Cellular characterization of a new irreversible inhibitor of *S*-adenosylmethionine decarboxylase and its use in determining the relative abilities of individual polyamines to sustain growth and viability of L1210 cells

Debora L. KRAMER,* Radii M. KHOMUTOV,† Yuriy V. BUKIN,‡ Alex R. KHOMUTOV§ and Carl W. PORTER*||

*Grace Cancer Drug Center, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263, U.S.A.,

†Institute of Molecular Biology and §Institute of Organic Chemistry, Academy of Sciences of the U.S.S.R.,

Moscow, U.S.S.R., and ‡Institute for Carcinogenesis, All Union Cancer Research Center of U.S.S.R.,

Academy of Medical Sciences, Moscow, U.S.S.R.

S-(5'-Deoxy-5'-adenosyl)methylthioethylhydroxylamine (AMA) is an irreversible inhibitor of *S*-adenosylmethionine (AdoMet) decarboxylase, which is designed to bind covalently the pyruvate residue at the enzyme active site. In the present study the cellular effects of AMA were characterized for the first time in cultured L1210 leukaemia cells. At the approximate IC₅₀ (concn. giving 50% inhibition; 100 μM), AMA decreased spermidine and spermine by more than 80% at 48 h while increasing putrescine more than 10-fold. As an indication of enzyme specificity, growth inhibition was fully prevented with exogenous spermidine. When compared with the irreversible inhibitor of ornithine decarboxylase, α-difluoromethylornithine (DFMO), at similar growth-inhibitory concentrations, AMA was less cytotoxic, as determined by colony-formation efficiency. In combination with AMA, DFMO eliminated the rise in putrescine and decreased growth in an additive manner. The near-total depletion of intracellular polyamine pools achieved with the drug combination provided an opportunity to examine the relative abilities of individual polyamines to support growth and viability. Of the three exogenously supplied polyamines, only spermidine fully sustained cell growth and viability at control values during incubations totalling 120 h. By contrast, spermine supported growth at 23% of control and viability at 8%. Putrescine was similarly ineffective, supporting growth at 13% of control and viability at 7%. The data indicate that, in L1210 cells, spermidine is apparently the preferred polyamine in growth-related functions and is capable of fully supporting cell growth by itself. However, because spermine and putrescine can also support growth to some extent, maximum interference with growth and viability is best achieved by strategies which deplete all three polyamine pools.

INTRODUCTION

As elucidated through studies with auxotrophic mutants (Steglich & Scheffler, 1982; Pohjanpelto *et al.*, 1981) and, more generally with specific enzyme inhibitors (Mamont *et al.*, 1978; Tang *et al.*, 1980), the natural polyamines are now known to be critically important in the initiation and maintenance of the proliferative state in eukaryotic cell types. Although recognized for approximately a decade, the relationship has not been unambiguously dissected further to delineate the specific contribution of the individual polyamines, putrescine (PUT), spermidine (SPD) or spermine (SPM), in supporting cell growth. To a large extent, this is attributable to our general inability to deplete cells of all polyamines or to deplete one polyamine pool selectively without concomitantly increasing either or both of the others.

Polyamine depletion has been most widely studied using the irreversible inhibitor of ornithine decarboxylase, α-difluoromethylornithine (DFMO; Metcalf *et al.*,

1978). Typically, cells are depleted of PUT and SPD, while SPM pools either increase or decrease slightly. Thus its usefulness in determining the precise role of SPD in cell proliferation has been limited by the presence of SPM. Although exogenous SPD is known to prevent DFMO-induced cytostasis fully (Porter & Bergeron, 1983), it is unclear whether this could also be accomplished in the absence of SPM or without limited cellular conversion of exogenous SPD into SPM. Studies using the specific inhibitor of SPD synthase, *S*-adenosyl-L-thio-1,8-diamino-octane (also known as AdoDATO), to evaluate SPD function have encountered similar problems (Pegg *et al.*, 1982). SPD pools are depleted, but those of PUT and SPM are increased.

The cellular consequences of SPM depletion have also been examined with somewhat conflicting results. Casero *et al.* (1984) and McGovern *et al.* (1986) treated cells with DFMO plus a SPD analogue to deplete SPM pools, and concluded that both SPD and SPM are required for cell growth. By contrast, Pegg & Coward (1985) used *S*-methyl-5'-methylthioadenosine to inhibit SPM synthase,

Abbreviations used: AdoMet, *S*-adenosylmethionine; AMA, *S*-(5'-deoxy-5'-adenosyl)methylthioethylhydroxylamine; DFMO, α-difluoromethylornithine; PUT, putrescine; SPD, spermidine; SPM, spermine.

|| To whom reprint requests should be addressed.

Fig. 1. Structural representation of AMA as an analogue of decarboxylated AdoMet

and found that cell growth continued even though SPM synthesis was blocked and SPM pools were markedly decreased. Neither study unequivocally resolved the relationship of SPM to cell growth, since in the former a potentially toxic polyamine analogue accumulated in cells, and in the latter a substantial increase in SPD pools may have masked the consequences of SPM depletion.

In this study we: (a) characterize, for the first time, the cellular effects of the irreversible inhibitor of AdoMet decarboxylase, *S*-(5'-deoxy-5'-adenosyl)methylthioethylhydroxylamine (AMA; Fig. 1; Khomutov *et al.*, 1983; Artamonova *et al.*, 1986) on growth and polyamine pools [Paulin (1986) has described the effects of AMA on bacterial enzymes; although the AMA was reported to be provided by our laboratory, we cannot verify the authenticity of the compound, since it was not obtained directly from us]; (b) use AMA in combination with DFMO to achieve a substantial depletion of the total cellular polyamine pool; and (c) employ a product-prevention strategy with the drug combination to delineate the relative roles of individual polyamines in sustaining growth and viability of cultured L1210 cells. Our findings indicate that SPD is clearly the preferred polyamine in growth-related functions of these particular cells.

MATERIALS AND METHODS

AMA and the SPD analogue *N*-(2-amino-oxyethyl)-1,4-diaminobutane were synthesized as described elsewhere (Khomutov & Khomutov, 1986). DFMO was generously provided by the Merrell Dow Research Institute (Cincinnati, OH, U.S.A.).

Murine L1210 lymphocytic leukaemia cells were grown in RPMI-1640 medium containing 10% (v/v) Nu-Serum (Collaborative Research, Lexington, MA, U.S.A.), 16 mM-Hepes and 8 mM-Mops as a buffer and 1 mM-aminoguanidine as an inhibitor of serum amine oxidases (Shore & Cohn, 1960). Exponentially growing cells were seeded into T-75 flasks at 3×10^4 cells/ml. Drug treatment with 1 mM-DFMO and/or 0.1 mM-AMA was initiated at the time of seeding and carried out for 24 h and 48 h incubations at 37 °C in a humidified atmosphere containing 5% CO₂. For extended incubations, cells treated for 48 h were reseeded at 5×10^4 cells/ml in fresh medium, and appropriate drugs were added to maintain similar conditions. In prevention studies, cells were continuously treated with either or both drugs plus 1 μ M- or 10 μ M-PUT, 2 μ M-SPD or 1 μ M- or 3 μ M-SPM during both the 48 h and the 120 h incubation periods. Extracellular concentrations of the exogenous polyamines were selected to ensure a sufficient, if not comparable, intracellular accumulation of each poly-

amine. By themselves, the polyamines were not toxic or growth-inhibitory to untreated cells. Cell number was determined by electronic particle counting (model ZF Coulter Counter; Coulter Electronics, Hialeah, FL, U.S.A.) and confirmed periodically with haemocytometer measurements. Cell viability was assessed by exclusion of Trypan Blue (0.5% in unbuffered 0.9% NaCl solution). The percentage control growth was calculated by dividing the final treated cell number minus the initial inoculum by the final untreated cell number minus the initial inoculum. Only viable (dye-excluding) cells were considered in this determination.

At 24, 48 and 120 h (i.e. 72 h after reseeding at 48 h), cells were taken for determinations of polyamine pool size and measurements of ornithine decarboxylase and AdoMet decarboxylase activities. Enzyme activities were based on determination of the ¹⁴CO₂ released from the ¹⁴C-labelled substrates (Porter *et al.*, 1985) and results were expressed as nmol/h per mg of protein. Protein was determined by a Bio-Rad (Richmond, CA, U.S.A.) detection assay. Polyamine pools were measured by h.p.l.c. as described by Porter *et al.* (1985) and expressed as nmol/10⁶ cells or as nmol/culture. The latter was obtained by multiplying the total number of cells per culture by the polyamine pool in nmol/10⁶ cells.

For enzyme-inhibition studies, untreated cell extracts were incubated for 30 min at 37 °C with various concentrations of AMA in the standard enzyme assay mixtures. The amount of ¹⁴CO₂ produced was measured and expressed as the percentage of enzyme activity in the absence of AMA.

Colony-formation-efficiency assays were performed as described by Chang *et al.* (1984). A semi-solid medium made up of 1.5% (w/v) methylcellulose [40 kPa · s (4000 cP); Fisher Scientific Co., Fair Lawn, NJ, U.S.A.] in RPMI 1640 containing 50 μ g of penicillin/streptomycin/ml, 1 mM-glutamine and 20% (v/v) heat-inactivated fetal-calf serum was used to determine the colony-forming efficiency of cells after 0, 24, 48 or 120 h of treatment. The cells (4000–10000) were thoroughly mixed with 1 ml of the methylcellulose mixture and placed in 35 mm-diam. dishes (Lux, Naperville, IL, U.S.A.). After 5–7 days of incubation at 37 °C in a humidified 5%-CO₂ atmosphere, the colonies (> 30 cells) were scored with a light microscope. Results were expressed as efficiencies (the number of colonies per dish divided by the number of cells plated) and as percentage of control colony-formation efficiency.

RESULTS

In addition to reacting with the pyruvate residue of AdoMetDC, the hydroxylamine moiety of AMA has the potential of combining with the pyridoxal phosphate of ornithine decarboxylase (Khomutov *et al.*, 1987a). In an enzyme extract from untreated cells this was found to be the case (Fig. 2); however, the differential between AMA inhibition of AdoMet decarboxylase occurred at concentrations 100–1000-fold lower than inhibition of ornithine decarboxylase. Thus, at relatively low intracellular concentrations, the inhibition is selective for AdoMet decarboxylase. Since this cannot be strictly controlled in cells, it must be assumed that both enzymes may have been differentially affected under the conditions of the present experiments.

The dose-response curve of L1210-cell growth to AMA

Fig. 2. Differential inhibition of (□) AdoMet decarboxylase and (■) ornithine decarboxylase activities in an enzyme extract from untreated L1210 cells by AMA

at 48 h (not shown) was relatively flat over a range of concentrations between 0.01 and 0.15 mM, with 50% inhibition obtained at between 0.05 and 0.10 mM. To allow for detection of enhanced growth effects in combination experiments with DFMO, a standard experimental concentration of 0.1 mM-AMA was selected which consistently inhibited cell growth by about 40% at 48 h. Over time (Fig. 3b), this produced a constant slowing of cell growth, which persisted for up to 144 h or approx. 12–14 cell doublings. As indicated by colony-formation efficiency, AMA was partially cytotoxic, since at 48 h clonogenicity was found to be 52% of control (Table 1). Although 0.1 mM-AMA was less effective than 1 mM-DFMO (11% of control) in this regard, both drugs may be more active at higher concentrations.

In cells treated with AMA for 48 h, PUT pools were increased approx. 16-fold, whereas SPD and SPM pools were decreased by about 80 and 90% respectively (Table 1). Since AMA is an analogue of decarboxylated AdoMet, it is possible that it could serve as an inhibitor or a substrate in the synthase reactions to form SPD and/or SPM analogues. The latter possibility seems unlikely, however, since in h.p.l.c. chromatograms there were no peaks in AMA-treated cell extracts which corresponded to that for the SPD analogue *N*-(2-amino-oxyethyl)-1,4-diaminobutane in a mixture of standards.

Consistent with the polyamine perturbations, AdoMet decarboxylase activity was found to be inhibited by 80%. A massive (14-fold) increase in ornithine decarboxylase activity occurred, which could be related to regulatory responses resulting from SPD and SPM depletion and/or to possible stabilization of the enzyme protein owing to reversible active-site reactivity with the inhibitor. Growth inhibition was specifically attributable to effects on AdoMet decarboxylase, as indicated by product-prevention studies. At 48 h, both exogenously supplied SPD and SPM prevented growth inhibition (Fig. 3a, Table 2); however, only SPD maintained colony-formation efficiency at control values (Table 2). This was apparent when prevention studies were extended to 120 h (Fig. 3, Table 2). SPD fully maintained cell growth at control values, but SPM did not. A similar specificity for SPD during extended incubations was also noted with DFMO-induced cytostasis (Fig. 3). Increasing SPM 3-fold to 3 μM (results not shown) failed to sustain growth or viability any more effectively than 1 μM-SPM in the presence of DFMO or AMA (Fig. 3). All polyamine concentrations, including SPM, were found to be non-toxic to control cells treated identically.

The combination of AMA and DFMO gave an approximately additive inhibition of cell growth at 48 h (Fig. 4). The effect was more impressive after reseeding, where cells treated with the combination underwent only

Fig. 3. Growth curves for untreated L1210 cells and for cells treated with 1 mM-DFMO (a) and 0.1 mM-AMA (b) in the absence or presence of 2 μM-SPD or 1 μM-SPM

For DFMO, 10 μM-PUT is also indicated. In all cases cells are treated for 48 h, then reseeded into fresh medium of identical composition and treated for a further 96 h. Viable-cell number refers to those cells which exclude Trypan Blue stain. Comparisons of growth inhibition are best made at 120 h (72 h after reseeding), when control cells are still in exponential growth. Data represent means of two experiments performed in duplicate. For all data points, s.d. (not shown) was not more than 10% of the mean.

Table 1. Relative cellular effects of AMA, DFMO and their combination in cultured L1210 cells

Data represent mean values from at least three separate experiments performed in duplicate; s.d. was not more than 10% of the mean, except when indicated by *, where it was not more than 15% of mean. Abbreviation: CFE, colony-formation efficiency, expressed as percentage of control, which was 25% in 1.5%-methylcellulose semi-solid medium.

Treatment	Time (h)	Growth inhibition (% of control)	CFE (% of control)	Polyamine pools (nmol/10 ⁶ cells)			Decarboxylase activities (nmol of CO ₂ /h per mg of protein)	
				PUT	SPD	SPM	Ornithine	AdoMet
Control	24	100	100	0.43	3.01	0.82	3.98	2.16
	48	100	100	0.37*	2.58	0.80	4.89	2.28
0.1 mM-AMA	24	89	60	5.61	0.85	0.25	41.10	0.31
	48	62	52	5.81	0.61	0.09	67.40*	0.47*
1.0 mM-DFMO	24	88	35	0.01	0.35*	0.87	1.27	3.52
	48	60	11	0.01	0.11	0.94	0.52	7.97*
AMA + DFMO	24	66*	28	0.51	0.91	0.24	3.02*	0.14
	48	31	2	0.39	0.58	0.15	0.80	0.29*

Table 2. Prevention of AMA-induced growth inhibition with exogenous polyamines

Data represent mean values from at least three separate experiments performed in duplicate; s.d. was not more than 10% of the mean, except when indicated by *, where it was not more than 15% of mean. Cells were treated for 48 h in the presence of AMA with or without polyamines, reseeded in fresh media of identical composition and incubated for a further 72 h. CFE is explained in Table 1 legend.

Treatment	Time (h)	Growth inhibition (% of control)	CFE (% of control)	Polyamine pools (nmol/10 ⁶ cells)			Polyamine pools (nmol/culture)		
				PUT	SPD	SPM	PUT	SPD	SPM
Control	48	100	100	0.37	2.58	0.80	3.48	22.28	7.99
0.1 mM-AMA	48	54	60*	5.81	0.52	0.09	34.61	2.41	0.50
+ 2 μ M-SPD	48	95	97*	3.46*	2.54	0.12	22.81	16.00	1.06
+ 1 μ M-SPM	48	92	61*	4.96*	0.26	1.58*	39.00	2.19	10.09
Control	120	100	100	0.30	2.51	0.91	3.75	42.22	14.44
0.1 mM-AMA	120	39	53	5.80	0.35	0.09	35.90	1.74	0.06
+ 2 μ M-SPD	120	96	105	5.74*	1.01	0.04	161.90*	27.95	1.17
+ 1 μ M-SPM	120	27	15	0.37*	0.09	1.55*	3.00	0.79	12.14

slightly more than a single doubling, after which viable cell number actually decreased. These findings are reflected in the colony-formation efficiency, indicating that, by 48 h, the drugs had become highly cytotoxic, with a 98% loss in cell viability (Table 1). In combination with AMA, DFMO prevented the increase in PUT pools seen with AMA alone, but failed to cause further decreases in SPD or SPM, at least at the drug concentrations used in these studies. The finding suggests that the greater growth inhibition seen with DFMO plus AMA compared with AMA alone may be largely attributable to depletion of PUT. This is further suggested in prevention studies with PUT described below.

The effective decrease in total cellular polyamine content, particularly SPD and SPM, affords an opportunity to evaluate the relative importance of individual polyamines in cell growth and viability. At 48 h both exogenously supplied SPD and SPM similarly prevented growth inhibition (Fig. 4), but differed remarkably in their ability to sustain cell viability, as indicated by colony formation efficiency (Table 3). The

consequences of the latter became especially apparent during extended incubations (120 h) in which cells treated with the drug combination plus SPM became growth-inhibited and lost cell viability, whereas those treated with SPD retained control growth characteristics and viability. At 1 μ M, PUT was similar to SPM in supporting cell growth and viability during 120 h incubations, but was significantly less effective during 48 h incubations (i.e. 39% versus 89% of control growth). Interestingly, and in contrast with effects on growth, PUT was much more effective than SPM (59% versus 10%) in supporting cell viability at 48 h, although the two gave similar results at 120 h (7% versus 8%). This is consistent with previous findings (Table 1) indicating that AMA-treated cells (containing PUT) establish more colonies than do DFMO-treated cells (containing SPM) under conditions of comparable growth inhibition. The distinction between PUT and SPM effects on growth and viability at 48 h was observed consistently during numerous experiments performed to test its validity, and may be related to the higher intracellular concentrations

Fig. 4. Growth curves for untreated L1210 cells and for cells treated with the combination of 0.1 mM-AMA and 1 mM-DFMO

Data for cells treated in the presence of 1 μ M-PUT, 2 μ M-SPD and 1 μ M or 3 μ M-SPM are also included. Cell treatment was as described in Fig. 3. Comparisons of growth inhibition are made at 120 h (72 h after reseeding) when control cells are still in exponential growth. Growth in the presence of 1 μ M- or 3 μ M-SPM was nearly identical and is represented as a single curve by the data for 1 μ M-SPM. Data represent the mean of three experiments performed in duplicate. For all data points, s.d. (not shown) was not more than 10% of the mean.

achieved with PUT. Increasing PUT concentrations from 1 to 10 μ M significantly enhanced its ability to support growth and viability (Table 3). This was not found to be the case for SPM, where increasing exogenous concentrations from 1 to 3 μ M had no significant effect on growth (Fig. 4).

Polyamine pool analysis on a per-cell basis indicated that exogenous PUT and SPM effectively maintain their respective intracellular pools while only slightly increasing those of others (Table 3). When assessed as nmol generated per culture (Table 3), SPD was found apparently to undergo limited back-conversion into PUT relative to control, and a small amount of forward conversion into SPM. However, since much larger intracellular pools of PUT were not as effective in supporting cell growth (Table 3, Fig. 4), it is doubtful that this small amount of back-conversion contributes significantly to the growth effects observed with SPD prevention. In cells treated with 1 μ M-PUT, a small amount was converted forward into SPD, but probably not enough to account for its effects on growth, whereas in SPM-treated cells there was no evidence of back-conversion into SPD or PUT. At higher PUT concentrations (10 μ M), an increased amount of SPD was formed. It is not clear whether this small amount is sufficient to account for the enhanced growth seen over that with 1 μ M-PUT.

DISCUSSION

Initial characterization of the cellular effects of AMA is consistent with its intended role as a pyruvate-reactive analogue of decarboxylated AdoMet (Fig. 1) and with its known action as an irreversible inhibitor of AdoMet decarboxylase (Khomutov *et al.*, 1987b). Polyamine pools are perturbed, as would be expected according to pathway considerations; PUT pools were increased remarkably, whereas those of SPD and SPM were depleted. Concomitantly, ornithine decarboxylase activity increased by approx. 10-fold. Although the latter may be due to a compensatory response to decreases in SPD and SPM pools, at least a portion of the increase could be due to enzyme stabilization as a result of complex-formation between the hydroxylamine of AMA and the pyridoxal phosphate at the reactive site of ODC (Fig. 2; Khomutov *et al.*, 1987a). The selectivity of AMA for polyamine biosynthesis was indicated by the ability of exogenous SPD to prevent growth inhibition fully.

Overall, the present findings with AMA are similar to those reported for the AdoMet-analogue inhibitors synthesized by Secrist (1987) and studied in L1210 cells by Pegg *et al.* (1988). Those analogues differ structurally, however, by having a nitrogen atom in place of the sulphur of AMA and, in some cases by having alternative terminal reactive groups. Both analogues are thought to react irreversibly with the active site of AdoMet decarboxylase. Like the enzyme substrate (AdoMet) and product (decarboxylated AdoMet), AMA has a positively charged sulphonium centre, which Pankaskie & Abdel-Monem (1980) have shown to be of critical importance in ensuring maximal complementarity between the inhibitor and the enzyme active site. Those authors report that, during chemical reduction of enzyme-inhibitor complex, sulphonium analogues, but not nitrogen analogues, achieve covalent binding of the enzyme pyruvoyl moiety. This difference may be of minimal consequence in cellular systems, however, since the IC_{50} value (concn. giving 50% inhibition) for AMA (50–100 μ M) is the same as that for the nitrogen analogues (Pegg *et al.*, 1988). Although AMA contains a sulphonium centre and bears structural similarity to decarboxylated AdoMet, we did not find h.p.l.c. evidence (i.e. a SPD analogue) to indicate that it participated as a substrate in the SPD synthase reactions.

An interesting distinction between the effects of AdoMet decarboxylase inhibition by AMA and ornithine decarboxylase inhibition by DFMO was that colony-formation efficiency was consistently more affected by DFMO than by AMA under conditions of comparable growth inhibition. We attribute this to the large PUT pool present in AMA-treated cells when they are seeded into the methylcellulose semi-solid media. Unlike the DFMO-treated cells, which presumably have to generate (by synthesis or back-conversion) a sizeable SPD pool in order to resume growth, the AMA-treated cells are primed with PUT to synthesize substantial quantities of SPD as soon as AMA diffuses away. This difference could be representative of a trend between inhibitors of ornithine decarboxylase and AdoMet decarboxylase. If so, then inhibitors of ornithine decarboxylase will be generally more cytotoxic as single agents than inhibitors of AdoMet decarboxylase.

The combination of AMA with the ornithine decarboxylase inhibitor DFMO affords a unique

Table 3. Effects of exogenous polyamines on growth, colony formation and polyamine pools of cells treated with DFMO plus AMA for 48 and 120 h

Data represent mean values from at least three separate experiments performed in duplicate: s.d. was not more than 10 % of the mean, except when indicated by *, where it was not more than 15 % of mean. Cells were treated for 48 h in the presence of DFMO and AMA with or without polyamines, reseeded in fresh media of identical composition and incubated for a further 72 h. CFE is explained in Table 1.

Treatment	Time (h)	Growth inhibition (% of control)	CFE (% of control)	Polyamine pools (nmol/10 ⁶ cells)			Polyamine pools (nmol/culture)		
				PUT	SPD	SPM	PUT	SPD	SPM
Control	48	100	100	0.37	2.37	0.85	3.48	22.28	7.99
DFMO + AMA	48	32	1	0.48	0.48	0.08	1.44	1.47	0.24
+ 1 μ M-PUT	48	39	59*	2.41	0.39	0.09	8.92	1.44	0.33
+ 10 μ M-PUT	48	63	60*	7.11	0.52	0.06	38.39	2.81	0.32
+ 2 μ M-SPD	48	91	114	0.27	1.95	0.11	2.30	16.49	0.94
+ 1 μ M-SPM	48	89	10	0.19	0.18	1.49*	1.58	1.49	12.37
Control	120	100	100	0.25	2.82	0.96	3.75	42.22	14.44
DFMO + AMA	120	4	1	0.37	0.22	0.11	0.41	0.24	0.12
+ 1 μ M-PUT	120	13	7	2.48	0.34	0.05	9.18	1.26	0.19
+ 10 μ M-PUT	120	43	31	6.12	0.40	0.05	56.48	3.66	0.46
+ 2 μ M-SPD	120	103	98	0.43	1.07	0.08	6.66	16.58	1.24
+ 1 μ M-SPM	120	23	8	0.01	0.05	1.87*	0.04	0.21	7.85

opportunity to evaluate the significance of individual polyamines in cell growth. The drug combination nearly depletes SPD and SPM pools, without an accumulation of PUT. Moreover, the pathway is effectively immobilized by the inhibitors, so that exogenously added PUT or SPD are not converted into SPD and SPM, respectively, allowing the sizes of individual polyamine pools to be controlled and correlated with their effects on cell growth. In utilizing the system, it became obvious that a more definitive assessment of polyamine participation was best attained when incubations were extended beyond 48 h after reseeding. Thus, although both SPD and SPM fully supported cell growth during the first four cell doublings (48 h), only SPD was capable of doing so under extended (72 h) incubations. The reason for the latency of this effect is uncertain.

In contrast with SPD, PUT and SPM were distinctly less effective in maintaining cell growth during the same extended incubation. Their relative inabilities to do so, however, were not attributable to limited intracellular concentrations, since exogenous PUT and SPM concentrations were adjusted to maintain their respective pools at approximately twice those of SPD (in SPD-treated cells). Increasing exogenous SPM 3-fold to the limits of toxicity failed to improve cell growth, while increasing exogenous PUT 10-fold did.

In general, the ability of individual polyamines to support cell growth was paralleled by their ability to sustain cell viability, as determined by the colony-forming efficiency. An interesting exception to this was seen during the initial 48 h treatment with DFMO, AMA and SPM, in which cell growth, but not cell viability, could be maintained at control rates. Whether exogenous SPM decreases conversion of intracellular SPD into SPM during the first 48 h of treatment, or actually fulfils cellular functions on a temporary basis, remains to be determined. After reseeding, however, cell growth fell to values more consistent with the cell viability.

Overall, the implications of these data are as follows: (a) cells exhibit a distinct structural specificity in their polyamine requirement, with SPD ranking as the preferred species; (b) in addition to serving as a precursor to SPD, PUT has growth-supporting capabilities of its own; (c) although SPM is the apparent end-product of the polyamine-biosynthetic pathway, it supports growth less effectively than does its precursor, SPD; (d) the presence of markedly increased PUT pools in AMA-treated cells and SPM pools in DFMO-treated cells probably compromises the anti-proliferative potential of these drugs; and (e) because all three polyamines support cell growth, the greatest anti-proliferative impact may be achieved with strategies which attain depletion of all three polyamine pools.

The apparent critical role of SPD in cell growth and viability is consistent with indications from previous studies with other inhibitors, but which, for reasons described in the Introduction, could not be regarded as conclusive. For example, treatment of cells with either DFMO (Mamont *et al.*, 1978) or the SPD synthase inhibitor *S*-adenosyl-L-thio-1,8-diamino-octane (Pegg *et al.*, 1982), depletes SPD pools and inhibits cell growth. Restoration of SPD reverses the effect. Contrariwise, decreasing SPM pools with *S*-methyl-5'-methylthio-adenosine at concentrations selective for inhibiting SPM synthase has no effect on cell growth (Pegg & Coward, 1985). In all cases, data from these studies imply that SPD is more critical to cell growth than is SPM, but the present studies demonstrate this relationship unambiguously.

The present indication that SPD is apparently the favoured polyamine for cell proliferation brings into question why cells should have SPM and a specific enzyme for its synthesis. Conceivably, it could represent an inactivated metabolite of SPD or, alternatively, serve as a reservoir for SPD which is retrievable via the back-conversion pathway involving spermidine/spermine

acetyltransferase and polyamine oxidase (Seiler *et al.*, 1981; Pegg *et al.*, 1981). Although cultured L1210 cells in the uninduced state have extremely low quantities of the transferase (Libby *et al.*, 1989), there exists evidence (Claverie *et al.*, 1987) that inhibition of polyamine oxidase enhances the antitumour effect of DFMO in animals, indicating that, under appropriate conditions, SPM can be back-converted to replenish SPD pools partially. It is also possible, of course, that the cellular role of SPM is peripheral to cell proliferation, and therefore not assessable by studies using growth inhibition as an endpoint. The fact that SPM pools tend to increase relative to those of SPD as cells stop dividing lends support to this reasoning and to the possibility that SPM may play a role in cell differentiation.

In summary, this study provides the following information: (a) at relatively low concentrations, AMA perturbs polyamine biosynthesis in a manner consistent with inhibition of AdoMet decarboxylase, (b) growth inhibition is attributable to selective inhibition of polyamine biosynthesis, (c) under conditions of comparable growth inhibition AMA is less cytotoxic than the ornithine decarboxylase inhibitor DFMO, apparently because of different effects on polyamine pools; (d) the combined inhibition of ornithine decarboxylase and AdoMet decarboxylase by DFMO and AMA, respectively, provides a near-depletion of the total intracellular polyamine pools; and (e) product-prevention studies with the drug combination indicate that SPD is the preferred polyamine in cell proliferation, and that both PUT and SPM have limited potential in this regard.

We gratefully acknowledge the skilled technical assistance of Mr. Edwin Kelly and the secretarial assistance of Ms. Anne Culligan and Ms. Jessie Crowe. This work was supported by grants CA-22153 and CA-24538 from the National Cancer Institute, Department of Health Education and Welfare, and by the Bilateral Scientific Exchange Program between U.S.A. and U.S.S.R.

REFERENCES

- Artamonova, E. Y., Zavalova, L. L., Khomutov, R. M. & Khomutov, A. R. (1986) *Bioorg. Khim.* **12**, 206–212
- Casero, R. A., Bergeron, R. J. & Porter, C. W. (1984) *J. Cell. Physiol.* **121**, 476–482
- Chang, B. K., Black, O. & Gutman, R. (1984), *Cancer Res.* **44**, 5100–5104
- Claverie, N., Wagner, J., Knodgen, B. & Seiler, N. (1987) *Anticancer Res.* **7**, 765–772
- Khomutov, A. R. & Khomutov, R. M. (1986) *Bioorg. Khim.* **12**, 1662–1674
- Khomutov, R. M., Zavalova, L. L., Shiru, V. I., Artamonova, E. Y. & Khomutov, A. R. (1983) *Bioorg. Khim.* **9**, 130–131
- Khomutov, A. R., Gabibov, A. G., Khurs, E. N., Tolosa, E. A., Shuster, A. M., Goryachenkova, E. V. & Khomutov, R. M. (1987a) in *Biochemistry of Vitamin B₆* (Korpela, T. & Christen, P., eds.), pp. 317–320, Birkhauser Verlag, Basel and Boston
- Khomutov, A. R., Kritsky, A. M., Artamonova, E. Y. & Khomutov, R. M. (1987b) *Nucleosides Nucleotides* **6**, 359–362
- Libby, P. R., Bergeron, R. J. & Porter, C. W. (1989) *Biochem. Pharmacol.*, in the press
- Mamont, P. S., Duchesne, M.-C., Grove, J. & Bey, P. (1978) *Biochem. Biophys. Res. Commun.* **81**, 58–66
- McGovern, K. A., Clark, R. S. & Pegg, A. E. (1986) *J. Cell. Physiol.* **127**, 311–316
- Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. & Vevert, J. P. (1978) *J. Am. Chem. Soc.* **100**, 2551–2553
- Pankaskie, M. & Abdel-Monem, M. M. (1980) *J. Med. Chem.* **23**, 121–127
- Paulin, L. (1986) *FEBS Lett.* **202**, 323–326
- Pegg, A. E. & Coward, J. K. (1985) *Biochem. Biophys. Res. Commun.* **133**, 82–89
- Pegg, A. E., Matsui, I., Seely, J. E., Pritchard, M. L. & Poso, H. (1981) *Med. Biol.* **59**, 327–333
- Pegg, A. E., Tang, K.-C. & Coward, J. K. (1982) *Biochemistry* **21**, 5082–5089
- Pegg, A. E., Jones, D. B. & Secrist, J. A., III (1988) *Biochemistry* **27**, 1408–1415
- Pohjanpelto, P., Virtanen, I. & Holtta, E. (1981) *Nature (London)* **293**, 475–477
- Porter, C. W. & Bergeron, R. J. (1983) *Science* **219**, 1083–1085
- Porter, C. W. & Bergeron, R. J. (1988) *Adv. Enzyme Regul.* **27**, 57–79
- Porter, C. W., Cavanaugh, P. F., Jr., Stolowich, N., Ganis, B., Kelly, E. & Bergeron, R. J. (1985) *Cancer Res.* **45**, 2050–2057
- Secrist, J. A., III (1987) *Nucleosides Nucleotides* **6**, 73–84
- Seiler, N., Bolkenius, F. N. & Rennerz, O. M. (1981) *Med. Biol.* **59**, 334–346
- Shore, P. A. & Cohn, V. H., Jr. (1960) *Biochem. Pharmacol.* **5**, 91–95
- Steglich, C. & Scheffler, I. E. (1982). *J. Biol. Chem.* **257**, 4603–4609
- Tang, K. C., Pegg, A. E. & Coward, J. K. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1371–1377

Received 13 July 1988/29 November 1988; accepted 2 December 1988