Growth Inhibition of Hormone-responsive and -resistant Human Breast Cancer Cells in Culture by $N^1, N^{12}$-Bis(ethyl)spermine


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ABSTRACT

Previous studies have documented differential sensitivity of human lung cancer and melanoma cell lines to the cytotoxic effects of $N^1, N^{12}$-bis(ethyl)spermine (BESpm). We show here that BESpm can significantly inhibit the growth of six human breast cancer cell lines with 50% inhibitory concentration in the $\mu$M range. The degree of inhibition does not correlate with estrogen receptor status. Detailed studies with estrogen receptor-positive MCF-7 and estrogen receptor-negative Hs578T cells show a similar dose-response curve with concentrations of 1–10 $\mu$M resulting in maximal growth inhibition. Growth inhibition in both lines is associated with an 8–12-fold induction of the polyamine catabolic enzyme, spermidine/spermine N$\gamma$-acetyltransferase, and a progressive decrease in intracellular polyamine levels over 6 days even though steady-state levels of BESpm are achieved within 24 h. Similar studies on WTMCF7 and AdR-MCF7 cells show that the acquisition of resistance to hormonal or doxorubicin therapy is not associated with resistance to the growth-inhibitory effects of BESpm. These results suggest that BESpm exerts similar growth-inhibitory effects against both hormone-responsive and -unresponsive human breast cancer cells, a finding which has significance for the potential use of polyamine analogues in treating human breast cancer.

INTRODUCTION

Intracellular polyamines play an important role in the proliferation of normal and malignant cells. The recognition of their critical role in cell growth and differentiation has led to the development of several inhibitors of polyamine biosynthesis, particularly DFMO, which is directed against ODC, the first enzyme in polyamine biosynthesis (1–5). Recently, however, attention has been focused on other steps in the polyamine-metabolic pathway as potential targets for intervention (6). In particular the $N^1,N^{12}$-bis(ethyl) analogues of spermine were found to down-regulate ODC, deplete intracellular polyamine pools, and inhibit cell growth, suggesting a failure to substitute for the depleted natural polyamines (7). BESpm is a representative compound for this family of agents and its cytostatic and cytotoxic effects have been studied in several tumor models (8, 9–12).

In addition to down-regulating the biosynthesis of the polyamines, BESpm induces the activity of the rate-limiting enzyme in polyamine catabolism, SSAT (9, 10). A possible role for SSAT in human breast carcinogenesis has been suggested by the finding that SSAT activity is significantly elevated in primary human breast cancer specimens compared with surrounding normal breast tissue (13). SSAT is induced in a variety of cell types in response to several different stimuli including hormonal stimulation and exposure to polyamines and polyamine analogues (14–19). Although many cell types respond to BESpm treatment with a severalfold induction of SSAT, a few respond with extreme induction of SSAT (9, 10). Recent work using human lung cancer and melanoma cell lines as model systems suggests that a correlation between SSAT superinduction and growth inhibition is observed in that bis(ethyl)polyamines are cytotoxic primarily to SSAT “superinducer” cell types. For example, BESpm-mediated killing of human lung cancer cell lines is typically associated with a >100-fold induction in SSAT activity to levels of >1000 pmol/mg protein/min within 24 h, whereas cell types which demonstrate cytostatic growth effects manifest a smaller induction of SSAT in response to BESpm treatment (9).

Metastatic breast cancer is a common disease. A major clinical problem is that tumors which are initially responsive to both hormonal and chemotherapeutic approaches generally progress to more aggressive forms which are poorly responsive to either category of agents (20). The need for antineoplastic agents with novel mechanisms of action is therefore great. Previous studies have shown that growth of established human breast cancer cell lines can be inhibited by DFMO in culture (21–24). Evidence that hormone-responsive, but not hormone-resistant, human breast cancer cells are sensitive to the antiproliferative effect of DFMO has been presented by some investigators (21, 22) and refuted by others (23). Thus the major goal of this work was to characterize the effects of BESpm on a panel of hormone-responsive and -unresponsive human breast cancer cells. In addition we sought to correlate any change in growth with the effects of BESpm on intracellular polyamine levels and the induction of SSAT at the enzyme and steady-state mRNA levels.

MATERIALS AND METHODS

Chemicals. BESpm was synthesized as previously described (25, 26). It was maintained as a 10 mM stock in 0.1 mM HCl and diluted in media for cell treatment as described below.

Cell Culture and Growth Studies. The acquisition and maintenance of the estrogen-responsive MCF-7, T47D, and ZR–75–1 and estrogen-unresponsive MDA-MB-231, MDA-MB-468, and Hs578T human breast cancer cell lines have been described previously (27). WTMCF7 and AdR-MCF7 cells were the gift of Dr. K. Cowan (National Cancer Institute, Bethesda, MD). The AdR-MCF7 cell line was selected by serial passage of WTMCF7 cells in the presence of increasing concentrations of doxorubicin and was maintained in 10 $\mu$M doxorubicin as described previously (28). AdR-MCF7 cells were passaged in doxorubicin-free medium for at least four passages before use. For cell growth studies cells were plated in quadruplicate 12-mm wells under routine culture conditions. The plating density ranged from 20,000 to 30,000 cells/well, depending on the particular cell line under study. After 24 h the medium was replaced by medium with 0.01 mM HCl vehicle or varying concentrations of BESpm. The final concentration of HCl did not exceed 0.01 mM. Medium was replaced every 3 days and the number of adherent cells was determined by Coulter Counter after 6 days. Results are expressed as $N_1/N_0$, where $N_0$ is the number of cells on the day of BESpm or vehicle addition and $N_1$ is the number of cells after 6 days of BESpm or vehicle (9).

Analysis of Polyamine Content and SSAT Activity. The polyamine content of treated and untreated cells was determined by the precolumn dansylation, reversed-phase high-performance liquid chromatographic methods of Kabra et al. (29). 1,7-Diaminoheptane was used as the internal standard. The level of detection of this method is 5 pmol of the individual polyamines. SSAT activity of cellular extracts was measured as reported previously (19).

Enzyme activity is expressed as pmol $N^1-[^3H]$acetylspermidine formed/mg protein/min. Protein concentrations were determined by the method of Bradford (30).
Northern Analysis. Total cellular RNA from BESpm-treated and untreated cells was isolated by lithium chloride-urea precipitation as described previously (31). Northern analysis of resulting total RNA was carried out by published methods (32); filters were hybridized to the full length human SSAT complementary DNA clone AP3/F7 (33). Equal loading of RNA was ascertained by ethidium bromide visualization of gels and subsequent hybridization of filters to a rat complementary DNA probe homologous to human glyceraldehyde phosphate dehydrogenase (34).

RESULTS

Effect of BESpm on Growth of Human Breast Cancer Cell Lines. The effect of chronic treatment with 10 μM BESpm on the growth of six human breast cancer cell lines was first examined as shown in Fig. 1. This concentration of BESpm was chosen based on previous work in lung carcinoma and melanoma cell lines where 10 μM was found to be cytotoxic in certain lines (8-10). BESpm inhibited the growth of all cell lines; the most sensitive cell line, Hs578t, demonstrated an 86% growth inhibition. However, no net cell loss was observed in any cell line examined, i.e., \( N_t/N_0 \) was never less than 1. There was no clear relationship between estrogen receptor status and sensitivity to BESpm. Also the intrinsic differences in growth rate between cell lines were not associated with clear differences in BESpm sensitivity in that the three estrogen receptor-negative cell lines which have a more rapid doubling time were not uniformly more sensitive than the three slower growing estrogen receptor-positive cell lines.

The possibility that higher doses of BESpm might be associated with enhanced cytotoxicity was next addressed. For these studies the MCF-7 and Hs578t cell lines were chosen as representative estrogen receptor-positive and estrogen receptor-negative cell lines, respectively. As shown in Fig. 2, BESpm inhibited the growth of both cell lines in a dose-dependent fashion up to 1–10 μM. However, treatment with a higher dose of 100 μM BESpm was not associated with a further decrease in cell growth. This flat dose response is typical of that seen with bis(ethyl)polyamine analogues (8, 10). A concentration of 10 μM was chosen for further studies.

BESpm Effect on SSAT Activity and mRNA Expression. Previous work has suggested that there may be an association between the level of SSAT and growth effects in BESpm-treated cells (9, 10). The effect of 10 μM BESpm on SSAT activity in MCF-7 and Hs578t cells was therefore examined (Fig. 3). The basal activity of exponentially growing MCF-7 cells was 97 ± 45 pmol (SEM)/mg protein/min and 10 μM BESpm treatment led to a steady increase in SSAT activity over 6 days to a maximum 13-fold induction. A similar basal SSAT activity of 62 ± 19 pmol/mg protein/min was seen in Hs578t cells and a maximum 6–8-fold induction in SSAT activity was seen with 6 days of BESpm treatment. However, the level of induction of SSAT in both cell lines after 24 h was only 2.5–3.5-fold over control and SSAT activity was less than 400 pmol/mg protein/min in each case. These findings in conjunction with the observation that BESpm treatment of both MCF-7 and Hs578t cells results in significant growth inhibition, but not net cell loss, support the postulate that the kinetics of induction of SSAT activity may be an important determinant of BESpm effects on growth.

The level of SSAT mRNA in BESpm-treated MCF-7 and Hs578t cells was also examined (Fig. 4A). The steady-state SSAT mRNA level paralleled the pattern of induction of enzyme activity. Approx-
ethidium staining, are 1.3 kilobases for SSAT and 1.8 kilobases for glyceraldehyde phosphate dehydrogenase (GAPDH). The approximate sizes for each complementary DNA probe for SSAT (55-47) or a rat complementary DNA probe for or vehicle control for 24 or 48 h were subjected to Northern analysis using the AP3/F7 response to BESpm. Ten μg of total RNA derived from cells treated with 10 μM BESpm for 24 or 48 h of BESpm treatment of either cell line; this correlates well with cells.

MCF-7 and Hs578t cells.

The approximate induction of SSAT activity at the same early time points.

Modulation of Intracellular Polyamine Pools by BESpm. One possible explanation for the relatively slow induction of SSAT activity in both MCF-7 and Hs578t cells could be a decreased ability of the cells to accumulate BESpm. Therefore the intracellular concentrations of natural polyamines and BESpm were measured in parallel with SSAT activity and mRNA levels (Table 1). BESpm accumulates rapidly in both cell lines such that a steady-state level is achieved within 24 h and maintained throughout 6 days of treatment. A >50% reduction of intracellular polyamine pools is reached within 24 h in both MCF-7 and Hs578t cells. However, maximal depletion of intracellular polyamine levels does not occur until between 3 and 6 days of treatment. Thus, although BESpm uptake is rapid, its effects on SSAT activity and intracellular polyamine pools are maximal only after several days of treatment of MCF-7 or Hs578t cells.

Effect of BESpm on Human Breast Cancer Cells Resistant to Chemohormonal Therapy. A common problem in the clinical management of breast cancer is the development of resistance to hormonal and cytotoxic agents. We therefore tested whether or not this resistance is associated with a change in sensitivity to BESpm. For these studies doxorubicin-sensitive WTMCF7 and doxorubicin-resistant AdrMCF-7 cells were used. Parental WTMCF7 cells have been shown previously to contain estrogen receptor and respond to exogenous 17β-estradiol while AdrMCF7 cells lack estrogen receptor and are estrogen independent (35). Thus these cell lines represent an in vitro model of hormone- and chemotherapy-resistant breast cancer. Treatment of WTMCF7 and AdrMCF7 cells with 0.1–100 μM BESpm was associated with a similar dose-dependent inhibition of cell growth in both cell lines and was maximal at 1–10 μM (Table 2 and not shown). Again no evidence of net cell loss was observed.

Measurement of SSAT activity showed that basal activity was increased in AdrMCF7 cells compared with WTMCF7 cells. Treatment with 10 μM BESpm for 24 h induced SSAT activity 4-fold in both cell lines (Table 2) but, as in the two cell lines examined initially, the actual SSAT activity did not exceed 400 pmol/mg protein/min. Continued treatment of WTMCF7 cells with BESpm resulted in further induction of SSAT activity to a level of 745 pmol/mg protein/min after 6 days. SSAT activity in AdrMCF7 cells did not change greatly with chronic BESpm treatment, reaching a maximum of 485 pmol/mg protein/min after 6 days. Fig. 4B again shows that the steady-state SSAT mRNA levels paralleled the pattern of induction of enzyme activity after 24 or 48 h. Basal SSAT mRNA level was about 5-fold greater in AdrMCF7 cells than in WTMCF7 cells. Treatment of WTMCF7 cells with 10 μM BESpm resulted in a 2.5-fold induction of SSAT mRNA after 24 h and 6.0-fold induction after 48 h. Similar treatment of AdrMCF7 cells was associated with a 2.5–3.5-fold increase in SSAT mRNA levels at the same time points.

Concurrent measurement of intracellular polyamine levels after 1, 2, 3, or 6 days of treatment showed evidence of depletion within 24 h of BESpm treatment in both cell lines although maximal depletion was not achieved until between 3 and 6 days of treatment (Table 2; other data not shown). A significant level of BESpm was attained in both cell lines within 24 h. However, steady-state levels of BESpm were not reached until 48 h in WTMCF7, and BESpm levels in AdrMCF7 cells continued to increase throughout a 6-day course of treatment (not shown). Taken together these results suggest that the estrogen-independent, doxorubicin-resistant AdrMCF7 cells retain the sensitivity to growth inhibition by BESpm which characterizes the parental WTMCF7 cell line although the time course of BESpm accumulation differs. Also the initial pattern of induction of SSAT

Table 1 Comparison of effects of 10 μM BESpm on intracellular polyamines in MCF-7 and Hs578t human breast cancer cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Put (nmol/mg)</th>
<th>Spd (nmol/mg)</th>
<th>Spm (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, 1 day</td>
<td>3.5</td>
<td>32.5</td>
<td>11.2</td>
</tr>
<tr>
<td>BESpm, 1 day</td>
<td>0.4</td>
<td>11.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Control, 2 days</td>
<td>3.3</td>
<td>28.2</td>
<td>9.2</td>
</tr>
<tr>
<td>BESpm, 2 days</td>
<td>3.0</td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Control, 3 days</td>
<td>4.4</td>
<td>36.0</td>
<td>13.5</td>
</tr>
<tr>
<td>BESpm, 3 days</td>
<td>0.2</td>
<td>2.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Control, 6 days</td>
<td>2.7</td>
<td>27.8</td>
<td>10.9</td>
</tr>
<tr>
<td>BESpm, 6 days</td>
<td>ND</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Hs578t</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Put</td>
<td>0.8</td>
<td>18.8</td>
<td>23.8</td>
</tr>
<tr>
<td>Spd</td>
<td>1.0</td>
<td>10.0</td>
<td>9.8</td>
</tr>
<tr>
<td>Spm</td>
<td>9.8</td>
<td>26.1</td>
<td>30.8</td>
</tr>
<tr>
<td>BESpm</td>
<td>0.2</td>
<td>4.7</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*Put, putrescine; Spd, spermidine; Spm, spermine; ND, not detected.*

GROWTH INHIBITION OF BREAST CANCER CELLS BY BESpm

Fig. 4. Steady-state mRNA levels of SSAT in various human breast cancer cell lines in response to BESpm. Ten μg of total RNA derived from cells treated with 10 μM BESpm or vehicle control for 24 or 48 h were subjected to Northern analysis using the AP3/F7 complementary DNA probe for SSAT (SSAT) or a rat complementary DNA probe for glyceraldehyde phosphate dehydrogenase (GAPDH). The approximate sizes for each mRNA, as determined by comparison to 28S and 18S ribosomal bands visualized by ethidium staining, are 1.3 kilobases for SSAT and 1.8 kilobases for glyceraldehyde phosphate dehydrogenase. A, MCF-7 and Hs578t cells. B, WT MCF-7 and Adr MCF-7 cells.
activity is similar in the two cell lines and neither shows evidence of SSAT superinduction to levels of >1000 pmol/mg protein/min after 24 h of BESpm treatment.

**DISCUSSION**

Previous studies have documented differential sensitivity of human cancer cell lines to the cytotoxic or cytostatic effects of several N,N'-bis(ethyl) analogues of spermine (8–10). We have shown that one analogue, BESpm, can inhibit the growth of six human breast cancer cell lines with 50% inhibitory concentrations in the μM range. Significantly, the degree of inhibition is not correlated with the estrogen receptor status of the cell line. Detailed studies with a representative estrogen receptor-positive cell line, MCF-7, and a representative estrogen receptor-negative cell line, Hs578t, showed a similar dose-response curve with concentrations of 1–10 μM resulting in maximal growth inhibition. Growth inhibition in both lines was associated with a steady increase in SSAT activity over 6 days of treatment to a maximum of 8–12-fold over control. However, SSAT activity was increased by <4 fold in both lines after 24 h with actual activities of 200–300 pmol/mg protein/min in both cell lines. Intracellular polyamine levels were also found to decrease progressively over the 6-day study period, even though steady state levels of BESpm were achieved within 24 h of treatment. Similar studies on WTMC7 and AdrMCF7 cells showed that the acquisition of resistance to hormonal therapy or doxorubicin chemotherapy was not associated with resistance to the growth-inhibitory effects of BESpm. A similar pattern of induction of SSAT activity and polyamine depletion was also observed in both cell lines although accumulation of BESpm was slower in these two cell lines than in MCF-7 and Hs578t cells.

The pivotal role of polyamine biosynthesis for growth of experimental breast cancers has been demonstrated in a variety of models. Our results suggest that polyamines are equally involved in growth of hormone-responsive and -unresponsive human breast cancer cell lines. The proliferative capacity of all cell lines studied was uniformly suppressed by increasing doses of BESpm. These findings are consistent with the work of Glikman et al. (23) who documented a similar degree of growth inhibition and polyamine depletion with DFMO, an inhibitor of ODC, in hormone-dependent MCF-7 and hormone-independent MDA-MB-231 cells.

Other studies have demonstrated differential sensitivity to BESpm treatment in a limited number of cell types. Based on studies in human lung cancer and melanoma cell lines, a possible link between SSAT induction and cytotoxicity has been suggested (8–10). Work in both model systems supports the hypothesis that BESpm has cytotoxic effects in those cell lines which manifest high inductions of SSAT activity to >1000 pmol/mg protein/min within 24 h of treatment. Our findings in human breast cancer cell lines are consistent with this hypothesis. None of the cell lines studied demonstrated cytotoxicity during the 6-day treatment period although growth inhibition was observed in all cases. The magnitude of induction of SSAT activity in MCF-7 and Hs578t cell lines was <5-fold after 24 h and absolute activity remained below 500 pmol/mg/min. This lack of superinduction was not due to failure to accumulate drug inasmuch as BESpm levels in MCF-7 and Hs578t cells treated with 10 μM BESpm for 24 h were equivalent to those in the most sensitive lung cancer cell lines studied under the same conditions. In addition, the extent of polyamine depletion after 24 h of BESpm treatment was similar in both breast cancer cell lines and sensitive large cell lung cancer cell lines like H157 and H460 (9). This is in accordance with previous findings that BESpm can down-regulate the polyamine biosynthetic pathway, particularly ODC, regardless of whether cytotoxicity is observed (7, 36). Finally, a direct relationship between SSAT induction and subsequent cytotoxicity has not yet been demonstrated; gene transfer studies using SSAT expression vectors will be necessary to address this possibility.

In this study steady-state mRNA levels for SSAT correlated well with SSAT activity level in the human breast cancer cell lines examined. This suggests that increased SSAT activity in human breast cancer cells after treatment with BESpm could result from increased transcription of SSAT mRNA alone. However, the possibility of other posttranscriptional and/or posttranslational mechanisms coming into play cannot be eliminated. Such nontranscriptional mechanisms have been demonstrated in human lung cancer and melanoma cell lines and are postulated to be responsible for the superinduction of SSAT in sensitive lung cancer and melanoma cell lines (37, 38). It is possible to speculate that the presence of these posttranscriptional controls is necessary for the SSAT superinducer phenotype and that the absence of these mechanism(s) is associated with the failure of BESpm to superinduce SSAT. Again detailed mechanistic studies on the precise role of SSAT and the control of SSAT induction by BESpm in relatively sensitive and resistant cells will be required to address this possibility.

From a therapeutic perspective a critical finding in our work is that BESpm inhibits the growth of a wide variety of human breast cancer cell lines. Both estrogen receptor-positive and estrogen receptor-negative cell lines manifested cytostatic responses. Moreover the development of multidrug resistance gene-mediated resistance to one chemotherapy agent, doxorubicin, did not modulate response to BESpm. Both parental WTMC7 and AdrMCF7 cells were growth inhibited by BESpm. It is of note, however, that the doxorubicin-resistant AdrMCF7 cells had a higher basal level of SSAT mRNA expression and enzyme activity and delayed uptake of BESpm when compared with parental WTMC7 cells.

**Table 2. Comparison of effects of 10 μM BESpm on WTMCF7 and AdrMCF7 human breast cancer cells**

<table>
<thead>
<tr>
<th>Cell line and treatment</th>
<th>Growth (N/N₀)</th>
<th>SSAT activity (pmol/mg protein/min)</th>
<th>Polyamines (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTMCF7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, 1 day</td>
<td>14</td>
<td>0.96</td>
<td>Put *</td>
</tr>
<tr>
<td>BESpm, 1 day</td>
<td>61</td>
<td>11.3</td>
<td>ND</td>
</tr>
<tr>
<td>Control, 6 days</td>
<td>5.2</td>
<td>2.4</td>
<td>Spd</td>
</tr>
<tr>
<td>BESpm, 6 days</td>
<td>7</td>
<td>26.8</td>
<td>ND</td>
</tr>
<tr>
<td>AdrMCF7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, 1 day</td>
<td>94</td>
<td>0.75</td>
<td>Spm</td>
</tr>
<tr>
<td>BESpm, 1 day</td>
<td>380</td>
<td>3.5</td>
<td>ND</td>
</tr>
<tr>
<td>Control, 6 days</td>
<td>4.6</td>
<td>1.4</td>
<td>BESpm</td>
</tr>
<tr>
<td>BESpm, 6 days</td>
<td>54</td>
<td>8.9</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Put, putrescine; Spd, spermidine; Spm, spermine; ND, not detected.
In conclusion our studies support the need for further evaluation of the N,N'-bis(ethyl) analogues of spermine like BESpm in xenograft models of human breast cancer. In addition the possibility that previous therapies may influence the response of a tumor cell to BESpm treatment should be further investigated.

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REFERENCES


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