Intracellular putrescine and spermidine deprivation induces increased uptake of the natural polyamines and methylglyoxal bis(guanylhydrazone)

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Inhibition of polyamine synthesis by α -difluoromethylornithine in cultured Ehrlich ascites-carcinoma cells rapidly enhanced the uptake of exogenous putrescine, spermidine and spermine from the culture medium. In tumour cells exposed to the drug for 2 days, the intracellular concentration of spermidine was decreased to less than 10% of that found in untreated cells. However, the strikingly stimulated transport system brought the concentration of spermidine to the control values in less than 2 h after supplementation of the cells with micromolar concentrations of the polyamine. In the absence of polyamine deprivation, tumour cells did not accumulate extracellular polyamines to any appreciable extent. Ascites-tumour cells deprived of putrescine and spermidine likewise concentrated methylglyoxal bis(guanylhydrazone) {1,1'-[(methylethanediyldene)dinitrilo]diguanidine} at a greatly enhanced rate. A previous 'priming' of tumour cells with difluoromethylornithine followed by an exposure of the cells to methylglyoxal bis(guanylhydrazone) resulted in a marked and rapid anti-proliferative effect.

Intracellular deprivation of natural polyamines by means of chemical intervention in their biosynthesis triggers a series of cellular mechanisms aimed to compensate for the polyamine depletion. In addition to changes directly attributable to an interaction of the inhibitor with the short-lived decarboxylases of polyamine synthesis (L-ornithine decarboxylase, EC 4.1.1.17; S-adenosyl-L-methionine decarboxylase, EC 4.1.1.50), which result in a stabilization of the enzymes (Harik *et al.*, 1974; McCann *et al.*, 1977; Stevens & McKinnon, 1977; Pegg *et al.*, 1973), the activity of adenosylmethionine decarboxylase, normally repressed by polyamines, is enhanced by putrescine and spermidine depletion, by a mechanism possibly related to the gene expression of the enzyme protein (Mamont *et al.*, 1978; Alhonen-Hongisto, 1980).

Furthermore, inhibition of spermidine and putrescine accumulation in cultured Ehrlich ascites-carcinoma cells by difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978), was also associated with a greatly enhanced accumulation of cadaverine, a diamine only occasionally found in animal cells (Alhonen-Hongisto & Jänne, 1980).

The idea of a general conservation of intracellular polyamines during the inhibition of their accumulation was supported by the finding that

BHK-21/C13 cells exposed to methylglyoxal bis(guanylhydrazone) {1,1'-[(methylethanediyldene)dinitrilo]diguanidine} or α -methylornithine excreted considerably less polyamines into the medium than did untreated cells (Melvin & Keir, 1978; Wallace *et al.*, 1979).

We now report that tumour cells, depleted of putrescine and spermidine, concentrate extracellular polyamines much more effectively than do untreated cells. The enhanced uptake of the natural polyamines during polyamine deprivation was accompanied by a strikingly increased transport of methylglyoxal bis(guanylhydrazone) into the tumour cells, which may have practical implications when designing combinations of these two drugs to achieve better antiproliferative response.

Experimental

Cell cultures

Ehrlich ascites-carcinoma cells were adapted to growth in suspension cultures in RPMI 1640 medium (Orion Diagnostica, Helsinki, Finland) supplemented with 5% (v/v) pooled human serum (Finnish Red Cross Transfusion Service, Helsinki, Finland) and antibiotics as described by Alhonen-Hongisto *et al.* (1979). The tumour cells were harvested by low-speed centrifugation, washed once

with 2 ml of 0.9% NaCl, suspended either in buffer (enzyme assays) or distilled water (chemical assays) and disintegrated ultrasonically.

Chemicals

[1,4-¹⁴C]Putrescine (sp. radioactivity 116 Ci/mol), [1,4-¹⁴C]spermidine (sp. radioactivity 120 Ci/mol), [1,4-¹⁴C]spermine (sp. radioactivity 105 Ci/mol) and [1,4-¹⁴C]ornithine (sp. radioactivity 59 Ci/mol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). *S*-Adenosyl-L-[1,4-¹⁴C]methionine was prepared enzymically by the method of Pegg & Williams-Ashman (1969). Methylglyoxal bis(guanyldrazon) was the product of Eka Chemie (Steinheim/Albuch, West Germany). DL- α -Difluoromethylornithine was a generous gift from Centre de Recherche Merrell International (Strasbourg, France).

Analytical methods

The activities of ornithine decarboxylase and adenosylmethionine decarboxylase were assayed as described by Jänne & Williams-Ashman (1971a,b respectively). Protein was measured by the method of Lowry *et al.* (1951) and DNA by the method of Giles & Myers (1965).

Polyamine uptake was determined by incubating the cells in the presence of labelled polyamines for 30–60 min, followed by washing the cells and measuring the radioactivity of all cellular polyamines. Under the conditions used, the incorporation of polyamines into the cells was linear with respect to both time and the number of the cells. Owing to the short pulse, virtually all the cellular radioactivity was found in the polyamine fractions. Polyamines were quantified from HClO₄ extracts after dansylation (5-dimethylaminonaphthalene-1-sulphonylation) by the method of Seiler (1970) with the modifications of Dreyfuss *et al.* (1973). The uptake of unlabelled methylglyoxal bis(guanyldrazon) was determined by incubating the cells in the presence of the drug (5 μ M) for 60 min and the drug concentrations were determined enzymically with the aid of adenosylmethionine decarboxylase isolated from baker's yeast (Seppänen *et al.*, 1980).

Cell densities were measured with an electronic particle counter (Coulter Electronics, Harpenden, Herts., U.K.). Cell volumes (for calculation of the intracellular drug concentrations) were measured as described in the instruction manual for the Coulter counter. Cell cultures were run in duplicate. The variation of cell densities between cultures was always less than 5%. Under the experimental conditions used, there was a good correlation between the cell number and the number of viable cells as determined by the dye-exclusion test, even after a prolonged (3 days) exposure of the cells to inhibitors of polyamine synthesis.

Results and discussion

When Ehrlich ascites-carcinoma cells (after dilution with fresh medium) were exposed to 6 mM-difluoromethylornithine, the peaks of ornithine decarboxylase activity induced by the change of medium were totally prevented (Fig. 1a). In agreement with previous reports (Mamont *et al.*, 1978; Alhonen-Hongisto, 1980), the activity of adenosylmethionine decarboxylase gradually increased in cells exposed to the inhibitor (Fig. 1a). Dilution of the tumour cells with new medium induced a rapid, yet transient, increase in the cellular transport of putrescine (Fig. 1b), a phenomenon described for some other animal cells stimulated to grow (Pohjanpelto, 1976). Addition of difluoromethylornithine concomitantly with the change of medium resulted in a marked and persistent enhancement of putrescine transport into the tumour cells (Fig. 1b). It is remarkable that the diamine uptake

Fig. 1. Effect of difluoromethylornithine on ornithine decarboxylase and adenosylmethionine decarboxylase activities (a) and on the uptake and conversion of putrescine (b) in Ehrlich ascites cells

The cells (initial density 0.14×10^6 /ml) were grown in the absence (---) or presence (—) of 6 mM-difluoromethylornithine. The enzyme activities were measured at time points indicated and are given as nmol of CO₂ released/mg of protein (a): ●, ornithine decarboxylase; ▲, adenosylmethionine decarboxylase. The radioactivity of cellular polyamines was measured at 30 min after the supplementation of the cultures with 1 μ M-[1,4-¹⁴C]-putrescine (0.5 μ Ci) and is expressed as $10^{-3} \times$ c.p.m./ μ g of DNA (b): ●, putrescine; ▲, spermidine.

doubled already during the very first 30 min of exposure (the time-point 0 actually represents the time period between 0 and 30 min, i.e. the first pulse used for the determination of putrescine transport). In control cells the cellular radioactivity derived from labelled putrescine was exclusively found in the diamine fraction, the conversion into spermidine being negligible, whereas in cells exposed to difluoromethylornithine putrescine was rapidly converted into spermidine (Fig. 1b), in parallel with increasing adenosylmethionine decarboxylase activity (Fig. 1a). In fact, the latter observation was evident before any marked enhancement of adenosylmethionine decarboxylase activity, obviously because of the higher specific radio-activity of putrescine in the drug-treated cells.

Similar increases also occurred in the uptake of cadaverine (results not shown) and, as demonstrated in Table 1, in the transport of the higher polyamines spermidine and spermine. The uptake of spermidine was increased by more than 5-fold in cells grown in the presence of difluoromethylornithine for 24 h, and by more than 10-fold when the exposure time was prolonged to 48 h (Table 1). The transport of spermine was likewise greatly enhanced in tumour cells grown in the presence of the inhibitor (Table 1). Inhibition of cell glycolysis by 1 mM-iodoacetate in cells previously exposed to 6 mM-difluoromethylornithine for 24 h resulted in a rapid decrease in the uptake of labelled spermidine (results not shown), indicating that the transport process was energy-dependent in these highly glycolytic cells.

The remarkable efficiency of the stimulated polyamine transport system is illustrated in Fig. 2. In this experiment, the tumour cells were first exposed to difluoromethylornithine for 48 h, a treatment that resulted in almost complete disappearance of cellular putrescine (control cells 158, drug-treated cells 15 pmol/mg of DNA) and a decrease of spermidine concentration by more than 90% in

comparison with cells grown in the absence of the drug (Fig. 2, time-point 0). The concentration of spermine remained virtually unchanged (Fig. 2) and seemed to be sufficient for the maintenance of an almost normal rate of cell division (control cultures 0.98×10^6 cells/ml, difluoromethylornithine-treated cultures 0.77×10^6 cells/ml at 48 h). Addition of $10 \mu\text{M}$ -spermidine to the culture medium at 48 h (time-point 0) brought about an exceedingly rapid normalization of the cellular spermidine content. Intracellular spermidine depletion was corrected in less than 2 h, during which time the cellular concen-

Fig. 2. Uptake of spermidine by Ehrlich ascites cells previously exposed to difluoromethylornithine Ehrlich ascites cells (initial density $0.17 \times 10^6/\text{ml}$) were grown in the absence (---) or presence (—) of 6 mM-difluoromethylornithine. At 48 h (time point 0) the cultures were supplemented with $10 \mu\text{M}$ -spermidine. The cellular contents of polyamines were determined at the time points indicated and are expressed as nmol/mg of DNA: ●, spermidine; ▲, spermine.

Table 1. Uptake of spermidine, spermine and methylglyoxal bis(guanyldihydrazone) of Ehrlich ascites-carcinoma cells The ascites cells (initial density $0.16 \times 10^6/\text{ml}$) were grown in the absence or presence of 6 mM-difluoromethylornithine. At each time point the cultures were supplemented with $5 \mu\text{M}$ -[1,4- ^{14}C]spermidine, -[1,4- ^{14}C]spermine or -methylglyoxal bis(guanyldihydrazone) and the cellular contents of polyamines and the drug were measured 1 h later.

Treatment	Time (h)	Uptake (nmol/ 10^6 cells)		
		Spermidine	Spermine	Methylglyoxal bis(guanyldihydrazone)
Control	0	0.33	0.18	0.09
Difluoromethylornithine	0	0.37	0.13	0.12
Control	24	0.66	0.36	0.03
Difluoromethylornithine	24	3.94	2.12	0.44
Control	48	0.72	0.29	0.10
Difluoromethylornithine	48	8.46	2.66	1.30

tration of the latter polyamine increased about 12-fold (Fig. 2). In contrast with the inhibitor-treated cells, supplementation of untreated cells with exogenous spermidine did not produce any appreciable changes in the intracellular concentrations of the polyamines (Fig. 2).

Another fact seen from Table 1 was the finding that, in addition to the increased uptake of the natural polyamines, difluoromethylornithine likewise greatly enhanced the uptake of methylglyoxal bis(guanyldiazotane), an inhibitor of adenosylmethionine decarboxylase (Williams-Ashman & Schenone, 1972) and a compound considered as an analogue of spermidine. The transport of methylglyoxal bis(guanyldiazotane) was stimulated to the same extent as that of the natural polyamines, i.e. by about 10-fold in cells grown for 48 h in the presence of difluoromethylornithine (Table 1). This observation strongly supports the view that the latter compound shares a common transport system with the natural polyamines (Dave & Caballes, 1973; Mihich *et al.*, 1974). Further, it appeared that this transport system was specific to polyamines and methylglyoxal bis(guanyldiazotane), since no enhancement of the transport of amino acids was observed under similar conditions (results not shown).

The strikingly increased intracellular accumulation of methylglyoxal bis(guanyldiazotane) in cells previously exposed to difluoromethylornithine is also shown in Table 2. When the tumour cells were grown in the presence of difluoromethylornithine or methylglyoxal bis(guanyldiazotane), or the combination of the two, for 3 days, difluoromethylornithine alone had little effect on cell proliferation, whereas 5 μ M-methylglyoxal bis(guanyldiazotane) was strongly antiproliferative (Table 2). When methylglyoxal bis(guanyldiazotane) was combined

with the inhibitor of ornithine decarboxylase, the latter compound only marginally enhanced the growth-inhibitory action of methylglyoxal bis(guanyldiazotane) (Table 2). Interestingly, tumour cells grown in the presence of methylglyoxal bis(guanyldiazotane) alone for 3 days appeared to contain higher drug concentrations than did cells exposed to the combination of the drugs for the same period of time, which most probably resulted from slower uptake of the drug related to more pronounced growth inhibition (Table 2). When the cells were first grown for 2 days in the absence of any drug and then supplemented with 5 μ M-methylglyoxal bis(guanyldiazotane) for an additional 24 h period, there was no difference in the total cell count in comparison with untreated controls (Table 2). However, when the cells were first grown in the presence of difluoromethylornithine for 48 h and thereafter exposed to 5 μ M-methylglyoxal bis(guanyldiazotane) for another day, not only was the intracellular concentration of methylglyoxal bis(guanyldiazotane) increased to very high values (about 9 mM, representing almost a 2000-fold gradient across the cell membrane), but cell growth was also immediately halted (Table 2). Thus a 'priming' of the tumour cells with difluoromethylornithine (or conceivably by any compound capable of producing profound polyamine depletion) may offer a possibility to increase rapidly the concentration of methylglyoxal bis(guanyldiazotane) to values not achievable by a continuous treatment with the drug alone.

The striking enhancement of polyamine transport associated with inhibition of endogenous polyamine synthesis represents a further cellular mechanism by which the cells attempt to compensate for intracellular polyamine deprivation. The remarkable efficiency of this induced transport may have

Table 2. *Effect of difluoromethylornithine and methylglyoxal bis(guanyldiazotane) on the proliferation of Ehrlich ascites cells*

The cells (initial density 0.09×10^6 /ml) were grown in the absence (A) or presence of 6 mM-difluoromethylornithine (B), 5 μ M-methylglyoxal bis(guanyldiazotane) (C) or the combination of the drugs (D) for 3 days, or the cultures were first grown in the absence (E) or presence (F) of difluoromethylornithine for 2 days and then supplemented with 5 μ M-methylglyoxal bis(guanyldiazotane) for an additional day.

Treatment	Duration of treatment (days)	$10^{-6} \times$ Cell number (ml)	Intracellular concn. of methylglyoxal bis(guanyldiazotane) (mM)
A. Control	3	0.960	—
B. Difluoromethylornithine	3	0.948	—
C. Methylglyoxal bis(guanyldiazotane)	3	0.264	4.3
D. Difluoromethylornithine + methylglyoxal bis(guanyldiazotane)	3	0.198	1.31
E. Control + methylglyoxal bis(guanyldiazotane)	2		
	1	1.000	1.62
F. Difluoromethylornithine + methylglyoxal bis(guanyldiazotane)	3		
	1	0.542	8.82

practical importance in the event that inhibitors of ornithine decarboxylase are used in the clinical treatment of cancer. Extracellular concentrations of polyamines in cancer patients (for references see Jänne *et al.*, 1978; Hospattankar *et al.*, 1980) are not greatly different from those required for a complete restoration after polyamine deprivation in cancer cells, which may render the chemotherapy with polyamine anti-metabolites totally ineffective.

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