Investigations of the mechanism by which mammalian cell growth is inhibited by N^1N^{12} -bis(ethyl)spermine

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N^1N^{12} -Bis(ethyl)spermine (BESM) and related compounds are powerful inhibitors of cell growth that may have potential as anti-neoplastic agents [Bergeron, Neims, McManis, Hawthorne, Vinson, Bortell and Ingeno (1988) *J. Med. Chem.* **31**, 1183–1190]. The mechanism by which these compounds bring about their effects was investigated by using variant cell lines in which processes thought to be altered by these agents are perturbed. Comparisons between the response of these cells and of their parental equivalents to BESM, N^1N^{11} -bis(ethyl)norspermine, N^1N^{14} -bis(ethyl)homospermine and N^1N^8 -bis(ethyl)spermidine were then made. It was found that D-R cells, an L1210-derived line that over-expresses ornithine decarboxylase, were not resistant to these compounds. This indicates that the decrease in ornithine decarboxylase is not critical for the action of the compounds on cell growth. Furthermore, although polyamine levels were decreased in the D-R cells, the content was not totally

depleted, indicating that such depletion is also not essential for the anti-proliferative effect. Two cell lines lacking mitochondrial DNA (human 143B206 cells and chicken DU3 cells) did not differ in sensitivity to BESM from their parental 143BTK[−] and DU24 cells. Furthermore, the inhibition of respiration in L1210 cells in response to BESM developed more slowly than the inhibition of growth. Thus it appears that the inhibitions of mitochondrial DNA synthesis and of mitochondrial respiration are also not primary factors in the anti-proliferative effects of these polyamine analogues. The inhibition of growth did, however, correlate with the intracellular accumulation of the analogues. It appears that the bis(ethyl)polyamine derivatives act by binding to intracellular target molecules and preventing macromolecular synthesis. The decline in normal polyamines may facilitate such binding, but is not essential for growth arrest.

INTRODUCTION

Polyamines are ubiquitous components of living cells, and the clear need for polyamine synthesis in order to maintain normal growth rates has led to much interest in the production of therapeutically useful agents that interfere with normal polyamine function (Pegg and McCann, 1982; McCann et al., 1987; Pegg, 1988, Jänne et al., 1991). These studies have revealed that specific inhibitors of polyamine biosynthesis are clearly useful as drugs for the treatment of parasitic protozoa and have considerable potential for a number of other applications. However, with certain exceptions, these inhibitors have not been particularly successful as anti-neoplastic agents. In part, this may be due to their inability *in vivo* to produce an adequate depletion of tumour-cell polyamines, owing to compensatory changes in cellular polyamine metabolism (Pegg, 1986, 1988). The content of polyamines in mammalian cells is regulated by the intracellular level of polyamines in a manner that maintains polyamine levels within a relatively narrow range. This regulation is brought about by a combination of the polyamine-mediated repression of the biosynthetic enzymes ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase, induction of the degradative enzyme spermidine/spermine N^1 -acetyltransferase, and changes in polyamine uptake and efflux mechanisms.

A second approach to this problem has been the synthesis of polyamine analogues. A number of such derivatives have been synthesized and found to be effective antiproliferative agents. These include a series of bis(ethyl)polyamine derivatives of varying length that can be thought of as spermine analogues in

which the terminal nitrogen atoms carry an ethyl group (Bergeron et al., 1988, 1989; Casero et al., 1989a,b; Prakash et al., 1990; Basu et al., 1990a, 1991; Bowlin et al., 1991; Bernacki et al., 1992; Chang et al., 1992). These agents and related compounds are under active development as anti-neoplastic agents, but the mechanism by which they interfere with cell growth is unclear.

Numerous cellular components have been suggested as targets of polyamine analogues by previous experiments. Firstly, these compounds mimic the action of the natural polyamine, spermine, in repressing the activity of the biosynthetic enzymes, ODC and *S*-adenosylmethionine decarboxylase (Porter et al., 1987, 1990). This decrease shuts off the biosynthesis of polyamines. Secondly, they also mimic the natural polyamines in their ability to induce spermidine/spermine N^1 -acetyltransferase and to cause the efflux of polyamines from the cell (Pegg et al., 1989; Casero et al., 1989a; Libby et al., 1991; Porter et al., 1991). These changes may lead to a more complete depletion of cellular polyamines than is achieved by the application of direct inhibitors of polyamine biosynthesis. However, it is by no means clear that the interference with polyamine synthesis and even the depletion of normal polyamines is responsible for the antiproliferative effects of these compounds. Evidence has been presented that N^1N^{12} -bis(ethyl)spermine (BESM) can replace spermine in its effects on protein synthesis and cell growth (Igarashi et al., 1990), and thus could take over these functions from the natural polyamine. A dramatic decrease in mitochondrial DNA has been observed in cells treated with these analogues (Bergeron et al., 1988; Vertino et al., 1991). This decrease is consistent with a complete inhibition of mitochondrial DNA synthesis and may indicate that the drugs

Abbreviations used: ODC, L-ornithine decarboxylase; DFMO, α -difluoromethylornithine; BESM, N^1N^{12} -bis(ethyl)spermine; BENSM, N^1N^{11} -bis(ethyl)norspermine; BEHSM, N^1N^{14} -bis(ethyl)homospermine; BESPD, N^1N^8 -bis(ethyl)spermidine; MGBG, methylglyoxal bis(guanyldihydrazone).

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have a primary effect on mitochondrial metabolism by a dysfunctional replacement of spermine at mitochondrial sites. Other studies have indicated that these polyamine analogues bind tightly to DNA and influence its structure (Basu et al., 1989, 1990b; Feuerstein et al., 1990) and that nuclear chromatin structure is altered in cells treated with N^1N^{14} -bis(ethyl)-homospermine (BEHSM) (Basu et al., 1992).

In the present work we have carried out experiments to determine the critical target sites of BESM and related compounds by using mutant cell lines which have alterations in some of these potential targets. Thus we have used D-R cells that enormously overproduce ODC (Pegg et al., 1988a) to determine whether repression of this enzyme is necessary for the action of BESM. We have also used cell lines lacking mitochondrial DNA (Desjardins et al., 1986; Morais et al., 1988; King and Attardi, 1989) to assess the importance of mitochondrial DNA synthesis as a target. The results indicate that the inhibition of cell growth did not correlate with either the inhibition of ODC or the effects on mitochondrial metabolism or on the depletion of polyamine levels. It appears that the accumulation of the analogues themselves and their binding to intracellular targets is responsible for their anti-proliferative effects.

MATERIALS AND METHODS

Materials

Bis(ethyl)polyamine derivatives were synthesized by published methods (Bergeron et al., 1988, 1989). Other biochemical reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. DFMO was provided by Marion Merrell Dow, Cincinnati, OH, U.S.A.

Cell culture

D-R (Pegg et al., 1988a) and L1210 cells were grown at 37 °C in suspension culture in RPMI-1640 medium containing 10 % NuSerum (Collaborative Research, Lexington, MA, U.S.A.) in an atmosphere of 5 % CO₂ and in the presence of 95 % relative humidity. Cells were seeded at 5×10^4 cells/ml for most experiments and passaged twice weekly. Stock cultures of D-R cells were supplemented with 10 mM α -difluoromethylornithine (DFMO), which was removed for 48 h before plating for experimentation.

Human 143BTK⁻ osteosarcoma cells and 143B206 cells, also referred to as ρ^0 206, which are 'petite' cells, depleted of mitochondrial DNA, derived from 143BTK⁻ cells (King and Attardi, 1989) were generously provided by Dr. G. Attardi, California Institute of Technology, Pasadena, CA, U.S.A. These cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 5 % fetal-bovine serum and supplemented with 50 μ g/ml uridine plus 100 μ g/ml pyruvate. Cells were passaged every fourth day with a change of medium every second day. For experiments, 143BTK⁻ cells were seeded at 1.5×10^5 cells/100 mm dish, whereas ρ^0 206 cells were seeded at 2×10^5 cells/100 mm dish, due to small differences in doubling times.

DU24 primary chick-embryo fibroblast cells and the DU3 petite cell line lacking mitochondrial DNA derived from them by selection with ethidium bromide (Desjardins et al., 1986) were generously provided by Dr. R. Morais, University of Montreal, Montreal, Quebec H3E 3J7, Canada. These cells were grown in monolayer in RPMI-1640 media containing 10 % fetal-bovine serum and supplemented with 4 μ g/ml uridine and 50 μ g/ml pyruvate. Cells were passaged every third day, and medium was

changed on each second day. Since the doubling time of DU3 petite cells was half that of the parental DU24 cells, DU24 cells were seeded at 2×10^5 cells/100 mm dish and DU3 cells were seeded at 4×10^5 cells/100 mm dish for experimentation.

Treatment of cells with bis(ethyl)polyamine derivatives and determination of polyamines

Cells were seeded at their respective densities as stated above. Analogue additions to the culture medium were made at 4 h after plating, at which time 1 mM aminoguanidine was added to inhibit serum amine oxidase and prevent degradation of extracellular polyamines. For determination of cell number, cells grown as monolayers were harvested by washing once in phosphate-buffered saline, followed by treatment with 0.25 % buffered trypsin and resuspension in media. Cell number was determined by electronic particle counting using a model Z_i cell counter (Coulter Electronics, Hialeah, FL, U.S.A.). Cell pellets were prepared for polyamine determination by deproteinization in a 200–500 μ l volume of 0.2 M HClO₄. Cell lysis was ensured by sonication for 15 s in a sonicating water bath. Protein was pelleted by centrifugation, and the polyamine content of the supernatant was then determined by h.p.l.c. as previously described, using ion-pair reverse-phase h.p.l.c. with detection by post-column derivative formation with *o*-phthalaldehyde (Pegg et al., 1988a). Owing to the inability of the analogues to react with *o*-phthalaldehyde, analogues were measured by using a method of pre-column derivative formation with dansyl chloride (Basu et al., 1990a). These analyses were carried out in the laboratory of Dr. L. J. Marton, Brain Tumour Research Center and the Department of Laboratory Medicine, University of California School of Medicine, San Francisco, CA, U.S.A. The protein pellets were resuspended in a small volume of 0.1 M NaOH, and protein was determined by the method of Bradford (1976). Polyamine and analogue levels were expressed as nmol/mg of protein.

Respiration measurements

L1210 cells were pelleted by low-speed centrifugation and washed with an equal volume of ice-cold sucrose buffer (250 mM sucrose, 10 mM MgCl₂, 20 mM Hepes, pH 7.1). The pellet was resuspended in one-fifth the original volume of sucrose buffer. Samples of cells were removed for determination of cell number and viability. Cell number was determined as described above. Viability was determined by Trypan Blue exclusion by using a 1:1 dilution of cells to dye. Cells were permeabilized by a 30 s treatment with 5 μ l of 10 % digitonin/10⁷ cells. Cells were quickly pelleted and resuspended at 10⁸ cells/ml in respiration buffer (250 mM sucrose, 10 mM MgCl₂, 20 mM Hepes, pH 7.1, 2 mM K₂HPO₄, 1 mM ADP and 5 mM potassium malate). Oxygen consumption was measured by a method adopted from Granger and Lehninger (1982), using a Clark electrode in a closed 1 ml chamber. The electrode signal was monitored on a chart recorder. Calibration was established by equilibrating 1.2 ml of respiration buffer at 37 °C with air in an open chamber, and then setting a 0–100 % scale with a closed chamber. Samples (0.1 ml) of the permeabilized cell suspension were added to 1.2 ml of air-saturated respiration buffer. The chamber was sealed with a stopper and additions were made through a capillary port in the following order: 100 nM rotenone was added to block malate-dependent respiration; oxygen consumption was initiated by addition of 5 mM potassium succinate and monitored over 4 min; respiration was terminated with 1 mM NaCN. The solubility of oxygen in air-saturated medium at 37 °C was taken

as 390 ng-atoms/ml. Oxygen consumption is expressed as ng-atoms of oxygen consumed/min per 10^6 cells.

RESULTS

Effect of bis(ethyl)polyamines on growth and polyamine content of D-R cells

In agreement with previous reports by Bergeron and colleagues (Porter and Bergeron, 1988; Bergeron et al., 1989), the growth of L1210 cells was significantly affected by bis(ethyl)polyamines, with the relative order of potency being BESPD < BENSM < BESM < BEHSM. Representative results are shown in Figure 1. The effect on growth was more evident at 48 and 72 h after exposure to the compounds, and the approximate IC_{50} values at 72 h of exposure were 35 μ M for BESPD, 15 μ M for BENSM, 1 μ M for BESM and 0.3 μ M for BEHSM. All of these compounds also inhibited the growth of D-R cells, and were at least as active in their inhibitory actions towards these cells as they were towards the parent L1210 cells (Figure 1). In fact, in every case

the D-R cells appeared to be slightly more sensitive, although this difference is small.

D-R cells, which derive their resistance to DFMO by expressing several-hundredfold higher levels of ODC, contain much higher levels of putrescine than do L1210 cells (Pegg et al., 1988a). These results therefore render it highly unlikely that the repression of ODC by the bis(ethyl)polyamines (Porter et al., 1987; Pegg et al., 1988b) can be responsible for their anti-proliferative action. This conclusion is supported by measurement of the intracellular levels of putrescine and polyamines present at 72 h after addition of these compounds (Table 1); the levels of putrescine and of total polyamines in the untreated D-R cells were much greater than those present in the untreated L1210 cells, which is consistent with previous observations (Pegg et al., 1988a). Spermidine content was somewhat higher in the D-R cells, but the spermine content was lower. The addition of the bis(ethyl)polyamines greatly decreased putrescine and spermidine contents in both cell lines, but in the D-R cells there was still a substantial amount of putrescine and/or spermidine remaining. There was a lesser, but still significant, decrease in spermine, but again there was no correlation between the extent of the decrease and the growth-inhibitory action of the bis(ethyl)polyamines. These results therefore suggest that these polyamine analogues act at a site other than the polyamine-biosynthetic pathway.

There was little difference between the two cell lines in their accumulation of the analogues, but it appeared to be slightly greater in the D-R cells. It was found that BEHSM and BESM were accumulated more extensively than BENSM in both lines. The anti-proliferative effect of these compounds therefore does correlate with the efficiency of uptake of the drug.

Figure 1 Effect of bis(ethyl)polyamines on growth of L1210 and D-R cells

L1210 cells (○) or D-R cells (■) were seeded at a density of 5×10^4 cells/ml and grown for up to 72 h in the presence of 25 μ M BENSM (a), 5 μ M BESM (b), 1 μ M BEHSM (c) or 50 μ M BESPD (d) as indicated.

Effect of BESM on respiration of L1210 cells

It has been shown that much of the mitochondrial DNA is lost from L1210 cells or 935.1 fibroblasts within 48–72 h of treatment with BESM (Bergeron et al., 1988; Vertino et al., 1991). This depletion is a result of the inhibition of mitochondrial DNA replication, followed by dilution of the DNA with cell division. Experiments were therefore carried out to explore the possibility that mitochondrial functioning was altered by these polyamine analogues. In order to determine if mitochondrial respiration was altered by the uptake of BESM, succinate-dependent respiration was measured in permeabilized L1210 cells after treatment with 10 μ M BESM. Oxygen consumption was measured at

Table 1 Intracellular content of polyamines in L1210 and D-R cells after exposure to bis(ethyl)polyamines

The compounds shown were added at 10 μ M and the cells were harvested 72 h later. Results are shown as means $\pm$ S.D. for 3–6 observations (N.D., not determined); *the total could not be determined, since BESPD was not quantified.

Cell line	Addition	Polyamine content (nmol/mg of protein)				
		Putrescine	Spermidine	Spermine	Analogue	Total
L1210	None	4.3 ± 0.1	34.6 ± 3.4	21.9 ± 5.8	0.0	60.8
	BESPD	0.2 ± 0.1	1.4 ± 0.2	6.1 ± 1.1	N.D.	*
	BENSM	0.4 ± 0.1	1.7 ± 0.1	2.5 ± 0.2	38.0 ± 3.2	42.6
	BESM	< 0.1	2.9 ± 2.7	10.2 ± 0.5	66.8 ± 4.0	79.8
	BEHSM	0.5 ± 0.2	< 1.0	4.3 ± 0.3	54.6 ± 1.4	60.3
D-R	None	60.1 ± 2.5	49.8 ± 2.3	10.9 ± 0.6	0.0	120.8
	BESPD	16.3 ± 2.5	18.4 ± 2.8	8.2 ± 1.0	N.D.	*
	BENSM	21.5 ± 2.5	8.2 ± 2.1	2.3 ± 0.2	52.3 ± 5.6	84.3
	BESM	1.8 ± 0.2	3.9 ± 2.8	8.1 ± 0.6	74.9 ± 3.0	88.7
	BEHSM	8.2 ± 0.2	2.2 ± 0.3	2.4 ± 0.1	77.6 ± 7.0	90.4

Figure 2 Effect of BESM on respiration of L1210 cells
Cells were treated with 10 μ M BESM, and at the times shown, samples were removed, permeabilized and oxygen consumption measured (●). Cell viability (■) was also measured as indicated. Respiration and viability are expressed as percentages of control values.

12–72 h after drug treatment (Figure 2). Although respiration was decreased slightly relative to control by 24 h, a significant loss of function was not observed until after 48 h of drug treatment. The small loss of respiration seen at early times appears to be insufficient to account for the striking effects on the cells brought about by BESM. Furthermore, the eventual decline in respiration from 80 % of control at 48 h to 8 % of control at 72 h was not associated with cell death. Viability, as measured by Trypan Blue exclusion, remained above 95 % up to 72 h. These results indicate that the early loss of mitochondrial DNA does not damage respiratory capabilities over the time period in which cell growth was affected. This is probably due to the relatively slow turnover of key proteins of the respiratory chain, such as cytochrome *c* oxidase, that are coded for by mitochondrial DNA.

Effect of bis(ethyl)polyamines on growth and polyamine content of cells lacking mitochondrial DNA

In order to determine if the loss of mitochondrial DNA has any direct and early effect on the anti-proliferative potential of these drugs, two ‘petite’ cell lines lacking mitochondrial DNA were examined in terms of cell growth and polyamine content after drug treatment. DU3 petite cells were derived by chronic exposure of normal chick-embryo fibroblast cells (DU24) to ethidium bromide (Desjardins et al., 1986; Morais et al., 1988). The second petite cell line, 143B206, also referred to as ρ^0 206, was

Figure 3 Effect of BESM on growth of 143BTK[−] and ρ^0 206 cells
The 143BTK[−] cells were seeded at 1.5×10^5 cells/100 mm dish, and ρ^0 206 cells were seeded at 2×10^5 cells/100 mm dish. The cells were treated with 1 μ M (a), 5 μ M (b) or 10 μ M (c) BESM (□, ■) or no addition (○, ●). At the times shown, the cell number was determined. Results are shown for 143BTK[−] (●, ■) and ρ^0 206 (○, □) cells.

derived from the human osteosarcoma cell line 143BTK[−] (King and Atardi, 1989). Both petite cell lines are dependent on glycolysis and were grown in the presence of pyruvate. Uridine was added, since these cell lines also require this for growth, owing to their deficiency in respiratory-chain-dependent dihydro-orotate dehydrogenase. The effect of BESM on the growth of the 143BTK[−] cell line and its petite counterpart ρ^0 206 is shown in Figure 3. The doubling times of these two cell lines were similar (16 h for ρ^0 206 and 13.3 h for 143BTK[−]). Addition of 1, 5 or

Table 2 Intracellular content of polyamines and BESM in 143BTK[−] and ρ^0 206 cells after treatment with BESM

Compounds shown were added at the concentrations shown and the cells were harvested 72 h later. Results are shown as means $\pm$ S.D. for 3–6 observations.

Cell line	BESM treatment	Polyamine content (nmol/mg of protein)				
		Putrescine	Spermidine	Spermine	BESM	Total
143BTK [−]	None	8.7 $\pm$ 1.3	30.5 $\pm$ 5.9	19.9 $\pm$ 2.9	0.0	59.1
	1 μ M	8.5 $\pm$ 0.7	22.1 $\pm$ 1.9	13.5 $\pm$ 1.2	28.1 $\pm$ 2.7	72.2
	5 μ M	< 1.2	3.3 $\pm$ 2.5	2.7 $\pm$ 0.4	69.8 $\pm$ 4.2	42.6
	10 μ M	< 1.2	2.8 $\pm$ 2.5	2.2 $\pm$ 0.2	62.2 $\pm$ 2.7	79.8
ρ^0 206	None	7.4 $\pm$ 1.0	28.5 $\pm$ 5.5	18.1 $\pm$ 3.4	0.0	54.0
	1 μ M	6.8 $\pm$ 2.5	17.2 $\pm$ 3.5	9.9 $\pm$ 2.2	26.4 $\pm$ 1.0	60.3
	5 μ M	1.5 $\pm$ 2.5	3.2 $\pm$ 0.2	2.2 $\pm$ 0.2	64.8 $\pm$ 6.8	71.7
	10 μ M	< 1.2	3.3 $\pm$ 2.3	2.3 $\pm$ 0.3	74.7 $\pm$ 5.2	81.8

Figure 4 Effect of MGBG on growth of (a) 143BTK⁻ and (b) $\rho^{\circ}206$ cells

The cells were seeded as in Figure 3 and treated with 0 (●), 0.3 μM (■), 1 μM (○) or 4 μM (□) MGBG, and the cell number was determined after the time shown.

Figure 5 Effect of BESM on growth of DU3 and DU24 cells

DU24 cells were seeded at 2×10^5 cells/100 mm dish and DU3 cells at 4×10^5 cells/100 mm dish. The cells were treated with 5 μM (○, ●) or 10 μM (□, ■) BESM as shown, and the inhibition of cell growth was plotted against the number of cell divisions. Results are shown for DU3 cells (○, □) and DU24 cells (●, ■).

10 μM BESM to cultures of these cells resulted in a proportional decrease in cell growth. Both cell lines showed an inhibition of cell growth to 55% of control at 48 h, and to approx. 65% of control by 72 h. Thus the petite cells show no greater resistance to the drug than do the cells from which they were derived, even though they contain no mitochondrial DNA. Table 2 shows the drug and polyamine levels in these two cell lines. These results indicate that both cell lines take up the drug equally well and approach saturating levels with addition of 5 μM BESM. Additionally, at each drug concentration given, the levels of putrescine, spermidine and spermine in 143BTK⁻ cells were very closely matched to the levels found in the petite cells. The fact that the petite cells are not resistant to drug action suggests that the early loss of mitochondrial DNA seen in L1210 cells after BESM treatment (Bergeron et al., 1988; Vertino et al., 1991) has no direct causal link to the loss of cell growth. However, the similarity in growth inhibition of the two cell lines does correlate with the similarities in polyamine depletion and drug accumulation, suggesting that these parameters may be critical for the antiproliferative effect of BESM.

Methylglyoxal bis(guanyldiazide) (MGBG) is another polyamine analogue which has a profound effect on mitochondrial function. Treatment of a variety of cell lines, including L1210 cells, with MGBG leads to gross ultrastructural changes in the mitochondria, impairment of mitochondrial oxygen consumption and inhibition of the replication of mitochondrial DNA (Feuerstein et al., 1979; Pleshkewych et al., 1980; Nass, 1984). However, when MGBG was added to cultures of $\rho^{\circ}206$ and 143BTK⁻ cells, there was no difference in the extent of growth inhibition (Figure 4). Thus, petite cells are not more resistant than normal cells to the anti-proliferative effect of MGBG, a result identical with that found with BESM.

In order to rule out the possibility that the results obtained with the human 143BTK⁻ cells and their derivative lacking mitochondrial DNA were not an anomaly due to some unknown property of these cells, comparisons were also made of the chick-embryo fibroblast lines DU24 and DU3. The latter cell line also lacks mitochondrial DNA. DU3 cells grew at one-half the rate of the DU24 cells, which have a generation time of 12 h. Due to the differences in doubling time, the effect of BESM on cell growth, expressed as percentage of control, was plotted against the number of cell divisions (Figure 5). The DU3 cells were not resistant to BESM. In fact, there was no difference in the

Table 3 Intracellular content of polyamines and BESM in DU24 and DU3 cells after treatment with BESM

Experimental details are the same as for Table 2.

Cell line	Treatment	Time (h)	Polyamines (nmol/mg of protein)				
			Putrescine	Spermidine	Spermine	BESM	Total
DU24	None	24	57.0 $\pm$ 6.9	94.3 $\pm$ 16.2	85.2 $\pm$ 3.9	0.0	99.7
	5 mM	24	28.5 $\pm$ 8.4	38.5 $\pm$ 4.2	37.3 $\pm$ 5.7	41.4 $\pm$ 4.6	141.7
	10 mM	24	19.5 $\pm$ 1.9	31.9 $\pm$ 2.4	33.7 $\pm$ 2.1	45.8 $\pm$ 4.2	136.0
DU3	None	24	36.9 $\pm$ 5.4	51.5 $\pm$ 1.5	34.3 $\pm$ 0.9	0.0	116.0
	5 mM	24	26.3 $\pm$ 7.5	36.7 $\pm$ 13.7	33.9 $\pm$ 5.8	39.7 $\pm$ 3.6	143.3
	10 mM	24	25.4 $\pm$ 4.7	30.3 $\pm$ 4.0	29.7 $\pm$ 6.9	43.9 $\pm$ 13.4	130.2
DU24	None	72	5.5 $\pm$ 0.4	26.4 $\pm$ 0.8	17.3 $\pm$ 1.2	0.0	54.6
	5 mM	72	2.3 $\pm$ 0.3	3.7 $\pm$ 0.4	9.3 $\pm$ 0.5	38.3 $\pm$ 1.6	53.1
	10 mM	72	2.5 $\pm$ 0.3	4.0 $\pm$ 0.3	9.1 $\pm$ 0.6	37.4 $\pm$ 4.1	52.8
DU3	None	72	9.1 $\pm$ 2.1	25.9 $\pm$ 1.6	12.4 $\pm$ 1.1	0.0	48.2
	5 mM	72	4.5 $\pm$ 0.5	5.3 $\pm$ 0.2	7.6 $\pm$ 0.3	36.6 $\pm$ 1.3	52.8
	10 mM	72	4.7 $\pm$ 0.3	5.6 $\pm$ 0.5	8.3 $\pm$ 0.3	41.3 $\pm$ 2.2	58.0

response to 5 μ M BESM, and the DU3 cells were slightly more sensitive than DU24 cells to 10 μ M BESM. The intracellular accumulation of BESM was the same in both cell lines (Table 3). In the absence of the drug, the levels of polyamine at 24 h were significantly higher in DU24 than in DU3 cells. However, addition of BESM led to a time-dependent decrease in all polyamine pools, and the final intracellular concentrations of polyamines in the two cell lines were not significantly different from one another. These results confirm in a different cell line that the absence of mitochondrial DNA does not influence the sensitivity of cells to BESM. It is therefore highly unlikely that the loss of mitochondrial DNA is a cause of the growth inhibition by polyamine analogues.

DISCUSSION

Our results demonstrate that the anti-proliferative activity of BESM and derivatives cannot be accounted for either by the early loss of mitochondrial DNA or by the loss of respiratory function. Both human (143B206) and chicken (DU3) petite cell lines responded in a similar fashion as their normal counterparts to treatment with BESM. Human and petite cells responded with a dose-dependent inhibition of growth (Figure 3), corresponding to a loss of natural polyamine pools (Table 2). However, polyamine levels were not significantly different from control levels at the lowest concentration of drug (1 μ M). This may be explained by the fact that the intracellular accumulation of drug was much lower than at higher drug doses. This is consistent with the idea that the uptake of drug and replacement at macromolecular binding sites are essential factors in the anti-proliferative activity of these polyamine analogues.

Chicken-derived petite (DU3) cells also showed no greater resistance to the anti-proliferative activities of BESM compared with normal cells (Figure 5). In fact, DU3 cells may have been somewhat more sensitive, and responded with a dose-dependent inhibition of growth, whereas the normal cells did not. However, the polyamine levels were again similar in normal and petite cells after 72 h of drug treatment (Table 3), although DU24 cells had gone through twice the number of cell divisions. This can be accounted for by the fact that the uptake of drug was similar, and the intracellular content of drug must reach saturating levels within the first cell doubling.

The fact that petite cells are not more resistant to BESM growth-inhibitory effects than the normal cells suggests that a decrease in energy pools is not a prerequisite to decreased growth, since the petite cells are not respiring. This agrees with results by Feuerstein et al. (1979) that growth inhibition of L1210 cells treated with MGBG could not fully be accounted for by depletion of ATP pools. Although one might expect that replication of mitochondrial DNA would be inhibited immediately in normal cells (Bergeron et al., 1988), respiration may still be near normal capacity for a considerable time after drug treatment, due to stability of respiratory proteins.

The small changes in oxygen consumption over 96 h of exposure to BESM (Figure 2) were not sufficient to account for the rapid loss of growth in L1210 cells. Thus, even in the absence of mitochondrial DNA, cells continue to respire, suggesting that a loss of mitochondrial DNA does not contribute greatly to the anti-proliferative action of these polyamine analogues.

Rather, the early loss of mitochondrial DNA in response to BESM treatment probably reflects the importance of polyamines in mitochondrial DNA stability (Tabor and Tabor, 1964), even more so than nuclear DNA, which is protected by histones, or perhaps reflects the importance of polyamine binding to regula-

tory proteins such as an initiation factor or a polymerase involved in DNA replication (Welter et al., 1989).

It is also clear that the repression of ODC activity cannot be responsible for the anti-proliferative action of BESM. The growth of D-R cells that greatly over-produce ODC was not refractory to the bis(ethyl)spermine derivatives (Figure 1), despite having significant residual polyamines (Table 1). This contrasts sharply with previous findings with the compound 1-amino-oxy-3-aminopropane, which was shown to have ODC as its principal cellular target by the cross-resistance of the DFMO-resistant D-R cells (Poulin et al., 1989). It should be noted that D-R cells do not have elevated levels of *S*-adenosylmethionine decarboxylase and that the repression of this enzyme could still be of importance in decreasing cellular spermidine and spermine contents. The results with D-R cells and the conclusion that ODC is not the critical target for the bis(ethyl)spermine analogues are in agreement with the recent report by Ghoda et al. (1992), showing that mammalian cell lines transfected with plasmids expressing the trypanosome ODC, which is not sensitive to repression by polyamine analogues were not resistant to BEHSM.

However, in fact the D-R cells were somewhat more sensitive than the parental L1210 cells to the bis(ethyl)spermine derivatives, despite having significantly higher polyamine levels after treatment. It therefore appears that there is no exact correlation between the depletion of polyamines and the growth inhibition. It is nevertheless quite conceivable that the decrease in the normal intracellular polyamines allows the binding of the analogues to critical intracellular sites to occur more readily. In this context, the enormous increase in spermidine/spermine *N*¹-acetyltransferase activity that occurs in response to the analogues (Casero et al. 1989a, 1990; Pegg et al., 1989; Libby et al., 1989; Chang et al., 1992) may be of major importance, since the acetylation decreases the charge on the polyamines, therefore decreasing their binding to cellular macromolecules, and the released acetylated polyamines are rapidly degraded or excreted.

The apparent increase in sensitivity to the analogues of the D-R cells may be related to the higher intracellular accumulation of the drugs in these cells (Table 1). The reason for this increase is not clear at present, but recent studies on the D-R cells have indicated that their capacity to take up exogenous polyamines is increased (R. Poulin and A. E. Pegg, unpublished work). Since the bis(ethyl)spermine derivatives are transported on the same carriers as natural polyamines (Porter and Bergeron, 1988; Byers and Pegg, 1989; Bergeron et al., 1989), this may explain the increased content. Uptake of BESM and derivatives is clearly absolutely necessary for their actions. Mutant CHO cells lacking the polyamine transport system are not affected by these drugs even at levels of 200 μ M (Byers and Pegg, 1989). Although BESM was able to mimic several of the actions of spermine, including its ability to restore growth of polyamine-depleted lymphocytes (Igarashi et al., 1990), it is likely that the high level of intracellular accumulation of these agents interferes with chromatin structure and function. The polyamine analogues bind strongly to DNA and chromatin (Feuerstein et al., 1988, 1989; Basu et al., 1990b; Edwards et al., 1991), and changes in cellular polyamine levels are clearly associated with alterations in chromatin structure (Snyder, 1989; Basu et al., 1992). It is possible that the high level of accumulation of the polyamine analogues leads to dysfunctional changes in chromatin. This process may well be enhanced by the decrease in natural polyamines that allows a greater binding of the analogues at nuclear sites.

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