Effects of the *S*-adenosylmethionine decarboxylase inhibitor, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine, on cell growth and polyamine metabolism and transport in Chinese hamster ovary cell cultures

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The regulation of polyamine transport and the roles of polyamine transport and synthesis in cell growth were investigated using cultured Chinese hamster ovary (CHO) cells and CHOMG cells which are mutants lacking polyamine-transport activity. Metabolically stable methylated polyamine analogues were used to measure polyamine accumulation, and the irreversible *S*-adenosyl-L-methionine decarboxylase inhibitor, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (AbeAdo), was used to inhibit synthesis. Exposure to AbeAdo led to a dose-dependent decrease in growth for both cell lines, although CHOMG cells were more sensitive. Intracellular putrescine levels were greatly increased in AbeAdo-treated CHO cells and to a lesser extent in CHOMG cells, whereas intracellular spermidine and spermine levels were substantially reduced in both. Treatment with AbeAdo increased putrescine content in the culture medium to a much greater extent in CHOMG cultures indicating that a portion of the excess putrescine synthesized in response to AbeAdo treatment is excreted, but that CHO cells salvage this putrescine whereas it is lost to CHOMG cells which cannot take up polyamines. AbeAdo treatment increased polyamine transport into CHO cells despite high intracellular putrescine, suggesting that spermidine and/or spermine, and not putrescine, are the major factors regulating transport activity. The accumulation of either 1-methylspermidine or 1,12-dimethylspermine was significantly increased by AbeAdo treatment. Accumulation was increased even further when protein synthesis was blocked by cycloheximide, indicating that a short-lived protein is involved in the regulation of polyamine uptake. In the presence of cycloheximide and AbeAdo or α-difluoromethylornithine, methylated polyamine derivatives accumulated to very high levels leading to cell death. These results show that the polyamine-transport system plays an important role in retaining intracellular polyamines and that down-regulation of the transport system in response to increased intracellular polyamine content is necessary to prevent accumulation of toxic levels of polyamines.

INTRODUCTION

All mammalian cells contain physiologically significant levels of the polyamines putrescine, spermidine and spermine [1,2]. The ability to synthesize polyamines endogenously and to take up exogenous polyamines appears to be ubiquitous and highly regulated such that cells maintain intracellular polyamine levels optimum for cell function [3–6]. Because putrescine synthesis is the first step in the polyamine-biosynthetic pathway, ornithine decarboxylase (ODC) has historically been the target for drugs designed to interfere with polyamine metabolism. This has resulted in several specific inhibitors of ODC, such as α-difluoromethylornithine (DFMO), being used to demonstrate the importance of polyamines in the initiation and maintenance of mammalian cell growth. These inhibitors have therapeutic potential for the treatment of proliferative pathologies such as protozoal infections and cancer [1,6–8].

Experiments involving depletion of intracellular polyamines by ODC inhibitors first identified the regulated nature of the transport system responsible for the uptake of polyamines by mammalian cells [9]. It is now widely accepted that this system is regulated by intracellular polyamine concentrations [5,10]. Little is known at present about the molecule basis of the polyamine-transport system or its regulation, although there is evidence for multiple systems with overlapping specificity [5,11]. Putrescine- and spermidine-transport systems from *Escherichia coli* consisting of multiple proteins have been cloned and expressed [12,13] but as yet, none of the relevant proteins or cDNAs from mammalian cells are available although transfection of human DNA fragments restoring transport to inactive mutant CHOMG cells has been reported [14]. An unstable protein, the synthesis of which is stimulated by intracellular polyamines, appears to be involved in the repression of polyamine transport [15]. Cell lines in which this repression is deranged have been described [16,17].

Recent interest in *S*-adenosylmethionine decarboxylase (AdoMetDC) as a target for interference with polyamine metabolism has resulted in the synthesis of some potent and specific AdoMetDC inhibitors [18–22] including 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine (AbeAdo). AbeAdo irreversibly inactivates AdoMetDC from bacterial, protozoal and mammalian sources [23–25] and the mechanism of inactivation appears to involve transamination of the pyruvate cofactor [26,27]. Experiments conducted using other inhibitors of AdoMetDC that have a short life in culture media indicated that inhibition of AdoMetDC for a limited duration reduced, but did not halt, cell proliferation [18,19]. These results were confirmed in experiments using more stable inhibitors including AbeAdo [20–22,28–30]. However, prolonged exposure of L1210

Abbreviations used: ODC, ornithine decarboxylase; AdoMetDC, *S*-adenosylmethionine decarboxylase; DFMO, α-difluoromethylornithine; AbeAdo, 5′-{[(*Z*)-4-amino-2-butenyl]methylamino}-5′-deoxyadenosine.

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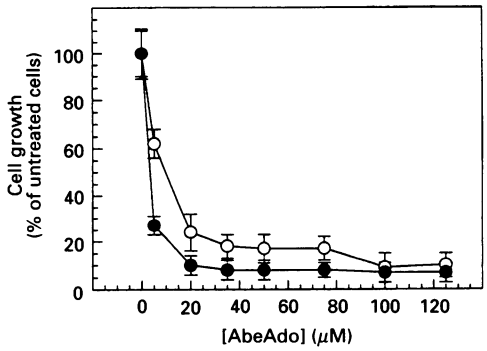


Figure 1 Effect of AbeAdo concentration on growth of CHO (○) and CHOMG (●) cells

AbeAdo was added to the cultures at the concentration shown and the cell number determined after 96 h. Results are expressed as the percentage of the number present in an untreated culture. Results are means ± S.D. for at least four observations.

cells to this drug resulted in cytostasis by the 12th day of treatment and subsequent cell death unless spermidine was provided [28,29]. It was suggested that the causative factor in this toxicity was prevention of the post-translational modification of the protein initiation factor eIF-5A to form hypusine by the absence of spermidine [28]. This modification is essential for the activity of eIF-5A which is known to be essential for cell viability and growth [31,32].

We now report the use of AbeAdo to study the effects of AdoMetDC inhibition on polyamine metabolism, polyamine transport and intracellular/extracellular polyamine distribution in Chinese hamster ovary (CHO) cells and in mutant CHO cells lacking polyamine-transport activity (CHOMG cells) [33]. The results indicate that putrescine does not appear to be effective in down-regulating polyamine transport and that this down-regulation is very important in maintaining the viability of CHO cells exposed to methylated polyamine analogues.

MATERIALS AND METHODS

Materials

AbeAdo and DFMO were generous gifts from the Merrell Dow Research Institute, Cincinnati, OH, U.S.A. [1,4-^{14}C]Putrescine and [1,4-^{14}C]spermine (111 μCi/μmol) were purchased from Amersham-Searle. 1-Methylspermidine and 1,12-dimethyl-

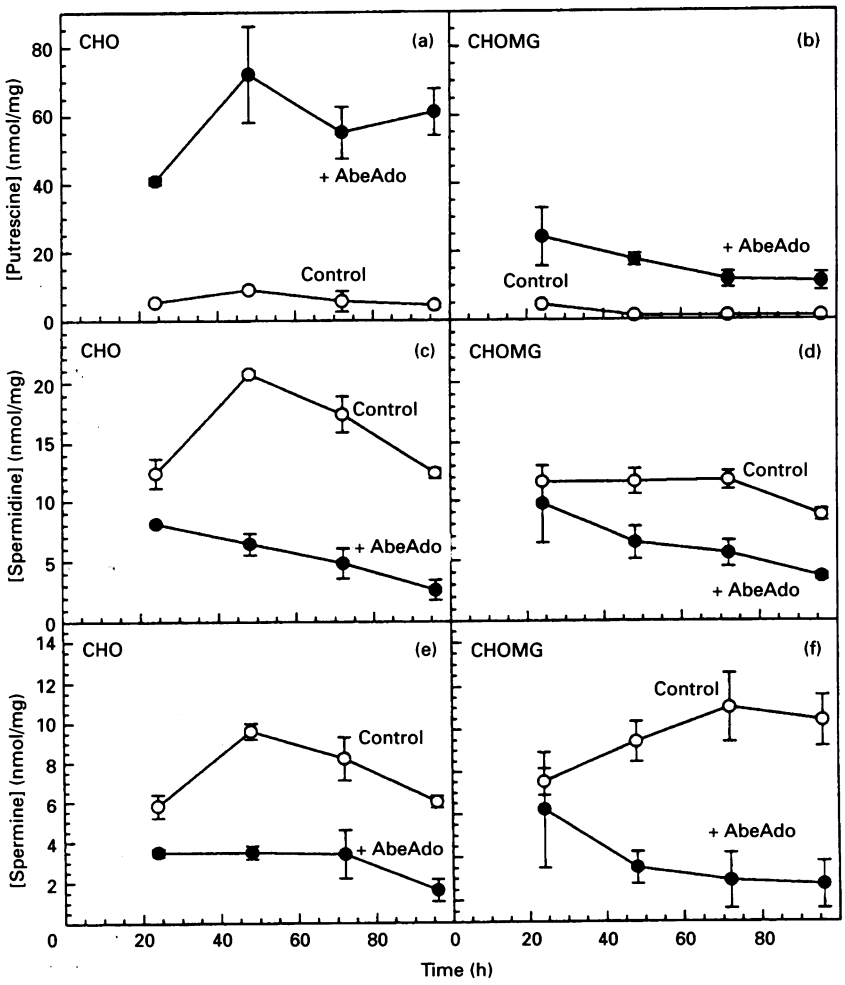


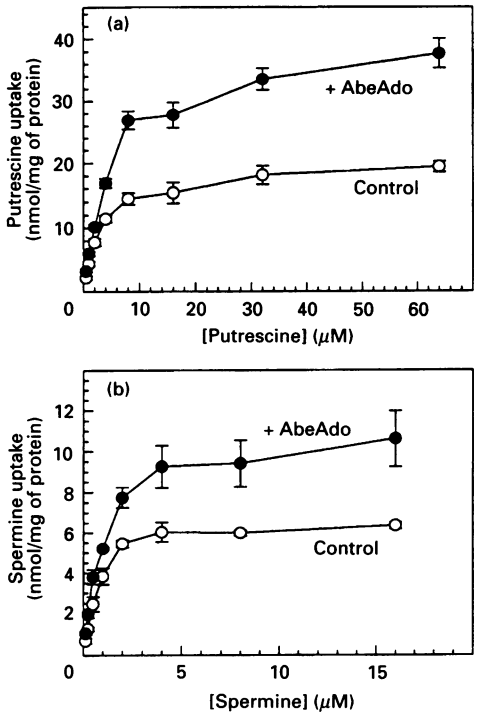
Figure 2 Polyamine content in CHO and CHOMG cells treated with AbeAdo

Cells were treated with 50 μM AbeAdo and the contents of intracellular putrescine (**a** and **b**), spermidine (**c**, and **d**) and spermine (**e** and **f**) were determined at the times shown for CHO cells (**a**, **c** and **e**) and CHOMG cells (**b**, **d** and **f**). Results are means ± S.D. for at least four observations. ○, Control; ●, + AbeAdo.

Table 1 Effect of AbeAdo on polyamine biosynthesis and distribution in CHO and CHOMG cell cultures

CHO and CHOMG cells were plated in the presence of 1 mM aminoguanidine and in the presence or absence of 50 μM AbeAdo. The intracellular polyamine contents of representative cultures at the time of plating were determined and were not significantly different, the totals being 1.7 nmol for CHO cells and 1.5 nmol for CHOMG cells. After 96 h, the cells and media from each culture were harvested for analysis of intracellular and extracellular polyamines respectively. Values are means ± S.D. for four individual cultures.

			Polyamine content (nmol/culture)			
Treatment	Cells	Site	Putrescine	Spermidine	Spermine	Total
Control, 96 h	CHO	Intracellular	0.67 ± 0.03	18 ± 1.0	21 ± 2.0	40
Control, 96 h	CHO	Extracellular	1.4 ± 0.3	0.64 ± 0.03	0.71 ± 0.15	2.8
Control, 96 h	CHOMG	Intracellular	0.13 ± 0.02	7.5 ± 0.4	15 ± 1.0	23
Control, 96 h	CHOMG	Extracellular	3.4 ± 0.5	7.2 ± 1.3	0.84 ± 0.03	11
AbeAdo, 96 h	CHO	Intracellular	17 ± 1.0	1.6 ± 0.1	0.55 ± 0.05	19
AbeAdo, 96 h	CHO	Extracellular	1.5 ± 0.1	0.49 ± 0.05	< 0.1	2.0
AbeAdo, 96 h	CHOMG	Intracellular	4.3 ± 0.3	2.4 ± 0.1	1.4 ± 0.6	11
AbeAdo, 96 h	CHOMG	Extracellular	31 ± 6.0	0.70 ± 0.21	< 0.1	32



Figue 3 Effect of AbeAdo on transport of spermine and putrescine into CHO cells

Uptake of labelled putrescine (**a**) or spermine (**b**) was measured as described in the Materials and methods section during a 30 min incubation with cells treated with 50 μM AbeAdo for 72 h (●) or control cells (○).

spermine were synthesized by Dr. J. R. Lakanen and Dr. J. K. Coward, Department of Chemistry, University of Michigan, Ann Arbor, MI, U.S.A. as previously described [33]. Chemical reagents were purchased from Fisher Scientific, Pittsburgh, PA, U.S.A. and Sigma Chemical Co., St. Louis, MO, U.S.A.

Determination of the effects of AbeAdo on cell growth and polyamine levels

Methylglyoxal bis(guanylhydrazone)-resistant CHOMG cells, which lack polyamine-transport activity, and their parental CHO cells were generously provided by Dr. W. Flintoff, Department of Microbiology, University of Western Ontario, Canada and grown as previously described using 1 mM aminoguanidine to block the activity of serum amine oxidases [14,34]. Cells were plated at 4×10^4 cells/ml in 35 mm dishes at 1 ml/dish. At 3 h after plating, 1 ml of medium containing AbeAdo at twice the desired final concentration was added. The cells were incubated for 96 h and then harvested utilizing a 0.025% (w/v) buffered trypsin solution, and counted using an electronic cell counter (Coulter Electronics). Separate dishes were used for polyamine analysis and cell counting at 24, 48, 72 and 96 h. Cells harvested for polyamine analysis were washed with ice-cold PBS and then gently scraped off the plate in 10% (w/v) trichloroacetic acid. Polyamines were also assayed in samples from the medium.

Measurement of polyamine-transport activity

Polyamine transport was assayed by measuring the uptake of [1,4-^{14}C]putrescine or [5,8-^{14}C]spermine [11]. Cells were plated in 24-well plates in the presence or absence of 50 μM AbeAdo for 72 h before addition of medium lacking serum and containing labelled polyamines. Cells were then incubated under growth conditions for 30 min, the medium was aspirated off and the cells were washed twice with 1 ml of ice-cold α-minimal essential medium (without fetal bovine serum) containing 1 mM putrescine and 1 mM spermine and then twice with 1 ml of ice-cold PBS. The washed cells were dissolved by sonication in 0.1 M NaOH. Portions of this solution were used for determination of radioactivity and protein.

Measurement of the accumulation of methylated polyamines

Cells were plated at 2×10^4–8×10^4 cells in 2 ml of medium in 35 mm dishes and grown for 72 h in control medium or medium containing 5 mM DFMO or 50 μM AbeAdo. The larger cell numbers were used with cultures containing these drugs so that approximately the same number of cells were present at 72 h. At this time, the medium was removed and replaced with fresh medium containing the same concentration of DFMO or AbeAdo and 10 μM 1-methylspermidine or 1,12-dimethylspermine. Cycloheximide (200 μM) was added to some dishes. The cells were returned to the incubator for various periods for 15 min to 12 h and then harvested, washed and the polyamine content determined. Controls were harvested at 0 min. These cultures contained no detectable methyl polyamines showing that the

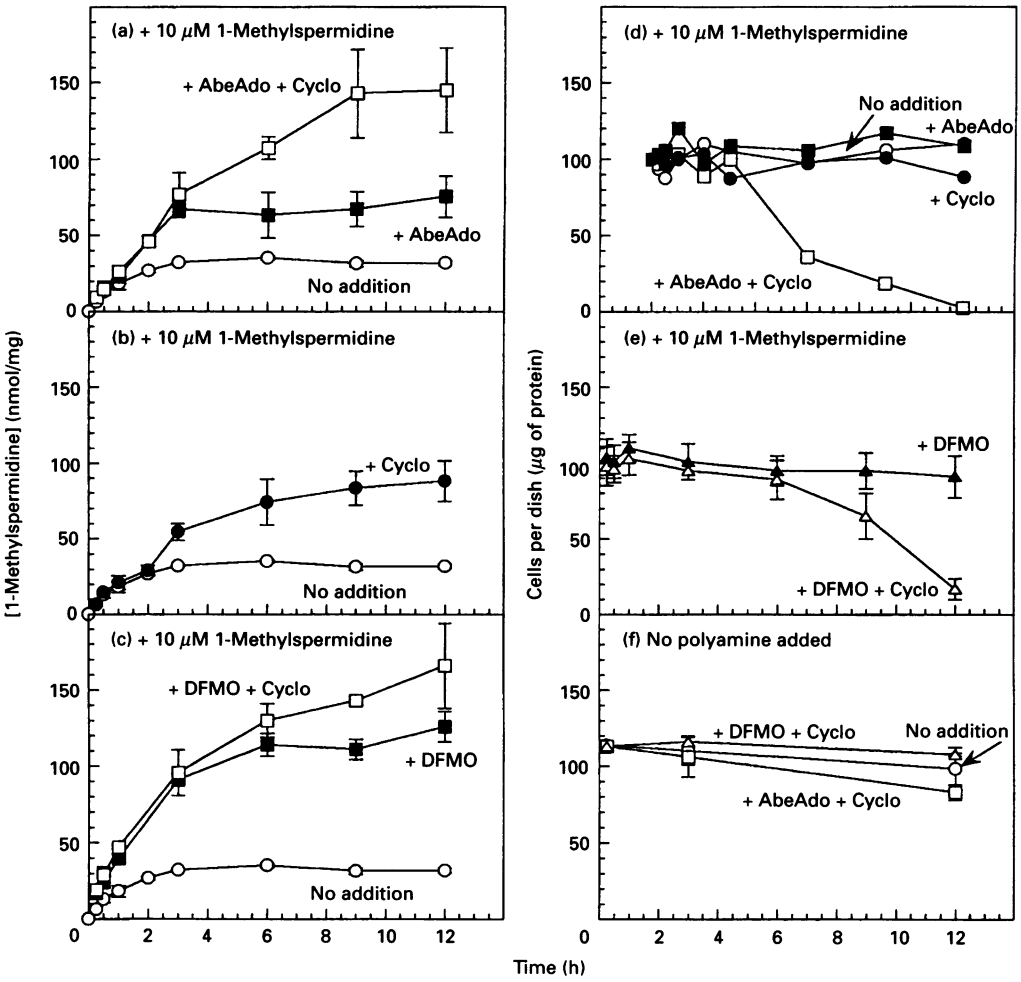


Figure 4 Effect of AbeAdo, DFMO and cycloheximide on uptake of 1-methylspermidine and growth of CHO cells

CHO cells were grown for 72 h in the presence of 50 μM AbeAdo or 5 mM DFMO as indicated. New medium containing the drugs and 1-methylspermidine (10 μM) was then added in the presence and absence of 200 μM cycloheximide (Cyclo) as indicated and the intracellular content of 1-methylspermidine measured at the times shown. (**a**) Effect of AbeAdo on 1-methylspermidine levels in the presence and absence of cycloheximide; (**b**) effect of cycloheximide on 1-methylspermidine levels; (**c**) effect of DFMO on 1-methylspermidine levels in the presence and absence of cycloheximide; (**d**) effect on cell growth of AbeAdo and cyclohexmide in the presence of 1-methylspermidine; (**e**) effect on cell growth of DFMO and cycloheximide in the presence of 1-methylspermidine; (**f**) effect on cell growth of DFMO and AbeAdo in the presence of cycloheximide but the absence of 1-methylspermidine. Results are means ± S.D. for at least six observations.

washing was sufficient to remove all of the medium from the cell pellets before polyamine analysis.

Determination of polyamines

Extracts from cells and media were deproteinized and putrescine, spermidine, 1-methylspermidine, spermine and 1,12-dimethylspermine separated and quantified using modifications [33,35] of the method described by Seiler and Knödgen [36]. Results are expressed as nmol of polyamine/mg of cell protein. Protein was measured by the method of Bradford [37].

RESULTS

Effects of AbeAdo on cell growth and polyamine levels

Growth of both CHO and CHOMG cells were reduced in a dose-dependent manner by a 96 h exposure to AbeAdo although CHOMG cells were more sensitive than CHO cells (Figure 1). The effects of AbeAdo on CHO cell growth could be prevented by exposure to either spermidine or spermine but AbeAdo-treated CHOMG cells could not be rescued by exogenous polyamines (results not shown). Altered growth rates of AbeAdo-treated CHO and CHOMG cells were evident within 48 h of exposure. The differences in sensitivity of CHO and CHOMG cells to AbeAdo were most pronounced at 72 h when either 5 μM or 50 μM AbeAdo reduced cell growth, expressed as a percentage of untreated controls, to 68% and 19% respectively for CHO cells and 48% and 11% respectively for CHOMG cells.

Exposure of CHO and CHOMG cells to 50 μM AbeAdo resulted in a significant increase in intracellular putrescine and decreases in spermidine and spermine (Figure 2). Although AbeAdo treatment elevated intracellular putrescine in both CHO and CHOMG cells within 24 h, putrescine levels were substantially higher in the CHO cells. Elevated CHO cell putrescine levels were maintained over the course of the experiment whereas the putrescine content in CHOMG cells peaked at 24 h and declined over the next 72 h. Spermidine and spermine levels were

Table 2 Effect of AbeAdo, DFMO and cycloheximide on polyamines and accumulation of 1-methylspermidine

CHO cells were grown for 72 h in the presence of 50 μM AbeAdo or 5 mM DFMO. New medium containing the drugs and 1-methylspermidine (10 μM) was then added in the presence and absence of 200 μM cycloheximide as indicated and the intracellular content of polyamines was determined at various times up to 12 h. The results are those obtained at 6 h shown as representative values. In contrast with 1-methylspermidine, the levels of which at various times are shown in Figure 4, the concentrations of the other polyamines did not change greatly over the 12 h period. Results are means ± S.D. for at least four determinations.

	Polyamine content (nmol/mg of protein)			
Addition	Putrescine	Spermidine	Spermine	1-Methylspermidine
None	10.3	18.9	9.9	–
1-Methylspermidine	8.3 ± 1.0	17.0 ± 1.3	10.0 ± 1.8	27.4 ± 1.9
1-Methylspermidine + AbeAdo	87.1 ± 10.1	3.5 ± 0.6	0.2 ± 0.1	59.1 ± 7.5
1-Methylspermidine + cyclohexmide	10.0 ± 1.4	14.6 ± 1.8	8.6 ± 1.0	73.4 ± 9.1
1-Methylspermidine + cycloheximide + AbeAdo	112 ± 8.9	3.8 ± 1.2	0.2 ± 0.1	98.9 ± 6.7
1-Methylspermidine + DFMO	0.4 ± 0.1	0.4 ± 0.3	11.7 ± 1.1	115 ± 9.0
1-Methylspermidine + cycloheximide + DFMO	0.6 ± 0.1	0.5 ± 0.2	9.1 ± 1.2	160 ± 11

substantially reduced in AbeAdo-treated CHO and CHOMG cells within 48 h and this became greater over the experimental period.

Although untreated CHOMG cells cultured for 96 h contained less intracellular polyamine than CHO cells, consideration of the polyamines accumulated in the media of these cultures showed that only a small fraction of this difference could be attributed to a lower amount of synthesis (Table 1). A much greater proportion of the polyamines formed was present in the medium of the CHOMG cells (32% compared with 7%). In AbeAdo-treated cells, where the majority of the cellular polyamine was putrescine, there was an even greater proportion of polyamine present in the medium. This amounted to 74% of the total in CHOMG cells compared with 10% in CHO cells. About 12% of the putrescine formed in AbeAdo-treated CHOMG cells was retained in the cell compared with 92% in the AbeAdo-treated CHO cells. Thus, although AbeAdo resulted in higher intracellular putrescine levels in CHO cells than in CHOMG cells, this difference appears to be due to differences in the intracellular/extracellular distribution of putrescine rather than to greater synthesis in the CHO cells. In fact, total polyamine synthesized by CHOMG cells in the presence of AbeAdo was 40.5 nmol/culture compared with 19.3 in CHO cells. The greater synthesis by CHOMG cells is probably due to the fact that much of the putrescine was excreted and is therefore not available to repress ODC which is known to be decreased by high intracellular putrescine levels [1–4].

Effect of AbeAdo treatment on polyamine-transport activity in CHO cells

Treatment of CHO cells with 50 μM AbeAdo for 72 h resulted in an increase in the apparent $V_{max.}$ for transport of both putrescine and spermine without a change in the apparent K_m which was 1.6 μM for spermine and 6.1 μM for putrescine (Figure 3). (These K_m values are in excellent agreement with those published previously [35].) The increase in transport activity in response to AbeAdo, which occurred despite intracellular putrescine levels higher than the total polyamine content of untreated cells, were similar to the changes in transport activity reported in CHO cells depleted of polyamines after treatment with DFMO or after polyamine starvation of mutants lacking ODC activity [10,35].

Effect of AbeAdo and cycloheximide on accumulation of methylated polyamines

In order to obtain more detailed information on the effects of AbeAdo on the accumulation of exogenous polyamines, use was made of two polyamine analogues, 1-methylspermidine and 1,12-dimethylspermine. These analogues replace the normal polyamines in growth but are not metabolized significantly in mammalian cells and are good substrates for the polyamine-transport system [33]. Exposure to AbeAdo significantly increased the rate of accumulation of 1-methylspermidine and also increased the resulting plateau level which was reached about 3 h after addition of the analogue (Figure 4a; Table 2). Treatment of the cells with cycloheximide at the time of addition of 1-methylspermidine also increased the rate of transport and led to a more prolonged period of uptake (Figure 4b). The combination of cycloheximide and AbeAdo increased 1-methylspermidine accumulation to an even greater extent leading to levels of more than 140 nmol/mg of protein (Figure 4a). This accumulation was severely toxic to the cells so that there were very few intact cells remaining by 12 h (Figure 4d). The toxicity was due to the uptake of the polyamine analogue as there was little effect of cycloheximide and AbeAdo on the cells when no exogenous polyamine was added (Figure 4f).

Exposure to DFMO for 72 h led to a larger increase in the accumulation of 1-methylspermidine than that seen with AbeAdo but there was also an additional increase and more continued accumulation when DFMO was combined with cycloheximide (Figure 4c; Table 2). The combination of DFMO, cycloheximide and 1-methylspermidine was also toxic because of the massive accumulation of 1-methylspermidine (Figure 4e). However, this toxicity was delayed compared with the effects of AbeAdo despite the fact that accumulation of 1-methylspermidine was greater with DFMO. This is likely to be related to the other changes in polyamines occurring in cells treated with these inhibitors. AbeAdo led to a very large increase in putrescine and an almost complete loss of spermine whereas DFMO abolished

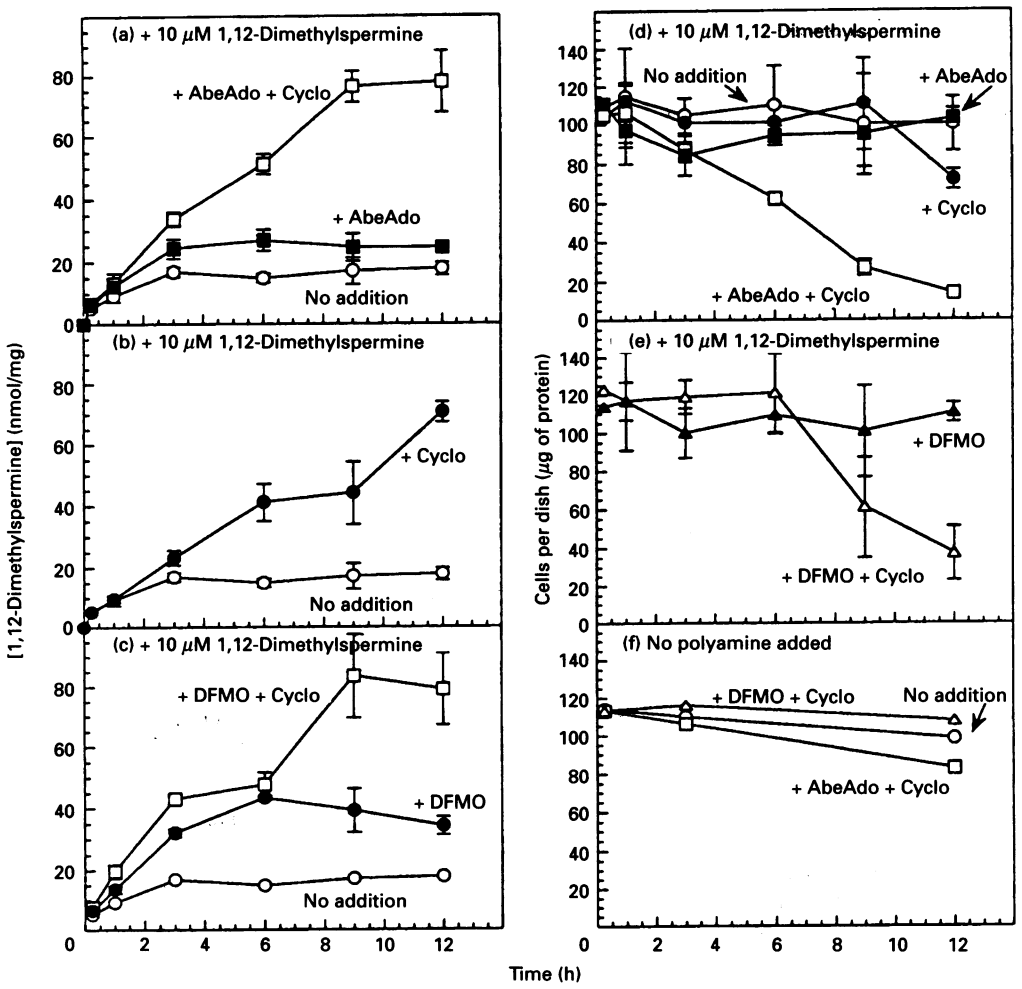


Figure 5 Effect of AbeAdo, DFMO and cycloheximide on uptake of 1,12-dimethylspermine and growth of CHO cells

CHO cells were grown for 72 h in the presence of 50 μM AbeAdo or 5 mM DFMO as indicated. New medium containing the drugs and 1,12-dimethylspermine (10 μM) was then added in the presence and absence of 200 μM cycloheximide (Cyclo) as indicated and the intracellular content of 1,12-dimethylspermine measured at the times shown. (**a**) Effect of AbeAdo on 1,12-dimethylspermine levels in the presence and absence of cycloheximide; (**b**) effect of cycloheximide on 1,12-dimethylspermine levels: (**c**) effect of DFMO on 1,12-dimethylspermine levels in the presence and absence of cycloheximide; (**d**) effect on cell growth of AbeAdo and cycloheximide in the presence of 1,12-dimethylspermine; (**e**) effect on cell growth of DFMO and cycloheximide in the presence of 1,12-dimethylspermine; (**f**) effect on cell growth of DFMO and AbeAdo in the presence of cycloheximide by the absence of 1,12-dimethylspermine. Results are means ± S.D. for at least six observations.

putrescine and spermidine without much effect on spermine. Exposure to 1-methylspermidine had little effect on polyamine levels during the time period of these experiments (Table 2).

Similar results were obtained when 1,12-dimethylspermine was used as the non-metabolizable polyamine (Figure 5a; Table 3). The uptake of 1,12-dimethylspermine was increased by AbeAdo treatment by about 50% in the rate and final plateau value of accumulation which was reached within 3 h in both the control and the AbeAdo-treated cells (Figure 5). Addition of cycloheximide increased the rate of uptake only slightly but accumulation continued over a 12 h period (Figure 5b) and the combination of AbeAdo and cycloheximide also led to continued accumulation of 1,12-dimethylspermine (Figure 5a). The combination of AbeAdo, cycloheximide and 1,12-dimethylspermine was highly toxic to the cells (Figure 5d).

Cells treated with DFMO showed a slightly greater increase in the accumulation of 1,12-dimethylspermine than cells treated with AbeAdo (Figure 5c; Table 3). The combination of cycloheximide with DFMO also led to a continued accumulation of 1,12-dimethylspermine to much higher levels (Figure 6c) and to a severe toxic effect (Figure 5d). However, as found with 1-methylspermidine, the toxicity in cells treated with cycloheximide and 1,12-dimethylspermine plus the inhibitor of polyamine biosynthesis occurred more rapidly with AbeAdo than with DFMO.

DISCUSSION

The striking difference in the content of polyamines in the medium between the CHO and CHOMG cells (Table 1) provides strong evidence that the polyamine-uptake system, which is inactive in the mutant CHOMG cells [14,34,35], plays an important role in maintaining cellular polyamine levels by recapturing polyamines lost from the cell. Polyamine efflux has been reported in several studies (e.g. [38–40]) but it is not clear whether this is an active or facilitated process or merely occurs by diffusion of the polyamines through the membrane. Such diffusion would only be likely to occur with the non-charged

Table 3 Effect of AbeAdo, DFMO and cycloheximide on polyamines and accumulation of 1,12-dimethylspermine

CHO cells were grown for 72 h in the presence of 50 μM AbeAdo or 5 mM DFMO. New medium containing the drugs and 1,12-dimethylspermine (10 μM) was then added in the presence and absence of 200 μM cycloheximide as indicated and the intracellular content of polyamines measured at various times up to 12 h. The results are those obtained at 6 h shown as representative values. In contrast with 1,12-dimethylspermine, the levels of which at various times are shown in Figure 5, the concentrations of the other polyamines did not change greatly over the 12 h period. Results are means ± S.D. for at least four determinations.

	Polyamine content (nmol/mg of protein)			
Addition	Putrescine	Spermidine	Spermine	1,12-Dimethylspermine
None	10.7 ± 0.4	18.3 ± 1.6	10.4 ± 1.2	
1,12-Dimethylspermine	8.4 ± 0.8	16.5 ± 1.6	10.2 ± 1.1	14.9 ± 1.4
1,12-Dimethylspermine + AbeAdo	108 ± 10	5.1 ± 0.3	< 0.1	26.9 ± 3.4
1,12-Dimethylspermine + cycloheximide	7.2 ± 0.5	20.4 ± 2.1	11.4 ± 0.9	41.1 ± 6.1
1,12-Dimethylspermine + cycloheximide + AbeAdo	81.5 ± 4.4	6.7 ± 0.2	< 0.1	51.3 ± 3.1
1,12-Dimethylspermine + DFMO	< 0.2	0.4 ± 0.1	12.2 ± 1.1	43.4 ± 1.2
1,12-Dimethylspermine + cycloheximide + DFMO	< 0.2	0.2 ± 0.1	10.5 ± 0.9	57.9 ± 4.2

form of the polyamines, and putrescine, which is excreted to the largest extent, has the highest proportion in this form. Spermine, which is not excreted significantly, has the lowest uncharged fraction. However, even with putrescine, there is only a minute amount present as the uncharged diamine at physiological pH, and in *E. coli*, a putrescine efflux mechanism has been identified [41]. Our results suggest that the uptake system is not involved in this efflux as CHOMG cells lack the uptake system but excrete polyamines into the medium.

A very large increase in putrescine accumulation is brought about by AbeAdo as a consequence of inhibition of synthesis of decarboxylated *S*-adenosyl-L-methionine, which is needed to convert putrescine into spermidine and spermine. In addition, ODC activity increases in response to the decline in levels of these higher polyamines so that total polyamine formed increases and a much greater proportion is in the form of putrescine. This very large amount of putrescine partially replaces spermidine and allows cell growth to continue at a reduced rate [28]. As putrescine is excreted more readily than the other polyamines, a significant proportion of cellular putrescine is transferred to the medium but CHO cells are able to reuptake this putrescine and thus maintain a very high intracellular putrescine level. The poor ability of putrescine to repress the uptake system allows accumulation to a very high level. CHOMG cells cannot take up extracellular putrescine and thus most of the polyamine synthesized by these cells in the presence of AbeAdo ends up in the medium (see Table 1). This may account for the greater antiproliferative effect of AbeAdo on CHOMG cells. These results (Table 1) also provide strong evidence for an important role of the polyamine-transport system in the retention of intracellular polyamines. If this is the major physiological role of this system in mammalian cells, it would explain the apparent paradox that cells that contain all of the enzymes necessary to synthesize polyamines *de novo* also have a system for their uptake.

It is well known that the activity of the polyamine-uptake system is altered in response to changes in intracellular polyamine levels. Decreases in polyamine concentration brought about by DFMO and other ODC inhibitors or polyamine starvation of cells lacking ODC stimulate the activity of the transport system [5,9,10,35]. The results in Figures 3–5 show clearly that this transport is increased in cells treated with AbeAdo despite the large rise in putrescine levels. Elevated transport activity in AbeAdo-treated cells in the presence of high putrescine levels is consistent with earlier results showing that the bis(ethyl) derivative of putrescine does not regulate transport activity [10] and that 10 μM putrescine had little effect on polyamine transport in L1210 cells treated with a mixture of ODC and AdoMetDC inhibitors [42].

Methylated polyamines are very useful for studying the polyamine-transport system and its effects on polyamine accumulation because they are not metabolized and are readily separated from native polyamines by h.p.l.c. [33]. Although they may be slightly less effective than the parent polyamines in bringing about the repression of polyamine transport when accumulated to high levels in the cell, the results of the experiments shown in Figures 4 and 5 show that this repression does occur unless cycloheximide is added to prevent protein synthesis. The results with both 1-methylspermidine and 1,12-dimethylspermine as transport substrates show clearly that the rate of uptake is enhanced by AbeAdo (or DFMO) and that this uptake is then reduced as the polyamines accumulated leading to a plateau level at about 6 h. This accumulation represents a substantial increase in total polyamine content of the cell but is not deleterious at least over the 12 h period studied.

The decline in the uptake system in response to accumulation of exogenous polyamine appears to be brought about by the synthesis of a regulatory protein, as exposure to cycloheximide increases uptake and allows continued accumulation to very high levels. This confirms the report of Mitchell et al. [15] showing that spermidine uptake increased in cycloheximide-treated cells. The nature of this protein, which must have a rapid turnover rate and be induced by polyamines, is not yet established but our results are consistent with the suggestion [43] that it is antizyme, a protein that regulates ODC activity by binding to it and enhancing its degradation. If antizyme is the regulatory protein, it would explain why polyamine uptake is poorly regulated and relatively unresponsive to polyamine content in cells with very high ODC levels [16,17] because in such cells all of the antizyme is probably bound to ODC.

The unregulated accumulation of methylated polyamine analogues in cells treated with AbeAdo plus cycloheximide leads to a rapid and severe toxicity with cell lysis (Figures 4 and 5). Such toxicity in response to high-level accumulation of

spermidine or 1-methylspermidine has been observed recently in D-R cells treated with polyamines after hypotonic shock [16]. As the level of 1,12-dimethylspermine reached was much lower than that of 1-methylspermidine (80 nmol/mg of protein compared with 150 nmol/mg), the spermine derivative appears to be more toxic. The toxicity of the methylated polyamine analogues is enhanced because they are not substrates for spermidine/spermine-N^1-acetyltransferase and thus cannot be degraded to putrescine by the acetyltransferase/polyamine oxidase system which normally acts to prevent the toxicity of excess polyamines [44].

The development of a toxic effect in response to accumulation of polyamine analogues was faster in cells treated with AbeAdo than in those treated with DFMO even though their accumulation was more rapid when DFMO was used. The major difference in these cells is that the AbeAdo-treated cells have very high levels of putrescine whereas putrescine is almost undetectable in cells treated with DFMO. Thus it appears that the presence of high levels of putrescine increases the toxicity of the analogues perhaps by occupying binding sites. Several polyamine analogues are currently being developed as antineoplastic agents. The mechanism(s) by which they bring about their effects are unknown but their accumulation and ability to be transported by the polyamine-transport system are clearly critical [45–48]. The use of conditions activating polyamine transport and minimizing its down-regulation and putrescine efflux may be important in the success of these agents.

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