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Properties of L1210 Cells Resistant to α-Difluoromethylornithine[1]

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ABSTRACT

L1210 cells were selected for resistance to the ornithine decarboxylase (ODC) inhibitor, α-difluoromethylornithine. When grown in the absence of the inhibitor, these cells possessed very high ornithine decarboxylase levels. These represented about 1 part in 300 of the soluble protein, which is several hundred times greater than the maximal value found in the original L1210 cells. The resistant cells contained at least 100-fold higher levels of ODC mRNA but the half-life of ODC (about 45 min) was not altered significantly. The resistant cells had much higher putrescine and cadaverine levels than control cells, but there was no significant difference in cellular spermidine or spermine content or in production of 5′-methylthioadenosine, which is a measure of polyamine synthesis. Addition of putrescine to the control or resistant cells had no effect on their content of spermidine and spermine but addition of decarboxylated *S*-adenosylmethionine increased the content of spermidine and spermine. These results indicate that ornithine decarboxylase is not the rate-limiting step in polyamine synthesis in these L1210 cells.

The growth of the α-difluoromethylornithine-resistant L1210 cells was inhibited when their ability to synthesize spermidine and spermine was blocked by the addition of the *S*-adenosylmethionine decarboxylase inhibitor, 5′-deoxy-5′-[*N*-methyl-*N*-(3-hydrazinopropyl)]aminoadenosine. Treatment with this compound produced a reduction of more than 85% in the production of 5′-methylthioadenosine and led to a large increase in the content of putrescine and a substantial decline in the content of spermidine and spermine. These results indicate the potential value of *S*-adenosylmethionine decarboxylase inhibitors as therapeutic agents in conditions where ODC inhibitors are ineffective.

INTRODUCTION

Polyamines appear to be essential for the normal growth and development of mammalian cells and there has been considerable recent interest in the possibility that substances interfering with the synthesis of polyamines may be useful antitumor agents (1–5). Most of these inhibitors have been directed against the activity of ODC[3] and many studies have shown that the blockade of ODC activity has an antiproliferative effect. Much of this work has been carried out with the enzyme-activated irreversible inhibitor, DFMO and this compound clearly has potential as a therapeutic agent (6–8). However, there have been several reports of the emergence of DFMO-resistant cells in cultures exposed to this drug for a long period of time (9–16). Such resistance has usually, but not always (5, 12, 13), been associated with amplification of the ODC gene and consequent overproduction of ODC. The cell lines containing such amplified genes have been useful tools for molecular biological studies of ODC (9, 10) but there has been little attention given to the actual properties of the DFMO-resistant cells in terms of polyamine metabolism and sensitivity to alternative inhibitors affecting other steps in the polyamine biosynthetic pathway. In the present work, we have derived a line of L1210 cells that is highly resistant to DFMO because of a very high content of ODC. The consequences with regard to polyamine metabolism of this overproduction, which appears to be stable in the absence of the drug, have been evaluated. Also, the effects of a potent inhibitor of *S*-adenosylmethionine decarboxylase on these cells have been examined. The results indicate that ODC is not the rate-limiting step in spermidine and spermine production by L1210 cells and that the proliferation of the D-R L1210 cells can be inhibited by blocking the activity of *S*-adenosylmethionine decarboxylase.

MATERIALS AND METHODS

Assay of Ornithine Decarboxylase Activity and mRNA Content. ODC activity was measured by measuring the release of $^{14}CO_2$ from L-[1-^{14}C] ornithine as described by Seely *et al.* (17). ODC protein was determined by radioimmunoassay (18). The content of ODC mRNA was determined by Northern and dot blot analysis using published methods for RNA extraction and hybridization with a cDNA probe for mouse ODC (19, 20). The half-life of ODC was measured after inhibition of protein synthesis by cycloheximide using the procedures described by Pegg *et al.* (21).

Cell Culture. Control L1210 cells were maintained and grown in suspension culture in RPMI 1640 medium containing 10% Nu-Serum (Collaborative Research, Inc., Lexington, MA) as described by Pera *et al.* (22). The DFMO-resistant cells were obtained by growing the cells in increasing concentrations of DFMO starting with 0.2 mM and proceeding through 0.5, 1, 5, 10, and 20 mM. The cells were maintained at each concentration of DFMO until their growth rate increased to that of the control cells without DFMO. The entire selection period was about 4 months. Cells were cloned by limiting dilution. Some of the experiments shown were carried out with the cells that were obtained after the 10 mM DFMO selection. The further selection by increasing the drug concentration to 20 mM DFMO did not produce cells having significantly greater levels of ODC.

Analysis of Diamines, Polyamines, and 5′-Methylthioadenosine. The cells were then harvested and extracts prepared for polyamine analysis as described (21). Appropriate aliquots of these extracts were used for the determination of intracellular putrescine, cadaverine, and polyamines. 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 excretion of putrescine, cadaverine, and polyamines by the cultures. The production of 5′-methylthioadenosine was measured using the same extracts and the same HPLC separation system.

Analysis was carried out using using an ion-pair reversed-phase separation (23). The content of putrescine, cadaverine, spermidine, and spermine was determined by post-column derivatization with *o*-phthalaldehyde (24). Intracellular diamines and polyamines were expressed as nmol/culture or as nmol/mg protein. Protein was determined by the method of Bradford (25). Extracellular polyamines were expressed as nmol/culture.

In order to determine 5′-methylthioadenosine, the eluate was monitored at 254 nm and the amount calculated from the peak heights using standard curves constructed with the authentic compounds. The amount of 5′-methylthioadenosine was expressed as nmol/culture.

Materials. L-[1-^{14}C]ornithine (55 Ci/mol) was purchased from NEN, Boston, MA. All biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, MO. DFMO was a generous gift from Dr. P.

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[3] The abbreviations used are: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; MHZPA, 5′-deoxy-5′-[*N*-methyl-*N*-(3-hydrazinopropyl)]aminoadenosine; D-R, DFMO-resistant L1210 cells; MTA, 5′-methylthioadenosine.

Table 1 *Ornithine decarboxylase activities in control and D-R L1210 cells*
Results are shown as mean ± SD for more than three estimations.

Hours	ODC activity (units/mg protein)[a]		mRNA content (units/μg RNA)[b]	
	Control cells	D-R cells	Control cells	D-R cells
6	12.3 ± 0.6	2254 ± 326	ND[c]	ND
24	22.4 ± 8	4349 ± 994	1 ± 0.3	120 ± 25
30	18.3 ± 0.8	5150 ± 919	ND	ND
48	10.0 ± 1.2	3671 ± 246	0.8 ± 0.2	130 ± 30
72	3.5 ± 1.1	1782 ± 95	ND	ND

[a] A unit of ODC activity produces 1 nmol of $^{14}CO_2$ in 60 min.
[b] The mRNA content was determined by scanning of Northern and dot blots with a densitometer and is expressed in arbitrary units which were set at 1 for the control cell RNA.
[c] ND, not determined.

P. McCann, Merrell Dow Research Institute, Cincinnati. MHZPA was synthesized by Secrist *et al.*[4] Plasmid pODC934, which contains an insert complementary to ODC mRNA, was a gift from Dr. F. G. Berger, University of South Carolina, Columbia, SC.

RESULTS

ODC Level of DFMO-resistant L1210 Cells. It is well known that DFMO at 1–5 mM greatly reduces the growth of L1210 cells (2, 22) and this effect was reproduced in our laboratory. Resistance to inhibitor was induced by growing the cells in increasing concentrations of DFMO with the cells maintained at each concentration until a normal growth rate was obtained. The cells increased their ODC activity as they acquired resistance to DFMO. When the resistant cells were grown in the absence of DFMO, the ODC activity 6 h after the addition of serum was 25 ± 4 units/mg for the cells resistant to 0.2 mM, 261 ± 82 units/mg for cells resistant to 1 mM, 2322 ± 393 for cells resistant to 5 mM and 2575 ± 458 for cells resistant to 10 mM DFMO.

The final D-R cell line grew at a rate similar to the control cells in the absence of the inhibitor but was unaffected by the presence of DFMO at concentrations of up to 20 mM. When grown in the absence of DFMO, the D-R cells had very high levels of ODC (Table 1) that were 200–400 times greater than in the control cells grown under the same conditions. This increase was entirely due to an increased amount of ODC protein rather than any change in its catalytic activity since measurements of the protein by radioimmunoassay gave identical results to the activity measurements (results not shown). Despite the greatly increased content of ODC protein, its half-life was not significantly altered in the D-R cells. The half-life was 41 min in the control cells and 50 min in the treated cells.

A substantial part, and possibly all of the increase in ODC protein, was due to an increased content of the mRNA. The ODC mRNA present in these cells was analyzed on Northern blots using a nick-translated cDNA probe and it was found to consist of a major species of about 2.2 kilobases and a faint minor species of 2.6 kilobases (results not shown). These sizes are in agreement with previous estimates of size of the ODC mRNA in L1210 cells (19). The increase in the level of mRNA was at least 100-fold as measured by densitometric scanning of Northern blots and dot blots (Table 1). This procedure for estimating the increase in mRNA content is not sufficiently accurate to be certain whether the change in mRNA is the sole factor responsible for the increase in ODC but it is clearly the major component in this increase.

[4] J. A. Secrist III, W. B. Forrister, T. H. Moss, E. L. White, and W. M. Shannon. Synthesis of substrate-analogue inhibitors of *S*-adenosylmethionine decarboxylase, manuscript in preparation.

Diamine, Polyamine, and 5′-Methylthioadenosine Content of D-R Cells. Analysis of the polyamine content of the D-R cells and the control cells grown in the absence of DFMO revealed a number of interesting differences. Firstly, the D-R cells, which have very high ODC levels, contained much higher amounts of putrescine and also contained cadaverine, which was not detected in the control cells (Table 2). Secondly, the medium from these DFMO-resistant cells contained large amounts of putrescine and cadaverine (Table 2). There was no significant difference in the spermidine and spermine content between the two cell lines (Table 2). Spermine and spermidine were not present in the medium in detectable concentrations.

These results show that, despite the much greater level of ODC in the DFMO-resistant cells, they do not form greater amounts of the polyamines, spermidine, and spermine. It could be argued that these polyamines are made in larger quantities and are then degraded but this possibility is ruled out by the measurement of MTA levels in the culture medium. As shown in Table 2, there was no difference in the production of MTA by the two cell lines. MTA is formed in the spermidine synthase and spermine synthase reactions and it is known that the L1210 cells used in these experiments do not degrade MTA but excrete it into the medium (1–3, 26–28). Thus, the production and excretion of MTA is a measure of the total extent of polyamine biosynthesis in these cells.

Effect of Addition of DFMO, Putrescine, or Decarboxylated *S*-Adenosylmethionine on Amine Content in Control and D-R Cells. The addition of 10 mM DFMO to control L1210 cells led to an inhibition of growth and to the reduction of cellular putrescine and spermidine to undetectable levels (Table 3). Addition of 10 mM DFMO to the D-R cells led to a striking decrease in putrescine and spermidine but the residual levels still amounted to 22 and 45% of the amounts found in the control L1210 cells and growth was not affected.

The addition of 100 μM putrescine to control or D-R cells increased intracellular putrescine but had no effect on spermidine and spermine (Table 3). These results also suggest that the supply of putrescine is not the limiting factor in the production of polyamines in L1210 cells. In contrast, the addition of 100 μM decarboxylated *S*-adenosylmethionine led to a significant increase in the content of both spermidine and spermine in L1210 cells and in the D-R cells. The increase in spermine in

Table 2 *Polyamine and MTA levels in D-R and control cells*

Cells[a]	Time (h)	Intracellular content of diamines and polyamines (nmol/culture)			
		Putrescine	Spermidine	Spermine	Cadaverine
Control	30	1.0 ± 0.1	4.6 ± 0.6	1.9 ± 0.2	<1
D-R	30	6.4 ± 0.6	5.5 ± 0.4	1.5 ± 0.2	1.4 ± 0.2
Control	48	2.5 ± 0.6	17.3 ± 1.7	5.5 ± 0.6	<1
D-R	48	17.4 ± 1.1	22.5 ± 1.5	5.0 ± 0.9	6.1 ± 0.7
Control	72	10.8 ± 1.0	77.2 ± 5.2	21.9 ± 2.9	<1
D-R	72	83.6 ± 11.1	70.8 ± 9.3	17.6 ± 0.8	18.1 ± 1.4

Cells[a]	Time (h)	Diamines and MTA in medium (nmol/culture)[b]		
		Putrescine	Cadaverine	MTA
Control	30	1.7 ± 0.2	<1	7.5 ± 2.3
D-R	30	10.4 ± 1.7	6.7 ± 0.8	9.1 ± 1.8
Control	48	2.3 ± 0.9	1.6 ± 0.2	26.6 ± 2.9
D-R	48	21.2 ± 2.9	29.1 ± 5.7	30.6 ± 1.1
Control	72	5.2 ± 1.7	1.1 ± 0.2	94.2 ± 2.6
D-R	72	89.1 ± 16.5	71.2 ± 14	101.3 ± 7.8

[a] The control and the DFMO-resistant cells grew at the same rate and there was no significant difference between them in the cell number at any time of culture. These numbers were 1.2×10^5/ml, 3.5×10^5/ml, and 14×10^5/ml at 30, 48, and 72 h, respectively.
[b] Results are shown as mean ± SD for more than three estimations.

Table 3 *Effect of putrescine and decarboxylated AdoMet on polyamines in control and D-R L1210 cells*

Results are shown as mean ± SD for more than three estimations.

Cell and treatment[a]	Polyamine levels (nmol/mg protein)		
	Putrescine	Spermidine	Spermine
Control	3.9 ± 1.1	23.9 ± 2.1	8.3 ± 1.3
Control + 10 mM DFMO	<0.1	<0.1	9.6 ± 1.4
Control + 100 μM decarboxylated AdoMet	3.0 ± 1.3	31.6 ± 6.9	12.3 ± 1.5
Control + 100 μM putrescine	7.4 ± 0.8	25.1 ± 1.7	6.1 ± 0.3
D-R	19.1 ± 2.7	22.5 ± 5.4	9.6 ± 2.4
D-R + 10 mM DFMO	0.9 ± 0.1[b]	13.1 ± 0.6	7.1 ± 0.9
D-R + 100 μM decarboxylated AdoMet	9.2 ± 0.4	29.1 ± 2.2	24.7 ± 6.8
D-R + 100 μM putrescine	80.2 ± 5.1	22.6 ± 5.3	7.6 ± 1.6

[a] Measurements were made after 40 h of culture in the presence of the compounds shown.

[b] All of the putrescine present in the medium of D-R cells (see Table 2) was abolished by the presence of 10 mM DFMO.

Table 4 *Effect of MHZPA on MTA production in control and D-R cells*

Results are shown as the mean for three estimations.

Treatment (h)	5′-Methylthioadenosine content (nmol/ml culture)			
	Control cells		DFMO resistant cells	
	No addition	+ 50μM MHZPA	No addition	+ 50μM MHZPA
48	2.36	0.36	2.20	0.32
72	7.95	1.0	7.19	0.72

Table 5 *Effect of 50 μM MHZPA on polyamines in D-R L1210 cells*

Results shown are the mean ± SD for four or more estimations or the mean of three estimations.

Cell and treatment	Time (h)	Polyamine levels (nmol/mg protein)		
		Putrescine	Spermidine	Spermine
D-R	24	31.6	27.0	6.8
D-R + MHZPA	24	110.2	11.2	1.8
D-R	48	24.9 ± 3.2	29.8 ± 3.6	7.5 ± 1.6
D-R + MHZPA	48	72.6 ± 6.3	9.6 ± 1.5	1.1
D-R	72	34.4	30.9	5.9
D-R + MHZPA	72	128.7	5.5	<1
Control	48	2.3 ± 0.2	27.2 ± 2.0	7.5 ± 1.4
Control + MHZPA	48	26.1 ± 7.7	11.3 ± 3.5	2.9 ± 0.8

the D-R cells in response to decarboxylated *S*-adenosylmethionine was particularly striking (2.6-fold).

Effect of Inhibition of *S*-Adenosylmethionine Decarboxylase in D-R Cells. The effects of MHZPA, an inhibitor of *S*-adenosylmethionine decarboxylase (28, 29), on polyamine production and the growth of the D-R cells were examined. As shown in Table 4, the addition of MHZPA to either the control cells or to the D-R cells led to a more than 90% inhibition of the production of MTA indicating that this inhibitor does effectively block polyamine synthesis in these cells. Analysis of the polyamine content in the cells showed that MHZPA caused a large drop in the content of spermine, a significant but slightly smaller decline in the content of spermidine and a massive increase in the content of putrescine (Table 5). The putrescine content in the D-R cells, which was already about eight times greater than in the control cells, was increased another 3- to 4-fold by the presence of MHZPA. These results are consistent with the blockage of the polyamine biosynthesis pathway by MHZPA at the *S*-adenosylmethionine decarboxylase step.

The addition of MHZPA to the control L1210 cells led to a decreased rate of cell growth (Table 6). This decrease in growth was similar to that produced by 5 mM DFMO. As expected, DFMO had no effect on the growth of the D-R cells but their growth was inhibited by MHZPA to the same extent as that of the control cells (Table 6). The addition of 5 μM spermidine

Table 6 *Effect of DFMO and MHZPA on growth of L1210 cells and D-R L1210 cells*

Results are shown as mean ± SD for five estimations.

Cells and treatment	Cell number[a] (% control)		
	24 h	48 h	72 h
Control	100	100	100
D-R	97 ± 8	106 ± 12	95 ± 9
Control + 5 mM DFMO	76 ± 11	47 ± 6	19 ± 4
D-R + 5 mM DFMO	105 ± 13	101 ± 19	98 ± 2
Control + 50 μM MHZPA	78 ± 3	47 ± 5	21 ± 3
D-R + 50 μM MHZPA	83 ± 12	42 ± 5	24 ± 6

[a] The actual control cell numbers were 0.9, 3.4, and 16 ($\times 10^5$/ml) at these time points and the cells had a doubling time of 10.8 h.

reversed the growth-inhibitory action of MHZPA on the D-R L1210 cells (results not shown).

DISCUSSION

The very high ODC activity found in the D-R cells when grown in the absence of the inhibitor corresponds to an ODC content of about one part in 200–400 of the total soluble protein. The ODC mRNA represents about 3.6% of the total mRNA in these D-R cells. [This was quantitated by comparing dot blots using a standard curve of authentic ODC mRNA synthesized *in vitro* from a plasmid containing a cDNA insert to ODC (30).] These cells are therefore comparable, or in some cases more active, in production of ODC to other DFMO-resistant cells that have been derived in several different laboratories (9–16). Although we have not checked this specifically, it is likely that the major cause of the increased production of ODC mRNA is gene amplification. The high level of ODC production in our D-R cells appears to be stable in the absence of DFMO and the large amount of ODC does not exert adverse effects on the rate of cell growth. These properties should render the cell lines particularly useful for studies of the regulation and function of ODC. It is particularly interesting that the rapid turnover of ODC protein is not affected in these cells. This suggests that any factors needed for the physiological degradation of the enzyme are either present in great excess or are increased coordinately with the ODC protein.

Overproduction of ODC leading to DFMO resistance has now been reported by a number of investigators and appears to be a relatively common phenomenon when a normal rate of cell growth is prevented by means of this inhibitor. Several groups have shown that the increased production of ODC provides a means by which some polyamines can be synthesized even in the presence of the inhibitor (11–16) but there have been no prior detailed studies of the consequences of the overproduction of ODC when the inhibitor is not present. Our finding of the presence of cadaverine in these cells is not entirely surprising since it is known that ODC will decarboxylate lysine although lysine is a much poorer substrate than ornithine (31, 32). Cadaverine is therefore only found in significant amounts in mammalian tissues, such as the mouse kidney, where ODC activity is very high (31). Lysine is present in quite large amounts in the RPMI medium used for the L1210 cells, and it is unlikely that it would become depleted by this reaction. However, it is possible that, under other culture conditions, the conversion of lysine to cadaverine by excess ODC could influence growth by reducing the availability of this essential amino acid.

It is clear from the results in Table 2 that, despite the very high content of putrescine in the D-R cells, there was no increase in the cellular content of spermidine and spermine. The possibility that excess of these polyamines are actually

made but are then degraded or excreted is ruled out by the findings that there was no spermidine or spermine in the medium and that 5′-methylthioadenosine excretion was also not different between the control and the D-R cells. Since ODC activity is several hundred times greater in the D-R cells than in the control cells this finding provides strong evidence that the activity of ODC is not the rate-limiting factor in spermidine and spermine production by these L1210 cells. This conclusion is reinforced by the results of the addition of putrescine or decarboxylated *S*-adenosylmethionine to the cells. Putrescine had no effect on the polyamine content but provision of decarboxylated *S*-adenosylmethionine increased the amount of spermidine and spermine. This implies that the activity of *S*-adenosylmethionine decarboxylase is the rate-limiting factor in the biosynthesis of polyamines in these cells. The very large increase in spermine seen in the D-R cells after the addition of decarboxylated *S*-adenosylmethionine is consistent with this interpretation since, once the aminopropyl donor has been provided, the excess of putrescine can be converted through spermidine into spermine that is the end-point of the pathway.

It should be noted that the original L1210 cells have quite high ODC activities and this conclusion concerning the rate-limiting factor may not apply to all other mammalian cell types. It is possible that in other cell types the excess production of spermidine and spermine in response to a high level of ODC expressed when DFMO is removed from a DFMO-resistant cell line may actually lead to a toxic effect. Excretion of excess polyamines or their degradation to putrescine by the polyamine-N^1-acetyltransferase/oxidase system (1, 33) may be necessary to prevent this. In our experiments with the D-R L1210 cells, where only the diamines were produced in excess, at least 50% of the putrescine and more than 75% of the cadaverine that was formed was excreted into the medium. Even the control L1210 cells were found to excrete putrescine (Table 2) although the amount released was much less than from the D-R L1210 cells. This finding, and the fact that addition of exogenous putrescine did not alter cellular spermidine content (Table 3 and Reference 34) are also consistent with the ODC step not being rate determining for spermidine synthesis in L1210 cells.

The D-R L1210 cells were found to be highly sensitive to the *S*-adenosylmethionine decarboxylase inhibitor, MHZPA (28, 29). This compound prevented the production of spermidine and spermine and led to a rise in putrescine above the already very high content present in these cells. MHZPA also blocked the growth of the cells as shown in Table 6. These findings strongly support the hypothesis that putrescine cannot fulfill the functions of spermidine and spermine that are needed for L1210 cell growth even when it is present in very high concentrations. They also indicate that it is possible that tumor cells resistant to ODC inhibitors by virtue of an increased content of ODC may easily be controlled by inhibitors of other steps in the pathway. These include MHZPA and other *S*-adenosylmethionine decarboxylase inhibitors (5, 28, 29) and possibly the aminopropyltransferase inhibitors (5, 35). Another approach would be to use the bis(alkyl)polyamines described by Porter and Bergeron and colleagues (2, 19, 36, 37) that repress both ODC and *S*-adenosylmethionine decarboxylase activities.

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