

# Relative effects of *S*-adenosylmethionine depletion on nucleic acid methylation and polyamine biosynthesis

Debora L. KRAMER,\* Janice R. SUFRIN\*†† and Carl W. PORTER\*

\* Grace Cancer Drug Center and † Department of Surgical Oncology, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263, U.S.A.

Treatment of cultured L1210 cells with 1 mM-L-2-amino-4-methoxy-*cis*-but-3-enoic acid (L-*cis*AMB), a methionine-analogue inhibitor of *S*-adenosylmethionine (AdoMet) synthetase (EC 2.5.1.6), produced a rapid and near-total depletion of AdoMet by 4 h. After this, the pools recovered to 60% of control by 48 h, apparently because of an increase in AdoMet synthetase activity. Both AdoMet depletion and the accompanying increase in synthetase activity were substantially enhanced by lowering methionine concentrations in the media from 100  $\mu$ M to 30  $\mu$ M, the minimal concentration that supports cell growth at control values. During a 4 h incubation in media containing 30  $\mu$ M-methionine, 1–5 mM-L-*cis*AMB depleted cellular AdoMet to undetectable values, and inhibited nucleic acid methylation by 44–72% and RNA methylation by 60–87%. Under these same treatment conditions, putrescine pools increased by about 3-fold, whereas spermidine pools decreased by only 20% and spermine pools remained the same. Pool changes were accompanied by a 2–4-fold increase in ornithine decarboxylase activities and AdoMet activities. Thus the rapid depletion of AdoMet pools by L-*cis*AMB results immediately in a decrease in methyl-transfer reactions involving nucleic acids, whereas, by contrast, biosynthesis of higher polyamines appears to be minimally affected, owing to compensatory increases in key enzyme activities.

## INTRODUCTION

The ubiquitous involvement of *S*-adenosylmethionine (AdoMet) as a methyl donor in transmethylation reactions and its critical role as an aminopropyl donor in polyamine biosynthesis combine with other regulatory functions to make it a pivotal molecule in cell metabolism. Increasingly, these functions appear to play a determining role in cellular processes related to the initiation, maintenance and cessation of cell growth and/or differentiation (Borchardt *et al.*, 1986; Cantoni & Razin, 1985). As a means to define more precisely its involvement in such processes, strategies designed to perturb AdoMet biosynthesis or metabolism have been proposed (Borchardt, 1980; Borchardt *et al.*, 1986; Chou *et al.*, 1977; Porter & Sufrin, 1986). Among these is the use of rationally designed methionine analogues as inhibitors of the key fabricating enzyme, AdoMet synthetase (EC 2.5.1.6) (Chou *et al.*, 1977). Owing to its availability and widespread use, the methionine analogue cyclo-leucine has become the best-known example of such a

methionine analogue (Lombardini *et al.*, 1970). More recent studies, however, have identified a novel and more potent AdoMet synthetase inhibitor, L-*cis*AMB (Fig. 1), whose specificity appears to relate to its close structural correlation with the enzyme-bound conformation of methionine (Sufrin & Lombardini, 1982; Sufrin *et al.*, 1982). Our continued interest in this compound derives from its usefulness as an experimental tool for probing the cellular consequences of AdoMet depletion (Porter *et al.*, 1984) and also from its potential as a lead compound in a synthetic programme to design methionine analogues as antiproliferative agents (Porter & Sufrin, 1986).

In a comparison study of the effects of four methionine analogues on AdoMet formation and polyamine metabolism, L-*cis*AMB appeared to be the most potent and specific compound (Porter *et al.*, 1984). Foremost among its effects in cultured L1210 cells was its ability to decrease cellular AdoMet pools by approx. 50% after a 48 h incubation (Porter *et al.*, 1984; Sufrin *et al.*, 1986). Subsequent studies examining the temporal sequence of events preceding this effect have led to the realization that L-*cis*AMB is a far more effective inhibitor in intact cells than was previously recognized. In particular, we now know that L-*cis*AMB is capable of depleting cellular AdoMet pools in less than 4 h. In the present study, we document this observation and determine its consequences on nucleic acid methylation and polyamine biosynthesis.

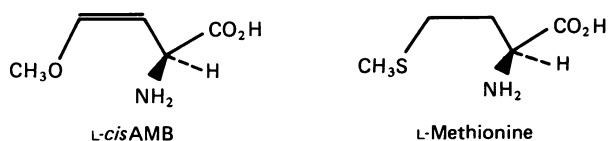


Fig. 1. Structural comparison of L-*cis*AMB with methionine

L-*cis*AMB is competitive with methionine as an inhibitor of L1210-cell AdoMet synthetase isoenzymes ( $K_i$  5.0 and 15.8  $\mu$ M; Sufrin *et al.*, 1986). The inhibitory activity of L-*cis*AMB is believed to relate to its structural resemblance to the enzyme-bound conformation of methionine (Sufrin *et al.*, 1982).

## MATERIALS AND METHODS

### Materials

L-*cis*AMB was synthesized by procedures published previously (Sufrin *et al.*, 1982).

† To whom reprint requests should be addressed.

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; L-*cis*AMB, L-2-amino-4-methoxy-*cis*-but-3-enoic acid; ODC, ornithine decarboxylase.

### Cell culture

Murine L1210 leukaemia cells were grown as a suspension culture in RPMI-1640 medium containing 16 mM-Hepes/8 mM-Mops as a buffer system and 10% Nu-Serum IV (Collaborative Research, Lexington, MA, U.S.A.) as a semi-defined serum substitute. Methionine concentrations in media were either 100  $\mu\text{M}$  (standard) or 30  $\mu\text{M}$ , excluding that contributed by the Nu-serum. Cells were grown in 25- or 75-cm<sup>2</sup> tissue-culture flasks in a total volume of 15 or 50 ml respectively, under a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. Cell number was determined by electronic particle counting (model ZF Coulter Counter; Coulter Electronics, Hialeah, FL, U.S.A.). Cells were treated with concentrations in the range 1–5 mM-L-*cis*AMB while in exponential growth. Incubations were carried out for periods of 0–72 h.

### AdoMet and decarboxylated AdoMet pools

The intracellular concentrations of AdoMet and decarboxylated AdoMet were measured in a 0.6 M-HClO<sub>4</sub> extract taken from 10<sup>7</sup> cells. Analysis of the extract was performed by a slight modification of the method of Zappia *et al.* (1980) as described elsewhere (Porter *et al.*, 1984).

### AdoMet synthetase activity

The AdoMet synthetase assay is a modification of that described by Kotb & Kredich (1985), which depends on the selective binding of AdoMet to phosphocellulose paper. Washed cells ( $4 \times 10^7$ ) were sonicated for  $2 \times 5$  s in 1 ml of a breaking buffer containing 10 mM-sodium Tes (pH 7.4), 0.5 mM-EDTA, 4 mM-dithiothreitol and 5  $\mu\text{M}$ -phenylmethanesulphonyl fluoride. The extract was centrifuged at 12000 *g* for 10 min and the supernatant assayed. The reaction mixture contained the following: 80 mM-Tes (pH 7.4), 50 mM-KCl, 40 mM-MgCl<sub>2</sub>, 10 mM-ATP, 10 mM-dithiothreitol, 0.5 mM-EDTA, 250  $\mu\text{M}$ -methionine and 0.125  $\mu\text{Ci}$  of L-[methyl-<sup>3</sup>H]methionine (58 mCi/mmol; Amersham Corp., Arlington Heights, IL, U.S.A.). To start the reaction, 20  $\mu\text{l}$  of enzyme extract was added to 80  $\mu\text{l}$  of the reaction mixture to give a final volume of 100  $\mu\text{l}$ . After incubation for 40 min at 37 °C, 30  $\mu\text{l}$  of each sample was applied to P-81 phosphocellulose paper discs, which were washed five times with water, placed on a Millipore filtering system for the final wash, and counted for radioactivity in a Beckman model LS1800 liquid-scintillation counter. AdoMet formation was linear with time and amount of enzyme extract up to 1 h, and the methionine and ATP concentrations remained saturating. The results were expressed as nmol of AdoMet formed/h per mg of protein.

### Methionine accumulation

Exponentially growing cells were resuspended in media containing 30  $\mu\text{M}$ -methionine 16 h before the addition of 0.6  $\mu\text{Ci}$  of [methyl-<sup>3</sup>H]methionine (80 Ci/mmol; New England Nuclear Corp.)/ml with or without the desired concentration of L-*cis*AMB. Samples (2 ml) were taken at 1 and 4 h, centrifuged (1200 *g* for 5 min), and washed with phosphate-buffered saline (0.8% NaCl, 0.115% Na<sub>2</sub>HPO<sub>4</sub>, 0.02% KH<sub>2</sub>PO<sub>4</sub>, 0.02% KCl, pH 7.4) containing 0.3 mg of unlabelled methionine/ml. The cell pellets were dissolved in 1 M-NaOH, and their

radioactivity was measured by scintillation counting after neutralization with 1 M-HCl. The results were ultimately expressed as percentages of control radioactivity (c.p.m.) per 10<sup>6</sup> cells.

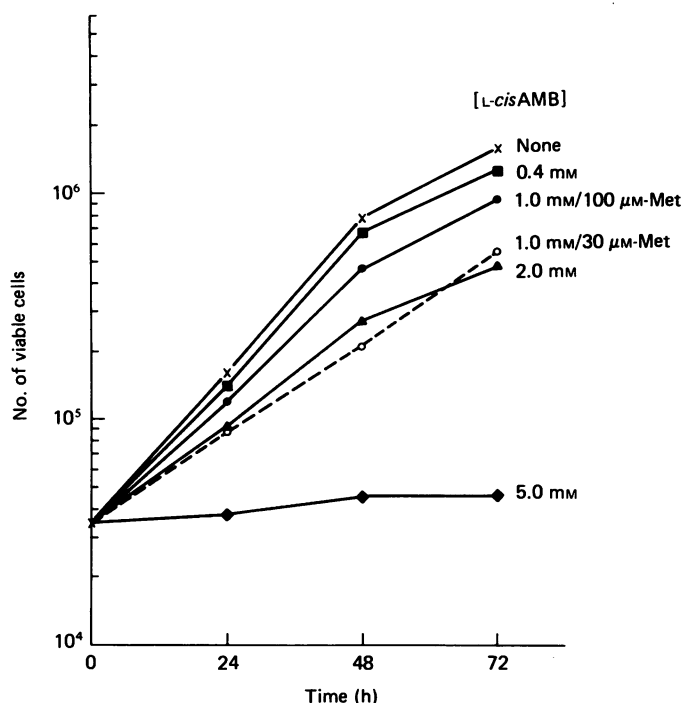
### Precursor incorporation

Exponentially growing cells were resuspended in media containing 30  $\mu\text{M}$ -methionine 16 h before addition of 0, 1 mM-, 2 mM- or 5 mM-L-*cis*AMB. At the end of 4 h the cells were washed and resuspended in fresh media to give  $5 \times 10^5$  cells/ml. The cell suspension was distributed (200  $\mu\text{l}$  per well) into a 96-well micro-titre plate (Falcon), and each of the following precursors were added: [<sup>3</sup>H]thymidine (0.3  $\mu\text{Ci}$ /well; 60 Ci/mmol; New England Nuclear Corp.), [<sup>3</sup>H]uridine (0.3  $\mu\text{Ci}$ /well; 17 Ci/mmol; ICN, Irvine, CA, U.S.A.). After 1 h incubation at 37 °C, the incorporation was halted by adding 100  $\mu\text{l}$  of phosphate-buffered saline with 2% (w/v) NaN<sub>3</sub> to each well. The cells were harvested on to paper discs (Whatman) by using an automated mash harvester, washed five times with 0.9% NaCl, and then washed once with 10% (w/v) trichloroacetic acid containing 10 mg of unlabelled thymidine or uridine/ml to displace unincorporated label. Radioactivity of discs was measured by scintillation counting, and the results were ultimately expressed as percentages of control radioactivity (c.p.m.) per 10<sup>5</sup> cells.

### Methylation of nucleic acids

A sample of cells ( $\sim 2 \times 10^7$ ) taken from each flask was pelleted (1200 *g* for 5 min, twice) to determine [<sup>3</sup>H]-methyl-group incorporation into nucleic acids by a method similar to that described by Pellicer *et al.* (1978). The pellets were resuspended in 5 ml of suspension buffer (10 mM-Tris/HCl, 2 mM-EDTA, 10 mM-NaCl, pH 8.0 containing 10% (w/v) SDS and 100  $\mu\text{g}$  of proteinase K (Sigma, St. Louis, MO, U.S.A.)/ml. After overnight incubation at 37 °C, a sample was extracted with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) three times and finally with chloroform/3-methylbutan-1-ol (24:1, v/v). The nucleic acids were precipitated by adding 0.2 M-sodium acetate and 2 vol. of cold ethanol and leaving the suspension at -20 °C overnight. The samples were centrifuged (3000 *g* for 10 min), washed once with ethanol, allowed to dry, and resuspended in 10 mM-Tris/HCl/1 mM-EDTA buffer (pH 7.5). One portion was counted for radioactivity, and another was used to determine the A<sub>260</sub>. To ensure a uniform suspension, the samples were boiled for 10 min to convert all the double-stranded DNA into single-stranded form. Results were ultimately expressed as percentages of control radioactivity (c.p.m.) per  $\mu\text{g}$  of nucleic acid.

To isolate RNA specifically, the method described by Cox (1968) was adapted with the following modifications. The cell pellets were resuspended in a guanidinium chloride buffer (8 M-guanidinium chloride, 10 mM-EDTA, 50 mM-Tris/HCl, pH 7.5). The suspension was passed 20 times by syringe through an 18-gauge needle to break the DNA sufficiently into small fragments. The lysate was adjusted to pH 5.0 with 1 M-acetic acid to a final concentration of 0.2 M, followed by addition of 0.5 vol. of ethanol, which preferentially precipitates the RNA, leaving the DNA in solution. These samples were stored at -20 °C overnight. The RNA was pelleted by centrifuging at 12000 *g* for 10 min and the pellets were resuspended in 6 M-guanidinium chloride/Tris/EDTA



**Fig. 2. Dose-dependence of growth inhibition of *L-cis*AMB over a 72 h period**

Note that decreasing methionine in the medium from the standard 100  $\mu\text{M}$  to 30  $\mu\text{M}$  substantially increased growth inhibition by 1 mM-*L-cis*AMB.

buffer, pH 7.5. The RNA was reprecipitated by adding acetic acid and 0.5 vol of ethanol, and then stored at  $-20^\circ\text{C}$  for 2 h or more. The pellets were then dissolved in distilled water, or 25 mM-EDTA, quantified by measurements of  $A_{260}$  and counted for radioactivity to determine incorporation. Results were ultimately expressed as percentages of control radioactivity (c.p.m.) per  $\mu\text{g}$  of RNA.

**Intracellular polyamine pools**

A sample of the same  $\text{HClO}_4$  extract used for AdoMet determinations was analysed by h.p.l.c in a system based on cation exchange and post-column derivative formation with *o*-phthalaldehyde as described by Porter *et al.* (1985).

**Decarboxylase activities**

Extracts for measurement of ornithine decarboxylase (ODC) activity were prepared by sonicating ( $2 \times 5$  s) control or treated cells in 10 mM-Tris/HCl (pH 7.2) containing 0.5 mM- $\text{Na}_2\text{EDTA}$ , 5 mM-dithiothreitol and 50  $\mu\text{M}$ -pyridoxal 5'-phosphate, followed by centrifugation at 20000 *g* for 20 min. ODC activity was determined by measuring the release of  $^{14}\text{CO}_2$  from L-[1- $^{14}\text{C}$ ]-ornithine (New England Nuclear Corp.) in the presence of saturating concentrations of pyridoxal phosphate (0.1 mM) by the methods of Pegg & Seely (1983). AdoMet decarboxylase activity was similarly assayed with *S*-adenosyl[carboxy- $^{14}\text{C}$ ] methionine (New England Nuclear Corp.) as substrate in the presence of saturating (3 mM) putrescine as described by Pegg & Poso (1983).

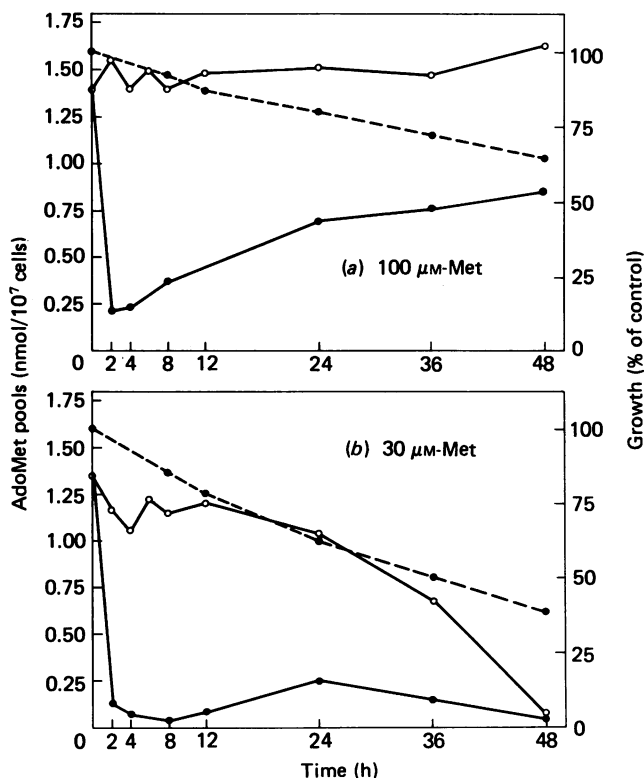
**RESULTS**

Dose-response curves for the effects of *L-cis*AMB on cell growth over the course of 72 h are presented in

Fig. 2. At doses up to and including 2 mM, growth inhibition increased progressively during the 0–48 h period, after which it decreased, so that cell growth during the 48–72 h period paralleled that of control cells. Cells treated with 5 mM-*L-cis*AMB were completely growth-inhibited for the entire 72 h period. Decreasing the methionine in the medium from the standard 100  $\mu\text{M}$  to 30  $\mu\text{M}$  [the minimal concentration that will support growth at control rate during a 48 h incubation (Sufrin *et al.*, 1986)] resulted in a substantial increase in growth inhibition, as exemplified for 1 mM-*L-cis*AMB in Fig. 2.

The time-dependence of *L-cis*AMB effects on intracellular AdoMet pools and cell growth over the course of a 48 h incubation are shown in Fig. 3. In the presence of standard methionine concentrations (100  $\mu\text{M}$ ) in media, AdoMet pools declined rapidly to about 20% of control at 2 h and then, after 4 h, recovered steadily to about 60% of control by 48 h. Throughout this same period, control AdoMet pools ranged between 1.25 and 1.80 nmol/ $10^7$  cells. Cell growth decreased steadily with *L-cis*AMB treatment and did not seem to reflect changes in AdoMet pools.

Decreasing methionine in media to 30  $\mu\text{M}$  enhanced both growth inhibition and AdoMet depletion (Fig. 3). In cells treated with *L-cis*AMB, AdoMet pools were



**Fig. 3. Time-dependence of *L-cis*AMB (1 mM) effects on L1210-cell growth and AdoMet pools in the presence of 100  $\mu\text{M}$  (a) or 30  $\mu\text{M}$  (b) methionine in the media**

Cell growth (●—●) is expressed as a percentage of control, and AdoMet pools in the presence (●—●) or absence (○—○) of drug are given as nmol/ $10^7$  cells; 30  $\mu\text{M}$ -methionine is the minimal concentration in media that will support cell growth at the same rate as with 100  $\mu\text{M}$  over a 48 h incubation. Data represents the mean values of two experiments performed in duplicate.

**Table 1. Effect of L-cisAMB-induced AdoMet depletion on AdoMet synthetase activity and its prevention by exogenous AdoMet**

Values represent means for at least two experiments performed in duplicate: n.d., not determined.

Treatment	[Methionine] in medium ( $\mu\text{M}$ )	AdoMet synthetase activity (nmol/per mg of protein)		
		4 h	24 h	48 h
None	100	29.6	23.4	24.7
1 mM-L-cisAMB	100	39.9	66.4	56.1
None	30	29.9	34.3	39.5
1 mM-L-cisAMB	30	35.3	(24.2)* 97.7	173.0
100 $\mu\text{M}$ -AdoMet	30	n.d.	20.1	—†
1 mM-L-cisAMB + 100 $\mu\text{M}$ -AdoMet	30	n.d.	57.0	—†

\* In the presence of 50  $\mu\text{M}$ -cycloheximide.

† By 48 h, 100  $\mu\text{M}$ -AdoMet became toxic to cells.

almost totally depleted by 2 h and remained so until 8 h, after which they increased slightly and then fell, by 48 h, to nearly undetectable values. It should be noted that, in the presence of restricted (30  $\mu\text{M}$ ) methionine, AdoMet pools in untreated cells also showed substantial decreases, which began after 24 h. They decreased steadily from 24 until 48 h to values similar to those seen in L-cisAMB-treated cells. Despite this decline, growth continued at the same rate as cells in 100  $\mu\text{M}$ -methionine during the 48 h period (Sufrin *et al.*, 1986), but decreased rapidly thereafter (results not shown).

At least two factors could account for the recovery of AdoMet pools in cells treated with 1 mM-L-cisAMB in 100  $\mu\text{M}$ -methionine: AdoMet synthetase activity could increase and/or L-cisAMB could be degraded. In order to determine whether the former was involved, enzyme activity was measured at various times during drug treatment (Table 1). At 24 h, 1 mM-L-cisAMB increased AdoMet synthetase activity about 3-fold in cells grown in 100  $\mu\text{M}$ -methionine and about 4-fold in 30  $\mu\text{M}$ -methionine. Concomitant treatment with 50  $\mu\text{M}$ -cycloheximide during this time completely prevented this stimulation in enzyme activity, indicating that new protein synthesis was required. In the presence of 30  $\mu\text{M}$ -methionine, enzyme activity increased steadily with L-cisAMB treatment during the entire 48 h period. The inclusion of non-toxic concentrations (100  $\mu\text{M}$ ) of AdoMet during treatment with L-cisAMB minimized the increase in AdoMet synthetase activity, indicating a possible causal relationship between AdoMet depletion and increased enzyme activity (Table 1).

It was of primary interest in this study to determine the effects of L-cisAMB on polyamine biosynthesis and nucleic acid methylation under conditions of maximal AdoMet depletion. From Fig. 3, these were selected to be treated with 1.0 mM-L-cisAMB for 4 h in 30  $\mu\text{M}$ -methionine. In order to study methylation effects, we first established that cellular accumulation of [*methyl*- $^3\text{H}$ ]methionine was not affected and that DNA and RNA syntheses were not decreased. Either effect could give an artifactual indication that methylation was inhibited by the drug. The data in Table 2 indicate that these conditions were met. During treatment with 1 mM-

**Table 2. Effect of L-cisAMB on cellular accumulation of [*methyl*- $^3\text{H}$ ]methionine and nucleic acid precursor incorporation**

Values represent the averages of three experiments in duplicate; all media contained 30  $\mu\text{M}$ -methionine

L-cisAMB treatment (4 h)	[ <i>methyl</i> - $^3\text{H}$ ]- Methionine accumulation (% of control*)		Precursor incorporation† (% of control)	
	1 h	4 h	Thymidine	Uridine
	1 mM	94	86	99
2 mM	96	87	93	92
5 mM	102	82	97	79

\* Control value: methionine accumulation 69 292 c.p.m./10<sup>6</sup> cells.  
† Control values: thymidine, 23 521 c.p.m./10<sup>5</sup> cells; uridine, 7633 c.p.m./10<sup>5</sup> cells.

2 mM- or 5 mM-L-cisAMB, the cellular accumulation of labelled methionine was not decreased by more than 18%, and nucleic acid synthesis, as determined by precursor incorporation, was not inhibited by more than 21%. At 1 mM-L-cisAMB, precursor incorporation was not affected at all.

Incorporation data with [*methyl*- $^3\text{H}$ ]methionine (Table 3) indicate that methylation of total nucleic acids was substantially decreased during a 4 h treatment with L-cisAMB in a dose-dependent manner. At 1 mM, where non-specific factors were most clearly excluded (Table 2), a 44% inhibition of total nucleic acid methylation was achieved (Table 3). Relative to total nucleic acids, methylation of RNA was consistently inhibited to a greater extent at all concentrations of drug. Thus, at 1 mM-L-cisAMB, RNA methylation was inhibited by 60%. Prevention studies with AdoMet were attempted in order to relate more closely L-cisAMB-induced depletion of AdoMet pools to decreases in nucleic acid methylation.

**Table 3. Short-term effects of L-cisAMB on AdoMet pools, nucleic acid methylation and polyamine biosynthesis**

All values represent the averages of at least four separate experiments performed in duplicate; all media contained 30  $\mu$ M-methionine.

L-cisAMB treatment (4 h)	AdoMet (nmol/10 <sup>7</sup> cells)	Methylation (% of control)*		Polyamine pools (nmol/10 <sup>7</sup> cells)			Decarboxylase activity (% of control)†	
		Total nucleic acids	RNA	Putrescine	Spermidine	Spermine	ODC	AdoMet
None	0.96	100	100	5.05	24.15	8.55	100	100
1 mM	< 0.05	56	40	17.00	20.10	7.30	315	258
2 mM	< 0.05	34	17	15.40	18.15	8.35	449	321
5 mM	< 0.05	28	13	15.80	18.60	8.60	269	224

\* Control values: 227 c.p.m./ $\mu$ g of total nucleic acid; 127 c.p.m./ $\mu$ g of RNA.  
† Control values: ODC, 5.01 nmol of CO<sub>2</sub>/h per mg; AdoMet decarboxylase, 2.55 nmol of CO<sub>2</sub>/h per mg.

Data, however, were not unambiguously interpretable since the net effect of exogenous AdoMet was to lower differentially the specific radioactivity of the radiolabelled AdoMet pools in control and L-cisAMB-treated cells and thus differentially to affect apparent methylation.

The effects of L-cisAMB-induced AdoMet depletion in decreasing intracellular polyamines were minimal (Table 3). Putrescine pools increased as might be expected, with a decrease in AdoMet pools or inhibition in AdoMet decarboxylase. Unexpectedly, however, spermidine pools decreased by only 25% and spermine pools remained generally the same. This inability of AdoMet depletion to decrease spermidine and spermine pools in a manner comparable with its effects on nucleic acid methylation probably relates to the rapid increases in ODC activity (3–4-fold) and particularly AdoMet decarboxylase activity (2–3-fold), the lead-in enzyme for AdoMet into the polyamine-biosynthetic pathway (Table 3).

## DISCUSSION

The development of methylation inhibitors has received considerable attention as a strategy for the design of chemotherapeutic agents (Borchardt *et al.*, 1986). Methionine analogues designed to inhibit AdoMet synthetase and thereby to limit the availability of the methyl donor, AdoMet, represent one such class of compounds. Currently, these are probably best represented by L-cisAMB, the most potent methionine-analogue inhibitor of AdoMet synthetase thus far identified (Lombardini & Sufrin, 1983). Its ability to accomplish this inhibition in intact cells has afforded a unique opportunity to delineate the effects of AdoMet depletion on polyamine biosynthesis and transmethylation reactions.

From previous data (Porter *et al.*, 1984), which indicated a 60% decrease in AdoMet by 48 h, it was expected that AdoMet pools would decrease gradually during the 48 h treatment with L-cisAMB. Indeed, it was found that they were rapidly depleted by 4 h and, in the presence of 100  $\mu$ M-methionine, gradually recovered during the next 44 h. Thus the effectiveness of L-cisAMB as an inhibitor of AdoMet synthetase in intact cells appears to be much greater than was originally reported

(Porter *et al.*, 1984; Sufrin *et al.*, 1986) and significantly better than another methionine analogue, cycloleucine, as reported by Caboche & Hatzfeld (1978). Although the latter also causes a depletion of cellular AdoMet pools, excessively high concentrations (up to 30 mM) are required to obtain such effects.

The recovery of AdoMet pools during treatment with L-cisAMB is potentially troublesome from a chemotherapeutic perspective and could be due to several possibilities. Included among these are: (a) decay of the inhibitor under cell-culture conditions, (b) compensatory increases in AdoMet synthetase activity, (c) decreased utilization of AdoMet or (d) any combination of the above. Our initial attempts to discern which of these mechanism(s) are involved indicate that an increase in AdoMet synthetase activity might be, at least partially, responsible. During the first 24 h of drug treatment, AdoMet synthetase increased nearly 3-fold in activity in 100  $\mu$ M-methionine and nearly 4-fold in 30  $\mu$ M-methionine. Experiments with cycloheximide (Table 1) indicate that this increase appears to be dependent on new protein synthesis. That this increase in enzyme activity is related to AdoMet depletion is strongly suggested by the finding that exogenous AdoMet partially prevents the effect in L-cisAMB-treated cells (Table 1). These findings relate directly to previous studies by Caboche (1977) and Jacobsen *et al.* (1980), which indicate that AdoMet synthetase activity increases severalfold when methionine in media is restricted. Further, Jacobsen *et al.* (1980) suggest that the increase is related to a post-transcriptional derepression of enzyme synthesis, which is mediated by methionine or one of its metabolites. It seems from our results and those of Caboche (1977) that this might be more specifically attributed to AdoMet or one of its metabolites, since, in both cases, methionine-analogue-induced AdoMet depletion, such as also occurs with methionine deprivation (Fig. 3), results in an increase in enzyme activity. Indeed, the prevention studies in Table 1 support this conclusion. Exogenous AdoMet lowers AdoMet synthetase activity in untreated cells grown in 30 mM-methionine to values comparable with those in cells grown in 100  $\mu$ M. Thus AdoMet is likely to be a critical regulator of AdoMet synthetase activity.

It is also possible that, because L-cisAMB is a reversible inhibitor of AdoMet synthetase, it might also stabilize

the enzyme against degradation and thereby extend its half-life in a manner similar to the effect of methylglyoxal bis(guanyldrazone) on AdoMet decarboxylase (Fillin-game & Morris, 1973; Pegg *et al.*, 1973). However, because AdoMet synthetase appears to have a relatively long half-life [ $\sim 30$  h; Caboche (1977)], the contribution of this phenomenon would probably be minimal over a 24 h period.

The increased effectiveness of *L-cis*AMB in depleting AdoMet pools under conditions of decreased methionine in media (Fig. 3) is consistent with our previous findings that it competes with methionine as an inhibitor of AdoMet synthetase (Sufrin *et al.*, 1982; Porter *et al.*, 1984). By contrast, *L-cis*AMB does not appear to compete with the amino acid for accumulation into L1210 cells (D. L. Kramer, unpublished work), so that any observed competition in intact cells is likely to occur at intracellular sites such as the enzyme itself. The importance of increased AdoMet depletion under conditions of restricted methionine is enhanced by the fact that  $30 \mu\text{M}$  is roughly equivalent to the methionine concentration of human serum (Fischer *et al.*, 1974). Moreover, we have determined previously (Sufrin *et al.*, 1986) that  $30 \mu\text{M}$  is the minimal concentration that will support growth at control ( $100 \mu\text{M}$ ) values during a 48 h incubation, although these conditions lead to AdoMet-pool depletion by the end of the incubation (Fig. 3). We note that growth of L1210 cells is inhibited by  $30 \mu\text{M}$ -methionine when incubations are extended beyond 48 h.

Given the ability of *L-cis*AMB to deplete AdoMet rapidly and almost totally in culture cells, it became of primary interest to determine the consequences of this effect in terms of the two major metabolic fates of AdoMet, transmethylation reactions and polyamine biosynthesis. Under conditions determined to be specific for assessing methylation of nucleic acids, *L-cis*AMB was found to inhibit total nucleic acid methylation and, to a greater extent, RNA methylation within 4 h (Table 3). The effect was dose-dependent, so that with 1–5 mM-*L-cis*AMB, nucleic acid methylation was decreased by 44–72% and RNA methylation by 60–87%. This is consistent with several previous observations concerning the preferential effects of cycloleucine on RNA methylation and synthesis, which, again, were accomplished at much higher drug concentrations (Caboche & Bachel-lerie, 1977; Caboche & Hatzfeld, 1978; Dimock & Stoltzfus, 1978; Prince *et al.*, 1986; Kajander *et al.*, 1986).

It is difficult to assess the effects of *L-cis*AMB on polyamine biosynthesis over such a short time course on the basis of polyamine pools alone. It appeared, however, that depletion of the polyamines, spermidine, and particularly spermine, was minimized by the observed compensatory increases in the rate-limiting enzymes, ODC and AdoMet decarboxylase activities. Indeed, in previous studies (Porter *et al.*, 1984), polyamine pools were examined after an extended period (48 h) of treatment with *L-cis*AMB, and the same trends prevailed: putrescine increased, while spermidine and spermine remained the same. Thus, under conditions of AdoMet depletion, polyamine biosynthesis seems to be conserved by enzyme regulatory mechanisms at the expense of transmethylation reactions involving nucleic acids and probably other molecules as well. Apparently any AdoMet which leaks through the biosynthetic blockade imposed by *L-cis*AMB is immediately utilized by the

polyamine pathway. The findings extend those of Eloranta & Raina (1972) who nutritionally altered AdoMet metabolism in rats and concluded that poly-amine metabolism was not primarily affected by changes in AdoMet pools. Similarly, Caboche & Hatzfeld (1978) speculated that polyamine biosynthesis would not be decreased by cycloleucine-induced AdoMet depletion, but did not measure it directly.

It has been suggested (Cantoni, 1986) that the partitioning of AdoMet between polyamine biosynthesis and transmethylation reactions may be determined by the relative affinity of *S*-adenosylhomocysteine for AdoMet decarboxylase and the various methyltrans-ferases. *S*-Adenosylhomocysteine is a by-product of AdoMet-mediated transmethylation reactions and has been shown to be a competitive inhibitor of most reactions where AdoMet serves as either a methyl donor or an aminopropyl donor (Borchardt, 1977). Ultimately, the metabolic partitioning of AdoMet may be determined by the activity of *S*-adenosylhomocysteine hydrolase, since this enzyme controls the intracellular concen-trations of the AdoMet metabolite. Our current data indicate that, although this hypothesis might obtain under steady-state conditions, the partitioning of AdoMet during AdoMet depletion seems to be regulated by increases in the activities of AdoMet synthetase, AdoMet decarboxylase and ODC. As the net effect of these influences, polyamine biosynthesis is spared at the expense of transmethylation reactions, at least, in proliferating cells.

It is uncertain whether the initial AdoMet depletion and related interference with transmethylation reactions is responsible for the growth-inhibitory effects of *L-cis*AMB. Previously, we demonstrated that growth inhibition by *L-cis*AMB preceded decreases in protein biosynthesis (Porter *et al.*, 1984), so its role as an amino acid antagonist is an unlikely basis for antiproliferative activity. Alternatively, the analogue may be incorporated into proteins and, although not inhibiting protein synthesis directly, could cause secondary effects related to protein structure and/or function. Although it is possible that *L-cis*AMB has other sites of action within the cell, it is equally possible that early interference with trans-methylation reactions by *L-cis*AMB might impart macromolecular alterations which eventually become growth-limiting to the cell.

We gratefully acknowledge the synthetic accomplishments of Dr. Vitauts Alks and Arthur Spiess in preparing *L-cis*AMB, the skilled technical assistance of Richard Beckmann, Dr. Sondra Berger, Edwin Kelly, John Miller and Paula Pera, and the secretarial assistance of Anne Giambelluca and Jessie Crowe. This investigation was supported by grants CA-13038 and CA-24538 from the National Cancer Institute, Department of Health and Human Resources. D. L. K. was supported as a Postdoctoral Fellow by training grant CA-09072 from the National Cancer Institute.

## REFERENCES

- Borchardt, R. T. (1977) in *The Biochemistry of Adenosyl-methionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H. G. & Schlenk, F., eds.), pp. 151–171, Columbia University Press, New York
- Borchardt, R. T. (1980) *J. Med. Chem.* **23**, 347–357

- Borchardt, R. T., Creveling, C. R. & Ueland, P. M. (eds.) (1986) *Biological Methylation and Drug Design*, Humana Press, Clifton
- Caboche, M. (1977) *J. Cell. Physiol.* **92**, 407–424
- Caboche, M. & Bachellerie, J. P. (1977) *Eur. J. Biochem.* **74**, 19–28
- Caboche, M. & Hatzfeld, J. (1978) *J. Cell. Physiol.* **97**, 361–370
- Cantoni, G. L. (1986) in *Biological Methylation and Drug Design* (Borchardt, R. T., Creveling, C. R. & Ueland, P. M., eds.), pp. 227–238, Humana Press, Clifton, NJ
- Cantoni, G. L. & Razin, A. (eds.) (1985) *Biochemistry and Biology of DNA Methylation*, Alan R. Liss, New York
- Chou, T.-C., Coulter, A. W., Lombardini, J. B., Sufrin, J. R. & Talalay, P. (1977) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H. & Schlenk, F., eds.), pp. 18–36, Columbia University Press, New York
- Cox, R. A. (1968) *Methods Enzymol.* **12B**, 120–129
- Dimock, K. & Stoltzfus, C. M. (1978) *Biochemistry* **17**, 3627–3632
- Eloranta, T. O. & Raina, A. M. (1972) *Biochem. J.* **168**, 179–185
- Fillingame, R. H. & Morris, D. R. (1973) in *Polyamines in Normal and Neoplastic Growth* (Russell, D. H., ed.), pp. 249–260, Raven Press, New York
- Fischer, J. E., Yoshimura, N., Aguirre, A., James, J. H., Cummings, M. G., Abel, R. M. & Deindoerfer, F. (1974) *Am. J. Surg.* **127**, 40–47
- Jacobsen, S. J., Hoffman, R. M. & Erbe, R. W. (1980) *J. Natl. Cancer Inst.* **65**, 1237–1244
- Kajander, E. O., Kubota, M., Carrera, C. J., Montgomery, J. A. & Carson, D. A. (1986) *Cancer Res.* **46**, 2866–2870
- Kotb, M. & Kredich, N. M. (1985) *J. Biol. Chem.* **260**, 3923–3930
- Lombardini, J. B. & Sufrin, J. R. (1983) *Biochem. Pharmacol.* **32**, 489–495
- Lombardini, J. B., Coulter, A. W. & Talalay, P. T. (1970) *Mol. Pharmacol.* **6**, 481–499
- Pegg, A. E. & Poso, H. (1983) *Methods Enzymol.* **94**, 234–239
- Pegg, A. E. & Seely, J. C. (1983) *Methods Enzymol.* **94**, 158–166
- Pegg, A. E., Corti, A. & Williams-Ashman, H. G. (1973) *Biochem. Biophys. Res. Commun.* **52**, 696–701
- Pellicer, A., Wigler, M., Axel, R. & Silverstein, S. (1978) *Cell* **14**, 133–141
- Porter, C. W. & Sufrin, J. R. (1986) *Anticancer Res.* **6**, 525–542
- Porter, C. W., Sufrin, J. R. & Keith, D. D. (1984) *Biochem. Biophys. Res. Commun.* **122**, 350–357
- Porter, C. W., Cavanaugh, P. F., Jr., Stolowich, N., Ganis, B., Kelly, E. & Bergeron, R. J. (1985) *Cancer Res.* **45**, 2050–2057
- Prince, D. L., Kotin, R. M. & Dubin, D. T. (1986) *Mol. Biol. Rep.* **11**, 51–55
- Sufrin, J. R. & Lombardini, J. B. (1982) *Mol. Pharmacol.* **22**, 752–759
- Sufrin, J. R., Lombardini, J. B. & Keith, D. D. (1982) *Biochem. Biophys. Res. Commun.* **106**, 251–255
- Sufrin, J. R., Lombardini, J. B., Kramer, D. L., Alks, V., Bernacki, R. J. & Porter, C. W. (1986) in *Biological Methylation and Drug Design* (Borchardt, R. T., Creveling, C. R. & Ueland, P. M., eds.), pp. 373–384, Humana Press, Clifton, NJ
- Zappia, V., Galletti, P., Porcelli, M., Manna, C. & Della Ragione, F. (1980) *J. Chromatogr.* **189**, 399–405

---

Received 23 February 1987/26 May 1987; accepted 17 June 1987