Biochemical Pharmacology, Vol. 33, No. 22, pp. 3639–3643, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

MECHANISM OF ACTION OF 5′-METHYLTHIOADENOSINE IN S49 CELLS

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(*Received* 23 *December* 1983; *accepted* 20 *March* 1984)

Abstract—5′-Deoxy-5′-methylthioadenosine, a naturally occurring co-product of polyamine biosynthesis, has been shown to inhibit a variety of biological processes. To investigate the mode of action of this nucleoside and to assess the involvement of cAMP in this action, the effect of methylthioadenosine on S49 wild type and two cAMP-related mutant cells was examined. The sulfur-containing nucleoside potently inhibited the growth of the parental strain (IC_{50} = 50 μM), whereas nearly 10-fold greater resistance was demonstrated by S49 adenylate cyclase deficient (IC_{50} = 420 μM) and S49 cAMP-dependent protein kinase deficient (IC_{50} = 520 μM) mutant cells. Methylthioadenosine was shown to competitively inhibit the S49-derived high-affinity cAMP phosphodiesterase (K_i = 62 μM) *in vitro*, whereas methylthioadenosine phosphorylase activity was equivalent in all three cell types. The intracellular levels of the regulatory nucleotide, cAMP, increased dramatically in the wild type (17-fold) and protein kinase deficient (6-fold) strains in response to 100 μM concentrations of the drug. It is concluded that the growth arrest produced by 5′-methylthioadenosine in S49 cells is primarily due to the inhibition of cAMP phosphodiesterase and the subsequent increase in cAMP levels that result.

5′-Deoxy-5′-methylthioadenosine is the co-product of the spermidine and spermine synthetase reactions of mammalian cells [1]. This sulfur-containing nucleoside has been shown to adversely affect several biological processes, such as lymphocyte blastogenesis [2] and the growth of murine hematopoietic cell lines [3] and virally transformed mouse fibroblasts [4]. Knowledge of the primary mode of action for the growth inhibition produced by methylthioadenosine is of interest because of the potential chemotherapeutic usefulness of the drug and several of its structural analogs. It is also of importance in ascribing a biological function to the nucleoside in mammalian cells. Some methylthioadenosine-induced biochemical effects have been described in attempts to elucidate the mechanism of action of this compound. Three of these effects are notable: (1) methylthioadenosine has been shown to potently inhibit sperimidine and spermine synthetase from a variety of sources [5, 6]; (2) Ferro and coworkers [7] have demonstrated the "suicide like" inactivation of erythrocyte *S*-adenosylhomocysteine hydrolase by methylthioadenosine; and (3) methylthioadenosine is a competitive inhibitor of the lymphocyte-derived high-affinity cAMP phosphodiesterase [8]. In light of these later findings, our attention has focused on the modulation of cAMP metabolism by methylthioadenosine. Our investigation was aided by the availability of two mutant cell lines which were originally selected for their resistance to either cAMP itself or substances which induce the synthesis of this growth regulatory nucleotide. The variant clones, S49 adenylate cyclase deficient [9] and S49 cAMP-dependent protein kinase deficient [10], have been useful as tools in elucidating the components of the cAMP-induced regulatory system. This system is composed of a protein kinase which, in the presence of cAMP, phosphorylates various enzyme substrates and brings about growth arrest. According to this model, if cAMP plays a central role in the biological effects of methylthioadenosine, the responses of these cAMP-related mutant cells to methylthioadenosine should be altered in characteristic ways.

Specifically, in this report we compare the effects of methylthioadenosine on parental wild type, adenylate cyclase deficient, and protein kinase deficient S49 cells. We confirm the previous findings of the inhibition by methylthioadenosine of the cAMP phosphodiesterase, and we provide evidence suggesting that this perturbation of cyclic nucleotide metabolism by methylthioadenosine plays an important role in determining its biological effects.

MATERIALS AND METHODS

Materials. 5′-Deoxy-5′-methylthioadenosine was synthesized according to the procedure of Kikugawa and Ichino [11]. [2,8-^{3}H]Adenosine 3′,5′-cyclic phosphate (27 Ci/mmole) was purchased from ICN. cAMP and 5′-nucleotidase purified from *Crotalus astrox* venom were purchased from Sigma. AG-1-X8 was obtained from BIO-RAD, the cAMP binding protein assay kit from Amersham (UK), and all cell culture supplies from Gibco. All other reagents were obtained from Sigma.

Cell lines and culture. S49 mouse lymphoma cells of the wild type and mutant phenotypes, protein kinase (24.6.1) and adenylate cyclase deficient (94.15.1), were obtained from Dr. Phillip Coffino (University of California, San Francisco). The cells were cultured in Dulbecco's modified Eagle's

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medium (DMEM) with 4.5 g/l of glucose, supplemented with 10% heat-inactivated horse serum [12].

Preparation of cell extracts. All cell extracts were made from logarithmic cultures. Suspensions of cells were centrifuged at 700 *g* for 5 min, and the supernatant fraction was discarded. The cell pellet was washed in 250 mM sucrose, 100 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) (pH 7.2), 3 mM 2-mercaptoethanol, and centrifuged at 4° at 700 *g* for 10 min. The cells were resuspended in 3 ml of the same buffer and gently fractured by the freeze–thaw method. After addition of dimethyl sulfoxide to 5%, the suspension was homogenized in a Dounce homogenizer until breakage was >99% as indicated by the Trypan blue dye exclusion test. The crude extract was centrifuged at 10,000 *g* for 10 min at 4°, and the supernatant fraction was collected and stored at −80° until utilized.

Analytical procedures. cAMP present in acid-soluble extracts of cells was quantitated using the competition binding assay kit of Amersham. The nucleotides were first separated from the perchlorate salts by the freon:alamine extraction method of Khym [13].

Enzyme assays. cAMP phosphodiesterase activities were measured by the two-step procedure of Thompson *et al.* [14]. Each reaction contained 40 mM Tris (pH 8.0), 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, [^{3}H]cAMP (300,000 cpm), and enzyme extract. Methylthioadenosine phosphorylase activity was determined by measuring the conversion of 5′-[$^{14}CH_3$]methylthioadenosine to 5-[$^{14}CH_3$] methylthioribose-1-phosphate [15].

RESULTS

Effect of 5′-methylthioadenosine on the proliferation of parental wild type and mutant S49 cells. The effect of the exogenous supplementation of methylthioadenosine on cultures of wild type and mutant S49 cells is shown in Fig. 1. Untreated cultures of each cell type proliferated with a generation time of 17–18 hr. Parental wild type cells, which are extremely susceptible to killing by cAMP [16], were likewise growth-inhibited by methylthioadenosine. Significant growth inhibition (30%) was observed at concentrations as low as 10^{-5} M in these cultures. Exhibiting an IC_{50} of 50 μM, the S49 wild type cells proved to be the most sensitive to methylthioadenosine of the three cell lines studied. By contrast, sensitivity was greatly reduced in mutant cell lines which are either incapable of synthesizing cAMP (adenylate cyclase deficient) or resistant to the effects of the regulatory nucleotide (protein kinase deficient). Adenylate cyclase deficient S49 cells demonstrated nearly 10-fold greater resistance to methylthioadenosine (IC_{50} = 420 μM) than the parental strain. Similarly, cAMP-dependent protein kinase deficient cells also were 10-fold more resistant (IC_{50} = 520 μM) to the growth inhibitory effect of the drug. These gross differences in the biological responses among these cell lines suggests a major involvement of cAMP in the inhibitory effects of methylthioadenosine.

Methylthioadenosine phosphorylase activities of S49 wild type and mutant cells. Differences in the sensitivity to methylthioadenosine between the wild type and mutant cells could have been due to an enhanced ability of the mutant cells to degrade the nucleoside. To examine this possibility, methylthioadenosine phosphorylase activities from logarithmic cultures of the three cell types were measured. The S49 wild type, S49 adenylate cyclase deficient, and S49 protein kinase deficient cells contained nearly equivalent methylthioadenosine phosphorylase activities: 36.5, 36.1, and 33.6 pmoles/min/mg protein respectively. The data indicate that the differences in sensitivity to methylthioadenosine

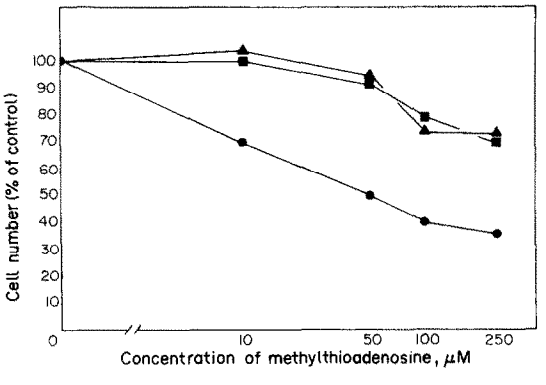

Fig. 1. Comparison of the inhibitory effect of 5′-methylthioadenosine on the proliferation of S49 parental wild type and mutant cells. Cells were seeded at 1 × 10^5/ml in Dulbecco's modified Eagle's Medium supplemented with 10% horse serum and variable concentrations of the nucleoside. Key: (●) S49 wild type; (■) S49 adenylate cyclase deficient; and (▲) S49 protein kinase deficient. The results represent the average of three independent experiments.

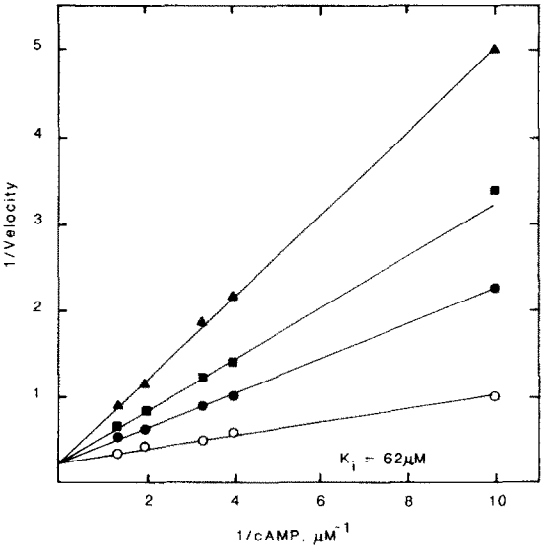

Fig. 2. Inhibition of high-affinity cAMP phosphodiesterase activity of S49 wild type by 5′-methylthioadenosine. Initial velocities were pmoles of [^{3}H]adenosine formed per mg of protein per min. Each point represents the average of three determinations. Key: (○) no inhibitor; (●) 62.5 μM; (■) 125 μM; and (▲) 250 μM.

Table 1. Comparison of cAMP phosphodiesterase activities of S49 wild type and mutant cells

Cell type	High-affinity cAMP phosphodiesterase specific activity* (pmoles/min/mg)	% Inhibited by methylthioadenosine†
S49 wild type	1.67	60.0
S49 adenylate cyclase deficient	1.53	63.5
S49 protein kinase deficient	1.19	51.4

* Specific activities were determined at 0.25 μM substrate.
† Assay conditions were: 40 mM Tris (pH 8.0), 5 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 0.25 μM [^{3}H]cAMP (300,000 cpm), enzyme extract, and 250 μM methylthioadenosine.

noted for the three strains were not due to differences in their abilities to degrade the nucleoside.

Inhibition of the high affinity cAMP-phosphodiesterase by 5'-methylthioadenosine. Previous kinetic analysis of S49 cell homogenates for cAMP phosphodiesterase activity established the presence of two enzyme forms: a high-affinity enzyme exhibiting a K_m value of 0.52 μM, and a second low-affinity enzyme exhibiting a K_m of 16.9 μM [17]. Zimmerman *et al.* [18, 19] have reported that methylthioadenosine and several of its structural analogs inhibit the high-affinity phosphodiesterase of murine cytolytic lymphocytes. To evaluate this possible site of action of methylthioadenosine in the S49 cell strains, we tested the inhibitory potency of the nucleoside on the high-affinity phosphodiesterase activity from homogenates of S49 wild type cells (Fig. 2). Our data confirm the previous estimate of the K_m value (0.50 μM) as well as the potent competitive inhibition exerted by methylthioadenosine on the enzyme ($K_i = 62$ μM).

Basal cAMP phosphodiesterase activities in S49 wild type and mutant cells. Since methylthioadenosine is a potent inhibitor of cAMP phosphodiesterase, the differences in the sensitivity to this substance between the S49 wild type and mutant cells could possibly reflect extreme variability in the levels of this enzyme among the three cell types. Activities of the high-affinity form measured in cell homogenates (Table 1) revealed only slight variation of these activities among the S49 cell strains (range: 1.19 to 1.67 pmoles/min/mg protein). These data suggest that the increased resistance of the S49 protein kinase deficient and S49 adenylate cyclase deficient cell lines to the inhibitory effects of methylthioadenosine was not due to an enhanced capacity to degrade cAMP.

The cAMP phosphodiesterase activities from the three cell types were also examined for their sensitivity to methylthioadenosine (Table 1). Under our standard assay conditions (0.25 μM cAMP and 250 μM methylthioadenosine), the activities of the wild type, protein kinase deficient, and adenylate cyclase deficient S49 cells were inhibited by, 60.0, 51.4 and 63.5% respectively. These data indicate that the high-affinity forms of the phosphodiesterase in these three cell types are very similar with respect to their activities and their sensitivities to methylthioadenosine.

Comparison of the cAMP response in S49 wild type and mutant cells after exposure to 5'-methylthioadenosine. S49 wild type and S49 protein kinase deficient cells that had been exposed to 100 μM methylthioadenosine for 48 hr exhibited markedly elevated levels of cAMP (Table 2). The response in the wild type strain was a 17-fold increase in the intracellular concentration of the nucleotide. Nearly the same intracellular concentration of cAMP was found in the S49 protein kinase deficient cells after 48 hr in medium containing methylthioadenosine, representing a 6-fold enhancement in the cAMP levels. Under these same conditions, the S49 adenylate cyclase deficient mutant cells did not contain detectable levels of cAMP. This is in agreement with the data of Coffino *et al.* [20] who also could not measure detectable levels of cAMP in this strain.

Table 2. Effect of methylthioadenosine addition on cAMP pool size in S49 wild type and mutant cells*

Additions	cAMP (pmoles/10^7 cells)		
	S49 wt	S49 cyc$^-$	S49 pk$^-$
None	0.31	Not detectable	1.15
Methylthioadenosine (100 μM)	5.56	Not detectable	6.26

* Cultures were seeded at 1×10^5 cells/ml in Dulbecco's modified Eagle's medium in the presence or absence of 100 μM 5'-methylthioadenosine. After 48 hr, the cells were harvested by centrifugation, and the nucleotides were extracted in perchloric acid. The solution was cleared of the perchorate salts by freon:alamine extraction, and cAMP levels were determined subsequently by the competition binding assay of Amersham. Cells were S49 wild type (S49 wt), S49 adenylate cyclase deficient (S49 cyc$^-$), and S49 protein kinase deficient (S49 pk$^-$). Results are the average of duplicate experiments where values did not vary more than 10%.

Higher concentrations of methylthioadenosine were not used in these studies due to the extreme cytotoxicity to the wild type strain.

DISCUSSION

The naturally occurring nucleoside, methylthioadenosine, has been described as a growth regu latory substance. Reports have demonstrated the cytostatic effects of this substance in murine T and B lymphocytes [3], as well as SV-40 infected mouse fibroblasts [4]. It has also been found to retard the progression of lymphocytes undergoing blastogenesis [2]. Previous studies designed to elucidate its mode of action indicate that methylthioadenosine may exert its effects via the potent inhibition of polyamine biosynthesis [5, 6]. Pegg *et al.* [4] and Raina *et al.* [21], however, have observed that the biologic effects of methylthioadenosine cannot be reversed by the exogenous addition of the polyamines spermidine or spermine. Inhibition of *S*-adenosylhomocysteine hydrolase activity also has been proposed as the primary site of action [22]. Zimmerman *et al.* [8], however, found that the inhibition by methylthioadenosine of lymphocyte-mediated cytolysis was unrelated to *S*-adenosylhomocysteine levels. On the basis of these results, it was suggested that other target sites must be considered. Zimmerman and coworkers [8] first proposed that this alternate site may be the high-affinity cAMP phosphodiesterase. Although this work described the competitive inhibition of the enzyme by methylthioadenosine, it did not access the significance of this site of action to the inhibitory action of the nucleoside.

A central aim of this study was to use mutants defective in cAMP-related functions to aid in determining the importance of cAMP in the growth inhibitory properties of methylthioadenosine. Like methylthioadenosine, high levels of cAMP arrest cellular growth [11] and influence a wide variety of biochemical processes [23]. The effects of this regulatory nucleotide are thought to be mediated through the activity of a cAMP-dependent protein kinase [12]. From this knowledge we predicted that, if cAMP mediated the effects of methylthioadenosine, then mutant cells incapable of synthesizing cAMP (S49 adenylate cyclase deficient) or incapable of responding to elevated levels of the nucleotide (S49 protein kinase deficient) should have reduced sensitivity to methylthioadenosine. Our data show that these mutants were nearly 10-fold more resistant to the cytotoxic action of the nucleoside than the parental wild type strain and that this difference was not due to differences in methylthioadenosine phosphorylase activity. Correlating to these effects were increased levels of cAMP (up to 17-fold) in the S49 wild type and S49 protein kinase deficient cells while the S49 adenylate cyclase deficient cell line did not contain measurable levels of the nucleotide. *In vitro* experiments also demonstrated that the inhibitory potency of methylthioadenosine towards the S49-derived cAMP phosphodiesterase ($K_i = 62\ \mu M$) was superior to that reported by Zimmerman *et al.* [8] in homogenates of murine cytolytic lymphocytes ($K_i = 225\ \mu M$).

Taken together, our results suggest that the perturbation of cAMP metabolism by methylthioadenosine represents a primary site of action of this compound in S49 cells. Mechanistically, it appears that methylthioadenosine competitively inhibits the cytosolic enzyme responsible for the degradation of cAMP, thereby leading to enhanced levels of the regulatory nucleotide and subsquent growth arrest.

It has been suggested that methylthioadenosine phosphorylase may be a useful target for chemotherapeutic or immunosupressive agents [24]. The data presented here indicate that nonmetabolizable structural analogs of methylthioadenosine which are also potent inhibitors of the cAMP phosphodiesterase should increase the effectiveness of such agents.

In conclusion, the primary growth regulatory properties of methylthioadenosine appear to be transmitted through the ubiquitous second messenger, cAMP. The significance of this finding and its relationship to the regulation of cell division by either or both of these compounds must await further investigation. Knowledge of possible changes in the metabolism of methylthioadenosine throughout the cell cycle should aid in this endeavor.

Acknowledgements—The authors wish to express their appreciation to Carlene Pelroy and Connie Zook for their excellent secretarial assistance; to Dave Jones for the synthesis of methylthioadenosine; and to R. J. Parker and J. A. Hughes for their careful review of the manuscript. A.J.F. is a recipient of a Public Health Service Career Development Award (CA 00617) from the National Institutes of Health. Partial support for M.K.R. was from the N. L. Tartar Research Fellowship Fund. This research was supported in part by Grant CA25756 from the National Cancer Institute and by a grant from the Medical Research Foundation of Oregon.

REFERENCES

1. A. E. Pegg and H. G. Williams-Ashman, *J. biol. Chem.* **244**, 682 (1969).
2. A. A. Vandebark, A. J. Ferro and C. L. Barney, *Cell Immun.* **49**, 26 (1980).
3. R. W. Wolford, M. R. MacDonald, B. Zehfus, T. J. Rogers and A. J. Ferro, *Cancer Res.* **41**, 3035 (1981).
4. A. E. Pegg, R. T. Borchardt and J. K. Coward, *Biochem. J.* **194**, 79 (1981).
5. R. L. Pajula and A. Raina, *Fedn Eur. Biochem. Soc. Lett.* **99**, 343 (1979).
6. H. Hibasami, R. T. Borchardt, S. Y. Chen, J. K. Coward and A. E. Pegg, *Biochem. J.* **187**, 419 (1980).
7. A. J. Ferro, A. A. Vandenbark and M. R. MacDonald, *Biochem. biophys. Res. Commun.* **100**, 523 (1981).
8. T. P. Zimmerman, G. Wolberg, C. J. Schmitges, L. M. Beachman, G. S. Duncan and R. D. Deeprose, in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Eds. E. Usdin, R. T. Borchardt and C. R. Creveling), pp. 627–35. Macmillan, London (1982).
9. H. R. Bourne, P. Coffino and G. M. Tomkins, *Science* **187**, 750 (1975).
10. P. Coffino, in *Progress in Cancer Research and Therapy* (Eds. S. Iacobelli, R. J. B. King, H. R. Lindner and M. E. Lippman), Vol. 14, pp. 287–94. Raven Press, New York (1980).
11. K. Kikugawa and M. Ichino, *Tetrahedron Lett.* 87 (1971).

12. P. Coffino, H. R. Bourne and G. M. Tompkins, *J. cell. Physiol.* **85**, 603 (1975).
13. J. X. Khym, *Clin. Chem.* **21**, 1245 (1975).
14. W. J. Thompson, G. Brooker and M. M. Appleman, *Meth. Enzym.* **38**, 205 (1974).
15. M. W. White, A. A. Vandenbark, C. L. Barney and A. J. Ferro, *Biochem. Pharmac.* **31**, 503 (1982).
16. H. R. Bourne, P. Coffino and G. M. Tomkins, *J. cell. Physiol.* **85**, 611 (1975).
17. V. M. Brothers, N. Walker and H. R. Bourne, *J. biol. Chem.* **257**, 9349 (1982).
18. T. P. Zimmerman, C. J. Schmitges, G. Wolberg, R. D. Deeprose and G. S. Duncan, *Life Sci.* **28**, 647 (1981).
19. T. P. Zimmerman, C. J. Schmitges, G. Wolberg, G. S. Duncan and R. D. Deeprose, *Proc. Am. Ass. Cancer Res.* **22**, 16 (1981).
20. P. Coffino, J. W. Gray and G. M. Tompkins, *Proc. natn. Acad. Sci. U.S.A.* **72**, 878 (1975).
21. A. Raina, K. Tuomi and R-L. Pajula, *Biochem. J.* **204**, 697 (1982).
22. A. J. Ferro, A. A. Vandenbark and M. R. MacDonald, *Biochem. Biophys. Res. Commun.* **100**, 523 (1981).
23. T. R. Soderling, T. P. Hickenbottom, E. M. Reimann, F. L. Hunkeler, D. A. Walsh and E. G. Krebs, *J. biol. Chem.* **245**, 6317 (1970).
24. T. M. Savaresse, G. W. Crabtree, and R. E. Parks, *Biochem. Pharmac.* **28**, 2227 (1979).