Effect of analogues of 5'-methylthioadenosine on cellular metabolism

Inactivation of S'-adenosylhomocysteine hydrolase by 5'-isobutylthioadenosine

Fulvio DELLA RAGIONE and Anthony E. PEGG
Department of Physiology, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, PA 17033, U.S.A.

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The effects of a number of nucleosides related to 5'-methylthioadenosine on the activities of S'-adenosylhomocysteine hydrolase, 5'-methylthioadenosine phosphorylase, spermidine synthase and spermine synthase were investigated. Both 5'-methylthioadenosine and 5'-isobutylthioadenosine gave rise to an enzyme-activated irreversible inhibition of S'-adenosylhomocysteine hydrolase, but 5'-methylthiotubercidin (5'-methythio-7-deaza-adenosine), 5'-deoxy-5'-chloroformycin, 5'-ethylthio-2-fluoroadenosine and 1,N6-etheno-5'-methylthioadenosine were totally ineffective in producing this inactivation. Of the nucleosides tested, only 5'-methylthioadenosine, 5'-methylthiotubercidin and 5'-isobutylthioadenosine were inhibitory towards the aminopropyltransferases responsible for the synthesis of spermine and spermidine. 5'-Methylthiotubercidin, 5'-deoxy-5'-chloroformycin and 5'-isobutylthioadenosine were inhibitors of the degradation of 5'-methylthioadenosine by 5'-methylthioadenosine phosphorylase, but only 5'-isobutylthioadenosine was also a substrate for this enzyme. These results suggest that the effects of 5'-isobutylthioadenosine of the cell may result from the combination of inhibitory actions on polyamine synthesis, 5'-methylthioadenosine degradation and S'-adenosylhomocysteine degradation. The resulting increased concentrations of S'-adenosylhomocysteine could bring about inhibition of methyltransferase reactions. A new convenient method for the assay of S'-adenosylhomocysteine hydrolase in the direction of synthesis is described.

AdoHcy is formed as a by-product in reactions involving methylation with S'-adenosylmethionine as a methyl donor (Cantoni, 1977). It is a powerful inhibitor of such reactions (Borchardt, 1980; Coward, 1981) and cellular methylation would be strongly inhibited if it were not rapidly removed. Such removal is brought about by AdoHcy hydrolase (EC 3.3.1.1), forming adenosine and homocysteine. Although this enzymic reaction is reversible and its equilibrium position favours synthesis of AdoHcy, the degradation is favoured in the cell because of the further metabolism of adenosine (de la Haba & Cantoni, 1959; Kredich & Hershfield, 1979; Duerre & Briske-Anderson, 1981).

Another nucleoside derived from S'-adenosylmethionine as a by-product of biosynthetic reactions is MTA. A number of reactions leading to MTA are known, but quantitatively the most important of these appears to be the synthesis of polyamines (Williams-Ashman et al., 1982; Kamatani & Carson, 1980). MTA is a very strong product inhibitor of the aminopropyltransferases responsible for polyamine production (Pajula & Raina, 1979; Hibasami et al., 1980), but its concentration in mammalian cells is very low because of metabolism by MTA phosphorlase (Pegg & Coward, 1981; Williams-Ashman, 1981; Williams-Ashman et al., 1982). This enzyme forms adenine and 5'-methylthioribose 1-phosphate, which are salvaged by conversion into adenine nucleotides and methionine respectively (Williams-Ashman et al., 1982; Backlund et al., 1982; Kamatani & Carson, 1981).

A considerable number of analogues of AdoHcy or MTA have been synthesized, and some of these compounds have quite potent pharmacological activity related to their abilities to serve as substrates or inhibitors of the enzymes involved in the synthesis or degradation of MTA and AdoHcy.
(Borchardt, 1980; Coward, 1981; Parks et al., 1981; Chiang et al., 1981; Robert-Géro et al., 1979). One of these compounds is SIBA, which has attracted particular attention because of its ability to inhibit a number of important processes including growth of protozoal parasites, viral replication, cell transformation by viruses and cell division (Robert-Géro et al., 1979; Bachrach et al., 1980). SIBA was originally synthesized by Lederer and colleagues as an analogue of AdoHcy, and it was found to inhibit a variety of methylases in vitro, albeit at very high concentrations (Enouf et al., 1979; Robert-Géro et al., 1979). However, SIBA is more closely related to MTA and is a substrate and competitive inhibitor of MTA phosphorylase (Carteni-Farina et al., 1979; Pegg et al., 1981; Parks et al., 1981; Williams-Ashman et al., 1982) and resembles MTA in having inhibitory activity towards aminopropyltransferases (Pegg et al., 1981; Hibasami et al., 1980, 1982) and cyclic AMP phosphodiesterases (Zimmerman et al., 1981). Such inhibitions occur at lower concentrations in vitro than inhibition of methylations and effects on polyamine metabolism can be demonstrated to occur in vivo (Pegg et al., 1981). Despite this, exposure of cells to SIBA also appears to lead to significant inhibition of methylation reactions (Vedel et al., 1978). This inhibition is surprising in view of the weak effect of SIBA in vitro. In the present work we have observed that SIBA leads to an irreversible inactivation of AdoHcy hydrolase. Such inhibition could therefore lead to a rise in cellular AdoHcy content and this could inhibit methylation indirectly. In contrast, it was found that MTT and 5'-deoxy-5'-chloroformycin, which are also inhibitors of MTA phosphorylase (Coward et al., 1977; Savarese et al., 1979, 1981), do not bring about direct inactivation of AdoHcy hydrolase. Comparisons of the effects of these drugs may therefore be valuable in assessing the relative importance of the various sites of action in their pharmacological effects. A new, particularly convenient method for the assay of AdoHcy hydrolase is also described.

Experimental

Materials

[2-3H]Adenosine (18 Ci/mmol) and S-adenosyl-L-[methyl-14C]methionine (57.3 Ci/mol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Labelled MTA and decarboxylated S-adenosylmethionine were prepared from S-adenosyl-L-[methyl-14C]methionine as described by Pegg et al. (1981). MTT was generously given by Dr. J. K. Coward, Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY, U.S.A. 5'-Deoxy-5'-chloroformycin, 5'-ethylythio-2-fluoro-adenosine and 1,N8-etheno-5'-methylthioadenosine were kindly given by Dr. H. G. Williams-Ashman, Ben May Laboratory for Cancer Research, University of Chicago, Chicago, IL, U.S.A. 2'-Deoxycoformycin was obtained from the Developmental Therapeutics Program of the National Cancer Institute. Cellex-P was purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Enzymes

S'-Adenosylhomocysteine hydrolase was prepared from rat liver by precipitation with (NH4)2SO4 as described by Kajander & Raina (1981), except that 15 mM-Tris/HCl, pH 7.4, containing 2 mM-mercaptopethanol was used as homogenization buffer. Proteins precipitated between 38 and 55% (NH4)2SO4 saturation were resuspended in 15 mM-Tris/HCl (pH 7.4)/2 mM-mercaptopethanol and dialysed for 24 h against the same buffer. Rabbit erythrocyte AdoHcy hydrolase was purchased from Sigma and was dialysed, before use, for 24 h against 15 mM-Tris/HCl (pH 7.4)/2 mM-mercaptopethanol. Rat liver MTA phosphorylase was prepared by the method of Ferro et al. (1979). Spermidine synthase and spermine synthase were purified from rat liver as previously described (Hibasami et al., 1980). The latter preparations were essentially free from MTA phosphorylase activity.

Assay of AdoHcy hydrolase activity

AdoHcy hydrolase was assayed in the direction of synthesis by following the formation of labelled AdoHcy from [2-3H]adenosine and L-homocysteine. The two labelled compounds were separated by ion-exchange chromatography on Cellex-P. For assay, 100 μl of incubation mixture containing 10 nmol of [2-3H]adenosine (30 c.p.m./pmol), 1 μmol of Tris/HCl, pH 7.4, 0.2 μmol of 2-mercaptoethanol, 0.5 μmol of L-homocysteine, 0.1 nmol of 2'-deoxycoformycin and the enzyme solution were incubated at 37°C for 10 min. The reaction was stopped by addition of 0.5 ml of 15 mM-HCl and then 0.4 ml of water was added. A sample (0.5 ml) was applied to a column (0.2 cm x 1 cm) of Cellex-P (H+ form) equilibrated with water. Adenosine was eluted with 10 μl of 10 mM-HCl, while AdoHcy was quantitatively retained. The latter compound was then eluted with 2 ml of 0.5M-HCl. The radioactivity present in the 0.5M-HCl eluate was assayed after addition of 10 ml of ACS II (Amersham Corp.). This method compares favourably with those previously reported in the literature involving t.l.c. (Hershfield, 1979) or paper discs (Kajander & Raina, 1981) for the separation of adenosine and AdoHcy. Those methods are more complicated and time-consuming and less suitable for the assay of a large number of samples. The
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Inactivation of AdoHcy hydrolase

A total volume of 0.25 ml containing 0.17 mg of enzyme, 2.5 μmol of Tris/HCl, pH 7.4, 0.5 μmol of 2-mercaptoethanol and the compounds indicated were incubated at 37°C. At various times, 5 μl samples were removed and added to 95 μl of the assay mixture for AdoHcy hydrolase activity, which was then measured as described above.

Assay of MTA phosphorylase and spermine synthase

These enzymes were assayed as previously described (Pegg et al., 1981). MTA phosphorylase activity was measured by following the production of 5-[methyl-14C]methylthioribose 1-phosphate from 5'-[methyl-14C]MTA. The incubation times were modified to obtain less than 10% of phosphorylase at all concentrations of MTA used, and all assays were conducted under conditions in which the reaction rate was linear with respect to time and enzyme concentration. Spermine synthase activity was measured by following the production of 5'-[methyl-14C]MTA from decarboxylated S-adenosyl[methyl-14C]methionine in the presence of spermidine (Hibasami et al., 1980). Spermidine synthase was assayed in the same way, but the spermidine was replaced with putrescine.

Results

Effects on AdoHcy hydrolase

When rat liver AdoHcy hydrolase was incubated with MTA or with SIBA, activity was lost, as shown in Fig. 1. The incubation and assay conditions were such that no MTA phosphorylase activity degrading these nucleosides was manifest, owing to the absence of phosphate and the removal of most of this activity during purification of the liver enzyme. Therefore the loss of AdoHcy hydrolase activity was not due to metabolites of MTA or SIBA. Also, the maximum amount of MTA or SIBA in the final assay mixture (25 μM) did not interfere with the reaction rate, indicating that these substances are only very weak direct inhibitors of the reaction. However, they were quite potent inactivators of the enzyme, with more than 80% of the activity being lost in a 20 min incubation (Fig. 1). This loss of activity appeared to be irreversible in that extensive dialysis (24 h against 2000 vol. of 15 mM-Tris/HCl/2 mM-2-mercaptoethanol, pH 7.4) did not restore the activity. By plotting the reciprocal of the inactivation rate against the reciprocal of the inhibitor concentration, K values of 0.047 μM for MTA and 0.042 μM for SIBA were calculated. The maximal rate of inactivation was calculated to be about 0.1 min⁻¹ for both the compounds, and the half-life of enzyme at infinite inhibitor concentration was 4.3 min in the presence of MTA and 4.9 min in the presence of SIBA.

The rat liver AdoHcy hydrolase could be protected against inactivation by SIBA or MTA by the presence of adenosine or AdoHcy, but not by homocysteine (Table 1). The first-order kinetics of inhibition, irreversibility and protection by substrates suggest that activity is lost by an enzyme-activated irreversible inhibition. No inactivation of rat liver AdoHcy hydrolase was produced by MTT, 1, N6-etheno-5'-methylthioadenosine, 5'-ethylthio-2-fluoroadenosine or 5'-deoxy-5'-chloroformycin when these nucleosides were preincubated with the enzyme solution at a concentration of 500 μM for 30 min at 37°C. The results obtained with the rat liver enzyme were confirmed with rabbit erythrocyte AdoHcy hydrolase (results not shown). Again, MTA and SIBA were powerful inactivators and the other compounds had no effect towards the enzyme from this source.

Effects on aminopropyltransferases

As shown in Fig. 2, 5'-deoxy-5'-chloroformycin,
Table 1. Effect of AdoHcy, adenosine or homocysteine on inactivation of AdoHcy hydrolase by MTA or SIBA

The AdoHcy hydrolase from rat liver was preincubated for 20 min with the inactivators and other compounds shown. Activity remaining was then assayed by removal of 5 µl portions to 95 µl of the standard assay mixture. When adenosine was added, 1 µM 2'-deoxycoformycin was added to prevent deamination by any adenosine deaminase present. This had no effect on the hydrolase.

<table>
<thead>
<tr>
<th>Inactivator</th>
<th>Compound added</th>
<th>AdoHcy hydrolase activity remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>Adenosine (20 µM)</td>
<td>95</td>
</tr>
<tr>
<td>None</td>
<td>AdoHcy (20 µM)</td>
<td>96</td>
</tr>
<tr>
<td>None</td>
<td>Homocysteine (2 mM)</td>
<td>102</td>
</tr>
<tr>
<td>MTA (100 µM)</td>
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<td>25</td>
</tr>
<tr>
<td>MTA (100 µM)</td>
<td>Adenosine (10 µM)</td>
<td>57</td>
</tr>
<tr>
<td>MTA (100 µM)</td>
<td>Adenosine (20 µM)</td>
<td>63</td>
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<tr>
<td>MTA (100 µM)</td>
<td>Homocysteine (2 mM)</td>
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<tr>
<td>SIBA (100 µM)</td>
<td>Adenosine (20 µM)</td>
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</tr>
<tr>
<td>SIBA (100 µM)</td>
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</tr>
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</table>

5'-ethylthio-2-fluoroadenosine and the 1,N6-etheno derivative of MTA had very little or no inhibitory action on the spermine synthase reaction, producing less than 30% inhibition at 200 µM concentrations. SIBA and MTT were inhibitors, as previously reported (Pegg et al., 1981; Raina et al., 1982; Hibasami et al., 1980), but are considerably less active than MTA itself (Fig. 2). Because of the complex kinetics of this enzyme, these results were expressed as the concentration needed to achieve a certain degree of inhibition of the enzyme assayed in the presence of 20 µM-decarboxylated S-adenosylmethionine and 0.5 mM-spermidine. A 50% inhibition was produced by 10 µM-MTA, 20 µM-
MTT or 160 μM-SIBA. Spermidine synthase was less strongly inhibited by these compounds than was spermine synthase, and more than 500 μM-SIBA was needed to achieve 50% inhibition, as previously reported by us (Pegg et al., 1981). More recently, Hibasami et al. (1982) have reported that both spermidine synthase and spermine synthase are inhibited by SIBA, but in their experiments 50% inhibition of spermidine synthase was produced by only 50 μM-SIBA. We have no clear explanation for this discrepancy, except for minor differences in the assay conditions.

Effects on MTA phosphorylase

SIBA was found to be a substrate for the rat liver MTA phosphorylase, in agreement with previous reports for the enzyme from human placenta (Carteni-Farina et al., 1979), sarcoma-180 cells (Savarese et al., 1979) and SV-3T3 fibroblasts (Pegg et al., 1981). SIBA was a competitive inhibitor of the decomposition of MTA by the rat liver enzyme, having a $K_i$ of 5.2 μM (result not shown). The $K_m$ for MTA in our experiments, which were carried out in 50 mM-phosphate, was 0.5 μM, in good agreement with the value of 0.47 μM for the rat liver enzyme reported by Ferro et al. (1979). Neither MTT nor 5'-deoxy-5'-chloroformycin were substrates for MTA phosphorylase, but, as shown in Figs. 3 and 4, they were competitive inhibitors, having $K_i$ values of 4.4 μM and 0.22 μM respectively. These results confirm the findings with enzyme from other sources (Coward et al., 1977; Pegg et al., 1981; Savarese et al., 1979, 1981; Parks et al., 1981) that these nucleosides may inhibit the degradation of MTA. As reported by Savarese et al. (1979, 1981), 5'-deoxy-5'-chloroformycin appears to be the most potent inhibitor, and our estimate of the $K_i$ for the liver enzyme is similar to the value of 0.3 μM obtained for the enzyme from sarcoma-180 cells. However, the $K_m$ for the enzyme from this source is reported to be 4 μM (Savarese et al., 1979, 1981), which is an order of magnitude greater than that for the liver enzyme.

Discussion

Although SIBA inhibits polyamine synthesis in vitro and in vivo (Pegg et al., 1981; Hibasami et al., 1982; Lawrence et al., 1982), its inhibitory effects on the growth of SV-3T3 cells are not reversed by the addition of polyamines (Pegg et al., 1981). This finding suggests that the cytostatic action arises from some other action, and although changes in cyclic nucleotide concentrations cannot be ruled out (Zimmerman et al., 1981; Schorderet-Slatkine et al., 1981), it has been believed that inhibition of cellular methylation reactions is responsible (Vedel et al., 1978; Robert-Géro et al., 1979). In fact, in several cell systems the incorporation of labelled methyl groups into nucleic acids appears to be strongly decreased by exposure to SIBA (Vedel et al., 1978). However, when SIBA was tested as an inhibitor of methyltransferases in vitro it was only weakly inhibitory (Enouf et al., 1979; Robert-Géro et al., 1979). The finding in the present study that SIBA produces an irreversible inhibition of rat liver or rabbit erythrocyte AdoHcy hydrolase provides an explanation for this discrepancy. The effect could be mediated by an increase in the intracellular concentration of AdoHcy, which is a powerful methyltransferase inhibitor (Borchardt, 1980; Coward, 1981). It is well documented that AdoHcy concentrations can be increased in the cell by exposure to irreversible inhibitors of AdoHcy hydrolase, such as 2'-deoxyadenosine (Hershfield & Kredich, 1980), 9-β-D-arabinofuranosyladenine (Helland & Ueland, 1982), periodate-oxidized adenosine (Hoffman, 1980) and carbocyclic 3-deaza-adenosine (Montgomery et al., 1982), or by raising the adenosine and homocysteine contents so that the AdoHcy hydrolase reaction favours synthesis (Kredich & Martin, 1977; Duerr & Briske-Anderson, 1981). These manipulations also inhibit cell growth and macromolecular methylation reactions.

Our experimental results with both rat liver and rabbit erythrocyte AdoHcy hydrolases indicate that MTA and SIBA have similar inactivating actions, producing rates of inactivation proportional to time. This confirms previous reports that the enzyme from human erythrocytes (Ferro et al., 1981) and human lymphoblasts (Fox et al., 1982) is inactivated by MTA. Our results indicate that SIBA is equally effective at bringing about this inhibition, and this inactivation is more likely to occur in vivo with the pharmacological doses of SIBA used than with the physiological concentrations of MTA, which are normally very low (Pegg & Coward, 1981; Williams-Ashman et al., 1982). Chiang et al. (1981) tested the effects of a large number of nucleosides on AdoHcy hydrolase from ox liver and concluded that the inhibition by SIBA was not linear with time of exposure. This discrepancy may be due to the different enzyme sources, since there was a striking difference in the response of the ox liver enzyme to adenine arabinoside (Chiang et al., 1981) compared with earlier studies with the enzyme from other sources (Hershfield, 1979).

The suicidal inhibition of AdoHcy hydrolase by MTA could be responsible for some of its anti-proliferative actions, but MTT and 5'-deoxy-5'-chloroformycin do not inhibit AdoHcy hydrolase directly and could act in this way only via their inhibition of MTA phosphorylase, which may raise the MTA concentration in the cells. The competitive inhibition of MTA phosphorylase by SIBA could also lead to increased concentrations of MTA.
However, it is important to note in this regard that the cellular membrane seems to be permeable to this nucleoside. An accumulation of MTA in the medium of cultures of cells lacking MTA phosphorylase has been reported (Kamatani & Carson, 1980), and a carrier-mediated transport mechanism for MTA has been described in human erythrocytes (Della Ragione et al., 1980). Thus at least three sites of action of SIBA in the cell are indicated by the present experiments. These are: direct inhibition of polyamine synthesis; increased concentrations of AdoHcy owing to inactivation of AdoHcy hydrolase; and possibly an increase in MTA, owing to inhibition of MTA phosphorylase.

Further studies of the effects on cellular polyamine, AdoHcy and MTA concentrations will be needed to evaluate these possibilities. Suitable methodology for measurement of AdoHcy and polyamines is available (Helland & Ueland, 1982; Pegg et al., 1981), but at present the lack of a very sensitive assay for MTA able to measure the intracellular concentrations in cultured cells, which are very low, is a major problem in this area. Development of a specific radioimmunoassay for this nucleoside (Pegg & Coward, 1981) may provide this methodology. The present work indicates the complexity of the action of substances such as SIBA, which may interfere with AdoHcy metabolism, methylations, polyamine synthesis, MTA metabolism and cyclic AMP metabolism. Such alterations could bring about profound changes in cellular adenine nucleotide and methionine content. The compound 5'-deoxy-5'-chlorouracil, which is a potent MTA phosphorylase inhibitor without any action on AdoHcy hydrolase or aminopropyltransferases, may be very useful to prevent the rapid intracellular degradation of SIBA or MTA.

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References


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