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# Selective inhibition of spermidine biosynthesis and differentiation by *S*-adenosyl-1,8-diamino-3-thiooctane in murine erythroleukemia cells

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The low molecular weight aliphatic polyamines putrescine, spermidine and spermine are required for optimal cell growth [1] and may be involved in differentiation [2]. In mammalian cells, the rate-limiting step in polyamine metabolism is the synthesis of putrescine by the enzyme ornithine decarboxylase (EC 4.1.1.17). Spermidine synthase (putrescine aminopropyltransferase, EC 2.5.1.16) catalyzes the synthesis of spermidine and 5′-methylthioadenosine from putrescine and decarboxylated *S*-adenosylmethionine (AdoMet). Similarly, spermine synthase (spermidine aminopropyltransferase, EC 2.5.1.22) requires spermidine and decarboxylated AdoMet to synthesize spermine [1].

The inhibition of ornithine decarboxylase by α-difluoromethylornithine (DFMO) inhibits putrescine biosynthesis, and consequently cell growth and replication [3]. Previous studies have demonstrated that the induction of erythroid differentiation in murine erythroleukemia (MEL) cells by dimethyl sulfoxide (DMSO), hexamethylene bisacetamide and butyric acid requires the presence of intracellular spermidine [4]. Furthermore, there is a highly significant relationship between intracellular spermidine levels and MEL cell proliferation. The depletion of spermidine by DFMO also inhibits the induction of heme synthesis and globin RNA [5]. However, the requirement for spermidine in differentiation may indirectly reflect the need for this polyamine in proliferation.

The design and synthesis of the compound *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO) [6, 7], a transition-state analog and potent inhibitor of spermidine synthase, have allowed the study of cellular systems devoid of spermidine. This agent has little, if any, inhibitory effect on the related enzyme spermine synthase. Metabolic studies have demonstrated that AdoDATO is transported in Novikoff hepatoma cells and that there is no metabolism of this inhibitor for up to 6 hr [8]. Incubation of AdoDATO with transformed mouse 3T3 fibroblasts or rat HTC hepatoma cells has demonstrated a dramatic reduction of cell growth and spermidine levels which is associated with an increase in intracellular putrescine and spermine [9].

The present paper describes the effects of the spermidine synthase inhibitor AdoDATO on cell growth and induction of differentiation in MEL cells.

## *Materials and methods*

*Cell culture and chemicals.* The MEL cell line PC4 (provided by Dr. D. Housman, Massachusetts Institute of Technology, Cambridge, MA) was grown in continuous suspension culture in α-Minimum Essential Medium (α-MEM) (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin, 100 units/ml streptomycin and 2 mM L-glutamine at 37° in a humidified 5% $CO_2$ atmosphere. Cell density was maintained between $2 \times 10^4$ and $5 \times 10^5$ cells/ml for continuous logarithmic growth. AdoDATO was synthesized as previously described [7], dissolved in phosphate-buffered saline (PBS) and the pH adjusted to 7.2–7.4 by the dropwise addition of HCl. MEL cells were grown in suspension culture in the presence of various concentrations of AdoDATO, 1.25% DMSO (Fisher Scientific, Boston, MA) and putrescine, spermidine or spermine (Sigma, St. Louis, MO). Cultures were scored for benzidine positive cells by the wet benzidine method [10], and heme concentration was determined utilizing a Perkin–Elmer MPF-4 fluorescence spectrophotometer [11]. Viability was monitored by trypan blue exclusion.

*Polyamine determination.* MEL cells ($2 \times 10^6$) were harvested and washed twice with PBS. Polyamines were extracted in 4% sulfosalicylic acid and measured by cation-exchange chromatography using a Beckman 121MB automated amino acid analyzer as previously described [4].

*Northern blot analysis.* MEL cytoplasmic RNA was isolated as described [12]. Cells were washed three times with ice-cold PBS and resuspended in ice-cold lysis buffer [0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–Cl (pH 8.6), 0.5% NP-40, 10 mM vanadyl–ribonucleotide complexes]. The lysates were layered over an equal volume of lysis buffer containing sucrose (24%, w/v) and NP-40 (1%), and centrifuged for 15 min in an Eppendorf microcentrifuge; the cytoplasmic layer was then collected. After proteinase K digestion, the cytoplasmic RNA was extracted with phenol/chloroform and precipitated with ethanol.

The RNA (15 μg) was dissolved in 40% deionized formamide, 2.2 M formaldehyde, 40 mM 3-(*N*-morpholino)-propane sulfonic acid (MOPS), 10 mM sodium acetate, and 1 mM EDTA. Samples were heated at 55° for 15 min and then quickly chilled at 0°. Gel electrophoresis was performed in 1% agarose containing 2.2 M formaldehyde, 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, and 0.5 μg/ml ethidium bromide at 50 V for 20 hr. The gel was then washed and transferred onto a nitrocellulose filter [13].

The filters were prehybridized at 42° for 18 hr in buffer consisting of 50% formamide, 5x SSC (1x SSC: 0.15 M sodium chloride, 0.015 M trisodium citrate), 0.1% SDS, 1x Denhardt's solution and denatured salmon sperm DNA (200 μg/ml). The RNA blots were then hybridized at 42° for 24 hr in the same buffer with nick-translated $^{32}P$-labeled globin DNA probe from plasmid pBR325 containing a mouse β-major globin DNA [14] (provided by Dr. P. Leder, Harvard Medical School, Boston, MA). The filters were washed twice with 2x SSC and 0.1% SDS at room temperature, and twice with 0.1x SSC and 0.1% SDS at 50°. The filters were exposed to X-ray film (Kodak X-Omat XAR-5) for 24 hr at −70° using an intensifying screen.

## *Results*

The effects of various concentrations of AdoDATO on MEL cell growth are shown in Fig. 1. AdoDATO concentrations from 50 to 200 μM produced a dose-dependent inhibition of cell proliferation, while lower concentrations had no detectable effect. A complete inhibition of proliferation occurred after 72 hr of exposure to 125 μM AdoDATO. These effects on growth were not associated with a significant loss of viability. Moreover, similar results were obtained with AdoDATO alone or in combination with DMSO.

The effects of AdoDATO on polyamine synthesis in MEL cells are shown in Fig. 2. Treatment of MEL cells with 125 μM AdoDATO for 24 hr resulted in a 2.9-fold increase in putrescine levels, a 95% decrease in spermidine, and a 1.6-fold increase in spermine. Similar patterns with decreased spermidine levels were observed during 96 hr. Furthermore, the addition of DMSO to untreated or Ado-

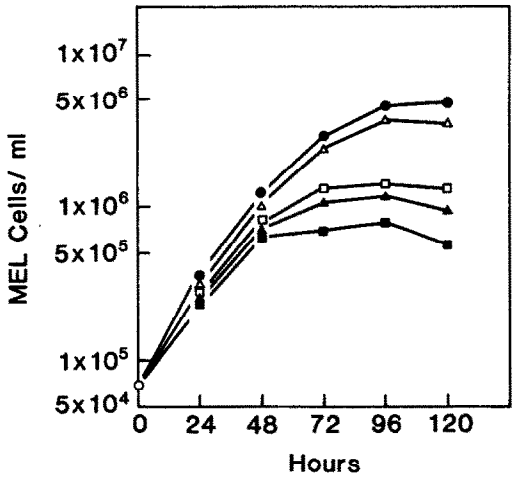


Fig. 1. Effects of various concentrations of AdoDATO on MEL cell growth. MEL cells ($7 \times 10^4$ cells/ml) were treated with various concentrations of AdoDATO and 1.25% DMSO. Key: control MEL cells (●), 50 μM AdoDATO (△), 100 μM AdoDATO (□), 125 μM AdoDATO (▲), and 200 μM AdoDATO (■).

DATO-treated cells did not alter significantly the polyamine levels.

We also monitored the effects of AdoDATO on the induction of MEL differentiation by DMSO. The concurrent addition of 100 μM AdoDATO and DMSO to MEL cells only partially inhibited the appearance of benzidine positive cells. After 96 hr, DMSO-treated cells were 71.7% benzidine-positive and AdoDATO/DMSO-treated cells were 47.4% benzidine-positive. However, pretreatment of MEL cells with AdoDATO for 24 hr, followed by the addition of DMSO, produced a dose-dependent inhibition of hemoglobin production as listed in Table 1. Under these experimental conditions, AdoDATO (125 μM) resulted in a nearly complete inhibition of heme synthesis (Table 1) and appearance of benzidine positive cells. Although benzidine staining was inhibited completely by 200 μM AdoDATO, cell viability decreased to 71%.

We next examined the effects of adding exogenous polyamines to AdoDATO-treated cells. The results are listed in Table 2. The addition of putrescine had no effect on AdoDATO-mediated inhibition of MEL cell growth or differentiation. In contrast, spermidine and spermine both

Table 1. Dose effects of AdoDATO on induction of MEL differentiation by DMSO

| AdoDATO (μM) | DMSO (%) | Viability (%) | Benzidine positive cells (% control) |
|---|---|---|---|
| 0 | 0 | >95 | 2.0 |
| 0 | 1.25 | >95 | 100 |
| 50 | 1.25 | >95 | 48.3 |
| 100 | 1.25 | 93 | 23.1 |
| 125 | 1.25 | 82 | 2.1 |
| 200 | 1.25 | 71 | <1.0 |

MEL cells in logarithmic phase were seeded at $7 \times 10^4$ cells/ml in the presence of various concentrations of AdoDATO. DMSO was added after 24 hr. Benzidine positive cells were determined 96 hr after the addition of DMSO.

reversed this inhibition in a concentration-dependent manner. Spermidine was relatively more effective, resulting in a nearly complete reversal of the effects of AdoDATO on MEL proliferation and differentiation.

The effects of AdoDATO treatment on the production of globin RNA were monitored by Northern blot analysis (Fig. 3). The treatment of MEL cells with 1.25% DMSO for 72 hr resulted in an increase in the level of cytoplasmic β-globin RNA. This induction of globin RNA was inhibited by pretreatment for 24 hr with 125 μM AdoDATO. Furthermore, these inhibitory effects of 125 μM AdoDATO on globin RNA production were partially abrogated by the addition of 5 μM spermidine.

*Discussion*

The involvement of polyamines in the relationship between proliferation and differentiation is complex. While polyamines are required for optimal cell growth, the importance of polyamines in differentiation is less certain. The polyamine spermidine is required for hormone-dependent milk protein synthesis in cultured mouse mammary tissue [15] and for the differentiation of 3T3-L1 fibroblasts into adipose cells [16]. However, in other systems, the involvement of polyamines may be secondary to their requirements for proliferation.

Previous studies have shown that DNA synthesis is required for erythroid cell precursor differentiation [17–19]. However, other studies have indicated that MEL differentiation may occur in the presence of inhibitors of DNA synthesis [20] and that DNA synthesis [21] or mitosis [22]

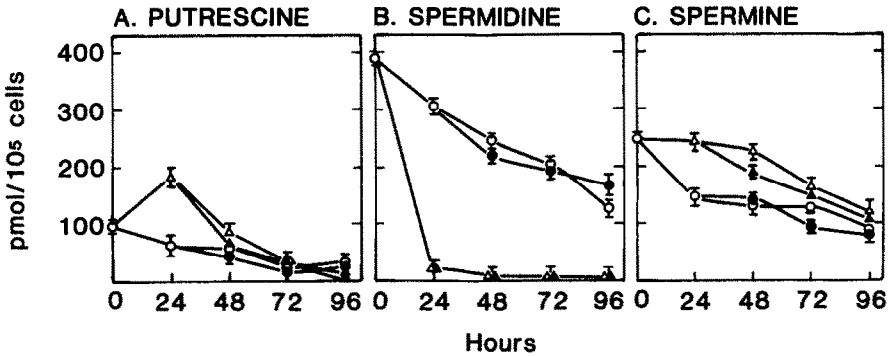


Fig. 2. Effects of AdoDATO on MEL intracellular polyamine levels. MEL cells ($1 \times 10^5$ cells/ml) were treated with 125 μM AdoDATO and 1.25% DMSO added after 24 hr. Control MEL cells (○), DMSO-treated cells (●), AdoDATO-treated cells (△) and AdoDATO/DMSO-treated cells (▲) were monitored for intracellular putrescine (A), spermidine (B), and spermine (C) at the indicated times. Each point represents the mean ± standard deviation of two separate determinations, each performed in duplicate.

Table 2. Effects of exogenous polyamines on AdoDATO-treated and DMSO-induced cells

| AdoDATO ($\mu$M) | Polyamine ($\mu$M) | Cell count ($\times 10^6$/ml) | % Benzidine positive cells | Heme (pmoles/5 $\times$ $10^5$ cells) |
|---|---|---|---|---|
| None | None | 4.0 | 79.9 ± 0.4 | 137.0 ± 6.5 |
| 125 | None | 1.6 | 5.2 ± 0.5 | 41.7 ± 2.1 |
| 125 | Putrescine, 50 | 1.3 | 5.0 ± 0.2 | 25.5 ± 0.7 |
| 125 | Putrescine, 5 | 1.7 | 3.8 ± 0.5 | 39.0 ± 2.2 |
| 125 | Putrescine, 0.5 | 1.7 | 3.2 ± 0.5 | 45.3 ± 0.5 |
| 125 | Spermidine, 50 | 4.4 | 72.8 ± 1.7 | 63.7 ± 4.5 |
| 125 | Spermidine, 5 | 4.3 | 68.1 ± 2.3 | 67.3 ± 1.9 |
| 125 | Spermidine, 0.5 | 3.0 | 39.4 ± 6.4 | 33.1 ± 3.1 |
| 125 | Spermine, 50 | 4.0 | 61.9 ± 1.6 | 88.0 ± 8.5 |
| 125 | Spermine, 5 | 4.0 | 50.0 ± 0.5 | 74.0 ± 1.7 |
| 125 | Spermine, 0.5 | 3.3 | 26.1 ± 0.2 | 38.3 ± 1.7 |

DMSO (1.25%) and polyamines were added 24 hr after treatment with AdoDATO. Cell counts, benzidine staining, and heme determination were performed 96 hr after the addition of DMSO. Values are mean ± S.E., N = 2.

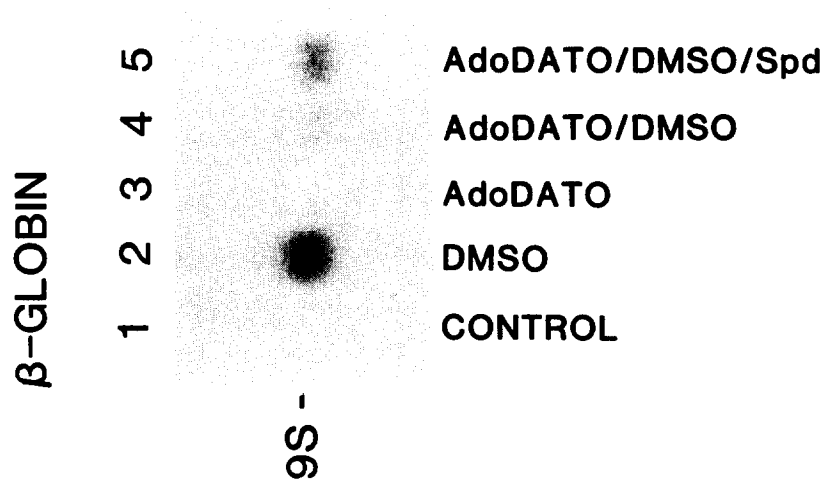


Fig. 3. Northern blot analysis of the effect of AdoDATO on MEL cellular $\beta$-globin RNA production. MEL cells were treated with 1.25% DMSO, 125 $\mu$M AdoDATO, and 5 $\mu$M spermidine. Total cellular RNA was extracted, isolated by agarose-formaldehyde gel electrophoresis, and transferred onto a nitrocellulose filter. Hybridization reactions were performed using a nick-translated $^{32}$P-labeled $\beta$-globin DNA probe. Lane 1, control MEL cells; Lane 2, DMSO-treated cells; Lane 3, AdoDATO-treated cells; Lane 4, AdoDATO/DMSO-treated cells; and Lane 5, AdoDATO/DMSO/Spermidine-treated cells.

is not required for commitment to MEL differentiation. We have demonstrated previously [4] that DFMO-mediated inhibition of MEL cell growth and differentiation is reversed by exogenous spermidine. However, DFMO-treated MEL cells were devoid of both intracellular putrescine and spermidine. The addition of exogenous putrescine with subsequent spermidine synthesis reversed the inhibitory effects of DFMO on MEL cell differentiation. However, the addition of exogenous spermidine allowed the expression of erythroid differentiation in DFMO-treated MEL cells without detectable levels of putrescine. These findings suggested that spermidine, and not putrescine, was required for induction of MEL differentiation.

In the present study, AdoDATO inhibited DMSO-induced MEL differentiation. This effect was associated with spermidine depletion but, in contrast to findings observed with DFMO, AdoDATO increased rather than decreased putrescine levels. Taken together, these findings demonstrate that putrescine alone is neither sufficient nor necessary for the expression of the mature phenotype. Thus, the polyamine spermidine is necessary for MEL differentiation. In other models studied, a different relationship between polyamines and cellular differentiation has been demonstrated. Inhibition of polyamine biosynthesis with DFMO or retinoic acid is associated with the induction of embryonal carcinoma cell differentiation [23, 24]. Depletion of the polyamine putrescine has been implicated with this event [25].

The possible requirement of cellular proliferation for differentiation to occur remains unclear. In our previous study [4], DFMO-treated MEL cells showed cytostasis after 24 hr of drug exposure. However, in the presence of AdoDATO, MEL cells continue to proliferate for 72 hr before cessation of cell growth. In both studies, DFMO and AdoDATO inhibited induction of differentiation. Recent

observations by us and others have shown that the addition of 5'-methylthioadenosine, the co-product of spermidine (and spermine) synthesis, to MEL cells inhibits differentiation without cytostasis [26, 27]. Further studies into the biochemical mechanisms involved in cell growth and gene expression are now necessary to better define these interrelationships.

In summary, the polyamines putrescine, spermidine and spermine are required for optimal cell growth and are implicated in differentiation. We have monitored the effects of AdoDATO, a transition-state analog and potent inhibitor of spermidine synthase, on the proliferation and differentiation of MEL cells. AdoDATO inhibited MEL cell proliferation in a concentration-dependent manner. Treatment of MEL cells with 125 μM AdoDATO for 24 hr resulted in a 2.9-fold increase in putrescine, a 95% decrease in spermidine and a 1.6-fold increase in spermine. The induction of MEL differentiation by dimethyl sulfoxide was inhibited by pretreatment with AdoDATO as monitored by benzidine staining, heme synthesis and globin RNA production. The addition of exogenous spermidine but not putrescine abrogated these inhibitory effects. These findings suggest that spermidine is necessary for MEL cell proliferation and differentiation.

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# Concentrations of the enantiomers of 5-hydroxymethtryptoline in mammalian urine: implications for *in vivo* biosynthesis

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5-Hydroxymethtryptoline (5-HMTLN, Fig. 1) represents the Pictet–Spengler condensation [1] product of serotonin and acetaldehyde and belongs to a class of compounds (β-carbolines) which have long been known to occur naturally in plants [2]. Members of the class have physiological and pharmacological effects in mammalian systems, including inhibition of the monoamine oxidase enzymes [3, 4], inhibition of monoamine reuptake mechanisms [5] and affinity for various monoamine binding sites [6–8]. Because acetaldehyde is a metabolite of ethanol, the presence of 5-HMTLN in mammals was originally implicated in connection with ethanol intake.

Surprisingly, however, 5-HMTLN was found to be a normal constituent of human urine [9], and rat tissues and body fluids [10]. 5-HMTLN exists as two stereoisomers because of the chiral carbon atom in the position which is created in the formation of the tricyclic structure (Fig. 1). Formation from a stereoselective process would be reflected in an unequal abundance of the two enantiomers and could be indicative of the direct participation of an