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Effects of inhibitors of spermidine and spermine synthesis on polyamine concentrations and growth of transformed mouse fibroblasts

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1. A number of compounds known to inhibit polyamine biosynthesis at various steps in the biosynthetic pathway were tested for their ability to inhibit growth and decrease polyamine concentrations in virally transformed mouse fibroblasts (SV-3T3 cells). 2. Virtually complete inhibition of growth was produced by the inhibitors of ornithine decarboxylase α-methylornithine and α-difluoromethylornithine and by the inhibitors of *S*-adenosylmethionine decarboxylase 1,1′-[(methylethanediylidene)dinitrilo]diguanidine and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine). The former inhibitors decreased putrescine and spermidine contents in the cells to very low values, whereas the latter substantially increased putrescine but decreased spermidine concentrations. The inhibitory effects of all of these inhibitors on cell growth could be prevented by the addition of spermidine, suggesting that spermidine depletion is the underlying cause of their inhibition of growth. 3. α-Difluoromethylornithine, which is an irreversible inhibitor of ornithine decarboxylase, was a more potent inhibitor of growth and polyamine production (depleting spermidine almost completely and spermine significantly) than α-methylornithine, which is a competitive inhibitor. This was not the case with the inhibitors of *S*-adenosylmethionine decarboxylase where 1,1′-[(methylethanediylidene)dinitrilo]diguanidine, a reversible inhibitor, was more active than 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine), an irreversible inhibitor. It is suggested that this effect may be due to the lesser uptake and/or greater chemical reactivity of the latter compound. 4. Various nucleoside derivatives of *S*-adenosylhomocysteine that inhibited spermidine synthase *in vitro* did not have significant inhibitory action against polyamine accumulation in the cell. These compounds, which included *S*-adenosylhomocysteine sulphone, decarboxylated *S*-adenosylhomocysteine sulphone, decarboxylated *S*-adenosylhomocysteine sulphoxide and *S*-adenosyl-4-thiobutyric acid sulphone did not inhibit cell growth or polyamine content until cytotoxic concentrations were added. 5. 5′-Methylthioadenosine, 5′-isobutylthioadenosine and 5′-methylthiotubercidin, which inhibit aminopropyltransferase activity *in vitro*, all inhibited cell growth and decreased spermidine content. Although these compounds were most active against spermine synthase *in vitro*, they acted in the cell primarily to decrease spermidine content. Cell growth could not be restored to normal values by addition of spermidine, suggesting that these nucleosides have another inhibitory action towards cellular proliferation. 6. 5′-Methylthioadenosine and 5′-isobutylthioadenosine are degraded by a phosphorylase present in SV3T3 cells, yielding 5-methylthioribose-1-phosphate and 5-isobutylthioribose-1-phosphate respectively, and adenine. This degradation appears to decrease the inhibitory action towards cell growth, suggesting that the nucleosides themselves are exerting the inhibitory action. 5′-Methylthiotubercidin, which is not a substrate for the phosphorylase and is a

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competitive inhibitor of it, was the most active of these nucleosides in inhibiting cell growth and spermidine content. 5'-Methylthiotubercidin and α-difluoromethylornithine had additive effects on retarding cell growth, but not on cellular spermine accumulation, also suggesting that the primary growth-inhibiting action of the nucleoside was not on polyamine production. 7. These results support the concept that 5'-methylthioadenosine phosphorylase plays an important role in permitting cell growth to continue by preventing the build-up of inhibitory intracellular concentrations of 5'-methylthioadenosine.

A number of agents that inhibit polyamine biosynthesis have been shown to cause inhibition of growth of cells in culture. These agents are nearly all inhibitors of ornithine decarboxylase, the only enzyme that forms putrescine in mammalian cells (Jänne *et al.*, 1978; Mamont *et al.*, 1978*a*,*b*; Pegg & McGill, 1979; Pegg & Williams-Ashman, 1980). The comparison of irreversible inhibitors of ornithine decarboxylase with competitive inhibitors has stressed the advantages of the former (Mamont *et al.*, 1978*a*,*b*). The only other widely used compound that interferes with polyamine production is methylglyoxal bis(guanylhydrazone), which is a potent, but irreversible, competitive inhibitor of *S*-adenosylmethionine decarboxylase, the enzyme forming decarboxylated *S*-adenosylmethionine, which is the source of the propylamine groups needed to convert putrescine into spermidine and spermine (Jänne *et al.*, 1978; Williams-Ashman & Pegg, 1980). In the present paper, we have tested the effects of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) on cell growth and polyamine synthesis. Previous studies has shown that this drug inactivates *S*-adenosylmethionine decarboxylase in a manner that could not be reversed by extensive dialysis (Pegg, 1978).

In the remainder of the experiments described in the present paper, we investigated the actions on cell growth and polyamine synthesis of a number of nucleosides that have inhibitory actions *in vitro* against spermidine and spermine synthases (Hibasami *et al.*, 1980*a*). The availability of compounds that could block the polyamine biosynthetic pathway at the later steps catalysed by these enzymes might be of value in the continued study of the role of individual polyamines in cellular proliferation. Another objective of the present experiments was to test whether 5'-methylthioadenosine, the other product of the spermidine and spermine synthase reactions, had an adverse effect on the cell growth and polyamine production. This nucleoside was a very potent inhibitor of the aminopropyltransferases, particularly spermine synthase (Hibasami & Pegg, 1978; Pajula & Raina, 1979; Hibasami *et al.*, 1980*a*). The presence of 5'-methylthioadenosine phosphorylase, which degrades this compound (Pegg & Williams-Ashman, 1969; Garbers, 1978), may be essential to prevent its inhibitory properties becoming manifest, but the extent to which this enzyme acts in this way *in vivo* is not yet known.

Materials and methods

S-Adenosyl-L-[*methyl*-^{14}C]methionine (sp. radioactivity 50–60 Ci/mol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A. Decarboxylated *S*-adenosylmethionine, both unlabelled and labelled in the methyl group, was prepared by the action of *S*-adenosylmethionine decarboxylase from *Escherichia coli* and purified as described by Pösö *et al.* (1976). Labelled 5'-methylthioadenosine was prepared from the *S*-adenosyl-L-[*methyl*-^{14}C]-methionine by heating at 90°C in 0.25 M-sodium citrate, pH 4, for 45 min and purified by chromatography on Dowex 50 (H^+ form; Schlenk & Zydek-Cwick, 1969). All other biochemicals were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. These included unlabelled *S*-adenosylmethionine and 5'-methylthioadenosine, which were purified by chromatography on Dowex 50 (H^+ form) before use. 5'-Methylthiotubercidin was synthesized as described by Coward *et al.* (1977). The sulphone and sulphoxide derivatives of *S*-adenosylhomocysteine, *S*-adenosyl-3-thiopropylamine and *S*-adenosyl-4-thiobutyric acid were prepared by procedures described by Borchardt & Wu (1974). 5'-Isobutylthioadenosine was obtained from Cal-Biochem, La Jolla, CA, U.S.A. 1,1'-[(Methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) was synthesized by the method of Baiocchi *et al.* (1963). 1,1'-[(Methylethanediylidene)dinitrilo]diguanidine ['methylglyoxal bis(guanylhydrazone) was purchased from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. DL-α-Difluoromethylornithine and DL-α-methylornithine were kindly provided by the Centre de Recherche Merrell International, Strasbourg, France. 2'-Deoxycoformycin and *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine were obtained from the Developmental Therapeutics Program of the National Cancer Institute. Media and reagents for cell culture were products of Flow Laboratories, Rockville, MD, U.S.A.

Cell culture

SV-40-virus-transformed 3T3 mouse embryo

fibroblasts were maintained in Dulbecco's modified Eagle's medium with 10% foetal calf serum as previously described (Bethell & Pegg, 1979). In some experiments, the cells were grown with 3% horse serum/2% foetal calf serum in place of the 10% foetal calf serum. Under these conditions, the cells grew slightly more slowly, but there was no difference in the response to the agents tested. For determination of the effect of the compounds on cell growth, 10^5 cells were seeded in 60-mm-diameter dishes. The number of cells was determined in a Coulter counter for duplicate samples from the same dish. All points shown are means for at least two separate experiments that agreed within $\pm 10\%$. Cytotoxic effects were tested as previously described (Bethell & Pegg, 1979).

Polyamine analysis

The medium was removed and the cells were washed twice with phosphate-buffered saline (6.8g of NaCl, 1.69g of Na_2HPO_4 and 0.2g of KH_2PO_4 in 1 litre) and then scraped from the plate with a 'rubber policeman' into 1–2ml of the phosphate-buffered saline. After centrifugation at 500*g* for 5min at 2°C, the phosphate-buffered saline was removed and the cells were resuspended in 0.2–0.4ml of 0.2M-$HClO_4$. The suspension was dispersed by two 10s bursts of a Heat-Systems-Ultrasonics Inc. sonicator with a micro-tip at 30–35W. The mixture was then centrifuged for 20min at 3000*g* and the supernatant was used for the assay of polyamines with a Dionex D 500 (Dionex, Sunnyvale, CA, U.S.A.) amino-acid analyser with fluorescence detection by the method of Seidenfeld & Marton (1979).

Assay of spermidine synthase and spermine synthase

The assay was carried out using enzymes purified from rat ventral prostate and the assay conditions described previously (Hibasami *et al.*, 1980*a*).

Assay of 5'-methylthioadenosine phosphorylase

Extracts from rat liver containing 5'-methylthioadenosine phosphorylase were prepared by the method of Garbers (1978) excluding the gel-filtration step. Extracts from the SV-3T3 cells were prepared by washing the cells twice with phosphate-buffered saline and then adding 1ml of 25mM-Tris/HCl, 0.1mM-disodium EDTA, 2.5mM-dithiothreitol, pH7.5. After swelling at 4°C for 15min, the cells were scraped from the plate and the sample was freeze-thawed twice in liquid N_2 to ensure complete cell lysis. The mixture was centrifuged at 17000*g* for 30min at 4°C in a Sorvall RC5 refrigerated centrifuge and the supernatant was used as a source of enzyme.

Enzyme assays were carried out by incubation at 37°C of a total volume of 0.2ml containing 50mM-sodium phosphate, 1mM-dithiothreitol, 100μM-5'-[*methyl*-^{14}C]methylthioadenosine (sp. radioactivity 1.22Ci/mol) and the enzyme extract. The final pH of the assay medium was 7.2. Some assays contained up to 20mM-Tris/HCl because of the large volume of cell extract added, but this did not affect the reaction. The amount of protein extract added was kept such that 1–6nmol of product was produced. Under these conditions, the reaction was proportional to the time of incubation for up to 30min and to the amount of protein added. The standard assay conditions used incubation for 15min. At the end of this time, the reaction was halted by the addition of 0.8ml of 0.3M-$HClO_4$. After centrifugation at 3000*g* for 10min, 0.5ml of the supernatant was applied to a small column of Dowex 50 (X2; H^+ form) in a Pasteur pipette plugged with glass wool and the effluent was collected. The column was eluted with 2ml of 0.1M-HCl and the combined eluates were counted for radioactivity at an efficiency of 59% in the presence of 10ml of Formula 947 scintillation fluid (New England Nuclear) in a Beckman model LS3133T liquid-scintillation counter. A zero-time unincubated sample contained about 120–140c.p.m., which was routinely subtracted from the assays. All assays had at least five times the radioactivity in this blank. Approx. 90% of the 5-methylthioribose 1-phosphate produced in the reaction was eluted in the 2.5ml counted. The remaining 10% could be eluted with a further 2.5ml of 0.1M-HCl, but this eluate also contained 150–200c.p.m. in the unincubated samples and, therefore, increased the blank value. The assays were therefore carried out using the first eluate only and were not corrected for the 90% recovery. Results were expressed as nmol of substrate degraded/mg of protein per 15min incubation. The protein content of the extracts was determined by the method of Bradford (1976) with the reagents obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A., using crystalline bovine serum albumin as a standard.

Results

Effects of inhibitors of ornithine decarboxylase

When cells were diluted into fresh medium containing various potential inhibitors, the effects on cell growth as measured by cell number were as shown in Table 1. α-Methyl- and α-difluoromethylornithine markedly decreased the growth of transformed fibroblasts, although quite high concentrations (1mM or greater) were needed to exert a maximal effect. Addition of 1–5mM-α-difluoromethylornithine produced the greatest decrease in cell growth without significant cytotoxicity. Table 1

Table 1. *Effect of inhibitors of various steps in the polyamine biosynthetic pathway on growth of SV-3T3 cells*
Abbreviation used: N.D., not determined.

Compound added	10^{-5} × Number of cells after 3 days	Polyamine content (nmol/10^6 cells)		
		Putrescine	Spermidine	Spermine
None	66	0.29	3.12	1.22
α-Methylornithine (5 mM)	11	0.02	0.51	1.21
α-Difluoromethylornithine (5 mM)	8	0.02	0.05	0.58
1,1′-[(Methylethanediylidene)dinitrilo]diguanidine (1 μM)	32	0.78	0.55	0.70
1,1′-[(Methylethanediylidene)dinitrilo]diguanidine (10 μM)	4	N.D.	N.D.	N.D.
1,1′-[(Methylethanediylidene)dinitrilo]bis-(3-amino-guanidine) (10 μM)	24	N.D.	N.D.	N.D.
1,1′-[(Methylethanediylidene)dinitrilo]bis-(3-amino-guanidine) (50 μM)	12	0.82	0.79	0.95
S-Adenosylhomocysteine sulphone (100 μM)	41	0.36	2.38	0.95
S-Adenosylhomocysteine sulphone (500 μM)	2*	N.D.	N.D.	N.D.
Decarboxylated *S*-adenosylhomocysteine sulphone (100 μM)	35	0.31	2.74	1.10
Decarboxylated *S*-adenosylhomocysteine sulphone (500 μM)	3*	N.D.	N.D.	N.D.
Decarboxylated *S*-adenosylhomocysteine sulphoxide (100 μM)	38	0.32	2.70	1.30
Decarboxylated *S*-adenosylhomocysteine sulphoxide (500 μM)	2*	N.D.	N.D.	N.D.
S-Adenosyl-4-thiobutyric acid sulphone (100 μM)	46	0.30	2.95	1.15
5′-Methylthioadenosine (25 μM)	38	N.D.	N.D.	N.D.
5′-Methylthioadenosine (100 μM)	11	0.28	2.90	1.24
5′-Methylthioadenosine (200 μM)	10	0.20	1.04	1.07
5′-Isobutylthioadenosine (200 μM)	19	0.32	1.61	0.81
5′-Methylthiotubercidin (10 μM)	16	N.D.	N.D.	N.D.
5′-Methylthiotubercidin (50 μM)	14	0.53	0.88	1.00
5′-Methylthiotubercidin (200 μM)	10	0.43	0.74	0.79

* Considerable cytotoxicity was evident in these cells.

also shows polyamine concentrations in SV-3T3 fibroblasts exposed to these compounds. Exposure to α-methyl- and α-difluoromethyl-ornithine led to almost complete depletion of putrescine. The irreversible inhibitor produced a similarly complete loss of spermidine and a substantial decline in spermine concentrations; α-methylornithine led to more than an 80% decrease in spermidine, but had little effect on spermine content. The possibility that the decrease in spermidine concentrations was responsible for the decreased growth rate was tested by adding spermidine to the growth medium. Because the serum in which the cells are grown contains oxidases that convert spermidine into a toxic product, 1 mM-aminoguanidine was also added. As shown in Fig. 1, the presence of aminoguanidine prevented the inhibitory effects of the added spermidine. The addition of spermidine completely reversed the growth inhibition by α-difluoromethylornithine (Fig. 2).

Effects of inhibitors of S-adenosylmethionine decarboxylase

Methylglyoxal bis(guanylhydrazone) was a very potent inhibitor of growth of SV3T3 cells, exerting a significant inhibitory effect at 1 μM concentration and producing almost complete inhibition at 10 μM. 1,1′-[(Methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) also was able to prevent growth of SV-3T3 fibroblasts, but required 5–10-fold higher concentrations to produce the same effect (Table 1). As shown in Fig. 2, the inhibitory effects of these compounds on cell growth could be reversed by the addition of spermidine, suggesting that their effects on cell growth were mediated via inhibition of spermidine synthesis. Another possible explanation for the reversal could be that spermidine interferes with the uptake of the inhibitor. However, 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) produced a considerable decline in the

activity of *S*-adenosylmethionine decarboxylase activity measured in undialysed homogenates *in vitro*. This inhibition was not decreased by exposure to spermidine (results not shown), suggesting that the concentration of the drug within the cell was not greatly decreased by the spermidine.

Effects of derivatives of S-adenosylhomocysteine

Recently, certain oxidized derivatives of *S*-adenosylhomocysteine were found by us to be quite potent inhibitors *in vitro* of mammalian aminopropyltransferases (Hibasami *et al.*, 1980*a*). These included *S*-adenosylhomocysteine sulphone, decarboxylated *S*-adenosylhomocysteine sulphone and decarboxylated *S*-adenosylhomocysteine sulphoxide. We have also observed significant inhibition *in vitro* by *S*-adenosyl-4-thiobutyric acid sulphone, whereas the corresponding sulphoxide of *S*-adenosyl-4-thiobutyric acid was less active (Table 2). All of the compounds mentioned above that showed significant inhibitory action were more active against spermidine synthase. When these nucleosides were tested at concentrations of 0.1 mM for their abilities to inhibit growth of SV-3T3 cells, they produced a moderate (30–47%) inhibition. Higher concentrations, such as 0.5 mM, led to complete inhibition, but produced cytotoxic effects and considerable cell lysis (Table 1). At the concentrations tolerated by the cells, *S*-adenosylhomocysteine sulphone decreased spermidine and spermine content of the fibroblasts only slightly. The other derivatives of *S*-adeno-

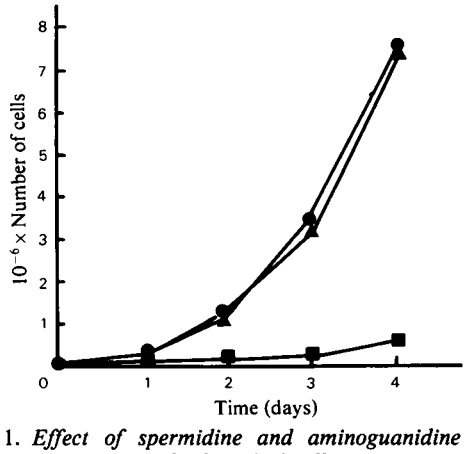

Fig. 1. *Effect of spermidine and aminoguanidine on growth of SV-3T3 cells*

The cells were seeded at a density of 10^5 in 60 mm dishes containing 4 ml of medium containing 10% foetal calf serum with no additions (●), 10 μM-spermidine (■) or 10 μM-spermidine + 1 mM-aminoguanidine (▲).

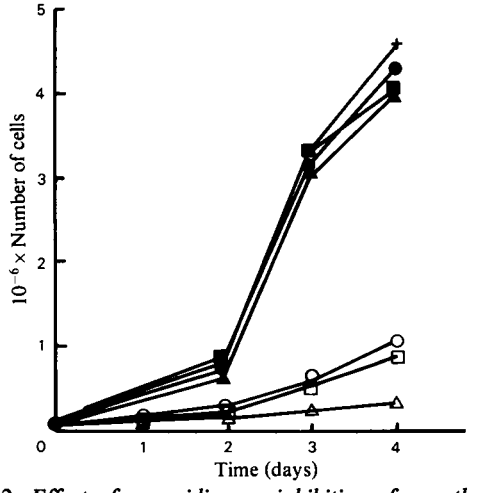

Fig. 2. *Effect of spermidine on inhibition of growth of SV-3T3 cells produced by 1,1'-[(methylethanediylidene)-dinitrilo]diguanidine, 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) and α-difluoromethylornithine*

Cells were seeded as described in Fig. 1 in medium containing 10% of foetal calf serum and 1 mM-aminoguanidine. The medium also contained no additions (+), 5 mM-α-difluoromethylornithine (○), 5 mM-α-difluoromethylornithine + 10 μM-spermidine (●), 50 μM-1,1'-[(methylethanediylidine)dinitrilo]bis-(3-aminoguanidine) (□), 50 μM-1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) + 10 μM-spermidine (■) and 10 μM-1,1'-[(methylethanediylidene)dinitrilo]diguanidine (△) or 10 μM-1,1'-[(methylethanediylidene)dinitrilo]diguanidine + 10 μM-spermidine (▲).

Table 2. *Inhibition of spermidine synthase and spermine synthase by various nucleosides*

Compound	Aminopropyltransferase activity (%)	
	Spermidine synthase	Spermine synthase
None	100	100
S-Adenosyl-4-thiobutyric acid sulphone (0.1 mM)	52	83
S-Adenosyl-4-thiobutyric acid sulphone (1.0 mM)	19	45
S-Adenosyl-4-thiobutyric acid sulphoxide (1.0 mM)	76	95
5'-Isobutylthioadenosine (0.1 mM)	78	61
5'-Isobutylthioadenosine (0.5 mM)	55	12

sylhomocysteine were even less effective in decreasing polyamine concentrations (Table 1).

Effects of 5'-methylthioadenosine and derivatives

We have shown previously that 5'-methylthioadenosine and 5'-methylthiotubercidin inhibit both aminopropyltransferases, but were more active against spermine synthase (Hibasami *et al.*, 1980*a*). The isobutyl analogue, 5'-isobutylthioadenosine, was also inhibitory towards spermine synthase (Table 2), although less potent than the parent compound. These alkyl thioethers were quite potent inhibitors of growth of SV-3T3 cells. Concentrations of 10 μM-5'-methylthiotubercidin and 25 μM-5'-methylthioadenosine significantly retarded growth and 200 μM concentrations of these compounds and 5'-isobutylthioadenosine produced more than 70% inhibition of the growth rate without appreciable toxicity (Table 1). Exposure to 5'-methylthiotubercidin led to a slight increase in putrescine content, a 75% decrease in spermidine and a small decrease in spermine concentration. 5'-Methylthioadenosine and 5'-isobutylthioadenosine also decreased spermidine content, but were less effective than 5'-methylthiotubercidin (Table 1). However, despite the significant decrease in cellular spermidine content, the growth-inhibitory effects of 5'-methylthioadenosine and 5'-methylthioadenosine (Fig. 3) and 5'-isobutylthioadenosine (results not shown) were not reversed by spermidine. Therefore, spermidine depletion is not the sole cause of the inhibition, although as shown by the results with the inhibitors of the decarboxylases, such depletion is sufficient to prevent cell growth. It is probable that the alkyl thioethers have another site of action. Since 5'-methylthioadenosine is a substrate for a phosphorylase, which degrades it to 5-methylthioribose 1-phosphate and adenine, there was some possibility that this degradation might influence the toxic effects on the cells. Extracts from SV-3T3 cells had quite high activities of this enzyme, amounting to about 70 nmol of substrate decomposed/15 min per mg of supernatant protein when assayed under the standard conditions described in the Materials and methods section. This activity is 5-fold that in rat liver, which when assayed under similar conditions had an activity of 16 nmol of substrate decomposed/15 min per mg of supernatant protein. [The 5'-methylthioadenosine phosphorylase activity of SV-3T3 cells changed only very slightly (by less than 30%) in response to growth after dilution in fresh medium or after serum stimulation of quiescent, serum-starved cells. These results differ from those

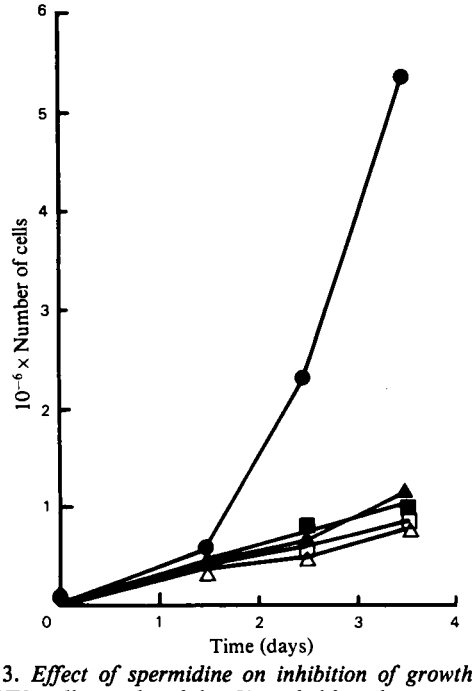

Fig. 3. *Effect of spermidine on inhibition of growth of SV-3T3 cells produced by 5'-methylthioadenosine and 5'-methylthiotubercidin*

Cells were seeded as described in Fig.1 in medium containing no additions (●), 100 μM-5'-methylthioadenosine (▲), 100 μM-5'-methylthioadenosine + 10 μM-spermidine (△), 50 μM-5'-methylthiotubercidin (■) or 50 μM-5'-methylthiotubercidin + 10 μM-spermidine (□).

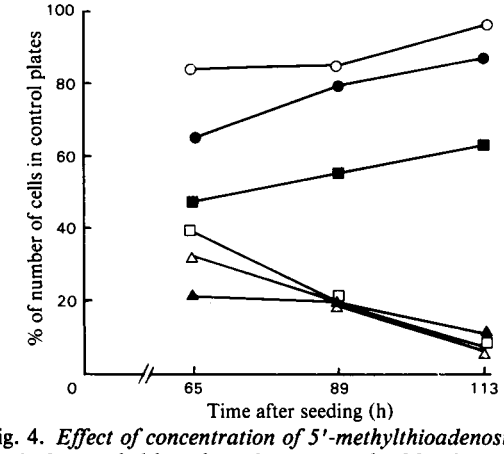

Fig. 4. *Effect of concentration of 5'-methylthioadenosine and of 5'-methylthiotubercidin on growth of SV-3T3 cells*

Cells were grown as described in the legend to Fig. 1 with the addition of alkyl thioethers indicated. At the times shown the number of cells was determined and expressed as a percentage of that present in dishes without any added inhibitor. Results are shown for 1 μM- (○), 10 μM- (□) and 50 μM- (△) 5'-methylthiotubercidin and for 10 μM- (●), 25 μM- (■) and 100 μM- (▲) 5'-methylthioadenosine.

of Ferro *et al.* (1979), who reported a 2-fold increase in 5'-methylthioadenosine phosphorylase activity in mitogen-stimulated lymphocytes.] The fibroblasts, therefore do have the ability to degrade 5'-methylthioadenosine and this may account for the apparent decrease in inhibition of the cell growth at later times when concentrations of less than 100 μM of 5'-methylthioadenosine were tested (Fig. 4). Higher concentrations of 5'-methylthioadenosine led to an increasing inhibition with time and this increase was also seen with all concentrations of 5'-methylthiotubercidin except 1 μM, which inhibited to only a very small extent (Fig. 4).

The effects of the 5'-methylthiotubercidin may be partly mediated via its ability to inhibit 5'-methylthioadenosine phosphorylase. As previously reported for the enzyme from rat prostate (Coward *et al.*, 1977), this compound, which was not a substrate for the phosphorylase from SV-3T3 cells, inhibited its action on 5'-methylthioadenosine (Table 3). Such inhibition could lead to a build-up of the 5'-methylthioadenosine generated intracellularly. The inhibition of fibroblast 5'-methylthioadenosine phosphorylase produced by 5'-methylthiotubercidin was competitive in nature and gave a K_i of about 95 μM compared with a K_m of about 40 μM (results not shown). These values are considerably lower than those reported for the phosphorylase from the rat ventral prostate (Coward *et al.*, 1977), but are not directly comparable owing to differences in the assay conditions. It should be emphasized that these present values were obtained in the presence of saturating phosphate concentrations, but analysis of the kinetics of rat liver 5'-methylthioadenosine phosphorylase suggests that it is an equilibrium ordered reaction in which 5'-methylthioadenosine is the first substrate to bind (Garbers, 1978). 5'-Isobutlthioadenosine also inhibited the degradation of 5'-methylthioadenosine (Table 3) with a K_i of about 50 μM. However, as reported by Carteni-Farina *et al.* (1979) and Savarese *et al.* (1979) for enzymes from human placenta and S-180 cells, 5'-isobutylthioadenosine was a substrate for the fibroblast phosphorylase and was degraded at a rate comparable with that of 5'-methylthioadenosine itself.

The combination of α-difluoromethylornithine and 5'-methylthiotubercidin was tested to see whether the compounds had an additive inhibitory effect on cell growth and whether a more complete depletion of spermidine could be produced than with α-difluoromethylornithine alone. When 0.25 mM-α-difluoromethylornithine was present, cell growth was

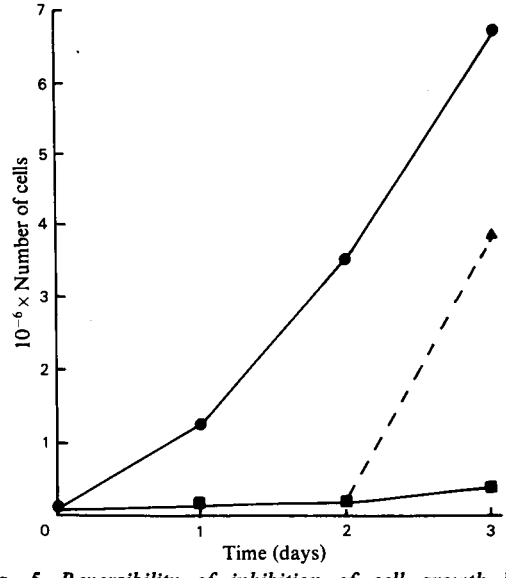

Fig. 5. *Reversibility of inhibition of cell growth by 5'-methylthioadenosine*

Cells were seeded as described in Fig. 1 in the presence (■) or absence (●) of 400 μM-5'-methylthioadenosine. After 2 days, the medium containing 5'-methylthioadenosine was removed from one set of plates and replaced with control medium (■——▲).

Table 3. *Effect of 5'-methylthiotubercidin and 5'-isobutylthioadenosine on degradation of 5'-methylthioadenosine by 5'-methylthioadenosine phosphorylase*

Addition	Activity (nmol of 5'-methylthioadenosine decomposed/15 min)	% of control
None	2.51	100
5'-Methylthiotubercidin (37 μM)	2.30	92
5'-Methylthiotubercidin (93 μM)	1.94	77
5'-Methylthiotubercidin (185 μM)	1.46	58
5'-Methylthiotubercidin (370 μM)	1.09	44
5'-Methylthiotubercidin (500 μM)	0.81	36
5'-Isobutyladenosine (50 μM)	1.81	72
5'-Isobutyladenosine (100 μM)	1.53	61
5'-Isobutyladenosine (250 μM)	1.05	42

inhibited by 80% when measured three days later. Addition of 0.05 mM-5'-methylthiotubercidin as well as the 0.25 mM-α-difluoromethylornithine increased the inhibition to 91%. However, after growth for either 3 or 8 days under these conditions there was no significant difference between intracellular polyamine content in the presence or absence of 5'-methylthiotubercidin (Table 4). Spermidine and putrescine content was greatly decreased, but there was only a small decline in spermine content. The decrease in growth rate produced by 5'-methylthiotubercidin even under conditions where it had no additional effect on polyamine concentration supports the hypothesis that the nucleoside has an effect on cell growth not related to inhibition of polyamine metabolism.

The inhibitory action of 5'-methylthioadenosine and 5'-isobutylthioadenosine on growth of SV-3T3 fibroblasts could conceivably have been mediated via the generation of adenine through the action of the phosphorylase described above and its subsequent conversion into toxic amounts of adenosine and adenine-containing nucleotides. The powerful toxicity of adenosine towards many mammalian cells is well known. This explanation is rendered unlikely by the experiment shown in Table 5. The inhibitory action of these nucleosides on cell growth was not increased when the powerful inhibitors of adenosine deaminase, deoxycoformycin or *erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine, were also present. These compounds considerably enhanced the toxicity of adenosine, presumably by preventing its degradation by cellular adenosine deaminase. Also, the inhibitory effects of 5'-methylthioadenosine could be abolished rapidly upon removal of the inhibitor (Fig. 5). These results combined with the studies with the metabolically stable 5'-methylthiotubercidin suggests that 5'-methylthioadenosine itself, or some metabolite not derived from the action of the phosphorylase, is responsible for the inhibitory action.

Discussion

The present work reports for the first time the actions of 1,1'-[(methylethanediylidene)dinitrilo]bis-

Table 4. *Effect of combinations of 5'-methylthiotubercidin and α-difluoromethylornithine on growth and polyamine content of SV-3T3 fibroblasts*

Treatment	Days	10^{-5} × Cell number	Polyamine content (nmol/10^6 cells) Putrescine	Spermidine	Spermine
Control	3	82	0.24	2.94	1.15
α-Difluoromethylornithine (0.25 mM)	3	16	0.05	0.14	0.96
α-Difluoromethylornithine (0.25 mM) + 0.05 mM-5'-methylthiotubercidin	3	7	0.06	0.12	1.02
Control	4.5	206	0.18	2.16	1.01
α-Difluoromethylornithine (0.25 mM)	8	185	0.04	0.11	0.74
α-Difluoromethylornithine (0.25 mM) + 0.05 mM-5'-methylthiotubercidin	10	179	0.04	0.08	0.67

Table 5. *Effect of adenosine deaminase inhibitors and 5'-methylthioadenosine or 5'-isobutylthioadenosine on growth of SV-3T3 cells*

Treatment	10^{-5} × Cell number
Control	130
5'-Methylthioadenosine (0.05 mM)	29
5'-Methylthioadenosine (0.05 mM) + 1 μM-deoxycoformycin	28
5'-Methylthioadenosine (0.05 mM) + 10 μM-*erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine	30
Adenosine (0.1 mM)	89
Adenosine (0.1 mM) + 1 μM-deoxycoformycin	37
Adenosine (0.1 mM) + 10 μM-*erythro*-9-[1-(1-hydroxyethyl)-heptyl]adenine	25
5'-Isobutyladenosine (0.1 mM)	48
5'-Isobutyladenosine (0.1 mM) + 10 μM-*erythro*-9-[1-(1-hydroxyethyl)heptyl]adenine	46
Deoxycoformycin (1 μM)	105
erythro-9-[1-(1-Hydroxyethyl)heptyl]adenine (10 μM)	99

(3-aminoguanidine), an inhibitor of *S*-adenosylmethionine decarboxylase, and of inhibitors of spermidine synthase and spermine synthase on growth of cultured fibroblasts. The action of these compounds was compared with that of α-methyl- and α-difluoromethylornithine and of 1,1′-[(methylethanediylidene)dinitrilo]diguanidine, compounds known to inhibit polyamine production and cell growth (see Mamont *et al.*, 1978*a*,*b*; Morris *et al.*, 1977; Seyfried & Morris, 1979; Heby *et al.*, 1977; Krokan & Eriksen, 1977; Sunkara *et al.*, 1979; Rupniak & Paul, 1978*a*,*b*; Hölttä *et al.*, 1979*a*,*b*). Our results using virally transformed mouse fibroblasts are in good agreement with these studies on other cells. The ornithine derivatives caused almost complete decline of putrescine and spermidine content and were powerfully anti-proliferative but the effect could be reversed completely by the addition of spermidine. In one respect, our findings differ slightly from those of others (summarized by Mamont *et al.*, 1978*a*). In the SV-3T3 cells, there was a significant, although not complete, decrease in spermine content, whereas [with the exception of mitogen-stimulated lymphocytes (Hölttä *et al.*, 1979*b*)] in other cells, spermine content was virtually unaffected by the drug.

1, 1′ - [(Methylethanediylidene) dinitrilo] bis - (3 - aminoguanidine), the derivative of 1,1′-[(methylethanediylidene)dinitrilo]diguanidine, which we had previously shown to be an irreversible inhibitor of *S*-adenosylmethionine decarboxylase (Pegg & Conover, 1976; Pegg, 1978) was somewhat less active than 1,1′-[(methylethanediylidene)dinitrilo]-diguanidine itself in inhibition of polyamine synthesis and cell proliferation in the SV-3T3 cells. However, at a slightly higher concentration, it had similar effects to 1,1′-[(methylethanediylidene)dinitrilo]-diguanidine producing an increase in putrescine and a substantial fall in both spermidine and spermine. The higher concentration required could be due to the greater chemical reactivity of 1,1′-[(methylethanediylidene)dinitrilo] bis-(3-aminoguanidine), which may combine with components in the culture medium or to a lower extent of uptake. 1,1′-[(Methylethanediylidene)dinitrilo]diguanidine is known to be concentrated quite extensively by an active-transport mechanism (Mandel & Flintoff, 1978; Mihich, 1975) and, although competitive, is a highly potent inhibitor of *S*-adenosylmethionine decarboxylase. Therefore, in cultured cells that cannot decrease the drug concentration by excretion or metabolism, 1,1′-[(methylethanediylidene)dinitrilo]-diguanidine is preferable in many ways to 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) as an experimental tool. 1,1′-[(Methylethanediylidene)dinitrilo] bis-(3-aminoguanidine) does have the advantage, however, that the degree to which the enzyme activity is irreversibly inactivated can be measured. This renders it easier to rule out the possibility that the means by which spermidine overcomes its inhibitory action is a prevention of drug uptake or even a counterflow out of the cell. This is a real possibility since 1,1′-[(methylethanediylidene)dinitrilo]diguanidine and polyamines share a common transport system (Mandel & Flintoff, 1978), but it is shown in the present work that spermidine could reverse the inhibitory effect of 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) on cell growth even when *S*-adenosylmethionine decarboxylase activity remained inhibited. There remains considerable controversy as to whether the inhibitory effects of 1,1′-[(methylethanediylidene)dinitrilo]diguanidine on cell growth are mediated via the depletion of polyamines or are exerted at another site in a manner that can be antagonized by spermidine. A careful study by Seyfried & Morris (1979) suggests that inhibition of DNA synthesis by this drug does result from polyamine depletion. On the other hand, Rupniak & Paul (1978*b*, 1980) and Hölttä *et al.* (1979*b*) argue that inhibition of growth may not be mediated through effects on polyamine metabolism and the drug has marked effects on the ultrastructure and function of mitochondria (Pathak *et al.*, 1977; Mikles-Robertson *et al.*, 1979; Porter *et al.*, 1979). It is clear, therefore, that this compound is not specific for *S*-adenosylmethionine decarboxylase, since it also produces a marked inhibition of diamine oxidase (Hölttä *et al.*, 1973; Pegg & McGill, 1978). 1,1′-[(Methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) is unfortunately not an improvement in this respect, since it is an even more persistent inhibitor of diamine oxidase, which could account for part of the rise in putrescine concentrations and is also active in producing mitochondrial damage (Pegg & McGill, 1978; Pathak *et al.*, 1978).

Although *S*-adenosylhomocysteine sulphone and its derivatives were quite potent inhibitors of spermidine synthase *in vitro* (Hibasami *et al.*, 1980*a*; Table 2), they were disappointingly ineffective in the cultured fibroblast system. It is possible that lack of uptake of the nucleoside or its rapid intracellular degradation prevents more extensive inhibition. However, higher concentrations were strongly cytotoxic. It appears that the activity of spermidine synthase in the cell is sufficient to provide normal synthesis rates at the maximal intracellular concentrations that could be tolerated by the cell.

The effect of 5′-methylthiotubercidin on cell growth and polyamine accumulation was quite surprising. *In vitro*, this compound and 5′-methylthioadenosine, which it might increase in concentration by inhibition of the phosphorylase, were more potent inhibitors of spermine synthase than of spermidine synthase. However, *in vivo*, these

nucleosides and 5′-isobutylthioadenosine led to a substantial decrease in spermidine content, but not of spermine. It is possible that under physiological conditions where decarboxylated *S*-adenosylmethionine concentrations are of the order of 1 μM (Hibasami *et al.*, 1980*b*) and spermidine concentrations exceed those of putrescine, spermidine synthase is the more sensitive site of action for these compounds. Another possibility is that the decline in spermidine concentrations is a secondary effect consequent on another action decreasing the growth rate. In any event, the inhibition of growth by 5′-methylthiotubercidin or 5′-methylthioadenosine must also involve some step not related to polyamine synthesis, since it could not be reversed by spermidine. At present, this step is entirely unknown. Recently, Ferro *et al.* (1979) reported that 5′-methylthioadenosine and 5′-methylthiotubercidin inhibited DNA synthesis and cellular proliferation in stimulated human lymphocyte cultures. This result is quite similar to that reported in the present paper for transformed fibroblasts, although the concentration of the nucleosides required for inhibition of lymphocyte proliferation was almost an order of magnitude greater than in the present experiments. Both results suggest that 5′-methylthioadenosine phosphorylase may play a critical role in decreasing the concentration of endogenously generated 5′-methylthioadenosine, which can be formed in several ways in addition to the action of the aminopropyltransferases synthesizing spermidine and spermine (see Pegg & Hibasami, 1979; Ferro, 1979). The build-up of this nucleoside must be prevented to permit normal cell growth. The normal intracellular concentration of 5′-methylthioadenosine is unknown, but is less than 5 μM (A. E. Pegg, unpublished work). Development of a method sensitive enough to permit measurement of the intracellular concentration of this nucleoside and the effect on it of 5′-methylthiotubercidin will be needed for further understanding of its role in cellular physiology.

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