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# Effect of Inhibitors of *S*-Adenosylmethionine Decarboxylase on Polyamine Content and Growth of L1210 Cells†

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ABSTRACT: Analogues of *S*-adenosylmethionine that were designed as inhibitors of *S*-adenosylmethionine decarboxylase were tested for their abilities to inhibit the purified enzyme from rat prostate. The most potent inhibitors were 5′-deoxy-5′-[*N*-methyl-*N*-[2-(aminooxy)ethyl]amino]adenosine (MAOEA) and 5′-deoxy-5′-[*N*-methyl-*N*-(3-hydrazinopropyl)amino]adenosine (MHZPA), which had $I_{50}$ values of 400 nM and 70 nM, respectively, when added directly to the assay medium under standard conditions. These compounds were irreversible inactivators of the enzyme, and more than 95% of the activity was lost within 15 min of exposure to 5 μM MAOEA or 0.5 μM MHZPA. Both inhibitors led to a large reduction in the content of decarboxylated *S*-adenosylmethionine in L1210 cells and to a substantial decrease in the production of 5′-(methylthio)adenosine by these cells. These results are consistent with their bringing about an inhibition of *S*-adenosylmethionine decarboxylase activity in the cell which leads to a reduction in the synthesis of spermidine and spermine. Analysis of the polyamine content in L1210 cells exposed to 100 μM MAOEA or 50 μM MHZPA showed that this was the case and that putrescine levels were greatly increased while spermidine and spermine content declined. The combined application of 100 μM MAOEA and 5 mM α-(difluoromethyl)ornithine (an ornithine decarboxylase inhibitor) to L1210 cells completely prevented the synthesis of putrescine, spermidine, and spermine for up to 48 h. The reduction in polyamine content brought about by MHZPA or MAOEA could be partially prevented by the addition of decarboxylated *S*-adenosylmethionine to the culture medium. These inhibitors also brought about an inhibition of cell growth which could be reversed by the addition of spermidine. These results indicate that inhibitors of *S*-adenosylmethionine decarboxylase block cell growth by means of their inhibition of the production of spermidine and that putrescine cannot satisfy the requirement for spermidine in the growth of L1210 cells. They also demonstrate that analogues of *S*-adenosylmethionine are taken up by some mammalian cells and can influence polyamine metabolism. Such compounds have considerable potential as therapeutic agents and for studies of the function of polyamines.

Recent results with inhibitors of ornithine decarboxylase suggest that the polyamine biosynthetic pathway is likely to be an important target for the design of chemotherapeutic agents (Pegg & McCann, 1982; Jänne et al., 1983; Sjoerdsma & Schechter, 1984; Porter & Sufrin, 1986; Pegg, 1986; Schechter et al., 1987). Such inhibitors lead to a virtually complete depletion of spermidine and putrescine but have little effect on spermine and produce an immense increase in the cellular content of decarboxylated *S*-adenosylmethionine. These changes lead to a reduction of cellular growth and alterations in cellular differentiation. The therapeutic value of ornithine decarboxylase inhibitors suggests that inhibitors of other steps in the polyamine biosynthetic pathway may also have pharmacological potential. The combination of inhibitors for different reactions in this pathway may have synergistic effects. Also, inhibitors of each of the steps would permit more

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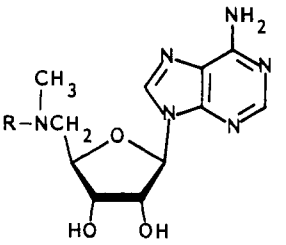
detailed studies on which of the changes described above are responsible for the effects on growth and differentiation when polyamine synthesis is shut off.

Inhibitors of *S*-adenosylmethionine decarboxylase may have advantages as substances with which to affect cellular polyamine content since this particular enzyme catalyzes the rate-limiting step in the production of spermidine and spermine and forms an important branch point which commits *S*-adenosylmethionine to a role in polyamine synthesis (Pegg, 1984a). The inhibitors that are currently available for this enzyme have been reviewed (Williams-Ashmann & Pegg, 1981; Pegg, 1983, 1986; Porter & Sufrin, 1986; Pegg & Williams-Ashman, 1987), but no compounds that are both potent and highly specific have been available. It has been known for some time that methylglyoxal bis(guanylhydrazone) (MGBG)[1] and a variety of its derivatives are strong inhibitors of *S*-adenosylmethionine decarboxylase (Williams-Ashman & Schenone, 1972; Corti et al., 1974), but the specificity of these compounds is in doubt because MGBG has been found to have many other effects on the cell and is known to enter cells by the polyamine transport system (Porter et al., 1981; Jänne et al., 1983; Kallio & Jänne, 1983; Williams-Ashman & Seidenfeld, 1986). Other substances that have been reported as inhibitors of *S*-adenosylmethionine decarboxylase include 1-(aminooxy)-3-aminopropane (Khomutov et al., 1985), guanethidine (Johnson & Taylor, 1980), phenothiazines (Hietala et al., 1983, 1984), and Berenil and Pentamidine (Karvonen et al., 1985; Bitonti et al., 1986), but their specificity has not been established.

At present, the available nucleoside inhibitors for *S*-adenosylmethionine decarboxylase are even more limited. A number of nucleosides that were relatively weak competitive inhibitors have been described (Pankaskie & Abdel-Monem, 1980; Yamanoha & Samejima, 1980; Kolb et al., 1982; Pegg & Jacobs, 1983). The most potent of these was *S*-methyl-5′-(methylthio)adenosine (Kolb et al., 1982; Pegg & Jacobs, 1983), which is clearly not specific since it is a more active inhibitor of spermine synthase (Pegg & Coward, 1985; Pegg et al., 1986). Reports of the synthesis of *S*-adenosylmethionine analogues that might act as irreversible inhibitors have been published by Kolb and Barth (1985), who synthesized 5′-[[3-(aminooxy)propyl]amino]-5′-deoxyadenosine, and by Khomutov et al. (1983), who synthesized *S*-(5′-deoxy-5′-adenosyl)[(methylthio)ethyl]hydroxylamine. Preliminary evidence that the latter compound does inactivate *S*-adenosylmethionine decarboxylase in vitro has been published (Khomutov et al., 1983; Artamonova et al., 1986; Paulin, 1986), but no evidence of its metabolic stability or ability to enter cells and affect their polyamine content was available. Recently, a number of compounds that were designed as active site directed reagents for *S*-adenosylmethionine decarboxylase have been synthesized (J. A. Secrist III, W. B. Forrister, T. H. Moss, E. L. White, and W. M. Shannon, unpublished results). In the present paper, we describe the inhibition of *S*-adenosylmethionine decarboxylase by several of these compounds and show that two of them did inhibit the synthesis of decarboxylated *S*-adenosylmethionine, spermidine, and spermine in L1210 cells. The inhibitors also produced an inhibition of growth which was reversible by the provision of spermidine. These compounds or derivatives are therefore promising candidates for selective inhibition of *S*-adenosylmethionine decarboxylase and evaluation of the role of polyamines in cellular physiology.



MAOEA, R = $CH_2CH_2ONH_2$

MAOPA, R = $CH_2CH_2CH_2ONH_2$

MHCPA, R = $CH_2CH_2CH_2NHC(=O)NHNH_2$

MHTPA, R = $CH_2CH_2CH_2NHC(=S)NHNH_2$

MHZPA, R = $CH_2CH_2CH_2NHNH_2$

FIGURE 1: Structures of compounds tested as inhibitors of *S*-adenosylmethionine decarboxylase.

## Materials and Methods

*Materials.* All biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, MO. *S*-Adenosyl[*carboxy*-$^{14}$C]methionine (50 Ci/mol) and L-[1-$^{14}$C]ornithine (55 Ci/mol) were purchased from New England Nuclear, Boston, MA. DFMO was a generous gift from Dr. P. P. McCann, Merrell Dow Research Institute, Cincinnati, OH. Decarboxylated *S*-adenosylmethionine was a generous gift from Dr. K. Samejima, Josai University, Japan. The syntheses of MAOEA, MAOPA, MHCPA, MHTPA, and MHZPA are reported elsewhere along with preliminary enzyme inhibition data with crude *S*-adenosylmethionine decarboxylase isolated from MRC 5 cells (J. A. Secrist III, W. B. Forrister, T. H. Moss, E. L. White, and W. M. Shannon, unpublished results). The structures of these inhibitors, which were all obtained as the sulfate salts, are given in Figure 1.

*Assay of S-Adenosylmethionine Decarboxylase, Ornithine Decarboxylase, and 5′-(Methylthio)adenosine Phosphorylase.* Prostatic *S*-adenosylmethionine decarboxylase was purified as described by Shirahata et al. (1985). Activity was measured by measuring the release of $^{14}CO_2$ from *S*-adenosyl[*carboxy*-$^{14}$C]methionine (Pegg, 1984b). The assay medium contained 3 mM putrescine, 1.25 mM dithiothreitol, 50 mM sodium phosphate buffer, pH 7.5, and 0.2 mM *S*-adenosyl-[*carboxy*-$^{14}$C]methionine (4 Ci/mol) in a total volume of 0.25 mL. Crude extracts from L1210 cells containing *S*-adenosylmethionine decarboxylase were prepared as described for SV-3T3 cells by Pegg (1984b). The L1210 cells were grown for 48 h in the presence of 0.4 mM *S*-methyl-5′-(methylthio)adenosine in order to increase the content of *S*-adenosylmethionine decarboxylase (Pegg et al., 1987). Ornithine decarboxylase was purified from mouse kidney and assayed as described by Seely et al. (1982). 5′-(Methylthio)adenosine phosphorylase activity using 5′-(methylthio)-adenosine, MHZPA, or MAOEA as substrates was measured by determination of the production of adenine as described by Savarese et al. (1981).

*Cell Culture.* SV-3T3 cells were grown in Dulbecco's modified Eagle's medium with 3% horse serum/2% fetal calf

[1] Abbreviations: MGBG, methylglyoxal bis(guanylhydrazone); DFMO, α-(difluoromethyl)ornithine; MAOEA, 5′-deoxy-5′-[*N*-methyl-*N*-[2-(aminooxy)ethyl]amino]adenosine; MAOPA, 5′-deoxy-5′-[*N*-methyl-*N*-[3-(aminooxy)propyl]amino]adenosine; MHCPA, 5′-deoxy-5′-[*N*-methyl-*N*-[3-(hydrazinecarboxamido)propyl]amino]-adenosine; MHTPA, 5′-deoxy-5′-[*N*-methyl-*N*-[3-(hydrazinethiocarboxamido)propyl]amino]adenosine; MHZPA, 5′-deoxy-5′-[*N*-methyl-*N*-(3-hydrazinopropyl)amino]adenosine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

serum as described by Pegg (1984b). L1210 cells were grown in suspension culture in RPMI-1640 medium containing 10% NuSerum (Collaborative Research, Inc., Lexington, MA) as described by Pera et al. (1986). In experiments in which polyamines were added to the cultures, 1 mM aminoguanidine was added to the medium to inhibit the action of oxidases present in the serum.

*Polyamine Analysis.* The cells were then harvested and extracted with 10% (w/v) trichloroacetic acid, and aliquots were used for the determination of polyamine content on an amino acid analyzer with fluorescence detection (Pegg, 1984b; Pegg & Coward, 1985). Results are expressed as nanomoles of polyamine content per $10^6$ cells or nanomoles of polyamine content per culture.

*Determination of the Intracellular Content of Decarboxylated S-Adenosylmethionine and the Production of 5′-(Methylthio)adenosine.* Aliquots of the cell culture medium were deproteinized by the addition of an equal volume of 10% perchloric acid followed by centrifugation to remove the protein. These aliquots were used to determine the production of 5′-(methylthio)adenosine by the cultures of L1210 cells, which lack 5′-(methylthio)adenosine phosphorylase (Williams-Ashman et al., 1982; Schlenk, 1983). The L1210 cells excrete 5′-(methylthio)adenosine into the medium, and its intracellular content was negligible compared to the total present in the medium. The content of *S*-adenosylmethionine and its decarboxylated derivative was determined with cell extracts, and these nucleosides were not detected in the medium. The cell extracts were prepared as for polyamine analysis. The content of 5′-(methylthio)adenosine, *S*-adenosylmethionine, and decarboxylated *S*-adenosylmethionine was determined with an ion-pair reversed-phase separation (Seiler, 1983). The aliquots (up to 0.25 mL) were mixed with 0.45 mL of buffer A (0.1M sodium acetate, 0.01 M sodium octanesulfonate, pH 4.5), centrifuged, and applied to a column (Beckman Ultrasphere ODS 5 micron; 4.6 mm × 25 cm protected by a 4.6 mm × 4 cm guard column of ODS-5S from Bio-Rad) equilibrated with a mixture of 90% buffer A and 10% buffer B (10 parts of 0.2 M sodium acetate and 0.01 M sodium octanesulfonate, pH 4.5; 3 parts of acetonitrile). The column was then eluted with a linear gradient of 90% buffer A/10% buffer B to 100% buffer B over 40 min at a flow rate of 1 mL/min at 35 °C. The eluate was monitored at 254 nm, and the amounts of the nucleosides were calculated from the peak heights with standard curves constructed with known amounts of the authentic compounds. The results for *S*-adenosylmethionine and decarboxylated *S*-adenosylmethionine were expressed as nanomoles of the nucleosides present per milligram of total protein in the cell extracts. Protein was determined by the method of Bradford (1976). The amount of 5′-(methylthio)adenosine was expressed as nanomoles per culture.

## RESULTS

*Inhibition of S-Adenosylmethionine Decarboxylase in Vitro.* The compounds were tested as inhibitors of the activity of purified *S*-adenosylmethionine decarboxylase from rat prostate and of a crude cell preparation from L1210 cells containing *S*-adenosylmethionine decarboxylase. When added directly to an assay mixture containing 0.2 mM *S*-adenosylmethionine, all of the compounds inhibited its decarboxylation (Figure 2). MHZPA was the most potent having an $I_{50}$ value of 0.07 $\mu$M. MAOEA and MAOPA were also strong inhibitors giving $I_{50}$ values of 0.4 $\mu$M and 0.9 $\mu$M, respectively. MHTPA ($I_{50}$ of 70 $\mu$M) and MHCPA ($I_{50}$ of 50 $\mu$M) were less active. Increasing the substrate concentration to 2.8 mM made little

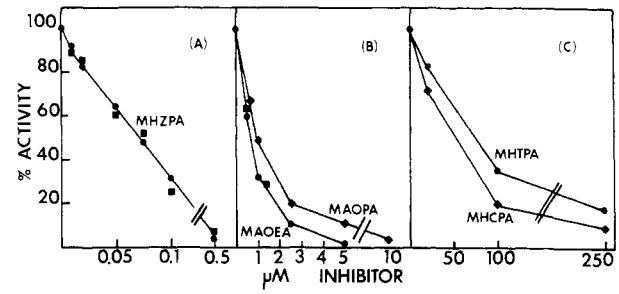



FIGURE 2: Inhibition of *S*-adenosylmethionine decarboxylase. The effects of MHZPA (panel A), of MAOEA and MAOPA (panel B), and of MHTPA and MHCPA (panel C) on the decarboxylation of *S*-adenosylmethionine were tested by adding the concentrations shown to the standard assay mixture which contained either prostatic (●, ◆) or L1210 cell (■) *S*-adenosylmethionine decarboxylase, 0.2 mM *S*-adenosyl[*carboxy*-$^{14}$C]methionine, 50 mM sodium phosphate, pH 7.5, 1.25 mM dithiothreitol, and 3 mM putrescine. After 30 min at 37 °C, the amount of $^{14}CO_2$ produced was measured and expressed as the percentage of that given by the enzyme in the absence of the inhibitors (% activity).

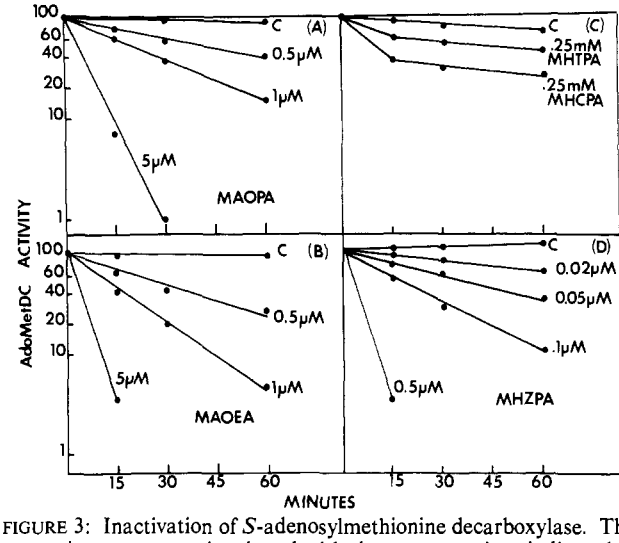



FIGURE 3: Inactivation of *S*-adenosylmethionine decarboxylase. The prostatic enzyme was incubated with the concentrations indicated of MAOPA (panel A), MAOEA (panel B), MHTPA or MHCPA (panel C), or MHZPA (panel D), for the time shown in the presence of 3 mM putrescine, 1.25 mM dithiothreitol, and 50 mM sodium phosphate, pH 7.5. Samples were then removed and diluted 25-fold with the same buffer, and aliquots were used for the assay of *S*-adenosylmethionine decarboxylase activity as described in Figure 2.

difference to the inhibitory effect of MAOEA and MHZPA when assayed under these conditions (results not shown). There was no significant difference in sensitivity to MAOEA and MHZPA between the prostatic and the L1210 *S*-adenosylmethionine decarboxylase (Figure 2).

A more detailed study of the inhibition was carried out by incubating the enzyme with the inhibitors in the absence of the substrate and determining the amount of enzymic activity remaining. The determination of activity was carried out after a large dilution of the mixture of enzyme and inhibitor so that the residual free inhibitor in the assay was negligible. As shown in Figure 3, MAOEA, MAOPA, and MHZPA were potent, irreversible inactivators of *S*-adenosylmethionine decarboxylase leading to a time- and concentration-dependent inactivation of the enzyme when assayed under these conditions. The half-life of the *S*-adenosylmethionine decarboxylase was about 12 min in the presence of 1 $\mu$M MAOEA, about 22 min in the presence of 1 $\mu$M MAOPA, and about 20 min in the presence of 0.1 $\mu$M MHZPA (Figure 3). MHTPA and MHCPA were much less active but also led to the loss of activity. In all cases, the loss of activity was not reversible

Table I: Effect of MAOEA and MHZPA on ODC Activity[a]

| concn added | % ODC activity left | concn added | % ODC activity left |
|---|---|---|---|
| 0 | 100 | 100 μM MAOEA | 67 |
| 5 μM MHZPA | 83 | 150 μM MAOEA | 48 |
| 50 μM MHZPA | 18 | 250 μM MAOEA | 30 |
| 200 μM MHZPA | 2 | | |

[a] Mouse kidney ornithine decarboxylase was assayed by incubation in an assay mix containing the inhibitor concentration shown, 40 μM pyridoxal phosphate, 50 mM Tris-HCl, pH 7.5, 0.2 mM L-[1-$^{14}$C]-ornithine, and 2.5 mM dithiothreitol. After 30-min incubation at 37 °C, the amount of $^{14}CO_2$ produced was measured and expressed as the percentage of that given by the enzyme in the absence of the inhibitors.

Table II: Effect of *S*-Adenosylmethionine Decarboxylase Inhibitors on Polyamine Levels in SV-3T3 Cells in Short-Term Experiment[a]

| addition | cell number × $10^{-6}$ | polyamine content (nmol/culture) putrescine | spermidine | spermine | total |
|---|---|---|---|---|---|
| control, 24 h | 2.4 | 1.7 | 7.1 | 2.2 | 11.0 |
| control, 30 h | 3.4 | 2.7 ± 0.2 | 11.5 ± 1.3 | 3.1 ± 0.4 | 17.3 |
| 100 μM MAOEA | 3.3 | 4.7 ± 0.7 | 8.5 ± 1.3 | 2.0 ± 0.3 | 15.2 |
| 250 μM MAOEA | 3.5 | 6.2 ± 0.6 | 8.0 ± 0.7 | 1.8 ± 0.2 | 16.0 |
| 5 mM DFMO | 3.5 | <0.2 | 8.3 ± 0.5 | 3.5 ± 0.4 | 11.8 |

[a] Total polyamine is the sum of putrescine, spermidine, and spermine. Results are shown as mean ± SE for at least four estimations. Inhibitors were added at 24 h and cells harvested at 30 h.

if the enzyme was dialyzed overnight prior to assay (results not shown).

MAOEA and MHZPA were also tested as potential inhibitors of ornithine decarboxylase with the purified enzyme from mouse kidney in an assay medium which contained 0.2 mM L-ornithine and 40 μM pyridoxal phosphate. Both compounds did produce inhibition of this activity with MHZPA being more active, but more than 100 times higher levels were needed than for the inhibition of *S*-adenosylmethionine decarboxylase (Table I).

*Effect of Inhibitors of S-Adenosylmethionine Decarboxylase on Polyamine Content of L1210 Cells and SV-3T3 Cells.* Initial experiments were carried out in which 75 μM MHTPA, MHCPA, MAOPA, and MAOEA were each added to exponentially growing cultures of SV-3T3 cells, and the polyamine content was determined 72 h later. There was no significant effect on the polyamine levels (results not shown). Further experiments on the SV-3T3 cells were carried out with MAOEA at 100 and 250 μM. The inhibitor was added after 24 h of growth, and cells were harvested 6 h later

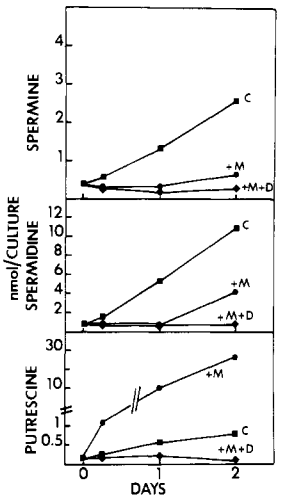



FIGURE 4: Effect of MAOEA on polyamine content of L1210 cell cultures. L1210 cells were grown for the time shown in the presence of 100 μM MAOEA (+M, ●), with 100 μM MAOEA plus 5 mM DFMO (+M+D, ◆), or with no addition (C, ■). The total polyamine present in the cell cultures is shown.

(Table II). Under these conditions there was a significant effect of MAOEA on the content of polyamines in the cells. Putrescine was increased by the inhibitor, and the spermidine and spermine content was reduced. These changes are consistent with it acting to block polyamine synthesis at the *S*-adenosylmethionine decarboxylase step (Table II). However, at longer times (results not shown) the effects of the inhibitor were no longer apparent. These results suggest that MAOEA does enter these cells and inhibit polyamine synthesis but that its effects are transient probably due to its metabolism or decomposition.

Similar experiments were carried out by studying the effect of MAOEA on polyamines in L1210 cells (Figure 4 and Table III). MAOEA (100 μM) had a much more striking effect on the polyamine content in L1210 cells than in SV-3T3 cells. As shown in Figure 4, the increase in spermidine and spermine which occurred over a 24-h period was blocked completely by MAOEA, and there was an extremely large increase in the content of putrescine. When 5 mM DFMO was also included in the culture, this increase in putrescine was also prevented, and there was no increase in cellular polyamine in the culture at all (Figure 4). Although the inhibitory effects of MAOEA on spermidine and spermine synthesis were still apparent at 48 h, there was some indication that this effect might be wearing off at the later time (Figure 4).

Table III shows the effect of 100 μM MAOEA and 50 μM MHZPA on polyamines in the L1210 cells expressed as polyamine per cell. There was a very large rise in cellular

Table III: Polyamine Levels in L1210 Cells Treated with MAOEA and MHZPA[a]

| time of treatment (h) | cell number × $10^{-5}$/mL | polyamine content (nmol/$10^6$ cells) putrescine | spermidine | spermine |
|---|---|---|---|---|
| 6 | 0.8 | 0.45 | 3.45 | 1.45 |
| 6 + 100 μM MAOEA | 0.8 | 2.66 | 2.24 | 1.32 |
| 24 | 3.0 | 0.39 ± 0.05 | 3.11 ± 0.32 | 0.91 ± 0.11 |
| 24 + 100 μM MAOEA | 1.9 | 10.7 ± 3.2 | 0.79 ± 0.17 | 0.33 ± 0.10 |
| 24 + 100 μM MAOEA + 100 μM decarboxylated *S*-adenosylmethionine | 2.3 | 6.1 | 1.67 | 0.32 |
| 24 + 50 μM MHZPA | 2.2 | 8.7 | 1.02 | 0.31 |
| 48 | 6.3 | 0.26 | 3.48 | 0.82 |
| 48 + 100 μM MAOEA | 5.0 | 10.0 | 1.94 | 0.23 |
| 48 + 50 μM MHZPA | 4.3 | 9.8 | 1.22 | 0.21 |
| 24* | 2.9 | 0.40 ± 0.11 | 3.11 ± 0.69 | 0.83 ± 0.21 |
| 24* + 100 μM MAOEA | 3.3 | 7.6 ± 0.5 | 0.66 ± 0.10 | 0.27 ± 0.06 |

[a] The cells were grown for 24 h prior to the addition of the drug. The values are shown as mean ± SD for four to nine estimations. Treatment marked * were for cells grown in 3% horse serum/2% fetal calf serum.

Table IV: Polyamine Levels in L1210 Cells Treated with Varying Doses of MHZPA[a]

| time of treatment (h) | cell number × $10^{-5}$/mL | polyamine content (nmol/$10^6$ cells) putrescine | spermidine | spermine |
|---|---|---|---|---|
| 24 | 3.3 | 0.84 ± 0.03 | 3.84 ± 0.11 | 0.82 ± 0.17 |
| 24 + 10 μM MHZPA | 3.4 | 3.64 ± 0.17 | 3.33 ± 0.15 | 0.48 ± 0.09 |
| 24 + 25 μM MHZPA | 2.3 | 6.04 ± 0.25 | 1.80 ± 0.34 | 0.37 ± 0.08 |
| 24 + 50 μM MHZPA | 2.6 | 7.03 ± 0.62 | 1.18 ± 0.12 | 0.39 ± 0.04 |
| 24 + 100 μM MHZPA | 2.6 | 6.94 ± 0.02 | 1.10 ± 0.07 | 0.37 ± 0.04 |
| 24 + 50 μM + 5 μM spermidine | 3.0 | 0.08 | 3.69 | 0.32 |
| 48 | 7.8 | 0.34 ± 0.08 | 2.60 ± 0.47 | 0.83 ± 1.1 |
| 48 + 10 μM MHZPA | 5.4 | 2.18 ± 0.18 | 2.85 ± 0.29 | 0.36 ± 0.04 |
| 48 + 25 μM MHZPA | 5.0 | 4.16 ± 0.29 | 1.83 ± 0.15 | 0.17 ± 0.03 |
| 48 + 50 μM MHZPA | 4.3 | 5.81 ± 0.33 | 1.00 ± 0.14 | 0.07 ± 0.03 |
| 48 + 100 μM MHZPA | 3.6 | 6.14 ± 0.88 | 0.75 ± 0.14 | 0.09 ± 0.02 |
| 48 + 50 μM MHZPA + 5 μM spermidine | 6.2 | 0.31 | 3.96 | 0.15 |
| 72 | 21.3 | 0.03 ± 0.01 | 2.46 ± 0.12 | 1.03 ± 0.08 |
| 72 + 10 μM MHZPA | 21.7 | 0.12 ± 0.02 | 2.67 ± 0.13 | 0.56 ± 0.04 |
| 72 + 25 μM MHZPA | 17.5 | 1.31 ± 0.12 | 3.05 ± 0.60 | 0.40 ± 0.14 |
| 72 ± 50 μM MHZPA | 11.3 | 4.92 ± 0.18 | 2.40 ± 0.15 | 0.24 ± 0.02 |
| 72 + 100 μM MHZPA | 10.9 | 6.82 ± 0.60 | 1.46 ± 0.21 | 0.11 ± 0.05 |
| 72 + 50 μM MHZPA + 5 μM spermidine | 20.1 | 1.48 | 3.25 | 0.24 |

[a] MHZPA (1 μM) was also tested but had no significant effect on the polyamine content. The drug was added at 6 h after reseeding the cells.

putrescine and a substantial fall in spermidine and spermine within a 24-h period in response to either compound. The inhibitory effect of MAOEA on the spermidine content of L1210 cells was partially prevented by the addition of 100 μM decarboxylated *S*-adenosylmethionine (Table III). The inhibitory effect of MAOEA on spermine content was still maximal at 48 h, but spermidine levels were beginning to recover (Table III).

MHZPA at 50 μM had very similar effects as 100 μM MAOEA except that the effect on spermidine may be more long lived (Table III). A more detailed dose-response study of the effects of MHZPA on polyamines in L1210 cells is shown in Table IV. The increase in putrescine and decrease in spermidine and spermine were clearly dose dependent, but 50 and 100 μM MHZPA had similar effects except at 72 h when the higher dose was more effective (Table IV). This may be due to the decomposition of the inhibitor over prolonged exposure. The addition of 5 μM spermidine to the cells treated with 50 μM MHZPA abolished the rise in putrescine and restored cellular spermidine content but did not restore spermine (Table IV).

The reason why the effects of MAOEA were much longer lived in the L1210 cells than in SV-3T3 cells is not clear at present. Most of the experiments with L1210 cells were carried out with cells grown in the presence of NuSerum, but the difference is not due to the degradation of the MAOEA by the serum used for SV-3T3 cells because the inhibitory effect was still manifest when the L1210 cells were grown in 3% horse serum/2% fetal calf serum, which is the serum that was used to support the growth of SV-3T3 cells (Table III, bottom two rows). One possibility is that MHZPA and MAOEA are degraded by 5′-(methylthio)adenosine phosphorylase since this enzyme is present in SV-3T3 cells and absent in L1210 (Williams-Ashman et al., 1982; Schlenk, 1983). Attempts were made to investigate whether MAOEA or MHZPA was a substrate for 5′-(methylthio)adenosine phosphorylase by incubating them at 0.2 mM concentrations with crude rat liver extracts containing this enzyme and looking for the production of adenine with the HPLC assay described by Savarese et al. (1981). No reaction was found, but the sensitivity of this method was such that a reaction rate of 7% of the rate of cleavage of 5′-(methylthio)adenosine itself, which was used as a positive control, was needed for detection.

Table V: Effect of MAOEA and MHZPA on Decarboxylated *S*-Adenosylmethionine Content of L1210 Cells[a]

| hours of treatment | inhibitor | *S*-adenosylmethionine (nmol/mg) | decarboxylated *S*-adenosylmethionine (nmol/mg) |
|---|---|---|---|
| | Experiment A | | |
| 6 | none | 1.6 | 0.19 |
| 6 | +100 μM MAOEA | 2.4 | <0.03 |
| 24 | none | 1.4 | 0.20 |
| 24 | + 100 μM MAOEA | 1.8 | 0.03 |
| 24 | + 50 μM MHZPA | ND | <0.03 |
| 48 | none | 1.4 | 0.15 |
| 48 | + 100 μM MAOEA | 1.2 | 0.06 |
| | Experiment B | | |
| 24 | none | 1.6 ± 0.3 | 0.14 ± 0.03 |
| 24 + 5 mM DFMO | none | 1.5 ± 0.2 | 1.9 ± 0.3 |
| 24 + 5 mM DFMO | + 100 μM MAOEA | 1.3 ± 0.2 | 0.16 ± 0.03 |
| 24 + 5 mM DFMO | + 50 μM MHZPA | ND | <0.03 |
| 48 + 5 mM DFMO | none | 1.2 ± 0.2 | 2.1 ± 0.2 |
| 48 + 5 mM DFMO | + 100 μM MAOEA | 1.0 ± 0.1 | 0.18 ± 0.04 |
| 48 + 5 mM DFMO | + 50 μM MHZPA | ND | <0.03 |

[a] Results shown for experiment A are the mean of three values per point. Results shown for experiment B in which the L1210 cells were grown in the presence of 5 mM DFMO are mean ± SD for four to five estimations. ND, not determined.

*Effect of Inhibitors of S-Adenosylmethionine Decarboxylase on Content of Decarboxylated S-Adenosylmethionine in L1210 Cells and SV-3T3 Cells.* Additional direct proof that MAOEA and MHZPA affected the activity of *S*-adenosylmethionine decarboxylase in L1210 cells was obtained by measuring the content of its immediate product, decarboxylated *S*-adenosylmethionine (Table V). A significant reduction of more than 80% was observed in cells treated for 6 and 24 h and a 60% reduction in cells treated for 48 h with MAOEA (Table V, experiment A). MHZPA was even more effective, and no decarboxylated *S*-adenosylmethionine was detected. However, these measurements are not very accurate because the decarboxylated *S*-adenosylmethionine level in the control cells is close to the limit of detection. A more accurate determination was obtained by looking at the effects of the inhibitors in cells also treated with DFMO since treatment with DFMO alone produces a large increase in decarboxylated *S*-adenosylmethionine (Table V, experiment B). MAOEA (100 μM) and MHZPA (50 μM) treatment for up to 48 h clearly reduced decarboxylated *S*-adenosyl-

Table VI: Effect of MHZPA on Production of 5′-(Methylthio)adenosine by L1210 Cells[a]

| hours of treatment | 5′-(methylthio)adenosine (nmol/culture) | |
|---|---|---|
| | control | +50 μM MHZPA |
| 24 | 2.4 ± 0.3 | 0.59 ± 0.12 |
| 48 | 11.8 ± 0.9 | 1.8 ± 0.4 |
| 72 | 39.7 ± 1.8 | 5.1 ± 0.5 |

[a] The medium alone did not contain any detectable content of 5′-(methylthio)adenosine prior to cell culture, and control experiments in which 5′-(methylthio)adenosine was added to the medium in the absence of cells indicated that this nucleoside was not degraded by enzymes present in the serum to any significant extent.

methionine levels by more than 90% in these cells.

*Effect of MHZPA on the Production of 5′-(Methylthio)-adenosine by L1210 Cells.* L1210 cells lack 5′-(methylthio)adenosine phosphorylase (Pegg & McCann, 1982; Schlenk, 1983; Pegg & Williams-Ashman, 1987) and are therefore unable to degrade this nucleoside which is produced in the spermidine and spermine synthase reactions. As previously reported for other cell lines lacking 5′-(methylthio)-adenosine phosphorylase (Kamatani & Carson, 1980, 1981), it was found that the 5′-(methylthio)adenosine that was made by the L1210 cells was excreted into the cell culture medium. The amount found in the cells was negligible compared to the high level found in the medium (results not shown). The addition of 75 μM MHZPA produced a substantial inhibition of the production of 5′-(methylthio)adenosine by the L1210 cells (Table VI). This inhibition amounted to 75–87%. This is slightly less than the reduction in decarboxylated *S*-adenosylmethionine seen in Table V, but it should be noted that, although it is clear that the majority of the 5′-(methylthio)adenosine is derived as a byproduct of polyamine synthesis, small amounts may be generated by metabolic reactions other than the aminopropyltransferases (Yamanaka et al., 1986).

*Effect of MAOEA and MHZPA on Growth of L1210 Cells.* Addition of 100 μM MAOEA or 50 μM MHZPA to L1210 cells had a significant effect on the growth rate, which became apparent at times after 24 h of exposure (Figure 5). This is consistent with the concept that it is the depletion of spermidine and/or spermine which is responsible for this reduction. Since the addition of 5 μM spermidine restored a normal growth rate (Figure 5) and also restored the intracellular spermidine content but not the spermine content (Table IV), these results suggest that replacement of the spermidine is sufficient for growth. However, addition of 5 μM spermine was also able to reverse the growth inhibition by 50 μM MHZPA (results not shown), and it appears that either polyamine is able to support growth. Decarboxylated *S*-adenosylmethionine at 100 μM only partially restored the growth rate (results not shown), but as indicated in Table III, this only partly overcame the reduction in spermidine synthesis. Higher doses of decarboxylated *S*-adenosylmethionine could not be tested owing to their toxicity toward the control cells in the absence of inhibitors. Addition of putrescine (5–50 μM) had no effect on the inhibition of growth by MHZPA or MAOEA.

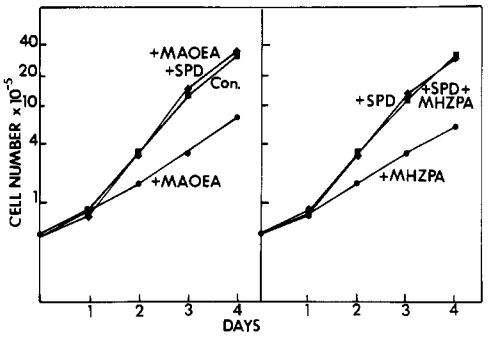



FIGURE 5: Effect of MAOEA and MHZPA on growth of L1210 cell cultures. The left panel shows results for the cells grown in the absence of additions (Con., ■), in the presence of 100 μM MAOEA (+MAOEA, ●), and in the presence of 5 μM spermidine + 100 μM MAOEA (+MAOEA+SPD, ◆). The right panel shows results for cells grown in the presence of 5 μM spermidine (+SPD, ◆), in the presence of 50 μM MHZPA (+MHZPA, ●), and in the presence of 5 μM spermidine + 50 μM MHZPA (+SPD+MHZPA, ■).

## Discussion

It is apparent from our results that both MHZPA and MAOEA are promising candidates for use as specific inhibitors of *S*-adenosylmethionine decarboxylase. These compounds are potent irreversible inactivators of the enzyme in vitro presumably because they resemble the *S*-adenosylmethionine substrate sufficiently to bind the active site. [Previous workers have found that competitive inhibitors of *S*-adenosylmethionine decarboxylase can be synthesized with a nitrogen atom in place of the sulfur showing that this replacement does not prevent binding at the active site (Pankaskie & Abdel-Monem, 1980; Kolb et al., 1982).] Once bound, the present inhibitors may form a covalent bond with the enzyme-bound pyruvate that is known to be the prosthetic group of *S*-adenosylmethionine decarboxylase. The rapid and permanent inactivation of the enzyme which they bring about should lead to the depletion of the cellular stores of decarboxylated *S*-adenosylmethionine and prevent the synthesis of spermidine and spermine provided that they are able to enter cells. Our results suggest that this is indeed the case in L1210 cells where the expected falls in the content of decarboxylated *S*-adenosylmethionine, 5′-(methylthio)adenosine, spermidine, and spermine did occur.

The mechanism by which these inhibitors are taken up is not known, and such positively charged nucleosides might be expected to be quite poor at passing through the cell membrane. However, it should be pointed out that there is good evidence that other similarly charged nucleosides [e.g., *S*-adenosyl-1,8-diamino-3-thiooctane and *S*-methyl-5′-(methylthio)adenosine] are taken up by mammalian cells (Pegg & Coward, 1985; Pegg, 1986). It is possible that the presence of a nitrogen atom in place of the sulfur has some advantages for the uptake of MHZPA and MAOEA over the corresponding sulfonium derivative since this would be expected to be even more strongly charged, but direct comparisons between these compounds will be needed to establish this. MHZPA and MAOEA are comparable to the sulfonium compound *S*-(5′-deoxy-5′-adenosyl)[(methylthio)ethyl]-hydroxylamine (Khomutov et al., 1983; Artamonova et al., 1986; Paulin, 1986) in their abilities to irreversibly inactivate *S*-adenosylmethionine decarboxylase, and it remains to be seen how the compounds compare in their cellular uptake and metabolic stability.

The presence of the *N*-methyl group in MAOEA, MAOPA, and MHZPA may increase their resistance to degradation and lessen their inhibitory action toward other enzymes such as ornithine decarboxylase and diamine oxidase. 5′-[[3-(Aminooxy)propyl]amino]-5′-deoxyadenosine, which lacks the *N*-methyl substituent, was almost as active as its methylated equivalent, MAOPA, as an inhibitor of AdoMetDC in vitro, but this compound was considerably less effective than MHZPA and MAOEA in reducing the content of spermidine and spermine in L1210 cells (unpublished observations).

The possibility that these nucleosides are subject to rapid metabolic degradation may limit their effectiveness unless modifications can be made to minimize this effect. The transient nature of the decline in polyamine synthesis brought

about by MAOEA in SV-3T3 cells and the apparent fall off in the effectiveness of MAOEA and MHZPA at longer times than 2 days in the L1210 cells suggest that these drugs are indeed degraded. At present we have no clear proof of the means by which this decomposition occurs, but the striking difference between the effects in the two cell lines tested provides a useful system to obtain further information. Since L1210 cells are deficient in 5′-(methylthio)adenosine phosphorylase activity (Williams-Ashman et al., 1982; Pegg & McCann, 1982; Schlenk, 1983; Pegg & Williams-Ashman, 1987) and SV-3T3 cells have high levels of this enzyme, it is conceivable that the inhibitors are degraded by 5′-(methylthio)adenosine phosphorylase. However, this seems unlikely since all of the known 5′-(methylthio)adenosine phosphorylase substrates lack polar groups attached to the 5′-deoxyadenosine (Schlenk, 1983; Pegg & Williams-Ashman, 1987), and we were unable to detect any degradation by rat liver 5′-(methylthio)adenosine phosphorylase. However, the assay method used was not sufficiently sensitive that this possibility can be totally ruled out, and further studies with radioactive inhibitors will be needed when these become available.

Another possibility is that the compounds are acetylated, but we were unable to find any reaction of them when incubated with acetyl-CoA and either spermidine/spermine-$N^1$-acetyltransferase or the histone/spermidine acetyltransferase, which is known to acetylate decarboxylated *S*-adenosylmethionine (Pegg, 1986). If metabolites can be isolated, it may prove possible to improve the effectiveness of the inhibitors by making chemical modifications that preserve their inhibitory action toward *S*-adenosylmethionine decarboxylase but reduce the potential for metabolic degradation.

Our results with MHZPA and MAOEA indicate that these compounds are themselves useful for studies of the regulation of polyamine biosynthesis and the role of polyamines in the growth of L1210 cells. Both compounds cause an inhibition of cell growth which is reversible by the addition of spermidine or spermine but not by putrescine. These results strongly suggest that it is the depletion of spermidine and spermine which leads to the reduction in cell growth and that putrescine (which is greatly increased by the *S*-adenosylmethionine decarboxylase inhibitors) cannot itself support growth except as a precursor of spermidine. Since these inhibitors have similar inhibitory effects toward growth as DFMO while having an opposite effect on the content of decarboxylated *S*-adenosylmethionine, it appears that it is the depletion of spermidine and spermine rather than the accumulation of decarboxylated *S*-adenosylmethionine which causes the inhibition of cell growth.

As shown in Table III, the addition of 100 $\mu$M decarboxylated *S*-adenosylmethionine partially reversed the reduction in cellular spermidine and spermine and growth inhibition by MAOEA. This effect was not complete, but the uptake of decarboxylated *S*-adenosylmethionine is likely to be poor, and only 50% of the material added, which is a mixture of the stereoisomers at the sulfonium pole, is likely to be biologically active. Higher concentrations of decarboxylated *S*-adenosylmethionine could not be used in the experiments because these had toxic effects towards control cells. This toxicity may be due to oxidation of the amino group of decarboxylated *S*-adenosylmethionine by oxidases in the culture medium, but this possibility was not investigated further.

Finally, it should be noted that the increase in putrescine brought about by MAOEA or MHZPA is greater than the decrease in spermidine and spermine. This result suggests that in addition to preventing the use of putrescine as a precursor of the polyamines its synthesis is enhanced as a consequence of polyamine depletion. Although MAOEA and, to a slightly greater extent, MHZPA do inhibit ornithine decarboxylase in vitro (Table I), it is clear from the large buildup of putrescine in cells treated with these inhibitors that they are unlikely to have any significant inhibitory effect on the enzyme in the cell at the doses used in these experiments. Several laboratories have reported that ornithine decarboxylase activity is reduced in response to the administration of putrescine, spermidine, or spermine and is increased in cells depleted of these amines by ornithine starvation or administration of competitive ornithine decarboxylase inhibitors (Pegg & McCann, 1982; Kahana & Nathans, 1985; Dircks et al., 1986; Hölttä & Pohjanpelto, 1986; McConlogue et al., 1986; Porter & Sufrin, 1986). Our results suggest that putrescine itself is not as effective at bringing about the reduction of ornithine decarboxylase activity as spermidine or spermine. Further studies in which the changes in ornithine decarboxylase activity in cells treated with these inhibitors are measured directly should be useful in understanding the regulation of this enzyme by polyamines.

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# Calcium-Induced Phase Separation Phenomena in Multicomponent Unsaturated Lipid Mixtures†

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ABSTRACT: The ability of calcium to induce phase separation in multicomponent lipid mixtures containing various unsaturated species of acidic and neutral phospholipids has been investigated by $^{31}P$ NMR, $^{2}H$ NMR, and small-angle X-ray diffraction techniques. It is shown that, in unsaturated (dioleoyl-) phosphatidylglycerol (PG)/phosphatidylethanolamine (PE) (1:1) and phosphatidic acid (PA)/phosphatidylcholine (PC) (1:1) mixtures, calcium is unable to induce lateral phase separation of the acidic and neutral lipids and that all the lipids adopt a hexagonal ($H_{II}$) phase in the presence of calcium. In multicomponent mixtures containing one or more acidic species the presence of cholesterol either facilitates calcium-induced lamellar to hexagonal ($H_{II}$) transitions for all the lipid components or, in systems already in a hexagonal ($H_{II}$) phase, mitigates against calcium-induced lateral phase separations. Further, cholesterol is shown to exhibit no preferential interaction on the NMR time scale with either PC, PE, or phosphatidylserine (PS) when the lipids are in the liquid-crystal state. The ability of cholesterol to directly induce $H_{II}$ phase formation in PC/PE mixtures is also shown to be common to various other sterols including ergosterol, stigmasterol, coprostanol, epicoprostanol, and androstanol.

The addition of calcium to liposomal systems composed of acidic (negatively charged) phospholipids mixed with net neutral lipids such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) can have two major effects on membrane morphology. The first of these, commonly called "lateral phase separation", refers to the ability of calcium to sequester the acidic species into separate bilayer domains which usually exhibit gel-state characteristics. Such effects have been observed in many binary mixtures, including PC/phosphatidylserine (PS) systems (Ohnishi & Ito, 1974; Johnson & Papahadjopoulos, 1975; van Dijck et al., 1978) and mixtures of PC and phosphatidic acid (PA) (Ohnishi & Ito, 1974; Galla & Sackmann, 1975), among others. It has been proposed that

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