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Apoptotic Signaling in Polyamine Analogue-treated SK-MEL-28 Human Melanoma Cells[1]

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ABSTRACT

N^1,N^{11}-Diethylnorspermine (DENSPM) is a polyamine analogue with clinical relevance as an experimental anticancer agent and the ability to elicit a profound apoptotic response in certain cell types. Here, we characterize the polyamine effects and apoptotic signaling events initiated by treatment of SK-MEL-28 human melanoma with 10 μM DENSPM. Maximal induction of the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase (SSAT) and polyamine pool depletion were seen by 16 h, whereas early apoptosis was first apparent at 36 h. Intermediate events related to apoptotic signaling were sought between 16 and 36 h. A loss of mitochondrial transmembrane potential ($\Delta\psi_m$) beginning at 24 h was followed by the release of cytochrome *c* into the cytosol at 30 h. Loss of mitochondrial integrity was accompanied by caspase-3 activation and poly(ADP-ribose) polymerase digestion from 30 to 36 h. The caspase inhibitor *Z*-Asp-2,6-dichlorobenzoyloxymethylketone rendered cells resistant to analogue-induced caspase-3 activation and reduced the apoptotic response in a dose-dependent manner. Because polyamine reduction achieved by inhibitors of polyamine biosynthesis inhibited growth but did not cause apoptosis, we looked for alternative polyamine-related events, focusing on induction of SSAT. Three DENSPM analogues that differentially induced SSAT activity but similarly depleted polyamine pools revealed a close correlation between enzyme induction and cytochrome *c* release, caspase activation, and apoptosis. Dose-dependent inhibition of polyamine oxidase, an enzyme that oxidizes acetylated polyamines generated by SSAT and releases toxic by-products such as H_2O_2 and aldehydes, prevented cytochrome *c* release, caspase activation, and apoptosis. Taken together, the findings indicate that DENSPM-induced apoptosis is at least partially initiated via massive induction of SSAT and related oxidative events and subsequently mediated by the mitochondrial apoptotic signaling pathway as indicated by cytochrome *c* release and caspase activation.

INTRODUCTION

Polyamine analogues, such as DENSPM,[3] have been designed and developed as potential anticancer agents (1). Because of their close structural similarity to the natural polyamines and their ability to mediate or exaggerate various polyamine regulatory responses, the analogues have been used as reagents for investigating polyamine function in cell physiology and proliferation. DENSPM, for example, is known to functionally substitute for polyamines in down-regulating the polyamine biosynthetic enzymes ODC and SAMDC, in suppressing polyamine transport, and in potently up-regulating the polyamine catabolic enzyme SSAT (2–4). Recently, we have used this analogue to probe polyamine-related events involved in cell cycle regulation. DENSPM treatment of MALME-3M human melanoma cells containing wild-type p53 induces a rapid G_1 arrest that correlates temporally with activation of the p53/p21$^{waf1/cip1}$/retinoblastoma checkpoint. By contrast, DENSPM treatment of SK-MEL-28 human melanoma cells containing mutated p53 causes significant apoptosis in the absence of cell cycle arrest (5). Whereas this is consistent with the proposed role of p53 in delaying apoptosis by G_1 arrest (6), the apoptotic pathways involved in this response and their relationship to various analogue effects on polyamine homeostasis have not yet been determined. For example, it is possible that the apoptotic effect is initiated by (*a*) polyamine pool depletion, (*b*) effects related to potent induction of SSAT, and/or (*c*) unforeseen direct effects of the analogue that may or may not be related to polyamine function.

Recent elucidation of the pathways involved in apoptotic signaling (7) has provided early end points for mapping analogue action and relating them to effects involving polyamine homeostasis. For example, caspases represent a family of cysteine proteases that are common downstream effectors of apoptosis. Their contributions are largely through cleavage of a variety of substrates such as PARP, inhibitor of caspase-activated deoxyribonuclease, Bcl-2, nuclear lamina, gelsolin, focal adhesion kinase, and p21-activated kinase 2 (8). Cytochrome *c* represents an upstream activator of the caspases that is released from the mitochondria into the cytosol, where it binds to Apaf-1 and initiates the caspase cascade (9–11). Cytochrome *c* release can be mediated by different mechanisms including loss of mitochondrial transmembrane potential ($\Delta\psi_m$), alteration of specific ion or protein channels, mitochondrial swelling, rupture of the outer membrane, and/or involvement of the Bcl-2 family proteins (12).

In this study, we investigate whether the mitochondrial apoptotic signaling events described above are involved in the cell death response seen in human melanoma SK-MEL-28 cells treated with the polyamine analogue DENSPM. Our findings indicate that activation of caspase-3 is critical for analogue-induced apoptosis, that cytochrome *c* is one of the main upstream activators of this pathway, and that oxidative events related to analogue induction of SSAT have probable initiating significance.

MATERIALS AND METHODS

Materials. The polyamine analogue DENSPM was provided by Parke Davis (Ann Arbor, MI). The DENSPM analogues N^1,N^{14}-diethylhomospermine (DE-444), 3,7,12,17-tetra-azanonadecane [N^1,N^{13}-diethyl(aminopropyl)-homospermidine] (DE-443), and N^1,N^{12}-diethylspermine (DE-343) were synthesized by Dr. Raymond Bergeron (University of Florida, Gainesville, FL). The inhibitor of PAO, N^1-methyl-N^2-(2,3-butadienyl)butane-1,4-diamine (MDL-72527), and SAMDC inhibitor 5′-{[(*Z*)-4-amino-2-butenyl] methylamino}-5′-deoxyadenosine (MDL-73811) were provided by Aventis Pharmaceuticals Inc. (Bridgewater, NJ). The ODC inhibitor DFMO was obtained from ILEX, Inc. (San Antonio, TX), the caspase inhibitor Z-D-DBMK was obtained from Alexis Biochemicals (San Diego, CA), and the antioxidant NAC was obtained from Sigma Chemical Co. (St. Louis, MO). CsA was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA).

Cell Culture. SK-MEL-28 human melanoma cells were maintained in a 5% CO_2 humidified incubator as monolayer cultures in RPMI 1640 supplemented with 10% Nu-Serum (Collaborative Research Products, Bedford, MA), 1 mM aminoguanidine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

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[3] The abbreviations used are: DENSPM, N^1,N^{11}-diethylnorspermine; CsA, cyclosporin A; DiAcSpm, N^1,N^{12}-diacetylspermine; NAC, *N*-acetylcysteine; ODC, ornithine decarboxylase; PARP, poly(ADP-ribose) polymerase; PAO, polyamine oxidase; Put, putrescine; SAMDC, *S*-adenosylmethionine decarboxylase; SSAT, spermidine/spermine N^1-acetyltransferase; Spd, spermidine; Spm, spermine; Z-D-DBMK, *Z*-Asp-2,6-dichlorobenzoyloxymethylketone; DFMO, α-difluoromethylornithine.

Polyamine Enzymes. ODC, SAMDC, and SSAT activities were assayed as described by Porter *et al.* (3). SSAT enzyme activity was expressed as pmol/min/mg protein.

Polyamine Pools. Intracellular polyamines and polyamine analogues were analyzed by high-performance liquid chromatography as described previously by this laboratory (13) with the following modifications. Dansylated samples (50 μl) were injected onto a 250 × 3.2-mm ID Econosil C18 column (5 μm particle size; Alltech, Deerfield, IL) with a column temperature of 50°C and eluted by a two-solvent gradient using a Waters 616 LC system (Waters, Milford, MA) and a Waters WISP 710B autosampler. Solvent A contained 55% of 10 mM ammonium phosphate/45% acetonitrile at pH 4.4. Solvent B contained 100% acetonitrile. At 0.9 ml/min, the gradient began at 100% solvent A and progressed linearly to 82% solvent B over 30 min with a 15-min hold. Compounds were detected using a McPherson FL-750 BX fluorescence detector (Acton, MA) with an excitation wavelength of 360 nm and an emission cutoff filter of 500 nm. The data were collected and analyzed using Waters Millennium 32 chromatography software version 3.05. Polyamine pools were expressed as pmol/10^6cells.

Western Blot Analysis. SK-MEL-28 cells were treated with 10 μM DENSPM for the indicated time. Cells were harvested, and proteins (40 μg/lane) were run on 7.5–15% SDS-PAGE gels followed by transfer to polyvinylidene difluoride membrane and immunoblotted with antibodies as indicated. Detection was performed using enhanced chemiluminescence from Amersham Pharmacia Biotech. (Arlington Heights, IL). Polyclonal rabbit anti-caspase-3, polyclonal rabbit antihuman caspase-8, anti-Bax, polyclonal rabbit anti-Bid, anti-Bcl-2, and anti-cytochrome *c* antibodies were purchased from PharMingen (San Diego, CA). The anti-PARP antibody was obtained from Boehringer Mannheim Corp. (Indianapolis, IN), and the monoclonal caspase-9 antibody and polyclonal Bcl-X_L antibody were bought from Oncogene Research Products (Boston, MA).

Cell Growth and Apoptosis. Control and analogue-treated cells were collected from flasks and counted by using a Model ZM Coulter (Coulter Electronics, Hialeah, FL). Apoptotic cells were detected by annexin V/FITC staining using a kit obtained from R&D Systems (Minneapolis, MN). Stained cells were analyzed using a Becton Dickinson FACScan (Flow Cytometry Facility, Roswell Park Cancer Institute), and data were analyzed using the Winlist program (Verity House, Topsham, ME).

Mitochondrial Transmembrane Potential. The electron gradient across the mitochondrial membrane space during normal respiration is called the mitochondrial transmembrane potential ($\Delta\psi_m$). Loss of $\Delta\psi_m$ was measured by flow cytometry after staining with a MitoCapture kit (Biovision Research Products, Palo Alto, CA). This technique uses a cationic dye that fluoresces red as it aggregates inside healthy mitochondria. In apoptotic cells, if the $\Delta\psi_m$ collapses, the dye stays as a monomer in the cytoplasm and emits green light. Cells were stained according to manufacturer's protocol and analyzed by FACScan.

Cytochrome *c* Release. Cells incubated in the presence and absence of 10 μM analogue for different periods of time were harvested for separation of mitochondria and cytosol according to the method reported by Yang *et al.* (14).

RESULTS

Induction of Apoptosis. We have previously reported that human melanoma SK-MEL-28 cells containing mutant p53 (15) undergo rapid apoptosis in response to DENSPM treatment (5). Here, we show that 10 μM DENSPM halted SK-MEL-28 cell growth at 24 h and that the majority of the cells were detached by 48 h (Fig. 1*A*). The annexin V assay measures phospholipid turnover from the inner to the outer lipid layer of the plasma membrane, an event typically associated with apoptosis (16). As shown in Fig. 2, the percentage of apoptotic cells was negligible at 24 h but became significant by 36 h, with cells appearing in the early and late apoptotic quadrants. Thereafter, apoptosis, as indicated by positive annexin V staining, increased to 52% by 48 h and to 85% by 72 h. As described below, major reductions in polyamine pools were apparent by 16 h.

In contrast to the effects of DENSPM, polyamine pool reduction produced by treatment with the polyamine biosynthetic inhibitors (5 mM DFMO plus 10 μM MDL-73811) did not induce apoptosis as indicated by annexin V staining after 96 h of treatment (Fig. 2). Rather, these compounds produced a sustained inhibition of cell growth that was first apparent at 48 h (Fig. 1*B*). Thus, the polyamine pool reduction produced by enzyme inhibitors (Table 1) was cytostatic rather than cytotoxic.

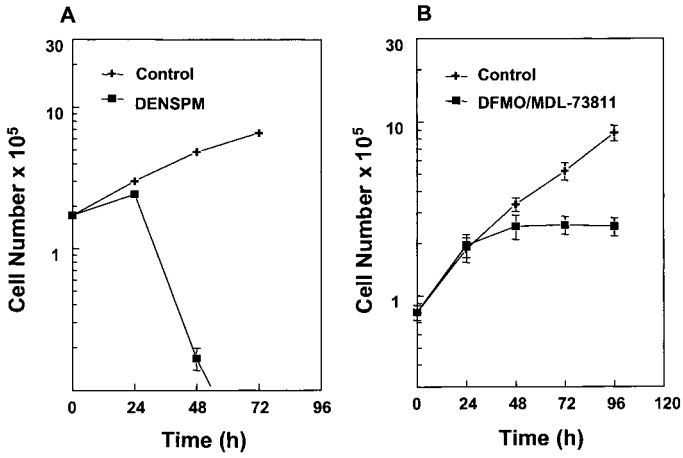

Fig. 1. Effects of treatment with (*A*) 10 μM DENSPM or (*B*) 5 mM DFMO plus 10 μM MDL-73811 on SK-MEL-28 cell growth. Note that cells treated with DENSPM undergo a profound cytotoxic response, whereas cells treated with DFMO plus MDL-73811 show a cytostatic response. Data represent mean values ± SD and are based on at least three separate experiments.

Polyamine Metabolism. On the basis of the above-mentioned findings, a temporal analysis of the effects of DENSPM on polyamine metabolism was performed from 0 to 28 h. The final time point was chosen to avoid the interfering influence of apoptotic events. Polyamine pools in SK-MEL-28 cells were somewhat unusual in that the basal Spm pool was ~4 times higher than that of Spd (Table 1). By comparison, the Spm:Spd ratio of MALME-3M human melanoma cells is closer to 1 (5). As shown in Table 1, polyamine pools were rapidly and profoundly affected by treatment with 10 μM DENSPM. By 8 h, intracellular Spd and Spm pools were reduced by >50%. By 16 h, these pools were almost completely depleted, whereas the Put pool was increased 5-fold, presumably because of back conversion of the higher polyamines by SSAT and PAO. As further evidence for SSAT activity, there was a substantial increase in the SSAT products, N^1-acetyl-Spd and N^1-acetyl-Spm. In addition, we noted the appearance of significant levels of DiAcSpm for the first time in DENSPM-treated cells (Fig. 3). In an earlier study of stably transfected cells that conditionally overexpress SSAT (17), we identified the same metabolite by spiking samples with authentic DiAcSpm kindly provided by Dr. Nikolaus Seiler (IRCAD, Strasbourg, France).

Activities of the biosynthetic enzymes ODC and SAMDC were suppressed by >80% at 8 h of DENSPM treatment (data not shown). By contrast, the activity of the catabolic enzyme SSAT rose from ~15 pmol/min/mg to ~4200 pmol/min/mg by 8 h of treatment (Table 1) and continued to rise rapidly until 16 h, where it plateaued at ~1500 times basal levels. The greatest decrease of polyamine pools and ~20% of the total SSAT induction occurred at 8 h, when intracellular analogue levels were comparable with the Spm pool in untreated cells. More specifically, DENSPM levels at 8 h were ~8000 pmol/10^6 cells, which was comparable to the basal Spm pool of ~7000 pmol/10^6 cells. DENSPM continued to accumulate steadily over the 28-h period and eventually attained an intracellular level that was ~4-fold that of the original Spm pool. Taken together, the data indicate that although DENSPM accumulates steadily, most of the analogue effects on polyamine metabolism were established during the first 16 h.

Caspase-3 Activation. Because our preliminary studies indicated that caspase effects occurred after 24 h, the present experiments

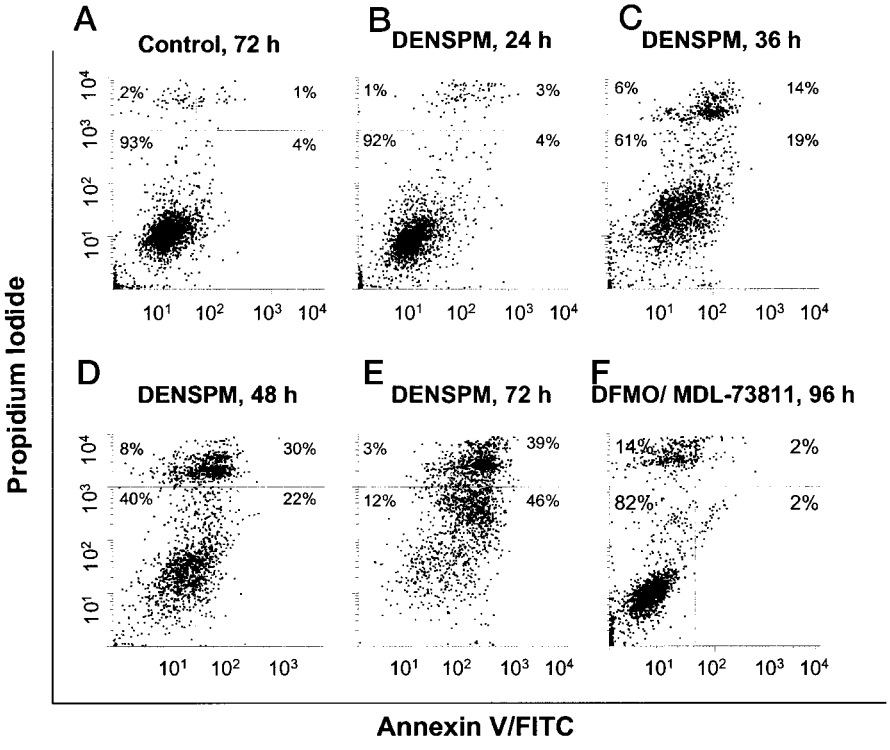

Fig. 2. Time-dependent induction of apoptosis by 10 μM DENSPM. For each histogram, the *bottom left quadrant* contains viable cells that do not stain for PI or annexin V. The *bottom right quadrant* contains early apoptotic cells that stain only for annexin V. The *top right quadrant* contains late apoptotic cells that stain for both PI and annexin V. The *top left quadrant* is necrotic cells that stain only for PI. Note that analogue-treated cells (*B–E*) undergo a rapid shift to positive annexin V staining between 24 and 48 h of treatment. By contrast, cells treated for 96 h with 5 mM DFMO plus 10 μM MDL-73811 (*F*) show no accumulation of positively stained cells. Data are representative of findings obtained in three separate experiments.

focused on the period from 24 to 36 h. As shown in Fig. 4, DENSPM-induced apoptosis was accompanied by activation of caspase-3 as indicated by the presence of M_r 20,000 and M_r 17,000 fragments at 30 h of treatment. The appearance of these protein bands at 30 h slightly preceded the onset of apoptosis at 36 h as indicated by annexin V staining (Fig. 2). As further confirmation of caspase-3 activation, we also assayed PARP cleavage (Fig. 4). Beginning at 30 h, there was a decline in the M_r 116,000 intact PARP protein accompanied by the appearance of a M_r 85,000 cleaved product that was fully evident at 36 h. Thus, PARP cleavage coincides with caspase-3 activation at 30 h and with onset of apoptosis at 36 h. We next examined whether either of two upstream proteases, caspase-8 and -9, was activated by DENSPM treatment. Caspase-8 is activated through death receptor-mediated apoptosis (18, 19) and is known to activate caspase-3 (20). The polyclonal antihuman caspase-8 antibody was used to measure both the proform (M_r 50,000) and the cleaved forms (40/36 doublet) of caspase-8. As shown In Fig. 4, caspase-8 was found in low abundance in these cells, and it did not appear to be activated. Examination of caspase-9 (Fig. 4) showed that the proform decreased steadily to 62% of control at 24 h, 64% at 30 h, and 7% at 36 h, suggesting that activation of caspase-3 is most likely mediated by caspase-9 rather than caspase-8.

We further tested whether caspase activation was causally linked to DENSPM-induced apoptosis by using the cell-permeable general caspase inhibitor Z-D-DBMK. SK-MEL-28 cells were treated with 10 μM DENSPM in the presence of Z-D-DBMK concentrations ranging from 10–200 μM. On the basis of data presented in Fig. 4, cells were treated for 36 h with 10 μM DENSPM to ensure obvious activation of caspase-3 and cleavage of PARP. As shown in Fig. 5, we found that Z-D-DBMK interferes with DENSPM-induced caspase-3 activation and PARP cleavage in a concentration-dependent manner (Fig. 5*A*). The effect was most apparent by the reduction of cleaved fragments accompanied by an increase in the intact proteins. Using these criteria, 100 μM Z-D-DBMK provided near total prevention of caspase-3 activation. It is significant, therefore, that this caspase inhibitor also protected cells from DENSPM-induced apoptosis as measured by annexin V staining at 48 h (Fig. 5*B*). Taken together with the temporal sequence data, the present findings establish a causal role for caspase-3 activation in DENSPM-induced apoptosis.

Cytochrome *c* Release. We next examined several upstream mitochondrial events that could contribute to caspase activation and DENSPM-induced apoptosis. Cytochrome *c* is normally located in the intermembrane space of mitochondria. When stimulated by apoptotic signals, cytochrome *c* is released into the cytosol, where it binds to Apaf-1 and initiates caspase activation. We focused on compartmental shifts in cytochrome *c* from 24 to 36 h in accordance with previous

Table 1 *Time-dependent effects of DENSPM and biosynthetic inhibitors on polyamine metabolism in SK-MEL-28 cells*

Data represent mean values from three separate experiments; SD < 20%.

Treatment	Treatment Time (h)	SSAT activity (pmol/min/mg)	Polyamine pools (pmol/10^6 cells)						
			Put	AcSpd[a]	Spd	AcSpm[a]	DiAcSpm	Spm	DENSPM
Control		15	50	<10	1565	<10	<10	7280	0
DENSPM (10 μM)	8	4230	80	1130	300	400	460	2870	8380
DENSPM (10 μM)	16	22335	250	1230	105	150	630	795	15465
DENSPM (10 μM)	20	25225	320	845	80	90	400	450	18440
DENSPM (10 μM)	24	24035	255	390	70	60	220	310	25265
DENSPM (10 μM)	28	23205	120	240	80	60	140	240	30220
DFMO (5 mM)/MDL-73811 (10 μM)	96	50	<10	<10	1310	<10	<10	500	0

[a] AcSpd, N^1-acetyl-Spd; AcSpm, N^1-acetyl-Spm.

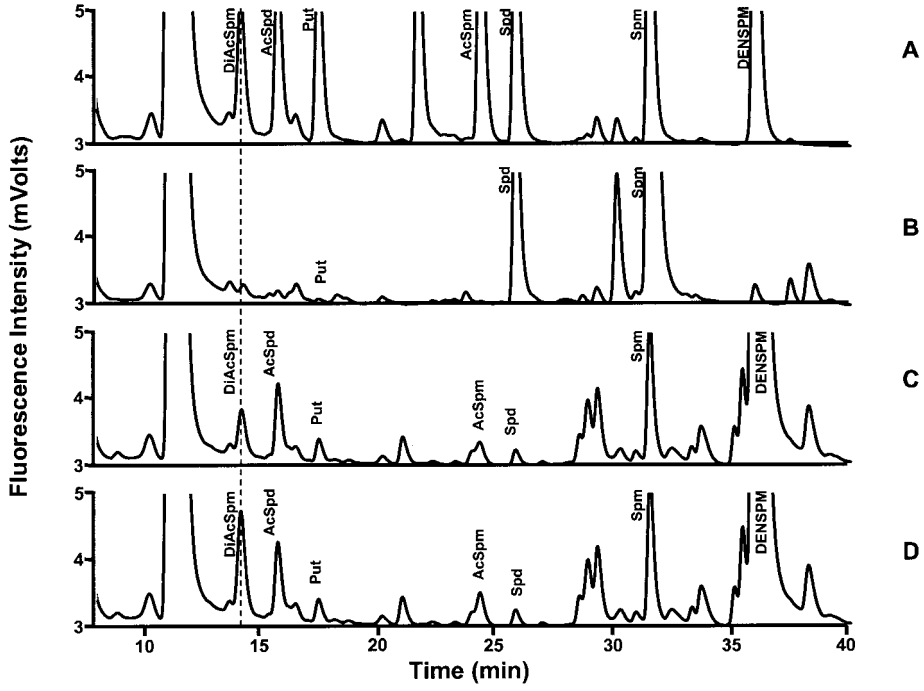

Fig. 3. Chromatographic detection and identification of an extra peak in DENSPM-treated SK-MEL-28 cells. *A* shows the high-performance liquid chromatography chromatogram of polyamine standards at 20 μM that includes DiAcSpm. *B* shows a chromatogram of untreated SK-MEL-28 cells. *C* shows a chromatogram of SK-MEL-28 cells treated with 10 μM DENSPM for 24 h. *D* shows the increase in the DiAcSpm peak after the extract used in *C* was spiked with DiAcSpm. Data are representative from three separate experiments.

findings. As shown in Fig. 6*A*, cytochrome *c* was largely located in the mitochondria of untreated cells. The protein shifted from the mitochondria to the cytosol beginning at 24 h and rapidly accumulated to significant levels by 30 h of treatment.

It is known that release of cytochrome *c* can be blocked by the antiapoptotic Bcl-2 family of proteins such as Bcl-2 and Bcl-X_L and promoted by the proapoptotic members such as Bax and Bid (14, 21–24). These proteins were examined for their possible involvement in cytochrome *c* release in DENSPM-treated SK-MEL-28 cells. We found no significant changes in total protein expression of Bid or Bcl-X_L during 0–36 h of treatment. Bax expression was barely detectable and did not appear to increase (data not shown). The data suggest that Bax, Bid, and Bcl-X_L may not be involved in DENSPM-induced apoptosis in these cells. It should be noted, however, that we observed a Bcl-2 cleaved product at 36 h of treatment (Fig. 6*A*). Both Bcl-2 cleavage and cytochrome *c* release were blocked by addition of the general caspase inhibitor Z-D-DBMK (Fig. 6*B*), suggesting that this cleavage may be mediated by caspases (25).

Loss of $\Delta\psi_m$ Precedes Cytochrome *c* Release. Disruption of the $\Delta\psi_m$ is an early event associated with apoptosis and has been suggested to be one of several factors responsible for cytochrome *c* release (26, 27). Using MitoCapture staining and flow cytometry, we analyzed the $\Delta\psi_m$ in DENSPM-treated SK-MEL-28 cells. In healthy cells, the dye accumulates and aggregates in mitochondria, where it fluoresces bright red. In apoptotic cells, however, it cannot enter mitochondria if the $\Delta\psi_m$ is altered and fluoresces bright green as a cytoplasmic monomer. Analogue treatment caused the loss of $\Delta\psi_m$ in a time-dependent manner (Fig. 7), as shown by the shift in the cell population from low to high green fluorescence. First apparent at 24 h, this effect preceded the release of cytochrome *c* at 30 h. These observations suggest that loss of $\Delta\psi_m$ may be associated with release of cytochrome *c* into the cytosol. To further examine the correlation of $\Delta\psi_m$ with cytochrome *c* release and its contribution to caspase-3 activation, we used CsA, a known inhibitor of the mitochondrial permeability transition pore, previously shown to block apoptosis in several other systems (28, 29). Preincubation of SK-MEL-28 cells with 5 μM CsA significantly reduced DENSPM-induced cytochrome *c* release, caspase activation, and apoptosis (Fig. 8), indicating that the majority of caspase-3 activation is occurring through cytochrome *c* release.

Differential Induction of SSAT. We next examined the role of SSAT induction in these newly defined apoptotic end points, building on our previously reported correlation between SSAT induction and apoptosis in SK-MEL-28 cells (5). For this purpose, we used a series of DENSPM analogues that similarly deplete polyamine pools but differ dramatically in their ability to induce SSAT (5). More specifically, we investigated whether these analogues will differentially induce cytochrome *c* release, caspase activation, and apoptosis in a manner that correlates with their differential effects on SSAT induction. As shown in Fig. 9, we found that analogues (DE-444 and DE-443) that induce

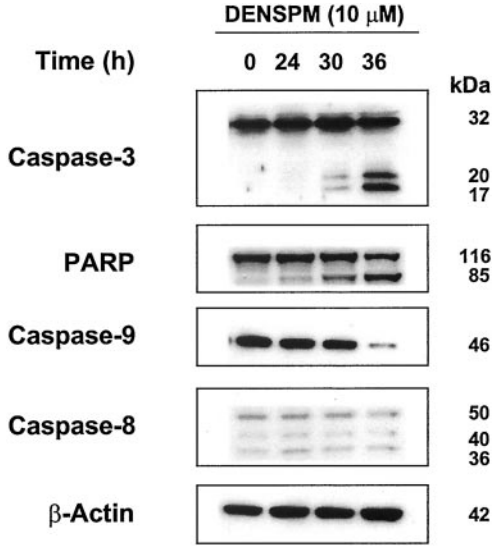

Fig. 4. Activation of caspase-3 and caspase-9 by DENSPM treatment. SK-MEL-28 cells were treated with 10 μM DENSPM for various times. Western analysis was performed by using antibodies to caspase-3, PARP, caspase-9, and caspase-8. β-Actin was used as an indicator of loading. Data are representative of findings obtained in three separate experiments.

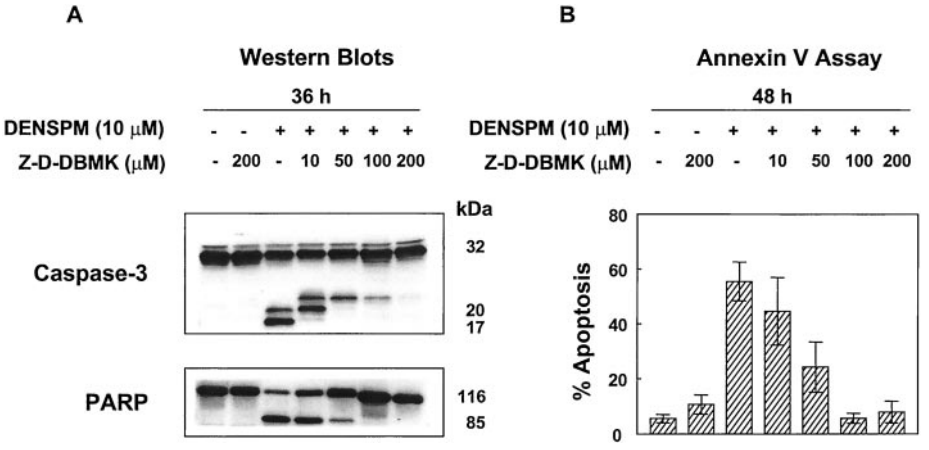

Fig. 5. Prevention of apoptosis by caspase inhibition. *A*, SK-MEL-28 cells were pretreated with varying concentrations of Z-D-DBMK for 1 h and then treated with 10 μM DENSPM for 36 h. Western blots were probed with antibodies to detect the amount of caspase-3 cleavage and PARP digestion. Each dose of Z-D-DBMK used alone was nontoxic to these cells. *B*, SK-MEL-28 cells were pretreated with varying concentrations of Z-D-DBMK for 1 h and then treated with 10 μM DENSPM for 48 h. Cells were harvested, and apoptosis was determined by annexin V staining. Data were graphed as the percentage of positively stained cells relative to the total population. Note that 100 μM of the general caspase inhibitor Z-D-DBMK can completely prevent DENSPM-induced caspase-3 activation and PARP digestion as well as reduce the percentage of annexin V-stained cells to that of untreated cells. Data shown here represent three separate experiments.

relatively low levels of SSAT (26- and 149-fold, respectively) failed to cause cytochrome *c* release or caspase-3 activation after 36 h of treatment. Similarly, they did not cause apoptosis by annexin V staining by 48 h. DE-343, which induced relatively high levels of SSAT (270-fold), did, similar to DENSPM, lead to cytochrome *c* release, caspase activation, and resulted in apoptosis in 34% of the cells. By comparison, DENSPM induced SSAT by 912-fold and caused apoptosis in 68% of the cells. We therefore deduce that differential induction of SSAT correlates with differential activation of caspase-3 and induction of apoptosis. Although not definitive, the findings strongly implicate massive SSAT induction as a fundamental effector of the apoptotic response by DENSPM.

SSAT Induction and H_2O_2 Liberation. H_2O_2 is a free radical oxidative species that has been frequently implicated in apoptotic responses (30, 31). Because H_2O_2 is liberated during the PAO reaction that follows SSAT in the polyamine catabolic pathway (32), we investigated whether this reactive by-product could be involved in apoptotic events associated with DENSPM treatment. Accordingly, cells were exposed to DENSPM in the presence of a sulfur-containing antioxidant, NAC (33), or a highly specific PAO inhibitor, MDL-72527 (34). Both compounds were expected to decrease apoptotic events if H_2O_2 was involved. As shown in Fig. 10, we observed that both NAC and MDL-72527 prevented cytochrome *c* release and caspase-3 cleavage as well as apoptosis in a dose-dependent manner. Toward this end, MDL-72527 was more efficient than NAC. More specifically, 40 mM NAC reduced annexin V-stained cells in DENSPM-treated cultures by 63%, and 250 μM MDL-72527 reduced apoptosis by 79%. The near complete prevention with MDL-72527 would suggest that the other metabolite generated by PAO, 3-acetamidopropanal, is also contributing to apoptosis. Cells treated with 5 and 20 mM NAC alone were similar to untreated cells; however, 40 mM NAC showed some toxicity by annexin V staining. NAC or MDL-72527 did not prevent cellular accumulation of DENSPM or its ability to reduce polyamine pool (data not shown). Combining both H_2O_2 modulators with DENSPM did not enhance their protective effects and gave results similar to that seen with DENSPM plus MDL-72527 alone (data not shown). Taken together with the differential analogue effects on SSAT, these findings suggest that a significant portion of DENSPM-induced apoptosis derives from analogue induction of SSAT and the downstream consequences of H_2O_2 and 3-acetamidopropanal liberation via the PAO reaction.

DISCUSSION

We reported previously that DENSPM causes rapid apoptosis in SK-MEL-28 human melanoma cells and G_1 arrest in MALME-3M melanoma cells (5). Although multiple regulatory events undoubtedly

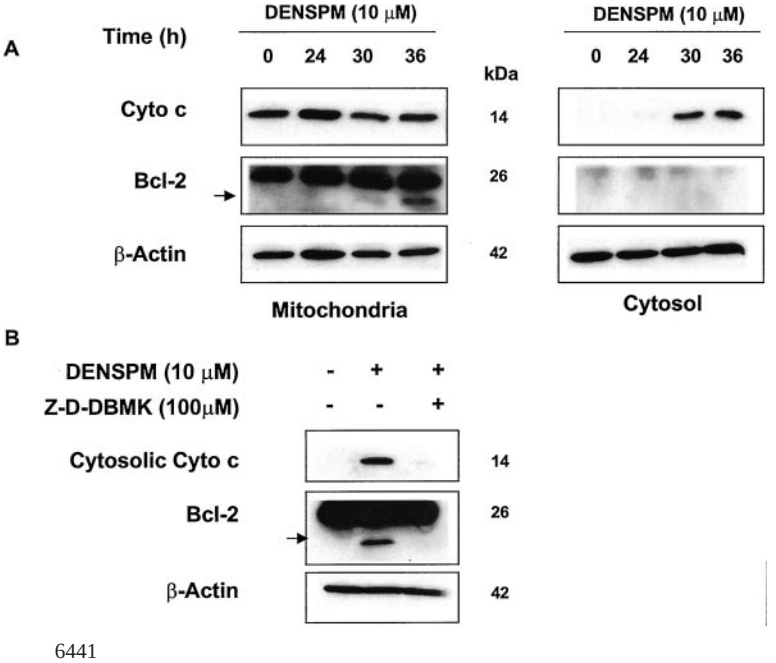

Fig. 6. Cytochrome *c* release and Bcl-2 cleavage. *A*, SK-MEL-28 cells were treated with 10 μM DENSPM for the indicated time. Mitochondria and cytosol were isolated as described in "Materials and Methods." Twenty μg of protein were loaded and analyzed by Western blot with the antibodies that detect cytochrome *c* (*Cyto c*) and Bcl-2. Note that cytochrome *c* is released from mitochondria to cytosol by 30 h of treatment. A cleaved form of Bcl-2 is detected in the mitochondria at 36 h. *B*, cells were pretreated with 100 μM Z-D-DBMK for 1 h followed by 10 μM DENSPM treatment for 36 h. Cells were harvested and processed as described above to measure cytosolic cytochrome *c* and mitochondrial Bcl-2 by Western blots. The *arrow* indicates cleaved Bcl-2. Data represent three separate experiments.

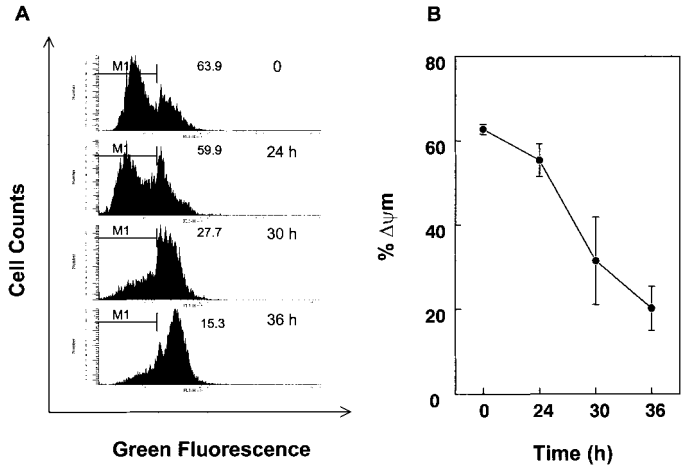

Fig. 7. Effect of DENSPM treatment on the mitochondrial transmembrane potential. Mitochondrial transmembrane potential ($\Delta\psi_m$) was analyzed using an assay based on the intensity of green fluorescence staining as described in "Materials and Methods." SK-MEL-28 cells were treated with 10 μM DENSPM from 24–36 h and harvested for flow cytometry. *A* shows histograms for untreated cells and treated cells at 24, 30, and 36 h. Each histogram profiles the number of cells relative to their green fluorescent intensity. Note that the majority of untreated cells (64%) were gated as M_1 with a low level of fluorescence. With treatment, there was a time-dependent shift in the cell population to a higher fluorescence intensity, reflecting a decrease in $\Delta\psi_m$. *B*, the average $\Delta\psi_m$ is graphically represented as the percentage of cells in M_1 over treatment time from two separate determinations performed in duplicate.

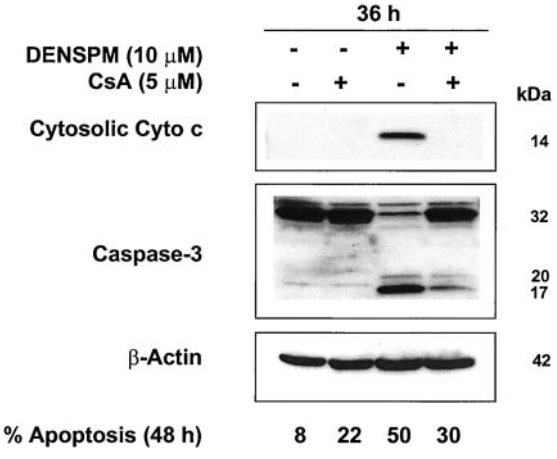

Fig. 8. Inhibition of DENSPM-induced apoptosis by CsA. SK-MEL-28 cells were pretreated with 5 μM CsA for 1 h and then treated with 10 μM DENSPM for 36 h. Cells were harvested to measure the amount of cytochrome *c* (*Cyto c*) in the cytosol and caspase-3 activation by Western blots. Apoptosis was measured after 48 h of treatment and shown as the percentage of positive annexin V-stained cells. Data represent three separate experiments. The SD is less than 20%.

converge to establish these outcomes, one determining factor that could be influential is the status of the *p53* gene, which is mutated in SK-MEL-28 cells and wild-type in MALME-3M cells (15). Whereas the role of this important gene is being investigated via transfection studies, the present inquiry was undertaken to characterize the apoptotic effectors and signaling pathways in SK-MEL-28 cells.

Time course analysis implicates disruption of polyamine homeostasis as the earliest DENSPM-mediated event. Major decreases in intracellular Spm and Spd pools began at 8 h and approached completion at 16 h. As shown previously (2–4), this pool depletion is preceded by down-regulation of the biosynthetic enzymes ODC and SAMDC and potent up-regulation of SSAT (up to a 1000-fold increase in activity). The induction of SSAT is further evidenced by a distinct rise in acetylated polyamines and by the increase in Put due to back conversion of Spm and Spd. In addition to monoacetylated polyamines, we also detected DiAcSpm, a novel metabolite recently identified in MCF-7 cells that conditionally overexpress SSAT (17). Although this metabolite was originally observed in bacterial and acellular systems (35, 36), this is the first report to describe its formation in analogue-treated mammalian cells. The cellular implications of DiAcSpm need to be further studied.

Having characterized the early polyamine-related responses, we sought

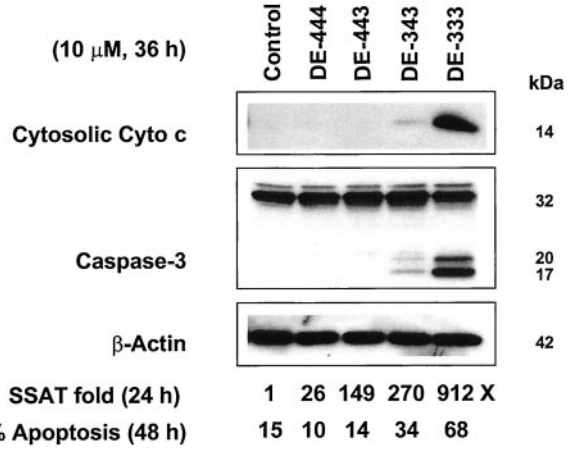

Fig. 9. Effects of differential SSAT induction on cytochrome *c*, caspase-3, and apoptosis. Cells were incubated with 10 μM DE-444, DE-443, DE-343, or DENSPM (*DE-333*) for 36 h and harvested to measure cytochrome *c* (*Cyto c*) release into the cytosol and caspase-3 activation by Western blots. Apoptosis was measured after 48 h of treatment and is shown as the percentage of positive annexin V-stained cells. Note that the extent of apoptosis by the analogues correlates with the level of SSAT induction. Data represent two separate experiments.

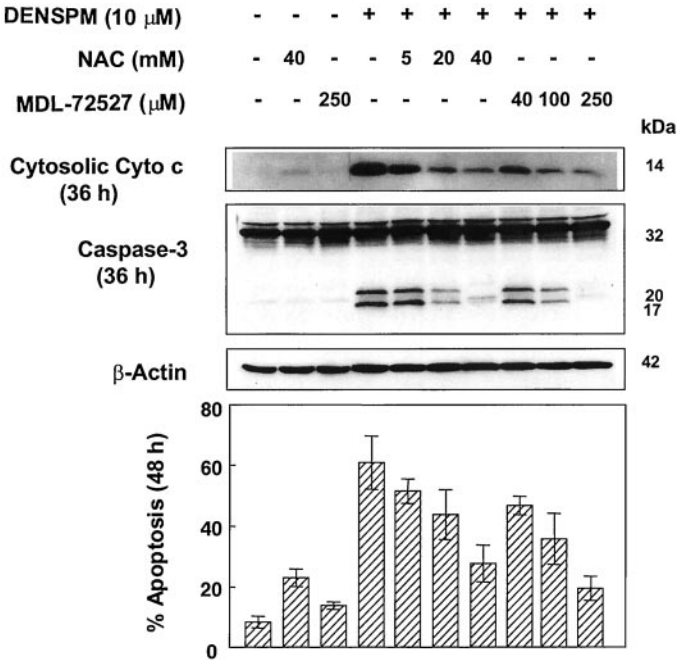

Fig. 10. Effects of modulators of H_2O_2 production on cytochrome *c*, caspase-3, and apoptosis. Cells were pretreated with various concentrations of NAC or MDL-72527 for 1 h before cotreatment with 10 μM DENSPM. At 36 h, cells were harvested to measure cytochrome *c* (*Cyto c*) release and caspase-3 activation by Western blots. Apoptosis was measured after 48 h of treatment and is shown as the percentage of positive annexin V-stained cells. Cells treated with 5 and 20 mM NAC and 40 and 100 μM MDL-72527 alone are not shown; in each analysis, these cells were similar to untreated cells. Note that treatment with 40 mM NAC or 250 μM MDL-72527 significantly prevented DENSPM-mediated effects on cytochrome *c* release, caspase-3 activation, and apoptosis. Data represent the averages with SDs from more than three separate experiments.

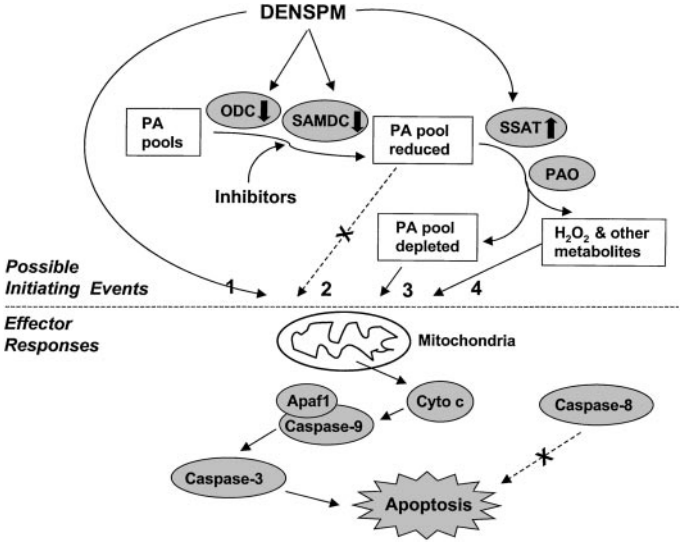

Fig. 11. Diagram of polyamine analogue effects on polyamine metabolism and pathways culminating in apoptosis. Four primary analogue effects are identified as possible initiating events leading to the activation of mitochondrial caspase signaling: *1*, direct analogue effects that may or may not be polyamine related; *2*, polyamine pool reduction that was excluded (designated by *x*) with polyamine inhibitor data; *3*, polyamine pool depletion; and *4*, induction of SSAT activity leads to elaboration of acetylated polyamines, which, when oxidized, give rise to H_2O_2 and acetamidopropanal.

to define the downstream signaling pathways that lead to apoptosis. Because impressive progress has been made in delineating apoptotic signaling, our strategy was to identify reliable end points and work backwards to investigate possible polyamine-related upstream events. We began with the caspases, a family of cysteine proteases regarded as the executioners of apoptosis (8). Our data demonstrate that caspase-3 is activated in DENSPM-treated cells and that it is a necessary component of apoptosis, as indicated by the finding that the general caspase inhibitor Z-D-DBMK interfered with apoptosis (Fig. 5). Further definition of upstream caspase effectors revealed that caspase-9, which is typically initiated by mitochondrial events (9, 10), was activated via cytochrome *c* release, whereas caspase 8, which is usually stimulated by cytosolic apoptotic events (37), was not activated.

An important role of mitochondria in apoptosis involves the liberation of factors such as cytochrome *c* into the cytosol (38), where it binds to Apaf-1 and activates the caspase cascade (9–11). Indeed, our studies found that the location of cytochrome *c* shifted from the mitochondria to the cytosol during DENSPM treatment and that this effect preceded the activation of caspase-3 (Fig. 6*A*). This is accompanied by a time-dependent loss of the mitochondrial transmembrane potential (Fig. 7). CsA, the inhibitor of membrane pores, not only prevented cytochrome *c* release by DENSPM but also reduced caspase activation and apoptosis (Fig. 8). Others have demonstrated that caspases can act in a feed-forward pathway to cleave and inactivate the antiapoptotic Bcl-2 protein to promote further cytochrome *c* release (25). Our finding that Bcl-2 cleavage temporally followed caspase activation and was inhibited by the caspase inhibitor (Fig. 6*B*) supports this feed-forward possibility. Interestingly, other Bcl-2 family proteins such as Bcl-X_L, Bax, and Bid were unaffected by DENSPM treatment (data not shown).

It was of interest to determine whether the observed apoptotic effects were related to analogue-induced polyamine perturbations. As noted above, we found that DENSPM caused potent SSAT induction by 16 h and complete polyamine pool depletion by 20 h, both of which happened before the apoptosis occurring between 24 and 48 h (Fig. 2). The data suggest that SSAT induction, polyamine pool depletion, or both could be initiating events in apoptosis. To discern the relative contribution of these two components (SSAT induction and polyamine pool depletion), we used polyamine analogues that differentially induce SSAT but similarly deplete polyamine pools and accumulate to comparable intracellular levels (5). As shown in Fig. 9, the analogues differentially affected apoptosis in a manner consistent with their ability to induce SSAT, but not with their ability to deplete polyamine pools. Thus, the data are consistent with the possibility that SSAT induction may contribute to apoptosis, a conclusion reinforced by studies with polyamine enzyme inhibitors. More particularly, treatment with both biosynthetic inhibitors, DFMO and MDL-73811, markedly lowered polyamine pools in the absence of SSAT induction and produced cell cycle arrest but not apoptosis. The relative contributions of complete pool depletion as well as direct analogue effects (Fig. 11) are still being investigated.

In the polyamine catabolic pathway, Spm or Spd is acetylated by SSAT and subsequently oxidized by PAO to form Spd and Put, respectively, plus stoichiometric amounts of acetamidopropanal and H_2O_2. At least two other groups have linked these metabolic effects to apoptosis by analogues (39, 40), and one group (40) has demonstrated possible caspase involvement. In the present study, we found that the PAO inhibition by MDL-72527 effectively blocked DENSPM-induced cytochrome *c* release, caspase activation, and apoptosis in a dose-dependent manner. This strongly suggests a contributing role for the by-products of oxidase reaction, H_2O_2 and acetamidopropanal. However, because prevention of DENSPM-induced apoptosis by PAO inhibition was not complete (~79%), it is possible that other contributing events may be involved. One possibility is 3-aminopropanal, a by-product of SSAT-independent Spm oxidation that has been shown to be involved in glial cell death induced by cerebral ischemia (41). In further support of the above-mentioned findings, we also observed that the glutathione precursor and antioxidant NAC (33) prevented DENSPM-induced apoptosis (~63%), presumably by reducing oxidative events related to H_2O_2. Taken together, the data strongly implicate oxidative products related to SSAT induction to be among the events leading to apoptosis via mitochondrial damage and the caspase cascade (Fig. 11). Although Ha *et al.* (42) reported cytochrome *c* release and caspase activation in cells treated with an asymmetrically modified polyamine analogue, these effectors were not linked to apoptosis *per se*. This particular class of analogue has been shown to uniquely affect tubulin polymerization (43), which could account for the apoptotic response.

Having begun to define the apoptotic pathways involved in analogue-induced apoptosis, we were interested in using these effectors as end point markers for determining other upstream signaling events. In an ongoing study,[4] we noted that Egr-1 mRNA was increased in DENSPM-treated SK-MEL-28 cells analyzed by microarray cDNA analysis and confirmed by Northern blots. This, in turn, implicated the mitogen-activated protein kinase pathway, which is known to activate Egr-1 transcription (44). We are currently investigating the role of mitogen-activated protein kinase signaling in SK-MEL-28 apoptosis.

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