STUDIES ON THE METABOLISM OF 5'-ISOBUTYLTHIOADENOSINE (SIBA)

Phosphorolytic cleavage by methylthioadenosine phosphorylase

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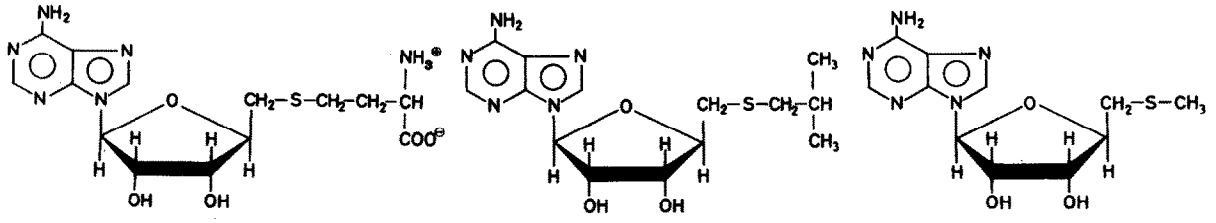
Received 13 June 1979

1. Introduction

5'-Isobutylthioadenosine, a synthetic analogue of S-adenosylhomocysteine [1], is a powerful antiproliferative drug. It has been reported to inhibit cell transformation induced by oncogenic RNA or DNA viruses [2,3], the growth of transformed mouse mammary cells [4], the mitogen-stimulated blastogenesis of lymphocytes [5] and the capping of herpes virus mRNA [6]. It has also been demonstrated that an antimalarial activity is exerted by SIBA against *Plasmodium falciparum* in cultures [7]. Evidences have been recently reported indicating a rapid degradation of SIBA in chick embryo fibroblasts, which has been attributed to S-adenosylhomocysteine hydrolase (EC 3.3.1.1) [8].

Despite the variety of effects exerted by SIBA at cellular level, the mechanism of action of the molecule is still largely obscure. It has been postulated that the antiproliferative action could be related to the inhibitory effect exerted by SIBA on tRNA methylases [9], on protein methylase I (EC 2.1.1.23) [9,10] and on SAH hydrolase [11]. These inhibition studies have been performed in view of a hypothesized similarity between SIBA and SAH [9] (see scheme 1).

In our opinion, however, the structure of SIBA resembles more 5'-methylthioadenosine, a natural product of S-adenosylmethionine metabolism, than



Abbreviations: MTA, 5'-methylthioadenosine; SIBA, 5'-isobutylthioadenosine; SAH, S-adenosylhomocysteine; MTI, 5'-methylthioinosine; SIBI, 5'-isobutylthioinosine; SAM, S-adenosylmethionine; MTR-1-phosphate, methylthioribose-1-phosphate

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SAH (see scheme 1). Therefore, the most probable targets for drug action are the enzymes involved in MTA metabolism.

The present study was undertaken to investigate the effect of SIBA on MTA phosphorylase (EC 2.4.2.1) which is the main enzyme involved in the catabolism of MTA in mammalian tissues [12–14].

2. Materials and methods

2.1. *Chemicals*

SAM was obtained from cultures of *Saccharomyces cerevisiae* [15] and isolated by ion-exchange chromatography [16]. S-Adenosyl-L-[Me-^{14}C]methionine was supplied by the Radiochemical Centre, Amersham, Bucks, UK; 5'-[Me-^{14}C]methylthioadenosine was prepared by acid hydrolysis of labelled SAM [17]. 5'-[Me-^{14}C]methylthioinosine was obtained from 5'-[Me-^{14}C]methylthioadenosine by enzymic deamination with non-specific adenosine deaminase from *Aspergillus oryzae* [16,18]. SIBA was supplied from Sefochem Fine Chemicals, Ltd., Emek, Hayarden, Israel; MTI and SIBI were prepared from MTA and SIBA, respectively, by enzymic deamination with a non-specific adenosine deaminase [16]. Adenine, SAH, hypoxanthine and dithiothreitol were supplied by Sigma Chemical Co., St. Louis, MO, USA. All other chemicals were the purest grade available from standard commercial sources.

2.2. *Biological sources*

Saccharomyces cerevisiae type 1 was furnished as dried yeast by Sigma Chemical Co. Human full term placentas were obtained from the Department of Obstetrics of Medical School, University of Naples.

2.3. *Enzymes*

MTA phosphorylase was purified from human full term placentas according to the method of Cacciapuoti et al. [12]. Non-specific adenosine deaminase from *A. oryzae* was purified from Sanzyme (Calbiochem, La Jolla, CA, USA) as described by Sharpless and Wolfenden [9]. Xanthine oxidase was purchased from Miles-Seravac, Kankakee, IL, USA.

2.4. *High performance liquid chromatography*

A Perkin Elmer liquid chromatograph, Model LC 65 T, equipped with an ultraviolet detector operating at 254 nm, was used. The column (25 X 4.6 mm I.D.) was prepacked with Partisil 10 SCX (Whatman). Integration was performed electronically using a Spectra Physic Minigrator. Injection was done via Model 70-10 sample injector valve and Model 70-10 loop filler part (Rheodyne Inc.).

Varying aliquots of a standard solution containing MTA, MTI, SIBA, SIBI, SAH, adenine and hypoxanthine were applied to a Partisil column equilibrated with ammonium formate buffer 0.5 M pH 4.0 using a Hamilton syringe (25–50 μl). The elution was carried out with the same solvent at room temperature and constant flow rate. Two different flow rates have been employed in order to separate the various investigated molecular species. As reported in fig.1 a good separation between adenine and SIBA or hypoxanthine and SIBI is achieved with a flow rate of 4 ml/min; to obtain a higher resolution between MTA, SAH and adenine or MTI and hypoxanthine a flow rate of 2 ml/min has been employed. Standard curves of MTA and its analogues and derivatives were constructed by comparing known amounts of standards to the peak areas produced by each compound. The curves appear linear over 50-fold amounts of standard. The sensitivity of this method allowed detection of amounts as low as 40 pmol. For quantitation of enzyme reaction mixtures, the changes in the peak areas of samples, compared to blanks, were determined and the obtained values were compared with standard curves.

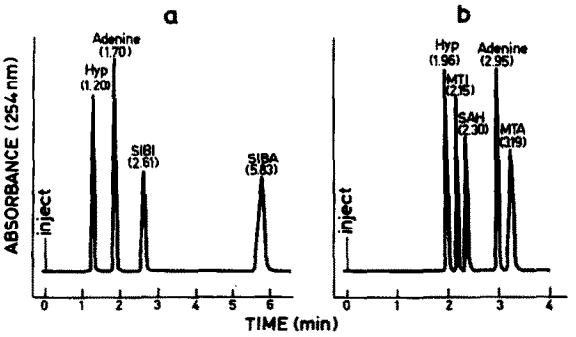

Fig.1.(a) HPLC separation of a mixture of hypoxanthine, adenine, 5'-isobutylthioinosine and 5'-isobutylthioadenosine. 20 μl of the mixture was injected onto a Partisil 10 SCX column at a flow rate of 4 ml/min. (b) HPLC separation of a mixture of hypoxanthine, 5'-methylthioinosine, S-adenosylhomocysteine, adenine and 5'-methylthioadenosine. 20 μl of the mixture was injected onto a Partisil 10 SCX column at a flow rate of 2 ml/min. The numbers within brackets refer to the retention times.

2.5. *Enzyme assay*

MTA phosphorylase activity was determined by measuring 5'-[Me-^{14}C]methylthioribose-1-phosphate obtained from labeled MTA [12]. The assay medium, unless otherwise stated, contained 20 μmol of potas-

sium phosphate pH 7.4, 0.15 μmol of 5′-[Me-^{14}C]-methylthioadenosine (74 mCi/μmol), 0.4 μmol of dithiothreitol and purified enzyme [12] in a total volume of 0.4 ml. The reaction was carried out at 37°C for 30 min, then terminated by the addition of 0.1 ml of 1 M trichloroacetic acid. The mixture was applied to a column (0.3 × 0.4 cm) of Dowex 50 (H^+ form) equilibrated with 0.2 M trichloroacetic acid: 5′-methylthioribose-1-phosphate is eluted with 2 ml of 0.2 M trichloroacetic acid. The cleavage of labeled MTI was followed by the above mentioned procedure, since its chromatographic behaviour on Dowex 50 (H^+ form) is similar to that of MTA. MTA phosphorylase activity was also determined by a second method with HPLC. This alternative assay procedure does not require labeled substrates like that previously described. An aliquot of 20 μl of the acidified reaction mixture was injected onto Partisil column. Figure 1 shows the detailed chronology of elution. The enzymatic cleavage of SIBA, SIBI and SAH was followed by the above procedure. Formation of adenine from SIBA and SAH was also measured by the increase in E_{305} in presence of the xanthine oxidase which converts adenine formed into 2,8-dihydroxyadenine [20].

3. Results

Preliminary experiments, not reported here, indicated an apparent inhibitory effect of SIBA on the phosphorolytic cleavage of MTA by MTA phosphorylase from human placenta ($I_{0.5}$ = 0.12 mM). Moreover when SIBA was assayed as substrate of MTA phosphorylase, a relevant cleavage of the molecule into adenine and isobutylthioribose-1-phosphate was observed. In fig.2 the effect is compared of various concentrations of MTA (fig.2a) and SIBA (fig.2b) on the reaction rate, at saturating levels of phosphate. The maximal rate of cleavage of MTA is observable at a substrate concentration of 0.25 mM. The double reciprocal transformation yielded a straight line (inset in fig.2a) from which an apparent K_m of 4.35 ± 0.3 × 10^{-5} M has been calculated. When SIBA was assayed as substrate (fig.2b) a maximal rate of cleavage was observed in presence of 0.11 mM thioether. From the double reciprocal plot in the inset an apparent K_m of 1.8 ± 0.2 × 10^{-5} M was calculated.

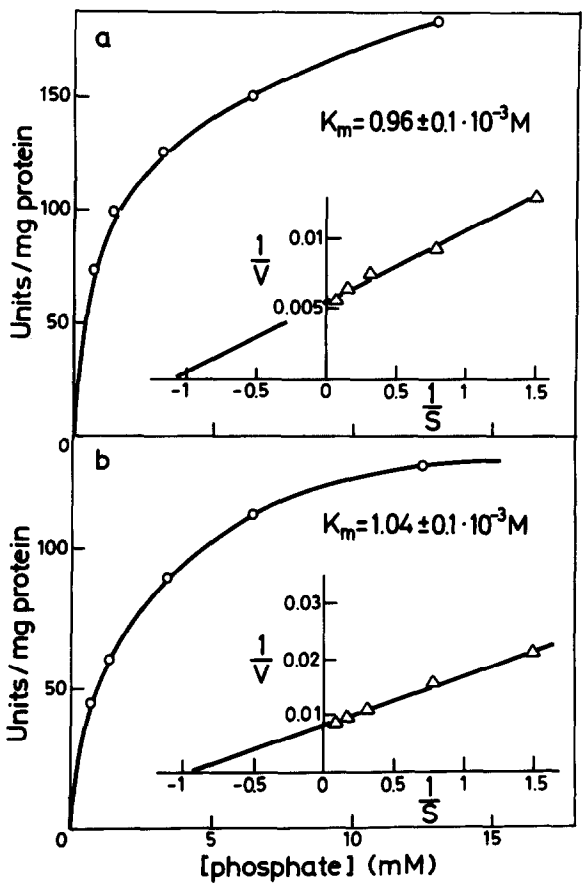

Fig.2.(a) Effect of 5′-methylthioadenosine concentration on MTA phosphorylase from human placenta. The assay was performed as indicated under Materials and methods except that MTA was added at the given concentrations. (b) Effect of 5′-isobutylthioadenosine concentration on MTA phosphorylase from human placenta. The assay was performed as indicated under Materials and methods except that 5′-isobutylthioadenosine was added at the given concentrations.

Data from Garbers [14] are indicative for a sequential mechanism of the reaction implying an ordered binding of MTA and phosphate to MTA phosphorylase. To investigate if the binding of SIBA influences the affinity of the second substrate the effect of phosphate concentration on the reaction rate at saturating levels of MTA and SIBA has been studied (fig.3). No activity is observable in the absence of phosphate. The apparent K_m values calculated from the double reciprocal plots in the inset do not differ significantly.

To investigate the relevance of the amino group of

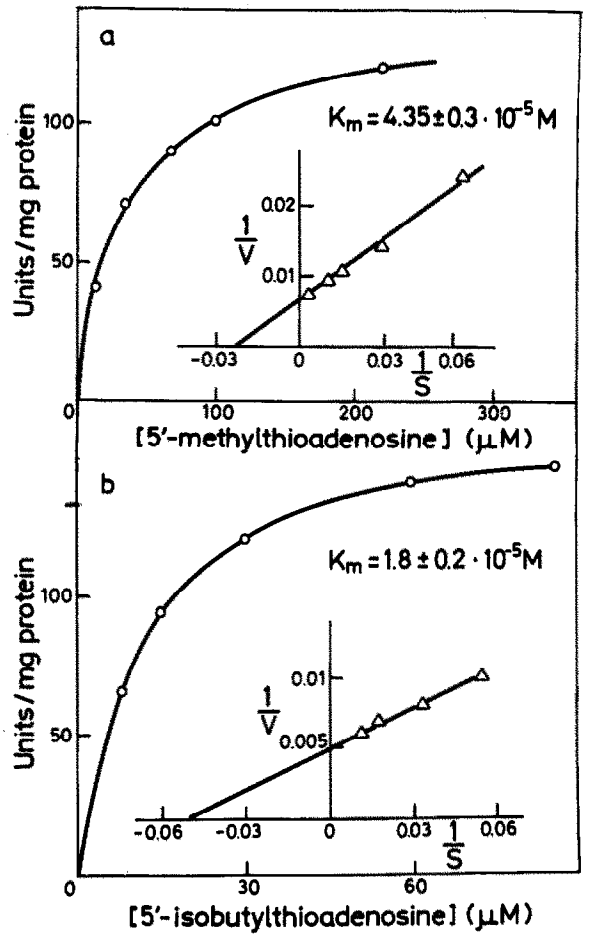

Fig.3.(a) Effect of the phosphate concentration on cleavage of 5'-methylthioadenosine by human placenta MTA phosphorylase. The assay was performed as indicated under Materials and methods except that phosphate was added at the given concentrations and the buffer was Tris–HCl 0.1 M pH 7.4. (b) Effect of phosphate concentration on cleavage of 5'-isobutylthioadenosine by human placenta MTA phosphorylase. The assay medium contained 0.15 μmol of 5'-isobutylthioadenosine, 0.4 μmol of dithiothreitol, 20 μmol of Tris–HCl, pH 7.4, and the purified enzyme (12) in a total volume of 0.4 ml. Potassium phosphate was added at the given concentrations. The assay was performed as indicated in Materials and methods.

SIBA on its binding to MTA phosphorylase, the deaminated analogues of SIBA and MTA have been assayed as substrates. The results reported in table 1 show that the replacement of the 6-amino group of SIBA and MTA by hydroxyl group results in 90% loss of activity.

Table 1
Specificity of 5'-methylthioadenosine phosphorylase

Substrate analogues	Enzyme activity (units/mg)	Relative activity (%)
5'-methylthioadenosine	125	100
5'-methylthioinosine	10.75	8.6
5'-isobutylthioadenosine	156	124
5'-isobutylthioinosine	11.36	9.1
S-adenosylhomocysteine	N.D.	–

Results are mean values of three separate experiments. The assays were performed as indicated under Materials and methods. N.D., not detectable

4. Discussion

The reported results indicate that SIBA is actively metabolized in mammalian tissues by MTA phosphorylase which converts the molecule into isobutylthioribose-1-phosphate and adenine. By comparing the two K_m values reported in fig.2 the affinity of the drug towards MTA phosphorylase is even higher than that of the natural substrate. It is interesting to note, in this respect, that the K_i of SIBA for tRNA methylases [2,9], protein methylase I [9,10] and SAH hydrolase [11] are significant higher when compared with the calculated K_m for MTA phosphorylase.

The cytostatic effect as well as the other pharmacological actions of the drug [2–7] could be therefore ascribed to the competition in vivo between SIBA and MTA. Moreover, the structural similarity of MTA and SIBA makes conceivable that the drug interferes also with the enzymes involved in MTA biosynthesis, i.e. S-adenosylmethionine lyase [21,22] and spermine/spermidine [23] or sym-nor-spermidine synthases [24,25].

It is noteworthy, in this respect, that the methylthio groups are probably involved in cell division [26,27] and evidences are reported in the literature [27], suggesting their origin from MTA according to the following equations:

$$\text{MTA} + \text{phosphate} \xrightarrow{\text{nucleosidase}} \text{MTR-1-phosphate} +$$

$$\text{adenine}$$

$$\text{MTR-1-phosphate} \xrightarrow{\text{methylthiolase}} \text{`}CH_3\text{-S'} +$$

$$[\text{ribose-1-phosphate}]$$

The data reported in this paper, indicating the formation of S-isobutylthioribose-1-phosphate from SIBA, suggest that SIBA could also interfere in the formation of methylthio groups essential for cell division.

With respect to the enzyme specificity, the results indicate the relevance of the adenine amino group of SIBA in the binding to the enzyme molecule. Moreover, the resistance of the deaminated analogue of SIBA to the enzymatic hydrolysis by MTA phosphorylase is paralleled by the lack of any cytostatic effects of this analogue [8].

Acknowledgements

The authors thank Mr A. Fusco for his skilled assistance in some of the kinetic experiments. This work was supported by a grant form the Consiglio Nazionale delle Ricerche, Rome, Italy; Project 'Control of Neoplastic Growth'.

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