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Effect of *S*-Adenosyl-1,12-diamino-3-thio-9-azadodecane, a Multisubstrate Adduct Inhibitor of Spermine Synthase, on Polyamine Metabolism in Mammalian Cells†

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Received May 8, 1989; Revised Manuscript Received June 22, 1989

ABSTRACT: The effects of the potent spermine synthase inhibitor *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDatad) on polyamine biosynthesis have been studied in transformed mouse fibroblasts (SV 3T3 cells) and in mouse leukemia cells (L1210). A dose-dependent decrease in intracellular spermine concentration was observed in both cell lines when grown in the presence of the inhibitor. A major difference in the effects seen in these two cell lines was the cytotoxicity observed in L1210 cells exposed to the inhibitor, which contrasted with little or no effects on growth of SV 3T3 cells treated similarly. Oxidative metabolism of the drug in L1210 cells was suggested by the fact that addition of aminoguanidine, an amine oxidase inhibitor, to the cell cultures ablated the cytotoxic effects of the inhibitor. Complete analysis of intracellular polyamines was carried out, together with analysis of *S*-adenosylmethionine, decarboxylated *S*-adenosylmethionine, and the inhibitor. These analyses revealed that, although the inhibitor had a dramatic effect on spermine biosynthesis in the cells studied, a compensatory increase in spermidine biosynthesis was observed. This resulted in no change in total polyamine concentrations in cells treated with inhibitors of either spermine synthase or spermidine synthase (Pegg et al., 1982) alone or in combination. In all cases, the concentration of the aminopropyl donor decarboxylated *S*-adenosylmethionine increased dramatically, thus allowing for the observed maintenance of total polyamine levels even in the presence of either one or both potent inhibitors of the aminopropyltransferases. Oxidative metabolism of the inhibitor complicates the interpretation of experiments carried out in the absence of amine oxidase inhibitors such as aminoguanidine. In addition, the results suggest that the extent of polyamine depletion achieved with AdoDatad and other aminopropyltransferase inhibitors could be significantly improved by combination with specific inhibitors of *S*-adenosylmethionine decarboxylase, which would prevent the compensatory increase in the decarboxylated *S*-adenosylmethionine pool size.

Inhibitors of polyamine biosynthesis have potential as chemotherapeutic agents and are also of considerable value as research tools to investigate the physiological function of polyamines. Potent and specific inhibitors have now been described for most of these enzymes, and the effects of exposure to cells to inhibitors of ornithine decarboxylase, *S*-adenosylmethionine (AdoMet)[1] decarboxylase, and spermidine synthase are well documented (Pegg & McCann, 1982, 1988; Tabor & Tabor, 1984; Porter & Sufrin, 1986; Pegg, 1986,

† This research was supported by Grants GM-26290 (to A.E.P.) and CA-37606 (to J.K.C. and A.E.P.) from the National Institutes of Health and by a postdoctoral fellowship from the Medical Research Council of Canada (to R.P.).
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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; AdoS$^+$(CH$_3$)$_2$, *S*-methyl-5′-deoxy-5′-(methylthio)adenosine [dimethyl(5′-adenosyl)-sulfonium perchlorate]; AdoDatad, *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane; DFMO, α-(difluoromethyl)ornithine; AdoDato, *S*-adenosyl-1,8-diamino-3-thiooctane; MTA, 5′-(methylthio)adenosine; MAOEA, 5′-deoxy-5′-[*N*-methyl-*N*-[2-(aminooxy)ethyl]amino]-adenosine; MHZPA, 5′-deoxy-5′-[*N*-methyl-*N*-(3-hydrazinopropyl)-amino]adenosine.

0006-2960/89/0428-8446$01.50/0

1988; McCann et al., 1987). However, selective inhibition of the production of spermine has been described only in a limited number of studies, most having been conducted by using *S*-methyl-5′-deoxy-5′-(methylthio)adenosine [$AdoS^+(CH_3)_2$].[1] Although this compound is a reasonably effective inhibitor of spermine synthase, it is not particularly potent and it is clearly not specific since it also blocks the activity of *S*-adenosylmethionine decarboxylase (Pegg & Coward, 1985; Pegg et al., 1986a). Recently, we have described the synthesis of *S*-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDatad)[1] and shown that this compound, designed as a mechanism-based inhibitor of spermine synthase, is a potent inhibitor of this enzyme in vitro (Woster et al., 1989). In the experiments described in the present paper, we have investigated the effect of AdoDatad on polyamine metabolism and cell growth in cultured mammalian cells. The results show that the compound is effective in depleting spermine but that it is subject to metabolism, which may limit its effectiveness and lead to other biological effects unrelated to the inhibition of spermine synthesis.

Materials and Methods

Materials. DFMO was kindly provided by Dr. P. P. McCann, Merrell Dow Research Institute, Cincinnati, OH. Decarboxylated *S*-adenosylmethionine was a generous gift from Dr. K. Samejima, Josai University, Japan. The syntheses of $AdoS^+(CH_3)_2$ (Tang et al., 1980), AdoDato (Tang et al., 1981), and AdoDatad (Woster et al., 1989) were carried out as previously reported. *S*-Adenosyl[*carboxyl*-^{14}C]methionine (50 Ci/mol) and [^{14}C(U)]sucrose (4.8 Ci/mol) were purchased from NEN, Boston, MA. [1-^{14}C]Acetyl-CoA (55 Ci/mol) was purchased from ICN Radiochemicals, Irvine, CA. All other biochemical reagents were obtained from Sigma Chemical Co., St. Louis, MO.

Assay of S-Adenosylmethionine Decarboxylase. Cell extracts were prepared, and the activity of AdoMet decarboxylase was determined by recording the release of $^{14}CO_2$ from *S*-adenosyl[*carboxyl*-^{14}C]methionine as previously described (Pegg, 1984).

Cell Culture. SV 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 3% horse serum/2% fetal calf serum as described by Pegg (1984). L1210 cells were grown in suspension culture in RPMI 1640 medium containing 10% Nu-serum (Collaborative Research, Inc., Lexington, MA) as described by Pera et al. (1986). In some experiments, 1 mM aminoguanidine was added to the medium to inhibit the action of oxidases present in the serum.

Polyamine Analysis. The cells were then harvested, extracted, and deproteinized with 10% (w/v) trichloroacetic acid (Pegg, 1984) and aliquots used for the determination of polyamine content by using an ion-pair reversed-phase HPLC separation and postcolumn derivatization with *o*-phthalaldehyde essentially as described by Seiler and Knödgen (1980, 1985). The aliquots (up to 0.25 mL) were centrifuged and applied to a column (Beckman Ultrasphere ODS 5 μm; 4.6 mm × 25 cm protected by a 4.6 mm × 4 cm guard column of ODS-5S from Bio-Rad) equilibrated with a mixture of 70% buffer A (0.1 M sodium acetate, 0.01 M sodium octanesulfonate, pH 4.5) and 30% buffer B (20 parts 0.2 M sodium acetate and 0.01 M sodium octanesulfonate, pH 4.5; 6 parts acetonitrile; 3 parts methanol). The column was then eluted with a linear gradient of 70% buffer A/30% buffer B to 100% buffer B over 40 min at a flow rate of 1 mL/min at 35 °C. The results were expressed as nmol/mg of protein or as fmol/cell. Protein was determined by the method of Bradford (1976).

Determination of Nucleosides. Decarboxylated AdoMet, acetylated decarboxylated AdoMet, MTA, AdoDatad and its metabolites were separated by ion-pair reversed-phase HPLC using the same conditions as those used for polyamines (Seiler, 1983). This system provided a good separation of all of the nucleosides of interest with retention times of 6.7 min for AdoMet, 9.5 min for acetylated decarboxylated AdoMet, 16.5 min for MTA, 30.0 min for decarboxylated AdoMet, 34.1 min for a metabolite of AdoDatad and for AdoDato (see below), and 42.2 min for AdoDatad. The eluate was monitored at 254 nm and the amount calculated from the peak heights using standard curves constructed with the authentic compounds except for the metabolites of AdoDatad for which no standards were available. It was assumed that the extinction coefficient of the metabolites was the same as for AdoDatad itself. The nucleosides were measured in extracts from the cells and from the culture medium. The extracts were prepared and deproteinized as for polyamine analysis.

Amounts of AdoDatad and its metabolite measured in L1210 cell extracts were corrected for extracellular water trapped in the cell pellet. Extracellular space was determined by adding [^{14}C]sucrose to the cell suspension and measuring the percentage of total added radioactivity left at the end of the harvesting procedure. This volume was estimated at 1.83 ± 0.23 μL of medium left in each total extract. This correction was not needed for the SV 3T3 cells, which grow as attached monolayers and could be washed more completely.

Acetylation of AdoDatad. The ability of AdoDatad and AdoDato to serve as substrates for acetyltransferases was determined as described by Pegg et al. (1986b). The assay medium contained 100 mM Tris-HCl, pH 7.8, 0.8 nmol of [1-^{14}C]acetyl-CoA, and either rat liver nuclear histone/polyamine acetyltransferase (Erwin et al., 1984) or cytosolic spermidine/spermine-N^1-acetyltransferase (Della Ragione & Pegg, 1982) as well as the amine substrate.

Results

Effect of AdoDatad on Polyamine Metabolism in SV 3T3 Cells. We have reported previously that AdoDatad is a powerful and specific inhibitor of spermine synthase (Woster et al., 1989). In order to test whether it was taken up by mammalian cells and was able to block the formation of spermine, various concentrations were added to SV 3T3 cells in culture, and the cellular polyamine content was determined after 72 h of growth (Table I). It can be seen from these results that there was a dose-dependent decrease in the content of spermine with a maximal 80% reduction occurring at 25 μM AdoDatad. AdoDatad was therefore considerably more potent in blocking spermine synthesis than $AdoS^+(CH_3)_2$, which has previously been used for this purpose but requires the addition of concentrations of 0.1–0.2 mM to achieve a maximal effect (Pegg & Coward, 1985) as shown in Table I. In parallel to the reduction of spermine, there was an increase in spermidine which is consistent with the polyamine biosynthesis pathway being blocked at the spermine synthase step which converts spermidine into spermine. There was also a reduction in the cellular putrescine concentration.

At the concentrations of AdoDatad below 25 μM, there was no effect on the growth of the SV 3T3 cells, suggesting that spermine synthesis is not essential for a normal growth rate (Table I). In the experiment described in Table I, cell growth was measured only over a 72-h period, but in other experiments no effect of 25 μM AdoDatad was seen over at least 10 population doublings. However, it cannot be ruled out that a requirement for spermine would be observed on longer exposure. Also, it can clearly be seen from Table I that the total

Table I: Effect of AdoDatad on Polyamine Levels in SV 3T3 Cells[a]

addition (total)	cell number × 10^{-6}	polyamine content (fmol/cell)			
		putrescine	spermidine	spermine	total
none	6.5 ± 0.3	0.49 ± 0.15	2.78 ± 0.31	1.34 ± 0.19	4.61
1 μM AdoDatad	6.6 ± 0.2	0.59 ± 0.16	2.74 ± 0.25	1.31 ± 0.15	4.64
5 μM AdoDatad	6.6 ± 0.4	0.25 ± 0.14	3.79 ± 0.30	0.73 ± 0.12	4.77
10 μM AdoDatad	6.2 ± 0.1	0.22 ± 0.06	4.01 ± 0.42	0.49 ± 0.08	4.72
25 μM AdoDatad	5.8 ± 0.3	0.18 ± 0.05	4.20 ± 0.35	0.28 ± 0.07	4.66
50 μM AdoDatad	5.3 ± 0.4	0.10 ± 0.02	4.55 ± 0.60	0.27 ± 0.08	4.92
100 μM AdoDatad	5.1	0.16	4.68	0.21	5.05
100 μM $AdoS^+(CH_3)_2$	6.5	0.26	4.52	0.30	5.08

[a] Cells were grown for 72 h in the presence of the compound shown, which was added 4 h after plating. Results are shown as the mean ±SD for at least five estimations or as the mean of three separate measurements.

Table II: Effect of AdoDatad on Polyamine Levels in L1210 Cells[a]

addition	polyamine content (nmol/mg of protein)			
	putrescine	spermidine	spermine	total
none	3.3 ± 0.4	22.4 ± 3.4	8.1 ± 1.3	33.8
5 μM AdoDatad	2.3 ± 0.6	28.6 ± 2.2	5.7 ± 0.9	36.6
10 μM AdoDatad	1.0 ± 0.2	29.9 ± 3.9	4.5 ± 0.8	35.4
20 μM AdoDatad	0.7 ± 0.4	34.8 ± 5.3	3.8 ± 1.0	39.3
50 μM AdoDatad	0.7 ± 0.2	35.2 ± 0.25	3.6 ± 0.6	39.5
100 μM AdoDatad	0.3 ± 0.1	30.3 ± 0.25	3.4 ± 0.6	34.0

[a] Cells were grown for 72 h in the presence of 1 mM aminoguanidine and the concentration of AdoDatad shown. Results are shown as the mean ±SD for at least four estimations. The results are given as nmol/mg of cell protein since the cell number was not determined directly in these experiments.

polyamine concentration was not affected by the drug because of the compensatory increase in spermidine, and it is certainly possible that the functions of polyamines needed for cell growth are fulfilled by the spermidine. Higher concentrations of AdoDatad had a significant growth inhibitory effect on the SV 3T3 cells, but this effect was not prevented by the provision of exogenous spermine (results not shown).

The content of polyamines in cultures of SV 3T3 cells grown for various times in the presence of 25 μM AdoDatad is shown in Figure 1. Over a 3-day period there was a substantial increase in the total spermine content of the control cultures, but there was very little increase in the spermine content in the presence of AdoDatad. After 3 days, the accumulation of spermine was reduced by about 90%. Over the same time period, there was an increase in spermidine (Figure 1B) and a decrease in putrescine (Figure 1C). These results confirm that the presence of AdoDatad strongly reduces the ability of the cells to synthesize spermine.

Results described below for studies on L1210 cells suggested that AdoDatad was degraded by serum oxidases and that this degradation was blocked by the addition to the cultures of 1 mM aminoguanidine. Such degradation of the drug may also take place in the SV 3T3 cultures although it did not produce any overt toxic effects. When the effects of AdoDatad on cellular spermine content in the SV 3T3 cells were determined in the presence and absence of aminoguanidine, it was found that the oxidase inhibitor increased the effectiveness of the AdoDatad such that a maximal effect was obtained with only 5–10 μM concentrations of AdoDatad (results not shown).

Effect of AdoDatad on Polyamine Metabolism and Growth of L1210 Cells. Addition of AdoDatad to L1210 cells growing in RPMI medium supplemented with Nu-serum produced toxic effects at all concentrations of the drug above 5 μM. This toxicity appears to be due to the oxidation of AdoDatad by serum oxidases present in the Nu-serum since it was prevented by the addition of 1 mM aminoguanidine to the culture medium (results not shown). In the presence of aminoguanidine, AdoDatad led to a dose-dependent reduction of spermine and

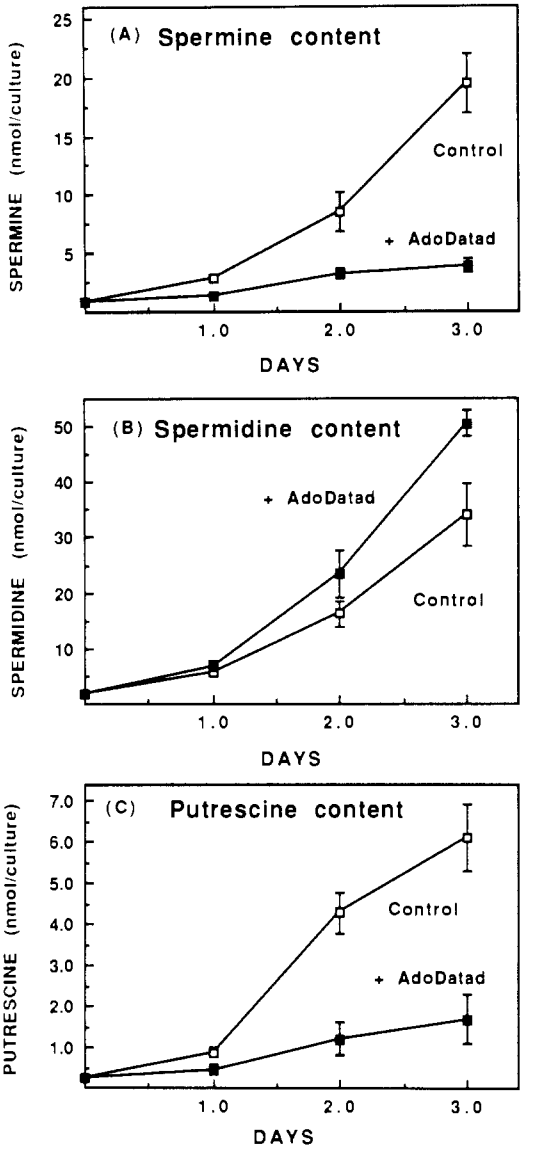

FIGURE 1: Time course of effect of AdoDatad on polyamine content in SV 3T3 cells. SV 3T3 cells were exposed to 25 μM AdoDatad at 4 h after plating, and the content of spermine (panel A), spermidine (panel B), and putrescine (panel C) in the culture was determined 1, 2, and 3 days later. Results are shown for control (□) and AdoDatad-treated (■) cells. Results are expressed as polyamine content in the entire culture, but there was no difference in cell number between the treated and the control cells. Results are shown ±SD. Where no SD is indicated, the value was too small to be distinct from the symbols.

increase in spermidine in L1210 cells (Table II) with a maximal effect at 10–20 μM concentrations of the drug. The reduction in cellular spermine levels brought about by AdoDatad was maintained for at least 142 h of culture provided

Table III: Effect of AdoDatad and Other Inhibitors on Content of Polyamines and Decarboxylated *S*-Adenosylmethionine in SV 3T3 Cells[a]

addition	polyamine or nucleoside content (nmol/mg)				
	putrescine	spermidine	spermine	decarboxylated AdoMet	acetylated decarboxylated AdoMet
none	4.7 ± 0.3	26.3 ± 1.2	15.0 ± 0.7	0.005	<0.005
10 μM AdoDatad	2.2 ± 0.2	36.1 ± 1.9	5.9 ± 0.4	0.11	0.03
25 μM AdoDatad	1.3 ± 0.2	38.8 ± 6.0	3.8 ± 0.5	0.13	0.04
100 μM AdoDato	9.1 ± 3.2	3.8 ± 0.7	25.7 ± 7.2	0.16	0.06
25 μM AdoDatad + 100 μM AdoDato	5.2 ± 0.7	29.6 ± 4.6	19.4 ± 3.3	0.44	0.17
5 mM DFMO	<0.2	1.0 ± 0.4	13.8 ± 1.4	1.63	0.36
5 mM DFMO + 50 μM AdoDatad	<0.2	2.9 ± 1.1	10.9 ± 1.3	1.75	0.42

[a] Cells were grown for 72 h in the presence of the compound shown, which was added 4 h after plating. Results are shown as the mean ±SD for at least five estimations or as the mean of three separate measurements. The results are given as nmol/mg of cell protein since the cell number was not determined directly in these experiments.

Table IV: Content of Decarboxylated AdoMet, AdoDatad, and Its Metabolite in SV 3T3 Cells Treated with AdoDatad[a]

addition	nucleoside content (nmol/mg of protein)		
	AdoDatad	metabolite	decarboxylated AdoMet
none	0.0	0.0	<0.01
1 μM AdoDatad	0.06	0.20	0.03
5 μM AdoDatad	0.11	0.38	0.08
10 μM AdoDatad	0.23	1.05	0.13
20 μM AdoDatad	0.97	1.25	0.15
50 μM AdoDatad	0.78	0.95	0.14
100 μM AdoDatad	0.96	0.99	0.16

[a] Cells were grown for 72 h in the presence of the concentration of AdoDatad shown, which was added 4 h after plating. Results are shown as the mean of three separate measurements. In this experiment the content of the acetyl derivative of decarboxylated AdoMet was not measured.

that the medium was changed after 3 days to permit continuous cell growth and replenishment of the drug (results not shown).

AdoDatad was inhibitory to the growth of L1210 cells even in the presence of aminoguanidine (Figure 2A). However, this effect may not be related to the reduction in spermine since the effect on growth increased with dose of the drug over the entire range of 5–100 μM whereas the effect on spermine was already maximal to 10–20 μM (Table II and Figure 2B). Furthermore, the inhibition of growth was not prevented by the addition of 1 μM spermine (Figure 2A) although this maintained the cellular spermine concentration (Figure 2B). One puzzling feature of these experiments is that the exposure to the higher concentrations of AdoDatad led to a clear increase in the amount of spermine present in the cells treated with 1 μM spermine. At present, we have no explanation for this effect although it should be noted that these concentrations of AdoDatad have a marked toxic effect on the cells and may lead to damage to the cell membrane.

Effects of AdoDatad on AdoMet Decarboxylase Activity, MTA Production, and the Cellular Content of Decarboxylated AdoMet. There was a dose-dependent increase in the content of decarboxylated AdoMet and its acetylated derivative in SV 3T3 cells treated with AdoDatad (Tables III and IV). This rise is likely to be due to an increased activity of AdoMet decarboxylase which is known to be negatively affected by the cellular spermine concentration (Pegg, 1986; Madhubala et al., 1988). In fact, there was a 3-fold rise in the activity of this enzyme (from 0.19 to 0.62 unit/mg of protein) in SV 3T3 cells treated with 25 μM AdoDatad for 72 h.

A slightly smaller (2.1-fold) rise in the activity of AdoMet decarboxylase was observed in L1210 cells treated with 10 μM AdoDatad for 72 h. In these cells, there was also a dose-dependent rise in the content of decarboxylated AdoMet, but

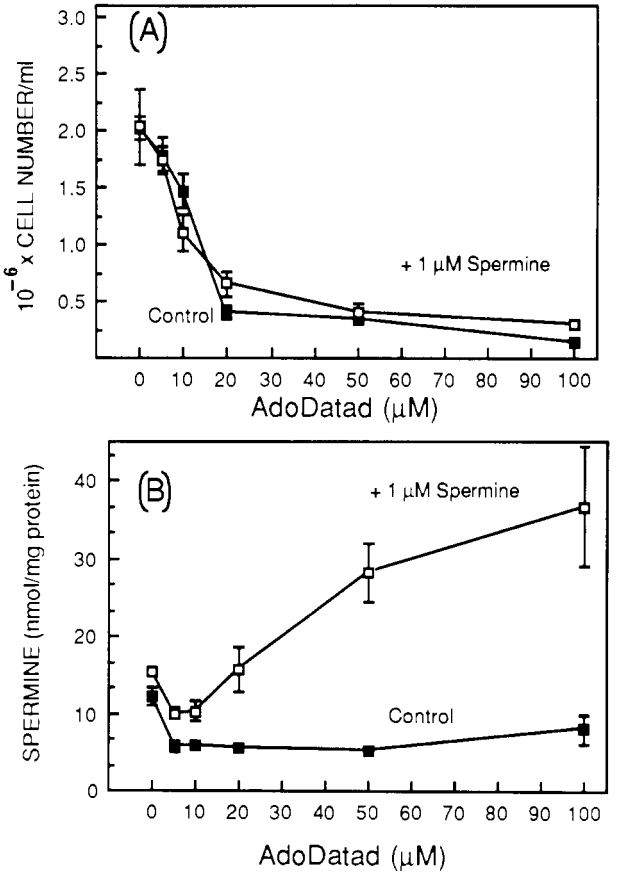

FIGURE 2: Effect of AdoDatad on growth and spermine content of L1210 cells. L1210 cells were exposed to the concentration of AdoDatad shown in the presence (□) and absence of 1 μM spermine (■). Panel A shows the cell number after 48-h exposure, and the panel B shows the spermine content after 72-h exposure. Results are shown ±SD. Where no SD is indicated, the value was too small to be distinct from the symbols. Aminoguanidine was added to cultures at 1 mM concentration.

there was no significant effect on the production of MTA (results not shown). The L1210 cells used in this study lack MTA phosphorylase, which is responsible for the very rapid degradation of MTA that occurs in most mammalian cells (Schlenk, 1983; Williams-Ashman et al., 1982). In cells lacking MTA phosphorylase, the production of MTA, which is excreted into the medium, provides an accurate reflection of the extent of aminopropyltransferase activity (Kamatani & Carson, 1980; Pegg et al., 1988). The lack of effect of AdoDatad on the production of MTA by the L1210 cells confirms that there is a compensatory synthesis of spermidine when spermine production is blocked.

Effect of Combination of AdoDatad and AdoDato on Cellular Polyamine Content. We have previously reported

that the addition of AdoDato, a potent inhibitor of spermidine synthase (Tang et al., 1980, 1981), to cultured SV 3T3 cells led to a decrease in spermidine but to a compensatory increase in spermine (Pegg et al., 1982). Since this is the reverse of the situation found on administration of AdoDatad, the effect of combinations of the two drugs was examined and is shown in Table III. Although each of the inhibitors added separately had their expected effect in altering cellular polyamine levels, when both drugs were present, the polyamine content was normalized. This result is, at first sight, quite paradoxical since the presence of two potent aminopropyltransferase inhibitors might be expected to greatly deplete cellular polyamines, but the explanation appears to reside in the marked rise in the cellular content of decarboxylated AdoMet (Table III). This rise allows the synthesis of spermidine and spermine to proceed even in the presence of the two inhibitors. A similar restoration of approximately normal levels of polyamines when both AdoDato and AdoDatad were applied was seen in L1210 cells (results not shown).

The effect of combining the administration of AdoDatad with that of DFMO, a widely used and potent ornithine decarboxylase inhibitor (McCann et al., 1987), were also examined. It is well-known that treatment with DFMO greatly depletes putrescine and spermidine but has little effect on spermine, and it was hoped that the further addition of AdoDatad would allow depletion of cellular spermine. However, as shown in Table III, there was no significant effect on AdoDatad on the DFMO treated cells, and this is likely to be due to the huge increase in decarboxylated AdoMet, which minimizes the effectiveness of the spermine synthase inhibitor.

Accumulation and Metabolism of AdoDatad. The accumulation and possible metabolism of AdoDatad by SV 3T3 or L1210 cells were examined by extraction of the nucleosides from cells and medium treated with the drug and analysis of the extracts by ion-pair reversed-phase chromatography (Seiler, 1983). AdoDatad was clearly present in the cells treated with it, confirming that this drug is able to pass across the cell membrane in SV 3T3 cells (Table IV) and in L1210 cells (Figure 3). In SV 3T3 cells, maximal intracellular concentrations of the drug also corresponded well with those levels giving the greatest depletion of cellular spermine (Table IV). In L1210 cells, AdoDatad continued to accumulate in the cells in a dose-dependent manner over the whole range of concentrations tested (Figure 3). In fact, in cells exposed to 100 μM external concentrations of AdoDatad, the intracellular content was about 50 μM on the basis of a cell volume of 4.5 pL/cell, as determined with ^3H_2O (R. Poulin, unpublished observations).

An additional peak representing a compound that appears to be a metabolite of AdoDatad was found in chromatograms of extracts from SV 3T3 cells treated with the drug (Table IV). The metabolite was also found in L1210 cells exposed to AdoDatad in the presence of aminoguanidine albeit in amounts less than the parent drug (Figure 3). In this experiment, the medium was also analyzed for the presence of this metabolite, and much higher amounts eventually accumulated in the medium than in the cells (Figure 3). Extracellular levels of the metabolite increased with the dose of the drug over the entire range studied. The intracellular levels increased with doses of AdoDatad up to 50 μM but then declined. This may be due to the toxic effects of the higher concentrations as described above.

One possible explanation for this result is that the AdoDatad was degraded by reaction with compounds present in the culture medium or metabolized by enzymes present in the

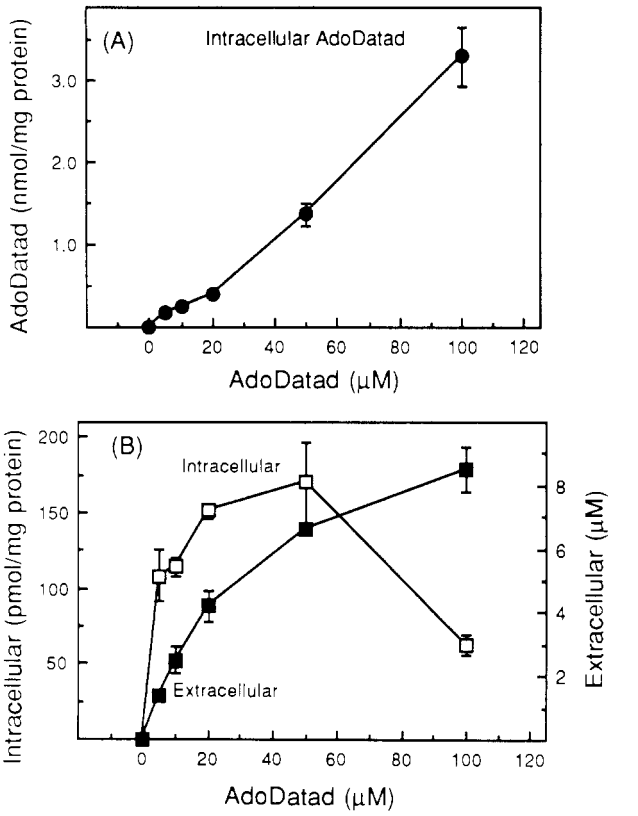

FIGURE 3: Uptake and metabolism of AdoDatad in L1210 cells. The cells were treated for 72 h with AdoDatad at the concentration shown, and the intracellular content of AdoDatad (panel A), and the intracellular (□) and medium (■) content of the metabolite of AdoDatad (panel B) were measured as shown. The values for intracellular content can be converted to approximate micromolar concentrations by multiplying by 12.5 (mg/L) for AdoDatad (panel A) and by 0.0125 (mg/L) for the metabolite (panel B). Results are shown ±SD. Where no SD is indicated, the value was too small to be distinct from the symbols. Aminoguanidine was added to cultures at 1 mM concentration.

serum added. In order to test this, 10 μM AdoDatad was incubated with the media used for the cell cultures in the presence and absence of serum and of aminoguanidine (Table V). The results show clearly that AdoDatad is converted to this metabolite (derivative 1, Table V) only when serum is present. The conversion occurred with either the Nu-serum or the horse serum/fetal bovine serum mixture. The concentration of this metabolite was always greater with the Nu-serum (Table V), but the formation in culture medium supplemented with the 3% horse serum/2% fetal bovine serum was variable from one batch of serum to another (results not shown). The addition of 1 mM aminoguanidine to the culture medium prior to the supplementation with AdoDatad prevented the formation of this compound by the serum enzymes (Table V).

The formation of derivative 1 was enhanced 2-fold when L1210 cells were grown for 48 h in serum-containing medium as compared to the production by the same medium in the absence of cells (results not shown). However, in shorter incubations, L1210 cells did not degrade AdoDatad to any significant extent over an 18-h period in the absence of serum whereas, under the same conditions, about 50% of the AdoDatad was degraded to derivative 1 in an aminoguanidine-sensitive reaction when serum was added to the cultures (Table VI). It is thus not yet clear whether intracellular metabolism can contribute to the degradation of AdoDatad.

Although the metabolite, derivative 1, has not been identified conclusively, it has an identical HPLC retention time with that

Table V: Content of AdoDatad and Derivatives in Media[a]

media	serum[b]	time (h)	aminoguanidine	nucleoside content (μM)[c] AdoDatad	derivative 1	derivative 2
RPMI 1640	none	72	none	9.6	ND[d]	0.11
RPMI 1640	none	72	1 mM	5.2	ND	1.1
RPMI 1640	10% Nu-serum	12	none	3.9	4.4	0.06
RPMI 1640	10% Nu-serum	72	none	1.7	6.0	0.07
RPMI 1640	10% Nu-serum	72	1 mM	7.0	ND	0.66
RPMI 1640	2% FBS/3% HS	72	none	9.6	ND	0.11
RPMI 1640	2% FBS/3% HS	72	1 mM	6.9	ND	0.07
DMEM	none	72	none	9.7	ND	0.06
DMEM	none	72	1 mM	9.4	ND	0.13
DMEM	2% FBS/3% HS	12	none	9.3	0.4	0.05
DMEM	2% FBS/3% HS	72	none	8.4	1.4	0.05
DMEM	2% FBS/3% HS	72	1 mM	9.4	ND	0.14
DMEM	10% Nu-serum	72	none	0.7	6.0	0.08
DMEM	10% Nu-serum	72	1 mM	9.3	ND	0.14

[a] Derivative 1 had an elution time of 34.1 min, and derivative 2 had an elution time of 39.1 min. AdoDatad was originally added to the culture medium at a concentration of 10 μM. The medium was incubated in the absence of cells in the cell culture incubator for 12 or 72 h as shown. The AdoDatad stock solution used for this experiment, which was made up in phosphate-buffered saline, pH 7.2, and had been stored at −20 °C, contained a small amount of derivative 2 corresponding to about 0.16 μM after addition to the medium. Recovery of metabolites was measured by estimating the peak heights of two different UV-absorbing compounds present in the growth medium alone. [b] 2% FBS/3% HS = fetal bovine serum/horse serum (2%/3% v/v). [c] These values are calculated by assuming that the metabolites have the same extinction coefficient as AdoDatad. Other derivatives which were not resolved sufficiently from serum components to be quantitated were also present in culture media without aminoguanidine. For these reasons, the sum of AdoDatad and derivatives does not add up to 10 μM. [d] ND = not detected.

Table VI: Degradation of AdoDatad by Stationary Cultures of L1210 Cells[a]

serum added	aminoguanidine	AdoDatad intracellular (nmol/mg)	AdoDatad extracellular (μM)	derivative 1 intracellular (nmol/mg)	derivative 1 extracellular (μM)
none	none	0.28 ± 0.05	23.9 ± 1.4	<0.05	<0.1
Nu-serum	none	0.16 ± 0.02	9.3 ± 0.1	0.14 ± 0.03	12.1 ± 0.6
none	1 mM	0.18 ± 0.02	25.8 ± 0.6	<0.05	<0.1
Nu-serum	1 mM	0.12 ± 0.02	19.7 ± 1.6	<0.05	2.6 ± 0.8

[a] A 4-day culture of L1210 cells was harvested, washed with 20 mL of serum-free RPMI 1640 medium, and resuspended at half the starting density (about 1.5×10^6 cells/mL) in 5 mL of culture fluid containing aminoguanidine as shown and either serum-free RPMI 1640 or the medium containing 10% Nu-serum as indicated. AdoDatad was added at 25 μM, and the cells were incubated for 18 h at 37 °C. The cells were then collected by centrifugation and the cell pellets and the media analyzed for the content of AdoDatad and derivative 1.

of AdoDato (34.1 ± 0.1 and 34.1 ± 0.2 min for 18 and 20 measurements, respectively), and when it was mixed with AdoDato, the mixture eluted as a single sharp peak. Moreover, AdoDato was itself degraded by serum-containing medium (but not in the absence of serum) to UV-absorbing material that was also found among the minor derivatives obtained from AdoDatad incubated in the presence of serum (see below). This degradation was completely prevented by aminoguanidine, making it likely that AdoDato is itself a substrate for serum amine oxidases.

A second compound (derivative 2) formed from AdoDatad was also found in the media incubated with 10 μM AdoDatad (Table V). Its concentration was increased significantly in the RPMI 1640 medium (but not in the DMEM) incubated in the presence of aminoguanidine but was reduced in the presence of serum. The origin of this derivative is unknown, but it may be due to the reaction of AdoDatad with H_2O_2 generated by the Hepes in the RPMI 1640 (Zigler et al., 1985). Other derivatives whose formation was prevented by addition of aminoguanidine were found to accumulate in AdoDatad-containing media. These could not be quantitated as the peaks were not fully resolved from other components in the serum, but they may account for the fact that the sum of AdoDatad and the derivatives 1 and 2 in Table V does not equal the starting concentration of AdoDatad.

It is also possible that AdoDatad might be metabolized by the formation of an *N*-acetyl derivative since it is known that both the polyamines themselves and decarboxylated AdoMet are subject to acetylation (Pegg et al., 1986b). AdoDatad and AdoDato were therefore tested for their ability to serve as a substrate in the reaction with [1-^{14}C]acetyl-CoA catalyzed by either the inducible spermidine/spermine N^1-acetyltransferase (Della Ragione & Pegg, 1982) or a nuclear enzyme preparation which is known to act on histones, polyamines, or decarboxylated AdoMet (Pegg et al., 1986). Both compounds were acetylated by the nuclear enzyme preparation, but neither were substrates for the spermidine/spermine N^1-acetyltransferase (Table VII). The radioactive product formed by the reaction of [1-^{14}C]acetyl-CoA with AdoDatad did chromatograph in the same position as derivative 1 when analyzed by HPLC, but more detailed comparisons were not possible owing to the small amount of material that could be synthesized in the enzymatic reaction in vitro.

Table VII: Acetylation of AdoDato and AdoDatad by Polyamine Acetylases

enzyme added	activity (cpm incorpd with 0.1 mM substrate shown) spermidine	AdoDato	AdoDatad
inducible spermidine/spermine-N^1-acetyltransferase	2357	5	7
nuclear histone/spermidine acetyltransferase	4994	239	170

Discussion

AdoDatad is the most potent inhibitor of spermine synthase that has been synthesized at present (Woster et al., 1989). The present results show that AdoDatad is able to enter mammalian cells and block the production of spermine. As a consequence of this inhibition, there is a rise in the intracellular content of spermidine and decarboxylated AdoMet but a fall

in the level of putrescine. This decline in putrescine may be mediated via a decrease in ornithine decarboxylase activity in response to the elevation of spermidine since ornithine decarboxylase is strongly repressed by spermidine (Pegg, 1986; Madhubala et al., 1988). Despite the falls in putrescine and spermine, the total polyamine content was not reduced because of the rise in cellular spermidine. This rise in spermidine may permit growth of SV 3T3 cells even though the capacity to produce spermine is impaired. The ability of a number of mammalian cell lines to grow at a normal rate in the presence of compounds interfering with spermine synthesis is now clearly established for three inhibitors of diverse chemical structures. In addition to the results shown here for AdoDatad, two other spermine synthase inhibitors, namely, $AdoS^+(CH_3)_2$ (Pegg & Coward, 1985) and *n*-butyl-1,3-diaminopropane (Baillon et al., 1989), do not lead to a reduction in cell growth. The inhibition of growth of L1210 cells by AdoDatad does not appear to be due to polyamine depletion since it was not reversed by exogenous spermine even though the cellular spermine content was restored to normal. Similar considerations apply to the growth inhibition in SV 3T3 cells when higher levels of the drug were used.

Although aminoguanidine, an inhibitor of the copper-containing amine oxidases (Seiler et al., 1985; Pegg, 1986), was added to the cell cultures to block the oxidation of AdoDatad by serum amine oxidases, it remains possible that the growth-inhibitory effects of higher doses of the compound are a secondary response to an oxidation product. The drug was considerably more cytotoxic toward L1210 cells when added in the absence of aminoguanidine, and as shown in Table V, the metabolism of AdoDatad to derivative 1 is suppressed by aminoguanidine. However, the derivative could still be detected in aminoguanidine-treated cultures, suggesting that inhibition of AdoDatad oxidation by aminoguanidine might become less efficient at higher AdoDatad concentrations (compare Tables V and VI) and for more extended incubation periods during which there may be some decomposition of the aminoguanidine. The formation of derivative 2, which may also contribute to cytotoxicity of high concentrations of AdoDatad, appears to be a chemical reaction involving AdoDatad and some components of the RPMI medium and was not prevented but rather was enhanced by aminoguanidine.

The terminal oxidation of naturally occurring polyamines such as spermine and spermidine leads to the production of unstable intermediates which can readily undergo β-elimination to generate acrolein (Kimes & Morris, 1971a). This production of acrolein, a highly reactive and toxic product, clearly contributes to the cytotoxicity of the oxidized polyamines (Kimes & Morris, 1971b; Kawase et al., 1982). Related compounds, which are oxidized by serum amine oxidases to form products that do not generate acrolein by this β-elimination, are in general less toxic toward cultured cells than natural polyamines and the other 3-substituted propylamines which can form acrolein (Kawase et al., 1982; Pegg, 1986). However, a number of 3-substituted propanals are also directly cytotoxic (Kawase et al., 1982), and the direct toxicity of the aldehydes generated by serum amine oxidases cannot be ruled out. Acrolein production could occur from oxidized AdoDatad via a reaction yielding also AdoDato, analogous to the conversion of spermine into spermidine and acrolein by bovine serum amine oxidases (Seiler et al., 1985; Kimes & Morris, 1971a). Our findings that derivative 1 and AdoDato have identical chromatographic properties and that AdoDato itself is subject to degradation by serum amine oxidase to products also formed during degradation of AdoDatad are consistent with the reaction scheme proposed above. Such metabolism may be prevented by minor changes to the structure such as the addition of an α-methyl group (Bey et al., 1987), which should leave the inhibitory action toward spermine synthase unchanged. The synthesis of such derivatives is presently being carried out.

Although it is not clear why the toxicity of AdoDatad is greater toward the L1210 cells than the SV 3T3 cells and the differential sensitivity of the L1210 cells is by no means excluded, it should be noted that the differences in the culture medium may well contribute to this phenomenon. The content of oxidases may be different between the Nu-serum (which is a semisynthetic serum substitute and contains 25% newborn bovine serum, giving a final concentration of 2.5% in the culture medium) and the 3% horse serum/2% fetal bovine serum used for the SV 3T3 cells. Also, the lower total protein concentration in the Nu-serum/RPMI medium used for the L1210 cells may afford less protection by serving as a reservoir to react with chemically reactive metabolites before they can be taken up by the cell than the medium used for the SV 3T3 cells.

In addition to the metabolism by oxidation, which may occur not only in the culture medium but also within cells, the possibility that AdoDatad may be metabolized by other routes must also be considered. In vitro, the drug and the related compound AdoDato may be subject to acetylation by the nuclear acetyltransferase. However, no intracellular metabolism of AdoDato was detected in Novikoff heptoma cells exposed to the drug for 6 h (Coward et al., 1983).

Very recently, Baillon et al. (1989) have reported the inhibition of spermine synthesis by *n*-butyl-1,3-diaminopropane. This substituted diamine was found to be a competitive inhibitor of mammalian spermine synthase with respect to the amine substrate and to cause a significant drop in the content of spermine in HTC cells. Although the specificity and metabolic stability of this compound has not yet been fully established, it may prove to be a useful addition to the available spermine synthase inhibitors. It is, however, likely to act as an inducer of the cytosolic spermidine/spermine N^1-acetyltransferase (Erwin & Pegg, 1986; Pegg, 1986), and the extent to which this induction might contribute to the fall in cellular spermine needs to be investigated.

The availability of two different spermine synthase inhibitors with diverse structures and distinct mechanisms of action should provide the research tools for detailed studies of the function of spermine in mammalian cells. Although the prevention of spermine synthesis appears to produce no effect on cell growth in the short term with the cell lines studied so far, the widespread occurrence of spermine synthase and the evolutionary development of a distinct aminopropyltransferase for the production of spermine suggest that this polyamine must play an essential role in some aspect of cellular physiology.

A significant problem confounding the use of aminopropyltransferase inhibitors to prevent the accumulation of cellular polyamines is the compensatory rise in the activity of AdoMet decarboxylase and hence in the production of decarboxylated AdoMet that occurs when the polyamine content is reduced (Pegg, 1984, 1986). Such an increase in AdoMet decarboxylase does occur in the cells treated with AdoDatad alone and to a much greater extent when spermidine is depleted by DFMO (Tables III and IV). This rise tends to overcome the inhibition by the drug. Therefore, AdoDatad did not have a significant effect on spermine levels in DFMO-treated cells, and for use in combination with DFMO in order to deplete

all of the cellular polyamines, an inhibitor such as *n*-butyl-1,3-diaminopropane which is a simple spermidine antagonist (Baillon et al., 1989) may be preferable. However, the combination of AdoDatad and DFMO with an AdoMet decarboxylase inhibitor such as MAOEA or MHZPA (Pegg et al., 1988) may be the most effective strategy for blocking the polyamine biosynthesis pathway with low concentrations of inhibitors. Such combinations could have a role in chemotherapy of tumors and other diseases that are sensitive to single agents blocking one step in the polyamine pathway (McCann et al., 1987; Pegg, 1988; Pegg & McCann, 1988). Another possibility for the pharmacological use of the spermine synthase inhibitors is suggested by the work of Marton, Seidenfeld, and colleagues, who have found that spermidine depletion by DFMO potentiated cell killing by DNA-alkylating and cross-linking agents such as 1,3-bis(2-chloroethyl)nitrosourea. The reduction of spermine which has a higher affinity for chromatin may be even more beneficial in this respect (Marton, 1987; Seidenfeld & Komar, 1985).

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