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Two polyamine analogs (BE-4-4-4 and BE-4-4-4-4) directly affect growth, survival, and cell cycle progression in two human brain tumor cell lines

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Abstract 1,14-*Bis*-(ethyl)-amino-5,10-diazatetradecane N^1,N^{11}-*bis*(ethyl)norspermine (BE-4-4-4) and 1,19-bis-(ethylamino)-5,10,15 triazanonadecane (BE-4-4-4-4) are two relatively new polyamine analogs synthesized for use as antineoplastic agents. In human brain tumor cell lines U-251 MG and SF-767, both agents inhibited cell growth, were cytotoxic, induced a variable G_1/S block, and depleted intracellular polyamines. Since intracellular polyamine depletion did not always correlate with growth inhibition, cell survival, or cell cycle progression, it cannot completely explain the effects of these agents on growth, survival, and cell cycle progression in U-251 MG and SF-767 cells.

Key words Polyamine analogs · Cell cycle · Glioma

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Introduction

Polyamines are involved in the growth and proliferation of all mammalian cells. Polyamine depletion inhibits cell growth [22]. Inhibitors of polyamine biosynthetic enzymes such as α-difluoromethylornithine (DFMO) [18] inhibit the growth of mammalian cells in culture and tumors in vivo [32]. The exact mechanism of this growth inhibition is unknown. However, it is known that 3T3 and WI-38 normal cells [31], human carcinoma cells [30] and 9L rat brain tumor cells [29] depleted of putrescine and spermidine by DFMO are growth-inhibited and blocked at the G_1 phase of the cell cycle; these effects can be reversed by providing exogenous polyamines. More recently developed polyamine analogs deplete all three intracellular polyamines [3, 4, 6, 8, 10, 11, 16, 24, 26, 28]. Aside from their ability to deplete putrescine, spermidine, and spermine, polyamine analogs differ from other inhibitors or regulators of polyamine metabolism in that the analog itself is present in intracellular fluid. Therefore, the analog might compete for polyamine binding sites both related and unrelated to polyamine synthesis or metabolism [3, 4, 6, 7, 17].

We have hypothesized that polyamines and polyamine analogs function biologically, at least in part, by interacting with DNA in chromatin. The polyamine analogs 1,14-*bis*-(ethyl)-amino-5,10-diazatetradecane N^1,N^{11}-*bis*(ethyl)norspermine (BE-4-4-4) and 1,19-*bis*-(ethylamino)-5,10,15-triazanonadecane (BE-4-4-4-4) differ in their interactions with DNA. BE-4-4-4 associates strongly with DNA in vitro but does not aggregate it [4, 5], whereas BE-4-4-4-4 both associates with and aggregates DNA more effectively than BE-4-4-4 [8]. Both analogs lower intracellular polyamine concentrations, but they differ in their effects on DNA conformation in vitro. Thus, the question arises: "Do polyamine analogs affect cellular processes by depleting polyamines and regulating their synthesis and/or

degradation or do these effects arise through an altogether different mechanism?" In this study, we approached this question by examining whether cell cycle progression, growth inhibition, and cell survival depend upon the polyamine-depleting abilities of BE-4-4-4 and BE-4-4-4-4. The effects of these drugs were studied at both equi-inhibitory (5 μ*M* BE-4-4-4-4 and 0.25 μ*M* BE-4-4-4) and equi-molar (5 μ*M*) doses in each cell line.

Materials and methods

Chemicals and reagents

BE-4-4-4 was a gift of R.J. Bergeron (University of Florida, Gainesville, Fl.) and BE-4-4-4-4 was synthesized by K. Samejima and A. Shirahata (Josai University, Sakado, Saitama, Japan). DFMO was a gift of the Marion-Merrell-Dow Research Institute (Cincinnati, Ohio). RNAase and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were of reagent grade, and deionized distilled water was used as solvent.

Cell lines and cultures

Human brain tumor cell lines U-251 MG and SF-767 were obtained from the National Cancer Institute (Frederick, Md.) and the Brain Tumor Research Center Tissue Bank (University of California, San Francisco, Calif.), respectively. Both lines were derived from grade 4 malignant gliomas; their doubling times were 1.1 and 1.8 days, respectively. They were grown in monolayer culture by slight modifications of procedures described elsewhere [23]. Approximately 3×10^5 cells were seeded in 75-cm^2 plastic flasks containing minimum essential medium supplemented with nonessential amino acids and 10% enriched calf serum (Gemini Bio-Products, Calabasas, Calif.); SF-767 was grown in 15 ml of this medium and U-251 MG in 24 ml.

Cell treatment

Stock solutions of BE-4-4-4, BE-4-4-4-4, and DFMO were prepared with Hanks' balanced salt solution and sterile-filtered immediately before use. Cells were seeded 1 day before drug treatment. The drugs were added on day 0. Cells were harvested by trypsinization on days 0, 1, 3, 5 and counted with an electronic particle counter. The doses of BE-4-4-4 and BE-4-4-4-4 that induced equivalent growth inhibition in U-251 MG and SF-767 cultures were determined as 0.25 μ*M* BE-4-4-4 and 5 μ*M* BE-4-4-4-4.

Polyamine measurement

Between 0.5×10^6 and 2×10^6 cells were collected from each sample, washed with isotonic phosphate buffer (pH 7.4), sonicated in 250 μl 8% sulfosalicylic acid, and dansylated. The polyamine content was determined by reverse-phase high-performance liquid chromatography [20].

Cytotoxicity

Cell survival at days 3 and 5 after drug treatment was determined by a colony-forming efficiency assay [13]. Cell suspensions (1 ml) containing a known number of cells were added to sterile 35 mm plastic wells (Costar, Cambridge, Mass.) to which 2×10^4 heavily irradiated (52.7 Gy) feeder cells in 4 ml medium had been added the previous day. After 10 days of incubation, colonies were stained with crystal violet. Colonies containing more than 50 cells were counted. The plating efficiency was calculated as the number of colonies per dish divided by the number of seeded cells per dish. The surviving fraction was calculated as the ratio of the plating efficiency of treated cells to that of control cells.

Flow cytometry

Cells (0.5×10^6 to 2×10^6) were fixed in 70% ethanol [12]. On the day of the experiment, cells were centrifuged, resuspended in phosphate buffered saline (PBS) containing 0.1 g/l calcium, treated with 250 μl RNAase solution (0.1 mg/ml in PBS containing calcium) for 1 h, and stained with 250 μl freshly prepared propidium iodide solution (0.1 μg/ml in PBS containing calcium). Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.). Cell cycle distribution was measured in 10,000 single cells with a doublet discriminator. The Becton Dickinson SFIT cell cycle algorithm was used to calculate the percentage of cells in each phase.

Results

Growth inhibition

The effects of BE-4-4-4, BE-4-4-4-4, and DFMO on the growth of U-251 MG and SF-767 cells are shown in Fig. 1. Growth inhibition was apparent by day 3; thereafter, little growth occurred in treated cells. In both cell lines, 5 μ*M* BE-4-4-4 was more potent than 5 μ*M* BE-4-4-4-4. U-251 MG cultures treated with BE-4-4-4 lost cells between days 3 and 5, while SF-767 cultures did not. This could indicate cell death or detachment of U-251 MG cells. In both cell lines, 5 μ*M* BE-4-4-4-4, 0.25 μ*M* BE-4-4-4, and 1 m*M* DFMO inhibited growth comparably.

Cell survival

The surviving fractions of SF-767 and U-251 MG are shown as a function of treatment time in Fig. 2. In both cell lines, 0.25 μ*M* BE-4-4-4 was less toxic than 5 μ*M* BE-4-4-4-4, and 5 μ*M* BE-4-4-4 was more toxic than 5 μ*M* BE-4-4-4-4. The toxicity of 5 μ*M* BE-4-4-4 was greater to SF-767 than to U-251 MG cells.

Polyamine levels

Polyamine and analog levels in control and treated SF-767 and U-251 MG cells are shown in Figs. 3 and 4, respectively. Putrescine levels were lower in control SF-767 cells than in control U-251 MG cells. In both cell lines, 24 h of treatment with 5 μ*M* BE-4-4-4-4 or 5 μ*M* BE-4-4-4 decreased putrescine and

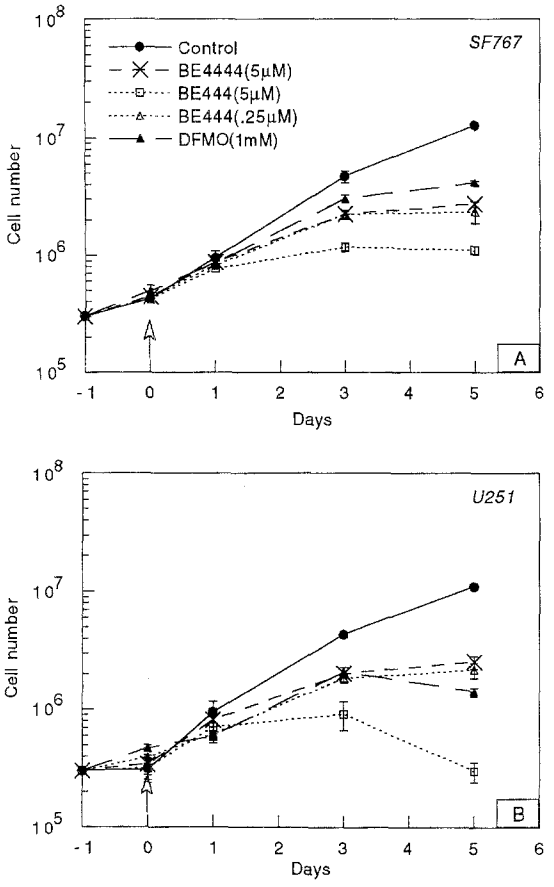

Fig. 1A, B Effects of 5 μ*M* BE-4-4-4-4, 5 μ*M* and 0.25 μ*M* BE-4-4-4, and 1 m*M* DFMO on the growth of SF-767 (**A**) and U-251 MG (**B**) human brain tumor cells. Values are an average of the results of three separate experiments. *Error bars*, where not visible, are smaller than the symbol size

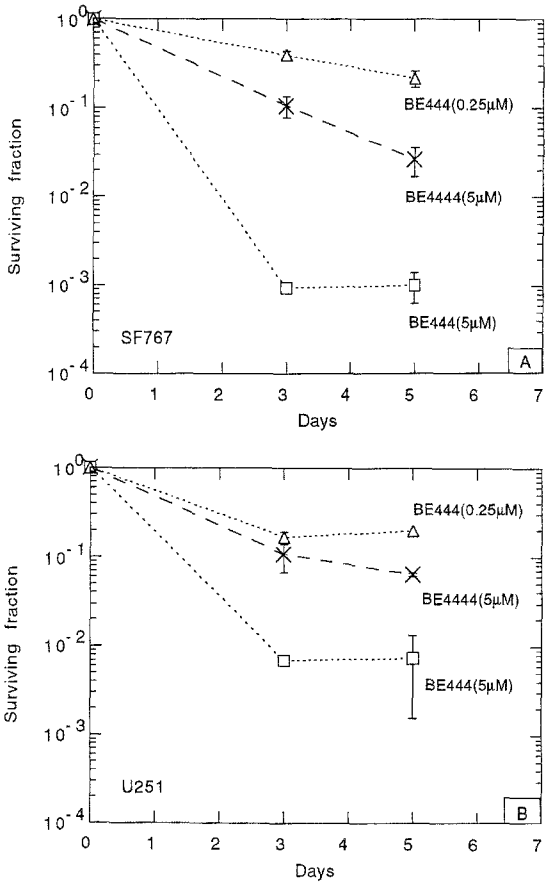

Fig. 2A, B Effects of 5 μ*M* BE-4-4-4-4 and 5 μ*M* and 0.25 μ*M* BE-4-4-4 on the survival of SF-767 (**A**) and U-251 MG (**B**) cells on days 3 and 5 of treatment as determined by an assay of colony-forming efficiency. Values are the average of the results of either three separate experiments or of two separate experiments

spermidine levels. By day 3, putrescine and spermidine were undetectable in both cell lines. In both cell lines 5 μ*M* BE-4-4-4-4 or 5 μ*M* BE-4-4-4 decreased spermine, but not to undetectable levels. At a concentration of 0.25 μ*M*, BE-4-4-4 had less effect on polyamine levels than 5 μ*M* BE-4-4-4-4 and 5 μ*M* BE-4-4-4 in both cell lines.

In SF-767 cells, the content of BE-4-4-4 and BE-4-4-4-4 plateaued 1 day after treatment. In U-251 MG cells treated with 5 μ*M* BE-4-4-4, the BE-4-4-4 content varied during the course of the experiment, whereas in cells treated with 0.25 μ*M* BE-4-4-4, it increased to a maximum near day 1 and then decreased; BE-4-4-4-4 levels plateaued after 1 day of treatment.

Cell cycle

The effects of polyamine analogs on the fractions of cells in the G_1, S, and G_2/M phases over time are shown in Figs. 5 and 6. In SF-767 cultures, 5 μ*M* BE-4-4-4-4 and 0.25 μ*M* BE-4-4-4 had similar effects on the cell cycle. The number of G_1-phase cells increased and reached a maximum at day 3 of treatment. This increase corresponded to a decrease in the S-phase fraction. There was no detectable change in the fraction of G_2/M-phase cells. Treatment with 5 μ*M* BE-4-4-4 accentuated these effects, and resulted in a small decrease in the G_2/M fraction. DFMO caused a slight decrease in the G_2/M fraction and a slight increase in the S-phase fraction.

In U-251 MG cultures, 5 μ*M* BE-4-4-4-4 and 0.25 μ*M* BE-4-4-4 had no effect on the cell cycle. Treatment with 5 μ*M* BE-4-4-4 increased the G_1 fraction and decreased the S-phase fraction between days 1 and 5 of treatment. The high percentage of control cells in G_1 on day 5 probably occurred because the culture had reached confluence or had depleted serum or growth factors.

Discussion

Our findings in this study support the idea that polyamine depletion alone is not completely responsible for alterations in cell cycle progression, survival, and

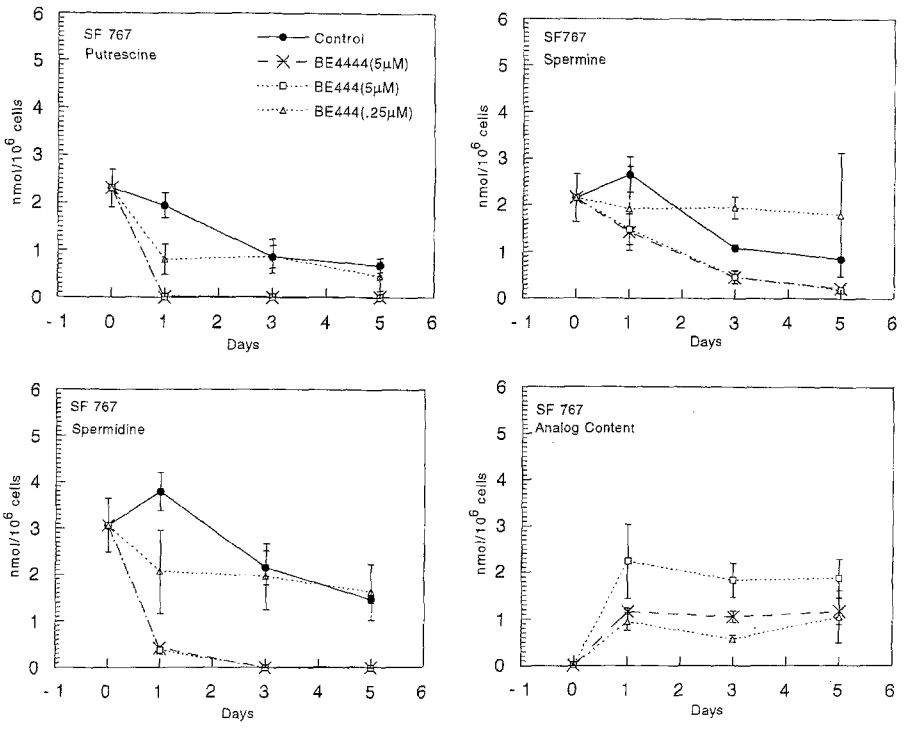

Fig. 3 Polyamine and analog levels of SF-767 cells treated with 5 μ*M* BE-4-4-4-4 and 5 μ*M* and 0.25 μ*M* BE-4-4-4. Values are the average of three separate experiments

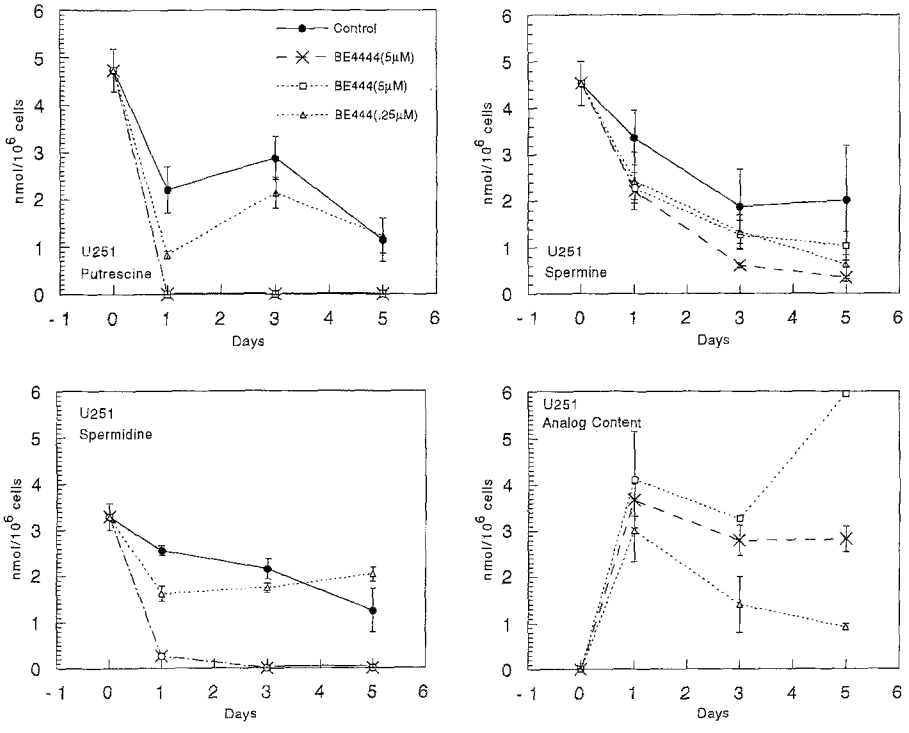

Fig. 4 Polyamine and analog levels of U-251 MG cells treated with 5 μ*M* BE-4-4-4-4 and 5 μ*M* and 0.25 μ*M* BE-4-4-4. Values are the average of either three separate experiments or of two separate experiments where results varied by less than 15%

growth rate [17] produced by the polyamine analogs BE-4-4-4-4 and BE-4-4-4-4 in the human brain tumor cell lines SF-767 and U-251 MG. This conclusion is based upon comparisons of the kinetics and dose dependence of alterations in polyamine levels, of polyamine depletion, cell cycle progression, survival, and growth rate in cells treated with polyamine analogs. It is also supported by evidence that the presence of polyamine analogs – not simple polyamine depletion – directly inhibits the growth of CHO cells [17] and various brain tumor cell lines [3, 4, 7, 8, 15].

In regard to the effects of BE-4-4-4-4 and BE-4-4-4-4 on the cell cycle, the G_1/S cell cycle block we observed in SF-767 and U-251 MG cells treated with 5 μ*M*

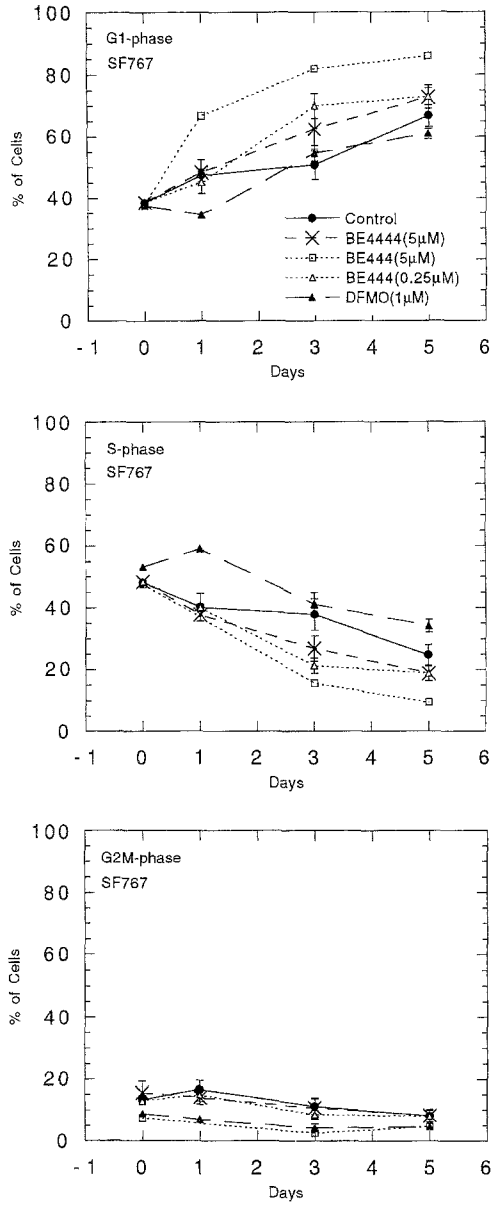

Fig. 5 Cell cycle effects of 5 μ*M* BE-4-4-4-4, 5 μ*M* and 0.25 μ*M* BE-4-4-4, and 1 m*M* DFMO on SF-767 cells. Values with error bars are the average of three separate experiments. Values without error bars are based on the average of two separate experiments where results varied by less than 15%

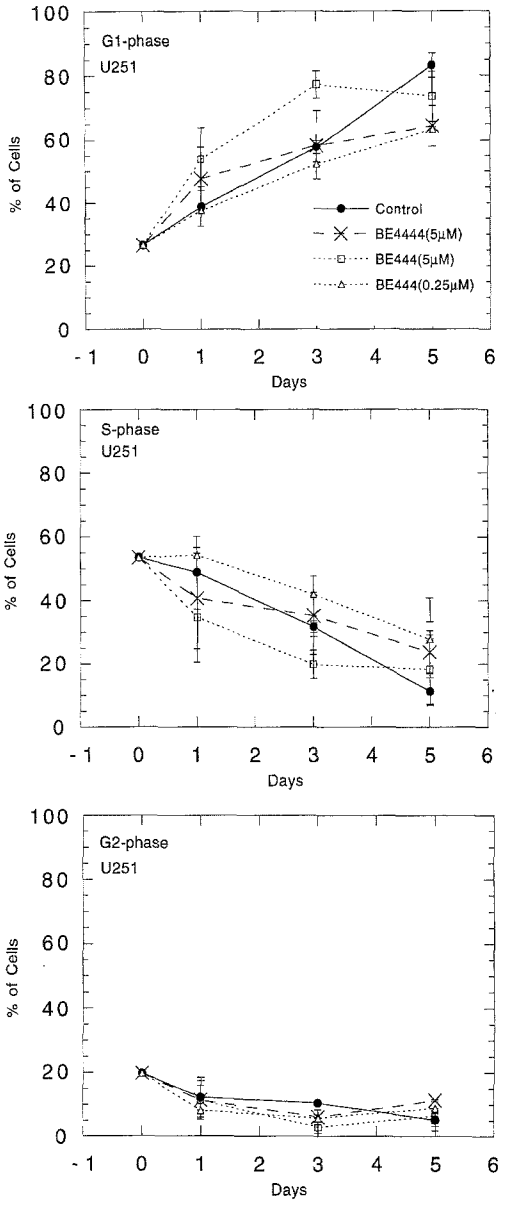

Fig. 6 Cell cycle effects of 5 μ*M* BE-4-4-4-4, 5 μ*M* and 0.25 μ*M* BE-4-4-4, and 1 m*M* DFMO on U-251 MG cells. Values with error bars are the average of three separate experiments. Values without error bars are based on the average of two separate experiments

BE-4-4-4 is consistent with a G_1/S block that it produced in U-87 MG cells [7]. Our results with DFMO, however, did not confirm the G_1/S block found in that study, although other patterns of cell cycle progression have been reported with this compound [2, 9, 19, 25, 30]. Koza and Herbst [21] have shown that cell cycle progression and DNA replication are blocked in DFMO-treated HeLa cells, implying that the polyamine depletion produced by DFMO is closely associated with the growth arrest produced by this drug. In contrast, we did not observe a close relationship between polyamine depletion and cell cycle progression in analog-treated, polyamine-depleted cells. Both 5 μ*M* BE-4-4-4 and 5 μ*M* BE-4-4-4-4 depleted putrescine, spermidine, and spermine in U-251 MG and SF-767 with nearly identical kinetics. Treatment with 5 μ*M* BE-4-4-4 blocked both SF-767 and U-251 MG at the G_1/S border, while 5 μ*M* BE-4-4-4-4 blocked each line much less or not at all (Figs. 5 and 6). Moreover, 0.25 μ*M* BE-4-4-4 altered polyamine levels much less than 5 μ*M* BE-4-4-4, but the effects of these two treatments on cell cycle progression were indistinguishable. Thus, the very similar patterns of polyamine depletion produced by 5 μ*M* BE-4-4-4 and 5 μ*M* BE-4-4-4-4 were not linked to similar cell cycle effects, and the very different patterns of polyamine depletion produced by

0.25 μ*M* BE-4-4-4 and 5 μ*M* BE-4-4-4-4 were linked to similar alterations in cell cycle progression. This is evidence that polyamine depletion associated with BE-4-4-4 and BE-4-4-4-4, in contrast to that produced by DFMO, is not directly correlated with cell cycle progression, at least in these two cell lines.

Nor did polyamine depletion correlate well with growth inhibition or with survival in either cell line studied. Although 5 μ*M* BE-4-4-4 and 5 μ*M* BE-4-4-4-4 produced similar kinetics and magnitudes of polyamine depletion in SF-767 and U-251 MG cells, the former treatment inhibited growth and was more cytotoxic than the latter. Furthermore, although 5 μ*M* BE-4-4-4-4 and 0.25 μ*M* BE-4-4-4 differed in their ability to deplete polyamines, they induced only small differences in growth or survival of SF-767 or U-251 MG cells. This result is consistent with the hypothesis that polyamine depletion resulting from treatment with these two polyamine analogs is not directly linked to their effects on survival and growth inhibition in SF-767 and U-251 MG cells. This hypothesis is further supported by the observation that the growth inhibitory effects of polyamine analogs are not related to their ability to decrease ornithine decarboxylase activity, deplete polyamines, or inhibit mitochondrial DNA synthesis [1].

The alterations in cell cycle progression, however, did correlate with growth inhibition and with survival in both cell lines. Treatment with 5 μ*M* BE-4-4-4 induced a G_1/S block in both cell lines whereas 5 μ*M* BE-4-4-4-4 and 0.25 μ*M* BE-4-4-4 did not, and 5 μ*M* BE-4-4-4 had more growth inhibitory and cell killing activity than either of the other treatments. This result is evidence that alterations in cell cycle progression, growth inhibition, and cell survival produced by BE-4-4-4 and BE-4-4-4-4 in SF-767 and U-251 MG result from a single effect. This effect cannot be totally dependent on intracellular polyamine concentration but it is more likely related to effects of the analogs on cell cycle progression. We have previously speculated that polyamine analogs act directly upon sites responsive to physiologic polyamines [3, 4, 6, 8], and have shown that polyamine analogs alter DNA conformation in vitro and chromatin conformation in tissue culture [3, 5, 7]. These effects may be responsible for the cell cycle block, growth inhibition, and cell kill. However, it remains unclear how the effects of polyamine analogs on chromatin condensation might inhibit cell cycle progression at the G_1 phase (or vice versa).

The intracellular accumulation of the polyamine analogs may partially explain the sensitivity to the analogs seen in each cell line. In SF-767, the intracellular analog level achieved with 0.25 μ*M* BE-4-4-4 is close to that achieved with 5 μ*M* BE-4-4-4-4. Similarly, intracellular BE-4-4-4 was nearly twice the level of BE-4-4-4-4 when both were given at 5 μ*M*. The higher levels achieved by BE-4-4-4 may translate into higher occupancy at binding sites. Alternatively, better binding at an intracellular site might be reflected in the higher intracellular level seen with BE-4-4-4. However, since the less sensitive cell line gauged by survival had higher levels of both analogs, the differential sensitivity is not completely explained by differences in intracellular analog levels.

It has been hypothesized by other investigators that polyamine analogs inhibit growth by inducing spermidine/spermine acetyl transferase (SSAT) activity, an enzyme important in polyamine metabolism [27]. This hypothesis may not hold true for all polyamine analogs in all cell lines [17]. In fact, BE-4-4-4-4 does not induce SSAT activity in U-251 MG and SF-767 cell lines [14], which is evidence against a general relationship between enhanced SSAT activity and the growth-inhibitory effects of all polyamine analogs. It remains to be determined whether the alterations in cell cycle progression, growth inhibition, and cytotoxicity in U-251 MG and SF-767 cells treated with BE-4-4-4 and BE-4-4-4-4 depend directly upon effects of the analogs on chromatin or other cellular component(s) responsible for cell cycle progression and growth.

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