Effects of chronic 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (AbeAdo) treatment on polyamine and eIF-5A metabolism in AbeAdo-sensitive and -resistant L1210 murine leukaemia cells

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We have previously reported that prolonged chronic exposure to the *S*-adenosyl-L-methionine decarboxylase (AdoMetDC) inhibitor, 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL 73811, AbeAdo), leads to cytostasis of L1210 cells [Byers, Ganem and Pegg (1992) *Biochem. J.* **287**, 717–724]. Further studies to investigate the mechanism by which these effects are brought about were carried out by comparing an L1210-derived cell line (R20) that is resistant to AbeAdo with the parent cells. The R20 cells were derived by two rounds of AbeAdo-induced cytostasis followed by rescue with exogenous polyamines. Cytostasis was induced in L1210 cells treated for 12 days with 10 μ M AbeAdo; however, exposure to up to 40 μ M AbeAdo did not induce cytostasis in R20 cells. Putrescine levels were elevated and spermine levels were depleted in both treated L1210 and treated R20 cells. Spermidine was depleted in treated L1210 cells but was only partly reduced in treated R20 cells. AdoMetDC activity was

below the limit of detection in treated L1210 cells but, although greatly reduced, could be measured in the treated R20 cells. The resistance of the R20 cells to the effects of AbeAdo on cell growth and spermidine depletion correlated with reduced AbeAdo accumulation by R20 cells. In the absence of spermidine synthesis, unhyposinated eukaryotic translation initiation factor 5A (eIF-5A) accumulated in AbeAdo-treated L1210 cells. There was no detectable accumulation of unhyposinated eIF-5A in R20 cells. Unhyposinated eIF-5A accumulated during AbeAdo treatment was depleted in L1210 cells rescued by exogenous spermidine. These findings are consistent with the hypothesis that AbeAdo-induced cytostasis is due to the loss of hypusinated eIF-5A. However, spermine was able to rescue AbeAdo-treated L1210 cells without significantly reducing the unhyposinated eIF-5A accumulated during AbeAdo treatment, suggesting that only a small amount of the unmodified protein must be hypusinated to restore cell growth.

INTRODUCTION

It has long been recognized that the activity of ornithine decarboxylase (ODC), the first enzyme in the polyamine-biosynthetic pathway that forms putrescine, is necessary for mammalian cell growth (Mamont et al., 1978; Pohjanpelto et al., 1981, 1985; Steglich and Scheffler, 1982). Many studies have now been carried out with inhibitors of this enzyme [reviewed by Pegg and McCann (1982), Danzin and Mamont (1987) and Pegg (1988)]. Putrescine is subsequently converted into spermidine by spermidine synthase, which catalyses the transfer of an aminopropyl group from decarboxylated AdoMet. The same aminopropyl donor is needed as a substrate for the action of spermine synthase, in order to convert spermidine into spermine. *S*-Adenosyl-L-methionine decarboxylase (AdoMetDC) is needed for the decarboxylation of AdoMet, and thus is required for spermidine and spermine synthesis. ODC inhibition not only directly prevents putrescine synthesis, but also indirectly limits spermidine and spermine production. Therefore when ODC is inhibited, putrescine and spermidine are depleted by their conversion into spermine. In the absence of intracellular putrescine and spermidine, the cells enter cytostasis, preventing the further dilution of intracellular spermine by cell growth. Although the polyamines are known to act as permissive factors in many biochemical reactions involved with cell growth, presumably via their ionic interaction with acidic biological macromolecules, a specific physiological reason for ODC-inhibitor-induced

cytostasis has not been determined. A knowledge of a cell's precise requirements for polyamines in proliferation would aid in identifying specific physiological functions for the polyamines. Putrescine itself may not be required for cell growth in the presence of an exogenous spermidine source (Porter and Bergeron, 1983). However, the use of aminopropyltransferase inhibitors to selectively alter intracellular spermidine or spermine levels has not conclusively defined the roles of these polyamines (Pegg et al., 1982; Pegg and Coward, 1985).

Inhibitors of AdoMetDC are therefore of major importance in designing experimental protocols to investigate the physiological role of the polyamines. Unfortunately, until recently, the use of such inhibitors has been limited by their lack of specificity [in the case of compounds such as methylglyoxal bis(guanyldiazide)] or lack of stability (in the case of active-site-directed inhibitors that form a covalent bond with the pyruvate prosthetic group of AdoMetDC) [reviewed by Pegg (1988)]. A more stable enzyme-activated irreversible inhibitor, AbeAdo, has now been synthesized (Casara et al., 1989). This compound has allowed a more detailed study of the effects of inhibition of AdoMetDC on cell growth (Seiler et al., 1991; Byers et al., 1992b) and shows considerable promise as a therapeutic agent (Bitonti et al., 1990; Seiler et al., 1991; Byers et al., 1992b).

Treatment of L1210 cells with AbeAdo depleted intracellular spermidine and spermine, greatly increased putrescine, and resulted in reduction of cell growth over a 12-day period followed by cytostasis and apparent loss of viability (Byers et al., 1992b).

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

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Similar reductions in cell growth in response to AdoMetDC inhibition have been reported previously (Pegg et al., 1988a; Kramer et al., 1989) but these experiments were of limited duration and failed to demonstrate the cytostatic result of prolonged AdoMetDC inhibition (Byers et al., 1992b). Because the onset of this cytostasis was delayed relative to spermidine and spermine depletion, it was suggested that elevated putrescine levels were able to support limited cell growth by fulfilling some general permissive physiological roles normally fulfilled by spermidine and spermine by virtue of their polybasic nature. However, after a time, a more specific spermidine- and/or spermine-requiring function apparently became unfilled leading to cytostasis. The hypusination of eukaryotic elongation factor 5A (eIF-5A), which requires the transfer of the 4-aminobutyl moiety of spermidine to the ϵ -nitrogen of the lysine at position 50, is the only known specific requirement for any of the polyamines in mammalian cells. Hypusination of eIF-5A is required for eIF-5A function *in vitro* (Park, 1989; Smith-McBride et al., 1989) and is required for normal yeast cell growth (Schnier et al., 1991). Therefore depletion of hypusinated eIF-5A in the absence of spermidine may be responsible for AbeAdo-induced cytostasis (Byers et al., 1992b). This theory was supported by the finding that spermidine and the spermidine analogue *N*-(3-aminopropyl)-1,4-diamino-*cis*-2-butene, which is a substrate for the eIF-5A hypusination reaction *in vitro*, were able to reverse the effect of AbeAdo but that spermidine analogues that are not substrates for this reaction could not rescue AbeAdo-treated cells.

In order to obtain more information on the mechanism by which exposure to AbeAdo affects cell growth, we have now derived from L1210 cells, a cell line, R20, that is resistant to the cytostatic effects of AbeAdo. This paper contains information on the mechanism of resistance and the effects of AbeAdo exposure on growth, polyamine metabolism and eIF-5A metabolism in L1210 and R20 cells.

MATERIALS AND METHODS

Chemicals

AbeAdo and MDL 72521, which were generous gifts from Marion Merrell Dow, Cincinnati, OH, U.S.A., were synthesized at Marion Merrell Dow Research Institutes, Cincinnati, OH, U.S.A. and Strasbourg, France (Bey et al., 1985; Casara et al., 1989). Dr. Bruce Ganem, Department of Chemistry, Cornell University, Ithaca, NY, U.S.A. synthesized the *N*-(3-aminopropyl)-1,4-diamino-*cis*-2-butene. All biochemical reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. and Fisher Scientific, Pittsburgh, PA, U.S.A.

Cell culture

Suspension cultures of L1210 cells were grown at 37 °C in RPMI-1640 medium containing 10 % (v/v) 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 *N*-(3-aminopropyl)-1,4-diamino-*cis*-2-butene were added to the medium, 1 mM aminoguanidine was also added to the cultures to prevent the toxic effects of exogenous polyamine degradation by serum oxidases. The cells were plated at densities such that the cultures would not reach confluence before harvest or passage. Control cells were plated at 5×10^4 cells/ml, and cells exposed to AbeAdo before passage were plated at higher densities that depended on the length of exposure. Typically, cells exposed to AbeAdo for 4 days or 8 days were plated at 2×10^5 cells/ml or 5×10^5 cells/ml respectively

for continued AbeAdo treatment. R20 cells exposed to AbeAdo for 12 days or longer were plated at 1×10^5 cells/ml.

Development of AbeAdo-resistant L1210 cells (R20 cells)

L1210 cells rescued from a 12-day exposure to 10 μ M AbeAdo by the addition of 10 μ M spermidine, 5 μ M spermine and 1 mM aminoguanidine to the media were found to be resistant to subsequent exposure to 10 μ M AbeAdo. However, these cells (R10 cells) were sensitive to a 12-day exposure to 20 μ M AbeAdo, exhibiting a reduction in growth similar to that seen in L1210 cells exposed to 10 μ M AbeAdo. R20 cells were derived from R10 cells exposed to 20 μ M AbeAdo for 12 days and then rescued with exogenous polyamines. The R20 cells were characterized further in an attempt to determine the mechanism of the growth-inhibitory effects of AbeAdo. R20 cells were maintained in the presence of 20 μ M AbeAdo. In order to ensure that there was no residual AbeAdo in these cells at the start of the experiment, R20 cells were grown in the absence of AbeAdo for 7 days before use in experiments in which the effects of AbeAdo on L1210 cells and R20 cells were compared.

Chronic AbeAdo treatment of L1210 and R20 cells

To determine the effect of chronic AbeAdo treatment on cell growth, polyamine metabolism and eIF-5A metabolism, L1210 and R20 cells were plated in the presence and absence of AbeAdo. The L1210 cells were exposed to 10 μ M AbeAdo and the R20 cells were exposed to 10, 20 or 40 μ M AbeAdo. These cells were passaged every 96 h throughout the remainder of the experiment and were maintained in media containing the appropriate AbeAdo concentration. The cells were counted using an electronic cell counter (Coulter Electronics) every 24 and 48 h after plating to determine cell-growth rates which were expressed as a percentage of the growth rate of untreated cells. Representative flasks of L1210 and R20 cells were harvested on days 4, 8 and 12, and R20 cells were also harvested on days 16 and 20.

Polyamine, AdoMet and AbeAdo quantification

Cells were harvested by centrifugation, washed twice with ice-cold PBS, and 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 μ m) 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 a reversed-phase Beckman Ultrasphere Ion Pairing 5 μ M C₁₈ h.p.l.c. column and post-column derivatization with *o*-phthalaldehyde (Fluka Chemical Corp., Ronkonkoma, NY, U.S.A.); fluorescence detection was as described previously (Seiler and Knödgen, 1985; Byers and Pegg, 1989). AdoMet and decarboxylated AdoMet were separated using the same method as for the polyamines but were detected by u.v. absorbance at 254 nm. AbeAdo was separated on the same h.p.l.c. column using conditions and u.v. detection as described previously (Byers et al., 1991).

Quantification of AdoMetDC activity and AdoMetDC protein

Cells washed with PBS were centrifuged and the cell pellet was resuspended in 50 mM Tris/HCl (pH 7.5) containing 2.5 mM dithiothreitol and 0.1 mM EDTA. The cells were lysed by several rounds of freeze-thawing, and the insoluble material was re-

moved by centrifugation at 17000 *g* for 20 min. The supernatant was assayed for AdoMetDC activity as reported previously (Pegg, 1984). The amount of AdoMetDC protein present in the cell extracts was determined by Western immunoblotting as described by Pegg et al. (1988b) except that the final detection was carried out using the ECLC system (Amersham International). The bands corresponding to AdoMetDC were quantified using a scanning laser densitometer. A standard amount of AdoMetDC protein was loaded on to one lane in each gel and used for the relative quantification of different blots.

Quantification of unhyposinated eIF-5A

The quantification of unmodified eIF-5A was described previously (Byers et al., 1992b). Briefly, a 45–75 % -satd. $(\text{NH}_4)_2\text{SO}_4$ protein fraction was prepared from samples in which unmodified eIF-5A was to be measured. This fraction was then incubated with a deoxyhypusine synthase preparation from untreated L1210 cells and $[\text{H}^3]$ spermidine. The ^3H -labelled eIF-5A formed in the sample was then separated on a 12.5 % (w/v) polyacrylamide gel, fixed and subjected to fluorography. A single band corresponding to eIF-5A (18000 M_r) was detected and quantified by densitometric scanning. Bands on different gels were related using an internal standard run on each gel. The relative absorbance units/ μg of protein in the extracts indicated the amount of unmodified eIF-5A/ μg of protein in a given sample. Results from different experiments were related to one another by expressing the amount of unmodified eIF-5A in a given sample as a percentage of the eIF-5A content of cells exposed to AbeAdo for 12 days.

RESULTS

Effects of chronic AbeAdo exposure on L1210 and R20 cell growth

The effect of chronic AbeAdo exposure on the growth of L1210 and R20 cells is shown in Figure 1. By day 5, the growth-inhibitory effects of AbeAdo on L1210 and R20 cells were

Figure 1 Effect of AbeAdo on growth of L1210 and R20 cells

L1210 cells were exposed to 10 μM AbeAdo for 16 days. R20 cells were exposed to 10 μM , 20 μM or 40 μM AbeAdo for 20 days. Every 4 days the cells were passaged into fresh media containing the appropriate concentration of AbeAdo at a cell density such that the cells did not reach confluence before the next passage. Untreated (no AbeAdo) L1210 and R20 cells were passaged along with the treated cells. Treated and untreated cells were counted 24 and 48 h after each plating and cell growth was expressed as: cell growth = $100 \times (\text{percentage increase in treated cells over 24 h} / \text{percentage increase in untreated cells over 24 h})$. The values represent means of triplicates. On days 4, 8 and 12 for L1210 and R20 cells and on days 16 and 20 for R20 cells, representative flasks for each group were harvested for analysis of polyamines and AbeAdo by h.p.l.c., analysis of AdoMetDC activity, and analysis of AdoMetDC protein by immunoblotting (Table 1).

Figure 2 Effect of chronic AbeAdo treatment of L1210 and R20 cells on intracellular polyamine levels

L1210 and R20 cells were exposed to AbeAdo as described in the legend to Figure 1. At the times shown, representative flasks for each group were harvested for analysis of polyamines, putrescine, spermidine or spermine as shown. The polyamine values plotted represent the means of triplicate measurements and in all cases the standard deviations were less than 15 % of the mean value. ●, L1210 cells; ○, L1210 cells + 10 μM AbeAdo; ■, R20 cells; □, R20 cells + 10 μM AbeAdo; △, R20 cells + 20 μM AbeAdo; ◇, R20 cells + 40 μM AbeAdo

approximately equal. However, by day 9, there was an obvious difference in the response and, by day 13, L1210 cells exposed to 10 μM AbeAdo stopped growing entirely, whereas R20 cells continued to grow. Further exposure of the R20 cells to AbeAdo did not result in the cytostasis seen in L1210 cells. By day 17, these cells were growing at almost 100 % the rate of untreated R20 cells. Only 10 μM AbeAdo was needed to bring about the cessation of growth of L1210 cells but the R20 cells continued to grow in the presence of 20 or 40 μM . At these higher concentrations, it appeared that the growth was slightly reduced at 5–9 days but then recovered at longer times (Figure 1).

Effects of chronic AbeAdo exposure on L1210- and R20-cell polyamine levels

Cells were harvested throughout the experiment depicted in Figure 1 and analysed for polyamine content (Figure 2). The polyamine contents of untreated L1210 and R20 cells were essentially identical. Putrescine levels were increased over 100-fold by day 4 in L1210 cells treated with 10 μM AbeAdo. Spermidine and spermine levels were greatly reduced in treated

L1210 cells by day 4 and these reduced levels were maintained throughout the experiment. There was an AbeAdo dose-dependent increase in putrescine in R20 cells that amounted to at least 25-fold, although none of the R20 cells had putrescine levels quite as high as the treated L1210 cells. Although spermidine levels in the treated R20 cells did show AbeAdo dose-dependent reductions by day 4, the magnitude of these reductions (64–85 %) were less than that observed in the treated L1210 cells (93 %). After day 4, a time-dependent increase in spermidine was observed in the treated R20 cells. By day 20, the reduction in spermidine was only 38–63 %. The rise in spermidine levels in treated R20 cells relative to those on day 4 correlated with the time-dependent increase in the growth rate of treated R20 cells shown in Figure 1. The reduction in spermine (more than 90 %) observed in treated R20 cells by day 4 was similar to the decrease in spermine observed in treated L1210 cells and was maintained throughout the experiment.

Effects of chronic AbeAdo exposure on L1210 and R20 cell AdoMetDC activity and AdoMetDC protein levels

Although the polyamine contents of untreated L1210 and R20 cells were virtually identical, the AdoMetDC activity and protein levels in untreated R20 cells were actually slightly lower than in untreated L1210 cells. AdoMetDC activities in untreated cells were 114–136 pmol of CO₂ released/30 min per mg of protein for the L1210 cells and 56–68 pmol of CO₂ released/30 min per mg of protein for the R20 cells. AdoMet protein levels in untreated R20 cells were significantly less than in untreated L1210 cells as determined by densitometric scanning of Western immunoblots.

When the cells were exposed to 10 μ M AbeAdo, AdoMetDC activity in the L1210 cells was reduced below the level of detection (0.3 % of the control activity) by day 4 and remained undetectable throughout the experiment (Table 1). AdoMetDC activity was also undetectable in the treated R20 cells on day 4 but a small amount of activity was found in the treated R20 cells at all time points later than day 4. This value was only 0.5–2.3 % of the activity of untreated cells. As would be expected with the inhibition of AdoMetDC activity observed in L1210 cells, decarboxylated AdoMet levels went from 0.079 ± 0.009 nmol/mg of protein in untreated L1210 cells to undetectable levels (< 0.005 nmol/mg of protein) in AbeAdo-treated L1210 cells.

As shown in Table 1, exposure to AbeAdo caused a substantial increase in the amount of AdoMetDC protein, as determined by immunoblotting. This increase varied between 3.3- and 26-fold and is probably due to the increased synthesis of AdoMetDC in response to the decline in spermidine and spermine since both polyamines are known to repress AdoMetDC (Pegg, 1988; Madhubala et al., 1988; White and Morris, 1989; Autelli et al., 1991). Most of this protein is clearly inactivated by reaction with the inhibitor since, as mentioned above, in all cases the activity is less than 2.5 % of the control values. The considerable variation in levels of AdoMetDC protein seen in the AbeAdo-treated cells may be an artifact since the immunoblotting technique used to measure the protein level is not very accurate for an unabundant cell protein such as AdoMetDC but taken together the results provide strong evidence for an increase in protein levels. However, there is no clear indication of a greater increase in the R20 cells than the L1210 cells when the response to 10 μ M AbeAdo is considered.

AbeAdo levels in L1210 and R20 cells

AbeAdo was detectable in treated L1210 cells on day 4 (Figure 3). The level of this drug in treated L1210 cells increased from

Table 1 Effect of chronic AbeAdo treatment on AdoMetDC activity and protein levels

Cells were treated as described in the legend to Figure 1, and, at the times indicated, AdoMetDC activities and the amount of AdoMetDC protein were measured. These were expressed as the percentage of the control value (in cells not treated with AbeAdo) at that time. These control activities were 114–136 nmol of CO₂ released/30 min per mg of protein for the L1210 cells and 56–68 pmol of CO₂ released/30 min per mg of protein for the R20 cells. The minimum level of detection for AdoMetDC was 0.3 pmol of CO₂ released/30 min per mg of protein. The values represent the means of triplicate measurements and the variation in samples is given in standard deviations. N.D., not determined.

AbeAdo Treatment	Concn. (μ M)	Period (days)	L120 cell extracts		R20 cell extracts	
			AdoMetDC activity (% of control)	AdoMetDC protein (% of control)	AdoMetDC activity (% of control)	AdoMetDC protein (% of control)
10		4	< 0.3	255	< 0.4	550
		8	< 0.3	425	1.6 ± 0.2	1700
		12	< 0.3	255	2.2 ± 1.2	330
		16	N.D.	N.D.	0.9 ± 0.2	500
		20	N.D.	N.D.	1.3 ± 0.1	400
20		4	N.D.	N.D.	< 0.4	700
		8	N.D.	N.D.	2.3 ± 0.9	2500
		12	N.D.	N.D.	1.0 ± 0.3	1200
		16	N.D.	N.D.	0.9 ± 0.4	700
		20	N.D.	N.D.	0.5 ± 0.2	800
40		4	N.D.	N.D.	< 0.4	1700
		8	N.D.	N.D.	1.6 ± 0.7	2600
		12	N.D.	N.D.	2.3 ± 0.6	2300
		16	N.D.	N.D.	0.9 ± 0.3	1900
		20	N.D.	N.D.	0.5 ± 0.2	900

day 4 to day 8 and was roughly the same on day 12 as on day 8. AbeAdo was detectable in treated R20 cells throughout the experiment. However, except for day 4, when L1210 cells treated with 10 μ M AbeAdo and R20 cells treated with 40 μ M AbeAdo had virtually identical AbeAdo contents, the levels of this drug in all treated R20 cells were lower than in treated L1210 cells throughout the experiment. After day 4, there was a time-dependent decrease in AbeAdo in treated R20 cells that correlated with the time-dependent increase in cell growth and spermidine levels. These results suggest that either the uptake process for AbeAdo is decreased in R20 cells or an efflux or degradation mechanism is increased and that this effect becomes more active with time of exposure to the drug.

AdoMet levels in L1210 cells

Large increases in trypanosome AdoMet levels correlate with the potent antitrypanosomal action of AbeAdo (Byers et al., 1991). AdoMet levels were assayed in treated and untreated L1210 cells to determine if these levels were elevated by the chronic AbeAdo treatment which induces cytostasis in these cells. AdoMet levels in untreated L1210 cells and in L1210 cells treated with 10 μ M AbeAdo for 12 days were 3.6 ± 0.6 nmol/mg of protein and 4.7 ± 0.6 nmol/mg of protein respectively.

Effect of chronic AbeAdo exposure and exogenously supplied polyamines on eIF-5A metabolism

L1210 cells were treated with 10 μ M AbeAdo for 12 days, after which time representative cells were exposed to 10 μ M spermidine or 10 μ M spermine. Both spermidine and spermine

Figure 3 Accumulation of AbeAdo in L1210 and R20 cells

L1210 and R20 cells were exposed to AbeAdo as described in the legend to Figure 1. At the times shown, extracts of the cells were analysed for their content of AbeAdo. The values plotted represent means $\pm$ S.D. of triplicate measurements. Where no S.D. is seen, the variation was less than the size of the symbol. AbeAdo was below the level of detection (< 0.03 nmol/mg of protein) in L1210 cells from cultures to which $10 \mu\text{M}$ AbeAdo was added immediately before the cell wash and harvest, showing that the measured values do represent intracellular drug and not material adsorbed on the cell surface. $\circ$, L1210 cells + $10 \mu\text{M}$ AbeAdo; $\square$, R20 cells + $10 \mu\text{M}$ AbeAdo; $\triangle$, R20 cells + $20 \mu\text{M}$ AbeAdo; $\diamond$, R20 cells + $40 \mu\text{M}$ AbeAdo.

Figure 4 Ability of exogenously supplied spermidine and spermine to rescue L1210 cells from cytostasis induced by chronic exposure to AbeAdo

L1210 cells were exposed to $10 \mu\text{M}$ AbeAdo for 12 days during which they were passaged every 4 days. On day 12, cells were passaged into media containing $10 \mu\text{M}$ AbeAdo, 1 mM aminoguanidine and, where indicated, $10 \mu\text{M}$ spermidine or $10 \mu\text{M}$ spermine. The cells were counted over the next 5 days to determine the effects of exogenous spermidine and spermine on the growth of cells exposed to AbeAdo. The cell numbers listed are means $\pm$ S.D. of six flasks/group.

reversed the AbeAdo-induced cytostasis, although spermine appeared to rescue the cells faster and allowed them to grow to a higher density (Figure 4). This is in agreement with an earlier study (Byers et al., 1992b) in which it was suggested that the

Table 2 Unhypusinated eIF-5A levels in AbeAdo-treated L1210 cells and the effects of spermidine, spermine and *N*-(3-propylamine)-1,4-diamino-*cis*-butene on these levels

L1210 and R20 cells were plated in the presence of $10 \mu\text{M}$ AbeAdo. On days 4 and 8, the cells were passaged into fresh media containing $10 \mu\text{M}$ AbeAdo. On days 4, 8 and 12, cells were harvested for analysis of unhyposinated eIF-5A. On day 12 representative cells were passaged into media containing $10 \mu\text{M}$ AbeAdo, 1 mM aminoguanidine and, where indicated, $10 \mu\text{M}$ spermidine, $10 \mu\text{M}$ spermine or $10 \mu\text{M}$ *N*-(3-aminopropyl)-1,4-diamino-*cis*-2 butene. On day 17 the cells were harvested for analysis of unhyposinated eIF-5A. The values are the means $\pm$ S.D. of multiple assays from three to six flasks. The minimum level of detection of unhyposinated eIF-5A in this experiment was 4% of the amount present in AbeAdo-treated cells on day 12.

Treatment	Unhypusinated eIF-5A (% of 12-day value)	
	L1210 cells	R20 cells
Untreated	< 4.0	< 4.0
$10 \mu\text{M}$ AbeAdo		
Day 4	21 ± 3	< 4.0
Day 8	101 ± 6	< 4.0
Day 12	100 ± 11	< 4.0
$10 \mu\text{M}$ Spermidine	< 4.0	Not determined
$10 \mu\text{M}$ Spermine	91 ± 21	Not determined
$10 \mu\text{M}$ <i>N</i> -(3-Aminopropyl)- 1,4-diamino- <i>cis</i> -2-butene	< 4.0	Not determined

cytostasis may result from the absence of adequate amounts of the hypusinated form of the protein-synthesis initiation factor, eIF-5A, when spermidine was not available to serve as a precursor of the post-translational modification that forms this product. In order to examine this possibility more closely, the unhyposinated eIF-5A content was determined in L1210 and R20 cells harvested after 4, 8 or 12 days of exposure to $10 \mu\text{M}$ AbeAdo (Table 2). The unhyposinated form could not be detected in untreated cells but rose rapidly in the treated L1210 cells, reaching a maximum at day 8. In contrast, unhyposinated eIF-5A was not found in the R20 cells at any time. Unhyposinated eIF-5A was undetectable in AbeAdo-treated L1210 cells rescued with spermidine or with the analogue *N*-(3-aminopropyl)-1,4-diamino-*cis*-2-butene, which is known to reverse the cytostatic effects of AbeAdo (Byers et al., 1992b). These results support the concept that cytostasis may result from the decline in the active form of eIF-5A and that a minimal amount of spermidine or an analogue capable of forming this product is needed to reverse cytostasis.

Unhyposinated eIF-5A was not significantly reduced in AbeAdo-treated L1210 cells rescued with spermine (Table 2). However, such treatment with spermine did lead to an increase in intracellular spermidine. The putrescine, spermidine and spermine levels in spermine-rescued AbeAdo-treated L1210 cells were 0.38 ± 0.02 , 4.0 ± 0.3 and 23 ± 5 nmol/mg of protein respectively compared with 30 ± 1 putrescine, < 0.2 spermidine and < 0.3 spermine in cytostatic AbeAdo-treated L1210 cells before the rescue. Thus it is possible that sufficient spermidine is made to allow some synthesis of hypusine. It is not clear how the spermidine is increased in spermine-treated cells. The increase could not be blocked by the addition of $20 \mu\text{M}$ MDL 72521, an inhibitor of polyamine oxidase (Bey et al., 1985) which might be expected to prevent the acetylase/oxidase interconversion pathway (Pegg, 1988). In this case the polyamine levels were putrescine, < 0.3 ; spermidine, 3.4 ± 0.3 ; spermine, 24 ± 4 nmol/mg of protein. Such treatment did not alter the effects of exogenous spermidine or spermine on the growth or on the

unhypusinated eIF-5A levels of AbeAdo-treated cell (results not shown).

DISCUSSION

Although AbeAdo has not yet been tested clinically, it has obvious potential as a therapeutic agent both as an antiparasitic agent (Bitonti et al., 1990) and, in combination with other treatments affecting polyamine metabolism, as an antitumour agent (Seiler et al., 1991). The antitrypanosomal activity of AbeAdo appears to be mediated by a rapid and massive (> 20-fold) increase in trypanosome AdoMet levels after AbeAdo treatment of trypanosome-infected animals (Byers et al., 1991). Acute AbeAdo treatment for 6 h, which profoundly increased AdoMet in the trypanosomes, did not change the level of this nucleoside in cultured mammalian cells (Byers et al., 1991), suggesting that AdoMet levels are more highly regulated in mammalian cells and that the antiproliferative effects of AbeAdo in mammalian cells are probably not exerted by changes in AdoMet levels. This is confirmed by our present finding that even after 12 days of AbeAdo treatment, at which time cytostasis is induced, AdoMet levels are not greatly increased in L1210 cells.

In view of the therapeutic potential of AbeAdo and the high specificity of its inhibition of AdoMetDC, both the mechanism by which the compound interferes with mammalian cell growth and the possible development of resistance to it are of considerable interest. We proposed previously that depletion of hypusinated eIF-5A during prolonged AbeAdo exposure may be the mechanism by which AbeAdo induces cytostasis (Byers et al., 1992b). The R20 cells described in this paper are the first report of AbeAdo-resistant cells, and their comparison with the parent L1210 cells provides useful insights into the mechanism of AbeAdo action.

Resistance to AbeAdo appeared to be readily acquired by L1210 cells. This resistance seems to be due to an ability of the R20 cells to continue to synthesize spermidine even in the presence of the inhibitor. In contrast with the situation with the ODC inhibitor, difluoromethylornithine, where resistance is usually acquired through gene amplification and subsequent overproduction of the target enzyme or an increase in enzyme stability [reviewed by Hayashi (1989) and Pegg (1989)], there was little indication that this was the case with R20 cells. In untreated R20 cells, AdoMetDC activity and AdoMetDC protein levels were actually slightly less than in untreated L1210 cells and the amount of AdoMetDC protein in the R20 cells treated with AbeAdo was only slightly increased and no more than in AbeAdo-treated L1210 cells. These results indicate that the AbeAdo resistance of the R20 cells is not due to overproduction of AdoMetDC. However, a low level of AdoMetDC enzyme activity was present in the treated R20 cells and could not be detected in the L1210 cells. It is likely that this residual activity is due to the lower concentration of AbeAdo in the R20 cells (Figure 3).

The mechanism that reduces AbeAdo accumulation in the R20 cells was not determined, although this issue should be addressed before clinical application of this drug is considered. A preliminary experiment demonstrated a difference in [^3H]AbeAdo accumulation between L1210 cells and R10 and R20 cells after a 5 min incubation with the labelled drug (T. L. Byers, unpublished work). Uptake of [^3H]AbeAdo by L1210 cells exposed to 0.01–100 μM drug for 5 min was not saturated, suggesting that AbeAdo accumulation in L1210 cells may not be transporter-mediated (Byers et al., 1992a). Therefore, it is unlikely that a

reduction in the uptake of the drug by the resistant cells is responsible for the reduced accumulation. Enhanced excretion of AbeAdo, similar to the excretion of chemotherapeutic agents by multidrug-resistant cells [reviewed by Beck (1987)], may be responsible for the reduced accumulation of the drug in AbeAdo-resistant cells. It is also possible that increased metabolism plays a role. AbeAdo is a substrate, albeit a very poor one, for diamine oxidase (Byers et al., 1992a). Enhanced degradation of AbeAdo by this pathway or other enzymes for which AbeAdo may be a substrate could reduce intracellular levels of the drug. It is interesting that the R20 cells appeared to develop further resistance to AbeAdo during the 20-day course of their exposure. This is based on the growth rates (Figure 1) and the time-dependent decrease in AbeAdo accumulation (Figure 3) of these cells over the course of the experiment. Furthermore, although the residual AdoMetDC activity did not increase appreciably with time, an increase in intracellular spermidine (Figure 3) did correlate with decreased intracellular AbeAdo and increased growth rates.

Exposure to AbeAdo had a dramatic effect on polyamine levels in both cell lines (Figure 2). The enormous increase in putrescine is likely to be due to both an increased activity of ODC (which is strongly repressed by spermidine and spermine) and the lack of decarboxylated AdoMet needed to convert it into spermidine. The decline in spermidine and spermine is expected from the absence of decarboxylated AdoMet. It is noteworthy that spermine levels were similar in both L1210 and R20 cells treated with AbeAdo whereas the treated R20 cells contained much higher amounts of spermidine. It is apparent that only a small fraction of the cellular AdoMetDC activity is needed for some spermidine production but that maintenance of the spermine level is more dependent on normal AdoMetDC activity. The difference in growth between the treated R20 and L1210 cells shows clearly that spermidine is able to prevent cytostasis.

The finding that unhypusinated eIF-5A did not accumulate in AbeAdo-treated R20 cells indicates that, despite reduced intracellular spermidine, there was enough spermidine to synthesize hypusine in these cells. The continued hypusination of eIF-5A in the R20 cells that were not susceptible to AbeAdo-induced cytostasis is consistent with depletion of hypusinated eIF-5A being responsible for this cytostasis in the AbeAdo-sensitive L1210 cells (Byers et al., 1992b). Under normal growth conditions, the rate of formation of hypusinated eIF-5A is limited by the availability of the unhypusinated precursor protein, and, in fact, unhypusinated eIF-5A is not detectable in cells with normal polyamine metabolism (Park et al., 1988; Table 2). However, as Park (1988) has shown with an ODC inhibitor and as we have shown with AbeAdo (Table 2), inhibition of polyamine biosynthesis results in spermidine becoming the limiting factor in hypusinated eIF-5A formation and in the accumulation of unhypusinated eIF-5A. This accumulation in AbeAdo-treated L1210 cells indicates that the synthesis of hypusinated eIF-5A did not occur, or was greatly reduced, and suggests that hypusinated eIF-5A pools present before AbeAdo treatment were subject to depletion by protein turnover and dilution through cell growth. The ability of exogenously supplied spermidine to deplete unhypusinated eIF-5A accumulated during AbeAdo treatment suggests that the hypusination of the protein in treated cells was limited by spermidine availability.

While spermidine was able to prevent and reverse the cytostatic effects of AbeAdo, spermine, which is not a substrate for the eIF-5A hypusination reaction (Park et al., 1981), was even better than an equal concentration of spermidine at reversing AbeAdo-induced cytostasis. Unhypusinated eIF-5A accumulated in L1210 cells during AbeAdo treatment was not significantly reduced by

exogenously supplied spermine. However, these results do not definitively refute a critical role for spermidine and hypusinated eIF-5A depletion in AbeAdo-induced cytostasis. Apparently, some spermine was converted into spermidine. Since the K_m for spermidine in the eIF-5A hypusination reaction is $0.08 \mu\text{M}$ (Wolff et al., 1990), it is likely that the small amount of spermidine formed would allow the reaction to proceed. Although we did not detect a significant decrease in unhypusinated eIF-5A accumulated during AbeAdo treatment, it should be remembered that this accumulation occurred over a 12-day treatment period and that it is likely that hypusination of only a small portion of this precursor might be required for the cells to resume growth. It is possible that growth does not absolutely require spermine but is optimal when spermine is present, which would explain why spermine was better able to rescue AbeAdo-treated cells than was an equal concentration of spermidine.

Chronic AbeAdo treatment appears to offer a unique model in which to study the role of hypusinated eIF-5A in cell proliferation. Kramer et al. (1989) reported that putrescine alone can support L1210 cell growth at 13% the control rate over a 5-day period. This is consistent with the hypothesis that the elevated putrescine levels in AbeAdo-treated L1210 cells were responsible for the limited growth of these cells in the absence of spermidine and spermine for 12 days (Byers et al., 1992b). Since ODC inhibitors result in a relatively rapid induction of cytostasis after an overall reduction in intracellular polyamine pools, it is unlikely that inhibition of ODC or AdoMetDC result in cytostasis for the same physiological reasons. We propose that ODC-inhibitor-induced cytostasis is due to an interruption of general and non-specific permissive functions that polyamines fulfil as polybasic anions, while AbeAdo-induced cytostasis is due to the loss of a specific spermine and/or spermidine-requiring function. Although further work is necessary to verify our hypothesis, our findings are consistent with the depletion of eIF-5A in the absence of spermidine being the cause of AbeAdo-induced cytostasis.

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