Effect of \(N^1,N^{14}\)-Bis(ethyl)homospermine on the Growth of U-87 MG and SF-126 Human Brain Tumor Cells

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ABSTRACT

The effect of the spermine analogue \(N^1,N^{14}\)-bis(ethyl)homospermine on the growth, polyamine levels, and survival of U-87 MG and SF-126 human brain tumor cells was examined in tissue culture. At concentrations of 10 \(\mu\)mol and above, \(N^1,N^{14}\)-bis(ethyl)homospermine inhibited growth significantly, caused a marked decrease in intracellular levels of the naturally occurring polyamines putrescine, spermidine, and spermine, and had a considerable cytotoxic effect on both cell lines after more than 96 h of treatment. In earlier studies we showed that the affinity of the analogue for calf thymus DNA was higher than the affinity of spermine, but that it did not aggregate DNA or release bound ethidium bromide from DNA as efficiently as spermine does. Therefore, the growth-inhibitory and cytotoxic effects of \(N^1,N^{14}\)-bis(ethyl)homospermine support our hypothesis that polyamine analogues that can enter cells, deplete intracellular levels of natural polyamines, and replace the natural polyamines from their binding sites on DNA without replacing function should act as antiproliferative agents.

INTRODUCTION

Mammalian cells contain the polyamines putrescine, spermidine, and spermine that are synthesized by a specific pathway (1). Inhibitors of polyamine biosynthesis have been used to explore molecular mechanisms of polyamine functions and to act as therapeutic agents (2). Studies with various inhibitors of the polyamine biosynthetic pathway have shown that polyamines are crucial for cell growth and proliferation (3). DFMO\(^3\) is a specific inhibitor of the enzyme ornithine decarboxylase, which acts early in the polyamine biosynthetic pathway. DFMO has been tested as an anticancer agent in tissue culture, animals, and humans (4, 5). Although DFMO has been a useful agent for both clinical and experimental studies, in most mammalian cells it does not deplete intracellular levels of all the naturally occurring polyamines (6). Drugs that can deplete intracellular levels of all naturally occurring polyamines have now been developed (7, 8); many of these drugs are polyamine analogues that may perform some intracellular functions of the natural polyamines and thus may have minimal effects on cell growth (9, 10). Therefore, the biological functions and structural features of polyamine molecules that are responsible for their intracellular functions need to be understood in greater detail in order to develop polyamine analogues that can inhibit cell growth and may have therapeutic significance.

The natural polyamines cause alterations in DNA conformation in vitro (11), the two most important of which are induction of the \(B\) to \(Z\) transition (11) and the introduction of bends in DNA before aggregation occurs (12, 13). There is evidence for similar effects in vivo, and we have suggested that the biological functions of polyamines are related in some way to these activities (8). Polyamine-induced DNA conformational changes are specific for nucleotide sequence (14, 15), and it is reasonable to imagine that both the \(B\) to \(Z\) transition and bending of specific DNA sequences induced by polyamines might be related to nucleosome phasing, chromosome condensation, and regulation of the transcriptional activity of specific genes. We have suggested that polyamine analogues that deplete intracellular polyamines but do not affect DNA conformation should act as inhibitory agents by replacing the structural but not the functional characteristics of natural polyamines (8).

The polyamine analogue BE-4-4-4 is an especially promising agent for tumor therapy. BE-4-4-4 appears to suppress the ornithine and S-adenosylmethionine decarboxylases, which inhibit the \(de novo\) synthesis of the natural polyamines (16). Very recently, it has been observed that BE-4-4-4 causes the export of intracellular polyamines in human colon tumor cells, which depletes cellular polyamine pools (17). This phenomenon, however, has not yet been observed in other cell lines. We have shown that BE-4-4-4 strongly associates with DNA in vitro but does not induce a bend in or cause aggregation of DNA,\(^4\) which according to our criteria makes it an excellent candidate as a growth-inhibitory agent. It has been reported recently (16) that BE-4-4-4 shows strong growth inhibition, cytotoxic activity, and a concomitant depletion of cellular polyamine pools in L1210 cells. We report here the effect of BE-4-4-4 on the growth, survival, and intracellular polyamine levels of two human brain tumor cell lines. The results of these studies provide support for our hypothesis regarding the correlation of structural and functional properties of polyamine analogues (8).

MATERIALS AND METHODS

Drugs. DFMO was generously provided by the Merrell-Dow Research Institute (Cincinnati, OH). BE-4-4-4 was synthesized as described (18).

Growth Curves. The human brain tumor cell lines U-87 MG and SF-126 were grown as monolayers in culture (19). Approximately 1.0 \(\times\) 10\(^6\) cells were seeded in 5 ml of minimal essential medium supplemented with nonessential amino acids and 20% fetal calf serum in multiple 25-cm\(^2\) plastic flasks. Stock solutions of BE-4-4-4 were prepared in Hanks’ balanced salt solution, the pH was adjusted to 7.4 with 5 M NaOH, and solutions were sterile filtered immediately before use. Cells were seeded 1 day before the addition of the analogue, harvested in triplicate at different times after treatment, and counted using an electronic particle counter. For reversal studies, appropriate concentrations of polyamines were added 1 day after addition of the analogue (2 days after cell seeding).

Polyamine Measurement. Between 0.5 \(\times\) 10\(^6\) and 1.0 \(\times\) 10\(^6\) cells were

Received 9/25/89; revised 2/10/90.

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\(^1\) Supported in part by NIH Program Project Grant CA-13525, National Cooperative Drug Discovery Group Grant CA-37606 (to M. P., L. J. M.), NIH Grants CA-41757 (to B. G. F.), and CA-49049 (to H. S. B.).

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\(^3\) The abbreviations used are: DFMO, \(\alpha\)-difluoromethylornithine; BE-4-4-4, \(N^1,N^{14}\)-bis(ethyl)homospermine; CFE, colony-forming efficiency; PE, plating efficiency.

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collected from each sample, washed with isotonic phosphate buffer (pH 7.4), sonicated in 250 μl of 8% sulfosalicylic acid, dianalyzed, and analyzed for polyamine content using high-performance liquid chromatography (20).

Cyto toxicity Studies. Cell survival at different times after drug treatment was determined with a CFE assay (21). One ml of appropriately diluted single cell suspensions containing known numbers of cells was added to plastic wells (35 mm diameter) to which 2.5 x 10^6 heavily irradiated (52.7 Gy) feeder cells in 4 ml of medium had been added the previous day. After 2 wk of incubation, colonies were stained with crystal violet. Colonies containing more than 50 cells were counted. The following calculations were made.

\[
PE = \frac{\text{no. of colonies/dish}}{\text{no. of cells seeded}} \\
\text{Surviving fractions} = \frac{\text{PE of treated cells}}{\text{PE of control cells}}
\]

RESULTS

Growth Effects. Effects of increasing concentrations of BE-4-4-4 on the growth of U-87 MG and SF-126 brain tumor cells are shown in Fig. 1. The analogue inhibited growth of both cell lines after 2 days of treatment (3 days after cell seeding). The growth-inhibitory effect increased directly with drug concentrations between 0.1 and 10 μM, but there was no appreciable difference in the growth-inhibitory effect at concentrations between 10 and 60 μM of analogue in either cell line.

Effect on Polyamine Levels. Polyamine levels of control and treated cells are listed in Table 1. Each value is the mean of results for 2 to 3 independent experiments. Spermidine and spermine levels of control U-87 MG cells were relatively higher than those of control SF-126 cells. In both cell lines at a concentration as low as 1 μM, BE-4-4-4 depleted putrescine and spermidine to nondetectable levels and spermine to 50% or less of the control levels by 2 days (3 days after seeding), although a small amount of spermidine was detected in U-87 MG cells for 2 to 4 days and in SF-126 cells for 2 days after treatment with 60 μM of the analogue. After 6 days of continuous treatment (7 days after cell seeding) with 10 or 60 μM BE-4-4-4, spermine was depleted to nondetectable levels. Significant amounts of BE-4-4-4 are taken up by both cell lines treated with as low as 1 μM of the analogue; for treatment with 10 μmol, however, the uptake is considerably higher for U-87 MG cells than for SF-126 cells. Because of the toxicity of the analogue, the yield of U-87 MG cells after 6 days of treatment with 60 μM of the analogue (7 days after cell seeding) was too low to allow accurate determination of polyamine concentrations.

The effect of 1 mM putrescine, 20 μM spermidine, or 20 μM spermine on the inhibitory action of BE-4-4-4 in both cell lines is shown in Figs. 2 and 3. Each exogenous polyamine only partially reversed the inhibitory effect in U-87 MG cells. With the possible exception of results for Day 7, there was no significant reversal of the growth of SF-126 cells.

Polyamine levels of cells treated with BE-4-4-4 and then treated with exogenous putrescine, spermidine, or spermine are listed in Table 2. In SF-126 cells, spermine reverted to near normal levels and spermidine reverted to near or above normal levels within 3 to 5 days of the addition of putrescine (5 to 7 days after cell seeding, see Table 2) and remained normal, while levels of putrescine remained above normal during the treatment period. In U-87 MG cells, however, spermidine and spermine levels were only partially restored by 1 day after putrescine addition, after which levels declined even though a significant amount of putrescine was taken up by these cells. Addition of 20 μM spermidine partially restored the spermidine and spermine levels, and 20 μM spermine partially restored the spermine level in both cell lines by 1 to 3 days after treatment (3 to 5 days after cell seeding, see Table 2). The replenished polyamine levels in SF-126 cells remained more or less constant after the addition of the respective polyamines until the end of the treatment period, but after an initial increase, the levels declined in U-87 MG cells. A small but significant amount of spermidine was detected in both cell lines after 3 days of treatment with spermine (5 days after cell seeding), but no detectable level of putrescine was observed in any of the cell lines treated with either spermine or spermidine. All exogenous polyamines appreciably decreased the intracellular concentration of BE-4-4-4 for U-87 MG cells in 1 day (3 days after cell seeding).

Table 1 Polyamine levels of human brain tumor cells treated with BE-4-4-4

<table>
<thead>
<tr>
<th>Cells</th>
<th>Polyamine levels (nmol/10^6 cells) on Days 3, 5, and 7 after seeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 3</td>
</tr>
<tr>
<td></td>
<td>Days 3</td>
</tr>
<tr>
<td></td>
<td>Days 3</td>
</tr>
<tr>
<td>U-87 MG</td>
<td>Putrescine</td>
</tr>
<tr>
<td></td>
<td>Spermidine</td>
</tr>
<tr>
<td></td>
<td>Spermine</td>
</tr>
<tr>
<td></td>
<td>BE-4-4-4</td>
</tr>
<tr>
<td>SF-126</td>
<td>Putrescine</td>
</tr>
<tr>
<td></td>
<td>Spermidine</td>
</tr>
<tr>
<td></td>
<td>Spermine</td>
</tr>
</tbody>
</table>

*ND*, nondetectable level.

**—**, cell yield too low to determine polyamine level (see text).
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Table 2 Polyamine levels of human brain tumor cells treated with 10 μM BE-4-4-4 followed by treatment with putrescine, spermidine, or spermine

<table>
<thead>
<tr>
<th>Cells</th>
<th>Control</th>
<th>BE-4-4-4 (10 μM)</th>
<th>BE-4-4-4 + putrescine* (1 mM)</th>
<th>BE-4-4-4 + spermidine* (20 μM)</th>
<th>BE-4-4-4 + spermine* (20 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Polyamine</td>
<td>3</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>87-MG</td>
<td>Putrescine</td>
<td>0.36</td>
<td>0.21</td>
<td>0.19</td>
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<tr>
<td></td>
<td>Spermidine</td>
<td>2.88</td>
<td>1.85</td>
<td>1.55</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Spermine</td>
<td>2.10</td>
<td>2.26</td>
<td>1.94</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>BE-4-4-4</td>
<td>4.88</td>
<td>8.82</td>
<td>7.22</td>
<td>ND</td>
</tr>
<tr>
<td>SF-126</td>
<td>Putrescine</td>
<td>0.59</td>
<td>0.55</td>
<td>0.25</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Spermidine</td>
<td>1.54</td>
<td>1.68</td>
<td>1.32</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Spermine</td>
<td>1.24</td>
<td>1.28</td>
<td>1.09</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>BE-4-4-4</td>
<td>2.06</td>
<td>3.94</td>
<td>3.86</td>
<td>3.27</td>
</tr>
</tbody>
</table>

* Polyamines were added 2 days after cell seeding (see text).

** ND, nondetectable level.

Fig. 2. Effect of 1 mM putrescine on the growth of U-87 MG (A) and SF-126 (B) cells treated with 10 μM BE-4-4-4 one day before the addition of putrescine: control (CON, ○); BE-4-4-4 alone (●); BE-4-4-4 plus putrescine (PUT) (△). Each point is an average of 2 to 3 observations. Error bars are smaller than the size of the symbols.

Fig. 3. Effect of 20 μM spermidine or spermine on the growth of U-87 MG (A) and SF-126 (B) cells treated with 10 μM BE-4-4-4 one day before the addition of polyamine: control (CON, ○); BE-4-4-4 alone (●); BE-4-4-4 plus spermidine (Sd, □); BE-4-4-4 plus spermine (Sm, ■). Each point is an average of 2 to 3 observations. Error bars are smaller than the size of the symbols.

Fig. 4. Effect of various concentrations of BE-4-4-4 on the survival of U-87 MG (A) and SF-126 (B) cells: 1 μmol (○); 10 μmol (●); 60 μmol (— — —). Each point is an average of 2 to 3 observations.

DISCUSSION

Six days of continuous treatment with 10 μM or higher concentrations of BE-4-4-4 depleted all measurable intracellular levels of the natural polyamines in both SF-126 and U-87 MG cells (Table 1). In contrast, a 24-h treatment of L1210 cells with 10 μM BE-4-4-4 depleted putrescine by 100%, spermidine by 80%, and spermine by 60% (16). The effect of BE-4-4-4 on the polyamine levels of any other cell line treated with the drug for more than 24 h has not been reported.

The amount of BE-4-4-4 taken up by either SF-126 or U-87 MG cells is directly proportional to the time of incubation and the extracellular concentration of the analogue up to a concentration of 10 μM. Extracellular concentrations of BE-4-4-4 greater than 10 μM had little additional effect on either the intracellular BE-4-4-4 level or the intracellular levels of natural polyamines. These results suggest that cells have a saturable mechanism for transporting the analogue across the membrane.

The growth-inhibitory effect (Fig. 1) and cytotoxicity (Fig. 4) of BE-4-4-4 parallel the extent of polyamine depletion induced by the analogue in U-87 MG and SF-126 cells. No significant difference in polyamine levels or in the growth-inhibitory effect for treatment with the analogue at concentrations between 10 and 60 μM was observed in either cell line. This suggests a correlation between polyamine depletion and growth inhibition by BE-4-4-4 in human brain tumor cells.

In an attempt to reverse the growth-inhibitory effect of BE-4-4-4, cells were treated with 1 mM putrescine, 20 μM spermidine, or 20 μM spermine. The concentrations of exogenous polyamines added were sufficient for total reversal of the DFMO-induced growth inhibition in 9L rat brain tumor cells (8) and U-87 MG human brain tumor cells, but could not reverse completely the inhibitory effects of BE-4-4-4 (Figs. 2 and 3). In the presence of exogenous polyamines, the intracellular concentration of the analogue was lower in U-87 MG cells (3) and for SF-126 cells in 5 days of treatment (7 days after cell seeding).

Effect on Cell Survival. Effects of 1, 10, and 60 μM BE-4-4-4 on cell survival are shown in Fig. 4. Both time- and dose-dependent cell kill was observed in U-87 MG and SF-126 cells. The analogue was relatively more toxic to SF-126 cells than to U-87 MG cells. In both cell lines the cell kill caused by 2 to 4 days of treatment with 60 μM of the analogue (3 to 5 days after cell seeding) was so high (more than 4 log) that viable colonies were not detectable.

Unpublished results.
than in SF-126 cells (Table 2). This may be the reason for relatively greater effectiveness of polyamines in the reversal of growth inhibition in U-87 MG cells than in SF-126 cells, even though polyamine levels were higher in SF-126 cells than in U-87 MG cells after addition of exogenous polyamines to medium (Table 2). In the presence of a high intracellular level of BE-4-4-4, partial replenishment of the cellular polyamine pool may not reverse the growth-inhibitory effect completely. We have shown that the binding constant of BE-4-4-4 to DNA is higher than that for spermine binding to DNA.4 We speculate that, in the presence of high concentrations of BE-4-4-4, polyamines fail to interact with their binding site(s) on DNA or other macromolecules and, therefore, cannot carry out their normal biological functions. The lowering of intracellular concentrations of BE-4-4-4 in both cell lines at some point after the addition of polyamines suggests that, in these cells at least, transport of BE-4-4-4 across the cell membrane probably involves the polyamine transport system.

Immunocytochemical studies using anti-spermine antibodies have shown an enhancement of the concentration of spermine in nuclei specifically when chromatin condensation occurs (22, 23), which suggests that spermine participates in this event. The mechanism by which the polyamines may function in this capacity could involve binding of polyamine to DNA and the introduction of bends at specific base sequences as we and others have observed in cell-free systems (14, 15). A polyamine analogue that does not bind and condense DNA as efficiently as natural polyamines should exhibit antiproliferative activity if it is able both to deplete intracellular polyamine levels and to remove them from their intracellular binding sites. Our earlier studies (17) showed that affinity of BE-4-4-4 for calf thymus DNA is higher than that of spermine, but that the analogue does not induce the bending or aggregation of DNA, or release bound ethidium bromide from the minor groove of DNA as efficiently as spermine does in vitro. Results presented here for two human cell lines and elsewhere for L1210 cells (16) show that BE-4-4-4 very effectively reduced intracellular polyamines, had a marked antiproliferative effect, and also had a significant cytotoxic effect on cells. These results provide support for our hypothesis that any polyamine analogue should act as an antiproliferative agent if it depletes intracellular polyamines. Further studies on the design and synthesis of other effective antineoplastic agents based on our hypothesis are in progress.

REFERENCES

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