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Selective Cellular Depletion of Mitochondrial DNA by the Polyamine Analog N^1,N^{12}-Bis(ethyl)spermine and Its Relationship to Polyamine Structure and Function

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SUMMARY

N^1,N^8-Bis(ethyl)spermidine (BESPD) and N^1,N^{12}-bis(ethyl)spermidine (BESPM) are minimally modified analogs of spermidine and spermine that deplete cellular polyamine pools by suppressing key polyamine biosynthetic enzymes. The consequences of polyamine depletion and the concomitant analog replacement of these pools were compared on two cellular DNA targets, mitochondrial DNA (mtDNA) and a defined nuclear DNA episome present in 935.1 mouse fibroblasts. The spermidine analog, BESPD, depleted cellular putrescine and spermidine pools, but not spermine pools, and had no effect on either DNA target. Treatment with the corresponding analog of spermine, BESPM, resulted in a near-total depletion of all three polyamine pools and a >80% reduction in the cellular content of mtDNA, without affecting the levels of the nuclear episome. Topological forms analysis by Southern blotting of mtDNA and episomal DNA from BESPM-treated cells failed to reveal any forms interconversion, indicating the absence of analog-induced single- or double-strand break damage to either DNA target. The growth-dependent loss of mtDNA is consistent with a rapid cessation of mtDNA replication and subsequent dilution of existing mtDNA copies by cell division. Similar decreases in polyamine pools and mtDNA were also observed in L1210 cells treated with BESPM. When a comparable level of polyamine depletion was produced in L1210 cells by specific enzyme inhibitors, there was no effect on the cellular content of mtDNA, and BESPD was not rendered capable of decreasing mtDNA levels. Because the analogs are structurally similar to the naturally occurring polyamines and would be expected to have similar binding properties, the loss in mtDNA may reflect dysfunctional replacement by BESPM at spermine-specific binding sites in the mitochondrion.

The endogenous polyamines PUT, SPD, and SPM are essential to the maintenance of eukaryotic cell proliferation. Although the precise molecular basis for this requirement is not well understood, there is considerable evidence to suggest that one such activity may involve the direct interaction of polyamines with DNA. SPD and SPM have been shown *in vitro* to protect DNA from heat- or alkali-induced denaturation and enzymatic degradation, to promote the condensation of DNA into organized structures, and to facilitate the transition of certain DNA sequences from a B helical conformation to the A and Z forms (reviewed in Ref. 1). Recent studies involving synthetic polyamine analogs indicate that polyamine interactions with DNA are highly structure and charge dependent (2–4). In addition, X-ray crystallographic data and computer modeling studies (5, 6) predict a rather specific interaction between SPM and DNA, which, according to molecular mechanics, could produce a bent DNA structure as a result of ligand binding (7).

Due to the noncovalent nature of polyamine interactions with macro-molecules, information concerning the intracellular binding sites and distribution of polyamines has been difficult to obtain. Studies addressing polyamine function have, therefore, been most effectively approached pharmacologically, through the use of either specific inhibitors of polyamine biosynthesis or auxotrophic mutants deficient in polyamine biosynthesis. Evidence from such studies suggests a role for polyamines in chromatin structure, on the basis of correlations between inhibitor-induced decreases in intracellular polyamine pools and various chromatin-related effects (8–10). We have previously demonstrated that certain bis(ethyl) derivatives of polyamines can also be very effective in depleting intracellular polyamine pools (11). Unlike inhibitors of biosynthesis, BESPD and BESPM deplete cellular polyamine pools by post-transcriptionally regulating the synthesis of one or both of the key polyamine biosynthetic enzymes, ODC and AdoMetDC (12,

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ABBREVIATIONS: PUT, putrescine; AdoMetDC, *S*-adenosylmethionine decarboxylase; AMA, *S*-(5′-deoxy-5′-adenosyl)methylthioethylhydroxylamine; BESPD, N^1,N^8-bis(ethyl)spermidine; BESPM, N^1,N^{12}-bis(ethyl)spermine; DFMO, α-difluoromethylornithine; kb, kilobase pairs; mtDNA, mitochondrial DNA; ODC, ornithine decarboxylase; PBS, phosphate-buffered saline; SPD, spermidine; SPM, spermine.

13). Polyamine depletion achieved by this approach differs from that of enzyme inhibitors by having, as an end result, the replacement of intracellular polyamine pools with a structurally similar analog. Because the regulation of the biosynthetic enzymes by the analogs appears to occur via mechanisms identical to those used by their natural polyamine counterparts (12, 13), the analogs seem able to substitute in at least certain polyamine-mediated functions. However, because the analogs do not support cell growth during polyamine depletion (11), dysfunctional replacement at other sites of polyamine activity can be inferred. Identification and characterization of those cellular sites could provide information concerning polyamine function.

The aim of the present study was to determine the effects of polyamine depletion and analog replacement of polyamines on two cellular DNA targets, episomal DNA and mtDNA. Because mtDNA and nuclear (episomal) DNA appear to be packaged differently (14), we propose that they may also be differentially sensitive to polyamine analogs or intracellular polyamine perturbations. Studies were carried out with transformed fibroblasts (935.1 cells) containing an 8.8-kb nuclear episomal element that is stably maintained as a nonintegrated, high copy number (100–200/cell), minichromosome (15). The cells have recently been shown to be a useful and relevant model for intracellular drug-chromatin studies (16). The results indicate that the SPM analog BESPM (but not the SPD analog BESPD) selectively depletes cellular mtDNA by apparently interacting in a structure-dependent manner with site(s) that may be involved in the replication of mtDNA.

Experimental Procedures

Materials. BESPD and BESPM were synthesized as previously described (17, 18). DFMO was generously supplied by the Merrell Dow Research Institute (Cincinnati, OH). AMA was synthesized (19) and generously provided by Drs. A. and R. Khomutov of the USSR Academy of Medical Sciences (Moscow) and, in part, synthesized and provided by Dr. B. Paul of this Institute. Pancreatic RNase A, proteinase K, and *Hae*II were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN), and DNase I was obtained from Cooper Biomedical (Malvern, PA). Dansyl chloride was purchased from the Pierce Chemical Co. (Rockford, IL). Nitrocellulose membranes were purchased from Schleicher & Schuell Inc. (Lincoln Park, NJ). SeaKem GTG agarose was obtained from FMC Bioproducts (Rockland, ME).

The 935.1 murine fibroblast cell line (15) and the pM19 plasmid, a pBR322 derivative that contains the entire pm19 episomal sequence, were gifts from Dr. G. Hagar of the National Cancer Institute (Bethesda, MD). The plasmid pAM1 is a pACYC177 derivative containing the entire mitochondrial genome from mouse LA9 cells (20) and was generously provided by Dr. D. A. Clayton of Stanford University (Palo Alto, CA). The L1210 murine leukemia cell line was obtained from the American Type Culture Collection (Rockville, MD).

Cell culture. The 935.1 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum and 1.1 mM glutamine (complete medium), in a humidified 5% CO_2 atmosphere at 37°. L1210 cells were maintained as suspension cultures in RPMI 1640 (GIBCO) supplemented with 10% Nu-serum IV (a semidefined serum substitute; Collaborative Research, Bedford, MA), in a humidified 5% CO_2 atmosphere at 37°.

For cell treatments, BESPD, BESPM, and AMA were dissolved in distilled water and DFMO was dissolved in 0.5 N NaOH; the solutions were sterilized before use by passage through a 0.22-μm filter. Logarithmically growing 935.1 cells were harvested by trypsinization and diluted into 60-mm or 100-mm tissue culture dishes, in a total volume of 5 or 12.5 ml of complete medium, respectively. Cells were allowed to attach overnight before the start of a 48- or 96-hr incubation time. L1210 cells were diluted to a final density of 3×10^4 cells/ml in 75-cm^2 tissue culture flasks, and the 48-hr incubation time started immediately. Cell numbers were determined by electronic particle counting (model ZM Coulter counter; Coulter Electronics, Hialeah, FL). Cell viability, as determined by trypan blue exclusion, remained above 90% for all treatments.

Polyamine pool determinations. 935.1 or L1210 cells were harvested, washed twice with ice-cold PBS, and extracted with 0.6 M perchloric acid at 1–2 × 10^7 cells/ml for 1 hr on ice. Amines in the acid-soluble extracts were dansylated as previously described, using diaminoheptane as an internal standard (21). A high performance liquid chromatographic method was used to separate the dansylated polyamines. Fifty microliters of the dansylated sample were injected (model AS-48 autosampler; Bio-Rad Laboratories, Richmond, CA) onto a 15-cm × 4.6-mm, 3-μm particle size Spherisorb phenyl column (Phase Sep Ltd, Deerside Industrial Park, UK). The polyamines were eluted at 1 ml/min, using a methanol gradient in 1.25 mM K_2HPO_4, pH 4.5.[1] The fluorescent dansyl compounds were detected by a Kratos FS 950 Fluoromat detector (ABI Analytical, Ramsey, NJ), with a 340-nm excitation filter and a 514-nm emission filter. Peak areas were calculated by a Perkin-Elmer LCI 100 integrator (Norwalk, CT), and the polyamines were identified and quantitated by comparison with known standards. Values were adjusted to nmol/10^6 cells.

ODC and AdoMetDC activities. Extracts from ~10^7 cells were prepared by sonication (5 sec) in 25 mM Tris (pH 7.5), 0.1 mM EDTA, 2.5 mM dithio-threitol, at 4°, and the extract supernatants were collected by centrifugation at 10,000 × *g* for 10 min. Measurement of ODC and AdoMetDC enzyme activities in the cell extracts was based on the catalytic release of [^{14}C]CO_2 from L-[1-^{14}C]ornithine and *S*-adenosyl-L-[*carboxy*-^{14}C]methionine (NEN Division, Dupont Company, Wilmington, DE), respectively, as previously described (22, 23). The total protein content of the extracts was measured using a Bio-Rad protein dye assay, according to the manufacturer's specifications (Bio-Rad Laboratories). Samples were assayed in duplicate, and values were corrected for nonenzymatic CO_2 release using values obtained from reactions in which the cell extract was omitted. Results were expressed as mean nmol of [^{14}C]CO_2 released/hr·mg of protein.

Dot blot and Southern analysis of mtDNA and episomal DNA. 935.1 cell monolayers in 60-mm tissue culture dishes were rinsed twice with 5 ml of PBS at 4° and lysed *in situ* with 0.6 ml of lysis buffer (1% sodium dodecyl sulfate, 10 mM Tris·HCl, 10 mM Na_2EDTA, pH 8.0) at room temperature. For L1210 cells, pellets containing 10^6 to 10^7 PBS-washed cells were similarly lysed. DNA in the lysates was purified by consecutive incubation with RNAse A (50 μg/ml at 37° for 4 hr) and proteinase K (100 μg/ml at 37° overnight), followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and two extractions with chloroform/isoamyl alcohol (24:1). DNA was recovered by ethanol precipitation at −20° and resuspended in 1 mM Tris·HCl, 0.1 mM EDTA, pH 8.0. DNA concentrations were determined from the UV absorbance at 260 nm.

The total level of mitochondrial or episomal DNA in experimental samples was quantitated using a dot blot procedure. Serial 2-fold dilutions from 0.3 μg of total cellular DNA were denatured by boiling for 5 min, followed by quick-cooling in ice water. The samples were brought to a final buffer concentration of 10× standard saline citrate (1.5 M NaCl, 0.15 M sodium citrate, pH 7) and dotted onto a nitrocellulose membrane, using a BIO-DOT vacuum manifold (Bio-Rad Laboratories). Specific DNAs bound to the membrane were detected by hybridization to either nick-translated [^{32}P]pAM1 (mitochondrial) or [^{32}P]pM19 (episomal) plasmid probe (specific activity, 1×10^8 cpm/μg)

[1] Gradient profile (in terms of the volume percentage of buffer B in a mix of buffers A and B) was as follows: step 1, from 0% B to 45% B over 7.5 min; step 2, from 45% B to 55% B over 7.5 min; step 3, from 55% B to 70% B over 20 min; step 4, from 70% B to 90% B over 10 min; and step 5, 90% B for 30 min; where buffer A is 30% methanol in 1.25 mM K_2HPO_4, pH 4.5, and buffer B is 80% methanol in 1.25 mM K_2HPO_4, pH 4.5.

(24), and the hybridized blots were exposed to Kodak XAR-5 film. The intensities of the dots on the resultant autoradiograph were quantitated in the linear film response range, using a Helena Quick-scan densitometer (detector voltage = 700 V) interfaced to an Apple IIc computer with a baseline correction and integration program (Helena Laboratories, Beaumont, TX). The intensity of the untreated control was considered to be 100%. Results were expressed as the sample intensity relative to that of the untreated control.

For Southern analysis, 0.5 μg of purified cellular DNA was electrophoresed on 0.7% agarose gels in 1× Tris/borate/EDTA buffer (89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3) containing 5 μg/ml ethidium bromide, at 100 V (5 V/cm) for 10 hr, with constant water-cooling (11°) and buffer recirculation. A full-length (16.3-kb) linear mtDNA marker, generated by *Hae*II restriction endonuclease digestion of pAM1, and DNA size markers ranging from 0.5 to 23 kb (Boehringer-Mannheim Biochemicals) were included on each gel. For DNase I studies, a 20-μl reaction containing 1 μg of purified DNA in 10 mM Tris·HCl, 10 mM NaCl, 3 mM $MgCl_2$, pH 7.4, was digested with DNase I, at final concentrations ranging from 0.1 to 3.2 ng/ml, at 37° for 5 min. The reaction was stopped by the addition of Na_2EDTA to 50 mM. One quarter of the reaction (0.25 μg of DNA) was electrophoresed as described above. After electrophoresis, the genomic DNA band was visualized using an UV transilluminator (model 3-3000; Fotodyne, New Berlin, WI) to check for consistent DNA loading in all lanes. DNA was transferred to nitrocellulose membranes by Southern blotting and hybridized either to nick-translated [^{32}P]pAM1 (mitochondrial) or to [^{32}P]AM19 (episomal) plasmid probe (specific activity, 1×10^8 cpm/μg) (23). Blots were exposed to Kodak XAR-5 film for autoradiography. The intensities of the mitochondrial or episomal bands corresponding to each topological form [supercoiled (form I), nicked circular (form II), and linear (form III)] were quantitated densitometrically, as described above, and the intensity of the form I band was expressed relative to the combined intensities of forms I, II, and III.

Results

At extracellular concentrations of 100 and 10 μM, respectively, BESPD and BESPM inhibited the growth of 935.1 cells to a similar extent over a 96-hr period (Fig. 1) but differentially affected polyamine biosynthesis. BESPD treatment resulted in a complete loss of detectable ODC activity and a 1.8-fold increase in AdoMetDC activity by 96 hr (Table I). At this time, PUT and SPD pools were almost completely depleted, whereas SPM pools were relatively unaffected (Table 1). BESPM treatment, in contrast, caused a significant decrease in both ODC and AdoMetDC activities. Like BESPD, BESPM treatment caused the near-complete depletion of cellular PUT and SPD pools and produced, in addition, an 85% decrease in cellular SPM pools (Table I). Although the effects of both BESPD and BESPM on polyamine metabolism in 935.1 cells appeared to be nearly complete by 48 hr, the cells continued to accumulate either analog for at least an additional 48 hr to an amount equivalent to about twice the original cellular polyamine content (Table 1). Because the extracellular concentrations of 100 μM BESPD and 10 μM BESPM resulted in comparable levels of analog accumulation and caused maximal polyamine depletion, they were used for all subsequent studies.

Thus, under the treatment conditions described above, the polyamine content of 935.1 cells treated with BESPD was composed essentially of a mixture of the SPD analog and the natural polyamine SPM, whereas in the case of BESPM treatment the polyamine content consisted almost entirely (~93%) of the SPM analog (Table 1). The effects of these polyamine perturbations on mtDNA and episomal DNA were investigated. Dot blot analysis of cellular DNA isolated from 935.1 cells

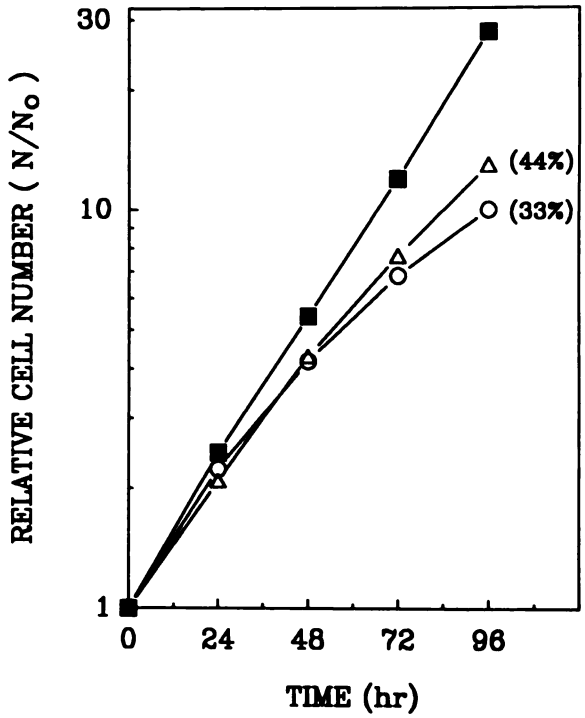

Fig. 1. Growth of 935.1 cells in the absence (■) or presence of 100 μM BESPD (△) or 10 μM BESPM (○). Mean of duplicate determinations from a representative experiment. *Numbers in parentheses*, percentage of control growth at 96 hr.

treated for up to 96 hr with BESPD revealed no significant change in the levels of either mtDNA or episomal DNA, relative to total cellular DNA (Fig. 2). To ensure that the extracellular concentration of BESPD was not a limiting factor in its ability to affect DNA, 935.1 cells were also treated with 1 mM BESPD. In spite of a 23% increase in the intracellular accumulation of BESPD, mtDNA and episomal DNA levels remained unaffected (data not shown).

In contrast, analysis of cellular DNA isolated from 935.1 cells that were treated with 10 μM BESPM revealed a time-dependent loss of the mitochondrial-specific sequences, relative to total cellular DNA (Fig. 3). Like the effects on polyamine pools, mtDNA depletion was nearly maximal by 48 hr and decreased only slightly over the next 48 hr, to 12% of control (Table 1). Importantly, the levels of episomal DNA in these samples were relatively unaffected over the same time period. In each case, mtDNA and episomal DNA content were quantitated relative to total cellular DNA. Because the amount of total DNA isolated per cell remained constant during BESPM (or BESPD) treatment for up to 96 hr (data not shown), the quantitation was considered to be a meaningful reflection of the cellular contents of mtDNA and episomal DNA.

To further characterize the effect of BESPM treatment on mtDNA, DNA isolated from BESPM-treated 935.1 cells was subjected to ethidium bromide-agarose gel electrophoresis and Southern blot analysis. For mtDNA, three topological forms of monomeric length were resolved, supercoiled circles (form I), nicked (relaxed) circles (form II), and linear (form III) (see Fig. 4A, *lane 1*). The identification of forms I, II, and III was aided by the use of DNase I to specifically introduce single-strand nicks into mtDNA. Because one single-strand break converts form I (supercoiled) to form II (nicked circular) molecules and, after more extensive damage, two opposing breaks convert form

TABLE 1

Effects of polyamine analogs on polyamine metabolism and mtDNA and episomal DNA content of 935.1 cells

Treatment	Time[a]	Decarboxylase activities[b]		Polyamine pools[c]				DNA content[d]	
		ODC	AdoMetDC	PUT	SPD	SPM	Analog	mtDNA	Episomal DNA
	hr	*nmol of CO_2/hr/mg of protein*		*nmol/10^8 cells*				*% of control*	
None	48	1.68	0.72	0.11 ± 0.05	2.56 ± 0.32	2.22 ± 0.44		100	100
100 μM BESPD	48	0.20	0.71	0.02 ± 0.01	0.52 ± 0	1.95 ± 0.57	4.52 ± 0.06	108	106
10 μM BESPM	48	ND[e]	0.14	ND	0.05 ± 0.04	0.27 ± 0.07	6.42 ± 2.48	24	115
100 μM BESPD	96	ND	1.26	ND	0.28 ± 0.01	1.89 ± 0.52	7.04 ± 1.06	106	109
10 μM BESPM	96	ND	0.22	ND	0.03 ± 0.01	0.26 ± 0.02	9.79 ± 1.91	12	113

[a] Exposure time. Total culture duration in each case was 96 hr.
[b] Mean of three independent experiments performed in duplicate (six experiments), where the standard error was less than 10% of the mean.
[c] Mean ± standard error of two to four independent determinations.
[d] Mean of two independent determinations, where the standard error was less than 10% of the mean.
[e] ND, none detected (<0.01 nmol of polyamine/10^8 cells or <0.01 nmol of CO_2/hr/mg of protein).

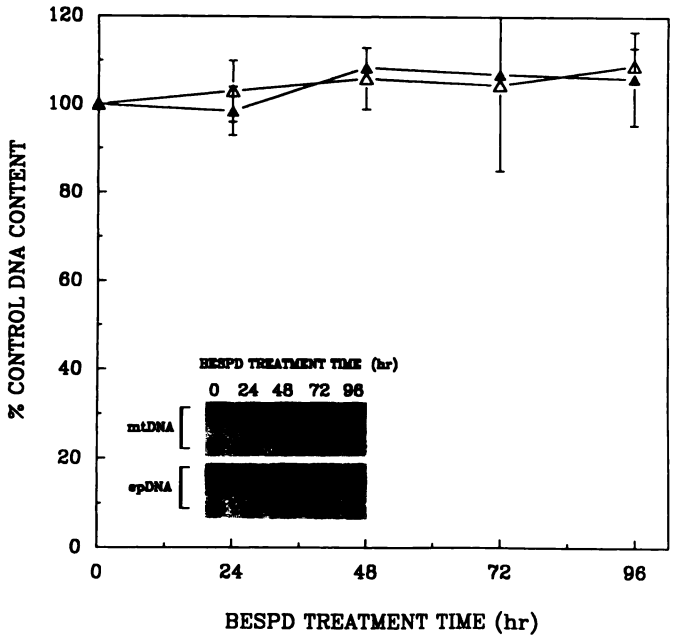

Fig. 2. Effects of BESPD treatment on the 935.1 cellular content of mtDNA (▲) and episomal DNA (△). Total cellular DNA was isolated from 96-hr cultures of 935.1 cells that were either untreated or treated for the last 24–96 hr with 100 μM BESPD. Four serial dilutions of 0.3 μg of total cellular DNA were dotted directly onto nitrocellulose filters. Duplicate dot blots were hybridized to nick-translated ^{32}P-pAM1 or ^{32}P-pM19 to probe for mtDNA or episomal DNA, respectively. The intensities of the dots on the resultant autoradiographs were quantitated by densitometric scanning and used to calculate the content of mtDNA or episomal DNA, assuming the intensity of the untreated control to be 100%. Data represent the mean ± standard error of determinations from two experiments. *Inset*, autoradiographs from a typical experiment. Depicted are representative DNA samples (*top*) and a 4-fold dilution (*bottom*).

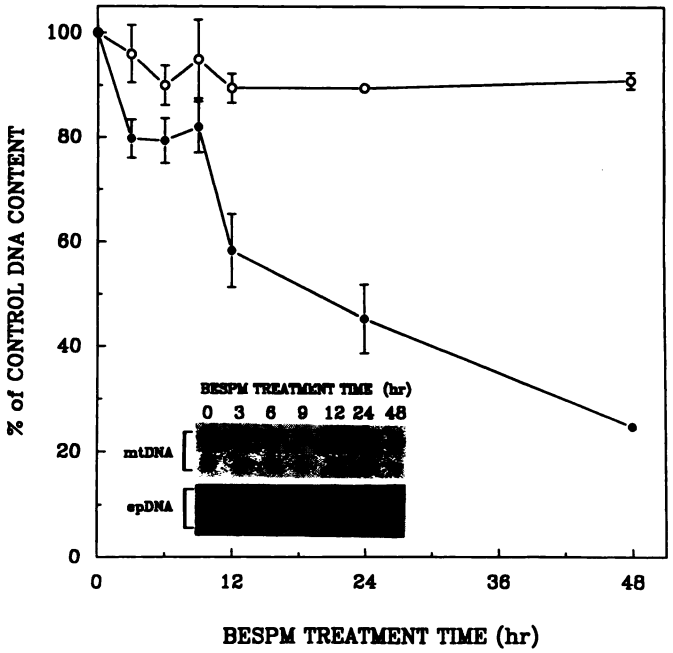

Fig. 3. Effects of BESPM treatment on the 935.1 cellular content of mtDNA (●) and episomal (○) DNA. Total cellular DNA was isolated from 48-hr cultures of 935.1 cells that were either untreated or treated for the last 3–48 hr with 10 μM BESPM, and DNA was analyzed for mtDNA or episomal DNA content as described in Fig. 2. Data represent the mean ± standard error of determinations from three experiments. Where not shown, *error bars* were smaller than the size of the *symbol*. *Inset*, autoradiographs from a typical experiment. Depicted are representative DNA samples (*top*) and a 4-fold dilution (*bottom*).

II to form III (linear) DNA, the relative change in mtDNA species on Southern analysis gives an indication of the nature of the form. Partial digestion of isolated 935.1 cell DNA with increasing concentrations of DNase I confirmed the identity of the three major topological isoforms (data not shown) and demonstrated the utility of forms analysis in the assessment of specific DNA damage (16). Other structures that were evident included nicked circles of dimeric length and several structures believed to be catenated circles. Presence of these higher forms are typical of the mtDNA population of cultured animal cells (25). Episomal DNA was resolved in this system predominantly as the supercoiled and nicked circular forms (see Fig. 4B, *lane 1*). Partial DNase I digestion of isolated 935.1 cell DNA and Southern analysis of the episomal DNA species confirmed the pattern of form I, II, and III migration (data not shown). An additional species, representing what has previously been described (16) as high molecular weight replicative intermediates (diffuse band in the center of the autoradiograph in Fig. 4B), was also observed.

Because supercoiled circular DNA molecules are sensitive to both single- and double-strand breaks, by conversion to the relaxed circular or linear forms, respectively, any decrease in the form I fraction (relative to the total of forms I, II, and III) is indicative of DNA damage. Southern blot analysis and quantitation of the topological forms of mtDNA from BESPM-

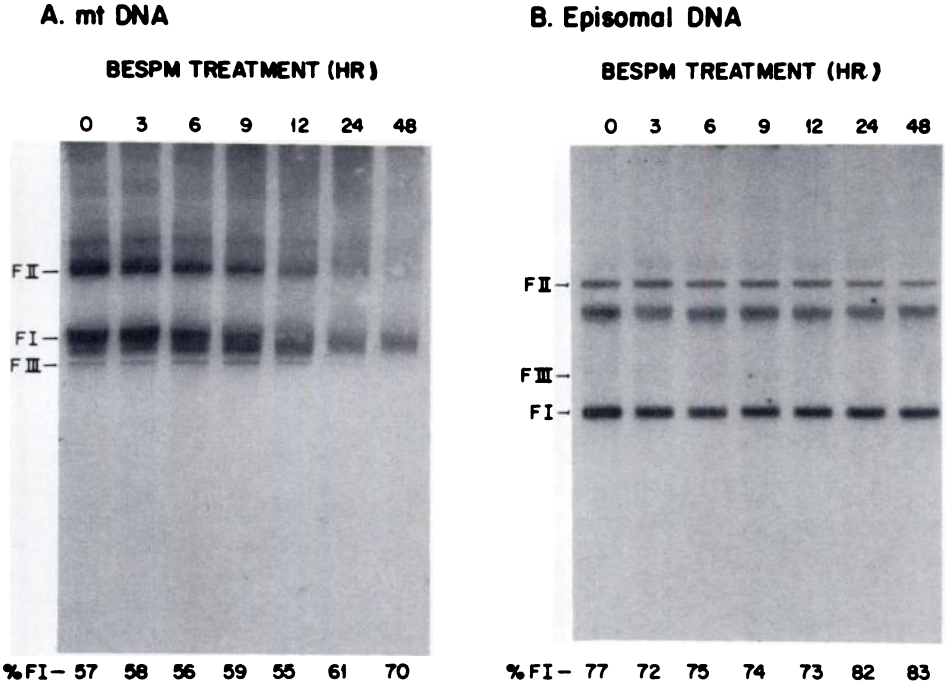

Fig. 4. Forms conversion analysis of mtDNA (A) and episomal DNA (B) from BESPM-treated 935.1 cells. Cells were treated with 10 μM BESPM as described in Fig. 3. Cellular DNA (0.5 μg) was electrophoresed, transferred to nitrocellulose, and hybridized to either nick-translated ^{32}P-pAM1 or ^{32}P-pM19 DNA, as described in Experimental Procedures. mtDNA and the episome migrated as supercoiled (form I) (*FI*), nicked circular (form II) (*FII*), and linear (form III) (*FIII*) DNA. High molecular weight species are discussed in the text. A linearized (16.3-kb) mtDNA marker and DNA size markers ranging from 0.5 to 23 kb were included on each gel to confirm the migration of form III mtDNA and episomal DNA (not shown). The intensity of the mtDNA and episomal DNA forms on the resultant autoradiographs were quantitated by densitometric scanning down each lane, and the percentages of forms I, II, and III in each sample were determined. The percentage of form I in each sample is displayed below the autoradiographs.

treated 935.1 cells revealed a 72% decrease in the combined intensities of forms I, II, and III after 48 hr of treatment, reflecting the overall loss in mtDNA species (Fig. 4A). However, within each sample the percentage of each form remained relatively the same for up to 24 hr of treatment. mtDNA from untreated cells was composed of 57% form I, 39% form II, and 4% form III molecules, whereas mtDNA from cells treated for 24 hr with BESPM contained 61% form I, 38% form II, and 2% form III molecules. After 48 hr of treatment, the levels of form III approached the limits of detection, accounting for the apparent absence of this form and the slight increase in the percentage of form I molecules (Figure 4A). The failure of forms analysis to demonstrate any decrease in the percentage of supercoiled mtDNA molecules indicates the absence of single- or double-strand damage as a result of BESPM treatment.

In addition, when BESPM at concentrations of up to 100 μM was added directly to control cell lysates and the DNA was isolated and analyzed in a similar manner, neither the structure nor the content of mtDNA was affected (data not shown). This confirmed that the loss of mtDNA was not due to the direct degradation of mtDNA by BESPM or to an artifact of the DNA-handling procedures. Southern blot analysis of episomal DNA from BESPM-treated cells showed it to be unchanged in structure and confirmed the lack of effect on episomal DNA levels (Fig. 4B). The data, thus, indicate that BESPM causes a selective loss of mtDNA from 935.1 cells in a manner that excludes BESPM-induced DNA damage.

When cell growth in the presence of BESPM was used to predict the dilution of cellular components attributable to cell division, it was found to correlate closely with the BESPM-induced depletion of mtDNA (Fig. 5). This observation is consistent with the idea that the cellular content of mtDNA that exists before treatment may be simply depleted via cell division. It also implies that the synthesis of new mtDNA is blocked and that this blockade must have occurred within the first several hours of BESPM exposure, because there was little

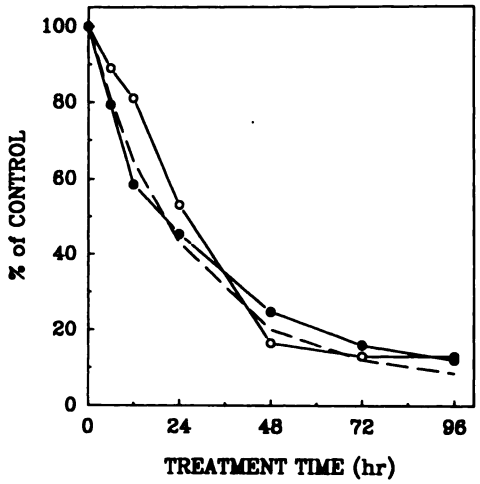

Fig. 5. Relationship between BESPM effects on 935.1 cell growth, mtDNA content, and SPM pools. Theoretical dilution of intracellular components based on cell growth in the presence of BESPM (– – –) was compared with the actual depletion of cellular mtDNA (●) and SPM (○) content with BESPM treatment time. Cell dilution was calculated from the mean cell growth from two experiments performed in duplicate. MtDNA content data are replotted from Fig. 3 and Table 1. The cellular SPM pool data represent the mean of three independent determinations.

delay in the onset of depletion. Depletion of the cellular SPM pool with BESPM treatment showed a similar time-dependent response (Fig. 5), indicating a possible correlation between SPM depletion and mtDNA depletion.

In order to examine the possible relationship between analog-induced polyamine (particularly SPM) depletion and the apparent inhibition of mtDNA synthesis, direct-acting inhibitors of polyamine biosynthetic enzymes were used to produce polyamine depletion in the absence of structural analogs. It has been previously shown that treatment of L1210 cells with the combination of AMA and DFMO depletes both cellular SPD and SPM pools (26). For these studies, the 935.1 cells could

not be used, because they exhibited an inherent resistance to AMA-induced polyamine depletion. Therefore, the L1210 murine leukemia cell line was studied. To ensure that the results would be comparable to those obtained in the 935.1 cell line, BESPD and BESPM were recharacterized in terms of their effects on polyamine metabolism and mtDNA. Although the control levels of the individual polyamines differed between 935.1 cells and L1210 cells, treatment of either cell line for 48 hr with BESPD or BESPM resulted in similar effects on polyamine pools. Likewise, the mtDNA contents of the two cell lines responded similarly to the analogs, indicating that depletion of mtDNA by BESPM was not peculiar to the 935.1 cell line (compare Tables 1 and 2). Southern analysis of mtDNA from BESPM-treated L1210 cells confirmed that the decrease in mtDNA content was, again, not due to damage to the mtDNA molecule (data not shown).

A comparison of the effects of the polyamine analogs and the biosynthesis inhibitors on cellular polyamine and mtDNA levels in the L1210 line is shown in Table 2. Concomitant treatment of L1210 cells with AMA and DFMO caused a >95% depletion of cellular SPD pools and a 76% decrease in SPM pools, a level of depletion similar to that achieved by BESPM alone (Table 2). Analysis of mtDNA content in inhibitor-treated cells revealed that it was unchanged (Table 2), indicating that mtDNA depletion is dependent on the presence of the spermine analog.

To determine whether BESPD might affect mtDNA under conditions in which the cellular SPM pools were depleted (i.e., as with BESPM), L1210 cells were exposed to the combination of AMA and BESPD. This treatment resulted in the most complete (>90% in each) depletion of cellular polyamines seen to date (Table 2). BESPD accumulated under these conditions to levels similar to those achieved using BESPD alone. Despite SPM depletion, mtDNA levels of AMA- plus BESPD-treated cells were not significantly lower than those of control cells (Table 2). Thus, under conditions of polyamine depletion comparable to those produced by the spermine analog BESPM, BESPD was still not able to reduce mtDNA content. The differential abilities of the polyamine analogs to interfere with mtDNA replication, therefore, are apparently not a function of their effects on the cellular levels of the natural polyamines but, rather, of specific structural differences.

Discussion

Inhibitors of polyamine biosynthesis provide a means to examine the cellular consequences of polyamine depletion. Polyamine analogs, such as BESPD and BESPM, extend this opportunity to the possibility of studying the consequences of polyamine depletion together with analog replacement at vacated polyamine binding sites. The 935.1 cell line used in these studies allowed for the direct comparison of analog effects on mtDNA, a circular non-histone-associated cytoplasmic DNA molecule, with those on a circular histone-associated episome contained in the cell nucleus. The most striking observation is that BESPM, but not BESPD, depletes mtDNA (but not episomal DNA).

In terms of analog replacement of intracellular polyamine pools, BESPM was the more effective of the two analogs. As noted in other cell lines (27, 28), BESPD invoked regulatory mechanisms (12) to suppress ODC but not AdoMetDC activity, which, in turn, led to depletion of PUT and SPD but not SPM pools (11). In contrast, and in accordance with previous findings (11, 13), BESPM suppressed both ODC and AdoMetDC activities and caused a near-total depletion of all three cellular polyamines. Only the near-total replacement of cellular polyamines produced by BESPM significantly affected either DNA target. BESPM caused a striking decrease in the cellular content of mtDNA, whereas BESPD did not. Furthermore, this effect was selective for mtDNA, because the levels of episomal DNA were unaffected by either BESPD or BESPM.

Several lines of evidence indicate that mechanisms other than analog-induced mtDNA damage seem to be responsible for the decrease in mtDNA content induced by BESPM. Firstly, topological forms conversion analysis of mtDNA from BESPM-treated cells demonstrated that, although decreased in content with time, there was no evidence of forms interconversion, indicating the absence of specific single- or double-strand damage. Secondly, BESPM was incapable of affecting the fraction of form I mtDNA when incubated with lysates from untreated cells, indicating the absence of direct lytic activity.

Comparison of the BESPM-induced pattern of mtDNA depletion (on a per cell basis) with the predicted loss based on dilution by cell division revealed a close correlation. In an independent study, Bergeron *et al.* (18) also found growth-dependent depletion of mtDNA in BESPM-treated L1210 cells.

TABLE 2

Relationship between polyamine pools and mtDNA content in L1210 cells treated with polyamine analogs and inhibitors

Treatment (48 hr)	*n*[a]	Polyamine pools[b]				mtDNA content[c]
		PUT	SPD	SPM	Analog	
		nmol/10⁶ cells				*% of control*
None	6	0.66 ± 0.10	2.76 ± 0.20	0.53 ± 0.07		100
100 μM BESPD	3	0.10 ± 0.06	0.44 ± 0.19	0.64 ± 0.15	6.18 ± 1.41	97
10 μM BESPM	2	ND[d]	0.06 ± 0.01	0.13 ± 0.02	4.93 ± 2.12	25
100 μM AMA	3	9.42 ± 1.07	0.37 ± 0.08	0.03 ± 0.01		100
1 mM DFMO	2	ND	0.02 ± 0.01	0.57 ± 0.23		101
100 μM AMA + 1 mM DFMO	2	0.33 ± 0.24	0.49 ± 0.29	0.07 ± 0.03		103
100 μM AMA + 100 μM BESPD	3	0.02 ± 0.02	0.14 ± 0.01	0.05 ± 0.02	5.64 ± 0.87	104

[a] *n*, number of independent determinations.
[b] Mean ± standard error of *n* determinations.
[c] Mean of *n* determinations, where the standard error was less than 10% of the mean.
[d] ND, none detected (<0.01 nmol/10⁶ cells).

These data, as well as the apparent absence of DNA damage, are consistent with a mechanism whereby mtDNA is diluted over the course of three to four cell divisions. In order to achieve such kinetics, an almost immediate cessation of mtDNA replication must occur. Other agents with demonstrated activity against mtDNA synthesis, such as ethidium bromide (29, 30, 31) and the active metabolite of 2′,3′-dideoxycytidine (32, 33), produce a similar depletion of mtDNA.

In the context of effects on polyamine pools, the observed BESPM-induced decrease in mtDNA could be the result of (a) the near-total depletion of cellular polyamines, including SPM, (b) the direct interaction of BESPM at a site in the mitochondrion, or (c) a combination of the above, whereby, in the absence of SPM, BESPM accesses a critical binding site. The first of these possibilities was excluded by the use of specific polyamine enzyme inhibitors, DFMO and AMA, in L1210 cells (the latter inhibitor does not work in 935.1 cells). Whereas BESPM decreased mtDNA in these cells in a manner comparable to that in 935.1 cells, the inhibitors did not, indicating that the BESPM effect is not mediated by polyamine depletion alone (Table 2). Whether the direct BESPM effect is dependent on the concomitant depletion of SPM (as in item c above) is a difficult question to address, because prevention studies involving the addition of exogenous SPM would result in competition of BESPM and SPM for uptake into the cells (11). It should be noted, however, that the inhibition of mtDNA synthesis appears to begin at a time when a significant pool of SPM still remains, suggesting that SPM depletion may not be a prerequisite for the effect of BESPM on mtDNA.

Taken together, the data are most consistent with the second of the possibilities given above, i.e., BESPM is capable of directly interfering at some binding site that may be involved in mtDNA replication. The ability of BESPM to interact with this site appears to be structure dependent, because even at higher intracellular concentrations, BESPD was unable to act similarly. Further, conditions of polyamine depletion comparable to those produced by BESPM failed to render BESPD active in this regard. Because there is considerable evidence to indicate that the polyamine SPM may be involved in mitochondrial respiration (34, 35), stability (36), and ion transport (37–39), BESPM may act at a site within the mitochondrion that is specific for tetramines and is located at the inner mitochondrial membrane (34). Alternatively, BESPM may bind directly to mtDNA, because the analog has been shown to be capable of affecting DNA conformation and thermal stability in *in vitro* systems (40). BESPM-induced changes in DNA structure could subsequently influence the binding of regulatory proteins involved in the initiation of mtDNA replication. Welter *et al.* (41) have described a curved DNA structure in front of the L strand origin of mtDNA replication, which interacts specifically with a mitochondrial protein thought to be an initiation factor. This is particularly interesting considering the predicted ability of SPM to produce DNA bending (7) and may explain the observed ability of BESPM to selectively interfere with mtDNA replication.

Given the difficulties encountered in assigning specific cellular functions to the polyamines, the identification of an unique site of action in the mitochondrion for an analog that is structurally similar to SPM represents a potentially relevant lead. Precedent for analog binding to a functional site is apparent in the ability of the analogs to substitute at those sites involved in regulation of ODC and AdoMetDC synthesis (13, 14). It is not unreasonable, therefore, to assume that the interaction of BESPM at the mitochondrion may involve dysfunctional replacement (or displacement) at a SPM binding site, particularly in view of the structural specificity implied by the inability of the SPD analog BESPD to interact in a similar manner. The differential activities of BESPD and BESPM in affecting mtDNA and in regulating decarboxylase levels (11, 12) suggest distinct biological functions for the different polyamine species. Thus, further characterization of the means by which BESPM apparently interferes with mtDNA replication may provide insight into the biological function(s) of SPM.

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