV. Effect of Combination Therapies with Two Polyamine Antimetabolites on the Spontaneous Metastasis of Lewis Lung Carcinoma Cells Inoculated into Footpads of Mice

Treatment	Dose (mg/kg)	Number of mice	Primary tumor (mg)	Incidence of metastasis	Number of pulmonary metastasis		
					Range	Mean±SE	Inhibition (%)
Control		8	586±38[b]	9/9	7–40	19.6±3.3	
DFMO[a]	1000	8	293±24**	7/7	2–11	5.6±1.3**	71
DFMO MGBG	1000 25	8	357±14**	5/8	0–4	1.6±0.5**	92
DFMO MGBG	1000 50	8	481±35	0/7	0	0**	100
DFMO EGBG	1000 50	8	428±38*	6/7	0–17	4.5±2.3**	77
DFMO EGBG	1000 75	8	511±23	5/8	0–7	2.0±1.0**	90

a) Polyamine antimetabolites were injected intraperitoneally.
b) Mean±SE; *, $P<0.05$; **, $P<0.01$.

Table V. Polyamine Contents in Primary Lewis Lung Carcinoma Cells in Footpads on the 12th Day Post-tumor Inoculation

Treatment	Dose (mg/kg)	Polyamines (nmol/mg protein)			Polyamine charge contents
		Putrescine	Spermidine	Spermine	
Control		0.53±0.05[a]	5.88±0.42	6.25±0.24	43.7
DFMO	1000	0.02±0.00**	1.29±0.12**	5.97±0.24	27.7
DFMO MGBG	1000 25	0.44±0.07	1.69±0.18**	6.60±0.30	32.4
DFMO MGBG	1000 50	1.28±0.26	5.64±0.39	5.32±0.23*	40.8
DFMO EGBG	1000 50	1.32±0.18*	3.36±0.20**	5.92±0.28	36.5
DFMO EGBG	1000 75	2.78±0.32**	4.29±0.56*	6.16±0.43	43.1

a) Mean±SE; *, $P<0.05$; **, $P<0.01$.

antimetastatic activity of the DFMO-EGBG combination was less than that of the DFMO-MGBG combination. When MGBG or EGBG was included in the therapy, the level of putrescine and spermidine in blood was higher than that of mice treated with DFMO (data not shown). These results indicate that the combined therapy with polyamine antimetabolites was not effective for inhibiting the cell growth of Lewis carcinoma cells, and that the enhancement of DFMO antimetastatic activity by MGBG (or EGBG) does not correlate with polyamine contents in primary tumors and blood.

Discussion

The experimental data show that the combined use of inhibitors of two different segments of polyamine biosynthesis is more effective than either drug alone in mice bearing P388 leukemia. The antitumor effect of polyamine antimetabolites correlated with the decrease of polyamine charge contents in the tumor cells. The polyamine charge contents were used as the index of growth-stimulating activity of polyamines, because the contents of the three polyamines in cancer cells changed differently and the effective concen-

tration required for the stimulation of macromolecular synthesis is in the order putrescine > spermidine > spermine.[21] However, the DFMO-MGBG combination was less effective than DFMO alone against Lewis lung carcinoma (Table IV). Another exception has been reported in the case of mammary EMT6 tumor-bearing mice.[22] A common characteristic of these two more resistant tumors is the finding that the polyamine charge contents were higher in tumor cells treated with DFMO-MGBG combination than in those treated with DFMO alone. This suggests that growth of tumors is proportional to the polyamine charge contents. Some possible explanations for these observations are as follows: (a) the concentration of MGBG in tumor cells may be low because the site of the tumor is far from the site of injection of MGBG and/or the MGBG transport activity of the tumor cell is low; (b) a low concentration of MGBG may induce synthesis of ornithine decarboxylase and/or S-adenosylmethionine decarboxylase rather than inhibition of the latter enzyme.

In the case of human stomach cancer cells xenotransplanted into nude mice, marked acceleration of tumor growth followed the cessation of the combined DFMO-MGBG therapy.[11] This accelerated growth was closely correlated with an increase of polyamine levels in the tumors.[23] Therefore a combination therapy, composed of polyamine antimetabolites and an antitumor drug with the ability to inhibit induction of ornithine decarboxylase and S-adenosylmethionine decarboxylase, may be a very promising approach. In the literature, there are a number of reports of effective combination therapies, which include polyamine antimetabolites being used with antitumor agents, such as mitomycin C,[11] adriamycin,[24] 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU),[25] and interferon.[26]

It should be noted that the antitumor effect of the DFMO-EGBG combination was less than that of the DFMO-MGBG combination, although EGBG is a more specific inhibitor of polyamine biosynthesis than is MGBG. This may be due to the induction of ornithine decarboxylase by EGBG, as evidenced by the increase of putrescine (Tables II and V). Since MGBG inhibits mitochondrial function[27] and protein synthesis,[28] the induction of ornithine decarboxylase by MGBG may be less than by EGBG.

When mice were fed a polyamine-free diet rather than a normal diet, a stronger antitumor effect of the DFMO-MGBG and DFMO-EGBG combinations was observed. This also supports the proposal that decrease of polyamine charge content in tumors parallels the antitumor effect of polyamine antimetabolites. Dietary control may be helpful for increasing the effect of cancer chemotherapy. In this connection, it should be mentioned that no difference in T/C between the controls of the normal and polyamine-deficient diet groups was observed. Since relatively high polyamine contents existed in the tumor cells of both control groups and the experiments were performed separately with different diets, the results may have been influenced by the difference in experimental conditions.

It has been reported that DFMO has antimetastatic activities.[16] This effect was not only confirmed by our experiments, but also the antimetastatic activity of DFMO was strengthened by the addition of MGBG or EGBG. However, the polyamine charge contents in the primary tumors and blood increased when either MGBG or EGBG was added to the DFMO therapy. This suggests that antimetastatic activity may not parallel the decreasing effect on polyamine charge contents. Recently, there have been reports that platelets may play important roles in cancer metastasis.[29, 30] This is based on the findings that cancer cells induce platelet aggregation and antiplatelet agents inhibit pulmonary metastasis. Thus, the antimetastatic activity of DFMO may be related to this chemical being able to induce thrombocytopenia.[31] In our preliminary experiments, the DFMO-MGBG or DFMO-EGBG combination decreased the number of platelets in mice, but they did not inhibit *in vitro* adenosine diphosphate-induced platelet aggregation (unpublished results). Further studies are necessary to elucidate the mechanism of the strong antimetastatic effect obtained by the combined use of two polyamine antimetabolites.

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