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, polyamine analogues have emerged as a novel class of experimental antitumor agents. The spermine derivative N',N'-diethylhomospermine (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'-acytlytransferase. Analogues accumulated to comparable intracellular concentrations and similarly affected cell growth with IC_{50} 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 G_1 block apparent as an accumulation of cells in G_1/G_0 and depletion of S-phase cells as well as a less restrictive G_2 block. By contrast, cytostatic analogues incompletely arrested cells in G_1, 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'-acytlytransferase 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 polyamine 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 constituent 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 polyamine 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 promising preclinical antitumor activity (4), the 5-adenosylmethionine decarboxylase inhibitor CDP-356 is undergoing Phase I clinical evaluations against solid tumors. Polyamine analogues have recently been identified that suppress rather than inhibit polyamine biosynthesis via endogenous regulatory mechanisms (5). Typically, SPM analogues, such as DE-333, down-regulate polyamine biosynthetic enzyme activities, suppress transport of polyamines, and potently up-regulate the catabolic enzyme SSAT (5). The net result of these effects is depletion of intracellular polyamine pools and accumulation of an analogue that is unable to substitute for natural polyamines in supporting cell growth. Due to promising preclinical findings (7-11), DE-333 is undergoing Phase I clinical evaluation against solid tumors and second generation analogues are being sought.

A number of polyamine analogues with functional and structural similarities to SPM have been synthesized and investigated in a variety of solid tumor systems (12-18). Recently, Bergeron et al. (19) synthesized a series of DE-333-like analogues that were used to investigate the biological significance of intra-amine carbon chain length, terminal nitrogen alkyl group size, and symmetry of methylene backbone. In a detailed analyses conducted in L1210 murine leukemia cells, structure-activity correlations were established between chain length and IC_{50} values and between terminal alkyl substituents and impact on transport potential, ODC, and S-adenosylmethionine decarboxylase activities. By far, the most profound structure-function distinction occurred with the mono- and dialkylated analogues in their varying abilities to induce SSAT. However, in L1210 cells, the most potent enzyme inducer, DE-333, increased SSAT activity by 15-fold as compared to 1000-fold in MALME-3M human melanoma cells (6).

The magnitude of the DE-333 response enabled Fogel-Petrovic et al. (20) to identify several of the molecular mechanisms responsible for SSAT induction. In the present study, we demonstrate that MALME-3M cell growth inhibition and cytotoxicity by DE-333 analogues involves cell cycle arrest and induction of apoptosis. The relationship of these events to regulatory responses, such as induction of SSAT, is also examined. Portions of these findings have recently been published in abstract form (21, 22).

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/mmole), L-1-14C]ornithine (50 μCi/mmole), and acetyl-1-[14C]CoA (60 μCi/mmole) 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 μM 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 IC_{50} 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 collected, 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 population was vortexed, 100 μl of 1 mg/ml propidium iodide (PI) and 0.1 mg/ml RNase (Gibco, Grand Island, NY) in 10 mM Tris-HCl pH 7.5 was added, and 500 μl of 20 mg/ml RNAse A (BBL, Cockeysville, MD) was added following at least 20 min at 37°C. The cell cycle profile was determined using a FACScan (Becton-Dickinson, Mountain View, CA) with an excitation wavelength of 488 nm and the acquisition was set at 200 laser gates with a pulse parameter of 200. An analysis of at least 35000 cells was performed.
sample was pelleted at 1200 rpm for 5 min, washed in PBS, and resuspended in 1 ml of propidium iodide buffer 
(0.1% sodium citrate, 0.02 mg/ml RNase, 0.37% NP40, 0.05 mg/ml propidium iodide (pH 7.4)), vortexed, and allowed to stand on ice for 30 min in the dark. Cells were pelleted and resuspended in the dye buffer to a concentration of 1-2 × 10^6 cells per ml. To ensure a single-cell suspension, each sample was passed through a 35 µm strainer cap fitted onto 12 × 75 mm polystyrene tubes (Falcon, Franklin Lakes, NJ) and kept on ice. Flow cell analysis was performed on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA), and dye emission was collected through a 575/26 band pass filter that records cellular events up to 10,000 relative to DNA content. DNA histograms were gated using LYSIS II software (Becton Dickinson, San Jose, CA). Model graphics data were generated using ModFit and Winlist Cell Cycle Analysis software (Verity Software House, Inc., Topsham, ME).

**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 IC_{50} 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 IC_{50} 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 IC_{50} 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 cytostatic 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 DIP-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 samples, 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 staining 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' hydroxyl termini of fragmented DNA in apoptotic nuclei. Cells undergoing 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 detached 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 analogues may underestimate apoptotic fragmentation.

**Cell Cycle Analysis.** Attached cells were analyzed by flow cytometry following 10 μM analogue exposure of 4 days or ~2 cell doublings (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 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 apoptotic cells. Time and dose analysis using DE-333, DE-444, and DP-343 showed significant increases on G2/M, 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 G1/G0, ~15% in S, and ~30% in G2/M.

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**Fig. 2.** Dose-dependent analogue effects on growth of MALME-3M cells treated for approximately two control cell doublings (96 h). Note that data are plotted according to percentage of initial cell number: toxicity is indicated when the final cell number falls below this level (lower broken line). Values for IC₅₀ are determined at one-half the difference between the initial cell number (100%) and the final cell number (340%) in control cultures (upper broken line). The data represent at least three separate determinations performed in duplicate.
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 diisopropyl 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 determining 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-

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

<table>
<thead>
<tr>
<th>Analogue</th>
<th>I_{50} dosea (at 4 days, in μM)</th>
<th>Cytotoxic dosea (at 4 days, in μM)</th>
<th>Extended growth responseb (10 μM for 8 days)</th>
<th>Percentage of mitotic versus apoptotic cells per 200 detached cellsc (10 μM for 6 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>~50</td>
<td>Cytostatic</td>
<td>52/1</td>
</tr>
<tr>
<td>DM-333</td>
<td>10</td>
<td>~30</td>
<td>Cytostatic</td>
<td>27/41</td>
</tr>
<tr>
<td>DM-344</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;1/88</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-344</td>
<td>0.5</td>
<td>1–3</td>
<td>Cytotoxic</td>
<td>&lt;1/66</td>
</tr>
</tbody>
</table>

*a* From data presented in Fig. 2.

*b* From data presented in Fig. 3.

*c* Detached cells were stained with H&E and scored for apoptotic and pyknotic nuclear characteristics. Remainder of cells displayed interphase or mitotic morphology. Slides from three separate experiments were scored twice (SD, <5%).
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 G0 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 MCF-7 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 seems 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

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 (% total cells)</th>
<th>S (% total cells)</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>
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<td>0</td>
<td>94</td>
<td>1</td>
<td>5</td>
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<td>DE-444</td>
<td>30</td>
<td>90</td>
<td>3</td>
<td>7</td>
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<tr>
<td>Dip-333</td>
<td>6</td>
<td>91</td>
<td>4</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.

** Data represent mean values from three separate experiments (SD, <5% for G1; <1% for S phase, and <1% for G2-M).

sponses during extended incubations. Such analogues are best represented by the four diethyl analogues plus DM-444 and DP-343. The dimethyl analogues, DM-343 and, to a lesser extent, DM-333, have an attenuated effect on cell cycle progression and apoptosis and are more cytostatic during extended incubations. In fact, the cellular responses observed by 4 days with the cytotoxic
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) in human non-small cell lung carcinoma and in breast carcinoma treated with DE-333 or a cyclopropyl analogue of SPM (47, 48). The linkage 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 cytotoxicly 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 diisopropyl group seems to define a structural limitation of binding sites involved in controlling these responses. Interestingly, the dipropyl analogue DP-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

### Table 3: Relative analogue effects on polyamine metabolism and uptake in MALME-3M cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% control growth</th>
<th>Regulatory responses</th>
<th>Intracellular pools</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10 μM, 2 days)</td>
<td></td>
<td>ODC (nmol/min/mg)</td>
<td>SSAT (nmol/min/mg)</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>2.2</td>
<td>40</td>
</tr>
<tr>
<td>DM-333</td>
<td>86</td>
<td>0.1</td>
<td>10,430</td>
</tr>
<tr>
<td>DM-343</td>
<td>85</td>
<td>0.15</td>
<td>3,355</td>
</tr>
<tr>
<td>DM-444</td>
<td>52</td>
<td>0.30</td>
<td>940</td>
</tr>
<tr>
<td>DE-333</td>
<td>54</td>
<td>0.03</td>
<td>49,945</td>
</tr>
<tr>
<td>DE-343</td>
<td>31</td>
<td>0.07</td>
<td>16,410</td>
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<td>DE-443</td>
<td>30</td>
<td>0.07</td>
<td>5,810</td>
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<td>DE-444</td>
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</tr>
<tr>
<td>DIP-333</td>
<td>68</td>
<td>1.44</td>
<td>10,570</td>
</tr>
<tr>
<td>DP-343</td>
<td>7</td>
<td>0.11</td>
<td>10,835</td>
</tr>
</tbody>
</table>

* Data represent mean values from five separate experiments; SD, <10%.
* Relative to control which includes acetylation activities in addition to SSAT.

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and in vivo, they will be further evaluated using molecular markers related to cell cycle death.

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REFERENCES

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

Debora L. Kramer, Mirjana Fogel-Petrovic, Paula Diegelman, et al.


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