Inhibition of the synthesis of polyamines and macromolecules by 5'-methylthioadenosine and 5'-alkylthiotubercidins in BHK21 cells

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5'-Methylthioadenosine and four 5'-alkylthiotubercidins were tested for their ability to inhibit polyamine synthesis *in vitro* and to decrease polyamine concentration and prevent growth of baby-hamster-kidney (BHK21) cells. 5'-Methylthioadenosine and 5'-methylthiotubercidin decreased the activity of spermidine synthase from brain to roughly the same extent, whereas brain spermine synthase was much more strongly inhibited by 5'-methylthioadenosine compared with 5'-methylthiotubercidin. These nucleoside derivatives also inhibited the growth of BHK21 cells and increased the concentration of putrescine. 5'-Methylthioadenosine decreased cellular spermine concentration, whereas 5'-methylthiotubercidin lowered the concentration of spermidine. The activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase were enhanced in cells grown in the presence of 5'-methylthiotubercidin. The growth inhibition produced by these nucleoside derivatives was not reversed by exogenous spermidine or spermine. 5'-Ethylthiotubercidin, 5'-propylthiotubercidin and 5'-isopropylthiotubercidin did not appreciably inhibit spermidine or spermine synthase *in vitro* or decrease the cellular polyamine content, but effectively prevented the growth of BHK21 cells. All nucleoside derivatives at concentrations of 0.2–1 mM caused a rapid inhibition of protein synthesis. It is concluded that the growth inhibition produced by 5'-methylthioadenosine and 5'-alkylthiotubercidins was not primarily due to polyamine depletion but other target sites, for instance the cellular nucleotide pool, cell membranes etc. must be considered.

Inhibitors of polyamine synthesis have recently become the object of intensive research because of their potential usefulness as antiproliferative agents and in the elucidation of the biological function of polyamines. Most of the compounds presently available are inhibitors of ornithine decarboxylase or *S*-adenosylmethionine decarboxylase (for references, see Jänne *et al.*, 1978). Many of these compounds, however, have unwanted effects on cellular polyamine metabolism, including stabilization and/or activation of the two decarboxylases, increase of the cellular concentration of putrescine or spermine, and inhibition of diamine oxidase, or effects probably unrelated to polyamine metabolism, for instance, acute inhibition of protein and nucleic acid synthesis and mitochondrial damage (Jänne *et al.*, 1978; Tuomi *et al.*, 1980; Heby & Jänne, 1981; Hölttä *et al.*, 1981). It is therefore important to find inhibitors acting at the propylamine transferase step and to develop agents of increased specificity.

Our observation (Pajula & Raina, 1979; Pajula *et al.*, 1979) that 5'-methylthioadenosine, a by-product of spermidine synthase and spermine synthase reactions, potently inhibited purified spermine synthase, suggested that methylthioadenosine analogues, for instance 5'-methylthiotubercidin, might also be effective inhibitors of spermine synthase *in vivo*. 5'-Methylthioadenosine is rapidly cleaved in animal cells to adenine and 5'-methylthioribose 1-phosphate by 5'-methylthioadenosine phosphorylase, an enzyme widely distributed in animal tissues (Pegg & Williams-Ashman, 1969a; Garbers, 1978; Zappia *et al.*, 1978), and is only found in minute amounts in tissues (Seidenfeld *et al.*, 1980; Pegg & Coward, 1981). In the present paper we show that 5'-methylthioadenosine and several 5'-alkylthiotubercidins, notably 5'-methylthiotubercidin, are able to modify polyamine synthesis and inhibit proliferation of BHK21 cells, an effect not readily reversible by exogenous polyamines added to the culture medium.

Similar observations have recently been reported by Pegg and his co-workers (Hibasami *et al.*, 1980; Pegg *et al.*, 1981). Our results also show that 5'-methylthioadenosine and 5'-alkylthiotubercidins rapidly inhibit cellular protein synthesis. Some of our observations have been discussed in a congress report (Raina *et al.*, 1981).

Experimental

Chemicals

1,4-Diaminobutane (putrescine) and spermidine, as their hydrochloride salts, were obtained from Calbiochem, Los Angeles, CA, U.S.A., and recrystallized twice from hot 6M-HCl/ethanol solution. Tubercidin, the 7-deaza analogue of adenosine, was also a product of Calbiochem. 5'-Alkylthiotubercidins were synthesized from tubercidin by published procedures (Borchardt *et al.*, 1976; Coward *et al.*, 1977) and purified by recrystallization. 5'-Methylthioadenosine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

DL-[2-¹⁴C]Methionine (sp. radioactivity 4.08 Ci/mol) was supplied by the New England Nuclear Corp., Boston, MA, U.S.A. L-[1-¹⁴C]Methionine (sp. radioactivity 60 Ci/mol), DL-[1-¹⁴C]ornithine (sp. radioactivity 59 Ci/mol) and L-[U-¹⁴C]leucine (sp. radioactivity 335 Ci/mol) were products from The Radiochemical Centre, Amersham, Bucks., U.K. Labelled S-adenosyl-L-methionine was synthesized essentially as described by Pegg & Williams-Ashman (1969b). Decarboxylated adenosyl-methionine was prepared from adenosylmethionine and purified as previously described (Raina & Hannonen, 1971).

Cell culture

Stock cultures of BHK21, a continuous cell line derived from baby hamster kidney, were maintained in Eagle's minimal essential medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% foetal-calf serum, penicillin (200 units/ml) and streptomycin (100 µg/ml) and passaged by trypsin treatment (Tuomi *et al.*, 1980). In the experiments the cells (3.5×10^5 cells/dish) were seeded on plastic Petri dishes (diameter 50 mm) in the above culture medium. After incubation for 4 h the medium was supplemented with the nucleoside derivative and other compounds specified in the experimental set-up. All values shown are means of at least two parallel cultures that agreed within $\pm 10\%$.

Analytical methods

At the end of the experiment the medium was removed, the cells washed twice with 0.9% NaCl, detached by scraping with a rubber policeman and collected by centrifugation. Polyamines were

analysed from a cold HClO₄ extract of the cell pellet by the dansylation method (Cohen *et al.*, 1969). DNA was determined from a hot HClO₄ extract as described by Giles & Myers (1965).

Protein synthesis was measured by treating the cultures with [U-¹⁴C]leucine (0.2 µCi/dish) for 1 h. Then the cells were washed twice with 0.9% NaCl containing a 100-fold excess of unlabelled leucine. Acid-soluble radioactivity was determined from a cold HClO₄ extract of washed cells. The acid-insoluble fraction was dissolved in 0.1 M-NaOH and counted for radioactivity in Aquasol (New England Nuclear Corp.). Protein was measured by the Lowry method (Lowry *et al.*, 1951), with bovine serum albumin as standard.

Enzyme assays

The activities of spermidine synthase (EC 2.5.1.16) and spermine synthase (EC 2.5.1.-) were assayed as previously described (Raina *et al.*, 1976; Pajula *et al.*, 1979). Spermine synthase and spermidine synthase were purified to homogeneity from bovine brain by affinity-chromatographic methods (Pajula *et al.*, 1979; Samejima *et al.*, 1982).

The activities of ornithine decarboxylase (EC 4.1.1.17) and S-adenosylmethionine decarboxylase (EC 4.1.1.50) were determined in 100 000 g_{av}. supernatant fraction of cell sonicated material as previously described (Eloranta *et al.*, 1976).

Results

Inhibition of propylamine transferases by 5'-methylthioadenosine and 5'-alkylthiotubercidins in vitro

We stated earlier in the present paper that crude tissue extracts contain high activities of 5'-methylthioadenosine phosphorylase (Pegg & Williams-Ashman, 1969a; Garbers, 1978) and that purified spermine synthase is potently inhibited by 5'-methylthioadenosine, a compound formed from decarboxylated adenosylmethionine in the propylamine transferase reaction (Pajula & Raina, 1979). Therefore, the degree of the inhibition of propylamine transferases by 5'-alkylthionucleosides might differ, depending on the purity of the enzyme preparation. Fig. 1 compares the effect of 5'-methylthioadenosine and 5'-methylthiotubercidin on the activities of crude and purified propylamine transferases, the former prepared from rat brain and the latter from bovine brain. In agreement with previous results obtained with rat prostatic enzymes (Hibasami *et al.*, 1980; Raina *et al.*, 1981), spermine synthase was much more sensitive to 5'-methylthioadenosine compared with spermidine synthase. Both crude and purified spermidine synthase was inhibited by 5'-methylthioadenosine and 5'-methylthiotubercidin to roughly the same extent, whereas

Fig. 1. Effect of 5'-methylthioadenosine and 5'-methylthiotubercidin on propylamine transferase activities. The enzymes used were crude $(\text{NH}_4)_2\text{SO}_4$ fraction [40–70% saturation of $(\text{NH}_4)_2\text{SO}_4$] from rat brain (○ and ▽) and propylamine transferases purified into homogeneity from bovine brain (● and ▼). The activities observed in the presence of 5'-methylthioadenosine (▽ and ▼) or 5'-methylthiotubercidin (○ and ●) are expressed as percentages of control. The values shown are means of duplicate assays that agreed within $\pm 5\%$. The concentration of decarboxylated S-adenosylmethionine in the assay mixture was $20 \mu\text{M}$.

only crude spermine synthase was inhibited by 5'-methylthiotubercidin. The inhibition by 5'-methylthioadenosine and 5'-methylthiotubercidin of crude spermidine and spermine synthases from bovine brain (results not shown) was very similar to that demonstrated in Fig. 1 for crude rat propylamine transferases. The difference in the inhibition by 5'-methylthiotubercidin between the crude and purified spermine synthase can be explained as follows. With crude enzyme preparation a linear reaction rate with time is obtained, as there is no accumulation of 5'-methylthioadenosine, whereas

with purified spermine synthase the reaction rate is non-linear, due to the accumulation of the nucleoside product (Pajula & Raina, 1979; Pajula *et al.*, 1979; R.-L. Pajula, unpublished work). In the latter case, a weak inhibitor, 5'-methylthiotubercidin, would abolish the inhibition produced by 5'-methylthioadenosine accumulating during the synthase reaction.

Other tubercidin derivatives (5'-ethyl-, 5'-propyl- and 5'-isopropyl-thiotubercidin) did not inhibit spermidine synthase or purified spermine synthase at all, whereas a slight inhibition (10–25%) was observed with crude spermine synthase (results not shown). These results suggest that of the 5'-alkylthiotubercidins known to be resistant to 5'-methylthioadenosine phosphorylase (Zappia *et al.*, 1978; Ferro *et al.*, 1979; Wolford *et al.*, 1981), 5'-methylthiotubercidin would be the most effective in decreasing the cellular polyamine pool.

Effect of 5'-methylthioadenosine and 5'-alkylthiotubercidins on the growth and cellular polyamines of BHK21 cells

As shown in Fig. 2, 0.1 mM-5'-methylthioadenosine and 0.5 mM-methylthiotubercidin inhibited the growth of BHK21 cells in culture. Contrary to the results obtained with α -difluoromethylornithine (Raina *et al.*, 1981), the inhibition of growth produced by 5'-alkylthionucleosides appeared fairly rapidly (i.e. within 24 h). 5'-Methylthiotubercidin, but not 5'-methylthioadenosine, prevented the increase in cellular spermidine concentration normally occurring after seeding the cells into a fresh medium, whereas only 5'-methylthioadenosine was able to decrease the concentration of spermine. Interestingly, both nucleosides increased putrescine concentration severalfold compared with the control cultures. This result was not as predicted by Heby & Jänne (1981) that 5'-methylthioadenosine would not change to cellular concentration of putrescine or spermidine. Pegg *et al.* (1981) have also observed some increase in putrescine concentration in SV-3T3 cells treated with 5'-methylthiotubercidin, but not in cells treated with 5'-methylthioadenosine.

Several inhibitors of polyamine synthesis are known to enhance the activities of ornithine and/or S-adenosylmethionine decarboxylases in cultured cells (for review, see Heby & Jänne, 1981). As seen in Fig. 3, 5'-methylthiotubercidin also increased the activity of both decarboxylases in BHK21 cells. It is not known whether the observed stimulation was due to increased synthesis or prolongation of the half-lives of the enzymes. In any case, it is in accord with the elevation of the putrescine concentration demonstrated in Fig. 2. The effect of other 5'-alkylthionucleosides on the two decarboxylases has not been studied in this system.

Fig. 2. Effect of 5'-methylthioadenosine and 5'-methylthiotubercidin on the accumulation of DNA and polyamines in BHK21 cells

Cells were grown in Eagle's minimal essential medium supplemented with 10% foetal-calf serum with no addition (○) or in the presence of 0.1 mM 5'-methylthioadenosine (Δ) or 0.5 mM 5'-methylthiotubercidin (□). The results of this experiment are representative of four experiments with 5'-methylthioadenosine and of six experiments with 5'-methylthiotubercidin. Each point represents the average of duplicate determinations of two separate cultures. The average deviations of single values from the means were 4%, 8%, 6% and 8% for DNA, putrescine, spermidine and spermine respectively.

The cellular regulation of *S*-adenosylmethionine decarboxylase activity is still a matter of dispute. The present observation of the increase of cellular putrescine and *S*-adenosylmethionine decarboxylase activity by 5'-methylthiotubercidin (Figs. 2 and 3) is of interest in connection with the suggestion by Sakai *et al.* (1980) that putrescine can act as a negative regulator of the decarboxylase in the cell. On the other hand, the recent reports by Alhonen-Hongisto (1980) and Mamont *et al.* (1981) have presented evidence for a negative control of *S*-adenosylmethionine decarboxylase by cellular spermidine and/or spermidine plus spermine, whereas putrescine might exert opposite effects (Mamont *et al.*, 1981). It should be noted that the observed effects of 5'-methylthiotubercidin on cellular polyamine metabolism, particularly the increase in the concentration of putrescine and in the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, are similar to those produced by methylglyoxal bis(guanyldiazide), which is known to increase the half-life of *S*-adenosylmethionine decarboxylase (see Heby & Jänne, 1981).

Fig. 3. Effect of 5'-methylthiotubercidin on ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities of BHK21 cells

BHK21 cells harvested from confluent cultures were seeded on plastic Petri dishes (diameter 100 mm) and grown for 24 h in the absence (○ and □) or presence (● and ■) of 0.5 mM 5'-methylthiotubercidin. At time points shown the cells were collected as described in the Experimental section and disrupted by sonication in 0.25 M-sucrose solution containing 0.1 mM-EDTA, 1 mM-dithiothreitol and 0.1 mM-pyridoxal phosphate. Each point represents the mean of two pooled samples, each obtained by combining cells of two to five separate cultures. ○ and ●, Ornithine decarboxylase; □ and ■, *S*-adenosylmethionine decarboxylase.

Table 1 shows that all four 5'-alkylthiotubercidins at a concentration of 0.2 mM clearly inhibited the growth of BKH21 cells, 5'-isopropylthiotubercidin being the most effective. As expected on the basis of inhibition of spermidine synthase and spermine synthase *in vitro* (see above), only 5'-methylthiotubercidin was able to decrease the cellular concentration of spermidine, whereas the other nucleoside derivatives tended to increase the concentration of spermidine and spermine. A higher concentration (1 mM; results not shown) of 5'-alkylthiotubercidins was clearly toxic to the cells, which is in accord with the report by Wolford *et al.* (1981). Our observations suggested that the growth inhibition produced by 5'-alkylthiotubercidins was not mediated by polyamine depletion. This hypothesis was strengthened by the observation that the growth inhibition produced by 5'-methylthiotubercidin was not reversed by the addition of spermidine to the culture medium (Table 2). The result (not shown) was the same when 5'-methylthioadenosine was used as the inhibitor. Also spermine and various combinations of spermidine and spermine were ineffective in reversion experiments. As seen in

Table 1. *Effect of tubercidin derivatives on the growth and polyamine concentrations of BHK21 cells*

BHK21 cells (3.5×10^5 cells/dish) were grown for 3 days in Eagle's minimal essential medium supplemented with 10% foetal-calf serum in the presence or absence of 0.2 mM-5'-alkylthiotubercidin. The values are means of duplicate assays of two to four separate cultures, which agreed within $\pm 4\%$ for DNA, $\pm 7\%$ for spermidine and $\pm 10\%$ for spermine. The number of cultures is shown in parentheses.

Tubercidin derivative	DNA ($\mu\text{g}/$ culture)	Spermidine ($\text{pmol}/\mu\text{g}$ of DNA)	Spermine ($\text{pmol}/\mu\text{g}$ of DNA)
None (4)	21.1	447	301
5'-Methylthiotubercidin (2)	13.6	349	382
5'-Ethylthiotubercidin (2)	13.2	533	402
5'-Propylthiotubercidin (2)	10.9	602	378
5'-Isopropylthio- tubercidin (2)	9.6	748	348

Table 2. *Effect of spermidine on the growth and cellular polyamine content of BHK21 cells grown in the presence of 5'-methylthiotubercidin*

BHK21 cells were grown in the presence of 1 mM-iproniazid and, where indicated, in the presence of 0.5 mM-5'-methylthiotubercidin and 10 μM -spermidine. The values are means of duplicate assays of two separate cultures.

Time (days)	Addition	DNA ($\mu\text{g}/$ culture)	Spermidine ($\text{pmol}/\mu\text{g}$ of DNA)	Spermine ($\text{pmol}/\mu\text{g}$ of DNA)
1	None	3.5	591	487
2	None	7.0	568	419
2	5'-Methylthio- tubercidin	5.5	367	444
2	5'-Methylthio- tubercidin + spermidine	5.5	509	447
3	None	14.1	484	295
3	5'-Methylthio- tubercidin	9.6	323	366
3	5'-Methylthio- tubercidin + spermidine	8.0	483	432

Table 2 and also demonstrated by radioactive spermidine (results not shown), 5'-methylthiotubercidin did not prevent the uptake of exogenous spermidine by the cells.

Inhibition of protein synthesis by 5'-alkylthiotubercidins

The possibility that 5'-alkylthiotubercidins might

Table 3. *Acute inhibition of protein synthesis by 5'-alkylthiotubercidins in BHK21 cells*

BHK21 cells (3.5×10^5 cells/dish) were grown for 48 h. Then a fresh medium supplemented with 0.2 mM-tubercidin derivative was added, and incubation was continued for 10 h before labelling the cells with [^{14}C]leucine (0.2 $\mu\text{Ci}/\text{dish}$) for 1 h. The radioactivity in proteins and in the acid-soluble pool was determined as described in the Experimental section. The results of this experiment are representative of three similar experiments. Values are means of at least two parallel cultures. The average and maximum deviation of single values from the means were 6% and 11% respectively. Numbers in parentheses are percentages of untreated cultures.

Tubercidin derivative	Concentration (mM)	10 ⁻² × Radioactivity (c.p.m./mg of protein)	
		Protein	Acid-soluble
None	—	679 (100)	15.2 (100)
5'-Methylthiotubercidin	0.2	764 (113)	18.2 (119)
	1.0	421 (62)	9.8 (65)
5'-Ethylthiotubercidin	0.2	606 (89)	14.9 (98)
	1.0	260 (38)	8.1 (53)
5'-Propylthiotubercidin	0.2	390 (58)	10.9 (72)
	1.0	131 (19)	4.1 (27)
5'-Isopropylthio- tubercidin	0.2	447 (66)	11.7 (77)
	1.0	295 (44)	8.8 (57)

have acute effects on macromolecular synthesis was studied by using amino acid incorporation into proteins after short-term treatment of cell cultures with the tubercidin derivatives as an indicator. As shown in Table 3, protein synthesis was clearly inhibited in BHK21 cells when grown for 10 h in the presence of 0.2 mM-5'-propyl- or 5'-isopropyl-thiotubercidin, whereas 5'-methylthiotubercidin was without effect at this concentration. However, at the concentration of 1 mM all the tubercidin derivatives strongly inhibited [^{14}C]leucine incorporation into proteins and also reduced the uptake (as measured by the radioactivity of the acid-soluble pool) of the amino acid by the cells. As previously reported (Raina *et al.*, 1981), the 5'-alkylthiotubercidins also markedly inhibited virus production in BHK21 cells at concentrations of 0.2–1 mM. It should be noted that the acute inhibitory effects were strongest with 5'-propyl- and 5'-isopropyl-thiotubercidin, compounds that neither appreciably inhibited the activities of propylamine transferases *in vitro* nor depleted the cellular polyamine pool.

Discussion

Depletion of cellular polyamines by specific inhibitors of polyamine synthesis in cultured cells

leads to a gradual decline of macromolecular synthesis and cessation of cell proliferation (for review, see Jänne *et al.*, 1978). The aim of this study was to test 5'-alkylthionucleosides as inhibitors of polyamine synthesis. Although 5'-methylthioadenosine and all four 5'-alkylthiotubercidins inhibited the growth of BHK21 cells, only two of the compounds, namely 5'-methylthioadenosine and 5'-methylthiotubercidin were able to inhibit propylamine transferases *in vitro* and to lower the cellular polyamine pool, indicating that the growth inhibition produced by these compounds was not due to polyamine depletion. This conclusion is further supported by the non-reversibility of the growth inhibition by exogenous polyamines and the acute inhibition of protein synthesis and virus production in BHK21 cells incubated in the presence of these nucleoside derivatives.

The mechanism of the antiproliferative action of 5'-alkylthioadenosines and 5'-alkylthiotubercidins remains unresolved. 5'-Methylthioadenosine is known to inhibit the growth of several cell lines, including BHK21 cells (the present study), transformed mouse fibroblasts (Pegg *et al.*, 1981) and murine lymphoid cells (Wolford *et al.*, 1981). This nucleoside derivative has been reported to inhibit several enzyme systems, for instance, methylation of histamine, acetylserotonin, DNA and protein, and to inhibit RNA synthesis in salivary glands of *Drosophila melanogaster* and transformation of lymphocytes (for references, see Ferro, 1979; Ferro *et al.*, 1979). In a recent study (Law & Miller, 1981) rapid changes in nucleolar morphology and interference with ribosomal RNA metabolism in cultured *Xenopus laevis* cells were noted. It has been suggested that growth-inhibitory properties of 5'-methylthioadenosine might be due to a 'suicide' inactivation of *S*-adenosylhomocysteine hydrolase by this nucleoside (Ferro *et al.*, 1981). There is some evidence to suggest that the nucleoside needs not to be degraded to inhibit cell growth (Pegg *et al.*, 1981; Wolford *et al.*, 1981). On the other hand, in BHK21 cells, exogenous 5'-methylthioadenosine (0.05 mM) is rapidly cleaved, possibly on entering the cell, and does not seem to accumulate in the cells even in the presence of 1 mM 5'-methylthiotubercidin, an inhibitor of 5'-methylthioadenosine phosphorylase (Eloranta *et al.*, 1982). However, it is difficult to exclude the possibility that degradation of the nucleoside taken up by the cells might continue during isolation of cells.

The site of action of 5'-methylthiotubercidin and other 5'-alkylthiotubercidins is also speculative. Some authors (Ferro *et al.*, 1979; Pegg *et al.*, 1981) suggest that the growth inhibition by 5'-methylthiotubercidin is indirect, i.e. due to the pile-up of cellular 5'-methylthioadenosine, and/or that these nucleosides have a common site of action (Ferro

et al., 1979). Acute changes in the cellular morphology, as demonstrated by Law & Miller (1981), and function (for instance, inhibition of protein synthesis and amino acid uptake reported in the present study) suggest that 5'-alkylthionucleosides at higher concentrations have several sites of action, e.g. the nucleotide pool and cell membranes. A careful analysis of these and other possible target sites seems desirable before introducing these compounds to chemotherapeutic trials, which has been recently suggested (Wolford *et al.*, 1981).

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