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METHIONINE SYNTHESIS FROM 5′-METHYLTHIOADENOSINE BY TUMOUR CELLS

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Abstract—Incubation of cytosolic fractions of some tumour and normal cell lines with 5′-methylthioadenosine (5′-MTA) resulted in the formation of methionine. Methionine formation only occurred in those cell lines possessing 5′-MTA phosphorylase. The kinetics of product formation indicated that 5′-MTA was first rapidly converted into 5-methylthioribose-1-phosphate, followed by its slower conversion into methionine. Methionine synthesis from 5′-MTA was increased in cells previously incubated in methionine-depleted medium supplemented with 0.1 mM L-homocysteine for 24 hr. For most cell lines methionine synthesis from 5′-MTA was linear for only a short time period, and was followed by a first order decrease in the rate of methionine synthesis. Methionine synthesized from 5′-MTA was extensively incorporated into cellular macromolecules suggesting that 5′-MTA may substitute for methionine as a one-carbon source. This was confirmed by growth experiments which showed that low concentrations of 5′-MTA could partially substitute for methionine for some, but not all, cell lines. Higher concentrations of 5′-MTA were growth inhibitory. It may be possible to use 5′-MTA to selectively 'rescue' cells from methionine deprivation produced by the enzyme L-methioninase.

5′-Methylthioadenosine (5′-MTA) is produced in stoichiometric amounts in the conversion of S-adenosylmethionine into the polyamines spermidine and spermine as well as from a number of other reactions [1–3]. In normal mammalian cells 5′-MTA does not accumulate in significant amounts [4] since it is rapidly cleaved into adenine and 5-methylthioribose-1-phosphate by 5′-methylthioadenosine phosphorylase [5]. The adenine produced in this way contributes between 85 and 97% of *de novo* synthesis in cultured human lymphoblasts [6]. Certain malignant mammalian cells have no detectable 5′-methylthioadenosine phosphorylase activity and would be expected to accumulate 5′-MTA with a consequent decrease in adenine formation [7, 8].

A pathway resulting in the formation of methionine from 5′-MTA has recently been shown in cell-free homogenates in rat liver [9] and certain mammalian cells grown in culture [10]. The kinetics of product formation indicated that 5′-MTA was rapidly converted to 5-methylthioribose-1-phosphate, followed by its slower conversion to methionine. Such a pathway may be important to cells under conditions of methionine deficiency. A number of malignant mammalian cells have been shown to be unable to survive when L-methionine in the culture medium was replaced by L-homocysteine [11–13]. For one such methionine auxotroph (Walker carcinoma) there was no stimulation of growth under such conditions in the presence of 5′-MTA [13].

The present report investigates the ability of such tumours to form methionine from 5′-MTA as well as the effect of 5-methylthioadenosine on growth under conditions of methionine deprivation.

MATERIALS AND METHODS

S-Adenosyl-L-[methyl-^{14}C]methionine (sp. act. 61 mCi/mmole) and [methyl-^{3}H]thymidine (sp. act. 5.0 Ci/mmole) were purchased from the Radiochemical Centre, Amersham. 5′-[Methyl-^{14}C]methylthioadenosine was prepared from S-adenosyl-L-[methyl-^{14}C]methionine by acid hydrolysis as described by Parks and Schlenk [14]. 5′-Methylthioribose was prepared from 5′-MTA by acid hydrolysis and purified by the method of Schlenk *et al.* [15]. Methionine-free medium was obtained from GIBCO Europe Ltd. and was supplemented with 0.1 mM folate and 7.5 μM vitamine B_{12}. Methionine was removed from foetal calf serum (GIBCO) by dialysis.

Cell culture and assay conditions

Tumour cells were grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum under an atmosphere of 10% CO_2 in air. The cells utilized in this study were Walker rat carcinoma (W.256), TLX5 mouse lymphoma, a mouse bladder carcinoma (MB), a hyman chronic myeloid leukaemia (K562), L1210 murine lymphocytic leukaemia and two normal cell lines, a human embryonic lung (L132) and normal sternal bone marrow (D98). Cell growth studies were performed in multi-well dishes (GIBCO). L-Homocysteine was freshly prepared from the thiolactone (Sigma) before each assay. Cell counts were made daily with a Coulter electronic particle counter and growth curves were constructed.

For enzyme assays cells were sedimented by low speed centrifugation (300 *g*), washed with 0.9 NaCl and sonicated in 15 mM phosphate buffer, pH 7.0,

containing 1 mM $MgCl_2$. A supernatant fraction prepared by centrifugation (10,000 *g*) was incubated with 40 μM 5′-[methyl-^{14}C]MTA in a total volume of 105 μl. The mixture was incubated at 37° for the indicated times and the reaction was stopped by the addition of 2 volumes of methanol. The reaction products were separated by ascending chromatography on cellulose thin-layer plates (F_{254}, Merck, Darmstadt) in a solvent mixture of 1-butanol:acetic acid:water (60:15:25). 5′-MTA was detected by u.v. absorbance, amino acids were detected by spraying with ninhydrin and sulphur-containing compounds were detected by potassium iodoplatinate [16]. Radioactivity on the chromatograms was determined by scraping into scintillation vials, eluting with 0.1 N HCl, and counting in PCS scintillation fluid. Protein was determined by the method of Lowry *et al.* [17] using bovine serum albumin as a standard. 5′-MTA phosphorylase was determined by the method of Toohey [7].

Effect of 5′-methylthioadenosine on [methyl-^{3}H]-thymidine incorporation in the absence of methionine

Cells (5×10^5/ml) were suspended in methionine-depleted media containing either 0.1 mM L-homocysteine or in L-homocysteine-supplemented media containing the concentrations of 5′-MTA given in Table 1. After 24 hr incubation in such depleted media 1 ml portions of the cells were incubated with [methyl-^{3}H]thymidine (5 μCi/ml) and the incorporation of radioactivity into acid-insoluble material was determined as previously described [18]. Bone marrow was prepared from normal and leukaemic subjects as previously described [18]. Patients were from the Department of Haematology, St. Thomas' Hospital, London.

Incorporation of 5′-[methyl-^{3}H]thioadenosine into proteins and nucleic acids

Cells were incubated in Dulbecco's modified Eagle medium together with 0.25 μCi/ml of 5′-[methyl-^{3}H]MTA. At various times cells were removed by trypsinization and sedimented by centrifugation. The cell pellet was treated with 1 ml of ice-cold 0.5 M perchloric acid, and the precipitate was washed four times by resuspension and centrifugation in 1 ml of 0.5 M perchloric acid. An aliquot of the acid supernatant, after neutralization with 5 N KOH, was counted in PCS scintillation fluid (Hopkin and Williams) to determine the acid-soluble radioactivity. A nucleic acid-soluble fraction (DNA + RNA) was prepared by heating one-half the acid precipitate at 70° for 20 min in 1 ml of 1.0 M perchloric acid, cooling rapidly on ice, and centrifuging at 600 *g* for 10 min at 4°. The 70° perchlorate hydrolysis was repeated on the remaining residue and after neutralization of a portion (1.6 ml) of the combined supernatant the radioactivity was determined as above. The radioactivity in the residue was determined after dissolution in 1 N NaOH and neutralization with 1 N HCl while the concentration of protein was determined by the method of Lowry *et al.* [17] using bovine serum albumin as a standard. The other half of the acid precipitate was used for the determination of RNA and protein. After incubating for 15 hr at 37° in 0.5 ml of 0.5 M KOH the residue was cooled in

Table 1. Effect of 5′-MTA on the incorporation of [methyl-^{3}H]thymidine into acid-insoluble material under conditions of methionine deprivation

Patient	[Methyl-^{3}H]thymidine incorporation (% of control)*					
	0.1 mM L-Hcy + 0.1 mM		0.1 mM L-Hcy + 0.2 mM			3 μM Met‡
Culture conditions	0.1 mM L-Hcy†	5′-MTA	5′-MTA	3 μM Met‡	+ 0.1 mM MTA	
Normal	95	109	73	80	76	
AML	77	74	76	91	100	
ALL	59	69	77	69	87	
CML	68	59	64	—	—	
AML	70	79	97	91	106	

* Mean of three determinations which did not differ by more than 5%.
† L-Homocysteine.
‡ L-Methionine.

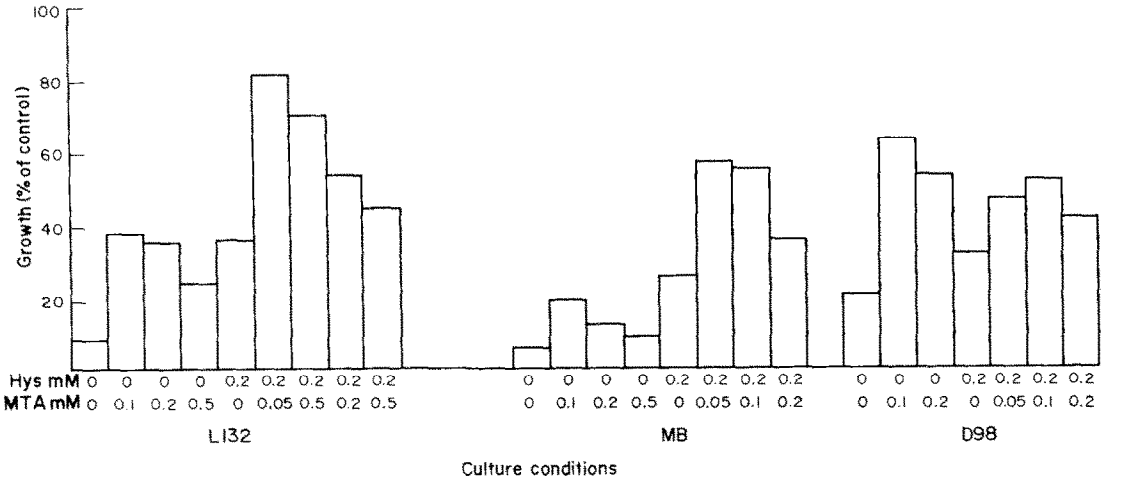

Fig. 1. Relative rate of growth of L132, MB and D98 in methionine-depleted medium containing L-homocysteine and/or 5′-MTA. Results are calculated from the linear part of the growth curves and are expressed as a percentage of a control culture growing in 0.3 mM L-methionine.

ice and 0.4 ml of 1 M perchloric acid was added. The supernatant fluid was neutralized and the radioactivity was determined as above.

RESULTS

Effect of 5′-MTA on growth

5′-MTA acted as a growth inhibitor towards all the cell lines used in this study. For most cell lines the ID_{50}s (dose required to produce 50% inhibition of growth) were similar (MB, 0.19 mM; L132, 0.22 mM; K562, 0.225 mM and D98, 0.3 mM) though W.256 was more sensitive to growth inhibition by 5′-MTA (ID_{50} 0.06 mM). The effects of 5′-MTA on growth of L132, MB and D98 under conditions of methionine deprivation are shown in Fig. 1. Growth rates are calculated from the linear part of the growth curves and are expressed as a percentage of a control culture growing in 0.2 mM L-methionine. In the absence of L methionine growth was minimal in all cell lines. Addition of 0.1 mM 5′-MTA caused a stimulation of growth in the three cell lines to a level similar to that seen by the substitution of 0.2 mM L-homocysteine. The stimulation of growth by 5′-MTA was greater in the two normal cell lines L132 and D98 than in the carcinoma MB. Increasing concentrations of 5′-MTA caused a reduction in the growth stimulation. A similar dose–response relationship was observed in the presence of 0.2 mM L-homocysteine. Growth of L132 was more responsive to stimulation by 5′-MTA than that of MB. Other cell lines such as W256 carcinoma, K562 leukaemia, L1210 leukaemia and TLX5 lymphoma showed no growth stimulation by 5′-MTA under conditions of L-methionine deprivation.

To investigate the ability of 5′-MTA to rescue human leukaemic marrow cells from methionine deprivation the incorporation of [methyl-^{3}H]thymidine into acid-insoluble material was used as a measure of proliferative activity. This technique was necessary because of the short *in vitro* lifetime of fresh marrow samples which precluded measurements on cell number. There was no effect of 5′-MTA on the uptake of [methyl-^{3}H]thymidine in either normal or methionine-depleted medium at the concentrations employed in the rescue experiments. In only one patient with acute lymphoblastic leukaemia (ALL) was there any evidence of stimulation of [methyl-^{3}H]thymidine incorporation into acid-insoluble material in the presence of 5′-MTA (Table 1). Normal bone marrow showed optimal incorporation in the presence of L-homocysteine alone.

Synthesis of [methyl-^{14}C]methionine from 5′-[methyl-^{14}C]methyl thioadenosine

When 5′-[methyl-^{14}C]MTA was incubated with a cytosolic preparation of various tumour cell lines it was metabolized rapidly to other radioactively labelled compounds. The data in Fig. 2 show a rapid disappearance of label corresponding to 5′-[methyl-^{14}C]MTA with a concordant rise in the level of 5-methylthioribose-1-phosphate. After 10 min incubation the peak of 5-methylthioribose-1-phosphate began to decrease and the peak corresponding to methionine began to increase. Confirmation that the labelled material was methionine was determined by addition of non-labelled methionine and crystallization from dilute ethanol in water. The labelled compound was found to crystallize with the methionine and the specific radioactivity was 606, 565 and 598 dpm/mg after three recrystallizations. The labelled material was found to co-chromatograph with authentic methionine on cellulose thin-layer sheets in butanol–acetic acid–water (60:15:25), butanol–acetic acid–water (4:1:1) and methanol–water–pyridine (20:5:1). The specific activity of 5′-MTA phosphorylase and methionine formation from 5′-MTA calculated from the linear part of the time course of the cell lines under study is shown in Table 2. Only those cell lines with the ability to cleave 5′-MTA by phosphorylase could synthesize methionine from 5′-MTA.

Methionine synthesis from 5′-MTA was increased in cells when L-homocysteine substituted for L-meth-

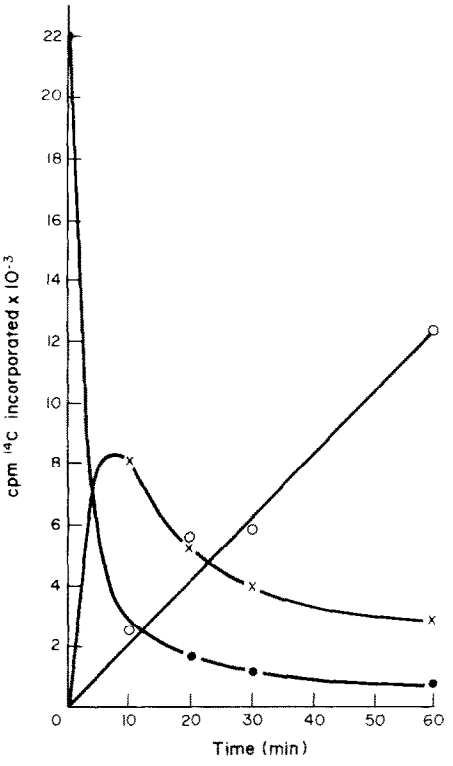

Fig. 2. Kinetics of product formation from 5′-[methyl-^{14}C]MTA by a cytosolic fraction of a mouse bladder carcinoma (MB). The assay mix was incubated at 37° as described in Materials and Methods and at various times the reaction was stopped by the addition of methanol. Portions of the reaction mix (25 μl) were chromatographed on cellulose tlc and the area of the chromatogram corresponding to standard 5′-methylthioadenosine (●—●), 5′-methylthioribose-1-phosphate (×—×) and methionine (○—○) were scraped off and the radioactivity for each was determined as in Materials and Methods.

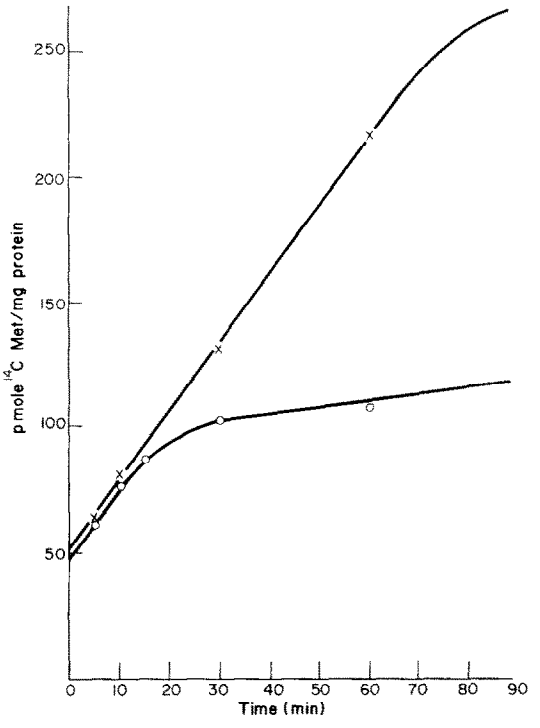

Fig. 3. Formation of methionine by cytosolic fractions of W.526 (○—○) and TLX5 lymphoma (×—×).

ionine for 24 hr (Table 2). This suggests an induction of methionine synthesis by this pathway to counteract the methionine deficit. A study of the time course of methionine synthesis from 5′-MTA by most cell lines (Fig. 3) showed a decrease in methionine synthesis with increasing time, which was first order (Fig. 4). The results in Table 3 show a greater decrease in the specific activity of methionine formation in cultures previously incubated for 24 hr in the absence of L-methionine, but in the presence of L-homocysteine. This suggests an incorporation of the labelled methionine into cellular macromolecules.

Incorporation of ^{3}H of 5′-[methyl-^{3}H]MTA into cellular macromolecules

Extracellular 5′-[methyl-^{3}H]MTA is taken up by cells and extensively incorporated into cellular macromolecules. The results in Fig. 5 show incorporation into DNA, RNA and protein of cell lines L132 and MB when they were incubated in methionine-free medium containing 0.05 mM MTA.

Table 2. Activity of 5′-MTA phosphorylase and rate of methionine formation from 5′-MTA by normal and tumour cell lines

Cell line	5′-MTA phosphorylase (nmole/hr mg protein ± S.E.M.)	Methionine formation from 5′-MTA (pmole/min/mg protein ± S.E.M.*) Normal media	Methionine-free medium + 0.1 mM L-Hcy†
K562	<10	0	0
L1210	<10	0	0
TLX5	22 ± 2	4.5 ± 0.5	NT‡
W256	36 ± 4	5.3 ± 0.4	13.8 ± 1.2
L132	43 ± 4	9.6 ± 0.6	11.6 ± 1.4
MB	70 ± 5	40 ± 5.0	85 ± 3.8
EJ	NT‡	14.8 ± 0.8	NT‡

* Rates of methionine formation were calculated from the linear part of the time course.

† Cells were *incubated* for 24 hr in Dulbecco's Eagle medium without methionine but containing 0.1 mM L-homocysteine.

‡ NT = not tested.

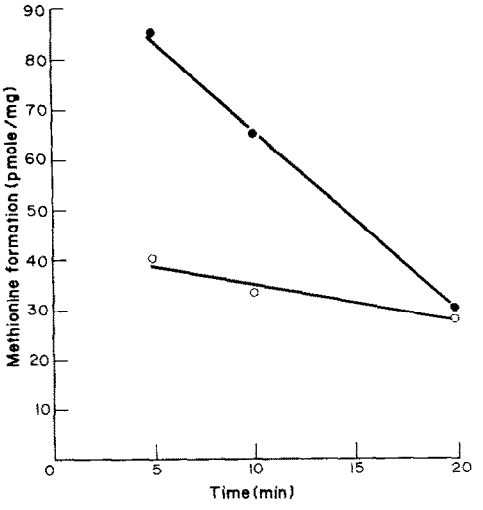

Fig. 4. Decrease in activity of [methyl-^{14}C]methionine formation from 5′-[methyl-^{14}C]MTA by cytosolic extracts of MB prepared from cells incubated 24 hr in medium containing either 0.2 mM L-methionine (○—○) or in methionine-free medium supplemented with 0.2 mM L-homocysteine (●—●).

Incorporation of label was linear over the time period investigated and was greatest in L132. The results in Table 4 show the rate of incorporation of ^{3}H from 5′-[methyl-^{3}H]MTA into DNA, RNA, and protein over a 24 hr period in both normal and methionine-free medium. The size of the acid-soluble pool of 5′-MTA was similar in both cell lines. However, L132 showed an enhanced incorporation of $C^{3}H_{3}$ into DNA and protein under conditions of methionine deprivation, whilst for MB incorporation under these culture conditions was either reduced or unaltered. These results suggest that 5′-MTA may substitute for cellular methionine as a one-carbon source.

Table 3. Decrease in specific activity of methionine formation from 5′-MTA

Cell line	Normal medium (pmole/min/mg protein)	Methionine-free medium* (pmole/min/mg protein)
L132	0.11 ± 0.02	0.35 ± 0.06
W256	0.21 ± 0.04	0.35 ± 0.08
MB	0.8 ± 0.1	3.8 ± 0.3

* Cells were incubated for 24 hr in Dulbecco's Eagle medium without L-methionine, but supplemented with 0.2 mM L-homocysteine before preparation of cytosolic extracts.

DISCUSSION

Formation of methionine from 5′-MTA may be particularly important for rapidly dividing tumour cells which synthesize relatively large amounts of polyamines [19]. In mammals, the first step in methionine synthesis from 5′-MTA is the formation of 5-methylthioribose-1-phosphate [9, 20] by the enzyme 5′-methylthioadenosine phosphorylase. The 5-methylthioribose-1-phosphate is converted to the α-keto acid of methionine, which is transaminated to methionine [20, 21]. Certain cell lines such as murine L1210 leukaemia and human K562 erythroleukaemia lack 5′-methylthioadenosine phosphorylase [10, 22] and would not be expected to synthesize methionine from 5′-MTA. For such cell lines 5-MTA was unable to substitute for methionine for growth. However, other cell lines such as W.256 have low levels of 5′-MTA phosphorylase and are able to synthesize methionine from 5′-MTA by broken cell preparations, but are unable to grow when 5′-MTA substitutes for methionine. Backlund and Smith [10] also found some cell lines with high levels of 5′-MTA phosphorylase which did not grow on 5′-MTA. In a study of two clones of the heterogeneous colon carcinoma cell line DLD-1, Savarese *et al.* [23] observed equal 5′-MTA phosphorylase in both clones A and D, whereas formation of methiodine from 5′-MTA was several-fold lower in clone D than in clone A and 5′-MTA failed to support growth of clone D cells. This lack of growth could be due to one of two reasons (1) Methionine synthesis from 5′-MTA was insufficient to meet the growth requirement of the cell line. The flux of methionine production from 5′-MTA *in vitro* is lower (about 10-times) [13] than that resulting from the methylation of homocysteine, though more of the methionine produced by the former appears to be incorporated into cellular macromolecules. (2) 5′-MTA may inhibit cell growth under the culture conditions. 5′-MTA has been shown to suppress the growth of murine lymphoid cell lines of both B- and T-cell origin in a reversible, non-toxic and dose-dependent fashion [24]. Growth inhibition may be associated with the ability of 5′-MTA to inhibit S-adenosylhomocysteine hydrolase [25], spermidine and spermine synthase [26]. In this study 5′-MTA inhibited the growth of all the cell lines investigated, though the ID_{50} was much lower for W.256. Thus, formation of methionine from 5′-MTA may be important, not only for the conservation of methyl groups in the cell, but also as a possible detoxification mechanism.

The mechanism for the formation of methionine from 5′-MTA in tumour cells appears to be similar to that operative in liver [9] with the initial rapid

Table 4. Rate of incorporation of ^{3}H from 5′-[methyl-^{3}H]MTA into DNA, RNA and protein in normal and methionine-depleted medium

Macromolecule	dpm/hr/mg protein L132 Normal	L132 Methionine-free	MB Normal	MB Methionine-free
DNA	14,443	59,375	37,869	19,591
RNA	16,211	13,102	13,768	18,377
Protein	17,239	46,153	22,147	21,640

formation of 5'-methylthioribose-1-phosphate, which is then converted by further reactions into methionine. For most cell lines formation of methionine is linear for only a short period of time and in intact cells the methyl group of 5'-MTA is incorporated into cellular macromolecules.

It seems unlikely that the methionine auxotrophy of certain malignant mammalian cells arises solely from differences in this salvage pathway for methionine synthesis, since no correlation appears to exist between the ability of a cell line to grow in a methionine-deficient, homocysteine-supplemented medium and the rate of formation of methionine from 5'-MTA. However the lack of 5'-MTA phosphorylase in a number of tumour cell lines [22] suggests the possibility of selective 'rescue' of normal cells by 5'-MTA from methionine deprivation induced by L-methioninase. In addition to the degradation of L-methionine by this enzyme, L-homocystine, L-homocysteine, L-cystine and L-cysteine are also rapidly degraded. Kreis *et al.* [27] have proposed the use of D-homocystine, which is resistant to the action of L-methioninase to 'rescue' normal cells from methionine deprivation. However, we have recently shown no differential 'rescue' of normal over leukaemic cells by D-homocysteine [28]. 5'-MTA would be expected to give complete protection of all normal cells and some tumours (such as MB) with an enhanced synthesis of methionine from 5'-MTA and a low methionine utilization.

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