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GROWTH INHIBITION BY METHIONINE ANALOG INHIBITORS OF S-ADENOSYLMETHIONINE BIOSYNTHESIS IN THE ABSENCE OF POLYAMINE DEPLETION

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Four methionine analog inhibitors of methionine adenosyltransferase, the enzyme which catalyzes S-adenosylmethionine biosynthesis, were tested in cultured L1210 cells for their effects on cell growth, leucine incorporation, S-adenosylmethionine (AdoMet) formation and polyamine biosynthesis. The IC_{50} values were as follows: selenomethionine, 0.13 mM; L-2-amino-4-methoxy-cis-but-3-enoic acid (L-cis-AMB), 0.4 mM; cycloleucine, 5 mM and 2-aminobicyclo[2.1.1]hexane-2-carboxylic acid, 5 mM. At IC_{50} levels, the analogs significantly reduced AdoMet pools by ~50% while not similarly affecting leucine incorporation or polyamine biosynthesis. In combination with inhibitors of polyamine biosynthesis, growth inhibition was greatly increased with methylglyoxal bis(guanylhydrazone), an inhibitor of AdoMet decarboxylase, but only slightly increased with α-difluoromethylornithine, an inhibitor of ornithine decarboxylase. Overall, the data indicate that the methionine analogs, and particularly L-cis-AMB, seem to inhibit cell growth by interference with AdoMet biosynthesis. Since polyamine biosynthesis is not affected, the antiproliferative effect may be mediated through perturbations of certain transmethylation reactions.

The critical involvement of S-adenosylmethionine in cell proliferation and function is a consequence of its participation in a wide spectrum of transmethylation reactions and in polyamine biosynthesis. The apparently widespread occurrence among human tumor cell lines, of methionine dependence,[1] a metabolic defect which has been correlated with alterations in AdoMet[2] biosynthesis, has led Hoffman and coworkers (1) to suggest that methionine dependence may represent an important and therapeutically exploitable aspect of malignancy. Thus, inhibition of AdoMet biosynthesis by

1 Methionine dependence is defined as the inability of cells to grow when methionine is replaced by its immediate precursor, homocysteine, in the culture medium.

2 Abbreviations used are: ABHCA, (+)-2-aminobicyclo[2.1.1]hexane-2-carboxylic acid; AdoMet, S-adenosyl-L-methionine; AdoSeMet,

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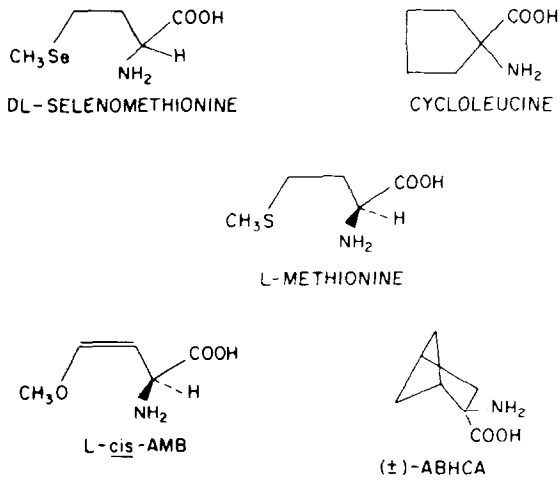

Figure 1. Structures of methionine and methionine analogs.

use of methionine analogs, might specifically provide a chemotherapeutic strategy for methionine dependent tumors. As support for this approach, Sufrin and Lombardini (3) have described differential sensitivities for methionine analogs among the methionine adenosyltransferase isozymes of hepatoma and liver tissues, suggesting a possible mechanism for selectively enhancing inhibition of tumor cell growth. Another aspect of AdoMet metabolism, polyamine biosynthesis, has also attracted attention as a target site for cancer chemotherapy (4-6).

In the present study, a series of methionine analog inhibitors of methionine adenosyltransferase (Fig. 1) have been examined with respect to their effects on protein synthesis, AdoMet pools, polyamine pools and growth of L1210 cells. Of the four methionine analogs tested, L-cis-AMB, cycloleucine and ABHCA are competitive inhibitors of the enzyme whereas selenomethionine is a mixed substrate/inhibitor (3,7,8).

MATERIALS AND METHODS

Chemicals. Cycloleucine, DL-selenomethionine and S-adenosyl-L-methionine iodide salt were obtained from Sigma Chemical Company, St. Louis, MO.

Se-adenosyl-L-methionine; dcAdoMet, decarboxylated AdoMet; DFMO, α-difluoromethylornithine; L-cis-AMB, L-2-amino-4-methoxy-cis-but-3-enoic acid; methionine adenosyltransferase, ATP:L-methionine S-adenosyltransferase (EC 2.5.1.6); MGBG, methylglyoxal bis(guanylhydrazone); Put, putrescine; RPMI 1640, Roswell Park Memorial Institute 1640 medium; Spd, spermidine; Spm, spermine.

L-cis-AMB and ABHCA were synthesized according to procedures published previously (8,9). DFMO was generously provided by the Merrell-Dow Research Center, Cincinnati, Ohio.

Cell Culture. Murine L1210 leukemia cells were maintained in logarithmic growth as a suspension culture in RPMI-1640 medium containing 2% HEPES-MOPS, 1 mM aminoguanidine and 10% NuSerum (Collaborative Research Inc., Lexington, MA). Cultures were treated while in logarithmic growth (0.5 to 1 x 10^5 cells/ml) with the methionine analogs at concentrations ranging from 0.01 mM to 10 mM. After 48 hr, cells were removed from tubes for counting and viability determinations. Cell number was determined by electronic particle counting (Model ZF Coulter counter; Coulter Electronics, Hialeah, FL). Cell viability was assessed by trypan blue dye exclusion (0.5% in unbuffered 0.9% NaCl solution).

Drug Interaction Studies. The ability of the methionine analogs to affect growth inhibition induced by the polyamine inhibitors, DFMO or MGBG, was studied by exposing cells to the analog at an IC_{50} concentration plus 5 mM DFMO or 1 μM MGBG for 24 or 48 hr. Cell growth and percent inhibition of growth were determined and compared to that achieved with DFMO, MGBG or the analogs alone.

Leucine Incorporation. Cells were treated with methionine analogs at the IC_{50} value for periods up to 48 hr. Treated and untreated cells were washed in RPMI-1640 medium and suspended at a density of 2 x 10^6/ml in medium containing 2 μCi/ml [^{3}H]-leucine (55 Ci/mmole, ICN, Chemical Radioisotope Div., Irvine, CA). The cells were incubated for 30 min at 37° then washed in phosphate buffered saline containing 5 mg/ml bovine serum albumin as a carrier protein and 1 mg/ml leucine to displace non-specifically bound [^{3}H]-leucine. The cells were then suspended in 1 N trichloroacetic acid. After 30 min, the acid extract was removed by centrifugation and the remaining precipitate dissolved in 1N NaOH. Following neutralization in 1N HCl, radioactivity content was determined by scintillation counting.

Polyamine Determinations. Polyamine pools were determined on cells treated for 48 hr in the presence or absence of the methionine analogs at the IC_{50} value. Cells were incubated, washed, and extracted for 30 min at 4° with 0.6M perchloric acid (0.5 ml/10^7 cells). High pressure liquid chromatography of the extract was performed as described elsewhere (5).

AdoMet Determinations. AdoMet levels were determined using 0.6 M perchloric acid extracts (0.5 ml/10^7 cells) obtained in the same manner as described for polyamine determinations. The perchloric acid extracts were chromatographed on Dowex 50(H^+) and prepared for high pressure liquid chromatography as described by Zappia et al. (10). Quantitation of AdoMet levels in each sample was done with isocratic elution by ammonium formate buffer, 0.5 M, pH 4.0, using a Varian 8500 liquid chromatograph equipped with a Whatman Partisil 10-SCX column, a Beckman Model 153 UV detector operating at 254 nm, and a Varian Model 485 integrator, essentially as described by Zappia et al. (10).

AdoMet Decarboxylase Activity. Cells treated for 48 hr in the presence or absence of methionine analogs at IC_{50} concentrations were pelleted, dried with a cotton swab, and resuspended in 0.4 ml of buffer containing 25 mM Tris (pH 7.5), 0.1 mM EDTA, and 5 mM dithiothreitol at 4°C. The cellular suspension was ultrasonicated and centrifuged at 8,000 x g for 3 min with a microcentrifuge. The resulting supernatant was assayed for AdoMet decarboxylase activity. The methods used for the determination of AdoMet decarboxylase activity were essentially those of Pegg and Williams-Ashman (11) as described elsewhere (12). The substrate, 1-[^{14}C] AdoMet (51 mCi/mmole) was obtained from New England Nuclear Corp. Data was recorded as

nmoles $^{14}CO_2$ released per hr per mg protein and then as percent of control.

RESULTS AND DISCUSSION

The potency of the analogs as growth inhibitors was assessed according to the inhibitory concentration required to reduce growth by 50% after 48 hr (IC_{50}). In all cases, the IC_{50} values were in the mM range (Table 1). When the analogs were ranked according to antiproliferative potency, the order correlated with the ability of the analogs to inhibit tumor-derived isozyme preparations of methionine adenosyltransferase (7,8). The correlation, though approximate, suggested that growth inhibition by the analogs was related to AdoMet depletion. When the growth of cells treated with the analogs at the IC_{50} levels was followed with time (Table 2), it was apparent that the greatest inhibition occurred during the first 24 hr (average, 44% inhibition) and much less during the second 24 hr (average, 10% additional inhibition).

Growth inhibition was found (Table 2) to precede protein inhibition (leucine incorporation) by 24 hr. While greatest growth inhibition occurred during the first 24 hr, inhibition of protein synthesis was not apparent until 48 hr and even then it was not as marked as growth inhibition. Thus, it appears that, although these compounds are indeed amino acid analogs,

Table 1. Correlation of Inhibition of Methionine Adenosyltransferase (MAT) Isozymes with L1210 Cell Growth Inhibition by Methionine Analogs

Analog	50% Inhibition Hepatoma MAT Isozymes*		50% Inhibition L1210 Cell Growth
	I	II	
	(mM)		(mM)
Se-methionine	0.006	0.006	0.13
L-cis-AMB	0.012	0.041	0.40
Cycloleucine	0.19	0.14	5.00
ABHCA	0.18	0.078	5.00

*Data from Lombardini and Sufrin (7) and Sufrin et al., (8).

Table 2. Time Dependence of Effects of Methionine Analogs on Growth, and Leucine Incorporation of Cultured L1210 Leukemia Cells

Analog	Growth (% Control)			Leucine Incorporation* (% Control)		
	12 hr	24 hr	48 hr	12 hr	24 hr	48 hr
None	100	100	100	100	100	100
0.13 mM Se-methionine	78	62	38	96	119	96
0.4 mM L-cis-AMB	85	58	43	90	113	85
5.0 mM Cycloleucine	62	44	43	89	114	71
5.0 mM ABHCA	76	59	58	93	109	60

*Control incorporation of ^{3}H-leucine during a 30 min incubation, 5020 CPM 10^6 cells after 12 hr, 2540 CPM/10^6 cells after 24 hr, and 2565 CPM/10^6 cells after 48 hr. Because of early nutrient depletion of the media by the faster growing control cells, leucine incorporation decreases at 24 hr giving the false impression by % of control expression that protein synthesis was increased in treated cells. Data represent the main of two experiments run in duplicate. In all cases standard deviation was less than 10%.

inhibition of protein biosynthesis is not among their primary metabolic effects especially with respect to cell proliferation.

As indicated in Table 3, the analogs have a significant effect on cellular AdoMet pools, presumably through inhibition of the methionine adeno-

Table 3: The Effects of Methionine Analogs on Growth, Polyamine and AdoMet Content, and AdoMet Decarboxylase (AdoMetDC) Activity in Cultured L1210 Leukemia Cells

Inhibitor (48 hr)	Growth (% Control)	AdoMet (% Control)	AdoMetDC (% Control)*	Polyamines (pmol/10^6 cells)		
				Put	Spd	Spm
None	100	100	100	490	2950	1170
0.13 mM Se-methionine	47	75+	107	430	3100	920
0.4 mM L-cis-AMB	49	38	246	750	2980	1190
5.0 mM Cycloleucine	53	62	140	560	2670	990
5.0 mM ABHCA	50	51	78	690	2740	1070

*Control AdoMet levels were 1.3 nmol/10^7 cells.

+AdoMet plus AdoSeMet.

syltransferase enzyme. Growth inhibition (~50%) correlated with AdoMet depletion (~50%) suggesting a causal relationship. Most active among the compounds was L-cis-AMB which, at IC_{50} level, reduced AdoMet pools by ~60%. Its potential usefulness as a tool for studying the cellular consequences of AdoMet depletion is apparent; its effects may be even more pronounced at higher concentrations which are expected to achieve near total depletion of AdoMet. The substrate/inhibitor, selenomethionine, may have been similarly active but we were unable to distinguish AdoMet from AdoSeMet in the analysis by high pressure liquid chromatography. Its ability to be utilized as a substrate by methionine adenosyltransferase (3), and its capacity to serve in subsequent transmethylation reactions and in polyamine formation as a substrate of AdoMet decarboxylase (13), make selenomethionine less well suited than L-cis-AMB as a tool for studying AdoMet metabolism.

It is well recognized that the product of the methionine adenosyltransferase reaction, AdoMet, serves as a donor in transmethylation reactions and in polyamine biosynthesis. It was unexpected, therefore, that although AdoMet pools were reduced to ~50% by the analogs, the polyamine pools were virtually unperturbed (Table 3). Following decarboxylation, AdoMet serves as an aminopropyl donor in the synthesis of spermidine and spermine. In its absence, putrescine pools should increase while spermidine and spermine pools decrease but, except for a modest increase in putrescine with two of the analogs, this did not occur. In the case of L-cis-AMB which brought about the greatest reduction in cellular AdoMet, a marked increase in AdoMet decarboxylase was observed. The polyamine pathway is exquisitely regulated (14) and this increase may represent a compensatory mechanism to assure appropriate fluxes through it.

In an attempt to further evaluate the relationship of AdoMet pools and polyamine biosynthesis, the analogs were tested with known polyamine inhibitors (Fig. 2). In combination with the inhibitor of ornithine decarboxylase, DFMO (15), the analogs exhibited only a slight increase in growth inhibition. However, in combination with MGBG, an inhibitor of AdoMet de-

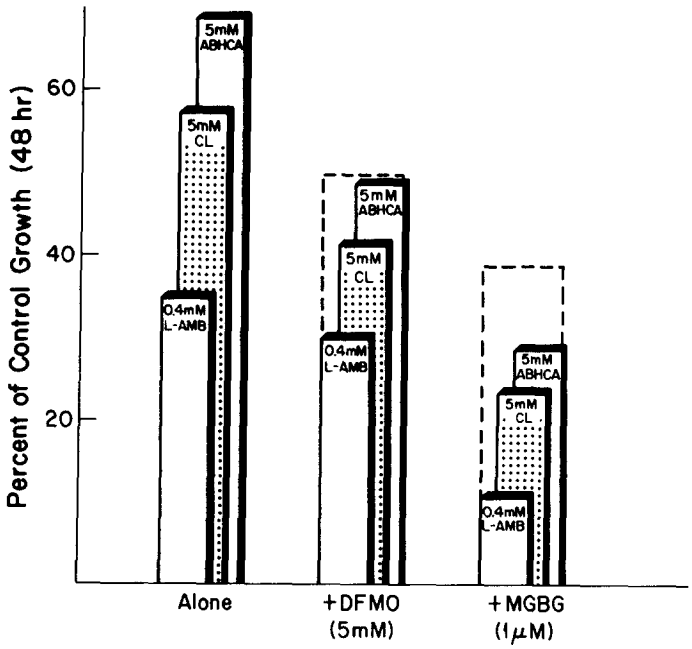

Figure 2. Effects of methionine analogs alone or in combination with polyamine inhibitors, DFMO or MGBG (at IC_{50} levels), on the growth of L1210 cells during a 48 hr incubation (CL, cycloleucine). Broken lines indicate growth inhibition with DFMO or MGBG alone.

carboxylase (16) growth inhibition was enhanced considerably as might be expected if a more complete blockade of AdoMet metabolism were established. This is consistent with the elevation in AdoMet decarboxylase produced by L-cis-AMB.

Overall, the data indicate that the methionine analogs, and particularly L-cis-AMB, have potential as antineoplastics and as experimental probes in cell physiology. Although their activity was found to be in the mM range, it should be noted that the analogs were tested in medium containing 100 μM methionine which may serve to effectively compete at the enzyme active site. By comparison, human serum levels are in the range of 20 μM. As for mechanism of action, several lines of evidence presented above suggest that the analogs elicit growth inhibition by perturbation of AdoMet pools. Although not measured directly, certain or all transmethylation reactions may be altered since the other major pathway for AdoMet metabolism, polyamine biosynthesis, was found to be unaffected.

This study establishes a causal relationship between AdoMet depletion and growth inhibition and shows that this occurs without concomitant alterations in polyamine biosynthesis. Moreover, these results were accomplished specifically and directly through the use of inhibitors of methionine adenosyltransferase. The unexpected finding that polyamine pools are not perturbed at levels of AdoMet which result in growth inhibition, indicates these interrelationships should now be further defined.

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