Inhibition of the Growth of Various Human and Mouse Tumor Cells by 1,15-Bis(ethylamino)-4,8,12-triazapentadecane

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ABSTRACT

Effects of 1,15-bis(ethylamino)-4,8,12-triazapentadecane (BE3333), the least toxic bis(ethyl)pentaneamine, on the growth of tumor cells were studied in in vitro systems and with tumor xenografts in mice. BE3333 suppressed ornithine decarboxylase and S-adenosylmethionine decarboxylase, induced spermidine/spermine N1-acetyltransferase, and thus decreased the amount of polyamines. BE3333 accumulated in cells at a concentration 3-5-fold that of spermine in control cells through the polyamine transport system. The accumulated BE3333 inhibited protein synthesis, especially mitochondrial protein synthesis, and decreased the amount of ATP. The inhibition of protein synthesis was correlated with the subsequent inhibition of cell growth. BE3333 showed inhibitory effects in in vitro systems against the growth of mouse FM3A mammary carcinoma cells, human SW480 and SW620 colon tumor cells, Lu-65A and A549 lung tumor cells, MCF-7 breast tumor cells, and MALME-3M and A375 melanoma cells at a range of 0.5-10 μM. Intravenous (30 mg/kg) or i.p. (50 mg/kg) daily injections of BE3333 for 5 or 7 days greatly suppressed the growth of human colon tumor SW620 xenotransplanted into nude mice. Similar antitumor activity was obtained with continuous infusion of BE3333 into the peritoneal cavity (80 mg/kg), but not with p.o. administration (200 mg/kg). BE3333 also showed inhibitory effects against the growth of lung tumors (Lu-65, Lx-1, Lc-1, and Lu-61), stomach tumors (Sc-6 and St-15), and melanoma (SEKI) xenotransplanted into nude mice. The results indicate that BE3333 is effective against both rapid- and slow-growing tumors, with reasonable short-term host toxicity.

INTRODUCTION

Since polyamine biosynthesis is closely related to cell growth, the possibility that inhibitors of polyamine biosynthesis may function as anticancer agents has been examined (1). Two chemicals, used widely as inhibitors in polyamine biosynthesis, are DFMO,2 an inhibitor of ODC (2), and MGBG, an inhibitor of SAMDC (3). These inhibitors of two different segments of polyamine biosynthesis have exhibited antitumor activity in experimental tumor studies (4, 5), and the anti-tumor effect of DFMO has been studied extensively (1). In addition, the combination of DFMO-MGBG has been shown to be more effective therapeutically than either drug alone in a number of in vivo model systems (6–11). The in vivo usefulness of these inhibitors, however, has been limited by regulatory homeostatic responses unique to the polyamine pathway. For example, in our experiments (10), combined treatment with DFMO and MGBG caused a remarkable regrowth after its termination.

As an alternative to specific inhibitors, bis(ethyl)polyamine analogues have been developed as antiproliferative reagents (12, 13). These reagents cannot only negatively regulate the synthesis of ODC and SAMDC (14), but can also induce SSAT activity (15, 16). The analogues could deplete intracellular polyamines almost completely, and the level of accumulation of the analogues was nearly equal to the original spermidine and spermine contents (17). Thus, the analogues were thought to inhibit cell growth through the depletion of polyamines. We found that N1,N12-bis(ethyl)spermine can substitute for the functions of spermine in various aspects and accumulates in cells at a concentration up to 5-fold that of spermine in control cells (18, 19); we suggested that not only polyamine deficiency but also the accumulation of N1,N12-bis(ethyl)spermine may be involved in the inhibition of cell growth (19). Recently, it has been reported that the inhibition of cell growth by N1,N12-bis(ethyl)spermine correlated with the intracellular accumulation of the analogue (20). We also found that the accumulated spermidine and spermine inhibited cell growth (21). The accumulated polyamines and bis(ethyl)polyamine analogues caused the inhibition of protein synthesis, especially mitochondrial protein synthesis, leading to a decrease in ATP (21, 22).

Since the inhibition by bis(ethyl)polyamines of cell growth in vitro systems was very potent, the antitumor activity of N1,N12-bis(ethyl)norspermine was examined against human MALME-3 melanoma xenografts, and it was found that the analogue fully suppressed growth of the melanoma (23, 24). As the degree of inhibition of cell growth by bis(ethyl)pentaneamines was stronger than that by bis(ethyl)-tetraamines in an in vitro system (22), we examined the antitumor activity of BE3333, the least toxic bis(ethyl)pentaneamine, against various human tumors xenotransplanted into nude mice, and found that the analogue has strong antitumor activities. In this regard, it has been reported recently that BE4444 also shows strong antitumor activities against human tumors xenotransplanted into nude mouse (25).

MATERIALS AND METHODS

Materials. BE3333 5HCl was synthesized by removal of the protecting groups from benzylated and p-toluenesulfonlated BE3333 prepared by N-alkylation of N1,N4,N7,N10-tetraethylspermine with N-ethyl-N-(3-bromopropyl)-p-toluenesulfonamide according to the method for the synthesis of pentaneamines (26), crystals from water-ethanol. The structure was confirmed by elemental analysis. Mouse mammary carcinoma FM3A cells were kindly supplied by Dr. H. Matsuzaki, Saitama University (Urawa, Japan). Human SW480 and SW620 cells were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). A375, MALME-3M, and MCF-7 cells were obtained from American Type Culture Collection (Rockville, MD). A549 and Lu-65A cells were supplied by Riken Cell Bank (Tsukuba, Japan) and the Japanese Cancer Research Resources Bank (Tokyo, Japan), respectively. Lc-1, Lu-61, Lu-65, Lx-1, SEKI, Sc-6, and St-15 cells were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). Cell Culture. Cells were grown in an environment of 5% CO2 in humidified air. Mouse FM3A mammary carcinoma cells were cultured in ES medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 50 units/ml streptomycin, 100 units/ml penicillin G, and 2% heat-inactivated FCS. Lu-65A lung tumor and MCF-7 breast tumor cells were grown in RPMI 1640...
Tumor growth data were expressed as tumor volumes (mm$^3$) calculated from the following equation: length $\times$ width$^2 \times 0.52$ (27). When tumor volumes reached 80 mm$^3$, BE3333 was daily given either i.v., i.p., or p.o. for 5 days (days 0–4) or for 7 days (days 0–6). Continuous infusion into the peritoneal cavity was also performed with Alza Model 1007 D micro pumps (Palo Alto, CA) according to the method of Siegell et al. (28). The statistical significance of differences in animal weight and tumor volume was assessed by the Student’s t test.

**Enzyme Assays.** FM3A cells (5 × 10$^5$) or tumor cells (50–200 mg) were homogenized with 0.3 ml 0.2 M HClO$_4$ and centrifuged for 10 min at 12,000 × g. The supernatant thus obtained was used for the enzyme assays. Assays of ODC, SAMDC, and SSAT were performed as described previously (30, 31) with some modifications (21). Assay for polyanamine transport was performed as described (32). The protein concentration was determined according to the method of Lowry et al. (33).

**Measurements of Polyamines, BE3333, and ATP.** FM3A cells (6 × 10$^5$) or tumor cells (10–50 mg) were homogenized with 0.3 ml 0.2 M HClO$_4$ and centrifuged for 10 min at 12,000 × g. The supernatant thus obtained was used for the following assays. Polyamines (putrescine, spermidine, and spermine) were measured as described previously (34). BE3333 was analyzed after dannylation according to the previous publication (22). ATP was measured using the luciferase enzyme system (35) after neutralization with 1 M KOH containing 50 mM K$_2$HPO$_4$.

**Analysis of Total and Mitochondrial Protein Synthesis.** FM3A cells (5 × 10$^5$) cultured in the presence of 10 μM BE3333 were incubated for 2 h with 3.7 MBq [35S]methionine in the presence and absence of emetine (0.2 mg/ml), which inhibits cytoplasmic protein synthesis specifically (36). The cells cultured in the presence of emetine were harvested, and total protein synthesis was measured by counting the hot trichloroacetic acid-insoluble radioactivity in the mitochondrial fraction. Mitochondrial protein synthesis was measured by counting the hot trichloroacetic acid-insoluble radioactivity in the mitochondrial fraction.

**RESULTS**

**Effect of BE3333 on the Growth of Mouse Mammary Carcinoma FM3A Cells in Culture.** In the previous communication (22), the effect of bis(ethyl)pentaamines on the growth of FM3A cells in culture was examined at relatively high concentrations (0.25–0.3 mM). In the present experiments (Fig. 1), the effect of BE3333 on the growth of FM3A cells was examined at concentrations of 0.3–100 μM. The inhibition of cell growth by BE3333 was observed to be dose dependent, and IC$_{50}$ was 0.48 μM. Furthermore, BE3333 caused cell death on day 6.

Polynamine and BE3333 contents and polynamine metabolizing enzymes in FM3A cells were treated with 10 μM BE3333 as measured (Table 1). BE3333 accumulated in cells at a concentration up to 5-fold that of spermine in control cells. The decrease in polynamine contents started with putrescine followed by decreases in spermidine and spermine. Thus, the decrease in polynamine contents at the initial phase by the treatment of cells with BE3333 paralleled the decrease in ODC and SAMDC activities. SSAT then increased in correlation with the decrease in spermine. BE3333 was probably transported by the polyanamine uptake system, since it inhibited spermidine uptake with almost the same efficiency as spermine (Fig. 2). The inhibition of mitochondrial protein synthesis was greater than that of total protein synthesis, and a decrease in ATP content was observed (Fig. 3). Swelling of mitochondria was also observed by electron microscopy in cells treated with BE3333 for 2 days (data not shown).

**Effect of BE3333 on the Growth of Human Tumor Cells in Culture.** IC$_{50}$ of BE3333 against various human tumor cells (colon adenocarcinoma, small cell lung carcinoma, lung adenocarcinoma, breast carcinoma, and melanoma) in culture was then measured (Table 2). The values obtained ranged from approximately 0.5 to 3 μM. There was a tendency that rapidly growing cells needed higher concentrations of BE3333 to inhibit cell growth. The only exception was MALME-3M melanoma cells, which grew slowly but the IC$_{50}$ value was rather high (1.16 μM).

### Table 1 Polyamine and BE3333 contents and polyamine metabolizing enzymes in FM3A cells

<table>
<thead>
<tr>
<th>Culture</th>
<th>Incubation time (h)</th>
<th>Polyamines (nmol/mg protein)</th>
<th>Enzyme activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Putrescine</td>
<td>Spermidine</td>
</tr>
<tr>
<td>Control</td>
<td>48</td>
<td>3.29</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>3.10</td>
<td>11.3</td>
</tr>
<tr>
<td>BE3333</td>
<td>6</td>
<td>1.02</td>
<td>7.84</td>
</tr>
<tr>
<td></td>
<td>(10 μM)</td>
<td>&lt;0.1</td>
<td>2.73</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>&lt;0.1</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* Each value is the average of two determinations.
Effect of BE3333 on the Growth of Human Tumors Xenotransplanted into Nude Mice. The antitumor effect of BE3333 was first examined with SW620 colon adenocarcinoma xenotransplanted into nude mice (Fig. 4). When BE3333 was given i.v. at a dose of 20–30 mg/kg/day for 7 successive days (days 0–6), the growth of tumors was strongly inhibited (Fig. 4A). However, the growth recovered gradually after cessation of the administration. When BE3333 was given i.p. at a dose of 50 mg/kg/day, a similar inhibitory effect to that with 30 mg/kg/day i.v. was obtained (Fig. 4D). The i.p. administration of 80 mg/kg/day was toxic, because all mice died by day 5. The i.p. administration of BE3333 at a dose of 30 mg/kg/day had a smaller inhibitory effect than at 50 mg/kg/day. Continuous infusion into the peritoneal cavity (50 or 80 mg/kg/day) by micro pumps also showed a strong antitumor effect (Fig. 4C). However, there was no

Table 2 Growth inhibitory activities of BE3333 against human tumor cell lines

<table>
<thead>
<tr>
<th>Tumor cells</th>
<th>Doubling time (h)</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW480 (colon)</td>
<td>39.0</td>
<td>0.60</td>
</tr>
<tr>
<td>SW620 (colon, lymphnod metastasis)</td>
<td>31.2</td>
<td>0.54</td>
</tr>
<tr>
<td>Lu-65A (lung carcinoma, small cell)</td>
<td>21.3</td>
<td>2.01</td>
</tr>
<tr>
<td>A549 (lung adenocarcinoma)</td>
<td>43.0</td>
<td>0.49</td>
</tr>
<tr>
<td>MCF-7 (breast)</td>
<td>20.0</td>
<td>2.98</td>
</tr>
<tr>
<td>MALME-3M (melanoma)</td>
<td>45.0</td>
<td>1.16</td>
</tr>
<tr>
<td>A375 (melanoma)</td>
<td>20.0</td>
<td>1.50</td>
</tr>
</tbody>
</table>

* Each value is the average of three determinations.
cultured FM3A cells (Table 1). On day 15, the amount of accumulated BE3333 had decreased in conjunction with the recovery of cell growth and the increase in polyamine content.

**DISCUSSION**

It has been reported that bis(ethyl)norspermine has antitumor activity against human melanoma xenografts (23, 24), and that BE4444 has antitumor activity against several human tumor xenografts (25). However, those bis(ethyl)polyamine derivatives are effective against relatively slow-growing tumors. In this study, we examined the effect of another derivative, BE3333, on the growth of various human tumors xenotransplanted into nude mice, and found that it is effective against both rapid- and slow-growing tumors. The i.v. administration (60 mg/kg) of bis(ethyl)norspermine was not effective against SW620 against both rapid- and slow-growing tumors. The i.v. administration of another derivative, BE3333, on the growth of various human tumors xenografted into nude mice, and found that it is effective against several human tumor xenografts (25). However, SSAT inhibition of cell growth by bis(ethyl)polyamine analogues correlated with their intracellular accumulation, and that the accumulation caused the inhibition of protein synthesis, especially mitochondrial protein synthesis, and then a decrease in ATP content (21, 22).

In the present study, we showed that BE3333 accumulated in the tumor cells xenotransplanted into nude mice at an amount about 3-fold that of spermine in control cells (Table 4). However, SSAT only showed a small increase. Taken together, our results strongly suggest that the antitumor activity of BE3333 is mainly due to the inhibition of protein synthesis, especially of mitochondrial protein synthesis. Still, the possibility that BE3333 may also be effective due to its multiple actions cannot be ruled out.

Although we obtained similar antitumor activity with i.p. (50 mg/kg) and i.v. (30 mg/kg) injections of BE3333, it should be noted that weight loss was more severe with the former. The rapid diffusion of BE3333 by i.v. injection may decrease the toxic effects of the drug. However, this is difficult to state conclusively because of differences in antitumor response (24). However, a direct correlation between SSAT induction and growth inhibition does not always exist for all cell lines and analogues (37). We recently reported with in vitro experiments that the inhibition of cell growth by bis(ethyl)polyamine analogues correlated with their intracellular accumulation, and that the accumulation caused the inhibition of protein synthesis, especially mitochondrial protein synthesis, and then a decrease in ATP content (21, 22).
in dosages and routes for delivery. When BE3333 was given p.o., it showed no antitumor effect at all, reflecting its insufficient absorption from the intestinal tract.

In the case of the antitumor activity caused by the polyamine deficiency of cells, such as with the combined therapy of DFMO and MGBG (6–11), the activity is essentially cytostatic, but not cytotoxic (38, 39). Thus, marked acceleration of tumor growth followed the cessation of therapy (10). The antitumor activity caused by bis(ethyl)polyamine analogues should be cytostatic judging from the experimental results obtained from cell culture of mouse F33A mammary carcinoma cells (18). However, tumor growth recovered after cessation of the drug treatment. Further studies will be necessary to clarify whether BE3333 possesses cytotoxic action in vivo.

ACKNOWLEDGMENTS

We thank Dr. K. Samejima for his encouragement during the course of this study and Dr. A. Tomura for his kind help in preparing the manuscript. Thanks are also due to Dr. H. Matsuzaki for his kind supply of mouse mammary carcinoma F33A cell lines.

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