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Synergistic Inhibition of Polyamine Synthesis and Growth by Difluoromethylornithine plus Methylthioadenosine in Methylthioadenosine Phosphorylase-deficient Murine Lymphoma Cells[1]

Hisashi Yamanaka, Masaru Kubota,[2] and Dennis A. Carson[3]

Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California 92037

ABSTRACT

The antiproliferative effects of the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO) are limited by the inability of the compound to deplete completely cellular polyamine pools. 5′-Deoxy-5′-methylthioadenosine (MeSAdo), the purine end product of the polyamine biosynthetic pathway, is an inhibitor of spermine and spermidine synthesis. Furthermore, a substantial number of human tumors are deficient in MeSAdo phosphorylase, and cannot degrade MeSAdo. It therefore seemed possible that DFMO and MeSAdo could interact synergistically to inhibit polyamine synthesis in MeSAdo phosphorylase-deficient malignant cells. To test this hypothesis, we have analyzed the effects of DFMO, in combination with MeSAdo, on polyamine synthesis and growth in a MeSAdo phosphorylase-deficient murine lymphoma cell line (R1.1-H), and a MeSAdo resistant mutant (R1.1-H3). Cultivation of the R1.1-H3 cells in medium containing 250 μM DFMO and 500 μM MeSAdo caused profound depletion of putrescine, spermidine, and spermine, and the accumulation of both decarboxylated *S*-adenosylmethionine and its acetylated derivative to levels that exceeded by nearly 3-fold the total cellular content of *S*-adenosylmethionine. Similarly, DFMO sensitized the lymphoma cells to the growth inhibitory effects of MeSAdo. Supplementation of the medium with putrescine, spermidine, or spermine partially protected R1.1-H3 cells from the DFMO-MeSAdo drug combination. It is conceivable that MeSAdo, or related nucleosides, may potentiate the cytostatic effects of DFMO toward MeSAdo phosphorylase-deficient tumors.

INTRODUCTION

The polyamine biosynthetic pathway represents a potential target for the development of cancer chemotherapeutic agents. All mammalian cells probably require polyamines for optimal growth. The onset of cellular proliferation is preceded by an increase in the activities of ornithine decarboxylase and AdoMet decarboxylase, the rate-limiting enzymes in polyamine synthesis. Compared to quiescent cells, rapidly growing tumors have elevated polyamine pools (1–3).

DFMO[4] is a potent enzyme-activated, irreversible inhibitor of ornithine decarboxylase (4). However, its ability to deplete cellular polyamine pools is limited (5–10). Exposure of cells to DFMO induces an increase in AdoMet decarboxylase activity, with a resultant accumulation of decarboxylated AdoMet (3, 9). In the presence of even small amounts of putrescine, the elevated decarboxylated AdoMet enables cells to synthesize enough polyamines to support growth. Recently, attempts have been made to augment to cytostatic activity of DFMO, by using it together with agents that affect the metabolism of spermidine or spermine (9–11).

MeSAdo is produced stoichiometrically during polyamine synthesis. MeSAdo is a potent endogenous inhibitor of spermine synthase, and can inhibit spermidine synthase as well (12, 13). Many tumor cell lines, and some leukemic cell populations taken directly from patients, are deficient in MeSAdo phosphorylase activity, and cannot metabolize MeSAdo (13–18). Cultivation of the enzyme-deficient tumor cells in MeSAdo-supplemented medium blocks spermine formation, but triggers a compensatory rise in ornithine decarboxylase and AdoMet decarboxylase (14, 18). Consequently, MeSAdo treatment is unable to completely deplete polyamine pools.

Considering the independent inhibitory effects of MeSAdo and DFMO on the polyamine biosynthetic pathway, it seemed possible that the two agents might act synergistically to block polyamine formation. In the present experiments, we have analyzed the effects of MeSAdo in combination with DFMO on polyamine synthesis in murine lymphoma cells that are either sensitive or resistant to the antiproliferative effects of exogenous MeSAdo. DFMO and MeSAdo synergized to cause profound polyamine depletion and growth inhibition. In lymphoma cells exposed to the DFMO plus MeSAdo combination, the acetylated derivative of decarboxylated AdoMet also accumulated to levels that exceeded by nearly 3-fold the entire cellular AdoMet content.

MATERIALS AND METHODS

Cell Lines. The R1.1-H murine T-lymphoma cell line is deficient in MeSAdo phosphorylase (17, 18). A secondary mutant, deficient in MeSAdo phosphorylase and resistant to MeSAdo (designated R1.1-H5), was selected and characterized as described previously (18). Another MeSAdo resistant clone, R1.1-H3, was selected at the same time as R1.1-H5. Both mutants were able to grow indefinitely in medium containing 500 μM MeSAdo. The R1.1-H3 mutant had a more stable phenotype than R1.1-H5 and was used for the present experiments (19). Unless otherwise stated, all cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 100 μM nonessential amino acids (complete medium; MA Bioproducts, Walkersville, MD) in a humidified atmosphere of 5% CO_2 in air. The heat-inactivated horse serum lacked detectable MeSAdo phosphorylase or polyamine oxidase activities (17). The population doubling time of the lymphoma cell lines was approximately 12–16 h (18, 19).

To determine the effects of MeSAdo and DFMO on growth, the lymphoma cells were suspended at a density of 1×10^5 cells/ml in complete medium, supplemented with MeSAdo, DFMO, or polyamines at the indicated concentrations. After 3 days culture, live cells were enumerated microscopically after the addition of 0.15% erythrosin B.

Measurement of Polyamines, AdoMet and Its Derivatives. R1.1-H3 cells were incubated with various concentrations of DFMO and Me-

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[2] Present address: Department of Pediatrics, Kyoto University Hospital, Shogoin, Sakyo-Ku, Kyoto 606, Japan.

[3] To whom requests for reprints should be addressed, at Dept. of Basic and Clinical Research (BCR4), Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037.

[4] The abbreviations used are: DFMO, α-difluoromethylornithine; MeSAdo, 5′-deoxy-5′-methylthioadenosine; AdoMet, *S*-adenosylmethionine; HPLC, high-performance liquid chromatography.

SAdo in medium containing 10% dialyzed, heat-inactivated horse serum. At intervals thereafter, aliquots containing 1×10^6 cells were collected by centrifugation ($400 \times g$, 5 min, 4°C), and were washed with ice-cold phosphate-buffered saline. Polyamines, AdoMet and its derivatives were extracted from cells with 0.4 N perchloric acid, followed by neutralization, as described previously (18). Putrescine and polyamine concentrations were measured fluorometrically using *o*-phthalaldehyde, after separation by reversed-phase ion-pair HPLC (20). AdoMet and its derivatives were also quantitated after separation by reversed-phase ion-pair HPLC (21). Since authentic acetylated decarboxylated AdoMet was not available, the concentration of the compound was estimated utilizing the standard absorbance curve for AdoMet as described by Pegg *et al.* (22).

Isotope Incorporation Studies. The incorporation of [^{3}H]ornithine into putrescine and polyamines was measured as previously described (18). Briefly, cells were incubated with 3 μCi/ml of L-[^{3}H]ornithine (s. a., 50 Ci/mmol; ICN, Irvine, CA) for the indicated time periods. Perchloric acid-extracted samples were fractionated by HPLC as described above, and the radioactivity in each fraction was measured by liquid scintillation spectrometry.

To label the acetylated derivative of decarboxylated AdoMet, R1.1-H3 cells were incubated for 16 h with either 5 μCi/ml of [2(*n*)-^{3}H]-methionine (s. a., 47 Ci/mmol; Amersham, Arlington Heights, IL) or with 12.5 μCi/ml of [^{3}H]acetate (s. a., 6 Ci/mmol; ICN, Irvine, CA), in medium supplemented with 500 μM MeSAdo. Methionine-free RPMI 1640 medium was used when the lymphoma cells were labeled with [^{3}H]methionine. DFMO (250 μM) was included in the medium during incubation with [^{3}H]acetate to prevent labeling of polyamines by the products of [^{3}H]acetate metabolism via the tricarboxylic acid and urea cycles.

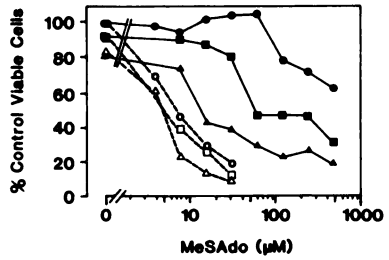

Fig. 1. Effect of DFMO on the growth inhibitory effect of MeSAdo. R1.1-H cells (*open symbols*) and R1.1-H3 cells (*closed symbols*), each at an initial density of 10^5 cells/ml, were cultured with 100 μM DFMO (□, ■), 250 μM DFMO (△, ▲) or without DFMO (○, ●) in the indicated concentrations of MeSAdo. After 72 h, viable cells were enumerated, and compared to control cultures that lacked both MeSAdo and DFMO. The final density in control cultures was 1.8×10^6 cells/ml. The percentage of control growth after exposure to 100 or 250 μM DFMO was 92 or 86% for R1.1-H, and 94 or 80% for R1.1-H3.

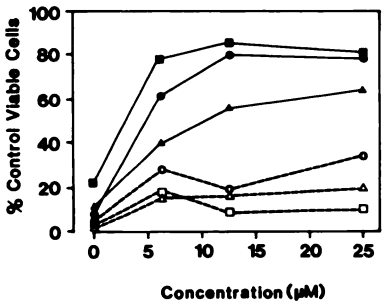

Fig. 2. Prevention of the growth inhibitory effect of MeSAdo plus DFMO by exogenous polyamines. R1.1-H cells (*open symbols*) and R1.1-H3 cells (*closed symbols*) were cultured with 100 μM MeSAdo and 250 μM DFMO in medium supplemented with the indicated concentrations of putrescine (○, ●), spermidine (□, ■) or spermine (△, ▲). After 72 h, viable cell numbers in the polyamine, MeSAdo, and DFMO-treated cultures were compared to control cultures that lacked both MeSAdo and DFMO.

RESULTS AND DISCUSSION

Exogenous MeSAdo at concentrations between 1 and 100 μM progressively inhibited the growth of the R1.1-H lymphoma cells (17, 18). Approximately 5 μM MeSAdo blocked their proliferation by 50% (Fig. 1). MeSAdo had much less effect on the proliferation of the R1.1-H3 mutant. The R1.1-H3 lymphoma cells were not defective in MeSAdo transport, since the nucleoside inhibited spermine biosynthesis and induced the accumulation of decarboxylated AdoMet. 250 μM DFMO caused only 20% inhibition of R1.1-H3 cell proliferation during 3 days culture. However, when R1.1-H3 cells were incubated with 100 μM or 250 μM DFMO, they became much more sensitive to MeSAdo toxicity (Fig. 1). In medium containing 250 μM DFMO, 10–15 μM MeSAdo blocked R1.1-H3 growth by 50%. In medium with 100 μM DFMO, 50 μM MeSAdo had a similar inhibitory effect. Supplementation of the medium with putrescine or polyamines partially protected R1.1-H3 cells from the cytostatic effect of the DFMO-MeSAdo drug combination (Fig. 2). These data suggest (*a*) that MeSAdo and DFMO acted synergistically to inhibit cell growth and (*b*) that the antiproliferative effects of the drug combination were related to depletion of polyamines.

Consistent with experiments in other systems (5, 9, 10), exposure of MeSAdo phosphorylase-deficient R1.1-H3 cells to DFMO caused putrescine and spermidine pools to decline, but had little effect on spermine content (Table 1). However, when 250 μM DFMO was included with 500 μM MeSAdo, the putrescine and spermine pools were depleted by more than 90%, and the spermidine pool by 70%. Thus, the DFMO-MeSAdo combination led to nearly total depletion of intracellular polyamines.

[^{3}H]Ornithine incorporation into polyamines was examined to show that the depletion of polyamine pools was related to a decreased synthetic rate. Previously, we reported that exogenous MeSAdo blocked for only 3–6 h the incorporation of [^{3}H]-ornithine into spermine in R1.1-H cells (18). The reinitiation

Table 1 *Putrescine and polyamine levels in R1.1-H3 lymphoma cells exposed to MeSAdo plus DFMO*[a]

R1.1-H3 lymphoma cells were cultured for 48 or 72 h at an initial cell density of 10^5 per ml in medium containing 10% dialyzed heat-inactivated horse serum and the indicated concentrations of MeSAdo, with or without 250 μM DFMO. Intracellular putrescine and polyamines were determined after derivatization with *o*-phthalaldehyde, described in "Materials and Methods."

	Putrescine		Spermidine		Spermine	
Treatment	None	DFMO	None	DFMO	None	DFMO
48 h						
None	0.60 ± 0.15	<0.10	2.71 ± 0.24	0.35 ± 0.10	0.68 ± 0.11	0.69 ± 0.12
MeSAdo (100 μM)	2.93 ± 0.21	<0.10	2.85 ± 0.66	0.75 ± 0.04	0.44 ± 0.12	0.35 ± 0.07
MeSAdo (500 μM)	4.41 ± 0.48	<0.10	2.24 ± 0.17	0.77 ± 0.14	0.34 ± 0.07	<0.10
72 h						
None	0.51 ± 0.33	<0.10	2.09 ± 0.26	0.16 ± 0.15	0.65 ± 0.08	0.61 ± 0.35
MeSAdo (100 μM)	1.88 ± 0.84	<0.10	2.40 ± 0.24	0.77 ± 0.12	0.40 ± 0.07	0.61 ± 0.27
MeSAdo (500 μM)	3.49 ± 0.46	<0.10	1.98 ± 0.24	0.63 ± 0.12	0.30 ± 0.04	<0.10

[a] Results expressed as nmol/10^6 viable cells ± SD (average of 3–6 different estimations).

of spermine synthesis was related to an increase in decarboxylated AdoMet levels (18). The same phenomenon was observed in the MeSAdo-resistant R1.1-H3 cells, when cultured with exogenous MeSAdo alone (Fig. 3). However, addition of DFMO together with MeSAdo totally prevented the resumption of spermine synthesis (Fig. 3). Indeed the combination of MeSAdo and DFMO, at cytostatic concentrations, inhibited the synthesis of all polyamines.

Decarboxylated AdoMet accumulated in the MeSAdo-treated lymphoma cells, in association with an induction of AdoMet decarboxylase activity (18), and a drop in AdoMet utilization for polyamine synthesis (14). Recently, Pegg and coworkers showed that the acetylated derivative of decarboxylated AdoMet was formed following treatment of SV-3T3 cells with DFMO (22). Fractionation by HPLC of extracts prepared from the MeSAdo-treated lymphoma cells revealed a major new UV absorbance peak, with a relative mobility similar to that reported for acetylated decarboxylated AdoMet (Fig. 4). Both decarboxylated AdoMet and the new peak were radiolabeled following incubation of R1.1-H3 lymphoma cells with [2(*n*)-^{3}H]methionine in MeSAdo-supplemented medium. In contrast, when the lymphoma cells were exposed to [^{3}H]acetate, in the presence of MeSAdo and DFMO, radioactivity was incorporated mainly into the new peak (Fig. 4). Time-course studies showed that the increase in decarboxylated AdoMet levels occurred within 3 h, and preceded the formation of the new metabolite (Fig. 5). Collectively, these results suggest that the unidentified compound that accumulated following MeSAdo-DFMO treatment was acetylated decarboxylated AdoMet.

Exposure of the R1.1-H3 lymphoma cells to the combination of MeSAdo plus DFMO engendered a massive increase in decarboxylated AdoMet and its acetylated metabolite to 140 pmol/10^6 cells each (Fig. 5). In lymphoma cells incubated with the DFMO-MeSAdo drug combination, the approximate cellular content of acetylated decarboxylated AdoMet was almost 3-fold greater than the AdoMet pool (50 pmol/10^6 cells). It is certainly possible that the marked elevation in acetylated decarboxylated AdoMet contributed to the enhanced toxicity toward the lymphoma cells of the DFMO-MeSAdo drug combination. The experiments of Pegg *et al.* suggested that histone acetylase was a principle enzyme catalyzing the formation of acetylated decarboxylated AdoMet, and that high concentrations of decarboxylated AdoMet impaired histone acetylation (22, 23). Histone acetylation is thought to be important in the maintenance of chromatin structure (24–26). Polyamine deprivation has been reported to cause chromosomal aberrations (27, 28). In cells depleted of polyamines, changes in histone acetylation patterns might have an enhanced effect on cell growth regulation.

In addition to MeSAdo, several 5′-modified derivatives of adenosine recently have been shown to inhibit spermine synthase *in vitro* (29). However, the most potent of the enzyme inhibitors, *S*-methyl-MeSAdo, did not cause sustained spermine depletion in intact cells, even in medium supplemented with

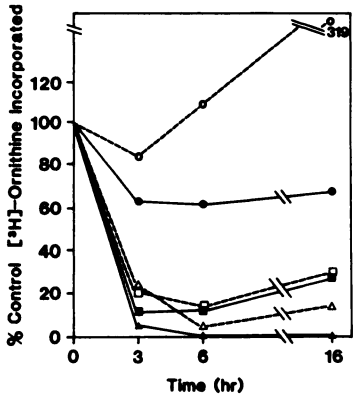

Fig. 3. Inhibition of polyamine synthesis by MeSAdo plus DFMO. R1.1-H3 cells (5 × 10^5 cells/ml) were incubated with [^{3}H]ornithine (3 μCi/ml) to label polyamine pools. In some cases, 500 μM MeSAdo (*open symbols*) or 500 μM MeSAdo plus 1 mM DFMO (*closed symbols*) were included in the culture medium. At the indicated time points, the radioactive counts incorporated into putrescine (○, ●), spermidine (□, ■) and spermine (△, ▲) were compared to control samples which lacked MeSAdo or DFMO. In control cultures incubated for 16 h with [^{3}H]ornithine, the incorporated counts were: putrescine 37,182 cpm, spermidine 76,740 cpm and spermine 4,898 cpm per 5 × 10^6 cells.

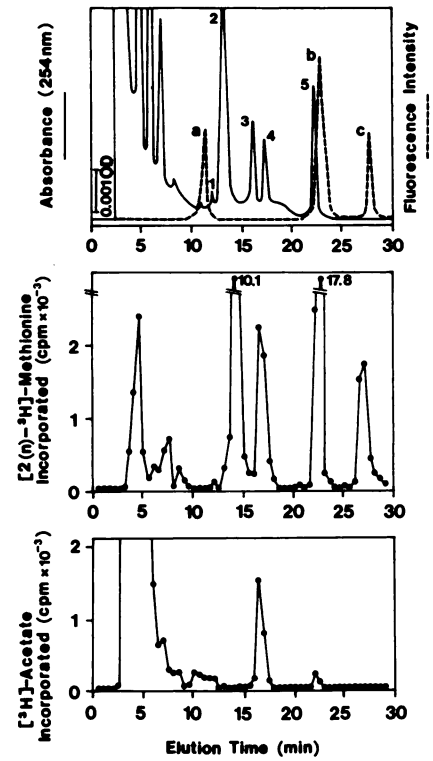

Fig. 4. Identification of acetylated decarboxylated AdoMet. R1.1-H3 cells were labeled with [2(*n*)-^{3}H]methionine (*middle*) or [^{3}H]acetate (*bottom*) in medium supplemented with 500 μM MeSAdo for 16 h. 1 mM DFMO was included during incubation with [^{3}H]acetate to prevent the labeling of polyamines by the products of [^{3}H]acetate metabolism. Acid-extractable radioactivity was fractionated by reversed-phase ion-pair HPLC and was compared with the elution profiles of UV absorbance (254 nm, *solid line*) and fluorescence intensity (*broken line*) shown at *top*. The peaks are *1*, *S*-adenosylhomocysteine; *2*, *S*-adenosylmethionine; *3*, acetylated decarboxylated *S*-adenosylmethionine; *4*, methylthioadenosine; *5*, decarboxylated *S*-adenosylmethionine; *a*, putrescine; *b*, spermidine; *c*, spermine.

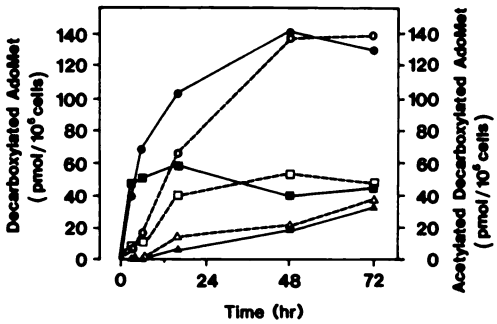

Fig. 5. Accumulation of decarboxylated AdoMet and acetylated decarboxylated AdoMet by MeSAdo and DFMO. R1.1-H3 cells were cultured with 250 μM DFMO (△, ▲), 500 μM MeSAdo (□, ■), or 250 μM DFMO plus 500 μM MeSAdo (○, ●). Decarboxylated AdoMet (*closed symbols*) and acetylated decarboxylated AdoMet (*open symbols*) were measured at the indicated times. In the control cultures without MeSAdo and DFMO, neither decarboxylated AdoMet nor acetylated decarboxylated AdoMet were detected.

DFMO (9, 29). Perhaps the greater efficacy (on a per cell basis) of MeSAdo is attributable to better cellular penetration, and to more profound impairment of spermine formation.

The practical utility of the MeSAdo-DFMO drug combination for the chemotherapy of MeSAdo phosphorylase-deficient neoplasms may be limited by the other potentially toxic effects of the thioether nucleoside (13, 19, 30). For instance, recent experiments indicate that MeSAdo may inhibit AdoMet-dependent aminocarboxypropyl transfer reactions that are involved in the posttranslational modification of elongation factor 2 (19), and perhaps of transfer RNA (30). Circumstantial evidence suggests that MeSAdo may interact with adenosine receptors on the plasma membrane (31). However, it is noteworthy that the amount of DFMO required to deplete polyamine pools in MeSAdo-supplemented medium was much lower than the concentrations that had been used in previous investigations (5, 9, 10). This finding encourages the search for other adenosine analogues that are potent and specific inhibitors of spermine and spermidine synthesis.

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