AN ABSTRACT OF THE THESIS OF

Michael W. White for the degree of Doctor of Philosophy in Microbiology presented on October 24, 1983

Title: The Effect and Metabolism of 5'-Methylthioadenosine in Normal and 5'-Methylthioadenosine Phosphorylase Deficient Mammalian Cells

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Abstract approved: _____
Adolph J. Ferro

5'-Deoxy-5'-methylthioadenosine (MTA) phosphorylase was purified 13.4-fold from human peripheral lymphocytes. The enzyme demonstrated normal Michaelis-Menten kinetics with K_m's of 26 uM and 7.5 mM for the two substrates, MTA and phosphate, respectively. Five structural analogs served as alternative substrates with K_m's ranging from 31 uM to 53 uM while two compounds, 5'-deoxy-5'-methylthiotubercidin (MTT) (K_i = 31 uM) and adenine (K_i = 172 uM) were inhibitory. These same analogs were examined as inhibitors of mitogen-induced human lymphocyte blastogenesis. MTT was found to be the most effective inhibitor of lymphocyte transformation with an I_{50} of 80 uM.

The antiproliferative effects of MTA and the MTA analogs, were further studied using two mouse cell lines, one 5'-methylthioadenosine phosphorylase deficient (MTAase$^-$) the

other 5'-methylthioadenosine phosphorylase containing ($MTAase^+$). All of the compounds were found to be growth inhibitory to both cell lines demonstrating that these compounds need not be degraded to exert their inhibitory effects. The effect of the nondegradable MTAase inhibitor, MTT, on the disposition of cellularly synthesized MTA was explored using both cell types. MTT inhibited the accumulation of exogenous MTA from $MTAase^-$ cells with no effect on intracellular MTA. In contrast, MTT caused a large accumulation of extracellular MTA with a concomitant smaller increase intracellularly in $MTAase^+$ cells.

The use of methotrexate (MTX)/MTA combined chemotherapy to selectively inhibit $MTAase^-$ cells was examined. The selective inhibition of $MTAase^-$ cells by the above strategy was found to be successful provided the serum used contained no MTAase activity. MTA chemotherapy with or without MTX and methionine deprivation growth conditions was examined as an alternative strategy. Using this regime $MTAase^-$ cells were found to be selectively inhibited despite high exogenous MTAase activity. $MTAase^-$ cells grown in 1 uM methionine, 100 uM MTA and fetal calf serum medium were 99% inhibited after three days. The data suggests that MTA as a source of methionine can be used to selectively inhibit $MTAase^-$ cells regardless of the exogenous MTAase activity.

The Effect and Metabolism of 5'-Methylthioadenosine in Normal and 5'-Methylthioadenosine Phosphorylase Deficient Mammalian Cells

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I dedicate this thesis to my wife, Cheryl, without whom I could not have survived, and to my son, Matthew.

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CONTRIBUTION OF AUTHORS

Lymphocyte transformation experiments in the chapter entitled "Structural Analogs of 5'-Methylthioadenosine as Substrats and Inhibitors of 5'-Methylthioadenosine Phosphorylase and as Inhibitors of Human Lymphocyte Transformation" were performed by Carolyn L. Barney within the laboratory of Dr. Arthur A. Vandenbark. All other experiments in this chapter were performed by Michael W. White within the laboratory of Adolph J. Ferro.

THE EFFECT AND METABOLISM OF 5'-METHYLTHIOADENOSINE IN NORMAL AND 5'-METHYLTHIOADENOSINE PHOSPHORYLASE DEFICIENT MAMMALIAN CELLS

INTRODUCTION

Biosynthesis of MTA

5'-Deoxy-5'-methylthioadenosine (MTA) is a naturally occurring sulfur nucleoside first discovered by Mandel and Dunham (59). The full chemical structure of MTA was elucidated by Zusuki (Figure 1) in 1924 (60). MTA is synthesized from S-adenosylmethionine by several biosynthetic pathways (36). In mammalian cells, the majority of MTA is synthesized during the biosynthesis of the polyamines spermidine and spermine (36). MTA is produced stoichiometrically with the propylamine transfer from decarboxylated S-adenosylmethionine, reactions catalyzed by spermidine and spermine synthases. It has been suggested that the MTA produced during polyamine biosynthesis is only a biproduct of this pathway. Williams-Ashman et. al. (39), however have suggested that the primary function of spermidine and spermine biosynthesis may be in the production of MTA which could act as a regulator of enzyme systems.

A possible minor pathway leading to MTA synthesis in mammalian cells is the enzymatic cleavage of S-adenosylmethionine to directly yield MTA and homoserine lactone. The cleavage of S-adenosylmethionine by this mechanism has been reported using

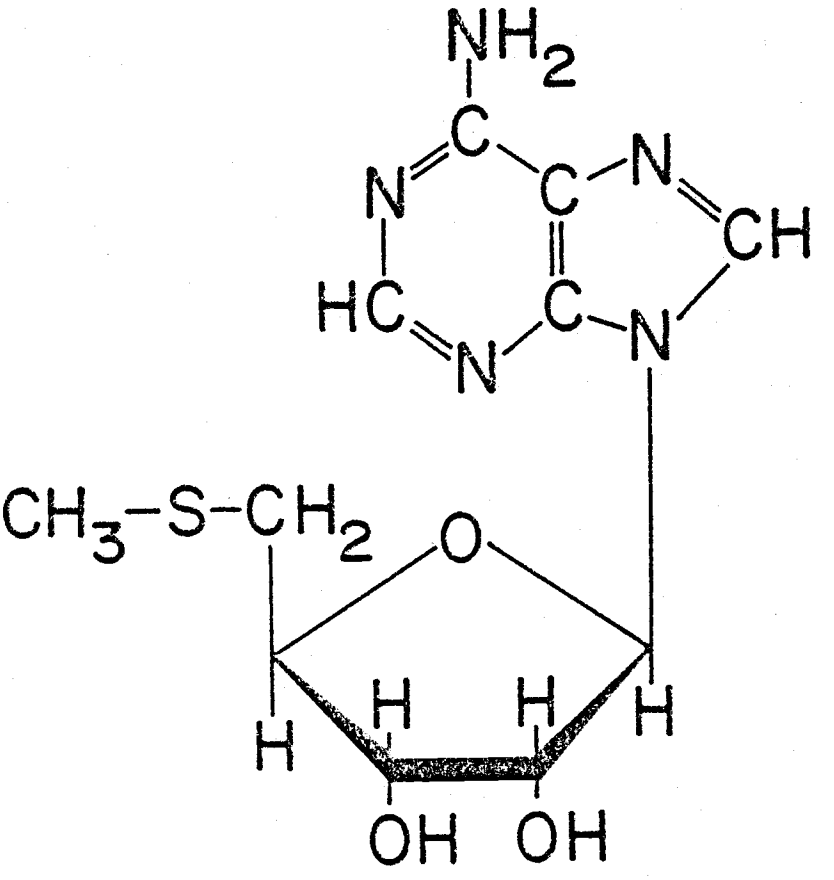


Fig. 1. The structure of 5'-deoxy-5'-methylthioadenosine

extracts prepared from some types of mammalian liver (61,62,63). This enzyme or enzymes, which has been named S-adenosylmethionine cyclotransferase or S-adenosylmethionine lyase, has not been characterized.

Removal of cellular MTA

In 1969, Pegg and William-Ashman (10) reported the existence of a MTA cleaving enzyme from rat ventral prostate. The enzyme, purified 30-fold, was completely dependent on phosphate in the assay medium. MTA phosphorylase, as this enzyme has been termed, degrades MTA into adenine and 5-methylthioribose-1-phosphate. MTA phosphorylase has been detected in all normal and some malignant mammalian tissues tested. The enzyme has been purified to near homogeneity from human placenta (6) and human prostate (7). Rat liver MTA phosphorylase was determined to have a molecular weight of 90,000 daltons (64), agreeing well with the molecular weight for the human placental enzyme (6). MTA phosphorylase purified 475-fold from calf liver (65) was found to have a molecular weight of 31,000 daltons by SDS gel electrophoresis indicating that the enzyme was composed of three subunits of approximately 30,000 daltons. Using rat liver MTA phosphorylase, Ferro et al. (23) determined a K_m for MTA to be 4.7×10^{-4}mM and a 0.2mM K_m for phosphate. Low micromolar K_m values for MTA have consistently been reported for mammalian MTA phosphorylases (7,9,43,45). This

agrees well with the role MTA phosphorylase plays in maintaining low intracellular MTA levels. While the polyamines spermidine and spermine may achieve millimolar levels within the cell (4), MTA levels are several orders of magnitude lower (>.2umoles/gm fresh weight (51)).

MTA is not a substrate for another purine degrading enzyme, purine nucleoside phosphorylase (10), nor has MTA been found to be a substrate for mammalian adenosine deaminase (9,10). Thus, MTA phosphorylase probably represents the only mechanism for catabolism in mammalian cells.

The primary route, in normal and some malignant tissues, for removal of cellular MTA is through degradation by MTA phosphorylase. In malignant tissues, lacking MTA phosphorylase, MTA is removed from the intracellular environment by excretion (49,53). Therefore, two mechanisms exist by which low cellular MTA levels are achieved, excretion or degradation, suggesting the importance the cell places on removing MTA.

Recycling of MTA

The products of the MTA phosphorylase are adenine and 5-methylthioribose-1-phosphate. The adenine produced from the degradation of MTA is the only source of free adenine in mammalian cells (66). The resulting adenine reenters the purine pool by the action of adenine phosphoribosyl-transferase which condenses 5-phosphoribosyl pyrophosphate (PRPP) with adenine to produce adenylate.

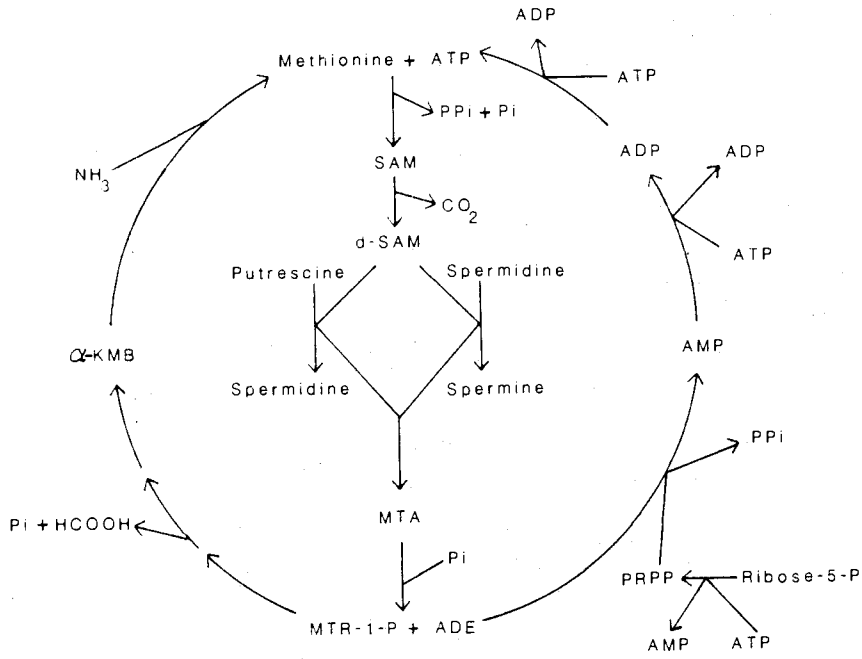
The recycling of the sugar-thio portion of MTA to methionine has been shown to occur in both prokaryotes and eukaryotes (37,67,69). Backlund and Smith (37) first demonstrated that cell free extracts of rat liver convert MTA to methionine. Using differentially labeled MTA they showed that the likely pathway is the formation of 5-methylthioribose-1-phosphate by MTA phosphorylase and the conversion of 5-methylthioribose-1-phosphate to methionine. The four carbons of the methionine skeleton were suggested to be derived from the pentose ring of 5-methylthioribose-1-phosphate. Backlund et al. (69) have also reported that 2-keto-4-methylthiobutyric acid was an intermediate and that the final step was a transamination reaction utilizing either glutamine or asparagine. Trackman and Abeles (38) have recently reported that the C-1 carbon of 5-methylthioribose-1-phosphate is released as formic acid by rat liver extracts. Thus, the pathway of MTA to methionine in mammalian cells involves first the degradation of MTA, by MTA phosphorylase, to 5-methylthioribose-1-phosphate, the opening of the pentose ring and the removal of the C-1 carbon as formic acid, a series of uncharacterized steps forming 2-keto-4-methylthiobutyric acid and a final amino transfer to produce methionine. Edwards et al. (57) have reported that adult rats fed a sulfate free diet could utilize MTA as a source of methionine and upon feeding rats

[^{35}S]-MTA, [^{35}S]-methionine was recovered in plasma and urine. The efficient recycling of the purine moiety of MTA back into the adenylate pool and the methylthio-sugar to methionine conserves essential moieties of S-adenosylmethionine and suggests that the only essential compounds required for polyamine synthesis are Ribose-5-P, ATP, putrescine, and an amino donor (Figure 2).

Antiproliferative Effects of MTA

Ferro (36) first described the cytostatic effect of MTA on human lymphocytes stimulated with mitogens, antigens or allogeneic cells. Thus, MTA belongs to a unique and relatively rare group of naturally occurring compounds which inhibit cellular proliferation. In many cellular systems, since the first observation by Ferro, MTA has been shown to be growth inhibitory. A brief list would include virally-transformed mouse fibroblasts (34), murine lymphoid cells in culture (43), and baby hamster kidney cells (44). The significance of MTA's antiproliferative effects cannot be overstated, the potential for chemotherapeutic exploitation has been suggested (51), yet the mechanism by which MTA is able to block cell growth remains elusive.

Several cellular sites at which MTA may act have been suggested by investigators seeking to explain MTA's antiproliferative effects. It has also been suggested that the products of MTA degradation are the true growth inhibitory agents (24). Despite numerous explanations, the image of MTA's effect on



Biosynthesis of spermidine and spermine

Balanced reaction

$$2\text{Putrescine} + 3\text{Ribose-5-P} + 9\text{ATP} + 3\text{NH}_3 =$$

$$\text{Spermidine} + \text{Spermine} + 6\text{ADP} + 3\text{AMP} + 15\text{Pi} + 3\text{CO}_2 + 3\text{HCOOH}$$

Figure 2. Summary of MTA metabolism in mammalian cells. Abbreviations: SAM, S-adenosylmethionine; d-SAM, decarboxylated SAM: MTA, 5'-methylthioadenosine; MTR-1-P, 5-methylthioribose-1-phosphate; ADE, adenine; KMB, 2-keto-4-methylthiobutyric acid; PRPP, 5-phosphoribosyl-1-pyrophosphate.

cellular metabolism will not reduce to black and white and in fact the interaction of MTA with cellular biochemistry is undoubtedly a complex picture. However, the metabolic sites at which MTA has been shown to have an effect certainly deserve mention.

MTA has been shown to exert an inhibitory effect on polyamine biosynthesis. MTA inhibits the activity of spermine synthase (34,44) and the exogenous addition of MTA decreased the spermidine content of virally transformed mouse fibroblasts (34) in addition to blocking the growth of these cells. However, the inhibition of cell growth by MTA could not be overcome by the addition of spermidine suggesting, another or additional sites at which MTA was acting.

The inhibition of cellular methylation has also been proposed to explain MTA's antiproliferative effects. MTA has been reported to inhibit protein methylase I of Krebs II ascites cells (70) and protein methylase II of human erythrocytes (71). Zappia et al. (72) have demonstrated the inhibitory effect of MTA on the histamine and acetylserotonin methyltransferases. Although MTA has been reported to directly inhibit some methyltransferase reactions, such as those listed above, non-physiological concentrations of MTA are usually required.

MTA, at physiological levels, has been reported to irreversibly inactivate S-adenosylhomocysteine hydrolase from human erythrocytes (35). A block in essential cell methylations by

MTA, therefore, may be through an indirect mechanism: the inhibition of S-adenosylhomocysteine hydrolase leading to a build up in S-adenosylhomocysteine, which is a known potent inhibitor of methyl transferase reactions. Recently it has been reported that MTA, at low micromolar levels, inhibited the incorporation of methyl groups into the DNA of transformed rat cells (75) and some human cell lines (76). MTA was not found to efficiently inhibit the activity of DNA methyltransferases in vitro nor was MTA found to alter S-adenosylhomocysteine levels. Since the incorporation of methyl groups into DNA was determined by the use of [^{3}H-methyl]-methionine, and MTA is a known precursor of methionine in mammalian cells, simple dilution of the methionine label may very likely explain the observations of these investigators.

Wolberg et al. (73) have found that MTA causes an increase in the intracellular level of c-AMP through the direct inhibition, by MTA, of the activity of c-AMP phosphodiesterase. Since alterations in c-AMP metabolism have been shown to adversely affect lymphocyte blastogenesis (74), this represents another possible mechanism by which MTA controls cell growth.

It has been suggested (24) that the products of MTA degradation, adenine and/or 5-methylthioribose-1-phosphate, are the agents reponsible for the antiproliferative effects attributed to MTA. The demonstration, in Sarcoma 180 cells, that MTA blocks PRPP accumulation and causes an increase in the adenylate pool

lends support to this hypothesis (24). However, as this thesis will show, MTA is strongly cytostatic to mammalian cells lacking MTA phosphorylase, indicating that MTA is the true cytostatic compound.

The mechanism through which MTA blocks cellular proliferation is most likely a combination of events already described and those yet to be discovered. Although this prediction is much like saying Mt. St. Helens will erupt again in the future, it is not taking a big risk to say MTA's effect on cell growth is probably multifaceted. Further, it is possible that the action of MTA at any particular site may be concentration dependent.

Mutations in MTA Metabolism

Mammalian cell lines deficient in MTA phosphorylase were first reported by Toohey (20). Since then Kamatani et al. (49) reported that 7 out of 31 human malignant cell lines tested were deficient in MTA phosphorylase. Recently, Kamatani et al. (55) have observed that leukemic cells from two patients afflicted with leukemia were MTA phosphorylase deficient. The incidence of MTA phosphorylase deficiency in malignant mammalian cells is not known, however it may be more common than first realized. MTA phosphorylase deficiency in normal tissue has yet to be described.

The molecular biology of the MTA phosphorylase deficiencies is uncharacterized. Kamatani et al. (77) have reported that the

MTA phosphorylase deficiency phenotype is recessive in intraspecies cell hybrids. The genetics of MTA metabolism in mammalian cells, especially with regard to mutant phenotypes, may be important in understanding MTA's effects in mammalian cells.

Preface to the text

The following chapters report investigations on the MTA phosphorylase from stimulated human lymphocytes and examine its substrate specificity. In this work MTA analogs have been used to study their effect on lymphocyte transformation and this information was correlated with the *in vitro* studies on the phosphorylase.

The effect of MTA and several MTA analogs on the proliferation of two murine lymphocyte cell lines, one containing an active MTA phosphorylase the other deficient in this enzyme, have been examined. In addition, the normal disposition of cellularly synthesized MTA, in these two cell lines, was investigated in an attempt to understand how a MTA phosphorylase deficient cell deals with the problem of MTA removal.

Since MTA phosphorylase deficiencies has thus far been found previously to occur in malignant cells *in vitro* and *in vivo*. I have examined whether the inability to degrade MTA can be exploited to selectively inhibit MTA phosphorylase deficient cells in culture.

Throughout the text the following abbreviations have

been used: MTA, 5'-deoxy-5'-methylthioadenosine, MTAase; MTA phosphorylase, MTAase$^-$; MTA phosphorylase deficient cell, MTAase$^+$; MTA phosphorylase containing cell.

STRUCTURAL ANALOGS OF 5'-METHYLTHIOADENOSINE AS SUBSTRATES AND INHIBITORS OF 5'-METHYLTHIOADENOSINE PHOSPHORYLASE AND AS INHIBITORS OF HUMAN LYMPHOCYTE TRANSFORMATION

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ABSTRACT

5'-Deoxy-5'-methylthioadenosine (MTA) phosphorylase was purified 13.4-fold from human peripheral lymphocytes. The enzyme demonstrated normal Michaelis-Menten kinetics with K_m's of 26 uM and 7.5 mM for the two substrates, MTA and phosphate, respectively. The rate of MTA degradation was temperature dependent, 47°C being the optimum temperature. Five structural analogs served as alternative substrates with K_m's ranging from 31 uM to 53 uM while two compounds, 5'-deoxy-5'-methylthiotubercidin (MTT) (K_i = 31 uM) and adenine (K_i = 172 uM) were inhibitory. These same analogs were examined as inhibitors of mitogen-induced human lymphocyte blastogenesis. MTT was found to be the most effective inhibitor of lymphocyte transformation with an I_{50} of 80 uM.

INTRODUCTION

The progression of cells from a quiescent to a proliferating state is accompanied by marked increases in the cellular levels of polyamines (1) as well as in the activities of the enzymes involved in their biosynthesis (2). 5'-Deoxy-5'-methylthioadenosine (MTA) is synthesized in mammalian tissue in stoichiometric quantities with the production of spermidine and spermine (3). MTA, however, does not appear to normally accumulate intracellularly. Seidenfeld et al. (4) recently demonstrated that MTA levels in rat tissue are one order of magnitude smaller than those of S-adenosylmethionine and nearly two orders of magnitude lower than the levels of spermidine plus spermine in the same tissue. The rapid degradation of MTA in mammalian tissue is accomplished via MTA phosphorylase, an enzyme shown to exist in a variety of normal and transformed cells (5-10) and to be distinct from purine nucleoside phosphorylase (10).

Vandenbark et al. (11) described the cytostatic action of MTA to human peripheral lymphocyte cultures stimulated with mitogens, antigens, or allogeneic cells. The MTA-mediated inhibitory effect also was shown to be dose-dependent and readily reversible. Ferro et al. (8) subsequently found that MTA phosphorylase activity markedly increased during blastogenesis and that the 7-deaza analog of MTA, 5'-deoxy-5'-methylthiotubercidin (MTT) was a more potent inhibitor of the lymphocyte

enzyme. The nonreversible inhibition of lymphocyte transformation by MTT, as compared to the reversibility of MTA, was suggested to be due, in part, to the analogs' resistance to degradation by MTA phosphorylase.

The present study was designed to further investigate the effects of MTA, MTT and additional structural analogs of MTA on lymphocyte transformation and to compare this to the substrate specificity of the MTA phosphorylase from human lymphocytes. In addition, a further characterization of the lymphocyte enzyme is described.

MATERIALS AND METHODS

Compounds. 5'-[^{14}C-methyl]Methylthioadenosine was prepared by the procedure of Schlenk et al. (12) from S-adenosyl-L-[^{14}C-methyl]methionine (>40 Ci/mmole) which was obtained from Amersham Corp., Arlington Heights, IL. MTA, tubercidin, adenosine, adenine and inosine were purchased from Sigma Chemical Co., St. Louis, MO. 5'-Ethylthioadenosine (ETA), 5'-propylthioadenosine (PTA), 5'-isopropylthioadenosine (iPTA), 5'-butylthioadenosine (BTA), 5'-isobutylthioadenosine (iBTA), 5'-methylthiotubercidin (MTT), 5'-methylthioinosine (MTI), and 5'-dimethylthioadenosine (DMTA) were synthesized by established methods (13-18).

Lymphocyte transformation. Lymphocyte transformation was carried out using purified cells from normal human donors as described previously (19).

Preparation of cell extracts. Purified lymphocytes were centrifuged at 1,500 x g for 15 min, washed and resuspended in 0.05 M sodium HEPES buffer (pH 7.2) containing 0.05 M K_2HPO_4, 3 mM mercaptoethanol and 10% glycerol. The cells were freeze-thawed five times in liquid nitrogen, centrifuged at 700 x g for 20 min, and then acidified to pH 4.0 and heated at 60°C according to the procedure of Toohey et al. (20). This procedure resulted in a 2.3-fold purification with 95% yield. The pH-heat treated extracted was precipitated with ammonium sulfate at 40, 50, and 60% saturations. The great majority of the enzyme activity was

found in the precipitate of the 60% saturation. This precipitate was dissolved in the HEPES buffer (pH 7.2) and dialyzed against 20 volumes of the same buffer. This final preparation represented a 13.4-fold purification and 30% yield, as compared to the crude extract, and was utilized as the source of enzyme in the MTA phosphorylase assays.

MTA phosphorylase assays. MTA phosphorylase activity was determined by: 1) measuring the conversion of [^{14}C-methyl]MTA to [^{14}C-methyl]5-methylthioribose-1-phosphate (MTR-1-P) (21) and/or 2) measuring the conversion of MTA to adenine. The standard reaction mixture for both methods, unless otherwise stated, contained in a total volume of 250 ul, 23 mM Na HEPES, 23 mM K_2HPO_4 (pH 7.2), 1.4 mM mercaptoethanol protein and either 70 uM [14C-methyl]MTA (6.6 x 10^6 cpm/umole) or 70 uM MTA. The assay mixture was incubated at 47°C for 30 min, terminated by the addition of 50 ul of 1.8 M trichloroacetic acid and the precipitate removed by centrifugation at 11,000 x g for 5 min. When [^{14}C-methyl]MTA was utilized as the substrate, a 0.2 ml aliquot of the centifuged reaction mixture was applied to a Dowex 50 H^+ x 4 (100-200 mesh) column (1 x 4 cm) equilibrated with 3N H_2SO_4. The product,[^{14}C-methyl]MTR-1-P was eluted directly into scintillation vials with 3 ml water and the radioactivity determined (21). When unlabeled MTA was used as substrate either MTA depletion or adenine formation was measured by high pressure liquid

chromatography. An aliquot of the centrifuged assay mixture was injected into a Waters model M-6000A HPLC system equipped with a Waters ubondapak C_{18} column and a model 440 detector (254 nm). The solvent used was 10 mM potassium acetate, 10% CH_3CN with Pic B7 (Waters paired complex), pH 4.0 at 1.0 ml/min and a pressure of 1,500 psi. Peak heights of MTA, its analogs and/or adenine were measured and concentration values determined from a standard curve run the same day. Standard curves were linear over a range of 0.01 to 1.0 nanomoles of MTA or adenine with r values typically greater than 0.99. Protein concentrations were determined by the commercially available BioRad protein assay using bovine serum albumin as a protein standard (22).

RESULTS

Initial experiments were designed to further characterize human lymphocyte MTA phosphorylase. Utilizing the 13-fold purified enzyme preparation, the effect of incubation temperature on enzyme activity was studied (Fig. 3). The data reveal that the rate of MTA breakdown was increased markedly by temperature elevation. At 4°, 23°, 37° and 47°C the degradation rate was linear throughout the 30 min incubation period, whereas at 62°C the rate was linear for only 20 min and at 81°C linearity did not exceed 10 min. Of the incubation temperatures which exhibited linearity throughout the 30 min period, the highest enzyme activity after 30 min was observed at 47°C. Under these conditions (47°C for 30 min) the rate of MTA degradation also was linear for the concentrations of protein and substrate used, and, therefore were utilized for all further experiments. The enzyme was quite stable under the standard assay conditions; when the 13-fold purified enzyme was preincubated for 30 min at 47°C and then assayed for activity after 30 min more of incubation at 47°C, no loss in activity was observed. At 62°C, however, loss in activity was observed beginning at 20 min of preincubation, while at 81°C, enzyme stability was decreased by 10 min preincubation.

To further characterize the lymphocyte enzyme, the effect of MTA and phosphate concentration on the reaction rate was determined. The reaction velocity of MTA phosphorylase showed

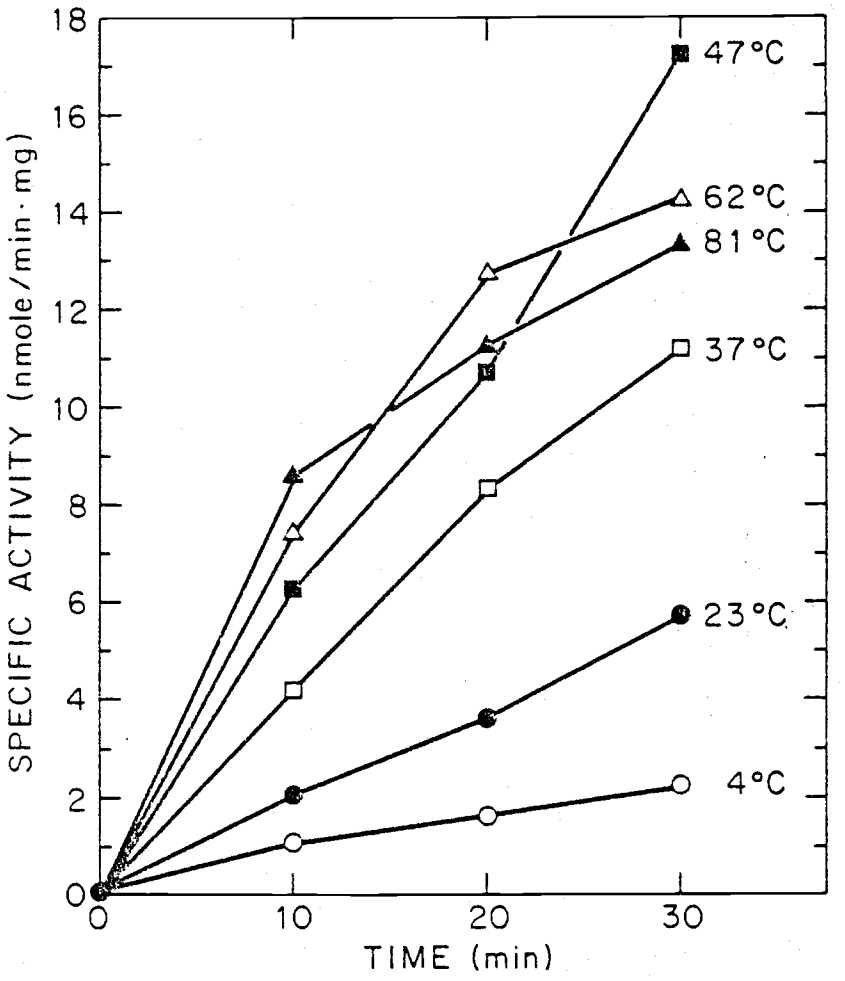

Fig. 3 Effect of temperature of incubation on MTA phosphorylase activity. Enzyme activity was determined by Method 1 as described in Materials and Methods at 4°C (O), 23°C (●), 37°C (□), 47°C (■), 62°C (△), and 81°C (▲) at the designated times. In each case controls were run to correct for any nonenzymatic breakdown of MTA.

normal Michaelis-Menten kinetics with either MTA or phosphate as the variable substrate. Apparent K_m values for MTA of 26 uM (Fig. 4) and for phosphate of 7.5 mM (Fig. 5) were calculated from double reciprocal plots.

To analyze the substrate specificity of MTA phosphorylase, several MTA analogs and derivatives were tested as substrates and inhibitors. Since the synthesized analogs were not radioactively labeled, a new method to measure MTA phosphorylase activity was devised. The reaction mixture and conditions were the same as for the radioactively labeled assay except that unlabeled substrates were substituted for the [$^{14}CH_3$]-MTA. HPLC, as described in Materials and Methods, was employed to measure the quantity of free adenine and/or the quantity of remaining substrate present in the reaction mixtures. Neither the enzyme preparation nor the analogs contained any detectable adenine. Under the conditions described, the retention times of MTA and some of its analogs by HPLC are shown in Table 1.

Of the compounds tested, six served as substrates for MTA phosphorylase (Table 2). Three of these compounds (MTA, iPTA and ETA) served as better substrates than did iBTA, BTA or PTA. The naturally occurring substrate, MTA, had the lowest K_m (26 uM) while the propyl analog had the highest K_m (53 uM). Neither the 7-deaza (MTT), dimethyl (DMTA), nor deaminated (MTI) analogs, nor adenine or adenosine served as substrates for MTA phosphorylase.

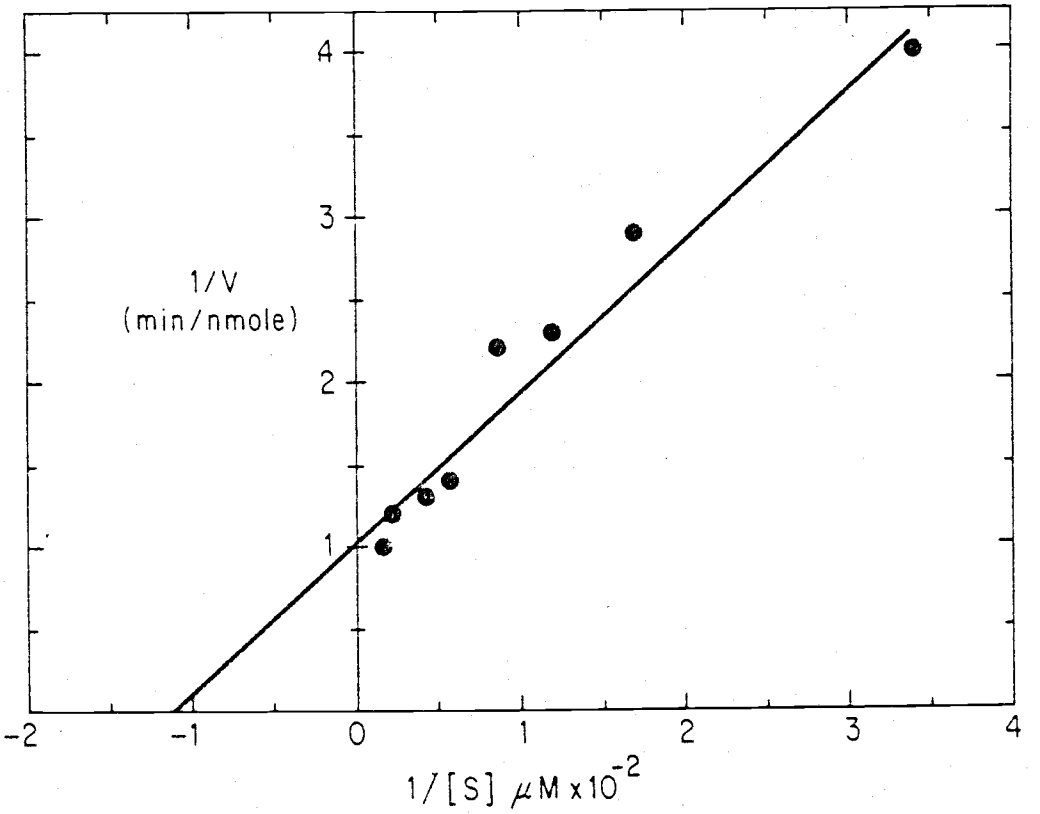

Fig. 4 Double-reciprocal plot of initial reaction velocity versus MTA concentration.

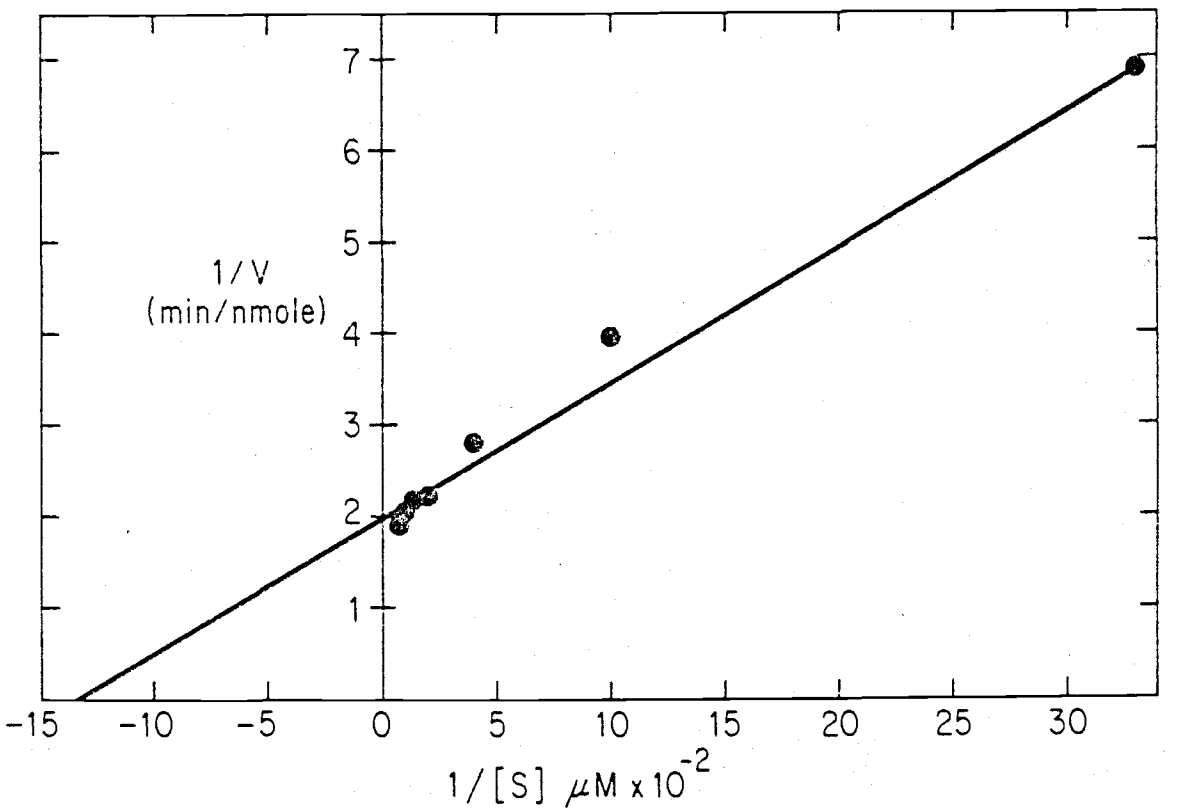

Fig. 5 Double-reciprocal plot of initial reaction velocity versus phosphate concentration.

Table 1. Retention times of MTA and some of its derivatives in potassium acetate-CH_3CN-Pic B7*

Compound	Retention time (min)
Adenine	3.2
Adenosine	3.3
5'-deoxy-5'-dimethylthioadenosine	4.9
5'-deoxy-5'-methylthioinosine	5.1
5'-deoxy-5'-methylthioadenosine	6.9
5'-deoxy-5'-ethylthioadenosine	11.4
5'-deoxy-5'-methylthiotubercidin	12.3
5'-deoxy-5'-isopropylthioadenosine	21.0
5'-deoxy-5'-propylthioadenosine	25.8
5'-deoxy-5'-isobutylthioadenosine	51.6
5'-deoxy-5'-butylthioadenosine	59.4

* Flow-rate, 1.0 ml/min.

Table 2. Kinetic constants of MTA and MTA analogs for MTA phosphorylase from human lymphocytes.*

Compound	K_m (uM)	K_i (uM)
5'-deoxy-5'-methylthioadenosine	26	-
5'-deoxy-5'-isopropylthioadenosine	28	-
5'-deoxy-5'-ethylthioadenosine	31	-
5'-deoxy-5'-isobutylthioadenosine	40	-
5'-deoxy-5'-butylthioadenosine	46	-
5'-deoxy-5'-propylthioadenosine	53	-
5'-deoxy-5'-methylthiotubercidin	-	31
Adenine	-	172
5'-deoxy-5'-dimethylthioadenosine	-	-
5'-deoxy-5'-methylthioinosine	-	-
Adenosine	-	-

* All K_m's and K_i's were determined by HPLC as described in Materials and Methods.

These latter compounds, therefore, were tested also for inhibitory activity. DMTA, MTI, and adenosine were without any significant effect, while MTT (K_i = 31 uM) and adenine (K_i = 172 uM) were found to be inhibitors of the lymphocyte enzyme activity.

MTA and its structural analogs were also tested as inhibitors of PHA-induced lymphocyte transformation as measured by [^{3}H]thymidine uptake. All compounds were added to the medium at the same time as the mitogen and were tested at various concentrations between 62.5 uM and 1 mM. Typical dose-response curves for MTA, iBTA (SIBA), and MTT are shown in Fig. 6 and the 50% inhibitory concentration (I_{50}) for each of these compounds was calculated (Table 3). The 7-deaza analog (MTT) was the most potent compound tested (I_{50} = 80 uM) and was followed by, in decreasing order of potency: BTA, iBTA, PTA, iPTA, MTA, ETA, and adenosine. 5'-Deoxy-5'-methylthioinosine (MTI), 5'-deoxy-5'-dimethylthioadenosine (DMTA), and adenine were not inhibitory (I_{50}'s = >1 mM). 5'-Methylthioribose-1-phosphate (1.0 mM) and 5-methylthioribose (1.0 mM) were also tested and found not to exert any inhibitory effects under the same conditions.

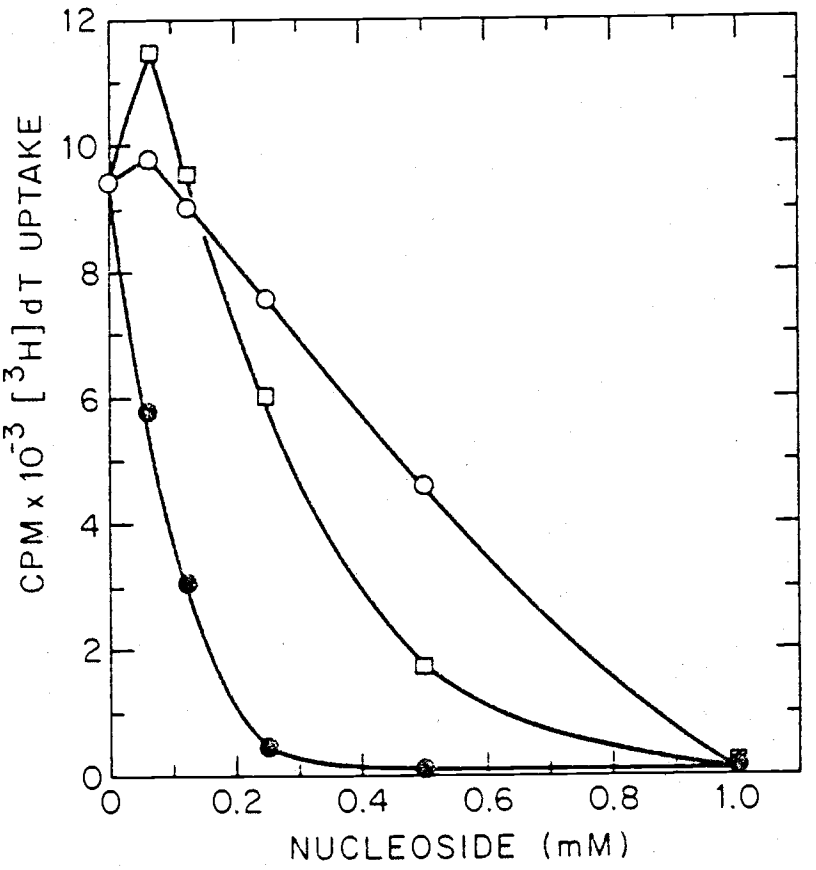

Fig. 6 Dose-dependent inhibition of PHA-stimulated human lymphocytes by MTA (O), SIBA (□), and MTT (●). Each point represents the mean of four replicate cultures. Individual values did not vary more than 10% from the mean.

Table 3. Effect of MTA and MTA analogs on lymphocyte transformation.*

Compound	I_{50} (uM)
5'-deoxy-5'-methylthiotubercidin (MTT)	80
5'-deoxy-5'-butylthioadenosine (BTA)	210
5'-deoxy-5'-isobutylthioadenosine (iBTA)	330
5'-deoxy-5'-propylthioadenosine (PTA)	370
5'-deoxy-5'-isopropylthioadenosine (iPTA)	450
5'-deoxy-5'-methylthioadenosine (MTA)	500
5'-deoxy-5'-ethylthioadenosine (ETA)	630
Adenosine	850
5'-deoxy-5'-dimethylthioadenosine (DMTA)	>1000
5'-deoxy-5'-methylthioinosine (MTI)	>1000
Adenine	>1000

* PHA (15 ug) was added at T_0 [^{3}H]-thymidine at T_{48}, and uptake of [^{3}H]-thymidine determined at T_{72}. Nonstimulated controls: 800 cpm ± 0.2; stimulated controls: 94,000 cpm ± 9.3. All compounds were added at T_0 at the following concentrations: 62.5, 125, 250, 500, and 1,000 uM.

DISCUSSION

The apparent K_m for MTA was found to be 26 uM, while the K_m for phosphate was 7.5 mM. The K_m for MTA, therefore, is similar to the values obtained from rat liver (23), human prostate (7) and sarcoma 180 cells (9), and suggests that the low levels of this nucleoside in mammalian tissues could be attributed to the high affinity that the phosphorylase has for its substrate. Cacciapuoti et al. (6) demonstrated that the rate of MTA breakdown by the human placenta enzyme was markedly increased by temperature elevation; a maximum rate of degradation occurred at 67°C when the reaction mixture was incubated for 60 min. We also have observed increased enzyme activity associated with increased temperature of incubation for the lymphocyte enzyme, but, in addition, have found that over a 30 min incubation period, the reaction is linear only at temperatures 47°C and below. At higher temperatures (62° and 81°C), the reaction is linear for only shorter periods of time (23 min and 10 min, respectively). Although the rate of degradation of MTA by the phosphorylase at 4°C is only 13% the rate at 47°C, this suggests that storage of cell extracts in phosphate-containing buffer at this low temperature will result in the degradation of any MTA present in the extracts. This may be particularly detrimental to those investigators wishing to determine the endogenous levels of this nucleoside.

The specificity of the human lymphocyte MTA phosphorylase for its substrate is rather strict compared to the E. coli enzyme (21). The replacement of the 6-amino group with a hydroxy group (MTI), the replacement of the bivalent sulfur in the thioether by a charged sulfonium group (DMTA), or the replacement of N-7 of adenine with a methinic radical (MTT) all result in complete loss of activity. Zappia et al. (7) have postulated that the human prostate MTA phosphorylase interacts with MTA at three sites: 1) the amino group of the adenine moiety, 2) N-7 of the purine ring, and 3) the sulfur atom in thioether conformation. Recently, however, Savarese et al. (24) found that 5'-deoxyadenosine was also a substrate for MTA phosphroylase from Sarcoma 180 cells, demonstrating that the substrate for the phosphorylase may be a non-sulfur containing compound. The human prostate enzyme also was found to be non-competitively inhibited by DMTA and it was suggested that this sulfonium compound may bind to a non-catalytic site on the enzyme (7). Our data with the human lymphocyte enzyme, however, is not consistent with this since DMTA was not found to be inhibitory. Whether or not this apparent difference are due to differences in tissue specificities has not yet been determined.

Coward et al. (16) found that MTT is an inhibitor but not a substrate of rat ventral prostate MTA phosphorylase, while Zappia et al. (7) reported similar data with the human prostate enzyme.

The data presented here are consistent with these reports. Three lines of evidence suggest that MTA need not be degraded to exert its inhibitory effect on lymphocyte blastogenesis: 1) Neither adenine, MTR, nor MTR-1-phosphate, degradation products of MTA, are inhibitory to lymphocyte transformation; 2) The 7-deaza analog, MTT, is not degraded by the human lymphocyte phosphorylase, yet it is still a potent inhibitor of the blastogenesis process. It is possible, however, that MTT and MTA have different modes of action and/or that MTT is degraded in vivo by a heretofore undefined pathway; 3) Of the compounds which served as substrates for the lymphocyte MTA phosphorylase, MTA, iPTA and BTA had lower K_m's than did iBTA, BTA, or PTA. If these K_m values are compared to the I_{50} values obtained for these same compounds, the group of compounds which served as the better substrates (MTA, iPTA, ETA) was also found to be the least inhibitory to the transformation process, while the group (iBTA, BTA, PTA) which had the higher K_m values was found to be more inhibitory to lymphocyte blastogenesis. Although this is not an exact correlation, it does suggest that MTA and its analogs need not be degraded to inhibit lymphocyte transformation. The inability to obtain a direct correlation between K_m and I_{50} values may be due to other requirements for inhibition, such as the rate of transport of each of the compounds and the specificity of the analogs at the site of action.

Adenine has previously been shown to be an inhibitor of MTA phosphorylase activity in rat lung (5) and rat liver (23) extracts. That adenine is an inhibitor of the in vitro human lymphocyte enzyme activity, yet has no effect on lymphocyte transformation is not surprising since adenine is found only in very low levels in human tissues due to its rapid conversion to AMP via adenine phosphoribosyl transferase (APRT). Individuals deficient in APRT activity have been shown to accumulate elevated levels of adenine and 2,8-dihydroxyadenine (25) but any effect of this deficiency on MTA phosphorylase activity remains to be elucidated.

5'-Deoxy-5'-isobutylthioadenosine (SIBA) is a powerful antiproliferative drug shown to inhibit the growth of transformed mouse mammary cells (26), cell transformation induced by oncogenic RNA or DNA viruses (27,28), mitogen-induced lymphocyte blastogenesis (29), and the capping of herpes virus mRNA (30). SIBA was originally synthesized as an analog of S-adenosylhomocysteine (31). Zappia and co-workers (32), however, have suggested that this nucleoside more closely resembles MTA and found that human placenta MTA phosphorylase utilized SIBA as a substrate. Our data are consistent with the contention that SIBA is an MTA analog in that the human lymphocyte MTA phosphorylase can also utilize SIBA as a substrate. The pharmacological actions of this drug may therefore be ascribed to its structural similarity to MTA. Our

data indicating that MTA need not be degraded to inhibit cellular proliferation also suggest, by analogy, that SIBA also need not be further metabolized to exert its cytostatic effects. It is noteworthy, in this respect, that MTA, ETA, and MTT were all found to be powerful in vitro inhibitors of both spermidine synthase and spermine synthase from the rat ventral prostate (33). Recently, MTA, MTT, and SIBA were each shown to decrease the content of spermidine in virally transformed mouse fibroblast cells; cell growth, however, could not be restored by the exogenous addition of spermidine (34). This suggests that these nucleosides have other inhibitory actions in addition to that observed on polyamine biosynthesis. Interestingly, we have found that MTA is an irreversible inactivator of S-adenosylhomocysteine hydrolase (35), which is the key enzyme involved in the degradation of S-adenosylhomocysteine, a potent inhibitor of transmethylation reactions.

ACKNOWLEDGEMENTS

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THE COMPARATIVE EFFECTS OF 5'-METHYLTHIOADENOSINE AND SOME OF ITS ANALOGS ON CELLS CONTAINING, AND DEFICIENT IN, 5'-METHYLTHIOADENOSINE PHOSPHORYLASE

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ABSTRACT

The antiproliferative effects of 5'-methylthioadenosine and the 5'-methylthioadenosine analogs, 5'-isobutylthioadenosine, 5'-deoxyadenosine, and 5'-methylthiotubercidin were examined using two mouse cell lines, one 5'-methylthioadenosine phosphorylase deficient the other 5'-methylthioadenosine phosphorylase containing. All of the compounds were found to be growth inhibitory to both cell lines demonstrating that these compounds need not be degraded to exert their inhibitory effects. A correlation was observed between the potency of the growth inhibitory effect and the ability of the cells to degrade these compounds. 5'-Methylthioadenosine, 5'-deoxyadenosine and 5'-isobutylthioadenosine, all of which are substrates for the 5'-methylthioadenosine phosphorylase *in vitro*, were more growth inhibitory to the 5'-methylthioadenosine phosphorylase deficient cells than to the 5'-methylthioadenosine phosphorylase containing cells, whereas, the 7-deaza analog, 5'-methylthiotubercidin, a nondegradable inhibitor of the 5'-methylthioadenosine phosphorylase was a more potent inhibitor of the 5'-methylthioadenosine

phosphorylase containing cell line. Due to the inhibition by 5'-methylthiotubercidin on 5'-methylthioadenosine phosphorylase *in vitro* the disposition of cellularly synthesized 5'-methylthioadenosine was explored using both cell types. 5'-Methylthiotubercidin inhibited the accumulation of exogenous 5'-methylthioadenosine from 5'-methylthioadenosine phosphorylase deficient cells with no effect on intracellular 5'-methylthioadenosine. In contrast, 5'-methylthiotubercidin caused a large accumulation of extracellular 5'-methylthioadenosine with a concomitant smaller increase intracellularly in 5'-methylthioadenosine phosphorylase containing cells. That cellularly synthesized 5'-methylthioadenosine as well as the cellular excretion of this nucleoside are altered in response to treatment with 5'-methylthiotubercidin suggests two possible sites at which 5'-methylthiotubercidin may exert its effect.

INTRODUCTION

The ubiquitous nucleoside 5'-deoxy-5'-methylthioadenosine is synthesized from S-adenosylmethionine via several metabolic pathways, most notably during the synthesis of the polyamines spermidine and spermine (36). In mammalian cells, 5'-methylthioadenosine is rapidly degraded to 5-methylthioribose-1-phosphate and adenine by the action of 5'-methylthioadenosine phosphorylase (10). Adenine can reenter the purine nucleotide pool, whereas 5-methylthioribose-1-phosphate is an intermediate in a recycling pathway leading back into methionine (37,38).

Williams-Ashman et. al. (39) first suggested that 5'-methylthioadenosine may act in some situations as a regulator of enzyme systems. Subsequently, 5'-methylthioadenosine has been shown to inhibit the activity of several enzymes including spermidine and spermine synthases (40,41), S-adenosylhomocysteine hydrolase (35), and the methyl esterification of membrane proteins (42). This naturally occurring nucleoside has also been found to inhibit the growth and proliferation of mammalian cells. Ferro (36) first described the cytostatic action of 5'-methylthioadenosine to human lymphocyte cultures stimulated with mitogens, antigens, or allogeneic cells. More recently, 5'-methylthioadenosine was found to inhibit the growth of virally transformed mouse fibroblasts (34), murine lymphoid cells in culture (43), and baby hamster kidney cells (44).

It has been suggested that the mechanism by which 5'-methylthioadenosine exerts its inhibitory effect is through the production of one or both of its degradation products, adenine and/or 5-methylthioribose-1-phosphate (9). Supporting this hypothesis is the observation that 5'-methylthioadenosine supplementation of Sarcoma 180 cells creates an increase in the intracellular levels of adenine nucleotides, especially ATP (24). The accumulation of these nucleotides, therefore, may be responsible for the growth inhibitory effects noted with 5'-methylthioadenosine.

Conversely, it has also been suggested that 5'-methylthioadenosine need not be degraded to exert its inhibitory effect. Coward et al. (16) synthesized 5'-methylthiotubercidin, the 7-deaza analog of 5'-methylthioadenosine, and found it to be a potent non-degradeable inhibitor ($K_i = K_m$) of the 5'-methylthioadenosine phosphorylase. Ferro et al. (8) utilized 5'-methylthiotubercidin to show that it was more potent than 5'-methylthioadenosine in inhibiting human lymphocyte blastogenesis, suggesting that 5'-methylthioadenosine, and not a degradation product, was the true inhibitory agent. Pegg et al. (34) also suggested that 5'-methylthioadenosine need not be degraded to be inhibitory. More recently, White et al. (45) utilized several analogs of 5'-methylthioadenosine which serve as substrates for the phosphorylase to show that, in general, those

substrates with the highest K_m values were the most potent inhibitors of lymphocyte blastogenesis, whereas, the substrates that were least inhibitory were also the best substrates.

Therefore, to further investigate the nature of the cytostatic compound responsible for the 5'-methylthioadenosine-mediated inhibition of cell growth and proliferation, we have utilized a mouse leukemia cell line (L1210-D) first shown by Toohey (20) to be deficient in 5'-methylthioadenosine phosphorylase activity. In this communication, we present data confirming that these L1210-D cells cannot degrade 5'-methylthioadenosine or other analogs of 5'-methylthioadenosine which are substrates for the 5'-methylthioadenosine phosphorylase. That these compounds inhibit the growth of both 5'-methylthioadenosine phosphorylase containing and 5'-methylthioadenosine phosphorylase deficient cells, confirms that they need not be degraded to exert their inhibitory effect. Furthermore, we also present data showing that the addition of 5'-methylthiotubercidin to 5'-methylthioadenosine phosphorylase containing cells causes the export of 5'-methylthioadenosine from these cells.

MATERIALS AND METHODS

Cells and culture conditions

BW5147, obtained from the Salk Institute, is an oubain resistant T-lymphoma cell line from AKR mice. The cells were grown in non-agitated suspension cultures using Dulbecco's modified essential medium containing 4 mM glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin, and 10% horse serum. L1210-D a methylthio-requiring mouse leukemia cell line was supplied by Dr. J. I. Toohey. Eagles minimal essential medium supplemented with 1.1 mg/% pyruvate, 3 mg/% glutamine, 1×10^{-4} M cysteine-S-S-CH_3 and 10% calf serum was used to grow L1210-D cells in nonagitated suspension cultures. Both cell lines were routinely passaged every three days in T-25 tissue culture flasks containing 10 ml of medium. Cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere. Cell number and viability was determined by the exclusion of trypan blue on a Neubauer hemacytometer.

Compounds

5'-[^{14}C-methyl]methylthioadenosine was prepared by the procedure of Schlenk et al. (12) from S-adenosyl-L-[^{14}C-methyl]methionine (>50 Ci/mmole) which was obtained from Amersham Corp., Arlington Heights IL. 5'-methylthioadenosine, adenine and tubercidin were purchased from Sigma Chemical Co., St Louis, MO. 5'-deoxyadenosine was purchased from United States Biochemical

Co. 5'-deoxy-5'-methylthiotubercidin and 5'-deoxy-5'-isobutylthioadenosine were synthesized by established methods (14). [^{35}S]-Methionine was purchased from Amersham Corp., while 5-methylthioribose-1-phosphate was prepared by the method of Ferro et al. (23).

Preparation of cell extracts

Cells were harvested at a saturation of greater than 1 x 10^6 cells/ml and centrifuged. The cell pellet was washed in Dulbecco's phosphate buffered saline, recentrifuged and the cells were resuspended in standard buffer containing 0.05 M sodium HEPES, 0.05 M K_2HPO_4, 3 mM mercaptoethanol, 1 x 10^{-4} M EDTA and 10% glycerol at pH 7.2. Breakage of the cells was accomplished by freeze-thawing five times in liquid N_2. The broken cell suspension was centrifuged at 700 x g for 20 min and the resulting supernatant used as the source of enzyme in the 5'-methylthioadenosine phosphorylase assays.

5'-Methylthioadenosine phosphorylase assays

Two methods, using radioactively labeled or unlabeled substrates, to determine 5'-methylthioadenosine phosphorylase activity were used as previously reported (45) with two modifications. All standard assay mixtures were incubated at 37°C for 30 min and enzyme assays using 5'-deoxyadenosine as the substrate were immediately neutralized with 1 M KOH following acidification with trichloroacetic acid and centrifuged to remove

the resulting trichloroacetate salts. Radioactive profiles of the MTA phosphorylase reaction using BW5147 and L1210-D extracts were prepared by stopping the reaction with the addition of 95% ethanol (2:1). The reaction mixture, after centrifugation, was spotted on Whatman No. 1 paper and developed in butanol:ethanol:H_2O (52:32:16). Radioactive profiles were determined on a Packard model 7201 radiochromatogram scanner.

Measurement of 5'-methylthioadenosine in growth media and cell extracts

5'-Methylthioadenosine was measured in growth media and cells by HPLC using a Waters Model M-6000A system equipped with a reverse phase ubondapak C18 column and a Model 440 detector (254 nm) and operated at a flow rate of 1.0 ml per min. Two mobile phases were employed; solvent A, 15% methanol and solvent B, 10 mM potassium acetate, 10% acetonitrile with Waters paired ion complex Pic B7 at a pH of 4.0. Media samples were prepared by extraction with 2 M perchloric acid (1:1), centrifuged and neutralized with 2 M KOH. Cells were extracted with 0.5 N perchloric acid (0.1 ml of perchloric acid per 1 x 10^6 cells), cell debris was removed by centrifugation and the supernatant neutralized with 2 M KOH. The above supernatants were used for analysis by HPLC. 5'-Methylthioadenosine is separated from adenine, 5'-deoxyadenosine, 5'-methylthiotubercidin and 5'-isobutylthioadenosine using either mobile system and with

retention times of 39 min and 18 min for solvent systems A and B, respectively.

Labeling of cellular 5'-methylthioadenosine using [^{35}S]-methionine

Cells were prelabled in medium containing [^{35}S]-methionine at 4.5 mCi/mmole for 2-4 doublings. The cells were harvested by centrifugation and resuspended in fresh [^{35}S]-medium prepared as stated above and containing either 0, 50 or 500 uM 5'-methylthiotubercidin. After 48 hrs in the new medium the cells were centrifuged, and washed 2 times with Dulbecco's phosphate buffered saline. Cells and media samples were extracted with perchloric acid as described above and 5'-methylthioadenosine was added to the extracts to a final concentration of 7.5 uM as unlabeled carrier. The 5'-methylthioadenosine spiked samples were then injected on the HPLC. The 5'-methylthioadenosine peak was collected, dissolved in a scintillation cocktail containing 0.4% PPO in toluene-Triton X-100 (2:1) and the radioactivity determined on a Beckman model LS8000 scintillation counter.

RESULTS

5'-Methylthioadenosine phosphorylase activity

5'-Methylthioadenosine, 5'-isobutylthioadenosine, 5'-deoxyadenosine, and 5'-methylthiotubercidin were all tested as substrates for the 5'-methylthioadenosine phosphorylase in extracts prepared from each cell line. 5'-Methylthioadenosine, 5'-deoxyadenosine and 5'-isobutylthioadenosine were substrates for the 5'-methylthioadenosine phosphorylase from BW5147 cell extracts. Enzyme activities (pmoles/mg protein/min), measured using the HPLC method previously described (45), were determined to be 68, 35 and 25 for the three compounds 5'-methylthioadenosine, 5'-deoxyadenosine and 5'-isobutylthioadenosine, respectively. Parks and coworkers (9) have previously shown that 5'-deoxyadenosine is a substrate for 5'-methylthioadenosine phosphorylase in extracts prepared from Sarcoma 180 cells. L1210-D cell extracts, conversely, lacked any detectable enzyme activity regardless of the substrate. 5'-Methylthiotubercidin was not a substrate for 5'-methylthioadenosine phosphorylase when either BW5147 or L1210-D cell extracts were utilized.

It has been suggested that under some conditions 5'-methylthioadenosine phosphorylase may be induced in 5'-methylthioadenosine phosphorylase deficient cells (46). Therefore, to explore this possibility, BW5147 and L1210-D cells were grown in the presence of 250 uM 5'-methylthioadenosine for

24 hrs, and the cell extracts analyzed for 5'-methylthioadenosine phosphorylase activity (data not shown). Again, the L1210-D cell extract was found to be completely devoid of 5'-methylthioadenosine phosphorylase activity and no induction of enzyme activity was observed in either cell line.

Not only were enzyme assays performed, but the reaction mixtures containing [$^{14}CH_3$]5'-methylthioadenosine were also analyzed by radiochromatogram scanning (Fig. 7). Only in the reaction mixtures containing the BW5147 extracts was 5'-methylthioadenosine degraded; 5-methylthioribose-1-phosphate was the only product found. Neither 5-methylthioribose-1-phosphate nor any other product were found when L1210-D cell extracts were utilized in the reaction mixture. Since radioactively labeled 5'-deoxyadenosine, 5'-isobutylthioadenosine, and 5'-methylthiotubercidin were not available to us, we utilized high pressure liquid chromatography to analyze the reaction mixtures containing these substrates. In all cases, no enzymatic degradation of any of these compounds were found with the L1210-D extracts. Investigators are cautioned, however, when stopping reaction mixtures containing 5'-deoxyadenosine with acid. Despite rapid neutralization, the HPLC scans indicated a significant amount of nonenzymatic breakdown of 5'-deoxyadenosine to adenine.

The possibility also existed that inhibitors of the 5'-

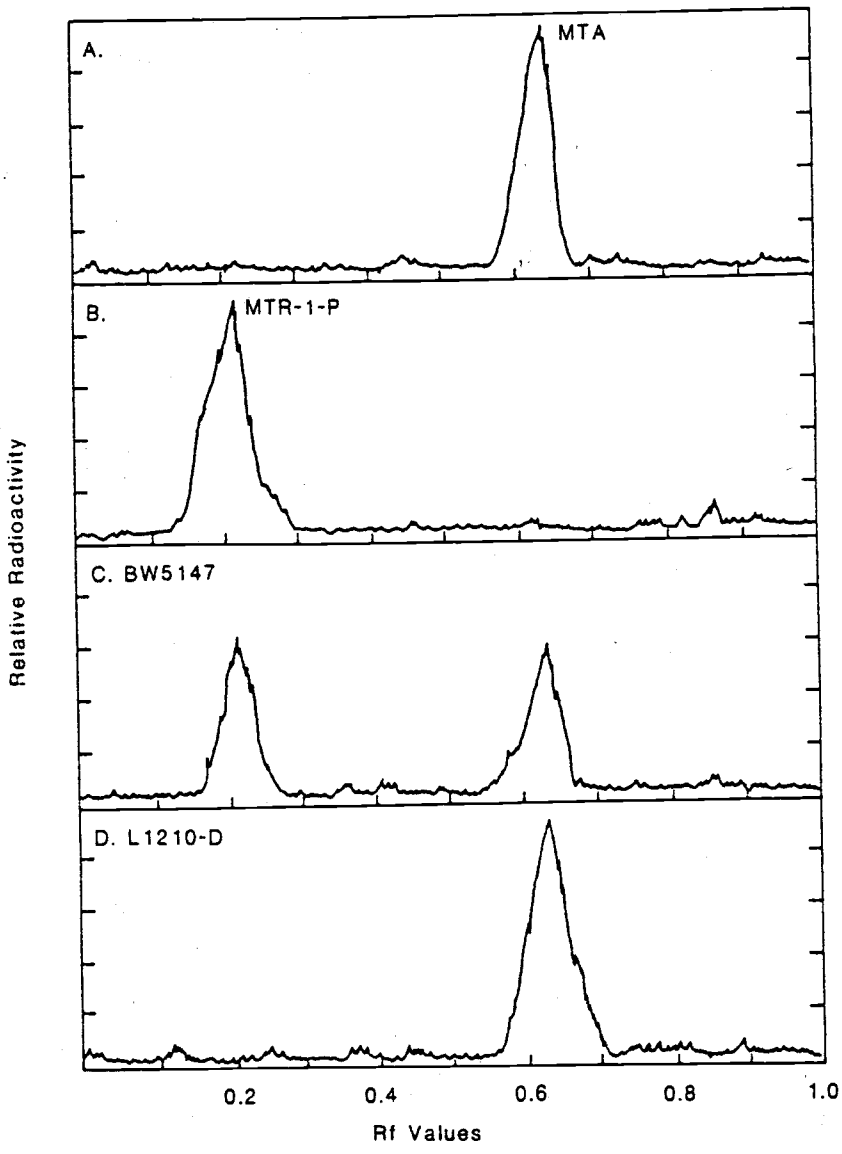

Fig. 7 Radioactivity profiles of paper chromatograms containing the products of the 5'-methylthioadenosine phosphorylase reaction using BW5147 and L1210-D cell extracts. Profiles presented represent (A) [$^{14}CH_3$]5'-methylthioadenosine, (B) [$^{14}CH_3$]5-methylthioribose-1-phosphate, (C) reaction mixture with BW5147 cell extract, (D) reaction mixture with L1210-D cell extract.

methylthioadenosine phosphorylase were released in L1210-D cells upon preparation of the crude cell extracts. Therefore, cell extracts from each cell line were mixed and 5'-methylthioadenosine phosphorylase activity measured. No decrease in BW5147 5'-methylthioadenosine phosphorylase activity was observed except that expected by simple dilution of the BW5147 cell extract (data not shown).

Disappearance of 5'-methylthioadenosine from growth media

That 5'-methylthioadenosine and its analogs do not serve as substrates in an *in vitro* assay for phosphorylase activity does not rule out the possibility of some *in vivo* degradation. To investigate this possibility, BW5147 and L1210-D cells were grown in media containing 100 uM 5'-methylthioadenosine. At various time intervals over 72 hr period, media samples were withdrawn and the decrease in 5'-methylthioadenosine measured by HPLC. Only in medium from BW5147 grown cells was the level of 5'-methylthioadenosine found to decrease (Fig. 8). 5'-Methylthioadenosine was depleted from the BW5147 medium at an approximate rate of 0.13 picomoles/cell/hr, to a final 26% decrease from the starting 5'-methylthioadenosine concentration. No measurable decrease in the concentration of 5'-methylthioadenosine was seen in medium from L1210-D grown cells over the entire 72 hr period.

The data demonstrate that BW5147 cells contain an active

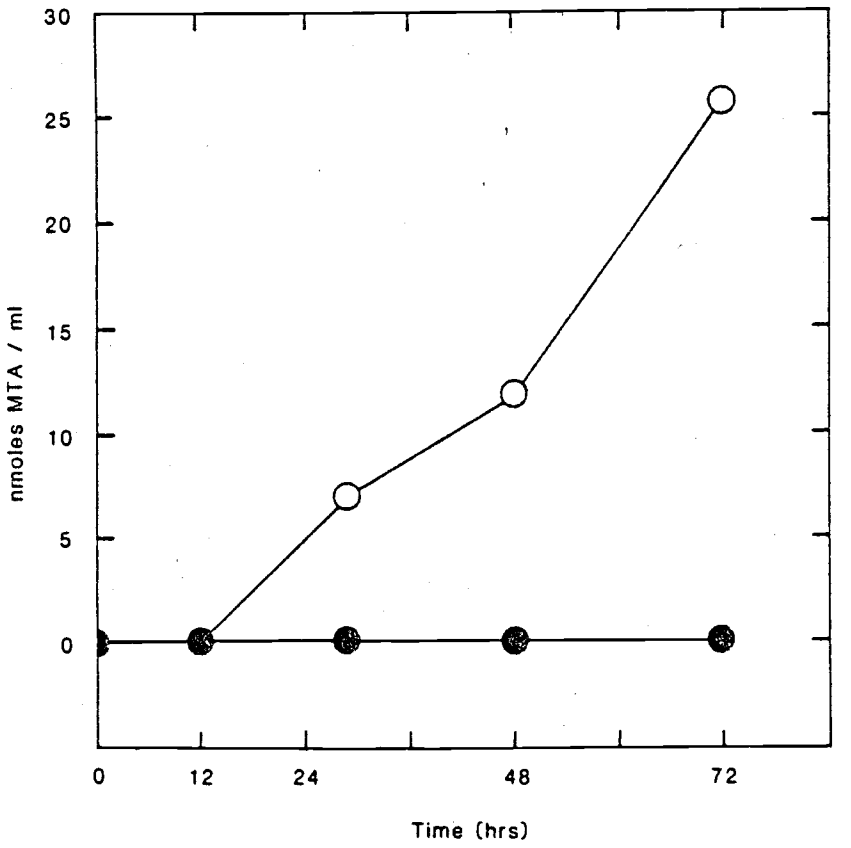

Fig. 8 Disappearance of 5'-methylthioadenosine from the growth medium of BW5147 and L1210-D cells. At various time intervals over a 72 hr period aliquots of medium containing either BW5147 (O) or L1210-D (●) cells were withdrawn and the decrease in the levels of 5'-methylthioadenosine in the medium was determined by HPLC analysis. The initial concentration of 5'-methylthioadenosine was 100 nmoles per ml of medium.

5'-methylthioadenosine phosphorylase which can utilize 5'-methylthioadenosine, 5'-isobutylthioadenosine, or 5'-deoxyadenosine as a substrate, but not 5'-methylthiotubercidin. L1210-D cells, on the other hand, are 5'-methylthioadenosine phosphorylase deficient and cannot utilize any of these compounds as substrates for the enzyme. The latter observation is in agreement with the data of Toohey (20).

Effect of 5'-methylthioadenosine, 5'-isobutylthioadenosine, 5'-dAdo and 5'-methylthiotubercidin on cell growth

The effect of 5'-methylthioadenosine and its analogs on the growth of BW5147 and L1210-D cells was followed over a 72 hr period. A typical response of these cells (at 100 uM) is illustrated in Fig. 9. Both the 5'-methylthioadenosine phosphorylase containing (BW5147) and 5'-methylthioadenosine phosphorylase deficient cells (L1210-D) were sensitive to growth inhibition by all of the compounds tested indicating that the compounds need not be degraded to be growth inhibitory. The further characterization of the effect of these nucleosides on cell growth is illustrated in Table 4 which shows the concentrations of the compounds which resulted in a 50% decrease in cell growth compared to controls without the inhibitors (I_{50}'s). Significant differences in potencies within the group of nucleosides tested and between the cell lines were apparent. The 5'-methylthioadenosine phosphorylase containing cells were most

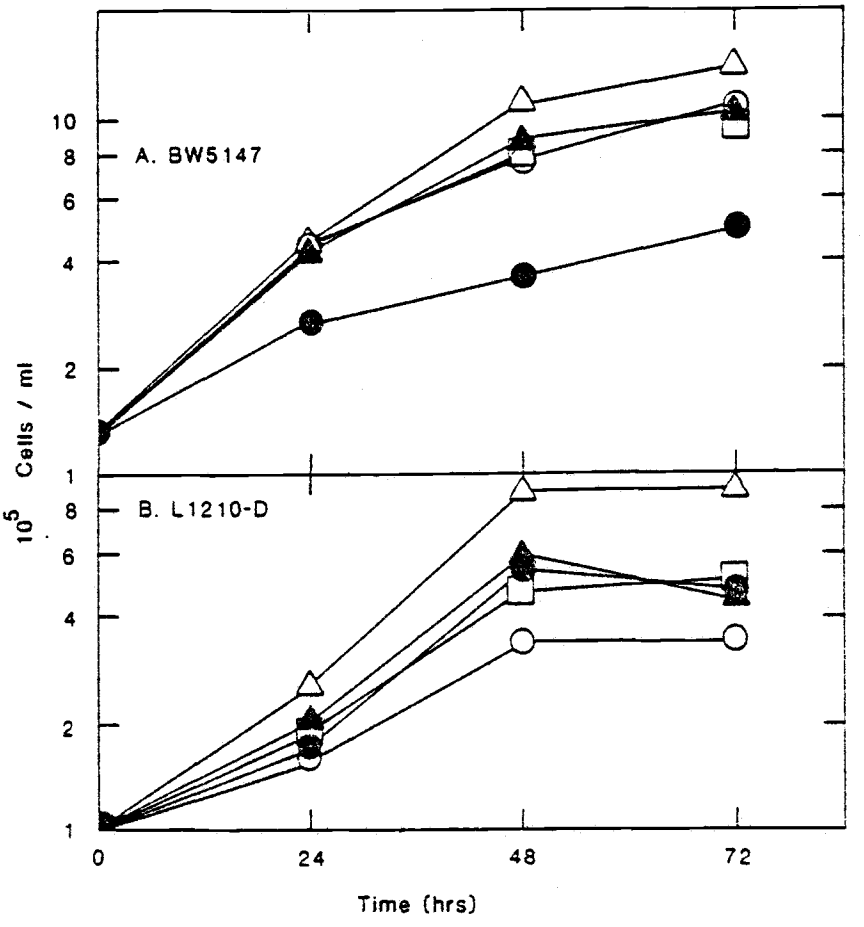

Fig. 9 Effects of 5'-methylthioadenosine, 5'-deoxyadenosine, 5'-isobutylthioadenosine and 5'-methylthiotubercidin on the cell proliferation of BW5147 (A) and L1210-D (B) cell lines. Log phase cells were inoculated (1 x 10^5 cells/ml) into normal medium (△) and media containing 100 uM 5'-methylthioadenosine (○), 5'-deoxyadenosine (▲), 5'-isobutylthioadenosine (□) and 5'-methylthiotubercidin (●). Cell number and viability were determined over a 3 day period.

Table 4 Effect of 5'-methylthioadenosine, 5'-isobutylthioadenosine, 5'-methylthiotubercidin and 5'-deoxyadenosine on the growth of BW5147 and L1210-D cells

The I_{50} values represent the concentration of each compound which resulted in a 50% decrease in cell growth as compared to controls. I_{50}'s were determined from complete dose response curves after 48 hrs of growth. The I_{50} presented are the average of three independent determinations. Individual determinations did not differ by more than 10% of the I_{50} value.

	I_{50} (uM)	
Compound	BW5147	L1210-D
5'-Methylthiotubercidin	<10	220
5'-Isobutylthioadenosine	250	100
5'-Methylthioadenosine	220	75
5'-Deoxyadenosine	450	187

sensitive to 5'-methylthiotubercidin, followed by 5-methylthioadenosine, 5'-isobutylthioadenosine, and 5'-deoxyadenosine, while the 5'-methylthioadenosine phosphorylase deficient cells were more inhibited by the parent compound, 5'-methylthioadenosine, followed in potency by 5'-isobutylthioadenosine, 5'-deoxyadenosine, and 5'-methylthiotubercidin. Those compounds which served as substrates for 5'-methylthioadenosine phosphorylase (5'-methylthioadenosine, 5'-isobutylthioadenosine, and 5'-deoxyadenosine) were all more inhibitory to the growth of the 5'-methylthioadenosine phosphorylase deficient cells (L1210-D) than to the 5'-methylthioadenosine phosphorylase containing cells (BW5147), whereas 5'-methylthiotubercidin, the non-degradeable analog of 5'-methylthioadenosine, was significantly more potent to the 5'-methylthioadenosine phosphorylase containing cells (I_{50} <10 uM) as compared to the 5'-methylthioadenosine phosphorylase deficient cells (I_{50} = 220 uM). The ability to degrade the nucleoside, therefore, renders the cells less sensitive to the growth inhibitory effect exerted by the compound.

Effect of 5'-methylthiotubercidin on 5'-methylthioadenosine levels

That 5'-methylthiotubercidin is not degraded by 5'-methylthioadenosine phosphorylase and is more inhibitory to the 5'-methylthioadenosine phosphorylase containing cells (BW5147)

than to 5'-methylthioadenosine phosphorylase deficient cells (L1210-D) suggest that this analog may be exerting its inhibitory effect by directly acting at the same site as 5'-methylthioadenosine and/or inhibiting the in vivo activity of 5'-methylthioadenosine phosphorylase, thus creating a pool of 5'-methylthioadenosine which results in a greater degree of inhibition. We, therefore, were interested in the effect of 5'-methylthiotubercidin on the normal disposition of 5'-methylthioadenosine. Both cells lines were pre-grown in medium containing [^{35}S]methionine for 2-4 doublings then transferred to fresh medium containing [^{35}S]methionine and either none, 50, or 500 uM 5'-methylthiotubercidin. After 48 hr the amount of [^{35}S]MTA which was excreted into the medium by the 5'-methylthioadenosine phosphorylase deficient cells was 48,000 cpm/ml (Table 5), whereas the medium from the 5'-methylthioadenosine phosphorylase containing grown cells contained only 1.25% this level. Media from 5'-methylthioadenosine phosphorylase deficient cells grown in the presence of 50 uM or 500 uM 5'-methylthiotubercidin contained decreasing quantities of 5'-methylthioadenosine, 15,650 cpm and 7,000 cpm, respectively. The opposite effect was observed with the 5'-methylthioadenosine phosphorylase containing cells; the presence of 5'-methylthiotubercidin resulted in an increase in the levels of 5'-methylthioadenosine in the media (1,730 cpm and 10,000 cpm at

Table 5 Effect of 5'-methylthiotubercidin on the disposition of cellularly synthesized 5'-methylthioadenosine

Cell and media extracts were prepared from [^{35}S]-methionine supplemented cultures containing none, 50 or 500 uM 5'-methylthiotubercidin. The values represent the amount of label over background found associated with the MTA isolated by HPLC.

Additions	Extracts	
	Cell (cpm per 1 x 10^7 cells)	Media (CPM per ml)
L1210-D cells		
5'-Methylthiotubercidin (uM)		
0	276	48,000
50	438	15,650
500	230	7,000
BW5147 cells		
5'-Methylthiotubercidin (uM)		
0	139	600
50	264	1,730
500	1,104	10,000

50 uM and 500 uM 5'-methylthiotubercidin, respectively). The concentration of endogenous 5'-methylthioadenosine in both cell types was low under all conditions. The only significant quantity of 5'-methylthioadenosine associated with the cell fraction (1,104 cpm/10^7 cells) was observed in BW5147 cells when grown in the presence of 500 uM 5'-methylthiotubercidin.

To assure that the [^{35}S]-label comigrating with unlabeled 5'-methylthioadenosine and collected by HPLC was not due to an overlapping [^{35}S]-labeled contaminant, fractions of complete HPLC runs were collected and counted. Two such scans with their corresponding radioactive histograms are shown in Fig. 10. 5'-Methylthioadenosine clearly separated from other ultraviolet-absorbing and [^{35}S]-labeled compounds. To further demonstrate that the [^{35}S]-labeled compound was 5'-methylthioadenosine, perchloric acid extracts were lyophilized, reconstituted in H_2O, and chromatographed by HPLC employing solvent A or solvent B. In each case, the ^{35}S -label cochromatographed with authentic 5'-methylthioadenosine.

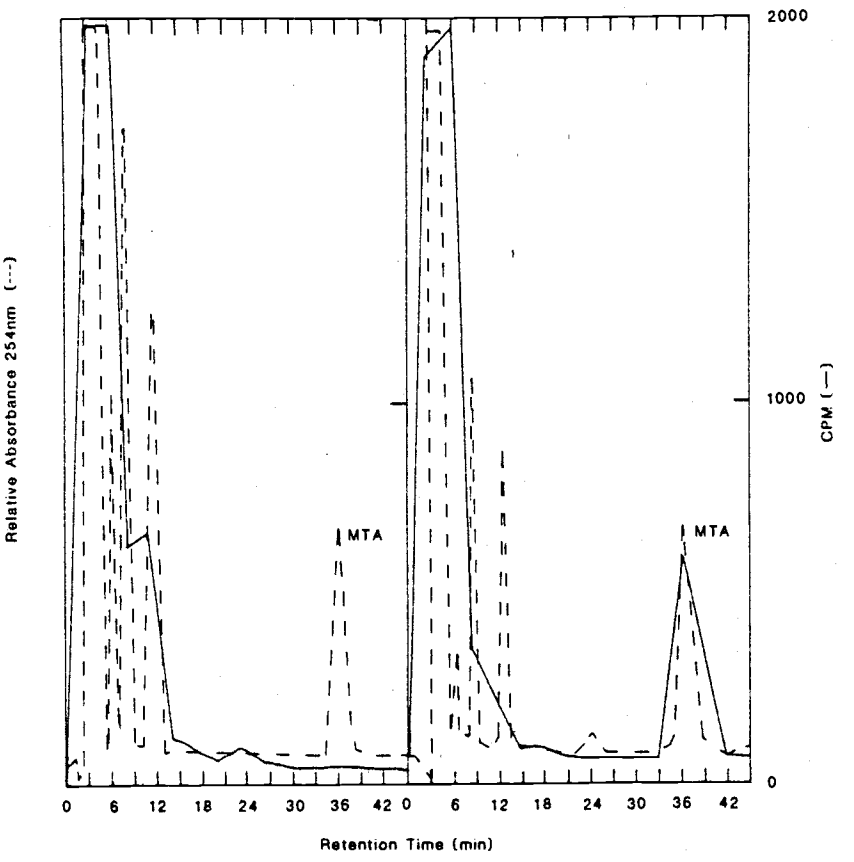

Fig. 10 HPLC (254 uM) and radioactivity profiles from [^{35}S]methionine supplemented media containing BW5147 or L1210-D cells. Media, without 5'-methylthiotubercidin, containing either BW5147 (left) or L1210-D (right) cells were prepared as described. Complete profiles at 254 nm were performed by HPLC, fractions collected (3 ml) and radioactivity determined. The U.V. absorbing peak for 5'-methylthioadenosine represents unlabeled 5'-methylthioadenosine spiked to a final concentration of 7.5 uM.

DISCUSSION

Toohey (20) found that some mammalian cells growing in culture lack 5'-methylthioadenosine phosphorylase activity. We have confirmed these observations and expanded them, to show that 5'-methylthioadenosine phosphorylase activity is also lacking under induction conditions (cells grown in the presence of 5'-methylthioadenosine) and that 5'-methylthioadenosine is not degraded via other metabolic pathways in these cells. In addition to 5'-methylthioadenosine, we have shown that these 5'-methylthioadenosine phosphorylase deficient cells (L1210-D) cannot degrade 5'-deoxyadenosine or 5'-isobutylthioadenosine. Further, when 5'-methylthioadenosine was added to the culture medium of L1210-D and BW5147 cells, only in the 5'-methylthioadenosine phosphorylase containing cells (BW5147) was the 5'-methylthioadenosine found to disappear from the medium, sugesting that 5'-methylthioadenosine is not metabolized in these 5'-methylthioadenosine phosphorylase deficient cells.

Our finding that 5'-methylthioadenosine, 5'-deoxyadenosine and 5'-isobutylthioadenosine, all shown to be substrates for the 5'-methylthioadenosine phosphorylase, inhibited the growth of the 5'-methylthioadenosine phosphorylase deficient cells, as well as the 5'-methylthioadenosine phosphorylase containing cells, confirms that these compounds need not be degraded to be inhibitory. That the 5'-methylthioadenosine phosphorylase deficient cells

were more sensitive to 5'-methylthioadenosine and 5'-deoxyadenosine than were 5'-methylthioadenosine phosphorylase containing cells is what would be expected if these compounds themselves and not their degradation products were the true inhibitory agents.

Schanchie et al. (47) has found that the exogenous supplementation of the 5'-methylthioadenosine analog, 5'-isobutylthioadenosine, caused the accumulation of both exogenous and endogenous 5'-methylthioadenosine in rat hepatocytes and suggested that 5'-isobutylthioadenosine, competed with intracellularly synthesized 5'-methylthioadenosine for the phosphorylase, thus inhibiting the degradation of 5'-methylthioadenosine. 5'-Methylthiotubercidin, the 7-deaza analog of 5'-methylthioadenosine, is a nondegradable competitive inhibitor of 5'-methylthioadenosine phosphorylase (16). Eloranta et al. (48) suggested that 5'-methylthiotubercidin does not perturb the metabolism of exogenously supplied 5'-methylthioadenosine and that the antiproliferative effects of 5'-methylthiotubercidin cannot be ascribed to a block at the phosphorylase and subsequent accumulation of 5'-methylthioadenosine. Contrary to this, we have found that 5'-methylthiotubercidin caused a large accumulation of exogenous 5'-methylthioadenosine, along with a smaller endogenous 5'-methylthioadenosine build up in 5'-methylthioadenosine phosphorylase containing cells. These fin-

dings are in accord with the data of Schanchie et al. (47) and suggest that like 5'-isobutylthioadenosine, 5'-methylthiotubercidin is inhibiting the in vivo degradation of 5'-methylthioadenosine. That 5'-methylthiotubercidin caused a build-up of endogenous 5'-methylthioadenosine in the 5'-methylthioadenosine phosphorylase containing cells but not the 5'-methylthioadenosine phosphorylase deficient cells, may account for the greater growth inhibition caused by this compound on the 5'-methylthioadenosine phosphorylase containing cell line.

The cellular export of 5'-methylthioadenosine in mammalian cell lines was first demonstrated by Kamatani and Carson (49) in human leukemic cells. We also have observed that the 5'-methylthioadenosine phosphorylase deficient cells export 5'-methylthioadenosine and in addition, that 5'-methylthioadenosine cannot be detected intracellularly. The rapid export of 5'-methylthioadenosine in these cells probably accounts for our inability to detect the disappearance of exogenously supplied 5'-methylthioadenosine. In the presence of 5'-methylthiotubercidin, however, the amount of 5'-methylthioadenosine excreted decreased dramatically in the phosphorylase deficient cells, yet even under these conditions 5'-methylthioadenosine was not found to accumulate endogenously. This suggests that 5'-methylthiotubercidin not only affects the catabolism of 5'-methylthioadenosine but also its biosynthesis.

Interestingly, the 5'-methylthioadenosine phosphorylase containing cell line, which normally does not export 5'-methylthioadenosine into the medium did so under the influence of 5'-methylthiotubercidin, indicating that the process of secretion of 5'-methylthioadenosine may be induced in cells which normally degrade 5'-methylthioadenosine once this degradation process has been blocked. Schanchie et al. (47) have indicated that S-adenosylhomocysteine is also secreted in cells where catabolism of this compound has been blocked. Whether the export of 5'-methylthioadenosine and S-adenosylhomocysteine is mediated by a common transport mechanism is unknown.

Pegg et al. (34) and Rajula and Raina (41) have reported that 5'-methylthioadenosine and 5'-methylthiotubercidin are inhibitors of spermidine and spermine synthase in vitro. In our study, L1210-D cells showed a marked inhibition of 5'-methylthioadenosine synthesis by 5'-methylthiotubercidin in vivo. These data support the hypothesis that inhibition of polyamine biosynthesis is the site of the antiproliferative action of 5'-methylthioadenosine. In contrast, Raina et al. (44) and Pegg et al. (34) have observed that the growth inhibition of mammalian cells by 5'-methylthioadenosine and 5'-methylthiotubercidin could not be reversed by the exogenous addition of spermidine and spermine. In addition, of the 5'-alkytubercidines tested by Raina et al. (44) only 5'-methyl-

thiotubercidin decreased cellular polyamine levels while all of the compounds were growth inhibitory. This suggests that another mechanism other than the inhibition of polyamine biosynthesis is responsible for the growth inhibitory effects. Ferro et al. (35) demonstrated that 5'-methylthioadenosine is a suicide inactivator of S-adenosylhomocysteine hydrolase in vitro whereas Zimmerman et al. (50) has observed that the 5'-methylthioadenosine structural analog, 5'-isobutylthioadenosine, is a competitive inhibitor of lymphocyte cyclic AMP phosphodiesterase. The possibility that the mode of action of 5'-methylthioadenosine is through inhibition of the S-adenosylhomocysteine hydrolase and/or cAMP metabolism deserves considerable attention.

Our study confirms the previous indirect evidence (34,43,45), that 5'-methylthioadenosine and its analogs need not be degraded to be inhibitory. This does not rule out additional effects caused by degration products, especially at high concentrations of these compounds, as has been observed by Savarese et al. (9). That the effect of 5'-methylthioadenosine and its analogs may have multiple sites of action has been suggested (51). Further, it is possible that the action at a particular site may be concentration dependent. The observation that mammalian cells in culture have more than one mechanism of removing 5'-methylthioadenosine indicates the importance the cell places on clearing this compound from the cellular environment.

THE SELECTIVE INHIBITION OF 5'-METHYLTHIOADENOSINE PHOSPHORYLASE DEFICIENT CELLS BY 5'-METHYLTHIOADENOSINE CHEMOTHERAPY AND METHIONINE DEPRIVATION

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ABSTRACT

The use of methotrexate (MTX)/5'-methylthioadenosine (MTA) combined chemotherapy to selectively inhibit MTA phosphorylase (MTAase) deficient cells was examined using growth media containing serum which varied in its level of MTAase activity. The chemotherapeutic strategy was successful when MTAase deficient ($MTAase^-$) cells were grown in medium containing horse serum which has no detectable MTAase activity. When serum containing MTAase activity was used as a component of growth medium, partial to complete loss of selective inhibition was observed. $MTAase^-$ cells grown in medium supplemented with calf serum were partially inhibited whereas no inhibition of cell growth was seen when cells were grown in a medium supplemented with fetal calf serum. Fetal calf serum contains two-folder higher MTAase activity than calf serum. Thus, the selective inhibition of $MTAase^-$ cells by the above strategy is successful provided there is no exogenous MTAase activity. MTX/MTA chemotherapy under methionine deprivation growth conditions was examined as an alternative strategy. Using this regime $MTAase^-$ cells were found to be selectively

inhibited despite high exogenous MTAase activity. Selective inhibition of MTAase$^-$ cells was methionine dependent; complete loss of growth inhibition was observed with the addition of 50 uM methionine. In addition, MTA chemotherapy under methionine deprivation successfully inhibited MTAase$^-$ cells with or without MTX. MTAase$^-$ cells grown in 1 uM methionine, 100 uM MTA and fetal calf serum medium were 99% inhibited after three days. The data suggest that MTAase$^-$ cells are unable to derive their methionine requirement from exogenous MTA and that providing MTA as a source of methionine can be used to selectively inhibit MTAase$^-$ cells regardless of the exogenous MTAase activity.

INTRODUCTION

Mammalian cell lines deficient in MTAase were first reported by Toohey (20). This enzyme catalyzes the degradation of MTA, which is synthesized from S-adenosylmethionine via several metabolic pathways (36). In mammalian cells, the majority of the MTA is produced concomitant during the synthesis of the polyamines spermidine and spermine (36). The removal of MTA by the cell is important since MTA has antiproliferative effects. Ferro (36) first described the cytostatic action of MTA on human lymphocyte cultures stimulated with mitogens, antigens or allogenic cells. More recently, MTA has been reported to inhibit the growth of several mammalian cell lines (11,34,44,52). The removal of MTA from the intracellular environment is accomplished either by excretion or degradation (53). MTAase catalyzes the phosphorolytic cleavage of MTA to 5-methylthioribose-1-phosphate and adenine (10). Adenine reenters the purine nucleotide pool through adenine-phosphoribosyl-transferase, whereas 5-methylthioribose-1-phosphate is an intermediate in a recycling pathway leading back into methionine (37). Since the first observation of a deficiency in MTAase, Kamatani et al. (55) reported that 7 out of 31 human malignant cell lines tested were deficient in MTAase. In addition, 5 of the 7 were human leukemic cell lines. Recently Kamatani et al. (55), have observed that leukemic cells from two patients afflicted with this disease were MTAase

deficient.

It has been proposed (54) that a chemotherapeutic strategy involving MTX and MTA could be used to selectively inhibit MTAase deficient ($MTAase^-$) cells. This strategy is based chiefly on the hypothesis that only MTAase containing ($MTAase^+$) cells can derive their purine requirement from MTA and, thereby, overcome the MTX block of de novo purine synthesis. Selective inhibition of MTAase deficient cells in culture, using the above regimen has been successful (54), however, as we will show, the selectivity of the above procedure is dependent on the cell culturing conditions. Further, we will propose two alternate chemotherapeutic strategies for the efficient selective inhibition of $MTAase^-$ cells.

MATERIALS AND METHODS

Chemicals

5'-[^{14}C-methyl]methylthioadenosine was prepared by the procedure of Schlenk et al. (12) from S-adenosyl-L-[^{14}C-methyl]methionine (>40 Ci/mmole) which was obtained from the Amersham Corp., Arlington Heights, IL. MTA was synthesized in our laboratory by established methods (13,14). Methotrexate, adenine, uridine and thymidine were purchased from Sigma Chemical Co., St. Louis, MO.

Cells and culture conditions

BW5147 (MTAase$^+$), obtained from the Salk Institute, is an oubain-resistant T-lymphoma cell line from AKR mice. The cells were maintained in non-agitated suspension cultures using Dulbecco's modified essential medium containing 4 mM glutamine, 100 units/ml penicillin, 100 ugm/ml streptomycin and 10% horse serum. L1210-D, (MTAase$^-$), a methylthio-requiring mouse leukemia cell line, was supplied by Dr. J. I. Toohey. Eagle's minimal essential medium supplemented with 1 mM sodium pyruvate, 1 x 10^{-4}M cysteine-S-S-CH_3 and 10% fetal calf serum was used to maintain L1210-D cells in nonagitated suspension cultures. Glutathione, a sulfhydryl reducing agent was added to a final concentration of 2 mM to all methotrexate growth experiments to stabilize MTAase *in vitro* (6,7). In all of the methotrexate growth experiments, BW5147 and L1210-D cells were cultured in

maintenance medium containing 0.5 uM methotrexate, 15 um uridine and thymidine with or without MTA (54). Methionine free medium was prepared using Gibco's MEM select-amine kit. Cell number and viability were determined by the exclusion of Trypan blue on a Neubauer hemacytometer.

MTA Phosphorylase assay

MTA phosphorylase activity was determined by the conversion of 5'-deoxy-5'-[methyl-^{14}C]methylthioadenosine to 5-deoxy-5-[methyl-^{14}C]methylthioribose-1-phosphate (8). Protein concentrations were determined as previously described (45).

Methionine levels in serum

Methionine levels in serum were determined by the HPLC separation of dansyl-methionine according to the procedure of O'Keefe and Warthesen (56). Serum samples were prepared by extraction with 1.5 N perchloric acid (1:1), centrifuged and neutralized with 2 N KOH. A standard solution of L-methionine 0.1 mg/ml treated identically as the samples was used for quantitation of methionine in serum. HPLC analysis was performed on a Waters Associates Model U6K injector 6000A solvent delivery system and 400 absorbance detector at 254 nm. A ubondapak C_{18} column (30 cm, 4 mm i.d.) and a mobile phase consisting of 25% acetonitrile and 0.01 M Na_2HPO_4 (pH 7.0) with a flow rate of 2 ml/min were used to separate dansyl-methionine from other interfering compounds. Methionine levels were determined by the com-

parison of measured peak heights to a standard curve run the same day. These curves were linear over a range of 50 to 500 picomoles of dansyl-methionine with r values typically greater than 0.99.

RESULTS

MTA rescue of MTAase$^-$ cells from MTX inhibition.

The ability of methylthioadenosine (MTA) to rescue cells from methotrexate (MTX) inhibition was examined using two murine cell lines, one containing MTAase (BW5147) and the other deficient in MTAase (L1210-D). Both cell lines were grown in a medium supplemented with 10% horse serum. Under these growth conditions, we found that only the MTAase$^+$ cell line was rescued from MTX inhibition by the addition of MTA. These results are similar to work done earlier (54) using human cell lines deficient in MTA phosphorylase and confirms the proposal that combined MTX and MTA therapy could be used to selectively inhibit MTAase$^-$ cells.

It has been observed by us and others (49) that some types of serum contain MTAase activity. We examined three sera used commonly in culturing mammalian cell lines for their MTAase activity. We found (Table 6) that while horse serum had no detectable activity, calf and fetal calf serum contained MTAase activity. Fetal calf serum had the highest activity, 2-fold higher than the activity found in calf serum.

The inhibition of MTAase$^-$ cells by combined MTX/MTA therapy is based on the inability of these cells to derive their purine requirement from MTA. Because of the high MTAase activity in some sera, we were interested in the selectivity of

Table 6. MTAase activity of various sera.

MTAase activity was determined as described in "Materials and Methods."

Serum	Enzyme Activity[a] (units x ml^{-1})
Horse	0
Calf	70
Fetal calf	140

[a] Units are expressed as picomoles product x min^{-1}.

this therapeutic scheme in growth media containing different types of serum. L1210-D cells (MTAase$^-$) were grown in media supplemented with 10% horse, calf or fetal calf serum and containing MTX. The ability of MTA to rescue cells from MTX inhibition was dependent on the MTAase activity of the serum used (Table 7). MTA was unable to rescue L1210-D cells grown in medium containing 10% horse serum while some rescue was seen in 10% fetal calf serum medium (39%) and complete rescue was observed in medium containing 10% fetal calf serum. In the absence of exogenous MTA the cells did not proliferate irrespective of the growth conditions.

MTA/MTX therapy in methionine deficient medium

MTA has been shown to be recyled to methionine by mammalian cells (37). We, therefore, examined the ability of MTA to rescue MTAase$^+$ and MTAase$^-$ cells from MTX inhibition in methionine deficient medium. Methionine levels of various sera were measured (Table 8) to determine the basal level of methionine in a methionine free medium containing 10% serum. Fetal calf serum was found to contain the highest level of free methionine (10 uM) of the sera tested. MTAase$^-$ and MTAase$^+$ cells were grown in methionine free medium containing either 10% horse serum (MTAase$^+$ cells) or 10% fetal calf serum (MTAase$^-$ cells). Under methionine deprivation the MTAase$^+$ cells were completely rescued from MTX inhibition by the addition of 15 uM MTA (Table 9).

Table 7. Ability of exogenous MTA to rescue MTAase$^-$ cells from MTX inhibition when grown in media containing horse, calf or fetal calf serum.

MTAase$^-$ cells (L1210-D) were cultured for 48 hrs in medium containing MTX/pyrimidine mixture with or without MTA and either 10% horse, calf or fetal calf serum as described in "Materials and Methods." Control cell numbers generally doubled 3-4 times in a 48 hr period.

Growth Condition	Growth (% of Control)[a,b]		
	Horse	Calf	Fetal Calf
MTX (0.5 uM)	0	0	38
MTX + MTA (15 uM)	0	39	152

[a]MTX and adenine (15 uM).

[b]$$\% \text{ of control} = \frac{T_F - T_i}{C_F - C_i} \times 100$$

T_i = initial cell number of the culture being tested

T_F = final cell number of the culture being tested

C_i = initial cell number of control.

C_F = final cell number of control.

Table 8. Methionine levels of serum.

Methionine levels in serum were determined by HPLC measurement of dansyl-methionine as described in "Materials and Methods."

Serum	Methionine Concentration (uM)
Horse	6.1
Calf	6.8
Fetal calf	10.0

Table 9. Ability of exogenous MTA to rescue MTAase$^+$ and MTAase$^-$ cells from MTX inhibition in methionine deficient medium.

MTAase$^+$ (BW5147) and MTAase$^-$ (L1210-D) cells were cultured for 48 hrs in methionine free medium containing MTX/prymidine mixture with or without MTA as described in "Materials and Methods." Horse serum at 10% was used to supplement the MTAase$^+$ cellular medium and 10% fetal calf serum was used with MTAase$^-$ cells.

Growth Condition	Growth (% of Control)[a,b] MTAase$^+$	MTAase$^-$
MTX (0.5 uM)	0	6
MTX + MTA (15 uM)	100	14

[a]MTX and adenine (15 uM).

[b]% of control was calculated as described in Table 2.

Conversely, MTAase$^-$ cells grew to only 15% of control in methionine deficient medium containing MTX and 15 uM MTA, indicating that MTA was unable to rescue MTAase$^-$ cells under methionine deprivation and MTX inhibition. In addition, the inability of MTA to rescue the MTAase$^-$ cells from MTX inhibition was methionine dependent (Table 10). MTAase$^-$ cells grown in medium containing only the basal level of methionine and the MTA/MTX combination grew to only 12% the control level, while cells grown in 50 uM methionine were completely rescued (148% of control) by MTA. The data indicate that while BW5147 cells (MTAase$^+$) can derive their methionine requirement from MTA, L1210-D cells (MTAase$^-$) are unable to use MTA as a source of this essential amino acid.

Selective inhibition of MTAase$^-$ cells by methionine deprivation

L1210-D cells (MTAase$^-$) are unable to use MTA as a source of methionine. Therefore, we devised a scheme for selective inhibition of these cells by methionine deprivation and MTA chemotherapy. L1210-D cells were grown in methionine free medium containing 10% fetal calf serum and either 0, 20 or 100 uM MTA. As Table 11 shows, L1210-D cells are inhibited 80% by methionine deprivation alone, while cells grown under methionine deprivation and 100 uM MTA are 99% inhibited. Thus the MTAase$^-$ cells cultured in fetal calf serum medium, which contains high MTAase activity, cannot be rescued by MTA utilizing this strategy.

Table 10. Dependence on methionine for MTA rescue for MTX inhibition in MTAase$^-$ cells.

MTAase$^-$ cells (L1210-D) were cultured for 72 hrs in methionine free medium containing MTX/pyrimidine mixture, MTA, 10% fetal calf serum and various concentrations of methionine.

Growth Condition	Growth (% of Control)[a,b]
MTX (0.5 uM)	0
MTX + MTA (15 uM) + Met (1 uM)[c]	12
MTX + MTA + Met (2 uM)	29
MTX + MTA + Met (5 uM)	52
MTX + MTA + Met (10 uM)	114
MTX + MTA + MET (50 uM)	148

[a] MTX, Adenine (15 uM) and Met (100 uM).

[b] % of control was calculated as described in Table 2.

[c] Represents basal level of methionine in medium containing 10% fetal calf serum.

Table 11. Inhibition of MTAase$^-$ cells by methionine deprivation and MTA chemotherapy.

MTAase$^-$ cells (L1210-D) were cultured for 72 hrs in methionine free medium with or without 100 uM methionine and either 0, 20 or 100 uM MTA. Fetal calf serum at 10% was used in all culture media.

Growth Conditions	Growth (% of Control)[a,b]
Met (101uM)	100
Met (1uM)[c]	20
Met (1uM)	
+ MTA (20uM)	20
+ MTA (100uM)	1
Met (101uM)	
+ MTA (20uM)	83
+ MTA (100uM)	45

[a] Met (101 uM).

[b] % of control was calculated as described in Table 2.

[c] Represents the basal level of methionine in medium containing 10% fetal calf serum.

Further, selective inhibition of MTAase$^-$ cells was accomplished without the addition of MTX.

DISCUSSION

Recent reports (20,49,54,55) indicate that the incidence of MTAase deficiencies occurring in malignant mammalian cells *in vitro* and *in vivo* may be more common than first realized. The potential for designing a chemotherapeutic treatment to select against these cells is significant. The first attempt at designing a chemotherapeutic strategy was based on MTAase$^-$ cells grown in a medium containing 10% horse serum (54). This choice was unfortunate because while efficient selectivity is obtained in horse serum medium, our data demonstrate that selectivity is lost when cells are grown in a medium containing exogenous MTAase activity. Additionally, our work with human serum has indicated that the MTAase activity would be intermediate between calf and fetal calf serum (pooled human sera MTAase activity, 98 picomoles x min^{-1} x ml^{-1}). The problem of selectivity using the above treatment has occurred in a cell culture system using 10% serum, greater loss of selectivity would be expected to occur in moving to a system with a higher concentration of serum. The use of a strategy which avoids the problem of serum MTAase activity would, therefore, be desirable.

An alternative approach to this problem is based on MTA's dual nutritional role. That MTA can serve as a source of purine has been established, however, MTA can also serve as a source of

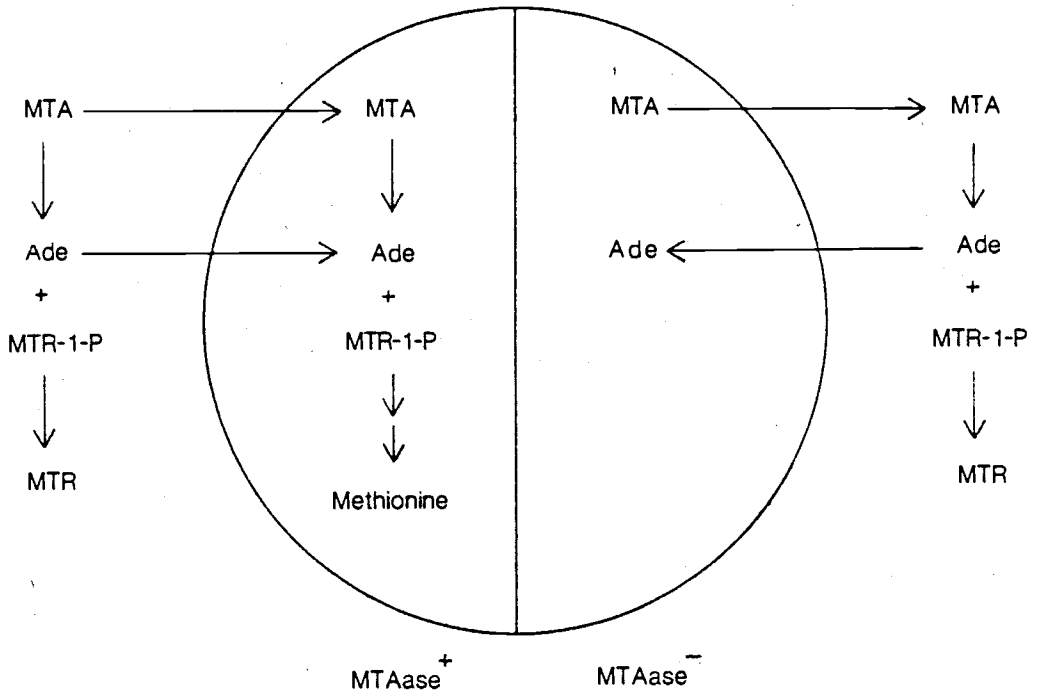

Figure 11. Metabolism of MTA in a cell culture system containing $MTAase^-$ or $MTAase^+$ cells and exogenous serum MTAase. Abbreviations: MTA, 5'-methylthioadenosine; ADE, adenine; MTR-1-P, 5-methylthioribose-1-phosphate; MTA, 5-methylthioribose.

methionine in MTAase$^+$ cells but not MTAase$^-$ cells (65). The metabolism of MTA in a cell culture system containing MTAase$^-$ or MTAase$^+$ cells and exogenous serum MTAase is depicted schematically in Fig. 11. A major difference is seen between MTAase$^+$ and MTAase$^-$ cells in the ability of MTAase$^+$ cells to generate intracellular 5-methylthioribose-1-phosphate, which can then be recycled into methionine. Our data show that MTAase$^-$ cells grown in methionine deficient medium are unable to use MTA as a methionine source suggesting that exogenously produced 5-methylthioribose-1-phosphate cannot be recycled to methionine, possibly due to the inability of cells to transport phosphorylated compounds. Previous studies in our laboratory have indicated that the phosphate moiety of exogenously produced 5-methylthioribose-1-phosphate is rapidly lost under cell culture conditions, and that the resulting 5-methylthioribose is unable to serve as a source of methionine in either MTAase$^+$ or MTAase$^-$ cells. Both cell types can recycle adenine into the purine pool and therefore, MTA can serve as a source of purine in either cell provided MTAase is present exogenously or endogenously.

Our findings show that MTAase$^-$ cells, despite the presence of exogenous MTAase, are growth inhibited when placed under methionine deprivation and are not rescued by the addition of MTA. In fact, the addition of 100 uM MTA causes a greater inhibition of MTAase$^-$ cell growth than methionine deprivation alone,

correlating well with MTA's known antiproliferative effects (34, 36,44,52). The opposite is observed for MTAase$^+$ cells, as they readily use MTA as a methionine source. Further, our data indicate that the above selective strategy is equally successful with or without the addition of methotrexate. Therefore, there may be no advantage in using methotrexate *in vivo*. However, the use of methotrexate in systems with low exogenous MTAase activity may improve the efficiency of selection.

The successful use of methionine deprivation and MTA chemotherapy to selectively inhibit MTAase$^-$ cells in culture does not assure success in the treatment of MTAase$^-$ tumors *in vivo*. However, several observations indicate that the above regimen may be a plausible approach. Edwards et al. (57) have reported that adult rats fed a sulfate free diet could utilize MTA as a source of methionine and upon feeding rats [^{35}S]-MTA, [^{35}S]-methionine was recovered in plasma and urine. In this report, we have used methionine deficiency as an integral part of our chemotherapeutic scheme *in vitro*. Dramatic reductions in human plasma methionine levels *in vivo* have been reported by Valle et al. (58) in the treatment of patients suffering the genetic disease homocystinuria. In human plasma, subnormal methionine levels were achieved using a nearly methionine free diet formulated by mixing protein-free Mead Johnson product 80056® and a Milner Labs. product Methionaid®. Additional studies are needed to improve our

understanding of the metabolism and effect of MTA in whole animals.

CONCLUSION

Our work with human lymphocyte MTAase suggests that this enzyme is typical of most mammalian MTAases examined (7,9,43,45). The apparent K_m for MTA was 26uM, and the enzyme exhibited a fairly strict substrate specificity. The enzyme demonstrated a considerable stability to acid pH and temperature extremes, with activity found at temperatures ranging from 4°C to 81°C and no loss of enzyme activity at a pH as low as 4.0 (personal observation). A correlation was demonstrated between the ability of MTA and several analogs to inhibit lymphocyte blastogenesis and their efficiency as MTAase substrates in vitro. Those analogs which were better MTAase substrates were also found to be the least inhibitory to lymphocyte blastogenesis, indicating that MTA or its analogs, and not a metabolic product of MTA was responsible for the inhibition of lymphocyte blastogenesis.

That MTA and not a metabolic product was responsible for the known antiproliferative effects was further demonstrated using two murine lymphoid cell lines, one $MTAase^+$ and the other $MTAase^-$. MTA and two analogs, which serve as alternate substrates for MTAase, were found to inhibit the growth of the $MTAase^-$ cell line. Further, the concentration of these compounds, which caused a 50% reduction in cell growth (I_{50}), was lower with the $MTAase^-$ cell line as compared to the $MTAase^+$ cell line. The ability of $MTAase^+$ cells to degrade these compounds

may, therefore, reduce their antiproliferative effects. An earlier report (49) indicated that MTAase$^-$ cells survive without an active MTAase by excreting MTA into the culture medium. Similarly, we found that our murine MTAase$^-$ cell line normally removes endogenously synthesized MTA by excretion. Interestingly, we observed that while the MTAase$^+$ cells do not normally excrete MTA, they do so under the influence of MTT, a potent inhibitor of MTAase. This suggests that both MTAase$^-$ and MTAase$^+$ cells contain the mechanism for excreting MTA.

The two murine cell lines, just described, were used to test several strategies for the selective inhibition of MTAase$^-$ cells based on the inability of these cells to degrade MTA. Our data indicated that a strategy utilizing MTA's ability to serve as a purine source was successful only if no exogenous MTAase activity was present in the culture medium. Since, horse serum was the only serum we tested which lacked MTAase activity (including human sera), an alternate strategy was proposed to avoid the problem of exogenous MTAase. This alternative regimen was based on the ability of MTA to serve as a methionine source. We found that the MTAase$^+$ cell line utilized MTA as source of methionine for growth and that the MTAase$^-$ cell line was unable to use MTA as a source of methionine despite high exogenous MTAase activity. We have now tested the human leukemic MTAase$^-$ cell line, CCRF-CEM, and found that it is also growth inhibited utilizing the above regimen (see Appendix).

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APPENDIX

Human MTA phosphorylase deficiency

Introduction

Very little is known concerning the incidence of MTA phosphorylase deficiency (MTAase$^-$) in human malignant cells. Only two reports have addressed this, and a total of 31 malignant cell lines (54) and 28 primary tumor samples (55) were examined. From this small sample 23% of malignant cell lines and 7% of primary tumor tissues have been found to be MTAase$^-$. These percentages increase when the survey is limited to leukemic tissue; 70% of human leukemic cell lines (5 of 7) and 10% of leukemic cells (2 of 20) from patients suffering this disease were found to be MTAase$^-$. The actual frequency of MTAase$^-$ in malignant tissues remains to be determined. However, whether the frequency is 70% or as low as 7% the potential for exploiting this deficiency to the detriment of MTAase$^-$ cells is significant. This appendix is intended to show that the chemotherapeutic strategy previously demonstrated to selectively inhibit a murine MTAase$^-$ cell line (L1210-D) also can be used to inhi-inhibit the growth of the human leukemic cell line, CCRF-CEM. In addition, the MTAase activity from 16 primary tumor tissues will be reported.

MATERIALS AND METHODS

Chemicals

5'-[^{14}C-methyl]methylthioadenosine was prepared by the procedure of Schlenk et al. (12) from S-adenosyl-L-[^{14}C-methyl]-methionine (>40 Ci/mmole) which was obtained from the Amersham Corp., Arlington Heights, IL. MTA was synthesized in our laboratory by established methods (13,14).

Cells and culture conditions

CCRF-CEM, obtained from Dr. C.K. Matthews, is a human T-lymphoblastoid cell line. The cells were maintained in suspension cultures using RPMI-1640 medium containing 100 units/ml penicillin, 100ugm/ml streptomycin and 10% fetal calf serum. Methionine free media was prepared using Gibco's MEM select-amine kit. A basal level of methionine (1uM), in the methionine deprivation experiments, was achieved by using methionine free medium containing 10% fetal calf serum. Our fetal calf serum contained 10uM free methionine (see Table 8). Cell number and viability were determined by the exclusion of Trypan blue on a Neubauer hemacytometer.

Primary tumor tissue extracts

Lymphocytes were prepared, from heparinized blood samples drawn from 11 leukemia patients, by density gradient centrifugation over Ficoll-Hypaque (19). The cells were washed, resuspended in minimal essential medium containing 10% fetal calf

serum and 10% DMSO and stored in liquid nitrogen. Solid tumor tissues from 5 patients were teased apart, the cells washed and stored as described above. All of the tumor samples were provided by Dr. John Fitchen, University of Oregon Health Science Center, Portland, Oregon. Cell extracts were prepared by first centrifuging the stored cell suspensions, resuspending the cells in MTAase buffer and freeze-thawing five times in liquid nitrogen. Cell debris was removed by centrifugation and the resulting supernatant used as the source of enzyme.

MTA phosphorylase (MTAase) assay

MTAase buffer contains 0.05M HEPES buffer (pH 7.2), 0.05M K_2HPO_4, 3mM mercaptoethanol and 5% DMSO. MTAase activity was determined by the conversion of 5'-deoxy-5'-[methyl-^{14}C]methylthioadenosine to 5-deoxy-5-[methyl-^{14}C]-methylthioribose-1-phosphate (8). Protein concentrations were determined as previously described (45).

RESULTS

MTAase of human malignant cells

Sixteen tumor samples were tested for MTAase activity (Table 12) and four samples were found to contain low or undetectable activity. These presumptive negative tissues consisted of one acute meylocytic leukemia, one acute lymphoblastic leukemia, one melanoma and one lung cancer. The viability of the 11 leukemia samples was examined by trypan blue exclusion and although the viability varied considerably no correlation was observed between viability and MTAase activity.

Inhibition of the human leukemic cell line, CCRF-CEM, by MTA chemotherapy and methionine deprivation

CCRF-CEM cells were grown in methionine free medium containing 10% fetal calf serum with or without 100uM methionine and either 0, 20 or 100uM MTA. As expected CCRF-CEM cells grew very poorly in methionine depleted medium (Figure 12). These cells were not rescued by the addition of MTA and in fact MTA caused a further inhibition of cell growth despite the exogenous MTAase activity present in fetal calf serum. As Table 13 demonstrates, CCRF-CEM cells were growth inhibited 64% (20uM MTA) and 66% (100uM MTA) under normal methionine concentrations (100uM). This suggests that the above regimen would be successful under incomplete methionine deprivation.

Table 12. MTA phosphorylase activity of human malignant cells

MTA phosphorylase assays were performed as described in "Material and Methods".

Cell extract	Tumor Type	Specific Activity (Picomoles x min^{-1} x mg^{-1})
GH-82	AML[1]	51
MG-82	AML	42
MB-82	AML	38
DP-83	AML	32
VC-82	AML	49
JC-82	AML	37
D-83	Lymphoma	37
TG-82	AML	48
HC-82-HJC	AML	50
G-82	Sarcoma	37
RS-82	AML	20
O-83	Melanoma	20
Cof-82	Lung cancer	5
SH-83	ALL[2]	4.5
C-82	Melanoma	0
JS-83	AML	0

[1]AML, acute myelocytic leukemia

[2]ALL, acute lymphoblastic leukemia

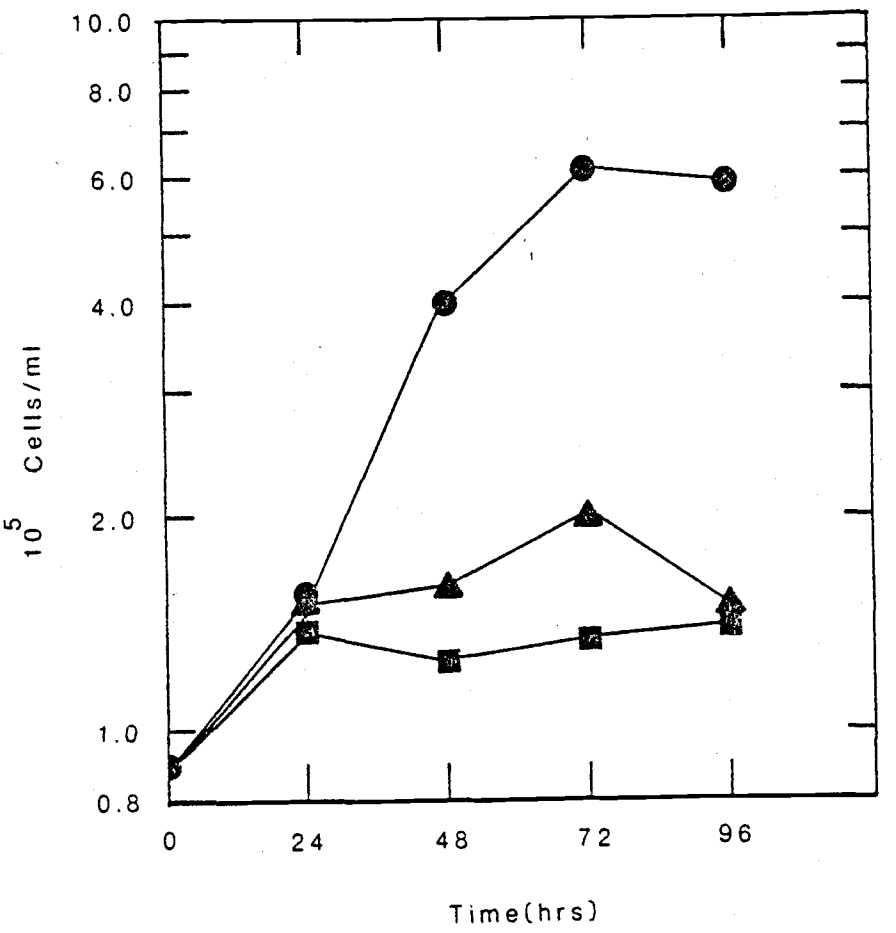

Figure 12. Inhibition of the human leukemic cell line, CCRF-CEM, by MTA chemotherapy and methionine deprivation. Log phase cells were washed with phosphate buffered saline and inoculated (1 x 10^5 cells/ml) into methionine free MEM, 10% fetal calf serum medium containing 101uM (●) or 1uM (▲) methionine or 1um methionine + 100uM MTA (■). Cell number and viability were determined over a 96 hr period.

Table 13. Growth inhibition of CCRF-CEM cells by MTA in the presence of 100 or 1 uM methionine

CCRF-CEM cells were cultured for 96 hrs in medium with none or with 100uM methionine (met) and either 0, 20, or 100 uM MTA. Fetal calf serum at 10% was used in all culture media.

Growth conditions	Growth (% of control)[a]
Met (101uM)	100
Met (1uM)	12
Met (1uM)	
+ MTA (20uM)	18
+ MTA (100uM)	12
Met (101uM)	
+ MTA (20uM)	38
+ MTA (100uM)	34

[a]% of control = 100 x (cells grown under the listed growth conditions/cells grown in medium containing 101uM methionine)

[b]Represents the basal level of methionine in methionine free medium containing 10% undialyzed fetal calf serum.

DISCUSSION

Growing evidence suggest that MTAase$^-$ malignant cells may be relatively common *in vivo*. The present study is the first to show a MTAase deficiency in an acute meylocytic leukemia or in a solid tumor. Thus far, 44 tumor tissues have been examined with the result of 6 tumors having low or undetectable MTAase activity. MTAase deficiency does not appear to be involved with any particular group of tumor tissues, as four different tumor types, both solid and fluid tumors, are among the 6 presumptive negatives. The types of tumors include; 3 acute lymphoblastic leukemias, 1 acute meylocytic leukemia, 1 melanoma, and 1 lung tumor. Several strategies (54,78) have been suggested for selective inhibition of MTAase$^-$ cells. We have demonstrated that MTA chemotherapy and methionine deprivation was successful against both murine and human MTAase$^-$ cells in culture and that the use of this regimen avoids the problem of exogenous MTAase activity.

Clearly further study of MTAase deficiencies in human malignancies are in order. A larger number of patients should be screened and a procedure for verifying presumptive negatives devised. In addition, the efficacy of the suggested chemotherapeutic strategies, developed in cell culture, should be examined with whole animals. Both of these concerns are currently being pursued by our laboratory in conjunction with Dr. John Fitchen's laboratory at the University of Oregon Health Science Center.