Cytostasis induced in L1210 murine leukaemia cells by the *S*-adenosyl-L-methionine decarboxylase inhibitor 5'-{[(*Z*)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine may be due to hypusine depletion

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The effects of inhibition of the capacity to form spermidine and spermine on cell growth were investigated using murine leukaemia L1210 cells and 5'-{[(*Z*)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL 73811, AbeAdo), an enzyme-activated irreversible inhibitor of *S*-adenosyl-L-methionine decarboxylase. Putrescine levels were increased 80-fold, and spermidine and spermine levels were greatly reduced after a 3-day exposure to a maximally inhibitory dose of 10 μ M-AbeAdo. Addition of AbeAdo to the culture medium inhibited the growth of L1210 cells measured 3 days later in a dose-dependent manner, but, even at a dose of 10 μ M, which was maximally effective, exposure to AbeAdo was not immediately cytostatic. However, the growth rate of L1210 cells chronically exposed to 10 μ M-AbeAdo declined steadily until day 12, when the cells stopped growing. L1210 cells exposed to AbeAdo for 12 days could not be rescued from cytostasis by removal of the drug from the culture, but could be rescued by exposure to exogenous spermidine or spermine, indicating that the growth-inhibitory effects of AbeAdo were a result of spermidine and/or spermine depletion. It is suggested that elevated intracellular putrescine in AbeAdo-treated cells sustained limited growth in the absence of physiological levels of spermidine and spermine until certain critical and specific physiological role(s) fulfilled by spermidine (and/or spermine) became deficient resulting in cytostasis. *N*-(3-Aminopropyl)-1,4-diamino-*cis*-but-2-ene, a spermidine analogue that is a substrate for deoxyhypusine synthase, was able to mimic the effects of spermidine in reversing AbeAdo-induced cytostasis. Spermidine analogues such as 5,5-dimethylspermidine, which are not substrates for deoxyhypusine synthase, were not active in this way. These results provide evidence that the formation of hypusine in the protein-synthesis initiation factor eIF-5A may be a critical role of spermidine essential for cell growth.

INTRODUCTION

Studies with specific inhibitors of ornithine decarboxylase (ODC), the first enzyme in the polyamine-biosynthetic pathway, and with mutants unable to synthesize polyamines, have demonstrated the importance of polyamines in the initiation and maintenance of mammalian cell growth (Mamont *et al.*, 1978; Steglich & Scheffler, 1982; Pohjanpelto *et al.*, 1985). Inhibitors of polyamine metabolism and polyamine analogues have been examined and found to have therapeutic potential for the treatment of cancer and protozoal infections (Sjoerdsma & Schechter, 1984; Bitonti *et al.*, 1985; Porter & Sufrin, 1986; Bacchi & McCann, 1987; Pegg, 1988; Casero *et al.*, 1989). Although it has been difficult to define specific functions for putrescine, spermidine and spermine, it is known that spermidine is required for formation of hypusine {*N*^ε-[4-amino-2(*R*)-hydroxybutyl]lysine} in the protein-synthesis initiation factor eIF-5A (formally eIF-4D) (Hershey *et al.*, 1990; Park *et al.*, 1991). Hypusine is formed as a post-translational modification of the eIF-5A precursor protein in two reactions catalysed sequentially by deoxyhypusine synthase and deoxyhypusine hydroxylase. The first of these involves the transfer of the 4-aminobutyl group from spermidine to a lysine residue in the eIF-5A precursor protein to produce deoxyhypusine (Park & Wolff, 1988). The second reaction is the hydroxylation of deoxyhypusine to form hypusine (Abbruzzese *et al.*, 1986).

S-Adenosyl-L-methionine decarboxylase (AdoMetDC) catalyses the rate-limiting step in the biosynthesis of spermidine and spermine. Thus AdoMetDC is an important target for inhibitors to affect cellular polyamine content. Although inhibitors of this enzyme have been identified previously (Williams-Ashman & Pegg, 1981; Kolb *et al.*, 1982; Pegg, 1983, 1984a, 1986; Porter & Sufrin, 1986; Pegg & Williams-Ashman, 1987; Pegg *et al.*, 1988), virtually none of these compounds is specific, potent or stable enough to study the regulation of polyamine biosynthesis or the role of the individual natural polyamines in the growth of cultured mammalian cells over prolonged periods. Pegg *et al.* (1988) and Kramer *et al.* (1989) both used recently developed irreversible inhibitors of AdoMetDC to demonstrate that depletion of spermidine and spermine reduces the growth of L1210 murine leukaemia cells in culture. Although the effects of these inhibitors on cell growth and viability were limited, these results, and studies in which inhibitors of spermidine synthase or spermine synthase were used (Ito *et al.*, 1982; Pegg *et al.*, 1982; Pegg & Coward, 1985; Nakashima *et al.*, 1986; Hibasami *et al.*, 1987, 1988), suggest that depletion of polyamines may be antiproliferative and have pharmacological potential. However, in all of the previous studies, the length of time of exposure to the inhibitor was limited. The drug 5'-{[(*Z*)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL 73811, AbeAdo), which is a rationally designed, specific and irreversible inhibitor of AdoMetDC from bacterial, protozoal and mammalian sources

Abbreviations used: AbeAdo, 5'-{[(*Z*)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL 73811); AdoMetDC, *S*-adenosyl-L-methionine decarboxylase; DFMO, α -difluoromethylornithine; eIF-5A, eukaryotic translation initiation factor 5A; ODC, ornithine decarboxylase.

(Casara *et al.*, 1989; Bitonti *et al.*, 1990; Danzin *et al.*, 1990), is a stable compound that is suitable for long-term cell-culture studies.

We now report the use of AbeAdo to study the effects of AdoMetDC inhibition on intracellular polyamine levels and on L1210 cell proliferation. Exposure to AbeAdo blocked the synthesis of spermidine and spermine, and the growth rate of treated cells declined over a 12-day time period, after which the cells no longer grew. This suggests that a physiological role normally filled by these polyamines became limiting after an extended growth in the absence of polyamine synthesis. Evidence is provided using analogues of spermidine differing in ability to take part in the deoxyhypusine synthase reaction that modification of eIF-5A may be this critical function.

MATERIALS AND METHODS

Materials

All biochemical reagents were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. DFMO, MDL 72527 [*N,N'*-bis(buta-2,3-dienyl)butane-1,4-diamine] and AbeAdo were synthesized at Marion Merrell Dow Research Institutes, Cincinnati, OH, U.S.A., and Strasbourg, France (Metcalf *et al.*, 1978; Bey *et al.*, 1985; Casara *et al.*, 1989). Polyamine analogues were synthesized as described previously (Nagarajan & Ganem, 1986, 1987). Purified rat liver polyamine oxidase enzyme was provided by Dr. Alan J. Bitonti, Marion Merrell Dow Research Institute, Cincinnati, OH, U.S.A.

Cell culture

The L1210 cells were obtained from Carl Porter, Roswell Park Memorial Institute, Buffalo, NY, U.S.A. These cells were grown at 37 °C in suspension culture in RPMI-1640 medium containing 10% Nu-Serum (Collaborative Research, Lexington, MA, U.S.A.) in an atmosphere of 5% CO₂ and in the presence of 95% relative humidity. The cells were passaged every 72–96 h. In experiments where spermidine, spermine or spermidine analogues were added to the medium, 1 mM-aminoguanidine was also added to the cultures to prevent the toxic action of exogenous polyamine degradation by serum oxidases.

SV-3T3 cells were grown as adherent cultures in Dulbecco's minimum essential medium supplemented with 3% (v/v) horse serum and 2% (v/v) fetal-calf serum at 37 °C in an atmosphere of 5% CO₂ and in the presence of 95% relative humidity (Pegg, 1984b).

Determination of polyamines

Cells were harvested by centrifugation and washed twice with ice-cold phosphate-buffered saline (137 mM-NaCl/16 mM-Na₂HPO₄/2 mM-KH₂PO₄, pH 7.4). The washed cell pellet was resuspended in 10% (w/v) trichloroacetic acid, the protein precipitate was removed by centrifugation, and the supernatant was filtered (0.22 µm-pore-size filter) before h.p.l.c. analysis for polyamines. Precipitated proteins were dissolved in 0.1 M-NaOH for measurement of protein content. Putrescine, spermidine and spermine were separated and quantified using modifications (Byers & Pegg, 1989; Byers *et al.*, 1991) of the method described by Seiler & Knödgen (1985). Briefly, a reverse-phase h.p.l.c. system was used to separate putrescine, spermidine and spermine and post-column derivatization with *o*-phthalaldehyde and fluorescence detection was used to quantify the polyamines.

Cell-growth experiments

In all cell-growth experiments, the cells were plated at densities such that the cells would not reach saturation before harvest or

passage. Control cells were plated at 5 × 10⁴ cells/ml; cells exposed to AbeAdo before passage were plated at higher densities that depended on the length of exposure. Typically, cells exposed to 10 µM-AbeAdo for 4 days or 8 days were plated at 2 × 10⁵ cells/ml or 5 × 10⁵ cells/ml respectively for continued AbeAdo treatment. In order to determine the effects of a range of AbeAdo concentrations on L1210-cell growth, the cells were plated at 5 × 10⁴ cells/ml in the presence of the drug. After 72 h, the cells were diluted and counted with an electronic cell counter (Coulter Electronics). The effect of the AdoMetDC inhibitor on cell growth was expressed as the percentage of the cell number in control cultures.

In experiments designed to determine the effect of chronic AbeAdo treatment on cell growth, and to determine the ability of spermidine and spermidine analogues to prevent these effects, L1210 cells were plated in the presence of AbeAdo. After 4 days, when a reduction in cell growth was evident, the cells were replated in media containing 1 mM-aminoguanidine in the presence or absence of 10 µM-AbeAdo and spermidine or spermidine analogues. These cells were counted every 24 h and passaged every 96 h throughout the remainder of the experiment. The cells were counted using an electronic cell counter. The content of aminoguanidine, AbeAdo and polyamines in the media was maintained with each passage. The growth of these cells over the periods of days 5–8, 9–12 and 13–16 were expressed as the percentage of cells present on days 5, 9 and 13 respectively.

In order to determine the ability of spermidine, spermine or polyamine analogues to reverse the cytostatic effects of AbeAdo, L1210 cells were exposed to 10 µM-AbeAdo for 12 days to induce cytostasis. On day 12, the cells were replated in the presence of media containing 1 mM-aminoguanidine in the presence and absence of 10 µM-AbeAdo and polyamines and polyamine analogues as indicated in the Figure legends. The cells were counted every 24 h for the next 5 days, and growth over this time period was expressed as the percentage of cells present on day 13.

Metabolism of AbeAdo

AbeAdo (50 µM) was incubated for up to 8 h with 25 mg of purified hog kidney diamine oxidase (Sigma)/ml or 7 mg of purified rat liver polyamine oxidase/ml in a 10 mM-Tris buffer, pH 7.5, in the presence or absence of 1 mM-aminoguanidine (a diamine oxidase inhibitor) or 10 µM-MDL 72527 (a polyamine oxidase inhibitor). After the incubation, trichloroacetic acid was added to 10% (w/v) and the proteins were precipitated by centrifugation and the supernatant was filtered (0.22 µm-pore-size filter). AbeAdo was quantified using a reverse-phase h.p.l.c. system and u.v. detection as described previously (Byers *et al.*, 1991).

Assay of deoxyhypusine formation

The deoxyhypusine formation assay was carried out by procedures based on the results obtained by Park & Wolff (1988). Cell extracts were prepared by freeze-thawing in buffer containing 25 mM-Tris/HCl, pH 7.5, 2.5 mM-dithiothreitol and 0.1 mM-EDTA. The pellet was removed by centrifugation at 17000 *g* for 20 min at 4 °C and the supernatant fractionated using (NH₄)₂SO₄. In order to obtain an extract containing deoxyhypusine synthase, the 0–45%-satd.-(NH₄)₂SO₄ fraction from control L1210 cells was used. The substrate, unmodified eIF-5A, was isolated in the 45–75%-satd.-(NH₄)₂SO₄ fraction from cells grown for 96 h in the presence of 5 mM-DFMO. The assay was carried out by incubating the extract in 0.2 M-glycine/NaOH buffer, pH 9.5, 0.1 mM-NAD⁺ and 10 µCi of [³H]spermidine (20.4 Ci/mmol) in a total volume of 0.15 ml for 1 h. The reaction was then stopped by addition of 50 µl of the electrophoresis denaturing buffer [12% (w/v) SDS/

0.5 M-Tris/HCl (pH 9.0)/10% (v/v) 2-mercaptoethanol/5 mM-EDTA/25% (v/v) glycerol] and heating at 100 °C for 3 min. The proteins were then separated on a 12.5%-(w/v)-polyacrylamide gel for 4 h, fixed, and subjected to fluorography. A single band of M_r about 18000, corresponding to eIF-5A, was detected, and the amount was quantified by densitometric scanning.

Assay of unmodified-eIF-5A content

The extract containing eIF-5A was prepared by taking the 45–75%-satd. $(\text{NH}_4)_2\text{SO}_4$ protein fraction from cells grown in the presence of the compounds being studied as indicated in the Tables. A deoxyhypusine synthase preparation from L1210 cells was used as the enzyme and the content of unmodified eIF-5A was determined by measuring the incorporation of radioactivity from [^3H]spermidine into the protein of M_r 18000 as described above. Results are expressed as units of [^3H]spermidine incorporation/ μg of protein isolated from the cells.

RESULTS

Effects of AbeAdo on cell growth and polyamine content

L1210 murine leukaemia cells exhibited a dose-dependent decrease in cell growth in response to 72 h AbeAdo treatment, although these cells did not stop growing over the course of the experiment. Exposure to 10 μM -AbeAdo maximally inhibited cell growth (Fig. 1). Exposure of cells to this dose of AbeAdo increased putrescine 80-fold, whereas spermidine and spermine were decreased 16- and 6-fold respectively (Table 1).

Although L1210 proliferation and intracellular spermidine and spermine levels were reduced by a 72 h exposure to AbeAdo, these cells did not stop growing, nor were spermidine and spermine totally depleted. To determine whether longer treatment with this inhibitor would result in cytostasis, possibly in conjunction with total depletion of both spermidine and spermine, L1210 cells were chronically exposed to AbeAdo. Cells exposed to 10 μM -AbeAdo and passaged every 96 h continued to grow over a 12-day time course, although the growth rate decreased over the course of this experiment (Fig. 2). After 12 days of AbeAdo exposure, intracellular putrescine levels were still elevated above control values (Table 1). Spermidine and spermine were reduced to below the level of detection in cells treated with AbeAdo for 12 days (Table 1).

Metabolism of AbeAdo

AbeAdo was not metabolized during incubation with purified rat liver polyamine oxidase (7 mg/ml) in the presence or absence of 10 μM -MDL 72527 (Bey *et al.*, 1985) (Table 2). AbeAdo was degraded when incubated with purified hog kidney diamine oxidase (25 mg/ml) (Table 2). This metabolism was inhibited by the presence of 1 mM-aminoguanidine, a competitive inhibitor of diamine oxidase (Seiler, 1987). These results suggest that AbeAdo may be a weak substrate for diamine oxidase, which is known to be present in bovine serum (Seiler, 1987). The L1210 cultures were supplemented with Nu-Serum, which contains 25% (v/v) newborn-calf serum. However, depending on the lot of Nu-Serum used to supplement the L1210 culture medium, addition of 1 mM-aminoguanidine to the media had only a slight effect, or no effect at all, on the 72 h dose response of L1210 cells to AbeAdo (results not shown). Thus, for the experiments presented here, aminoguanidine was not added to L1210 cell cultures unless the cells were also exposed to polyamines or polyamine analogues.

Effects of exogenous polyamines on growth of cells treated with AbeAdo

The ability of exogenously supplied spermidine and spermine

to reverse or prevent the effects of AbeAdo were examined in order to determine if the growth-inhibitory effects of AbeAdo were directly related to the reduction of these polyamines. Cells exposed to 10 μM -AbeAdo for 12 days doubled about 8–10 times, but after 12 days the cells no longer grew and rapidly lost viability. Even when washed with fresh media and replated in the absence of the drug, cells previously exposed to 10 μM -AbeAdo for 12 days would not grow unless 'rescued' with exogenous spermidine or spermine (Table 3 and Fig. 3). Exogenous spermine allowed for a more rapid recovery of cell growth in AbeAdo-treated cultures than equal concentrations of exogenous spermidine (Fig. 3). Cultures exposed to 10 μM -AbeAdo for 16 days could not be rescued with exogenous polyamines, suggesting that these cells had lost viability.

Intracellular polyamine levels in rescued cells were examined to provide insight into the different abilities of spermidine and spermine to rescue AbeAdo-treated cells. Putrescine levels in rescued cells were reduced from the greatly elevated values in

Fig. 1. Response of L1210 cell growth to a 72 h exposure to AbeAdo

L1210 cells were plated at 5×10^4 cells/ml in media in the presence and absence of the indicated concentration of AbeAdo. Cell number was determined at 72 h. The effect of cell growth was expressed as a percentage of the cell number in the control cultures, which had been grown for 72 h in the absence of AbeAdo. The results are means $\pm$ S.D. for three or four dishes.

Table 1. Polyamine levels in L1210 cells exposed for 3 or 12 days to 10 μM -AbeAdo and in L1210 cells exposed to 10 μM -AbeAdo for 12 days and then rescued with 2 μM -spermidine or 2 μM -spermine

L1210 cells were plated in the presence of 10 μM -AbeAdo. The cells were passaged every 4 days at a density such that they would not reach confluence before the next passage. On days 3 and 12, representative flasks (triplicates) were harvested for polyamine analysis. On day 12, cells in representative flasks (triplicates) were exposed to 1 mM-aminoguanidine and 10 μM -AbeAdo in the presence and absence of 2 μM -spermidine or 2 μM -spermine. Cells exposed to only AbeAdo and aminoguanidine did not grow over the next 4 days, although the cells exposed to spermidine and spermine did grow and were harvested on day 17 for polyamine analysis. The values shown are means $\pm$ S.D. for three or four flasks.

Treatment	Level (nmol/mg of protein)		
	Putrescine	Spermidine	Spermine
Control	0.9 $\pm$ 0.2	21.1 $\pm$ 1	5.7 $\pm$ 0.9
10 μM -AbeAdo			
Day 3	72 $\pm$ 4	1.3 $\pm$ 0.2	1.0 $\pm$ 0.3
Day 12	30 $\pm$ 1	< 0.2	< 0.3
Rescue			
2 μM -Spermidine	8.7 $\pm$ 0.3	5.9 $\pm$ 0.1	< 0.3
2 μM -Spermine	13.7 $\pm$ 2.3	0.8 $\pm$ 0.1	2.8 $\pm$ 0.1

Fig. 2. Effect of a 12-day exposure to 10 μ M-AbeAdo on L1210 cell growth and the ability of spermidine or spermidine analogues to prevent this effect

L1210 cells were exposed to 10 μ M-AbeAdo for 96 h before treatment with spermidine or spermidine analogues. After the initial 96 h period, the cells were passaged and exposed to spermidine, saturated *gem*-dimethylspermidine analogues and aminoguanidine as indicated: group 1, 10 μ M-3,3-dimethylspermidine, 10 μ M-AbeAdo and 1 mM-aminoguanidine; 2, 10 μ M-5,5-dimethylspermidine, 10 μ M-AbeAdo and 1 mM-aminoguanidine; 3, 10 μ M-1,1-dimethylspermidine, 10 μ M-AbeAdo and 1 mM-aminoguanidine; 4, 10 μ M-8,8-dimethylspermidine, 10 μ M-AbeAdo and 1 mM-aminoguanidine; 5, 10 μ M-2,2-dimethylspermidine, 10 μ M-AbeAdo and 1 mM-aminoguanidine; 6, 10 μ M-spermidine, 10 μ M-AbeAdo and 1 mM-aminoguanidine; 7, 10 μ M-AbeAdo and 1 mM-aminoguanidine; 8, 10 μ M-AbeAdo only; 9, no addition after initial 96 h AbeAdo exposure. These treatments were maintained for each group throughout the remainder of the experiment. The cells were passaged every 96 h and plated at 1×10^5 cells/ml and cells were counted every 24 h. The values shown are the mean values for three flasks, except for group 4, where a single flask was used owing to limited availability of this spermidine analogue. Results are shown for the first passage (a), second passage (b) and third passage (c) after the initial 96 h treatment with AbeAdo. Note that cells in group 6 (spermidine, aminoguanidine and AbeAdo) and group 9 (no addition after initial 96 h AbeAdo exposure) were confluent before 96 h in each passage.

Table 2. Metabolism of AbeAdo incubated with purified hog kidney diamine oxidase or purified rat liver polyamine oxidase

Purified hog kidney diamine oxidase (25 mg/ml) or purified rat liver polyamine oxidase (7 mg/ml) in 10 mM-Tris, pH 7.5, was incubated with 50 μ M-AbeAdo at 37 $^{\circ}$ C in the presence or absence of 1 mM-aminoguanidine for the indicated period of time. At the end of the incubation, trichloroacetic acid was added to give a final concentration of 10% and the precipitated proteins were sedimented by centrifugation and the supernatant was filtered. The AbeAdo content of the supernatant was determined by h.p.l.c. analysis. ND, not determined.

Additions	Incubation period (h)...	AbeAdo content (% of initial)		
		1	4	8
Diamine oxidase		64	< 6	< 6
Diamine oxidase + 1 mM aminoguanidine		100	91	90
Polyamine oxidase		ND	ND	100
Polyamine oxidase + 10 μ M-MDL 72527		ND	ND	100

undetectable in these cells (Table 1). Rescue with spermine in the presence of AbeAdo resulted in a significant increase in intracellular spermine and appearance of low, but detectable, levels of spermidine.

Use of polyamine analogues by deoxyhypusine synthase

We have reported that a number of *gem*-dimethylspermidine and some unsaturated spermidine derivatives are able to support the growth of DFMO-treated cells in short-term experiments (Nagarajan *et al.*, 1988; Pegg *et al.*, 1991). In order to check whether these compounds are able to replace spermidine in the deoxyhypusine synthase reaction, the content of non-hypusinated eIF-5A was measured in cells treated with DFMO alone or DFMO plus spermidine or DFMO plus the spermidine derivatives.

As Table 4 shows, there was a large increase in the content of unmodified eIF-5A when SV-3T3 cells were grown in the presence of DFMO for 72 h. This increase was completely prevented by the addition of spermidine. However, none of the *gem*-dimethylspermidine derivatives was able to block the rise in unmodified eIF-5A.

Deoxyhypusine synthase required NAD⁺ for activity and forms 1,3-diaminopropane in stoichiometric amounts with the eIF-5A-deoxyhypusine. Studies of the enzyme are consistent with a mechanism involving the dehydrogenation of spermidine between C-5 and the secondary nitrogen at position 4, the cleavage of this intermediate to form 1,3-diaminopropane and an imine between the ϵ -amino group of the modified lysine and the 4-aminobutyl group from spermidine, and, finally, the reduction of this intermediate to form deoxyhypusine (Wolff *et al.*, 1990). The compounds tested as substrates included 5,5-dimethylspermidine, in which both hydrogen atoms attached to C-5 are replaced by methyl groups. It is therefore impossible for this compound to enter into the dehydrogenation reaction, and it is not surprising that it cannot substitute for spermidine in this reaction. The inability of the 1,1-dimethyl and 3,3-dimethyl derivatives to substitute for spermidine cannot be explained in this way, and suggests that the deoxyhypusine synthase is relatively specific for spermidine and cannot tolerate such additions. This conclusion is supported by the results of experiments in which the activity of deoxyhypusine synthase was studied *in vitro* (Table 5). Activity was assayed by measuring the incorporation of radioactivity

treated cells to near control values (Table 1). Intracellular spermidine levels in cells rescued with exogenous spermidine were within the physiological range, although spermine was

Table 3. Reversal of AbeAdo-induced cytostasis by spermidine, spermine and *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene

L1210 cells were grown in the presence of 10 μ M-AbeAdo for 13 days, by which time cell growth was minimal (the cell density was 3.5×10^5 cells/ml on day 11 and 4.2×10^5 cells/ml on day 13). On day 13, the cells were washed and resuspended in media at a density of 1×10^5 cells/ml. The cells were exposed to spermidine, spermidine analogues or spermine and AbeAdo as indicated above. All cultures contained 1 mM-aminoguanidine. Cell numbers were determined at 24, 72, 96, 120 and 144 h by using a Coulter counter, and these values expressed as a percentage of the cell number present at 24 h. The values above represent the mean $\pm$ S.D. for three flasks, except for groups treated with *N*-(3-aminopropyl)-1,4-diamino-*trans*-but-2-ene or 8,8-dimethylspermidine, where only one flask was used, owing to the limited availability of the spermidine analogue. In all cases except the last ('None') 10 μ M-AbeAdo was also present.

Addition(s)	Time (h)...	Cells present (% of those present at 24 h)			
		72	96	120	144
10 μ M- <i>N</i> -(3-Aminopropyl)-1,4-diamino- <i>cis</i> -but-2-ene		121 $\pm$ 15	136 $\pm$ 22	152 $\pm$ 18	192 $\pm$ 19
10 μ M- <i>N</i> -(3-Aminopropyl)-1,4-diamino- <i>trans</i> -but-2-ene		98	100	118	103
10 μ M- <i>N</i> -(3-Aminopropyl)-1,4-diaminobut-2-yne		116 $\pm$ 10	108 $\pm$ 7	113 $\pm$ 9	119 $\pm$ 11
10 μ M- <i>N</i> ¹ <i>N</i> ⁴ -bis-(3-aminopropyl)-1,4-diamino- <i>cis</i> -but-2-ene		104 $\pm$ 2	120 $\pm$ 27	129 $\pm$ 39	128 $\pm$ 16
10 μ M-3,3-Dimethylspermidine		114 $\pm$ 23	106 $\pm$ 16	107 $\pm$ 22	100 $\pm$ 24
10 μ M-5,5-Dimethylspermidine		111 $\pm$ 13	111 $\pm$ 15	117 $\pm$ 13	109 $\pm$ 11
10 μ M-1,1-Dimethylspermidine		103 $\pm$ 19	106 $\pm$ 19	116 $\pm$ 21	107 $\pm$ 17
10 μ M-8,8-Dimethylspermidine		115	98	116	104
10 μ M-2,2-Dimethylspermidine		110 $\pm$ 14	110 $\pm$ 14	120 $\pm$ 9	114 $\pm$ 11
10 μ M-Spermidine		113 $\pm$ 15	140 $\pm$ 26	242 $\pm$ 63	493 $\pm$ 124
2 μ M-Spermine		132 $\pm$ 19	223 $\pm$ 39	491 $\pm$ 101	1230 $\pm$ 230
10 μ M-AbeAdo only		111 $\pm$ 12	108 $\pm$ 8	126 $\pm$ 4	132 $\pm$ 8
None		119 $\pm$ 9	107 $\pm$ 9	124 $\pm$ 9	120 $\pm$ 25

Fig. 3. Comparison of the ability of spermidine and spermine to reverse AbeAdo induced cytostasis

L1210 cells were exposed to 10 μ M-AbeAdo for 12 days. On day 12, the cells were washed and resuspended at 1×10^5 cells/ml in media containing 1 mM-aminoguanidine (Δ) or 1 mM-aminoguanidine plus 10 μ M-AbeAdo in the absence ($\blacktriangle$) or presence of 0.5 μ M-spermidine ($\circ$), 2 μ M-spermidine ($\bullet$), 0.5 μ M-spermine ($\square$) or 2 μ M-spermine ($\blacksquare$). Each consisted of three group flasks of cells. The cells were counted at the times indicated, and results are expressed as a percentage of the cell number present at 24 h.

Table 4. Content of unmodified eIF-5A in SV-3T3 cells treated with DFMO and spermidine analogues

Except for cells grown for 72 h in the absence of DFMO (*), all other cultures were grown for 72 h in the presence of 5 mM-DFMO, and at 4 h after the DFMO, the compounds shown were added at a concentration of 10 μ M.

Compound	Unmodified eIF-5A (units/ μ g of protein)
Not treated*	< 0.5
DFMO	16.2 $\pm$ 2.9
DFMO + spermidine	< 0.5
DFMO + 1,1-dimethylspermidine	13.8 $\pm$ 3.4
DFMO + 3,3-dimethylspermidine	15.1 $\pm$ 2.4
DFMO + 5,5-dimethylspermidine	17.5 $\pm$ 3.2
DFMO + <i>N</i> -(3-aminopropyl)-1,4-diaminobut-2-yne	16.1 $\pm$ 3.8
DFMO + <i>N</i> -(3-aminopropyl)-1,4-diamino- <i>cis</i> -but-2-ene	0.7 $\pm$ 0.5
DFMO + <i>N</i> -(3-aminopropyl)-1,4-diamino- <i>trans</i> -but-2-ene	0.9 $\pm$ 0.4
DFMO + <i>N</i> ¹ <i>N</i> ⁴ -bis-(3-aminopropyl)-1,4-diamino- <i>cis</i> -but-2-ene	18.4 $\pm$ 2.1

norspermine and *sym*-homospermidine were inhibitory to this reaction, but the tested *gem*-dimethylspermidines were much less effective. In fact only the 1,1-dimethyl and 8,8-dimethyl compounds, in which the methyl groups are as far away from the internal cleavage site as possible, showed any interaction at all, and even with these compounds concentrations of more than 100 μ M were needed to give 50 % inhibition of the incorporation of spermidine.

The effects of the addition of unsaturated spermidine derivatives to DFMO-treated SV-3T3 cells on the content of unmodified eIF-5A are shown in Table 4. Both the *cis* and the

from ³H-labelled spermidine into eIF-5A. The ability of compounds related to spermidine to inhibit this incorporation was measured to identify compounds that were either good inhibitors of the reaction or alternative substrates for it. As previously reported by Park & Wolff (1988), 1,3-diaminopropane, *sym*-

Table 5. Ability of polyamine analogues to inhibit deoxyhypusine synthase *in vitro*

The compounds were added at 10, 100 or 500 μM to the standard assay mixture, which contained 3.3 μM -[^3H]spermidine as a substrate for deoxyhypusine synthase, and the results are expressed as the percentage incorporation of label into eIF-5A when no addition was made. A number of other compounds, including N^1N^8 -bis(ethyl)spermidine, N^1N^{11} -bis(ethyl)norspermine, N^1N^{12} -bis(ethyl)spermine, N^1N^{14} -bis(ethyl)homospermine, 3,3,10,10-tetramethylspermine, 1,1,12,12-tetramethylspermine and N^1N^4 -bis-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene were tested and found to be inactive as inhibitors (< 20% inhibition at 500 μM).

Analogue	Concn. (μM)...	Deoxyhypusine synthase activity (% of control)		
		10	100	500
1,3-Diaminopropane		8	0	0
<i>sym</i> -Homospermidine		54	8	0
<i>sym</i> -Norspermine		26	6	0
1,1-Dimethylspermidine		96	61	18
2,2-Dimethylspermidine		104	109	89
3,3-Dimethylspermidine		101	116	109
5,5-Dimethylspermidine		104	114	109
8,8-Dimethylspermidine		102	94	54
<i>N</i> -(3-aminopropyl)-1,4-diaminobut-2-yne		77	35	0
<i>N</i> -(3-Aminopropyl)-1,4-diamino- <i>cis</i> -but-2-ene		25	2	0
<i>N</i> -(3-Aminopropyl)-1,4-diamino- <i>trans</i> -but-2-ene		3	0	0

Fig. 4. Ability of *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene to reverse AbeAdo-induced cytostasis

L1210 cells were grown in the presence of 10 μM -AbeAdo for 12 days. On day 12 the cells were replated at 2×10^5 cells/ml in media containing 10 μM -AbeAdo and 1 mM-aminoguanidine. Representative flasks of cells were also exposed to 10 μM -*N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene. On day 16 the cells were replated at 2×10^5 cells/ml in media containing 10 μM -AbeAdo and 1 mM-aminoguanidine and cells exposed previously to *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene were maintained in the presence of the drug. Cells in these cultures were counted on a Coulter counter at the times indicated and are expressed as a percentage of the cell number present on day 17. The values above represent the means $\pm$ S.D. for four flasks.

trans isomers of an unsaturated spermidine derivative containing a double bond between C-6 and C-7 were as active as spermidine itself in reducing the content. By contrast, the derivative containing a triple bond at this position was completely inactive. When the *cis* and the *trans* isomers were tested for their effects on

deoxyhypusine synthase activity *in vitro* (Table 5), it was found that both compounds were effective inhibitors of the incorporation of spermidine into eIF-5A. A more detailed comparison of the two isomers showed that the *trans* derivative was clearly more active, with 50% inhibition being achieved at 0.2 μM , whereas 4 μM of the *cis* derivative was needed (results not shown). These results suggest strongly that these compounds can be incorporated into eIF-5A. The apparent ability of the enzyme to use either isomer contrasts with spermine synthase, which acts only on the *cis* derivative (Pegg *et al.*, 1991). The spermidine derivative containing a triple bond at the C-6-7 position was inhibitory towards deoxyhypusine synthase (Table 5), but was far less active, requiring 45 μM for 50% inhibition. This result and the inability of *N*-(3-aminopropyl)-1,4-diaminobut-2-ene to affect the content of unmodified eIF-5A when added to cell cultures (Table 4) indicate that this compound is not a substrate for deoxyhypusine synthase.

Effects of polyamine analogues on the growth of cells treated with AbeAdo

In experiments designed to determine whether depletion of hypusine in eIF-5A had a role in inducing cytostasis in AbeAdo-treated cells, spermidine analogues with differing abilities to act as substrates for the deoxyhypusine synthase were tested for their ability to prevent or reverse the antiproliferative effects of AbeAdo. The growth-inhibitory effects of chronic AbeAdo exposure were prevented and reversed by spermidine, but not by several *gem*-dimethylspermidine analogues of spermidine (Fig. 2 and Table 3) which, as described above, do not serve as substrates for this reaction.

When the three unsaturated spermidine derivatives were tested in the same way, it was found that *N*-(3-aminopropyl)-1,4-diaminobut-2-yne and the *trans* isomer of *N*-(3-aminopropyl)-1,4-diaminobut-2-ene were not active (Table 3). However, limited growth was evident in previously cytostatic cells after 144 h exposure to the *cis* isomer of *N*-(3-aminopropyl)-1,4-diaminobut-2-ene (Table 3), and full reversal of the AbeAdo-induced cytostasis was obtained if exposure to this analogue was continued (Fig. 4).

DISCUSSION

The effects of AbeAdo on L1210-cell intracellular polyamine levels are consistent with the compound's ability to inhibit AdoMetDC. Spermidine and spermine were reduced, whereas putrescine was greatly increased. It should be noted that the increase in putrescine brought about by AbeAdo treatment is greater than the concurrent decrease in spermidine and spermine. This is probably due to the increased activity of ODC, which is known to be strongly repressed by spermidine and spermine (reviewed by Pegg, 1988).

The decreased growth rate of L1210 cells observed within 72 h of AbeAdo treatment is consistent with the findings of Pegg *et al.* (1988) and Kramer *et al.* (1989), who reported similar growth effects in L1210 cells treated with less stable AdoMetDC inhibitors. However, our observation of L1210 cell cytostasis in response to prolonged exposure to an AdoMetDC inhibitor has not been reported previously. The effect of AbeAdo on cell growth appears to be directly attributable to the effects of this inhibitor on spermidine and spermine biosynthesis in that the antiproliferative effects of this drug were reversed and prevented by exogenous spermidine or spermine. In the presence of AbeAdo, L1210 cells rescued from cytostasis with spermidine had undetectable spermine levels, suggesting that spermidine alone can support growth. Spermidine levels were low, but detectable in cells rescued with spermine. This low level of

spermidine in spermine rescued cells was presumably due to the back conversion of spermine into spermidine via the sequential action of spermidine:spermine *N*¹-acetyltransferase and polyamine oxidase (Pegg, 1986). Treatment of L1210 cells for 96 h with 5 mM-DFMO, which inhibits ornithine decarboxylase and depletes intracellular putrescine and spermidine, before treatment with 10 μ M-AbeAdo, caused the cells to stop growing 3 days after the addition of the AdoMetDC inhibitor (T. L. Byers, unpublished work). The synergistic effect of depleting putrescine and spermidine with DFMO before AbeAdo treatment provides additional evidence that AbeAdo influences cell growth via its effects on polyamine biosynthesis.

It is possible that greatly elevated putrescine levels sustained limited growth in AbeAdo-treated cells by fulfilling many of the cellular requirements for polyamines. However, the eventual cytostasis and loss of viability suggest that there is one or more specific physiological roles of spermidine and/or spermine which putrescine cannot satisfy. The best characterized specific function for any of the polyamines in mammalian cells is the ability of spermidine to serve as a substrate for the post-translational formation of hypusine in eIF-5A. This is the only protein known to contain hypusine. Hypusine is formed at lysine-50 of eIF-5A by the sequential actions of deoxyhypusine synthase and deoxyhypusine hydroxylase. Formation of hypusine is essential for eIF-5A to function in protein synthesis (Park, 1989; Smith-McBride *et al.*, 1989) and has been shown to be necessary for normal yeast-cell growth (Schnier *et al.*, 1991). Thus it is possible that AbeAdo-induced cytostasis could be due to the depletion of the hypusinated form of eIF-5A in the absence of spermidine. This hypothesis is consistent with the finding that polyamine analogues that are not substrates for deoxyhypusine synthase *in vitro* could not prevent or reverse the antiproliferative effects of prolonged AbeAdo treatment. Apparently, growth in AbeAdo-treated cells is limited by more than a general need for polybasic aliphatic amines. *N*-(3-Aminopropyl)-1,4-diamino-*cis*-but-2-ene, which was a substrate for deoxyhypusine synthase, did show limited ability to reverse AbeAdo-induced cytostasis. This is consistent with the effect being a result of hypusinated eIF-5A depletion.

Even though *N*-(3-aminopropyl)-1,4-diamino-*trans*-but-2-ene was also a substrate for deoxyhypusine synthase, this spermidine analogue did not rescue AbeAdo-treated cells. One possible explanation for this would be that the *cis* configuration in the unsaturated deoxyhypusine-eIF-5A may better resemble the conformation of the normal 4-aminobutyl group that does the *trans* configuration. This could allow deoxyhypusine hydroxylase to make more easily the further modification needed for activity and/or render the final product more active in protein synthesis. A second possibility is that spermine (or a spermine analogue) is also needed for prolonged growth in addition to the hypusination of eIF-5A. It is known that spermine synthase is highly specific with regard to the configuration of the spermidine it will use as an aminopropyl acceptor and will only act on the *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene isomer to form a spermine derivative (Pegg *et al.*, 1991). However, the ability to act as a spermine analogue precursor is an improbable explanation for the difference in the ability of the *trans* and *cis* isomers of *N*-(3-aminopropyl)-1,4-diaminobut-2-ene to support growth in the presence of AbeAdo, since AbeAdo inhibits AdoMetDC, which provides the aminopropyl donor substrate for spermine synthase. Furthermore, *N*¹*N*⁴-bis-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene, the spermine analogue of *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene, cannot be converted back into the spermidine *cis* isomer (Pegg *et al.*, 1991) and was not able to rescue the AbeAdo-treated cells (Table 3). This is consistent with its inability to support the hypusination of eIF-5A (Table 4).

The concept that both the availability of spermine and the formation of hypusine in eIF-5A from spermidine are needed for prolonged cell growth and viability may explain why spermine, which is not a substrate for the hypusination reaction, was better able than spermidine to reverse the effect of AbeAdo. Cells rescued with spermine did contain low levels of spermidine, and since the *K_m* for spermidine in the eIF-5A reaction is 0.08 μ M (Wolff *et al.*, 1990), it is likely that these cells were able to form sufficient hypusine in eIF-5A. The fact that mammalian cells normally contain not only spermidine but also spermine suggests that there is some physiological advantage in the presence of both polyamines.

It has long been known that ODC inhibition depletes the content of putrescine and spermine and that this, in turn, induces a cessation of mammalian cell growth. Two aspects of ODC-inhibitor-induced cytostasis are different from that produced by AbeAdo. First, the blockage of cell growth produced by ODC inhibitors occurs relatively rapidly after putrescine and spermine depletion, whereas AbeAdo-induced cytostasis is delayed relative to spermidine and spermine depletion. Secondly, cells in ODC inhibitor-induced cytostasis remain viable, whereas cells treated with AbeAdo rapidly lose viability after cell growth ceases. If depletion of hypusinated eIF-5A is the cause of AbeAdo-induced cytostasis, cells treated with ODC inhibitors may simply not continue to divide for long enough to deplete hypusinated eIF-5A. This is consistent with the results of Nagarajan *et al.* (1988), who found that 5,5-dimethylspermidine, which cannot take part in the deoxyhypusine-formation reaction, was able to support the growth of DFMO-treated SV3T3 cells. We suggest that the differences in the cytostasis caused by the inhibition of ODC or AdoMetDC are a result of the effects on total polyamine levels. Although inhibition of ODC reduced total intracellular polyamine levels, AdoMetDC inhibition actually increases total intracellular polyamines by elevating putrescine biosynthesis. The ODC inhibitor-induced cytostasis that is associated with the reduction of total intracellular polyamine levels may be the result of a general interruption of many relatively non-specific and permissive functions fulfilled by polyamines. The greatly elevated putrescine levels in AbeAdo-treated cells may fulfil many of these functions and thus support cell growth, although at a reduced rate, in the absence of physiological concentrations of spermidine and spermine. Under such conditions, AbeAdo-treated cells continue to grow only for as long as any specific and critical physiological function requiring spermidine or spermine are fulfilled. The results presented here are consistent with the possibility that the depletion of hypusinated eIF-5A is the factor leading to cytostasis and a rapid loss of viability in such AbeAdo-treated cells.

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REFERENCES

- Abbruzzese, A., Park, M. H. & Folk, J. E. (1986) *J. Biol. Chem.* **261**, 3085-3089
- Bacchi, C. J. & McCann, P. P. (1987) in *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies* (McCann, P. P., Pegg, A. E. & Sjoerdsma, A., eds.), pp. 317-344, Academic Press, New York
- Bey, P., Bolkenius, F. N., Seiler, N. & Casara, P. (1985) *J. Med. Chem.* **28**, 1-2
- Bitonti, A. J., Bacchi, C. J., McCann, P. P. & Sjoerdsma, A. (1985) *Biochem. Pharmacol.* **34**, 1773-1777
- Bitonti, A. J., Byers, T. L., Bush, T. L., Casara, P. J., Bacchi, C. J., Clarkson, A. B., McCann, P. P. & Sjoerdsma, A. (1990) *Antimicrob. Agents Chemother.* **34**, 1485-1490
- Byers, T. L. & Pegg, A. E. (1989) *Am. J. Physiol.* **26**, C545-C553

- Byers, T. L., Bush, T. L., McCann, P. P. & Bitonti, A. J. (1991) *Biochem. J.* **274**, 527–533
- Casara, P., Marchal, P., Wagner, J. & Danzin, C. (1989) *J. Am. Chem. Soc.* **111**, 9111–9113
- Casero, R. A., Ervin, S. J., Celano, P., Baylin, S. B. & Bergeron, R. J. (1989) *Cancer Res.* **49**, 639–643
- Danzin, C., Marchal, P. & Casara, P. (1990) *Biochem. Pharmacol.* **40**, 1499–1503
- Hershey, J. W. B., Smit-McBride, Z. & Schnier, J. (1990) *Biochim. Biophys. Acta* **1050**, 160–162
- Hibasami, H., Sakurai, M., Maekawa, S. & Nakasima, K. (1987) *Anticancer Res.* **7**, 1213–1216
- Hibasami, H., Kawase, M., Tsukada, T., Maekawa, S., Sakurai, M. & Nakasima, K. (1988) *FEBS Lett.* **229**, 243–246
- Ito, H., Hibasami, H., Keshior, S., Nagai, J. & Hidaka, H. (1982) *Cancer Lett.* **15**, 229–235
- Kolb, M., Danzin, C., Barth, J. & Calverie, N. (1982) *J. Med. Chem.* **25**, 550–556
- Kramer, D. L., Khomutov, R. M., Bukin, Y. V., Khomutov, A. R. & Porter, C. W. (1989) *Biochem. J.* **259**, 325–331
- Mamont, P. S., Duchesne, M. C., Grove, J. & Bey, P. (1978) *Biochem. Biophys. Res. Commun.* **81**, 58–66
- Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. & Vevort, J. P. (1978) *J. Am. Chem. Soc.* **100**, 2551–2553
- Nagarajan, S. & Ganem, B. (1986) *J. Org. Chem.* **51**, 4856–4861
- Nagarajan, S. & Ganem, B. (1987) *J. Org. Chem.* **52**, 5044–5046
- Nagarajan, S., Ganem, B. & Pegg, A. E. (1988) *Biochem. J.* **254**, 373–378
- Nakashima, K., Tsukada, T., Hibasami, H. & Maekawa, S. (1986) *Biochem. Biophys. Res. Commun.* **141**, 718–722
- Park, M. H. (1989) *J. Biol. Chem.* **264**, 18531–18535
- Park, M. H. & Wolff, E. C. (1988) *J. Biol. Chem.* **263**, 15264–15269
- Park, M. H., Wolff, E. C., Smit-McBride, Z., Hershey, J. W. B. & Folk, J. E. (1991) *J. Biol. Chem.* **266**, 7988–7994
- Pegg, A. E. (1983) *Methods Enzymol.* **94**, 239–247
- Pegg, A. E. (1984a) *Cell Biochem. Funct.* **2**, 11–15
- Pegg, A. E. (1984b) *Biochem. J.* **224**, 29–38
- Pegg, A. E. (1986) *Biochem. J.* **234**, 249–262
- Pegg, A. E. (1988) *Cancer Res.* **48**, 759–774
- Pegg, A. E. & Coward, J. K. (1985) *Biochem. Biophys. Res. Commun.* **133**, 82–89
- Pegg, A. E. & Williams-Ashman, H. G. (1987) in *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies* (McCann, P. P., Pegg, A. E. & Sjoerdsma, A., eds.), pp. 33–48, Academic Press, Orlando, FL
- Pegg, A. E., Tang, K. C. & Coward, J. K. (1982) *Biochemistry* **21**, 5083–5089
- Pegg, A. E., Jones, D. B. & Secrist, J. A. III (1988) *Biochemistry* **27**, 1408–1415
- Pegg, A. E., Nagarajan, S., Naficy, S. & Ganem, B. (1991) *Biochem. J.* **274**, 167–171
- Pohjanpelto, P., Hölttä, E. & Jänne, O. A. (1985) *Mol. Cell. Biol.* **5**, 1385–1390
- Porter, C. W. & Sufrin, J. R. (1986) *Anticancer Res.* **6**, 525–542
- Schnier, J., Schwelberger, H. G., Smit-McBride, Z., Kang, H. A. & Hershey, J. W. M. (1991) *Mol. Cell. Biol.* **11**, 3105–3114
- Seiler, N. (1987) in *Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies* (McCann, P. P., Pegg, A. E. & Sjoerdsma, A., eds.), pp. 49–77, Academic Press, New York
- Seiler, N. & Knödgen, B. (1985) *J. Chromatogr.* **339**, 45–57
- Sjoerdsma, A. & Schechter, P. J. (1984) *Clin. Pharmacol. Ther.* **35**, 287–300
- Smit-McBride, Z., Dever, T. E., Hershey, J. W. B. & Merrick, W. C. (1989) *J. Biol. Chem.* **264**, 1578–1583
- Steglich, C. & Scheffler, I. E. (1982) *J. Biol. Chem.* **257**, 4603–4609
- Williams-Ashman, H. G. & Pegg, A. E. (1981) in *Polyamines in Biology and Medicine* (Morris, D. R. & Marton, L. J., eds.), pp. 407–436, Marcel Dekker, New York
- Wolff, E. C., Park, M. H. & Folk, J. E. (1990) *J. Biol. Chem.* **265**, 4793–4799

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