The role of hypusine depletion in cytostasis induced by S-adenosyl-L-methionine decarboxylase inhibition: new evidence provided by 1-methylspermidine and 1,12-dimethylspermine

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The abilities of the natural polyamines, spermidine and spermine, and of the synthetic analogues, 1-methylspermidine and 1,12-dimethylspermine, to reverse the effects of the S-adenosyl-L-methionine decarboxylase inhibitor 5'-[[*(Z)*-4-aminobut-2-enyl]methylamino]-5'-deoxyadenosine (AbeAdo) on L1210-cell growth were studied. L1210 cells were exposed to AbeAdo for 12 days to induce cytostasis and then exposed to spermidine, spermine, 1-methylspermidine or 1,12-dimethylspermine in the continued presence of AbeAdo. AbeAdo-induced cytostasis was overcome by the natural polyamines, spermidine and spermine. The cytostasis was also reversed by 1-methylspermidine. 1,12-Dimethylspermine had no effect on the AbeAdo-induced cytostasis of chronically treated cells, although it was active in permitting growth of cells treated with the ornithine decarboxylase inhibitor, α -difluoromethylornithine. The initial 12-day exposure to AbeAdo elevated intracellular putrescine levels, depleted intracellular spermidine and spermine, and resulted in the accumulation of unmodified eukaryotic translation initiation factor 5A (eIF-5A). Exposure of these cells to exogenous spermidine, which is the natural substrate for deoxy-

hypusine synthase, resulted in a decrease in the unmodified eIF-5A content. 1-Methylspermidine, which was found to be a substrate of deoxyhypusine synthase *in vitro*, also decreased the levels of unmodified eIF-5A in the AbeAdo-treated cells. Although spermine is not a substrate of deoxyhypusine synthase, spermine was converted into spermidine in the L1210 cells, and spermine addition to AbeAdo-treated cells resulted in the appearance of both intracellular spermine and spermidine and in the decrease in unmodified eIF-5A. Exogenous 1,12-dimethylspermine, which was not metabolized to spermine or to 1-methylspermidine and was not a substrate of deoxyhypusine synthase *in vitro*, did not decrease levels of unmodified eIF-5A. The finding that AbeAdo-induced cytostasis was only reversed by polyamines and polyamine analogues that result in the formation of hypusine or an analogue in eIF-5A is consistent with the hypothesis [Byers, Wiest, Wechter and Pegg (1993) *Biochem. J.* **290**, 115–121] that AbeAdo-induced cytostasis is due to the depletion of the hypusine-containing form of eIF-5A, which is secondary to the depletion of spermidine by inhibition of S-adenosyl-L-methionine decarboxylase.

INTRODUCTION

The active form of the protein initiation factor eIF-5A contains hypusine {*N*'-[4-amino-2(*R*)-hydroxybutyl]lysine} at residue 50 [1–3]. The hypusine residue, which is unique to eIF-5A, is formed as a post-translational modification of the eIF-5A precursor protein in a two-step process. The first step is deoxyhypusine formation, which involves the transfer of the 4-aminobutyl group from spermidine to lysine-50 in the eIF-5A precursor protein by deoxyhypusine synthase [4]. The second step is the hydroxylation of deoxyhypusine by deoxyhypusine hydroxylase [5]. The level of modified eIF-5A correlates with the growth state of some mammalian cells, and the presence of hypusine in eIF-5A has been shown to be essential for cell viability and growth in yeast [1–3,6–9].

Prolonged exposure to the S-adenosyl-L-methionine decarboxylase inhibitor 5'-[[*(Z)*-4-aminobut-2-enyl]methylamino]-5'-deoxyadenosine (AbeAdo) [10,11] results in the depletion of intracellular spermidine and the accumulation of the unmodified form of eIF-5A, followed by cytostasis [12,13]. It has been suggested that this cytostasis is due to the depletion of hypusine in eIF-5A, which occurs due to the absence of spermidine, the

natural substrate required for hypusine formation. This evidence included the observation that, of the many polyamine analogues tested, the only one able to mimic the effects of spermidine in reversing AbeAdo-induced cytostasis was a synthetic substrate for deoxyhypusine synthase, *N*-(3-aminopropyl)-1,4-diaminocis-but-2-ene [12]. The association between AbeAdo-induced cytostasis and hypusine depletion was supported by the finding that AbeAdo-resistant L1210 cells did not accumulate unmodified eIF-5A [13]. However, spermine, which is not a substrate for deoxyhypusine synthase, was active in reversing the effects of AbeAdo [12,13]. It was suggested that the metabolism of spermine to spermidine by the sequential action of spermidine/spermine *N*¹-acetyltransferase and polyamine oxidase [14,15] was responsible for the reversal of the AbeAdo-induced cytostasis by providing spermidine for the hypusine formation on the unmodified eIF-5A precursor, and that the presence of spermine merely optimized the growth permitted by the active form of eIF-5A. However, the respective roles of spermidine and spermine in reversing AbeAdo-induced cytostasis could not be distinguished directly, because of the conversion of spermine into spermidine [13]. Experiments have therefore been carried out using methylated derivatives of spermidine and spermine that closely

Abbreviations used: AbeAdo, 5'-[[*(Z)*-4-aminobut-2-enyl]methylamino]-5'-deoxyadenosine (MDL 73811); eIF-5A, eukaryotic translation initiation factor 5A; ODC, ornithine decarboxylase; DFMO, α -difluoromethylornithine; MDL 72527, *N*¹*N*⁴-bis-(buta-2,3-dien-1-yl)butane-1,4-diamine; MDL 72521, *N*¹-methyl-*N*⁴-(buta-2,3-dien-1-yl)butane-1,4-diamine; dcAdoMet, decarboxylated S-adenosylmethionine.

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resemble their natural counterparts, but which are not readily interconverted [16]. The results obtained support the hypothesis that AbeAdo-induced cytostasis is due to depletion of hypusine in eIF-5A in the absence of spermidine, and that this cytostasis may only be reversed by the provision of spermidine or spermidine analogues that are substrates of deoxyhypusine synthase.

MATERIALS AND METHODS

Materials

All biochemical reagents were purchased from Fisher Scientific, Pittsburgh, PA, U.S.A., and Sigma Chemical Co., St. Louis, MO, U.S.A. AbeAdo [10], the ornithine decarboxylase (ODC) inhibitor α -difluoromethylornithine (DFMO) [17], and the polyamine oxidase inhibitors N^1N^4 -bis(buta-2,3-dien-1-yl)butane-1,4-diamine (MDL 72527) and N^1 -methyl- N^4 -(buta-2,3-dien-1-yl)butane-1,4-diamine (MDL 72521) [18] were generously given by Marion Merrell Dow, Cincinnati, OH, U.S.A. 1-Methylspermidine, 1-methylspermine and 1,12-dimethylspermine were synthesized as described previously [16].

Cell culture

L1210 murine leukaemia cells were grown at 37 °C as suspension cultures in RPMI-1640 medium containing 10% NuSerum (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. Aminoguanidine (1 mM) was added to culture media containing spermidine, spermine or polyamine analogues to prevent the toxic action of exogenous polyamine degradation by serum oxidases.

CHOMG cells, which lack polyamine-transport activity [19], and their parental CHO cells were originally provided by Dr. W. Flintoff, Department of Microbiology, University of Western Ontario, Canada. These cells were grown as monolayers in alpha-MEM (Gibco) with 10% foetal bovine serum, 12 µg/ml penicillin and 12 µg/ml streptomycin at 37 °C in 10% CO₂ and 93% humidity [20].

Determination of polyamines

L1210 cells were harvested by centrifugation and washed twice with ice-cold PBS. CHO cells were harvested as previously described [20]. The washed cell pellets were resuspended in 10% (w/v) trichloroacetic acid, precipitated protein was removed by centrifugation, and the supernatant was filtered (0.22 µm pore) before h.p.l.c. analysis for polyamines. The precipitated protein was dissolved in 0.1 M NaOH for measurement of protein content. Putrescine, spermidine, spermine, 1-methylspermidine and 1,12-dimethylspermine were separated by reverse-phase h.p.l.c. and quantified by using post-column reaction with *o*-phthalaldehyde and fluorescence detection [16,21,22].

Cell growth experiments

The abilities of 1-methylspermidine and 1,12-dimethylspermine to support the growth of CHO and CHOMG cells depleted of putrescine and spermidine by exposure to DFMO was tested as follows. The cells were plated at 4×10^4 cells in 1 ml of medium per 35 mm-diam. dish and incubated for 72 h in the presence of 5 mM DFMO. The medium was then changed to medium

containing DFMO and the polyamine being tested, and the cell number was counted every 24 h for the next 4 days.

In order to determine the ability of spermidine, spermine, 1-methylspermidine or 1,12-dimethylspermine to reverse the cytostatic effects of AbeAdo, L1210 cells were exposed to 10 µM AbeAdo for 12 days to induce cytostasis. The cells were plated at densities such that they would not reach confluence before harvest or passage as previously described [12], and were passaged every 4 days. After the initial 12 days of exposure to AbeAdo, the cells were re-plated at 2×10^5 cells/ml in media containing 10 µM AbeAdo and 1 mM aminoguanidine in the absence of presence of spermidine, spermine, 1-methylspermidine or 1,12-dimethylspermine. These cells were counted every 24 h with an electronic cell counter and passaged after 96 h. The content of aminoguanidine, AbeAdo and polyamines or polyamine analogues in the media was maintained with each passage. The growth of these cells over the periods of days 13–17 and 18–22 was expressed as the percentage of cells present on days 13 and 18 respectively.

Assay of deoxyhypusine formation and of unmodified eIF-5A content

Formation of deoxyhypusine *in vitro* was determined with [³H]spermidine as a substrate and measuring the incorporation of radioactivity into protein of *M_r* 18000 [12]. The amount of unmodified eIF-5A present in cell extracts was determined by incubating extracts from the cells with deoxyhypusine synthase and [³H]spermidine [13].

RESULTS

Abilities of 1-methylspermidine and 1,12-dimethylspermine to reverse growth inhibition by DFMO in CHO and CHOMG cells

When CHO cells were grown in DFMO for 4 days, the growth rate was greatly diminished, spermidine was decreased by > 95% and putrescine was completely depleted. Both 1-methylspermidine and 1,12-dimethylspermine could reverse the effect on growth (Table 1). When added at 10 µM concentrations, both of the methylated polyamines were as effective as spermidine in supporting growth for 3 days (results not shown). 1,12-Dimethylspermine was not converted into any metabolites. 1-Methylspermidine was converted into 1-methylspermine to a small extent (Table 1). The importance of the polyamine-transport system for the uptake of the methylated polyamine derivatives is shown by their inability to increase the growth rate of CHOMG cells treated with DFMO (Table 1). CHOMG cells lack the polyamine-transport system [19,20].

Effects of 1-methylspermidine and 1,12-dimethylspermine on deoxyhypusine synthase activity

The ability of 1-methylspermidine or 1,12-dimethylspermine to compete with spermidine in the deoxyhypusine synthase reaction was determined *in vitro*. Cell extracts containing deoxyhypusine synthase were incubated with [³H]spermidine and unmodified eIF-5A, and the formation of ³H-labelled eIF-5A was determined. The inhibition of this incorporation when the unlabelled polyamine analogues were added indicates whether they are potential alternative substrates for deoxyhypusine synthase. 1-Methylspermidine inhibited [³H]spermidine incorporation, whereas 1,12-dimethylspermine did not (Table 2).

Effects of spermidine, spermine, 1-methylspermidine and 1,12-dimethylspermine on AbeAdo-induced cytostasis

As previously reported [12], the growth of L1210 cells exposed to

Table 1 Effect of methylated polyamines on growth of CHO and CHOMG cells treated with DFMO

The cells were grown in 5 mM DFMO for 4 days. The compounds shown were then added at 10 μ M concentrations and the cells grown for a further 3 days before harvesting and the determination of cell number and polyamine content. Putrescine and spermidine were decreased by more than 95% in the DFMO-treated cells to levels of 0.3 ± 0.1 nmol/mg for spermidine and < 0.2 nmol/mg for putrescine. The polyamine levels in CHO cells which were not treated with DFMO were 10.7 nmol/mg of protein for putrescine, 18.3 nmol/mg for spermidine and 10.4 nmol/mg for spermine. The polyamine levels in untreated CHOMG cells were not significantly different from those in CHO cells. Results are shown as means $\pm$ S.D. for 4 estimations. N.D., not detected (the detection limit was about 0.2 nmol/mg).

Addition	Cells	Polyamines (nmol/mg)				Growth (fold increase)
		Spermine	1-Methylspermidine	1-Methylspermine	1,12-Dimethylspermine	
None	CHO	9.5 ± 2.7	N.D.	N.D.	N.D.	1.1
Spermidine	CHO	$5.3 \pm 2.9^*$	N.D.	N.D.	N.D.	7.1
1-Methylspermidine	CHO	6.6 ± 2.8	31.0 ± 5.2	3.1 ± 1.1	N.D.	7.2
1,12-Dimethylspermine	CHO	1.4 ± 0.6	N.D.	N.D.	39.2 ± 6.1	6.8
None	CHOMG	8.9 ± 2.2	N.D.	N.D.	N.D.	1.1
Spermidine	CHOMG	7.8 ± 2.0	N.D.	N.D.	N.D.	1.1
1-Methylspermidine	CHOMG	7.3 ± 2.1	0.6 ± 0.2	N.D.	N.D.	1.3
1,12-Dimethylspermine	CHOMG	8.2 ± 0.8	N.D.	N.D.	N.D.	1.1

* These cells also contained spermidine at a level of 24.3 ± 3.4 nmol/mg.

Table 2 Effect of methylated polyamines on formation of deoxyhypusine in eIF-5A

1-Methylspermidine and 1,12-dimethylspermine were added at the indicated concentrations to the standard deoxyhypusine synthase assay mixture which contained ^{3} H-labelled spermidine as a substrate for the formation. The results are expressed as a percentage of incorporation of ^{3} H label into eIF-5A in the absence of either 1-methylspermidine or 1,12-dimethylspermine. Results are the means of triplicate assays which agreed within $\pm 10\%$.

Concn. of analogue (μ M)	Incorporation of [^{3} H]spermidine into eIF-5A (%)	
	1-Methylspermidine	1,12-Dimethylspermine
0	100	100
1	113	107
10	96	115
100	22	123
500	9	100

10 μ M AbeAdo decreased over a 12-day exposure and ceased completely by day 10 of exposure. The cell number of cultures plated in the presence of AbeAdo and maintained in the presence of the drug remained constant between days 10 and 12. On day 12, some of the AbeAdo-treated cells were re-plated in the presence of 10 μ M AbeAdo and 1 mM aminoguanidine with the addition of spermidine, 1-methylspermidine, spermine or 1,12-dimethylspermine, and growth was determined over the next 9 days (Figure 1). (The cells were re-plated and provided with fresh medium at day 17.) Cells exposed just to AbeAdo did not grow at all, and cells exposed to spermidine or spermine were rescued from cytotaxis as previously described [12,13]. The maximal effect on growth was observed at concentrations of 2 μ M spermine or 10 μ M spermidine. Addition of 10 μ M spermine or higher concentrations had a toxic effect on the cells. Although initially the effect of spermidine was less than that of spermine, at longer times spermidine was more effective (Figure 1). This may be due to the more rapid accumulation of spermine in the

Figure 1 Effects of polyamines and methylated analogues on the growth of L1210 cells treated with AbeAdo

L1210 cells were exposed to 10 μ M AbeAdo for 12 days. On day 12, portions of the cells were passaged in the presence of 10 μ M AbeAdo, 1 mM aminoguanidine, and in the presence and absence of the indicated polyamines as shown. On day 17, the cells were passaged again and maintained under the same conditions. The cells were counted on the days indicated, and the number listed is expressed as a percentage of that present 24 h after plating. The values are shown as means $\pm$ S.D. for counts from four separate flasks for each point.

Table 3 Effects of polyamines and methylated analogues on intracellular polyamine levels and eIF-5A metabolism in AbeAdo-treated L1210 cells

L1210 cells were exposed to 10 μ M AbeAdo for 12 days. On day 12, some cells were harvested for measurements. The remainder were passaged in the presence of 10 μ M AbeAdo, 1 mM aminoguanidine and the indicated polyamines, and harvested at 17 days for measurement of polyamine levels and unmodified eIF-5A as shown. Results are shown as means $\pm$ S.D. for 4–6 estimations.

Addition	Polyamines (nmol/mg of protein)				Unmodified eIF-5A (density/ μ g of protein)
	Putrescine	Spermidine	Spermine	Analogue	
Control L1210 cells*	3.2 $\pm$ 0.4	18 $\pm$ 4	4.3 $\pm$ 0.7	–	< 0.2
None (12 days)	79 $\pm$ 24	< 0.5	< 0.5	–	16.7 $\pm$ 3.5
None (17 days)	68 $\pm$ 7	< 0.5	< 0.5	–	25.0 $\pm$ 5.0
10 μ M spermidine	6.8 $\pm$ 2.1	36 $\pm$ 5	< 0.5	–	0.2 $\pm$ 0.1
10 μ M 1-methylspermidine	3.9 $\pm$ 1.6	< 0.5	< 0.5	30 $\pm$ 1†	8.3 $\pm$ 2.7
2 μ M spermine	66 $\pm$ 16	2.3 $\pm$ 1.1	6.5 $\pm$ 1.3	–	13.2 $\pm$ 3.0
2 μ M 1,12-dimethylspermine	21 $\pm$ 2	< 0.5	< 0.5	7.5 $\pm$ 1.4‡	26.0 $\pm$ 3.0

* L1210 cells not exposed to AbeAdo.

† 1-Methylspermidine. No 1,12-dimethylspermine or 1-methylspermine was detected in these cells.

‡ 1,12-Dimethylspermine. No 1-methylspermidine or 1-methylspermine was detected in these cells.

Table 4 Effects of inhibitors of polyamine oxidase on the formation of spermidine from spermine in AbeAdo-treated L1210 cells

L1210 cells were exposed to 10 μ M AbeAdo for 12 days. The indicated compounds were then added and AbeAdo was maintained at 10 μ M for a further 5 days. The cells were harvested at 17 days for measurement of polyamine levels. Results are shown as means $\pm$ S.D. for 4–6 estimations.

Addition	Polyamines (nmol/mg of protein)		
	Putrescine	Spermidine	Spermine
None (12 days)	46 $\pm$ 2	< 0.5	< 0.5
25 μ M MDL-72527	56 $\pm$ 3	< 0.5	< 0.5
25 μ M MDL-72527 + 2 μ M spermine	58 $\pm$ 3	2.3 $\pm$ 0.1	3.8 $\pm$ 0.4
25 μ M MDL-72527 + 10 μ M 1,12-dimethylspermine	28 $\pm$ 9	< 0.5	< 0.5
50 μ M MDL-72521	43 $\pm$ 4	< 0.5	< 0.5
50 μ M MDL-72521 + 2 μ M spermine	73 $\pm$ 11	3.1 $\pm$ 0.7	6.2 $\pm$ 1.8
50 μ M MDL-72521 + 10 μ M 1,12-dimethylspermine	33 $\pm$ 8	< 0.5	< 0.5

AbeAdo-treated cells. 1-Methylspermidine clearly reversed the effects of AbeAdo, although less well than the natural polyamines. However, 1,12-dimethylspermine was not able to reverse the AbeAdo-induced cytostasis when added at 2 μ M (Figure 1) or higher concentrations (results not shown).

Effects of spermidine, spermine, 1-methylspermidine and 1,12-dimethylspermine on polyamine and eIF-5A metabolism in cells chronically treated with AbeAdo

Cells were exposed to AbeAdo for 12 days and portions were harvested. Other portions were then exposed to AbeAdo in the presence of spermidine, spermine, 1-methylspermidine or 1,12-dimethylspermine for 5 days and then harvested on day 17. The cell pellets were then used for analysis of polyamine and unmodified eIF-5A levels (Table 3). Putrescine levels were greatly elevated, whereas spermidine and spermine levels were depleted

by day 12 of AbeAdo exposure and a virtually identical polyamine profile was detected in cells exposed to AbeAdo for 17 days. Putrescine levels were depressed by spermidine, 1-methylspermidine and to a much lesser extent by 1,12-dimethylspermine, but not by spermine. This decrease is presumably due to the repression of ODC, which is known to be negatively regulated by polyamines [23–25]. Spermidine levels were restored to above normal values by the addition of spermine. There was a modest rise in spermidine in the cells treated with spermine. The methylated polyamines accumulated in the cells treated with them, but did not give rise to any metabolites, confirming their usefulness as inert analogues of the parent polyamines. The unmodified form of eIF-5A was undetectable in the control L1210 cells, but accumulated to a large extent in the cells treated with AbeAdo. Addition of spermidine completely reversed this accumulation, and both 1-methylspermidine and spermine also led to a significant decrease in the amount of unmodified eIF-5A. However, 1,12-dimethylspermine had no effect on the content of unmodified eIF-5A (Table 3).

The formation of deoxyhypusine in eIF-5A in response to spermine is presumably due to the production of spermidine from spermine in the treated cells. Attempts were made to block the conversion of spermine into spermidine by the addition of inhibitors of polyamine oxidase [18,26], but these attempts were unsuccessful. Even in the presence of 25 μ M MDL 72527 or 50 μ M MDL 72521, there was an increase in spermidine in the spermine-treated cells (Table 4). No spermidine was formed in cells treated with 1,12-dimethylspermine.

DISCUSSION

The ability of the C-methylated analogues, 1-methylspermidine and 1,12-dimethylspermine, to induce the B–Z transition in poly(dC–dG), poly(5-MedC–dG) and small oligodeoxynucleotides has been studied by c.d. spectroscopy (B. Varnado and J. K. Coward, unpublished work). In addition, the pH-dependence of the ^{13} C-n.m.r. spectra of the C-methylated analogues has been determined (C. J. Voci and J. K. Coward, unpublished work). The comparisons of both the c.d. and ^{13} C-n.m.r. spectra of methylated versus non-methylated polyamines demonstrated that C-methylation α to the terminal amino groups results in little, if any, perturbation in polyamine structure, as monitored

by their ability to interact with either oligodeoxynucleotides (c.d.) or protons (n.m.r.). These results suggest that these compounds are useful analogues for the investigation of the function of polyamines. Their utility is supported by the results in Tables 1 and 2. The inability of these methylated polyamines to enter CHOMG cells shows that they are transported by the polyamine-transport system, and the lack of conversion of 1,12-dimethylspermine into other polyamines confirms that this derivative is not metabolized [16]. In CHO cells treated with DFMO, there was a small extent of conversion of 1-methylspermidine into 1-methylspermine. This is consistent with the weak activity of 1-methylspermidine as a substrate for spermine synthase [16] and the fact that decarboxylated *S*-adenosyl-methionine (dcAdoMet), which is used by spermidine synthase to form spermine, accumulates to very high levels in DFMO-treated cells [23–25]. In the AbeAdo-treated L1210 cells, there was no conversion of 1-methylspermidine into 1-methylspermine (Table 3), due to the complete blockage of the formation of dcAdoMet in these cells. The inability of DFMO to deplete spermine totally in CHO cells (Table 1) is similar to that found in many other experiments with many different cell types, including L1210 (reviewed in [23–25]). It is due to a number of factors, including: (a) the lack of complete inhibition of ODC; (b) the large increase in dcAdoMet, which ensures that any residual putrescine is efficiently converted into spermidine and then into spermine; and (c) the greatly decreased rate of cell growth which occurs within, at most, 2–3 days in the spermidine- and putrescine-depleted cells. This decrease in cell division leads to the maintenance of the spermine pools, since the degradation rate of spermine is very slow in cells exposed to inhibitors of polyamine formation [24]. Both 1-methylspermidine and 1,12-dimethylspermine are able to overcome the growth inhibition by DFMO (Table 1, and [16]). However, only 1-methylspermidine could overcome the cytostasis occurring after extended exposure to AbeAdo (Figure 1).

The results support the idea that cytostasis induced by the *S*-adenosyl-L-methionine decarboxylase inhibitor AbeAdo is a result of the depletion of hypusine in eIF-5A that occurs in the absence of spermidine [12,13]. The greatly elevated intracellular putrescine levels sustain limited cell growth in AbeAdo-treated cells by fulfilling many of the non-specific biochemical roles of spermidine and spermine. For this reason, the L1210 cells initially grow better in the presence of AbeAdo than in the presence of DFMO, since the ODC inhibitor prevents most of the synthesis of putrescine and spermidine [12,23,24]. However, the growth of AbeAdo-treated cells that occurs in the absence of spermidine results in the depletion of eIF-5A containing hypusine and the build-up of the unmodified form of eIF-5A. Since hypusine addition to eIF-5A has been shown to be required for normal yeast cell growth (see below), it is likely that the depletion of hypusine in eIF-5A in AbeAdo-treated cells interferes with cell growth and results in cytostasis and cell death. This hypothesis is supported by the fact that AbeAdo-induced cytostasis is reversed by spermidine, 1-methylspermidine and *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene [12,13], all of which are substrates of deoxyhypusine synthase, and by the observation that 1,12-dimethylspermine and other polyamine analogues [12] that are not substrates of deoxyhypusine synthase do not reverse this cytostasis.

Although many polyamine analogues relieve the growth inhibition produced by the ODC inhibitor DFMO in short-term experiments lasting only 1–3 days [24,25], only two polyamine analogues capable of reversing AbeAdo-induced cytostasis have been identified: *N*-(3-aminopropyl)-1,4-diamino-*cis*-but-2-ene [12] and 1-methylspermidine. The ability of 1-methylspermidine

to support the growth of cells chronically treated with AbeAdo is of particular interest, since (a) it was not converted into a spermine derivative and (b) the methyl group on the terminal carbon of the aminopropyl portion of the analogue does not appear to prevent the transfer of the 4-aminobutyl group to lysine-50 on eIF-5A by deoxyhypusine synthase, forming deoxyhypusine. The observation that 1-methylspermidine decreases elevated unmodified eIF-5A levels in previously AbeAdo-treated L1210 cells (Table 3) provides good evidence that this polyamine analogue is a substrate for deoxyhypusine synthase. This is consistent with the finding that 1-methylspermidine acts as a competitive inhibitor of the incorporation of labelled spermidine into eIF-5A by deoxyhypusine synthase (Table 2), which agrees with the recent report by Jakus et al. [27]. Although 1-methylspermidine is less active than spermidine in this assay *in vitro*, it should be noted that it accumulates to a high level in the treated cells (Tables 1 and 3), well above the reported K_m values for spermidine in the deoxyhypusine synthase reaction (which are 1–4.5 μM [4,27]). The fact that 1-methylspermidine allows growth of AbeAdo-treated cells in the absence of formation of a spermine analogue indicates that there is no absolute requirement for spermine in L1210 cells under these culture conditions.

Although spermine is not a substrate for deoxyhypusine synthase [28], provision of exogenous spermine reversed AbeAdo-induced cytostasis (Figure 1, and [12,13]). However, analysis of intracellular polyamines revealed that cells exposed to exogenous spermine contained both spermine and spermidine. The appearance of spermidine in these cells is most likely the result of the retroconversion of spermine into spermidine via the sequential action of spermidine/spermine *N*¹-acetyltransferase and polyamine oxidase [14,15,25,26], but attempts to test whether spermine, in the absence of spermidine, could reverse AbeAdo-induced cytostasis were thwarted by the inability of two different polyamine oxidase inhibitors, MDL 72521 and MDL 72527, to prevent the conversion of exogenous spermine into spermidine. This is quite surprising, in view of the published potency of these inhibitors against polyamine oxidase [18,26,29,30], and raises the possibility that another pathway for the conversion of spermine into spermidine exists. Such a pathway has not been described, but one possibility would be that spermidine formation occurs via the further metabolism of the terminal oxidation product *N*⁸-(2-carboxyethyl)spermidine, which is known to be formed from spermine by mammalian enzymes [31,32].

The inability of polyamine oxidase inhibitors to prevent the back-conversion of exogenous spermine into spermidine precluded the use of these inhibitors to distinguish between spermine and spermidine as 'depot forms' of the aminobutyl moiety appended to a specific lysine residue of eIF-5A during deoxyhypusine biosynthesis. However, the use of 1-methylspermidine and 1,12-dimethylspermine allowed us to distinguish between spermidine and spermine in hypusine biosynthesis, as monitored by the reversal of AbeAdo-induced cytostasis. Thus 1,12-dimethylspermine, a non-metabolizable analogue of spermine [16], was not converted into 1-methylspermidine in the treated cells and did not decrease the content of unmodified eIF-5A. The inability of 1,12-dimethylspermine to reverse AbeAdo-induced cytostasis and the apparent inability of this compound to act as a substrate for deoxyhypusine synthase is consistent with the hypothesis that spermine must be metabolized to spermidine for hypusine formation and subsequent cell growth of AbeAdo-treated cells.

Several papers (reviewed in [2,3]) have demonstrated that the formation of the hypusine in eIF-5A correlates with cell growth. The elegant studies by Hershey and colleagues have shown clearly that eIF-5A containing hypusine is needed for cell viability

in yeast [1,6–9]. The original identification of eIF-5A as a protein-synthesis initiation factor and the demonstration that the presence of hypusine in the protein was needed for its ability to stimulate methionyl-puromycin synthesis [1,28] have focused attention on the possibility that the key role of this protein in cell growth is related to protein synthesis. However, recent studies in which the rapid depletion of eIF-5A was engineered in yeast by replacing the endogenous gene with one containing a labile form of eIF-5A under the control of an inducible promoter have indicated that protein synthesis was only slightly affected for 5 h, whereas cell division stopped within one generation (2 h) of the decline in eIF-5A [33]. These results raise the possibility that eIF-5A containing hypusine may play some other role in cell-cycle regulation, but the selective stimulation of the translation of a small number of mRNAs coding for key proteins is not ruled out. The possible regulatory importance of eIF-5A is also suggested by the discovery that it is phosphorylated in yeast [34,35], although the role of such phosphorylation in its activity remains to be established.

The sensitivity of cell proliferation to perturbation of eIF-5A metabolism suggests that eIF-5A may present a target for chemotherapy. AbeAdo is capable of indirectly depleting the functional form of eIF-5A in cell culture, but AbeAdo resistance due to decreased drug accumulation was readily acquired in cultured cells [13] and AbeAdo is rapidly metabolized in rats and mice [36]. Thus, alternative approaches to perturb hypusine formation and eIF-5A function may be desirable. The fungicide guazadine was found to be inhibitory to deoxyhypusine synthase [37]. This finding has led to the development of some other guanyl derivatives of diamines and polyamines, including *N*¹-guanyl-1,7-diaminoheptane, as effective inhibitors of deoxyhypusine synthase [27], but their selectivity has not been demonstrated. Inhibition of deoxyhypusine hydroxylase by metal-chelating agents and other agents [38] has also been reported, and these agents arrest cell-cycle progression [2,3], but the relationship of this effect to the lack of hypusine formation has not yet been established. Another interesting possibility to probe hypusine function and to produce such inhibitors would be to design polyamine analogues that are substrates for deoxyhypusine synthase, but which result in inactive modifications of eIF-5A. Such a compound could theoretically compete with spermidine for unmodified eIF-5A and result in the depletion of functional eIF-5A. A possible lead compound in this approach is *N*-(3-aminopropyl)-1,4-diamino-*trans*-but-2-ene, which appeared to be a substrate for deoxyhypusine synthase, but, unlike its *cis*-isomer, did not support the growth of AbeAdo-treated cells [12]. It is therefore possible that the *trans*-unsaturated deoxyhypusine was not recognized by deoxyhypusine hydroxylase, and/or the aberrantly modified eIF-5A was not functionally active. Chronically AbeAdo-treated cells offer a unique model in which to study the role of hypusine and eIF-5A in cell proliferation, and may provide a model for the examination of the therapeutic potential of perturbation of this hypusine metabolism and function.

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REFERENCES

- Hershey, J. W. B., Smit-McBride, Z. and Schnier, J. (1990) *Biochim. Biophys. Acta* **1050**, 160–162
- Park, M. H., Wolff, E. C. and Folk, J. E. (1993) *BioFactors* **4**, 95–104
- Park, M. H., Wolff, E. C. and Folk, J. E. (1993) *Trends Biochem. Sci.* **18**, 475–479
- Wolff, E. C., Park, M. H. and Folk, J. E. (1990) *J. Biol. Chem.* **265**, 4793–4799
- Abbruzzese, A., Park, M. H. and Folk, J. E. (1986) *J. Biol. Chem.* **261**, 3085–3089
- Smit-McBride, Z., Dever, T. E., Hershey, J. W. B. and Merrick, W. C. (1989) *J. Biol. Chem.* **264**, 1578–1583
- Smit-McBride, Z., Schnier, J., Kaufman, R. J. and Hershey, J. W. B. (1989) *J. Biol. Chem.* **264**, 18527–18530
- Schnier, J., Schwelberger, H. G., Smit-McBride, Z., Kang, H. A. and Hershey, J. W. B. (1991) *Mol. Cell. Biol.* **11**, 3105–3114
- Schwelberger, H. G., Kang, H. A. and Hershey, J. W. B. (1993) *J. Biol. Chem.* **268**, 14018–14025
- Casara, P., Marchal, P., Wagner, J. and Danzin, C. (1989) *J. Am. Chem. Soc.* **111**, 9111–9113
- Danzin, C., Marchal, P. and Casara, P. (1990) *Biochem. Pharmacol.* **40**, 1499–1503
- Byers, T. L., Ganem, B. and Pegg, A. E. (1992) *Biochem. J.* **287**, 717–724
- Byers, T. L., Wiest, L., Wechter, R. S. and Pegg, A. E. (1993) *Biochem. J.* **290**, 115–121
- Seiler, N. (1987) *Can. J. Physiol. Pharmacol.* **65**, 2024–2035
- Casero, R. A. and Pegg, A. E. (1993) *FASEB J.* **7**, 653–661
- Lakonen, J. R., Coward, J. K. and Pegg, A. E. (1992) *J. Med. Chem.* **35**, 724–734
- Metcalfe, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. and Vevert, J. P. (1978) *J. Am. Chem. Soc.* **100**, 2551–2553
- Bey, P., Bolkenius, F. N., Seiler, N. and Casara, P. (1985) *J. Med. Chem.* **28**, 1–2
- Mandel, J. and Flintoff, W. F. (1978) *J. Cell. Physiol.* **97**, 335–344
- Byers, T. L., Wechter, R., Nuttall, M. and Pegg, A. E. (1989) *Biochem. J.* **263**, 745–752
- Seiler, N., Bolkenius, F. N. and Knöden, B. (1985) *Biochem. J.* **225**, 219–226
- Byers, T. L. and Pegg, A. E. (1989) *Am. J. Physiol.* **257**, C545–C553
- Pegg, A. E. and McCann, P. P. (1982) *Am. J. Physiol.* **243**, C212–C221
- Pegg, A. E. (1986) *Biochem. J.* **234**, 249–262
- Pegg, A. E. (1988) *Cancer Res.* **48**, 759–774
- Bolkenius, F. N., Bey, P. and Seiler, N. (1985) *Biochim. Biophys. Acta* **838**, 69–76
- Jakus, J., Wolff, E. C., Park, M. H. and Folk, J. E. (1993) *J. Biol. Chem.* **268**, 13151–13159
- Park, M. H., Wolff, E. C., Smit-McBride, Z., Hershey, J. W. B. and Folk, J. E. (1991) *J. Biol. Chem.* **266**, 7988–7994
- Bolkenius, F. N. and Seiler, N. (1987) *Biochim. Biophys. Acta* **923**, 125–135
- Sarhan, S., Quemener, V., Moulinoux, J. P., Knöden, B. and Seiler, N. (1991) *Int. J. Biochem.* **23**, 617–626
- Seiler, N., Bolkenius, F. N. and Rennert, O. M. (1981) *Med. Biol.* **59**, 334–346
- van den Berg, G. A., Nagel, G. T. and Muskiet, F. A. J. (1985) *J. Chromatogr.* **339**, 223–231
- Kang, H. A., Schwelberger, H. G. and Hershey, J. W. B. (1993) in *Protein Synthesis and Targeting in Yeast* (Tuite, M., ed.), NATO Series H, vol. 71, pp. 123–129, Springer Verlag, Berlin
- Kang, H. A., Schwelberger, H. G. and Hershey, J. W. B. (1993) *J. Biol. Chem.* **268**, 14750–14756
- Klier, H., Wohl, T., Eckerskorn, C., Magdolen, V. and Lottspeich, F. (1993) *FEBS Lett.* **334**, 360–364
- Byers, T. L., Bush, T. L., McCann, P. P. and Bitonti, A. J. (1991) *Biochem. J.* **274**, 527–533
- Murphey, R. J. and Gerner, E. W. (1987) *J. Biol. Chem.* **262**, 15033–15036
- Abbruzzese, A., Hanauske-Abel, H. M., Park, M. H., Henke, S. and Folk, J. E. (1991) *Biochim. Biophys. Acta* **1077**, 159–166