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Inhibition by Derivatives of Diguanidines of Cell Proliferation in Ehrlich Ascites Cells Grown in Culture

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The anti-proliferative effects of 1,1′-[(methylethanediylidene)dinitrilo]diguanidine [methylglyoxal bis(guanylhydrazone)] and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) have been studied in Ehrlich ascites carcinoma cells grown in suspension cultures. Both compounds are potent inhibitors of *S*-adenosyl-L-methionine decarboxylase from the tumour cells. In the presence of putrescine (but not in its absence), the inhibition produced by 1,1′-[methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) was apparently irreversible, as judged by persistent depression of the enzyme activity even after extensive dialysis. The two compounds produced similar increases in adenosylmethionine decarboxylase activity, which resulted from a striking stabilization of the enzyme in cells grown in the presence of the drugs. The inhibitory effect of the two diguanidine derivatives on the synthesis of DNA and protein became evident after an exposure of 4–8h. At that time, the only change seen in tumour polyamines in cells grown in the presence of the inhibitors was an increase in cellular putrescine. To find out whether the compounds initially interfered with the energy production of the tumour cells, the cultures were grown in the presence of uniformly labelled glucose, and the formation of lactate, as well as the oxidation of the sugar into CO_2, were measured. The activation of glycolysis upon dilution of the tumour cells with fresh medium and the subsequent formation of labelled CO_2 were similar in control cells and in cells exposed to methylglyoxal bis(guanylhydrazone), 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) or diaminopropanol. Only a marginal decrease in the cellular content of ATP was found in cells exposed to the inhibitors for 24h. The diguanidine-induced growth inhibition was fully reversed by low concentrations of exogenous polyamines. However, the possibility remained that the reversal by polyamines was due to a decrease of intracellular diguanidine concentration. Our results indicate that the mode of action of 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) is fully comparable to that of methylglyoxal bis(guanylhydrazone), as regards stabilization of adenosylmethionine decarboxylase and the appearance of growth inhibition in Ehrlich ascites cells. The data tend to support the view that both compounds apparently have an early anti-proliferative effect unrelated to polyamine metabolism.

Intracellular deprivation of polyamines in animal cells, brought about by specific inhibitors of their biosynthesis, has had a major impact on the elucidation of the physiological functions of putrescine, spermidine and spermine.

There are a great number of chemical compounds capable of blocking the formation of polyamines *in vivo*, either in cultured animal cells or in intact animals (for references see Williams-Ashman *et al.*, 1976; Jänne *et al.*, 1978). The oldest of these inhibitors apparently is methylglyoxal bis(guanylhydrazone), which was already used clinically in cancer chemotherapy (e.g. Regelson & Holland, 1961) before being discovered to be a potent inhibitor of eukaryotic adenosylmethionine decarboxylase (Williams-Ashman & Schenone, 1972). It became almost immediately apparent that this compound could be used in cultures of animal cells to produce intracellular polyamine deprivation (Kay & Pegg, 1973; Otani *et al.*, 1974; Fillingame *et al.*, 1975; Boynton *et al.*, 1976; Krokan & Eriksen, 1977). In support of the idea that the known anti-proliferative properties of methylglyoxal bis-(guanylhydrazone) were based on its ability to

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inhibit the accumulation of higher polyamines were the observations indicating that a supplementation of exogenous polyamines protected various cells against the anti-proliferative action of the compound (Fillingame *et al.*, 1975; Boynton *et al.*, 1976; Hölttä *et al.*, 1979*a*). However, over the past years, experimental evidence has been obtained suggesting that methylglyoxal bis(guanylhydrazone) may exert metabolic effects unrelated to polyamine metabolism. These include early mitochondrial damage (seen in leukaemia L1210 cells as degenerative vacuolization) (Dave *et al.*, 1978), powerful inhibition of mammalian diamine oxidase (Hölttä *et al.*, 1973) and an apparent dissociation of its initial anti-proliferative action from polyamine depletion in certain cells (Newton & Abdel-Monem, 1977; Hölttä *et al.*, 1979*b*). Furthermore, although the methylglyoxal bis(guanylhydrazone)-induced growth inhibition can be reversed by addition of low concentrations of exogenous polyamines (spermidine and spermine, but not putrescine), this does not necessarily mean that the reversal resulted from correction of the intracellular polyamine deprivation, since polyamines (spermidine and spermine) seem to compete with the drug for a common cellular uptake system (Mihich *et al.*, 1974).

After the profound anti-proliferative effects of methylglyoxal bis(guanylhydrazone) became evident, a large number of its derivatives was synthesized (Baiocchi *et al.*, 1963). Among these was 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) which possesses an extra amino group in each of its guanidine moieties. This compound was found by Pegg & Conover (1976) to be a strong inhibitor of eukaryotic adenosylmethionine decarboxylase, producing, unlike methylglyoxal bis(guanylhydrazone), an irreversible inhibition of the enzyme activity (Pegg & Conover, 1976; Pegg, 1978). In analogy with methylglyoxal bis(guanylhydrazone), 1,1′-[(methylethanediylidene)dinitrilo]-bis-(3-aminoguanidine) powerfully inhibited the activity of diamine oxidase (Pegg & McGill, 1978). In connection with its initial synthesis (Baiocchi *et al.*, 1963), 1,1′-[(methylethanediylidene)dinitrilo]-bis-(3-aminoguanidine) was not reported to possess any protective action in mice bearing L1210 leukaemia. In combination with 1,3-diaminopropane (an inhibitor of ornithine decarboxylase), but not alone, 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) inhibited the synthesis of DNA in regenerating rat liver (Wiegand & Pegg, 1978) and in Ehrlich ascites cells *in vivo* (Alhonen-Hongisto *et al.*, 1979). Apart from a short mention (Pegg, 1978), there are no experimental data describing the effects of 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) on cultured animal cells.

We have now compared the effects exerted by methylglyoxal bis(guanylhydrazone) and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine on Ehrlich ascites carcinoma cells grown in culture. These two diguanidine derivatives are strikingly similar as regards their potency to inhibit adenosylmethionine decarboxylase activity and polyamine accumulation as well as to depress macromolecular synthesis and cell growth. Unlike methylglyoxal bis(guanylhydrazone), 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) acts as an irreversible inhibitor of adenosylmethionine decarboxylase from ascites carcinoma cells, but only when the enzyme is exposed to the drug in the presence of putrescine. An inhibition of DNA and protein synthesis in tumour cells grown in the presence of either diguanidine derivative became evident well before any decreases in the concentrations of polyamines could be seen.

Experimental

Culture conditions

Ehrlich ascites cells were maintained in the peritoneal cavity of female mice (weighing about 30g) by weekly inoculations. The cells were adapted to grow in suspension cultures over a period of 3–6 weeks. Either minimum essential medium or suspension medium (Gibco Bio-Cult, Glasgow, Scotland, U.K.), both supplemented with 10% (v/v) horse serum and 2mM-glutamine, were used. Stock cultures were maintained in 75cm² Falcon flasks at 37°C without any agitation. Experimental cultures were grown in sterile 30-ml plastic tubes placed horizontally.

Preparation of cell extracts

The cells were harvested by low-speed centrifugation (2000*g* for 3min), suspended in 25mM-Tris/HCl buffer, pH7.4, containing 5mM-dithiothreitol and 0.1mM-EDTA and disintegrated ultrasonically (Branson Sonifier, 3×5s at half-maximum power). After centrifugation for 30min at 100000*g*, the resulting supernatant fractions were used for the assay of ornithine decarboxylase and adenosylmethionine decarboxylase activities.

Chemicals

S-Adenosyl-L-[1-^{14}C]methionine was prepared enzymically by the method of Pegg & Williams-Ashman (1969). DL-[1-^{14}C]Ornithine (specific activity 59Ci/mol), [^{14}C]leucine (sp. act. 342Ci/mol), [^{3}H]thymidine (sp. act. 26Ci/mmol) and uniformly labelled [^{14}C]glucose (sp. act. 333Ci/mol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). Methylglyoxal bis(guanylhydrazone) was obtained from Aldrich-Europe (Beerse, Belgium) and 1,3-diamino-2-propanol was a product of Fluka A.G. (Buchs S.G., Switzerland). 1,1′-[(Methylethanediylidene)dinitrilo]bis-(3-amino-

guanidine) was synthesized by the method of Baiocchi *et al.* (1963).

Analytical methods

Ornithine decarboxylase (Jänne & Williams-Ashman, 1971*a*) and adenosylmethionine decarboxylase (Jänne & Williams-Ashman, 1971*b*) activities were assayed by published methods. Polyamines were determined after dansylation by the method of Seiler (1970) as modified by Dreyfuss *et al.* (1973).

The synthesis of DNA in ascites cells was measured by determining the incorporation of radioactive thymidine into acid-hydrolysable material. The incubation time was 15 min. The reaction was linear both as regards time and the number of cells used. Similarly, protein synthesis was measured by determining the incorporation of labelled leucine into trichloroacetic-acid-insoluble material. The content of DNA was measured after acid hydrolysis by the method of Giles & Myers (1965) and protein by the method of Lowry *et al.* (1951). Radioactive lactate (derived from uniformly labelled glucose) was determined as described by Hoskins & Patterson (1968). The cell densities were counted with the aid of an electronic particle counter (Coulter Electronics), and were periodically verified by counting under a microscope. The viability of the cells was tested by a dye-exclusion test (Black & Berenbaum, 1964). Cellular ATP content was measured with the aid of a Lumac Celltester M 1030 (Lumac Systems A.G., Basel, Switzerland). The assays were carried out according to the manufacturer's applications bulletin (Medical Applications, Lumac Systems A.G., 1979).

Results

Inhibition of adenosylmethionine decarboxylase by methylglyoxal bis(guanylhydrazone) and 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine)

When crude cytosolic fractions obtained from Ehrlich ascites cell were incubated in the presence of 10 μM-methylglyoxal bis(guanylhydrazone) or 30 μM-1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) the activity of adenosylmethionine decarboxylase was profoundly inhibited. However, if the cytosol was exposed to the drugs in the absence of putrescine, the inhibited enzyme activity was largely recovered by extensive dialysis for more than 2 days (Fig. 1*a*). In the presence of putrescine (1 mM), 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine), but not methylglyoxal bis(guanylhydrazone), inhibited adenosylmethionine decarboxylase activity in a manner that was not reversed

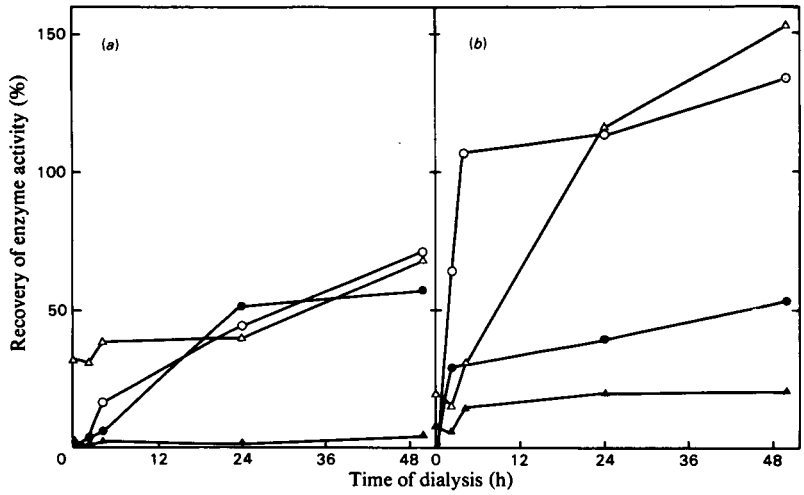

Fig. 1. *Effect of putrescine on the reversibility of diguanidine-induced inhibition of adenosylmethionine decarboxylase from Ehrlich ascites cells (a) and from rat liver (b)*

Cytosol fractions were exposed to methylglyoxal bis(guanylhydrazone) (10 μM) or 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) (30 μM) for 30 min at 37°C in the presence or absence of 1 mM-putrescine. Thereafter the cytosol fractions were dialysed against 25 mM-Tris/HCl buffer, pH 7.4, and the enzyme activity was measured at the time points indicated, in the presence of 2.5 mM-putrescine. Relative enzyme activities refer to cytosol fractions treated in the same way but in the absence of diguanidines. ●, Methylglyoxal bis(guanylhydrazone) + putrescine; ○, methylglyoxal bis(guanylhydrazone); ▲, 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) + putrescine; △, 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine).

by dialysis (Fig. 1*a*). A comparable inhibition of adenosylmethionine decarboxylase activity was obtained when rat liver cytosol was used as the source of the enzyme (Fig. 1*b*). Again, the inhibition of the enzyme activity produced by the drugs in the absence of putrescine could be completely removed by extensive dialysis. In the presence of the diamine, methylglyoxal bis(guanylhydrazone), and especially 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine), caused a more or less irreversible disappearance of the enzyme activity (Fig. 1*b*). Thus the 'irreversibility' (Pegg & Conover, 1976; Pegg, 1978) of the 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine)-induced inhibition of adenosylmethionine decarboxylase activity critically depends on the presence of putrescine, the activator of the reaction. These results are in accordance with those obtained by Williams-Ashman & Schenone (1972).

Inhibition of growth of cultures of Ehrlich ascites cells by diguanidine derivatives

At concentrations (5 and 15 μM) sufficient to inhibit adenosylmethionine decarboxylase activity, methylglyoxal bis(guanylhydrazone) and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) decreased the concentration of spermidine and spermine (as expressed per mg of DNA) in the tumour cells, while markedly raising the concentration of putrescine (Table 1). An increase in cell number was markedly prevented in cultures grown in the presence of diguanidine derivatives, as was the synthesis and accumulation of DNA and protein (Table 1). The incorporation of radioactive orotic acid into alkali-hydrolysable material, however, proceeded as in the absence of the inhibitors (results not shown).

1,3-Diamino-2-propanol, an indirect inhibitor of mammalian ornithine decarboxylase (Kallio, 1978; Piik *et al.*, 1978), was included in the experiments for comparative purposes. This drug effectively inhibited the formation of putrescine and spermidine, while the concentration of spermine was only moderately decreased (Table 1). Despite the fact that diaminopropanol decreased the concentration of total polyamines (spermidine plus spermine) more profoundly than did the diguanidine derivatives, its antiproliferative action was not nearly as profound as exerted by the diguanidine derivatives (Table 1). This finding may indicate that the growth-inhibiting effects of methylglyoxal bis(guanylhydrazone) and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) were not entirely based upon polyamine deprivation, or that diaminopropanol, as an amine, partly fulfills the requirements for natural polyamines.

A commonly reported 'side effect' of methylglyoxal bis(guanylhydrazone) is its ability to

Table 1. *Effect of diaminopropanol, methylglyoxal bis(guanylhydrazone) and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) on polyamine content and macromolecular synthesis in Ehrlich ascites cells grown in culture*

Ehrlich ascites cells were grown (initial density 0.16×10^6 cells/ml; DNA content 5.37 μg/culture and protein content 0.30 mg/culture) in the absence (control) or presence of 3 mM-diaminopropanol, 5 μM-methylglyoxal bis(guanylhydrazone) (MGBG) or 15 μM-1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) (MBAG) for 2 days.

Treatment	Polyamine content (nmol/mg of DNA)			[^{3}H]Thymidine incorporation (c.p.m./μg of DNA)	DNA content (μg/culture)	[^{14}C]Leucine incorporation (c.p.m./μg of protein)	Protein content (mg/culture)	$10^{-6} \times$ Cell density (cells/ml)
	Putrescine	Spermidine	Spermine					
Control	126	830	430	11600	15.6	72.5	0.51	0.38
Diaminopropanol	38.3	222	307	8570	14.5	46.6	0.51	0.29
MGBG	600	697	225	2810	12.0	33.0	0.39	0.22
MBAG	425	509	186	2360	11.8	31.3	0.39	0.21

stabilize adenosylmethionine decarboxylase against intracellular degradation. The stabilization results in a paradoxical accumulation of the enzyme in cells exposed to the drug (Pegg *et al.*, 1973; Fillingame & Morris, 1973; Pegg & Jefferson, 1974; Pegg, 1979). As shown in Table 2, not only methylglyoxal bis(guanylhydrazone) but also 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) strikingly increased adenosylmethionine decarboxylase activity (when assayed after sufficient dilution or dialysis of the enzyme preparation), in cultures grown in the presence of the drug. Pegg (1979) recently found that while methylglyoxal bis(guanylhydrazone) strikingly increased both the activity and the amount of enzyme protein, 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) only resulted in an accumulation of antigen with no catalytic activity. The reasons why 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) also produced a paradoxical rise in enzyme activity in Ehrlich ascites cells are not known.

Interestingly, in tumour cells grown in the presence of diaminopropanol, the activity of adenosylmethionine decarboxylase was likewise increased, though not as dramatically as in the presence of the diguanidine derivatives (Table 2). An enhancement of adenosylmethionine decarboxylase activity has been shown to occur in cultured cells exposed to inhibitors of ornithine decarboxylase (Mamont *et al.*, 1978), which do not influence adenosylmethionine decarboxylase activity *in vitro*, but rapidly deplete cells of spermidine. As reported earlier for methylglyoxal bis(guanylhydrazone) (Hölttä *et al.*, 1973; Heby *et al.*, 1973), 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) also produced a substantial increase in ornithine decarboxylase activity (Table 2).

The striking increase in adenosylmethionine decarboxylase activity in ascites cells grown in the presence of the diguanidine derivatives was expectedly based upon a dramatic prolongation of the half-life of the enzyme. In control cultures, the activity of adenosylmethionine decarboxylase decayed in the presence of cycloheximide with an apparent half-life of 22 min, whereas in cultures exposed to either diguanidine derivative the apparent half-life was extended to 2–8 h (Table 3). Unexpectedly, diaminopropanol also appeared to increase the stability of adenosylmethionine decarboxylase, yet the lengthening of the half-life was not nearly as striking as that produced by the diguanidines (Table 3). The difference of the half-lives may be attributable to the fact that the diguanidines increased the activity of adenosylmethione decarboxylase much more than did diaminopropanol. The variations between the half-lives of adenosylmethionine decarboxylase from cells treated with diguanidines may reflect the toxic effects of prolonged exposure to cycloheximide.

Onset of the anti-proliferative action of methylglyoxal bis(guanylhydrazone) and 1,1′-[(methylethanediylidene)dinitrilo]bis - (3 - aminoguanidine) and its relation to polyamine accumulation

Decreases in cellular concentrations of spermidine and spermine in cultures grown in the presence of methylglyoxal bis(guanylhydrazone) or 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) became apparent only after an exposure of the cells to the drugs for 12 h or more (Fig. 2). Interestingly, before any changes were seen in the concentrations of higher polyamines, enhanced putrescine accumulation had almost reached its

Table 2. *Effect of diaminopropanol, methylglyoxal bis(guanylhydrazone) (MGBG) and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) (MBAG) on ornithine decarboxylase and adenosylmethionine decarboxylase activities in Ehrlich ascites cells grown in culture*

For experimental details see Table 1.

Treatment	Enzyme activity (pmol of CO_2 produced/mg of protein)	
	Ornithine decarboxylase	Adenosylmethionine decarboxylase
Control	82	274
Diaminopropanol	1.7	1100
MGBG	306	12100
MBAG	264	11900

Table 3. *Effect of diaminopropanol, methylglyoxal bis(guanylhydrazone) and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) on the half-life of adenosylmethionine decarboxylase*

Stock cultures were diluted with fresh medium to an initial density of 0.19×10^6 cells/ml and were grown in the absence or presence of 3 mM-diaminopropanol, 10 μM-methylglyoxal bis(guanylhydrazone) (MGBG) or 30 μM-1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) (MBAG) for 2 days. Adenosylmethionine decarboxylase activity was assayed after an exposure of the cultures to cycloheximide (10 μg/ml) for 0–240 min.

Treatment	Half-life of adenosylmethionine decarboxylase (min)	
	Expt. 1	Expt. 2
Control	22	23
Diaminopropanol	46	48
MGBG	126	378
MBAG	228	486

maximum (Fig. 2). This finding does not support the commonly expressed view that methylglyoxal bis-(guanylhydrazone)-induced accumulation of putrescine would be based upon a block of the conversion of the diamine to spermidine. In comparison with the diguanidines, diaminopropanol induced a more rapid fall in tumour spermidine concentration (Fig. 2).

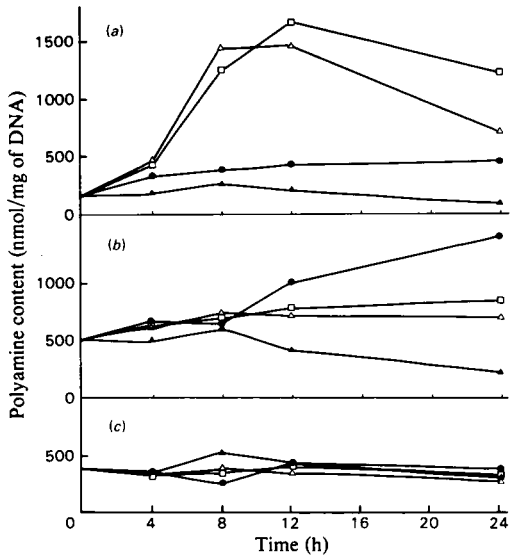

Fig. 2. *Effect of diaminopropanol, methylglyoxal bis-(guanylhydrazone) and 1,1'-[(methylethanediylidene)-dinitrilo]bis-(3-aminoguanidine) on polyamine accumulation in Ehrlich ascites cells grown in culture*

The cells were diluted to an initial density of 0.18×10^6 cells/ml and grown in the absence (●) or presence of 10 μM-methylglyoxal bis(guanylhydrazone) (△), 30 μM-1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) (□), or 3 mM-diaminopropanol (▲). The concentrations of the polyamines were measured at time points indicated. (*a*) Putrescine; (*b*) spermidine; (*c*) spermine.

Table 4 shows the development of the depression of DNA and protein synthesis in cultures grown in the presence of methylglyoxal bis(guanylhydrazone), 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) or diaminopropanol. The incorporation of labelled precursors into macromolecules (DNA and protein) was distinctly inhibited after an exposure of the cultures to diguanidines for 8 h, i.e. well before any changes were observed in the concentrations of spermidine and spermine (Fig. 2). Even though diaminopropanol decreased the total concentration of tumour polyamines more markedly and rapidly in comparison to the diguanidines, the former compound only marginally depressed macromolecular synthesis (Table 4). The reasons for this apparent dissociation of the early anti-proliferative actions of diguanidines from polyamine deprivation are not known.

Effect of inhibitors of polyamine biosynthesis on the degradation of glucose in Ehrlich ascites cells

It has been reported that, preceding its anti-proliferative action, methylglyoxal bis(guanylhydrazone) exerts a deleterious effect on mitochondria in leukaemia L1210 cells (Dave *et al.*, 1978). In order to get some idea of the mitochondrial function of tumour cells exposed to diguanidines for short periods of time, the capacity of the cells to degrade uniformly labelled glucose was determined. As seen in Fig. 3(*a*), none of the experimental drugs produced any significant changes in the oxidation of glucose to CO_2 over the first 24 h, during which time an inhibition of protein and DNA synthesis had already developed (Table 4). The activation of glycolysis (production of lactate), upon dilution of the tumour cells with fresh medium, likewise occurred as in the absence of inhibitors (Fig. 3*b*). Determination of the ATP content of cells exposed to diguanidines or diaminopropanol for 24 h revealed the following results (triplicate measurements from duplicate cultures): control cells, 4.08; methylglyoxal bis-(guanylhydrazone), 3.52; 1,1'-[(methylethanediyl-

Table 4. *Effect of methylglyoxal bis(guanylhydrazone) (MGBG), 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) (MBAG) and diaminopropanol on development of macromolecular synthesis in Ehrlich ascites cells grown in culture*

Experimental details are as in Fig. 2.

Time (h)	Thymidine incorporation (c.p.m./μg of DNA) in cells treated with				Leucine incorporation (c.p.m./μg of protein) in cells treated with			
	Control	MGBG	MBAG	Diaminopropanol	Control	MGBG	MBAG	Diaminopropanol
0	4120	—	—	—	33.0	—	—	—
4	7450	5400	5790	6750	44.4	43.0	39.1	42.7
8	9410	5050	6640	7470	41.6	27.4	27.3	27.4
12	16900	6520	10240	14700	37.7	17.8	19.9	25.0
24	9100	2870	4730	6620	48.9	14.8	17.1	37.0

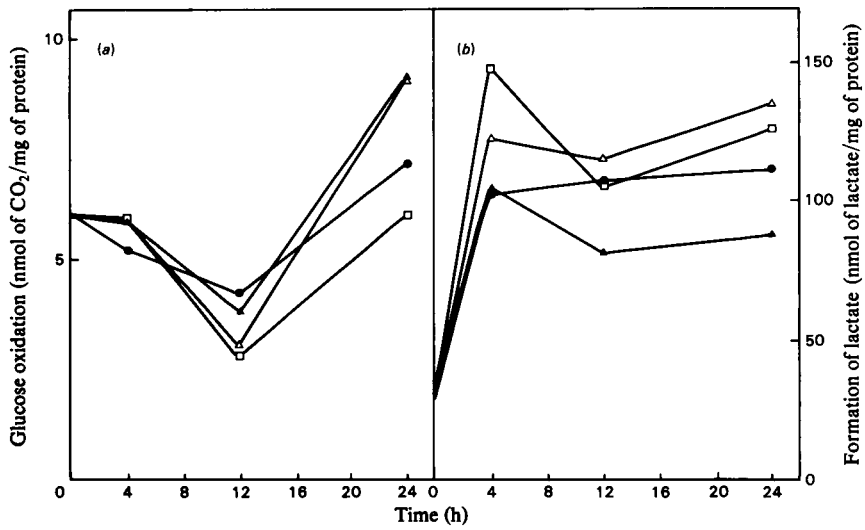

Fig. 3. *Effect of diaminopropanol, methylglyoxal bis(guanylhydrazone) and 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) on the oxidation of glucose to CO_2 (a) and the formation of lactate (b)*

Experimental details are as in Fig. 2. △, Methylglyoxal bis(guanylhydrazone); □, 1,1'-[(methylethanediylidene)-dinitrilo]bis-(3-aminoguanidine); ▲, diaminopropanol; ●, control.

idene)dinitrilo]bis-(3-aminoguanidine), 3.52; diaminopropanol, 3.31 pg/cell, respectively. Thus the content of ATP in the tumour cells exposed to the inhibitors was only marginally (14–19%) decreased. It thus appears that inhibition of energy production (mitochondrial or glycolytic) is not an early metabolic effect, in comparison with the impaired synthesis of macromolecules, in Ehrlich ascites cells exposed to diguanidine derivatives.

Reversal of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine)-induced growth inhibition by polyamines

1,1' - [(Methylethanediylidene)dinitrilo]bis - (3 - aminoguanidine) not only prevented any increases in cell number, but in many experiments also induced a gradual fall of the original cell density, and loss of viability (Fig. 4). Just as reported for methylglyoxal bis(guanylhydrazone) (for references see Jänne *et al.*, 1978), the anti-proliferative action of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) could be prevented by spermine and spermidine, while putrescine was in this respect only partially active (Fig. 4).

A straightforward interpretation of the reversal of the diguanidine-induced toxicity by spermidine and spermine as a correction of intracellular polyamine shortage is complicated by the fact that higher polyamines apparently interfere with the uptake of methylglyoxal bis(guanylhydrazone) (Mihich *et al.*, 1974). Because the concentrations of diguanidines

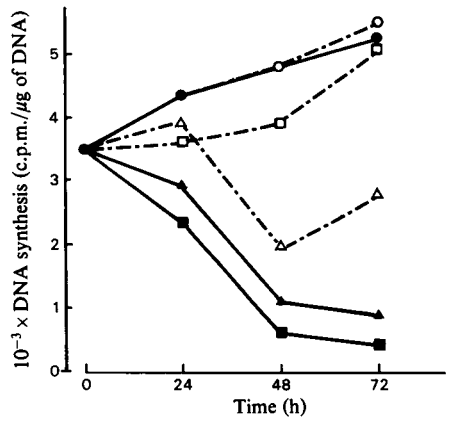

Fig. 4. *Effect of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) and methylglyoxal bis-(guanylhydrazone) on DNA synthesis in cultured Ehrlich ascites cells*

The cells were grown (initial density 0.44×10^6 cells/ml) in the absence (●) or presence of 30 μM-1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) (▲) or 10 μM-methylglyoxal bis-(guanylhydrazone) (■) with or without 0.1 mM-putrescine, spermidine or spermine. The incorporation of labelled thymidine into DNA was measured every 24 h. △, 1,1'-[(Methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) + putrescine; □, 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) + spermidine; ○, 1,1'-[(methylethanediylidene)dinitrilo]bis - (3 - aminoguanidine + spermine.

used were very low, thus making it difficult to directly measure the intracellular concentrations of the drugs, the possible effects of polyamines on diguanidine uptake were investigated indirectly. As shown earlier (Table 2), both diguanidines produce a dramatic increase in adenosylmethionine decarboxylase activity (provided that the activity is measured after sufficient dilution or dialysis to remove the inhibitors). Polyamines alone did not increase adenosylmethionine decarboxylase activity, as seen Table 5. When the tumour cells were grown in the presence of 30 μM-1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine), the activity of adenosylmethionine decarboxylase was increased 100-fold (Table 5). The anti-proliferative effect of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) was largely prevented by spermidine or spermine even at a concentration of 20 μM (Table 5). Spermidine, at concentrations from 20 to 50 μM, did not modify the 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine)-induced increase in adenosylmethionine decarboxylase to any appreciable extent. However, spermine, at a concentration of 20 μM, slightly reduced the 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine)-induced enhancement of the enzyme activity, as did higher concentrations of spermidine (100–300 μM). The latter finding apparently implies that higher concentrations of spermidine, and especially of spermine, partly interfered with the transport of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) into the cells. However, even though the disappearance of enhanced enzyme activity in the presence of high concentrations of polyamines can be taken as a proof of impaired drug uptake, the relationship between enhanced adenosylmethionine decarboxylase activity and the intracellular concentration of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) is not clear. In fact, our results (not shown) seem to indicate that although at low and non-inhibitory concentrations of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-amino-

Table 5. *Reversal of the anti-proliferative effect of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) by polyamines*

Ehrlich ascites cells (initial density 0.17×10^6 cells/ml) were grown in the absence or presence of increasing concentrations of putrescine, spermidine or spermine with or without 30 μM-1,1'-[(methylethanediylidene)dinitrilo]-bis-(3-aminoguanidine) (MBAG).

Treatment	Concentration of amine (μM)	Adenosylmethionine decarboxylase activity (nmol of CO_2/mg of protein)	DNA content (μg/culture)	Protein content (μg/culture)	$10^{-6} \times$ Cell density (cells/ml)
A. Control	—	0.30	13.5	424	0.566
+ putrescine	20	0.00	13.4	434	0.582
+ spermidine	20	0.13	13.9	411	0.580
+ spermine	20	0.10	13.5	388	0.580
+ putrescine	50	0.21	15.4	460	0.590
+ spermidine	50	0.25	14.8	469	0.580
+ spermine	50	0.11	15.2	462	0.596
+ putrescine	100	0.23	16.3	525	0.646
+ spermidine	100	0.23	14.3	460	0.596
+ spermine	100	0.11	15.3	462	0.596
+ putrescine	300	0.31	14.6	479	0.608
+ spermidine	300	0.26	15.5	479	0.600
+ spermine	300	0.12	14.1	430	0.548
B. With MBAG	—	33.8	5.8	216	0.300
+ putrescine	20	67.9	5.0	176	0.316
+ spermidine	20	32.1	10.9	334	0.540
+ spermine	20	14.1	12.0	355	0.496
+ putrescine	50	46.4	7.1	197	0.362
+ spermidine	50	33.4	12.6	372	0.490
+ spermine	50	14.5	12.2	348	0.516
+ putrescine	100	71.4	6.2	202	0.336
+ spermidine	100	22.0	11.1	374	0.514
+ spermine	100	11.4	12.5	395	0.540
+ putrescine	300	45.2	9.8	300	0.378
+ spermidine	300	11.5	12.6	393	0.548
+ spermine	300	5.0	12.7	344	0.536

guanidine) the enzyme activity rapidly decreased, there was no linear correlation between the activation of the enzyme and extracellular diguanidine concentrations. The possibility thus remained that low polyamine concentrations impaired the uptake of the drug.

Discussion

The present results show that the properties of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) are strikingly similar to those of methylglyoxal bis(guanylhydrazone) when used in cell cultures. Although 1,1'-[(methylethanediylidene)-dinitrilo]bis-(3-aminoguanidine) certainly differs from methylglyoxal bis(guanylhydrazone) as regards its irreversible mode of action in the presence of putrescine, both compounds share many common features. These include the marked stabilization of adenosylmethionine decarboxylase and an apparent competition with higher polyamines for a common (or similar) cellular uptake system.

Methylglyoxal bis(guanylhydrazone) was the first inhibitor of the biosynthetic enzymes of polyamines to be employed systematically to induce intracellular polyamine deprivation in rapidly dividing animal cells. Over the past few years, more experimental data have accumulated suggesting that methylglyoxal bis(guanylhydrazone) can exert metabolic effects, which may not be related to polyamine metabolism, in various cells.

When potent inhibitors of ornithine decarboxylase became available, it was possible to compare the relationship of polyamine deprivation, induced by inhibitors with different modes of action, to the development of anti-proliferative effects. Newton & Abdel-Monem (1977) showed that, when L1210 leukaemia cells were grown in the presence of either α-methylornithine or methylglyoxal bis(guanylhydrazone), the accumulation of spermidine was prevented to a roughly comparable extent, yet only methylglyoxal bis(guanylhydrazone) decreased the content of DNA in the tumour cells. Similarly, we (Hölttä *et al.*, 1979*b*) could demonstrate distinct dissociation of the early anti-proliferative actions of methylglyoxal bis(guanylhydrazone) from polyamine depletion in human fibroblasts grown in the presence of methylglyoxal bis(guanylhydrazone) or difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978). In spite of the similar inhibition of polyamine accumulation produced by the drugs, only methylglyoxal bis(guanylhydrazone) depressed the synthesis of DNA during the first cell cycle. A delay of 2 days was required before any changes of DNA synthesis were observed in cultures grown in the presence of difluoromethylornithine (Hölttä *et al.*, 1979*b*). Pertinent to these findings was the observation of Rupniak & Paul (1978) indicating that low concentrations (1 μM) of methylglyoxal bis(guanylhydrazone), although preventing any increases in spermidine and spermine accumulation in rat liver cells and fibroblasts upon medium replacement, did not influence the early enhancement of thymidine incorporation or increases in labelling index. In some cell types (BHK cells) the effect of methylglyoxal bis(guanylhydrazone) on cell growth could be partly reversed by an excess of magnesium ions (Melvin & Keir, 1979) indicating that the requirements of intracellular polyamines can be met by these inorganic cations, or that methylglyoxal bis(guanylhydrazone) somehow interacts with intracellular magnesium ions.

Our present results with Ehrlich ascites cells likewise support the idea that the early anti-proliferative effects, not only of methylglyoxal bis(guanylhydrazone) but also of 1,1'-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine), can be dissociated from intracellular polyamine deprivation. This conclusion is based on the experimental findings showing that the diguanidine derivatives inhibited the incorporation of labelled thymidine into DNA and that of leucine into protein well before any changes (except an enhancement of putrescine accumulation) in polyamine concentrations were observed (Table 4). Moreover, exposure of the tumour cells to diaminopropanol, a potent inhibitor of ornithine decarboxylase (Kallio, 1978; Piik *et al.*, 1978), resulted in an earlier and more pronounced decrease in total polyamines, than did the diguanidines, with less marked anti-proliferative changes. The fact that most of the effects exerted by methylglyoxal bis(guanylhydrazone) on living cells can be reversed by simultaneous addition of spermidine or spermine, has been taken as proof for a polyamine-mediated action of this compound. However, this does not necessarily mean that the reversal of the cytostatic action of methylglyoxal bis(guanylhydrazone) is based upon an intracellular correction of polyamine shortage, since polyamines and methylglyoxal bis(guanylhydrazone) compete for a common uptake system in various cells (Mihich *et al.*, 1974; Mandel & Flintoff, 1978). There are only a few reports in which this point has been properly examined, but an interference of polyamines with methylglyoxal bis(guanylhydrazone) at the level of the cell membrane has been excluded (Feil *et al.*, 1977; Melvin & Keir, 1979). We have approached the problem by using the striking enhancement of adenosylmethionine decarboxylase in cells grown in the presence of the drugs as an indirect measure for their cellular uptake. Although less pronounced enhancement of adenosylmethionine decarboxylase activity in the presence of polyamines indicates reduced drug uptake, the presence of marked accumulation of the enzyme cannot be taken as a

direct proof of unaltered intracellular 1,1′-[(methylethanediylidene)dinitrilo]bis - (3 - aminoguanidine) concentration.

The fact, however, remains that the anti-proliferative action of both methylglyoxal bis(guanylhydrazone) and 1,1′-[(methylethanediylidene)dinitrilo]bis-(3-aminoguanidine) developed before any decreases in the concentrations of higher polyamines. When searching for those metabolic actions of these compounds which are unrelated to polyamine metabolism, a tempting possibility would be an early impairment in energy-yielding processes, especially since it has been reported that methylglyoxal bis(guanylhydrazone) inhibits oxidative phosphorylation and cell respiration (Pine & DiPaolo, 1966) and induce mitochondrial damage that precedes growth arrest (Dave *et al.*, 1978). This possibility, however, was not supported by the present data obtained with Ehrlich ascites cells. The oxidation of glucose to CO_2 (which is an indicator of mitochondrial function), and the activation of glycolysis upon change of medium proceeded virtually normally during the first day of exposure, at the time when the anti-proliferative action and polyamine deprivation were already established. Moreover, the cellular contents of ATP were only marginally decreased in cells exposed to the drugs for 24 h.

Even though no metabolically mediated disturbances by diguanidines have been identified so far, it is fairly possible that these basic compounds, just like natural polyamines, interact directly with some cellular components or structures. It has been actually reported that methylglyoxal bis(guanylhydrazone) (and spermine) could interact with eukaryotic DNA resulting in a decrease in template activity (Brown *et al.*, 1975). Although this effect was seen only *in vitro* at millimolar concentrations of the drug, i.e. two orders of magnitude higher than those usually employed in cell cultures, it should be taken into consideration that some mammalian cells appear to possess enormous capacity to concentrate methylglyoxal bis(guanylhydrazone) intracellularly from the medium (Mandel & Flintoff, 1978).

A concentration gradient across the cell membrane as high as 800-fold can be achieved (Mandel & Flintoff, 1978). Accordingly, a 5 μM concentration of methylglyoxal bis(guanylhydrazone) in the medium could result in something like 4 mM intracellular concentration of the drug, which already markedly impairs template activity (Brown *et al.*, 1975). However, no morphological changes in chromatin were observed in L1210 cells grown in the presence of 0.1–10 μM-methylglyoxal bis(guanylhydrazone) (Pathak *et al.*, 1977). In agreement with the decreased template activity was the observation of Dave *et al.*, (1978) indicating that treatment of L1210 cells with methylglyoxal bis(guanylhydrazone) results in a destabilization of DNA as judged by enhanced release of radioactivity from pre-labelled DNA in the presence of the drug.

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