Effects of Novel Spermine Analogues on Cell Cycle Progression and Apoptosis in MALME-3M Human Melanoma Cells

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ABSTRACT

On the basis of encouraging preclinical findings, polyanine analogues have emerged as a novel class of experimental antitumor agents. The spermine derivative N,N,N,N'-diethylnorspermine (DE-333, also known as DENSPM) is currently undergoing Phase I clinical trials against solid tumors. A series of systematically modified DE-333 analogues differing in intra-amine carbon distances and in N-alkyl terminal substituents (i.e., methyl, ethyl, and propyl) were evaluated in MALME-3M human melanoma cells, a cell line known to be cytotoxically affected by DE-333 and especially responsive to analogue induction of the polyamine catabolic enzyme spermidine/spermine N,N-acetyltransferase. Analogues accumulated to comparable intracellular concentrations and similarly affected cell growth with IC50 values in the 0.5-1.0 μM range. During prolonged incubations, diethyl and dipropyl analogues were cytotoxic, whereas two dimethyl analogues were cytostatic. Cell cycle analysis following treatment with the cytotoxic analogues revealed a prominent G1 block apparent as an accumulation of cells in G1 and depletion of S-phase cells as well as a less restrictive G2 block. By contrast, cytostatic analogues incompletely arrested cells in G1, leaving a significant number of S-phase cells. Morphological and immunocytochemical analysis of detached cells revealed a far greater proportion of apoptotic cells with cytotoxic analogues than with cytostatic analogues. Although spermidine/spermine N,N-acetyltransferase activity was differentially induced by the analogues, there was no obvious correlation with cell cycle effects. Overall, these data indicate a previously unrecognized combined effect of polyanine analogues on cell cycle progression and apoptosis. On the basis of structure-function relationships, these activities may be manipulated to optimize therapeutic efficacy.

INTRODUCTION

The intracellular polyamines PUT, SPD and SPM are constitutive components of all mammalian cells, and their intracellular availability is critical for cell proliferation. Thus, interference with polyamine biosynthesis has emerged as an important anticancer strategy involving polyanine analogues and specific inhibitors of key biosynthetic enzymes. At present, the ODC inhibitor DFMO is being clinically evaluated as a chemopreventive agent (1-3), and on the basis of encouraging preclinical findings, polyamine analogues have recently synthesized as a chemopreventive agent (1-3), and on the basis of structure-function relationships, these events may be manipulated to optimize therapeutic efficacy.

MATERIALS AND METHODS

Materials. The polyamine analogues were synthesized according to Bergeron et al. (19, 23). Analogue nomenclature is defined according to Fig. 1. MALME-3M human melanoma cells were obtained from the American Type Culture Collection (Rockville, MD). Radioactive [3H]SPD (17.6 Ci/mmol), L-[1-14C]ornithine (50 mCi/mmol), and acetyl-L-[14C]CoA (60 mCi/mmol) was purchased from New England Nuclear (Boston, MA).

Cell Culture. MALME-3M human melanoma cells were maintained as monolayer cultures growing in RPMI 1640 containing 10% Nu-Serum (Collaborative Research Products, Bedford, MA) and 1 mM aminoguanidine, as described previously (24). Under these conditions, MALME-3M cells have a doubling time of ~50 h. Cells were seeded 24 h prior to treatment with polyamine analogues, and growth inhibition was assessed by dose-response curves at 4 days. Other assays were determined on cells treated with 10 μM analogue for time periods as indicated. Cell number was determined electronically. Data were expressed as a percentage of the initial seeding density to define IC50 values as well as the cytotoxic dose.

Flow Cytometry. Cell cycle analysis was performed on attached and detached cells following treatment for ~2 control cell doublings (4 days) with 10 μM analogue. Cells were counted, washed in PBS, and resuspended in 1 ml of 70% ethanol in PBS. Samples were stored at ~20°C until they were stained using a modification of the procedure described by Krishan (25). Each cell

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The abbreviations used are: PUT, putrescine; SPD, spermidine; SPM, spermine; SSAT, SPD/SPM N,N-acetyltransferase; ODC, ornithine decarboxylase; DE-333, N,N,N,N'-diethylnorspermine (also known as DENSPM, N,N,N,N'-diethylnorspermine); DM-333, N,N,N,N'-dimethylspermine; DFMO, α-difluoromethylornithine; DIP-333, N,N,N,N'-dipropylspermine; DE-333, N,N,N,N'-diethylhomospermine (also known as DEHSPM); DM-444, N,N,N,N'-di-n-propylspermine; DE-444, N,N,N,N'-diethylnorspermine; DE-333, N,N,N,N'-diethylnorspermine; DE-333, N,N,N,N'-diisoeiylhomospermine; DE-333, N,N,N,N'-dimehylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-dimehylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylspermine; DE-333, N,N,N,N'-di-n-propylsperme
Enzyme Activity Assays. Following treatments with 10 μM analogue for 2 days, ODC and SSAT activities were determined from acid extracts of cells processed using a high-performance liquid chromatography system described previously (28).

SPD Transport. Cells were seeded into six-well plates at a density of 5 × 10^4 cells/well and allowed to grow in the presence or absence of 10 μM analogue for 2 days. Medium was decanted, and the cells were washed with PBS on ice. The cells were exposed to 5 μM H^2-SPD (305 pmol/cpm) in 2 ml of serum-free media for 20 min at 37°C. Following incubation, the cells were washed three times with PBS on ice and dissolved in 1 n NaOH and neutralized with 1 n HCl, and a portion was removed for radioactive counting and protein determinations (Bio-Rad). Results were expressed as pmol/min/mg.

Assay Time Line. Biochemical parameters, including polyamine enzymes and pools, analogue accumulation, and SPD transport were determined following treatment with 10 μM analogue for 2 days. Growth inhibition curves and cell cycle analysis were determined on the attached cells after ~2 control cell doublings (4 days). Analysis for apoptosis among attached and detached cell populations was carried out following 6-day incubations unless otherwise indicated. Extended growth curves evaluating cytotoxicity based on growth kinetics were carried out by continuous exposure to 10 μM analogue for 8 days or by replating treated cells at 6 days and continuing treatment for 4 additional days.

**RESULTS**

Analogue Accumulation. Relative analogue accumulation in cells was determined following a 2-day treatment with 10 μM analogue (Table 3). The substantial differences in structure did not alter their transport characteristics; in fact, all of the analogues accumulated to similar intracellular concentrations (i.e., 5000–6000 pmol/10^6 cells). Interestingly, these levels were nearly equivalent to the summed total of natural polyamines in untreated cells (6020 pmol/10^6 cells). Thus, the differences described below on cell growth or regulatory responses for this panel of analogues are not due to differences in cellular accumulation.

Cell Growth. Because DE-333 is regarded as the parent compound in this study, our analysis begins with the diethyl series. The relative similarity in the abilities of DE-333, DE-343, and DE-444 to inhibit the growth of MALME-3M cells has been reported previously (7). As indicated in Fig. 2 and 3 and summarized in Table 1, this similarity has been confirmed and extended in the present study. DE-333, DE-343, and DE-443 displayed very similar IC50 values (Table 1; Fig. 2), which were slightly lower than that for DE-444. Determination of doses required to produce cytotoxicity was performed by analyzing dose-response data relative to the initial cell number (Fig. 2). Cytotoxicity was suggested at the concentration at which cell number fell below 100%, the initial seeding density. As indicated by this analysis and by cell growth effects during extended incubations (Fig. 3), all four of the diethyl analogues were cytotoxic to MALME-3M cells at 10 μM.

Whereas the IC50 values for DM-444 were similar to those of the diethyl analogues (Table 1), the response curve for DM-333 and DM-343 was shifted to the right (Fig. 2; Table 1), with an IC50 value ~10 times higher than the other analogues. Thus, although the intra-amine carbon distance of the diethyl analogues had only minor consequences on their growth-inhibitory potential, it produced much greater effects in the di methyl series. The growth inhibition at concentrations of 10 μM or less was cytotstatic for DM-333 and DM-343 but cytotoxic for DM-444, as indicated by the extended incubations (Fig. 3).

In the case of the dipropyl analogues, the dose-response curve for DP-333 shifted to the right of that for DE-333, and the compound was found to be less cytotoxic (Figs. 2 and 3; Table 1). By contrast, the analogue DP-343 was >3-fold more potent than DE-333 and ranked as the most cytotoxic of this analogue series. This was shown most definitively by the extended growth curves. Whereas the other ana...
contained varying proportions of viable interphase cells, which presum-
ably had just undergone mitosis and not yet reattached. In control sam-
ple, 52% of the detached cells were in various phases of mitosis, and
~1% were apoptotic (Table 1). The remainder of the cells were viable
and displayed characteristic interphase morphology. Following treatment
with 10 μM of the cytotoxic analogues for 6 days, mitotic cells were
rarely seen among the detached cells, suggesting analogue interference
with cell cycling. In addition, these analogues markedly increased the
proportion of apoptotic cells as detected by pyknotic or fragmented
chromatin and dark-staining nuclei by H&E. Immunocytochemical stain-
ing with the ApopTag kit was used to confirm apoptotic nuclei seen by
H&E staining (Fig. 4). These nuclei stained deep brown due to the
peroxidase reaction product of digoxigenin antibody bound to the 3'-
hydroxy termini of fragmented DNA in apoptotic nuclei. Cells undergo-
ing necrosis with swollen and diffuse cytoplasmic and eosinophilic
nuclear staining were only infrequently observed (i.e., <5%) under all
treatment conditions. Neither apoptotic nor necrotic cells were seen
among attached cells, even in cultures in which >70% of the detached
cells were found to be apoptotic (i.e., with DM-444). Thus, on the basis
of cellular morphology, the analogues appear to limit cell growth by
interrupting cell cycling and simultaneously inducing apoptosis. The
exceptions to this were found in cells treated with the three cytostatic
analogues. In DM-333- and DM-343-treated cells, the number of de-
tached cells remained few in number, similar to untreated cultures, and
both apoptotic and mitotic cells were observed, indicating an incomplete
cell cycle arrest. These two analogues became cytotoxic after replating
and continued treatment with a significant increase in detached apoptotic
cells (data not shown).

Apoptosis was also studied in detached cells by the DNA laddering
assay, which produced only high molecular weight DNA fragments
(data not shown). The findings are consistent with earlier reports by
others (29) indicating that SPM, and presumably SPM analogues,
interfere with this assay by aggregating and condensing the apoptotic
DNA, causing anomalous sedimentation with intact DNA. Thus,
agarose gel assays of DNA from cells treated with polyamine ana-
logues may underestimate apoptotic fragmentation.

Cell Cycle Analysis. Attached cells were analyzed by flow cytom-
etry following 10 μM analogue exposure of 4 days or ~2 cell dou-
blings (Table 2). In control cultures or those treated with 10 μM SPM,
~15% of the cells were found to be in S phase. The major effect of
most analogues involved a near-total depletion of cells in S-phase
cells together with an increase of cells in G1/G0, indicating a G1 block
(Table 2; Fig. 5). The significant percentage of cells remaining in the
G2-M population indicated the presence of a G2 block. The exceptions
to these findings include DM-333 and DiP-333, which were less
effective in depleting cells in S phase and displayed characteristic interphase morphology. Following treatment
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G2-M population indicated the presence of a G2 block. The exceptions
to these findings include DM-333 and DiP-333, which were less
effective in depleting cells in S phase, and DM-343, which had no
apparent effect on cell cycle progression. Fig. 5 shows flow cytometry
data for attached and detached cells treated with DE-333, which is
representative of cytotoxic analogues. The majority of detached cells
eluted as a sub-G1 peak, which is considered characteristic of apop-
totic cells. Time and dose analysis using DE-333, DE-434, and DP-
343 showed significant increases on G1/G0, and decreases in S phase
as early as 1 day after treatment using analogue concentrations as low
as 1 μM (data not shown).

To test for their ability to support cell cycle progression, treatment
with DM-333 and DM-343 was extended to 6 days. S-phase cells
decreased to 4 and 5%, respectively. After replating and extending
treatment for 10 days, detached cells appeared that were >95%
apoptotic. Thus, these two cytostatic analogues displayed "cytotoxic"
characteristics with extended treatment.

Because only a small proportion (i.e., ~15%) of MALME-3M cells
are found in S phase, the analogue was examined in cells having a
much larger proportion of cells in S phase. The cell cycle distribution
of human MCF-7 breast carcinoma cells is typically ~55% in G0/G1,
33% in S phase, and 12% in G2-M phase. Following treatment for 4 days with 3 μM DP-343, this distribution shifted to 93% in G1, 2% in S phase, and 5% in G2-M. Thus, the G1 block was more obviously confirmed in a second human cell line.

**Polyamine Metabolism.** To determine analogue effects on key polyamine metabolic and regulatory responses, MALME-3M cells were treated for 2 days with 10 μM analogue, an assay time that precedes the appearance of detached cells for all analogues except DP-343. ODC activity and SPD transport activity, which are known to be negatively regulated by natural polyamines and polyamine analogues (6, 28), were used to evaluate the regulatory potential of the analogues. All but one of the analogues effectively down-regulated both ODC and transport by at least 80% (Table 3). Despite accumulating to similar intracellular levels, the disisopropyl analogue DiP-333 decreased these activities by only 20–30%. DP-343 behaved similarly to the ethyl and methyl analogues.

Induction of SSAT activity was more differentially affected by the analogues than any other parameter studied. Of all of the analogues, DE-333 remained the most potent inducer of the enzyme, increasing activity by >925-fold by 2 days. Because the SSAT enzyme activity assay also measures other acetylases as part of basal activity determined (6), the analogue-induced fold increase obtained by dividing the induced activity by the basal activity following analogue induction underestimates the actual rise in SSAT activity. The four diethyl analogues induced SSAT activity in a manner that correlated very closely with the number of 3-carbon intra-amine units. Thus, the rank order of analogues according to their ability to induce SSAT activity was DE-333 > DE-343 > DE-443 > DE-444 (Table 3) and a similar rank order was observed for the dimethyl analogues. As indicated above, these differences were unrelated to intracellular analogue accumulation. Of the three symmetrical N-alkyl substituents, the diethyl groups were most effective in inducing the enzyme. For example, DM-333 and DiP-333 were much less effective than DE-333. Although these structural distinctions indicate sensitive regulatory responses to these analogues, there was no apparent correlation between individual analogue ability to induce SSAT activity and inhibit cell growth. As indicated by DM-444 and DE-444, it would appear that a 20-fold increase in SSAT induction is sufficient to markedly lower SPD and SPM pools, providing that ODC is down-regulated. In this regard, the extent of SSAT induction tended to correlate with the residual pool of SPM (Table 3) except for DiP-333, which is unable to down-regulate polyamine biosynthesis.

**DISCUSSION**

A novel series of systematically modified polyamine analogues has been used to dissect out various structure-function relationships involved in regulatory responses and inhibition of cell growth. Perhaps the most novel of these is that growth inhibition in MALME-3M cells comprises two separable but probably related growth components, which are interference with the cell cycle progression and induction of apoptosis. On the basis of growth curves, cellular morphology, cytochemical staining, and flow cytometric evidence, we have shown that, despite achieving almost identical intracellular concentrations, the analogues differentially affect these two processes and that those that effectively accomplish both effects produce cytotoxic re-

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**Table 1. Antiproliferative activity and effects of polyamine analogues in MALME-3M Cells**

<table>
<thead>
<tr>
<th>Analogue</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; dose&lt;sup&gt;a&lt;/sup&gt; (at 4 days, in μM)</th>
<th>Cytotoxic dose&lt;sup&gt;b&lt;/sup&gt; (at 4 days, in μM)</th>
<th>Extended growth response&lt;sup&gt;c&lt;/sup&gt; (10 μM for 8 days)</th>
<th>Percentage of mitotic versus apoptotic cells per 200 detached cells&lt;sup&gt;d&lt;/sup&gt; (10 μM for 6 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM-333</td>
<td>10</td>
<td>~50</td>
<td>Cytostatic</td>
<td>52/1</td>
</tr>
<tr>
<td>DM-343</td>
<td>10</td>
<td>~30</td>
<td>Cytostatic</td>
<td>27/41</td>
</tr>
<tr>
<td>DM-444</td>
<td>0.5–1.0</td>
<td>5–10</td>
<td>Cytotoxic</td>
<td>26/29</td>
</tr>
<tr>
<td>DE-333</td>
<td>0.5–1.0</td>
<td>5–10</td>
<td>Cytotoxic</td>
<td>&lt;1/51</td>
</tr>
<tr>
<td>DE-343</td>
<td>0.5–1.0</td>
<td>5–10</td>
<td>Cytotoxic</td>
<td>&lt;1/61</td>
</tr>
<tr>
<td>DE-443</td>
<td>0.5–1.0</td>
<td>5–10</td>
<td>Cytotoxic</td>
<td>&lt;1/64</td>
</tr>
<tr>
<td>DE-444</td>
<td>1–5</td>
<td>10</td>
<td>Cytotoxic</td>
<td>&lt;1/68</td>
</tr>
<tr>
<td>DiP-333</td>
<td>3–5</td>
<td>~30</td>
<td>Cytostatic</td>
<td>7/11</td>
</tr>
<tr>
<td>DiP-343</td>
<td>0.5</td>
<td>1–3</td>
<td>Cytotoxic</td>
<td>&lt;1/66</td>
</tr>
</tbody>
</table>

<sup>a</sup> From data presented in Fig. 2.
<sup>b</sup> From data presented in Fig. 3.
<sup>c</sup> Detached cells were stained with H&E and scored for apoptotic and pyknotic nuclear characteristics.
<sup>d</sup> Remainder of cells displayed interphase or mitotic morphology. Slides from three separate experiments were scored twice (SD, <5%).
CELL CYCLE ARREST AND APOPTOSIS BY SPERMINE ANALOGUES

**Cell Cycle Arrest and Apoptosis by Spermine Analogues**

**Fig. 4. Light micrographs showing the morphological assessment of apoptosis.** Detached (A) and attached (B) cells collected from a MALME-3M cell culture treated for 4 days with 10 μM analogue were immunocytochemically stained using the Apoptag kit described in “Materials and Methods” and counterstained with hematoxylin. The nuclei containing diffuse chromatin (those indicated by * in A) and all of the nuclei in B that only reacted with hematoxylin are indicative of interphase cells. The nuclei containing pyknotic (condensed and fragmented) chromatin stained very dark brown by the in situ tunnel procedure and are indicative of apoptotic cells. Bar, 10 μm; magnification, ×750.

**Table 2 Relative effects of analogues on MALME-3M cell cycle progression**

<table>
<thead>
<tr>
<th>Analogue (10 μM, 4 days)</th>
<th>% control growth</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>78</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>DM-333</td>
<td>25</td>
<td>89</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>DM-343</td>
<td>23</td>
<td>79</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>DM-444</td>
<td>10</td>
<td>90</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>DE-333</td>
<td>0</td>
<td>94</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>DE-343</td>
<td>0</td>
<td>95</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>DE-443</td>
<td>0</td>
<td>94</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>DE-444</td>
<td>30</td>
<td>90</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>DIP-333</td>
<td>6</td>
<td>91</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>DP-343</td>
<td>0</td>
<td>96</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

*Values were determined from cell numbers represented in Fig. 3.*

**Fig. 5. Cell cycle histograms of attached and detached MALME-3M cells following treatment with 10 μM DE-333 for two control cell doublings (4 days).** The results are representative of those obtained with other cytotoxic analogues. The majority of the detached cells following treatment eluted in a sub-G1 peak characteristic of apoptotic cells. The percentages of cells in each phase of the cell cycle are indicated to the right of the histograms.

**Spermine analogues required 10 days to appear with the methyl analogues.** Thus, in comparing the 333 and 343 analogue series, it would seem that the methyl group is less effective than the ethyl or propyl groups at inducing cell cycle arrest and apoptosis. It is possible that on the basis of their smaller size, the methyl groups may allow these analogues to at least partially substitute for the natural polyamine SPM in supporting cell growth. Precedent for this is provided by Yang et al. (30), who demonstrated that dimethyl SPM analogues in which the methyl substituents are attached to α carbons deplete natural polyamine pools but do not inhibit cell proliferation, apparently because they are able to at least partially substitute for the natural polyamines in supporting growth.

Evidence for cell cycle arrest was based on the decline in morphologically detectable mitotic cells in the detached cell population and, more specifically, by flow cytometric findings showing an accumulation of cells in G1 phase together with a depletion of cells in S phase, indicating a G0 or G1-S phase block. The effect was observed in MALME-3M cells and confirmed in MCF7 human breast carcinoma cells, which display a larger proportion of S-phase cells. To our knowledge, this is the first report of phase-specific interference with cell cycle progression by a panel of DE-333-related polyamine analogues. The effect could be related to analogue depletion of polyamine pools, a direct analogue effect at alternative sites, or the combination of these two possibilities. Induction of polyamine biosynthesis is known to be activated in late G1 during cell proliferation (31–33), and an early report (34) using a relatively nonspecific inhibitor of S-adenosylmethionine decarboxylase described a distinct G1 block following polyamine depletion. Studies with specific ODC inhibitors, such as DFMO, describe a rise in G1 with a concomitant loss of S-phase cells (35–39); however, these effects generally resulted in slowing the cell cycle traverse rather than inducing a phase-specific block. Also, these effects with DFMO seem to be cell type specific, because others (40, 41) have reported an accumulation of S-phase cells with no apparent G1 block. Unless used in combination (42), most inhibitors of polyamine biosynthetic enzymes invariably deplete one or two polyamine pools while producing an increase in another. DFMO, for example, typically increases SPM pools while depleting PUT and SPD pools. By contrast, analogues are capable
of depleting all three polyamine pools, and this could be responsible for the more obvious G1 arrest seen here. The possibility that the analogues may have a direct effect elsewhere in the cell must also be considered (43–45). Some indication of direct analogue effects is provided by DM-333, which, like the other analogues, totally depleted the natural polyamine pools but failed to arrest completely in G1. At a minimum, the fact that structurally similar analogues have been identified that differentially affect the cell cycle should prove especially useful in future drug design.

Cell cycle G1 arrest, such as seen here, is known to be related to events leading to the initiation of programmed cell death. The present observations fully support a relationship between interference with cell cycle progression and apoptosis, because only those analogues that produced a complete G1 arrest gave rise to a significant apoptotic response, whereas analogues that failed to yield a complete G1 arrest do not. Whether this relationship is mediated via the classical G1 checkpoint events involving Rb and/or p53 function (46) remains to be determined. The finding that polyamine analogues are capable of inducing programmed cell death has been reported previously by Casero and coworkers (47, 48). The importance of these effects to cell cycle perturbations was demonstrated in the present study.

One possible determinant of apoptosis is induction of SSAT, the most profoundly altered biochemical response seen with analogue treatment. The basis for suspecting such a relationship is founded on the observation that cell types that induce massive amounts of SSAT, such as certain human non-small cell lung carcinoma and melanoma cell lines, are invariably cytotoxicity affected by analogues, whereas those cell lines that do not induce massive amounts of SSAT tend to be cytostatically affected (6, 12, 49, 50). As reported here and elsewhere (6, 47, 48), this relationship does not seem to hold when comparing analogues instead of cell lines. Analogues such as DE-444 and DM-444 were the poorest inducers of SSAT, but they initiated the highest levels of apoptosis. Conversely, Dip-333, a potent inducer of SSAT, was relatively ineffective at initiating programmed cell death. These observations tend to argue against the suggestion that H2O2 liberated by the SSAT-related enzyme polyamine oxidase might be responsible for apoptosis in these cells (51). Two counter-arguments are that even small amounts of SSAT induction could result in sufficient H2O2 production to trigger apoptosis or that certain analogues might actually protect the cells from oxidative damage induced by H2O2 production.

A major goal of this study was to probe the structural constraints of various polyamine-regulatory responses, particularly SSAT induction, which is greatly exaggerated in the MALME-3M cells. In a separate report (24), we have shown that SSAT mRNA accumulation and enzyme stabilization responded differentially to analogue structure, and each contributed to the overall enzyme response. The importance of aminopropyl moieties in SSAT induction was indicated by the diethyl analogue series, in which the activity ranking was related to the number of aminopropyl groups contained within each analogue of this series: DE-333 > DE-343 > DE-443 > DE-444. In addition, ethyl alkylation of the terminal amines was found to be more effective than either methyl or propyl groups at inducing SSAT activity. Down-regulation of ODC and transport were more uniformly affected by the analogues. One unanticipated exception to this was provided by Dip-333. Despite up-regulating SSAT activity in similarity to the other analogues, Dip-333 was quite inefficient at down-regulating ODC and polyamine transport activities. Because by comparison DE-333 is very effective at regulating ODC and transport, the disopropyl group seems to define a structural limitation of binding sites involved in controlling these responses. Interestingly, the dipropyl analogue DE-343 behaved like the other analogues.

On the basis of antitumor activity and minimized host toxicity (7–11), DE-333 has recently entered clinical trials and is regarded as the lead compound of this analogue program. Despite considerable structural variation, none of the eight analogues examined here was found to be more potent than the parent compound DE-333 at inducing SSAT activity. Some analogues, however, were more effective cytotoxic agents. It is possible that these analogues may have even more potent antitumor activity and thus qualify for second-generation status. For example, we have shown previously that, although DE-333, DE-343, and DE-444 are similar in their in vitro antiproliferative activity, their toxic dose levels in animals vary rather widely, so that DE-333 has the best chemotherapeutic window (7). In this regard, it is possible that dimethyl analogues that elicit a cytostatic response in MALME-3M cells may exhibit different toxicity profiles and potencies from diethyl and dipropyl analogues, which elicit cytotoxic responses in vitro. Of particular interest was the finding that analogues that exhibited a slowly developing antiproliferative effect in vitro showed reduced host toxicity in vivo (19). In addition, the current study reveals that antiproliferative activity of the analogues can be subclassified with respect to effects on cell cycle and apoptosis. The in vivo implications of this finding have not yet been evaluated. The various functional distinctions described here could also prove useful in altering the pharmacological properties of analogues by structural design.

In summary, we have extended earlier findings with this same analogue series by identifying certain structure-function relationships relevant to regulatory responses and more significantly, on parameters critically related to cell growth and survival. With respect to the latter, we have found that analogue-mediated growth inhibition comprises two separable but probably related growth components, interference with the cell cycle progression, and induction of apoptosis and that analogues that effectively accomplish both induce a cytotoxic response in MALME-3M cells. Because these distinctions may allow for a better understanding of analogue modes of action both in vitro
in vivo, they will be further evaluated using molecular markers related to cell death processes before conclusions are drawn.

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REFERENCES


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