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The Role of Mitogen-activated Protein Kinase Activation in Determining Cellular Outcomes in Polyamine Analogue-treated Human Melanoma Cells[1]

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ABSTRACT

The clinically relevant polyamine analogue N^1,N^{11}-diethylnorspermine (DENSPM) inhibits cell growth by down-regulating polyamine biosynthesis, up-regulating polyamine catabolism at the level of spermidine/spermine N^1-acetyltransferase (SSAT), and depleting intracellular polyamine pools. Among human melanoma cell lines, the analogue causes rapid apoptosis in SK-MEL-28 cells and a sharp G_1 arrest in MALME-3M cells. This study reveals that DENSPM potently activates the mitogen-activated protein kinase (MAPK) pathways in melanoma cells and investigates the role of this response in determining cellular outcomes. Onset of apoptosis was preceded by an intense phosphorylation of the MAPKs, including extracellular signal-regulated kinase 1/2, c-Jun NH_2-terminal kinase, and p38 in both SK-MEL-28 and MALME-3M cells. A panel of DENSPM analogues differing only in their ability to induce SSAT was used to show that MAPK activation was causally linked to induction of SSAT activity and related oxidative events. The latter was confirmed with the polyamine oxidase inhibitor MDL-75275 and the antioxidant *N*-acetyl-L-cysteine, which when used in combination with DENSPM, decreased MAPK activation and as previously shown, reduced apoptosis. The MAP/extracellular signal-regulated kinase-1 inhibitor PD 98059 reduced activation of all three kinases but failed to alter apoptosis in DENSPM-treated SK-MEL-28 cells. By contrast, the inhibitor prevented p21*waf1/cip1* induction and enhanced apoptosis in MALME-3M cells as indicated by accelerated caspase-3 activation and positive annexin V staining. The generality of this effect was demonstrated in DENSPM-treated A375 and LOX human melanoma cells. Taken together, the importance of the MAPK pathways in determining the biological response to DENSPM treatment is dependent on the genetic environment of the cell.

INTRODUCTION

The dependence of tumor cell growth on polyamines and the increased biosynthetic activity found in tumors relative to surrounding normal tissues (1, 2) provide a rationale for targeting these molecules by chemotherapeutic agents. DENSPM[3] is a polyamine analogue that has undergone clinical evaluation against solid tumors, including malignant melanoma (3, 4). Such analogues typically down-regulate the polyamine biosynthetic enzymes ODC and SAMDC and potently up-regulate the catabolic enzyme SSAT (5, 6), culminating in polyamine pool depletion and inhibition of cell growth. Recent studies from this laboratory (7, 8) have shown that DENSPM causes an initial G_1 arrest and delayed apoptosis in MALME-3M human melanoma cells and a rapid and intense apoptotic response in SK-MEL-28 human melanoma cells. We now seek to understand the basis for this differential response and, in particular, to identify the signaling pathways that lead to or modulate apoptosis. Results from gene profiling studies (reported here) with SK-MEL-28 and MALME-3M cells have focused our attention on EGR-1 signaling and the upstream MAPK pathways.

EGR-1, an immediate-early growth response gene, can act as a positive or negative regulator of gene transcription depending on the cell type (9) and is involved in many cellular functions, including cell proliferation, differentiation, and apoptosis (10). The functional complexity of EGR-1 may be explained by the fact that >30 genes are transcriptionally regulated by EGR-1, including p53, tumor grow factor β, platelet-derived growth factor-A, insulin-like growth factor-II, and metalloproteinase (11–15). Recent studies have demonstrated that activation of the MAPK pathway ERK1/2 can activate the ets factor, Elk-1, which, in concert with serum response factor, participates in initiation of *EGR-1* gene transcription (16), thus placing EGR-1 downstream of MAPK activation. P38/JNK1 activation has been similarly implicated in EGR-1 induction (17).

Activation of MAPK pathways is essential for signal transduction in response to numerous environmental stimuli (18). ERK1/2, JNK, and p38 kinases are three main MAPK pathways that can be activated by growth factors, DNA damage, cytokines, oxidant stresses, UV light, anticancer drugs, and osmotic shock (18). All three MAPK pathways can be differentially activated, and their involvement in apoptosis is highly context- and model dependent. For example, ERK1/2 activation is required for apoptosis in some systems (19–21), whereas ERK1/2 activation correlates with cell survival in others (22–24). Similarly, JNK or p38 activation differentially affects cell growth or apoptosis, depending on the stimuli and cell system (25–28). The linkage between polyamines and the MAPK proteins has not yet been defined. One study demonstrated subtle activation of MAPKs by polyamine pool reduction (29), whereas others found MAPK activation in response to polyamine excess (30, 31). Although these studies suggest that the MAPK effectors may be sensitive to changes in polyamine pools, the role of these signaling pathways in mediating cellular outcomes during analogue-induced perturbations in polyamine metabolism remains unknown.

The studies presented here are designed to identify signaling pathways involved in determining differential cellular responses to DENSPM. More particularly, the results suggest that distinct effectors linked to MAPK signaling modulate apoptosis in certain, but not all, melanoma cell lines.

MATERIALS AND METHODS

Materials. The polyamine analogue DENSPM was provided by Parke Davis (Ann Arbor, MI), whereas DE-444, DE-443, and DE-343 were kindly provided by Dr. Raymond Bergeron (University of Florida, Gainesville, FL). The inhibitor of PAO, MDL-72527, and the SAMDC inhibitor, MDL-73811, were provided by Aventis Pharmaceuticals, Inc. (Bridgewater, NJ). The ODC inhibitor DFMO was obtained from ILEX, Inc. (San Antonio, TX). SB 203580

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[3] The abbreviations used are: DENSPM, N^1,N^{11}-diethylnorspermine also known as DE-333; DE-343, N^1,N^{12}-diethylspermine; DE-443, 3,7,12,17-tetra-azanonadecane [N^1,N^{13}-diethyl(aminopropyl)homospermidine]; DE-444, N^1,N^{14}-diethylhomospermine; DFMO, α-difluoromethylornithine; EGR-1, early growth response-1; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MDL-72527, N^1-methyl-N^2-(2,3-butadienyl)butane-1,4-diamine; MDL-73811, 5′-{[(Z)-4-amino-2-butenyl] methylamino}-5′-deoxyadenosine; MEK-1, MAP/ERK kinase-1; NAC, *N*-acetyl-L-cysteine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; SAMDC, *S*-adenosylmethionine decarboxylase; SSAT, spermidine/spermine N^1 acetyltransferase; ZRP, zyxin-related protein.

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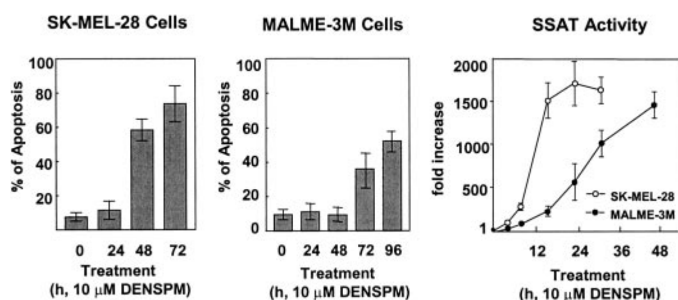

Fig. 1. Induction of apoptosis by DENSPM in SK-MEL-28 and MALME-3M cells. Cells were treated with 10 μM DENSPM for the indicated time and harvested for flow analysis to determine the percentage of cells that stain positive for annexin V. The third panel compares DENSPM induction of SSAT activity in both cell lines and shows that the activity expressed as fold increase is significantly increased before apoptosis. We have previously shown (7) that the two cell lines accumulate similar levels of the analogue. These data represent three separate experiments.

was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA), PD 98059 from Cell Signaling Technology, Inc. (Beverly, MA), U 0126 from Promega (Madison, WI), and the antioxidant NAC from Sigma (St. Louis, MO).

Cell Culture. SK-MEL-28, MALME-3M, and LOX 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) and 1 mM aminoguanidine to prevent polyamine oxidation. A375 human melanoma cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) and 1 mM aminoguanidine.

Polyamine Pools. Intracellular polyamines and polyamine analogues were analyzed by high-performance liquid chromatography as described previously (8).

cDNA Expression Array Analysis. A cDNA macroarray system (Atlas; Clontech, Palo Alto, CA) was used to compare the relative levels of RNA expressed in human melanoma SK-MEL-28 and MALME-3M cells in the presence or absence of 10 μM DENSPM for 24 h. The procedures are described in the manufacturer's protocol. Briefly, total cellular RNA was isolated and contaminating DNA was removed by treatment with DNase I. Poly(A)$^+$ RNA was enriched with biotinylated oligo(dT) and streptavidin magnetic beads. RNA templates bound to beads were subjected to a cDNA synthesis primer mixture and [α-^{32}P]dATP. Purified cDNA probes (>6.5 × 10^6 cpm) were hybridized to the arrayed cancer cDNA membranes under recommended conditions. Washed membranes were exposed to a phosphorescent imaging screen. The position of the hybridization signals was identified using the orientation grid provided. Signals were quantified using AtlasImage software. Changes in genes of interest were confirmed by Northern and Western blot analysis.

Northern Blot Analysis. Northern blot analysis was carried out as described by Fogel-Petrovic *et al.* (32). Briefly, total RNA was extracted with RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). RNA samples (10 μg/lane) were separated on 1.5% agarose/formaldehyde gels and transferred to membrane. RNA was hybridized to ^{32}P-labeled cDNA encoding human *EGR-1*. After exposure to X-ray film, membranes were washed in stripping buffer [2 mM EDTA (pH 8.0), 0.1% SDS] for 15–20 min at 75°C and hybridized again with human GAPDH cDNA. The GAPDH signal was used as an internal control for evaluating RNA loading and for quantitating changes in mRNA expression.

Western Blot Analysis. SK-MEL-28 cells or MALME-3M cells were treated with 10 μM DENSPM for various times. Cells were harvested, and total lysates (40 μg protein/lane) were run on 7.5–15% SDS-PAGE gels, followed by transfer to polyvinylidene difluoride membrane and immunoblotted with phospho-specific antibody against the activated ERK1/2 or p38 (New England Biolabs, Beverly, MA) or a phospho-specific antistress-activated protein kinase/JNK (Calbiochem-Novabiochem Corp.) that recognizes all three JNK isoforms. Rabbit polyclonal antibodies against ERK-1, JNK1, and p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anticaspase-3 was obtained from PharMingen (San Diego, CA). Rabbit polyclonal EGR-1 antibody was generously provided by Dr. Frank Rauscher, III (Wistar Institute, Philadelphia, PA). Detection was performed with enhanced chemiluminescence from Amersham Pharmacia Biotechnology (Arlington Heights, IL). Protein was determined by the standard Bio-Rad assay, and β-actin (Sigma) was used to equalize for variations in loading.

Apoptosis Assay. The percentage of apoptotic cells was determined 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 evaluated using the Winlist program (Verity House, Topsham, ME).

RESULTS

Effects on Polyamine Pools and Metabolism. We have recently characterized the temporal sequence of analogue effects on polyamine pools and metabolism, cell growth, and apoptosis that take place in DENSPM-treated SK-MEL-28 and MALME-3M human melanoma cells (7, 8). SK-MEL-28 cells treated with 10 μM DENSPM are profoundly apoptotic by 36 h with no evidence of prior cell cycle arrest (8). By contrast, similarly treated MALME-3M cells undergo a sharp G_1 arrest by 12–16 h that is sustained for 72 h and then followed by apoptosis (Ref. 7; Fig. 1). In both cell lines, DENSPM induces large amounts of SSAT activity (*i.e.,* >200-fold) before apoptosis (Fig. 1), which together with PAO, leads to the release of high levels of hydrogen peroxide (H_2O_2) and reactive aldehydes within the first 24 h (7). Our laboratory and others (8, 33, 34) have shown that the oxidative products contribute significantly to the apoptotic response. Having defined the apoptotic signaling pathways (8), we focus here on upstream effectors that modulate or determine cellular responses in these two melanoma cell lines.

cDNA Expression Array. To identify candidate effector molecules, we applied a commercially available cDNA expression array with 500 cancer-related genes. Comparisons were based on expression levels of apoptosis-prone SK-MEL-28 cells and G_1 arrest-prone MALME-3M cells treated for 24 h with 10 μM DENSPM. The expression of several candidate genes affected to levels >3-fold were confirmed by Northern and Western blot analysis. DENSPM induced RhoA, a GTP binding protein, and ZRP in both melanoma cell lines, whereas EGR-1, a transcription factor involved in multiple cellular functions (10), was mainly induced in SK-MEL-28 cells. Northern and Western blot analysis showed that EGR-1 mRNA and protein levels were increased by 48- and 7-fold, respectively, in DENSPM-treated SK-MEL-28 cells after 24 h, whereas increases of only 3- and 2-fold, respectively, were observed in MALME-3M cells (Fig. 2). This led us to investigate the possibility that EGR-1 and possible events such as activation of stress pathways might account for differences in cellular outcomes.

Induction of MAPKs by DENSPM. Because EGR-1 has previously been linked to apoptosis (35) via the MAPK pathways (16, 17), we investigated their contribution to DENSPM-induced apoptosis.

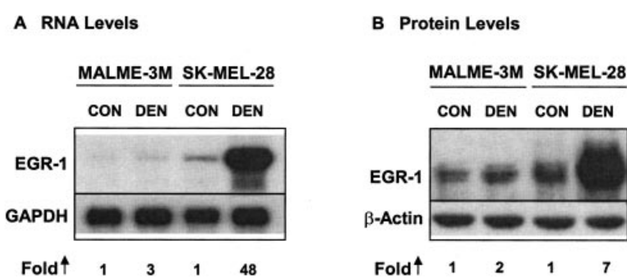

Fig. 2. Induction of *EGR-1* mRNA and protein by DENSPM. SK-MEL-28 and MALME-3M cells were treated with 10 μM DENSPM and harvested at 24 h for (*A*) Northern or (*B*) Western blot analysis of EGR-1. Loading was normalized by using GAPDH for Northern and β-actin for Western blots.

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SK-MEL-28 cells were treated with 10 μM DENSPM for various times and then analyzed for activated ERK1/2, JNK, and p38 using phospho-specific antibodies. As shown in Fig. 3*A*, we found ERK1/2 phosphorylation began to increase at 16 h and rose steadily (~10-fold) during the 28-h treatment period. In addition, p38 was phosphorylated in a similar manner reaching its maximum (~7-fold) at 24 h. JNK activation appeared to be more dramatically affected (~50-fold) than the other two because of low basal levels. In each case, the total protein levels of the MAPKs were not changed by DENSPM treatment (Fig. 3*B*).

SSAT Induction and MAPKs Activation. We next used DENSPM analogues to investigate the basis for this response. These particular analogues similarly down-regulate polyamine biosynthesis and deplete polyamine pools but differentially induce polyamine catabolism via SSAT (7). We previously relied on these analogues to link SSAT induction with caspase activation and apoptosis in SK-MEL-28 cells (7, 8). Thus, SK-MEL-28 cells were treated with one of four analogues (DE-444, DE-443, DE-343, and DENSPM) for 24 h and assayed for ERK1/2, JNK, and p38 phosphorylation. As shown in Fig. 4*A*, there was a general correlation between induction of SSAT and analogue activation of ERK1/2, JNK, and p38. However, these responses seemed to require some threshold level of SSAT induction between that achieved by DE-443 and DE-343. Furthermore, the two highest inducers of SSAT, DE-343 (270-fold) and DENSPM (912-fold), induced different levels of apoptosis but similar levels of MAPK activation (Fig. 4*A*), suggesting the latter involves a saturable event. Thus, although potent induction of SSAT activity appears to contribute to MAPK activation, it seems to participate within a fixed range of SSAT induction.

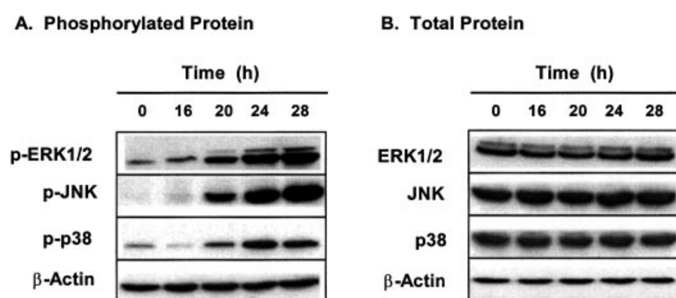

Fig. 3. Activation of MAPK pathways by DENSPM in SK-MEL-28 cells. Cells were treated with 10 μM DENSPM and harvested at various times for Western blot analysis. Blots were probed with (*A*) phospho-specific ERK1/2, JNK, or p38 antibodies or with (*B*) ERK1/2, JNK, or p38 antibodies. β-Actin was used as a loading control. Data represent three separate experiments.

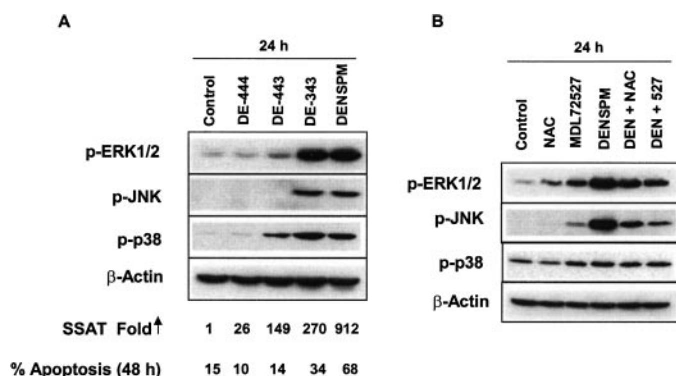

Fig. 4. SSAT induction and MAP kinase activation. *A*, SK-MEL-28 cells were treated with 10 μM DE-444, DE-443, DE343, or DE-333 (DENSPM) and harvested at 24 h for Western blot analysis or and harvested at 48 h for annexin V staining. The fold induction of SSAT determined previously for each analogue (7) is shown for comparison. Annexin V data represent the percentage of positively stained cells determined from three separate determinations (8). *B*, cells were pretreated with 20 mM NAC or 100 μM MDL-72527 (527) for 1 h followed by 10 μM DENSPM (DEN) treatment and harvested at 24 h for Western blot analysis.

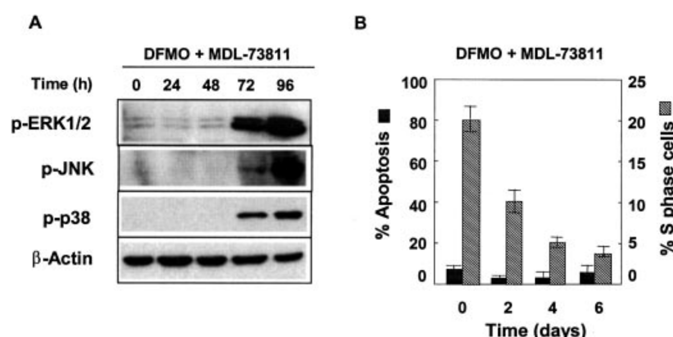

Fig. 5. Effects of polyamine inhibitors on MAPK activation and apoptosis. SK-MEL-28 cells were treated with 5 mM DFMO plus 10 μM MDL-73811 and (*A*) harvested at 0–96 h for Western blot analysis or (*B*) and harvested at 3, 4, or 6 days for annexin V staining to determine percentage of apoptotic cells (*left axis*) and flow analysis to determine percentage of S-phase cells (*right axis*). These data represent three separate experiments.

It is possible that SSAT induction and MAP kinase activation are linked in two ways: (*a*) via the oxidative products (*e.g.,* H_2O_2 and aldehydes) generated by the SSAT coupled with PAO and/or (*b*) via enhanced polyamine pool depletion caused by the SSAT-facilitated export of polyamines out of the cell. Evidence to support the former possibility was sought using the antioxidant NAC and the PAO inhibitor MDL-72527. In combination with DENSPM, either 20 mM NAC or 100 μM MDL-72527 reduced DENSPM-induced ERK1/2 and JNK activation (Fig. 4*B*). There was no apparent effect on p38 activation, although uncontrollable variability in the basal levels of phospho-p38 (control levels in Figs. 4 and 5*A*) made this interpretation difficult. Thus, liberation of oxidative products via the SSAT-mediated increase in acetylated substrates for PAO to act upon, as well as induction of the oxidases themselves (36–38) appears to contribute significantly to MAPK activation. We have previously found that these same agents also prevent caspase cleavage and apoptosis (8).

The other possibility noted above is that SSAT induction contributes to MAPK via the ability of SSAT to accelerate and increase polyamine pool depletion. In SK-MEL-28 cells, exposure to DENSPM rapidly reduces the combined Spd and Spm pools by 64% at 8 h and 90% at 16 h (8). To examine the role of polyamine pool depletion in MAPK activation, we used dual polyamine inhibitors to deplete pools without inducing SSAT. Thus, the combined treatment of SK-MEL-28 cells with the ODC inhibitor, DFMO at 5 mM, and the SAMDC inhibitor, MDL-73811 at 10 μM, reduced the total polyamine pool content by 45% (*i.e.,* from 4205 to 2315 pmol/10^6 cells) at 48 h. Pools continued to decrease with a maximum reduction of ~60–70% by 96 h of inhibitor treatment (8). As shown in Fig. 5, the inhibitor combination led to potent activation of ERK1/2, JNK, and p38 that was first evident at 72 h and additionally increased at 96 h (Fig. 5*A*). Despite MAPK activation, the inhibitors failed to cause apoptosis, even when treatment is extended for 6 days (Fig. 5*B*). Instead, the cells arrested in G_1 and G_2-M as shown by the time-dependent loss of cells transiting through S phase from 2 to 6 days (Fig. 5*B*).

Role of MAPK Activation in Apoptosis in SK-MEL-28 Cells. To examine the role of ERK1/2 in apoptosis, SK-MEL-28 cells were pretreated with a nontoxic concentration (20 μM) of the MEK-1 inhibitor, PD 98059, for 1 h and then cotreated with 10 μM DENSPM and the inhibitor for 24 h. As shown in Fig. 6*A*, PD 98059 fully prevented activation of ERK1/2. Unexpectedly, activation of both JNK and p38 was also reduced using standard pharmacological con-

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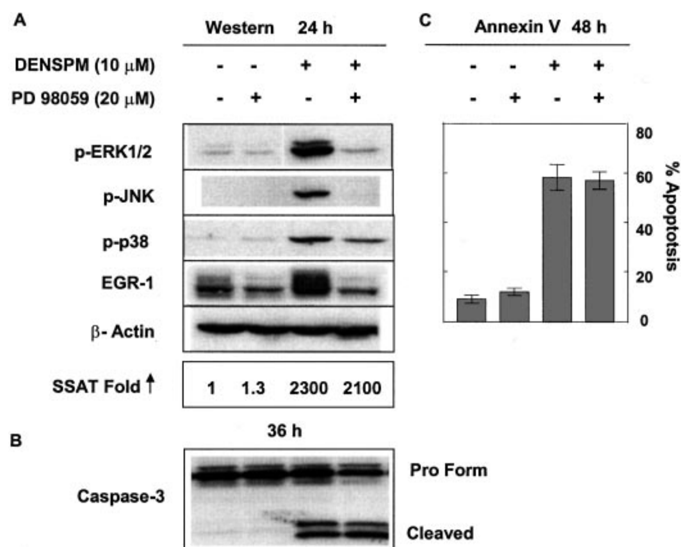

Fig. 6. PD 98059 prevents DENSPM-induced MAPK activation and EGR-1 induction but not apoptosis. SK-MEL-28 cells were pretreated with 20 μM PD 98059 for 1 h followed by 10 μM DENSPM and (*A*) harvested at 24 h or (*B*) at 36 h for Western blot analysis and SSAT activity expressed as fold increase relative to control (Lane 1). β-Actin was used to equalize for variations in loading. *C*, to determine apoptosis, cells were pretreated with 20 μM PD 98059 for 1 h followed by 10 μM DENSPM and harvested at 48 h for annexin V staining. Data represent three separate experiments.

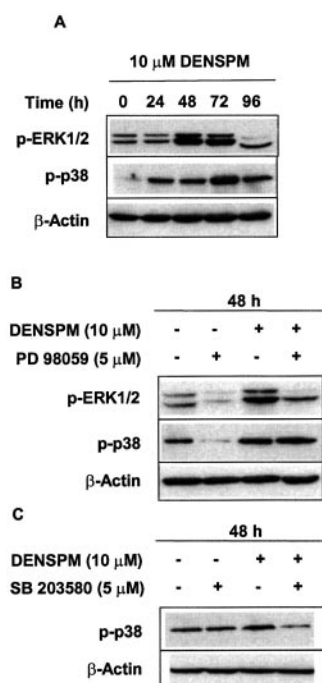

Fig. 7. DENSPM activation of MAPK pathways in MALME-3M cells. *A*, cells were treated with 10 μM DENSPM from 0 to 96 h and harvested for Western blot analysis. Blots were probed with phospho-specific ERK1/2, JNK, and p38. Cells were pretreated with (*B*) 5 μM PD 98059 or with (*C*) 5 μM SB 203580 for 1 h followed by 10 μM DENSPM and harvested at 48 h for Western analysis. β-Actin was used as a loading control.

centrations of PD 98059 (21). DENSPM induction of EGR-1 was also prevented by the MEK-1 inhibitor (Fig. 6*A*), indicating, in similarity to findings by others (16, 17), that the transcription factor is downstream from MAPK signaling. In contrast, SSAT induction by DENSPM was unaffected by PD 98059 (data not shown), indicating that this event is upstream of MAPK activation.

Although PD 98059 effectively prevented phosphorylation of ERK1/2 and JNK, as well as induction of EGR-1 (Fig. 6*A*), the inhibitor failed to abrogate DENSPM-induced apoptosis. Neither caspase-3 activation (Fig. 6*B*) nor annexin V staining (Fig. 6*C*) was affected by combining this MEK-1 inhibitor with DENSPM. The effects of other MAPK inhibitors were also evaluated. The MEK-1 inhibitor, U 0126, at doses of 5 and 10 μM blocked activation of all three MAPKs but proved toxic to cells when used alone (data not shown). The p38 inhibitor SB 203580 also failed to affect DENSPM-induced apoptosis (data not shown). Thus, the MAPK inhibitor data indicate that the potent activation of MAPKs and induction of EGR-1 are not directly involved in mediating or modulating DENSPM-induced apoptosis in SK-MEL-28 cells.

MAPK Activation and Apoptosis in MALME-3M Cells. We next examined whether MAPK activation might modulate cellular outcome in MALME-3M cells. When treated with DENSPM, these cells also induce high levels of SSAT activity (Fig. 1) and typically undergo a distinct G_1 arrest followed by apoptosis that occurs much later than apoptosis in SK-MEL-28 cells (7, 39, Fig. 1). As shown in

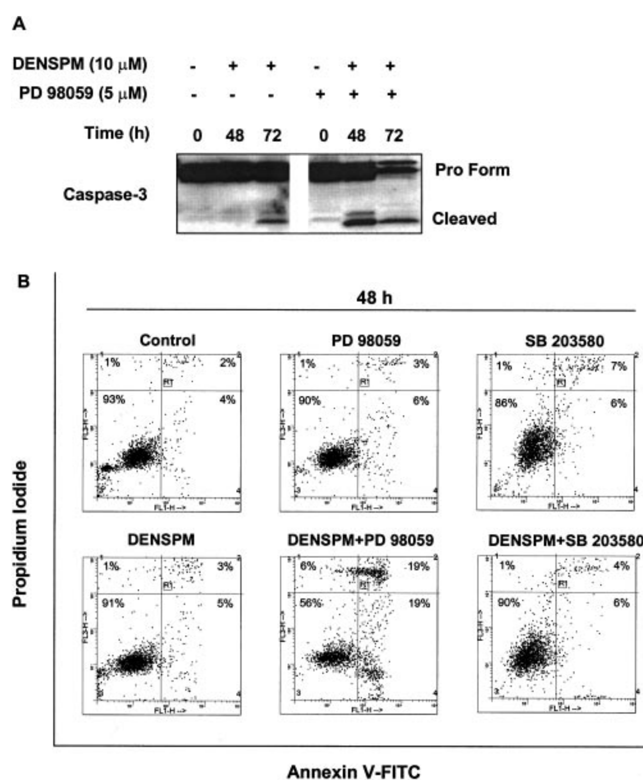

Fig. 8. Effects of MAPK inhibition on DENSPM-induced apoptosis in MALME-3M cells. *A*, cells were pretreated with 5 μM PD 98059 for 1 h followed by 10 μM DENSPM and harvested at 48 or 72 h for Western blot analysis. *B*, cells were pretreated with either 5 μM PD 98059 or 5 μM SB 203580 for 1 h followed by 10 μM DENSPM and harvested at 48 h for annexin V staining. These data represent three separate experiments.

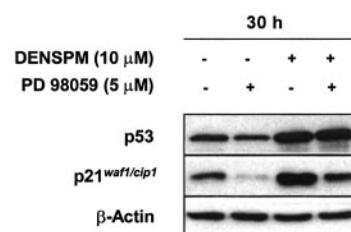

Fig. 9. Effects of MAPK inhibition on DENSPM-induced p53 and $p21^{waf1/cip1}$ expression in MALME-3M cells. Cells were pretreated with 5 μM PD 98059 for 1 h followed by 10 μM DENSPM and harvested at 30 h for Western blot analysis to detect p53 or $p21^{waf1/cip1}$ proteins.

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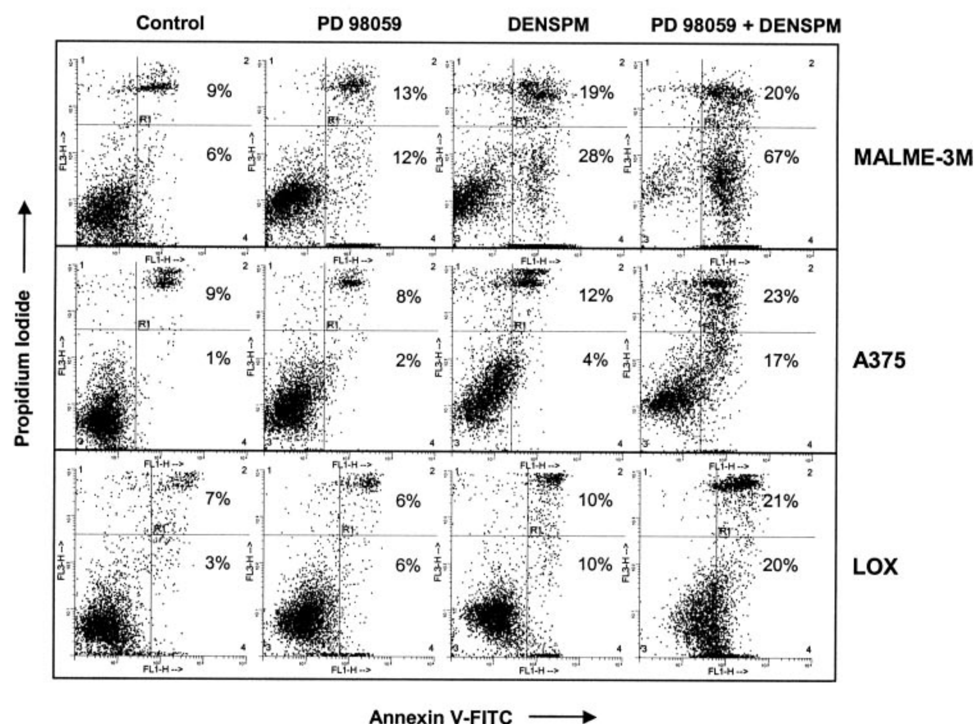

Fig. 10. Effects of MAPK inhibition on DENSPM-induced apoptosis in human melanoma cell lines. MALME-3M, A375, and LOX human melanoma cell lines that contain wild-type p53 were treated for 96 h with 5 μM PD 98059, 10 μM DENSPM, or the drug combination, stained with annexin V, and analyzed by flow cytometry. Note that MAPK inhibition increases DENSPM-induced apoptosis as indicated by a counterclockwise shift from the *bottom left quadrant*. Percentages in the *bottom right* and *top right quadrants* indicate the proportion of early and late apoptotic cells, respectively, in the total cell population. Note that the MALM-3M data in Fig. 8*B* was at 48 h as compared with 96 h shown here.

Fig. 7*A*, both ERK1/2 and p38 phosphorylation were increased during DENSPM treatment, whereas JNK (data not shown) remained in the inactive state. When cells were treated with 10 μM DENSPM and 5 μM PD 98059, activation of ERK1/2 was selectively prevented because p38 activation was not affected (Fig. 7*B*). Significantly, there was a marked enhancement of caspase-3 activation and apoptosis in these cells. In cells treated with DENSPM alone, caspase-3 activation was not apparent until 72 h as compared with 48 h in cells treated with DENSPM plus PD 98059 (Fig. 8*A*). This correlated closely with results of annexin V staining where at 48 h, only 8% of the DENSPM-treated cells were apoptotic as compared with 39% of the cells treated with DENSPM and PD 98059 (Fig. 8*B*). When used alone, the MEK-1 inhibitor had no significant effect on caspase activation or annexin V staining. In contrast to these effects, the p38 inhibitor SB 203580 at 5 μM prevented p38 activation (Fig. 7*C*) but did not enhance DENSPM-induced apoptosis (Fig. 8*B*). Taken together, these data indicate that activation of ERK1/2 (but not p38) protects cells from the DENSPM apoptotic cascade.

MAPK Activation and p21$^{waf1/cip1}$ Induction. DENSPM is known to induce p21$^{waf1/cip1}$ in MALME-3M cells via p53-dependent and -independent mechanisms, leading to a profound and sustained G_1 arrest (7). Thus, we next examined whether MAPK activation might exert its antiapoptotic effect by contributing to p21$^{waf1/cip1}$ induction. As shown in Fig. 9, PD 98059 reduced DENSPM-induced p21$^{waf1/cip1}$ induction without affecting p53 stabilization, indicating that p53-independent increases in p21$^{waf1/cip1}$ are at least partially attributable to MAPK activation. Taken together with earlier data, these findings suggest that MAPK protection from DENSPM-induced apoptosis is attributable to a p21$^{waf1/cip1}$-mediated delay via cell cycle arrest. Because PD 98059 itself induces G_1 arrest in MALME-3M cells (data not shown), cell cycle kinetics could not be evaluated with the combined treatment.

MAPK Activation and Apoptosis in Melanoma Cell Lines. Finally, we examined the ability of MAPK activation to delay apoptosis in A375 and LOX, two melanoma cell lines that, in similarity to MALME-3M, contain wild-type p53 and tend to undergo growth arrest as opposed to apoptosis when treated with DENSPM.[4] At 72 h, 10 μM DENSPM activated ERK1/2 in both A375 and LOX cells (data not shown). As shown by annexin V staining at 96 k (Fig. 10), the inclusion of PD 98059 during DENSPM treatment nearly doubled the proportion of apoptotic cells in all three cell lines.

DISCUSSION

It is well known that the antitumor effects of chemotherapeutic drugs are at least partially mediated by apoptosis. DENSPM represents the first polyamine analogue to undergo clinical evaluation against solid tumors. To design second generation analogues and to more effectively deploy DENSPM, the mechanisms underlying drug sensitivity need to be understood. We previously reported that DENSPM could cause G_1 arrest followed by a delayed apoptotic response in certain melanoma cells (7) and rapid apoptosis mediated by cytochrome *c* release and caspase activation in others (8). In another study,[4] we showed that SSAT-dependent down-regulation of the apoptotic inhibitory protein, survivin, is causally involved in the apoptosis of melanoma cells. Here, we additionally investigate the role of MAPK activation in determining cellular responses to this novel class of anticancer agents.

The present studies were guided by a gene-profiling lead indicating that the transcription factor EGR-1 was potently induced by DENSPM in apoptosis-prone SK-MEL-28 cells as confirmed in Fig. 2. Because EGR-1 has been reported to reside downstream of the MAPKs (16, 17), we examined whether these stress responses might also be affected by DENSPM treatment and observed an early and impressive

[4] Y. Chen, D. L. Kramer, F. Li, and C. W. Porter. Loss of inhibitor of apoptosis proteins as a determinant of polyamine analogue-induced apoptosis in human melanoma cells, Oncogene, in press.

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activation of ERK1/2, JNK, and p38 in SK-MEL-28 cells. Experiments with structural analogues of DENSPM confirmed that differential induction of SSAT activity in these cells correlated with differential activation of the MAPK proteins. We reason that the two events could be causally linked via (*a*) SSAT-mediated increases in polyamine pool depletion or (*b*) SSAT-enhanced polyamine catabolism and the liberation of by-products such as H_2O_2 and reactive aldehydes. In this regard, others have shown that H_2O_2 gives rise to reactive oxygen species that have been implicated in apoptosis (40) and MAPK activation (41, 42). Consistent with this possibility, we observed a significant reduction in MAPK activation when cells were cotreated with DENSPM and the PAO inhibitor MDL-72527 or the antioxidant NAC. The data imply that the MAPK pathways respond, in part, to oxidative activities produced by analogue-induced SSAT and its downstream partner enzyme, PAO. Recently, we and others (36, 37) identified an analogue-inducible novel spermine oxidase that is capable of oxidizing spermine directly. Because this oxidase also produces reactive by-products and because it is analogue inducible and also inhibited by the PAO inhibitor (37), it might also contribute to the MAPK response.

Findings examining a role for polyamine pool depletion in MAPK activation also proved interesting. Polyamine pool depletion achieved in the absence of SSAT induction resulted in profound MAPK activation that was coincident with G_1-G_2-M cycle arrest but not apoptosis. Although the MAPK response to polyamine inhibitors was impressive, it occurred much later (~72 h) than that seen with DENSPM (~16 h), indicating that accelerated polyamine pool depletion by SSAT induction together with PAO oxidative events could contribute to the rapid MAPK response to DENSPM. Still, the results tend to disconnect MAPK activation from apoptosis in these particular cells.

The various MAPKs are differentially involved in apoptosis because of the fact that they are capable of posttranslationally modifying molecules that either activate or inactivate apoptotic proteins. For example, in paclitaxel-treated MCF-7 cells, JNK was found to phosphorylate Bcl-2 to inactivate its antiapoptotic function (43) and in *cis*-platinum-treated A2780 human ovarian carcinoma cells, activated ERK1/2 phosphorylated p53 (44). The connection between ERK1/2 and caspase activation was previously established by a study showing that the MEK-1 inhibitor PD 98059 prevented poly(ADP-ribose) polymerase cleavage (45), indicating that ERK1/2 is upstream of this effector. We used a similar strategy and found that PD 98059 reduced DENSPM activation of all three MAPKs, as well as EGR-1, suggesting cross-talk between these pathways (46) and that the MAPKs are upstream of EGR-1. Significantly, the MEK-1 inhibitor failed to alter caspase activation and annexin V staining in SK-MEL-28 cells, indicating that MAPK and EGR-1 activation were unrelated to the apoptotic response. Although differing from the previously cited literature, this finding is consistent with those of others who have similarly failed to link MAPK activation with the apoptotic machinery (47, 48). Thus, the relationship appears to be highly context- and cell type dependent.

Although MAPK activation was independent of apoptosis in SK-MEL-28 cells, it appears to play a survival function in MALME-3M cells. As in SK-MEL-28 cells, DENSPM caused a profound activation of ERK1/2 in these melanoma cells (Fig. 7*A*). The functional significance of this response was revealed by the finding that cotreatment with PD 98059 accelerated DENSPM-induced apoptosis at the levels of caspase-3 activation and annexin V staining. An important distinction that may account for this cell line differences is the fact that p53 is mutated and nonfunctional in SK-MEL-28 cells and fully functional in MALME-3M cells (49).

Previously, we reported that induction of $p21^{waf1/cip1}$ in DENSPM-treated MALME-3M cells is a biphasic phenomenon that is initiated by p53 and sustained by p53-independent events (7). We now propose that the latter is at least partially because of MAPK activation and that the resulting prolongation in G_1 arrest serves to delay apoptosis. This is supported by the observation that induction of $p21^{waf1/cip1}$ was decreased by PD 98059, whereas induction of p53 was not. Indeed, others have reported that ERK1/2 activation is a regulator of the DNA damage response (44) and that stimulation of ERK1/2 can induce $p21^{waf1/cip1}$, leading to cell cycle arrest (50). The generality of this paradigm was extended to two additional melanoma cell lines, LOX and A375, both of which contain wild-type p53 and resist DENSPM-induced apoptosis. In similarity to findings with MALME-3M cells, PD 98059 accelerated apoptosis in both cell lines.

In summary, potent activation of MAPK by the polyamine analogue DENSPM was not obviously linked to apoptosis in SK-MEL-28 cells but was found to play an protective role in MALME-3M cells. In the latter case, it appears to contribute to the induction of $p21^{waf1/cip1}$ thereby prolonging and/or intensifying G_1 arrest, and delaying apoptosis. Thus, the importance of the MAPK pathways (in particular ERK1/2) in determining the biological response to DENSPM treatment is clearly dependent on the genetic environment of the cells, and it appears that p53 may play a role in this regard. It is possible that the MAPK inhibitors now under clinical development (51, 52) may be used to enhance melanoma responsiveness to DENSPM providing, of course, that such agents do not similarly enhance host toxicities.

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