Interaction between Polyamines and the Mitogen-Activated Protein Kinase Pathway in the Regulation of Cell Cycle Variables in Breast Cancer Cells

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Abstract

Inhibition of polyamine biosynthesis with α-difluoromethylornithine (DFMO) has been shown to inhibit proliferation of breast cancer cells although its mechanism of action has not been fully elucidated. To address this issue, we tested the effects of DFMO on cell cycle variables of MDA-MB-435 human breast cancer cells in culture. We also focused on the possible mediatory role of the mitogen-activated protein kinase (MAPK) pathway on the cell cycle effects of DFMO because this compound has been shown to activate MAPK signaling. We found that DFMO caused a p53-independent increase in p21 and its association with cyclin-dependent kinase (cdk)-2 and decreased cdk-2 protein as well as its phosphorylation on Thr160. In addition, DFMO markedly suppressed the expression of the full-length and low molecular weight forms of cyclin E. These effects of DFMO were reversible with exogenous putrescine, thus indicating that they are specifically mediated through polyamine depletion. Cdk-2 activity was drastically reduced in DFMO-treated breast cancer cells which exhibited a reduction in retinoblastoma (Rb) phosphorylation and protein. As a predictable consequence of these effects, DFMO caused a G1-S block. In addition, DFMO inhibited G2-M transition, most likely as a result of its induction of p21 expression. Inhibition of the MAPK pathway with PD98059 or U0126 blocked the DFMO-induced induction of p21 and the reduction of cdk-2 protein. PD98059 reversed the G2-M block induced by DFMO (probably as a result of suppression of p21) but not the G1-S arrest. MDA-MB-435 cells treated with PD98059 or U0126 in the presence and absence of DFMO exhibited a marked increase in the expression of p27 and its association with cdk-2, a decrease in phosphorylation of cdk-2 on Thr160, and a decrease in cyclin E expression. As predicted, PD98059 treatment reduced cdk-2 activity and Rb phosphorylation while reversing the decrease in Rb protein induced by DFMO. Neither DFMO nor PD98059, either alone or in combination, reduced cdk-4 activity despite a marked induction in p15 expression caused by DFMO. Our results indicate that activation of the MAPK pathway accounts for some of the effects of DFMO on cell cycle events of breast cancer cells. Inhibition of the MAPK pathway, however, does not reverse the cell cycle arrest induced by DFMO because of activation of alternative mechanisms leading to suppression of cdk-2 activity. (Cancer Res 2005; 65(23): 11026-33)

Introduction

The diamine, putrescine, and the polyamines spermidine and spermine are small aliphatic amines that play a major role in cancer biology including in breast cancer (1, 2). Considerable evidence provided by our and other laboratories has shown that polyamines play a critical role in breast cancer cell proliferation (3). Inhibition of ornithine decarboxylase (ODC), the first and rate-limiting enzyme in polyamine biosynthesis, with α-difluoromethylornithine (DFMO) has been shown to exert a potent growth inhibitory action in a variety of experimental breast cancer systems both in vitro and in vivo (4–6). In human breast cancer, ODC activity has been shown to be increased compared with normal breast tissue (7, 8) and associated with adverse prognostic features (8, 9) and decreased overall survival (8, 10). Based on these findings, ODC is being targeted both for the treatment (11) and prevention (12) of breast cancer. Despite the well-documented growth inhibitory action of DFMO in numerous preclinical breast cancer models, the mechanisms mediating its antiproliferative effects in numerous preclinical breast cancer models remain largely unknown.

To address this issue, a major focus of this work was to evaluate the effects of DFMO on cell cycle–related events. We conducted our experiments in the hormone-independent MDA-MB-435 human breast cancer cell line in which we have recently shown that DFMO, in addition to inhibiting proliferation, significantly suppressed invasiveness in vitro and inhibited metastasis (13, 14).

In this experimental system, we have shown that the anti-invasive effect of DFMO was causally linked to activation of the mitogen-activated protein kinase (MAPK) pathway because DFMO increased extracellular signal-regulated kinase (ERK) phosphorylation and its anti-invasive action was reversed by the MAPK/ERK kinase (MEK) inhibitor PD98059 (15). Therefore, another major goal of these experiments was to test whether activation of the MAPK pathway also played a role in the antiproliferative effects of DFMO with specific reference to cell cycle–related events.

Materials and Methods

Cell culture and reagents. The hormone-independent human breast cancer cell line MDA-MB-435 was kindly provided by Dr. Janet Price (M.D. Anderson Cancer Center of the University of Texas, Houston, TX). The cells were cultured in DMEM/F12 medium (Life Technologies, Inc., Grand island, NY) supplemented with 5% fetal bovine serum, 1% nonessential amino acids, 1 mmol/L sodium pyruvate, and 0.52% sodium bicarbonate solution and maintained at 37°C in an incubator with a 5% CO2 humidified atmosphere. Cells were subcultured following incubation at 37°C for 5 minutes with 0.125% trypsin and 2 mmol/L EDTA in Ca2+/Mg2+-free Dulbecco’s PBS. DFMO was kindly supplied by Ilex Oncology (San Antonio, Texas). PD98059 was purchased from Calbiochem (San Diego, CA). U0126 was purchased from Cell Signaling Technology, Inc. (Beverly, MA).
3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium growth assay. Cells were plated at 1,000/100 μl of medium per well in 96-well plates. Following overnight incubation, the adherent cells were incubated under the experimental treatments indicated in the figure legends. The medium was changed every 2 to 3 days. At the end of the incubation time, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) solution (Cell Titer 96 AQ One Solution Cell Proliferation Assay, Promega Corp., Madison, WI) were added to each well separately and allowed to incubate for 3 hours at 37°C. Absorbance was determined with an ELISA multwell reader at 490 nm (16). For each experimental treatment, three replicate wells were used.

Cell cycle analysis. The cells were exposed to the experimental treatments indicated in the figure legends. The cells were washed and resuspended in cold PBS and incubated in ice-cold 70% ethanol on ice for 3 hours. The cells were then centrifuged at 1,500 rpm for 10 minutes and resuspended in PI master mix (40 μg/mL propidium iodide and 100 μg/mL RNase in PBS) at a density of 0.5 × 10^6/mL and incubated at 37°C for 30 minutes before analysis with flow cytometry.

Western blots. Cellular extract proteins (100 μg) were fractionated by 4% to 12% gradient SDS-PAGE gel electrophoresis and transferred to Immobilon membranes (Osmonics, Minnetonka, MN). Membranes were probed with the following primary antibodies, as indicated in the figure legends: anti-p21 (BD Biosciences PharMingen, San Diego, CA), anti-p27 (BD Biosciences PharMingen), anti-retinoblastoma (Rb) (BD Biosciences PharMingen), anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin-dependent kinase (cdk)-2 (Santa Cruz Biotechnology), anti-phospho-ERK-1/-2 (Promega), anti–ERK-1/-2 (Santa Cruz Biotechnology), and anti–h-actin (Sigma, St. Louis, MO). Goat anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP; Cappel, Aurora, OH) or goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) was used as secondary antibody. Antibody-bound proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Rockford, IL).

Cyclin-dependent kinase-2 immunoprecipitation studies and kinase assay. The cells were harvested in ice-cold cell lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 2 μg/mL antipan, 2 μg/mL leupeptin, 2 μg/mL apoatin, 1 μg/mL pepstatin A, 1 mmol/L Na3VO4, 1 mmol/L NaP2O7, 10 mmol/L NaF, 1 μmol/L okadaic acid, 1 mmol/L phenylmethylsulfonyl fluoride]. Five hundred micrograms of protein extract and 40 μL of cdk-2 primary antibody agarose conjugate (Santa Cruz Biotechnology) were incubated at 4°C for 2 hours with mixing. The pellets of all samples were divided evenly in two parts. One aliquot was washed four times with lysis buffer and resuspended in 40 μL of 2× protein sample buffer for Western blotting. Samples were boiled for 5 minutes and fractionated by 4% to 12% gradient SDS-PAGE gel electrophoresis and transferred to Immobilon membranes. The membranes were probed with anti–phospho-cdk-2(Thr160) (Cell Signaling), anti-cdk-2, anti-p21, or anti-p27 antibodies as described above. Another aliquot was used for the cdk-2 kinase assay. It was washed twice with lysis buffer and twice with kinase assay buffer (25 mmol/L HEPES, pH 7.4.

Figure 1. Effect of DFMO and/or PD98059 on cell proliferation and cell cycle. A, cell proliferation. MDA-MB-435 cells were incubated in the presence or absence of 1 mmol/L DFMO and/or 50 μmol/L PD98059 in 96-well plates. On days 0, 3, and 5, MTS assays were done and absorbance (OD) was determined with an ELISA multwell reader at 490 nm. Columns, mean of six replicate wells; bars, SD. *, P < 0.01, versus control (ANOVA followed by Dunnett’s multiple comparison test). B, cell cycle. MDA-MB-435 cells were incubated in the presence or absence of 1 mmol/L DFMO and/or 50 μmol/L PD98059 for 6 days and then were analyzed with flow cytometry. Columns, mean of three replicates; bars, SD. *, P < 0.01, versus control; †, P < 0.01, versus PD98059 + DFMO (ANOVA followed by Dunnett’s multiple comparison test). Three replicate experiments were done with similar results.
10 mmol/L MgCl2, 1 mmol/L EDTA, 0.1 mmol/L Na3VO4) and resuspended in 50 μL of kinase buffer containing 50 μmol/L cold ATP, 6 μCi [γ-32P]ATP, and 1 μg glutathione S-transferase (GST)-Rb (Santa Cruz Biotechnology) as a substrate. The kinase reaction was done at 30°C for 30 minutes and was stopped by adding 60 μL of 2× protein sample buffer. The samples were boiled for 5 minutes and loaded on 4% to 20% gradient SDS-PAGE gel. The gel was dried and exposed to X-ray film to check for phospho-Rb bands. The intensity of the bands was quantitated using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA).

Cyclin-dependent kinase-4 immunoprecipitation studies and kinase assay. The cells were harvested in ice-cold cell lysis buffer, then 2 mg protein extract and 40 μL cdk-4 primary antibody agarose conjugate (Santa Cruz Biotechnology) were incubated at 4°C for 2 hours with mixing. The pellets of all samples were divided evenly in two parts. One aliquot was washed four times with lysis buffer and resuspended in 40 μL of 2× protein sample buffer for Western blotting. Samples were boiled for 5 minutes and fractionated by 4% to 12% gradient SDS-PAGE gel electrophoresis and transferred to Immobilon membranes. The membranes were probed with anti-cdk-4 antibody. Another aliquot was used for the cdk-4 kinase assay. It was washed twice with lysis buffer and twice with kinase assay buffer and resuspended in 30 μL of kinase buffer containing 50 μmol/L cold ATP, 6 μCi [γ-32P]ATP, and 1 μg GST-Rb as a substrate. The kinase reaction was done at 30°C for 30 minutes and was stopped by adding 60 μL of 2× protein sample buffer. The samples were boiled for 5 minutes and loaded on 4% to 20% gradient SDS-PAGE gel. The gel was dried and exposed to X-ray film to check for phospho-Rb bands. The intensity of the bands was quantitated using a Phosphor-Imager.

Polynamine measurements. Cellular levels of polyanimes were measured by high-pressure liquid chromatography as previously described (13, 17).

Results

Effect of α-difluoromethylornithine and/or PD98059 treatment on cell proliferation and cell cycle. We have previously shown that inhibition of ERK phosphorylation with the MEK inhibitor PD 98059 reversed the anti-invasive effect of DFMO in the Matrigel assay (15). Therefore, we were interested in testing the effect of PD98059 on the antiproliferative effect of DFMO in MDA-MB-435 breast cancer cells in liquid culture. We chose the concentration 1 mmol/L DFMO because it has been found by us and other investigators to consistently suppress cellular polyamine levels in virtually every breast cancer cell line tested (see ref. 18 for review). As shown in Fig. 1A, DFMO and PD98059 individually significantly suppressed MDA-MB 435 cell proliferation. However, in contrast to our findings in the invasion assay, PD98059 administration did not reverse the antiproliferative effect of DFMO. To determine the treatment effects on cell cycle, MDA-MB-435 cells were incubated in the presence or absence of DFMO and/or PD98059 for 6 days and were then analyzed with flow cytometry. As shown in Fig. 1B, both DFMO and PD98059 individually significantly suppressed MDA-MB-435 cell proliferation. However, in contrast to our findings in the invasion assay, PD98059 administration did not reverse the antiproliferative effect of DFMO.

Figure 2. Effect of DFMO and/or PD98059 treatment on the expression of cdk inhibitors and p53. MDA-MB-435 cells were incubated in the presence or absence of 1 mmol/L DFMO and/or 50 μmol/L PD98059 for 1, 2, 3, 4, and 6 days. One hundred micrograms of cell extract proteins were fractionated by 4% to 20% gradient SDS-PAGE gel electrophoresis and then transferred to Immobilon membranes which were probed with antibodies against p15, p16, p21, p27, p53, and β-actin. Three replicate experiments were done with similar results.

Figure 2.
DFMO induction of p21 whereas it stimulated p27 (see below), which is known to cause a G1-S arrest but does not affect G2-M transition.

**Effect of α-difluoromethylornithine and/or PD98059 treatment on cell cycle–related proteins.** Because a major effect of DFMO and PD98059 on cell cycle was to induce a G1-S arrest, we were interested in evaluating the effects of these two compounds on the expression of cell cycle–related proteins involved in G1-S transition. As can be seen in the time-course study depicted in Fig. 2, DFMO treatment induced a marked increase in p21 expression, which was already detectable by day 3. This effect seemed to be p53 independent because the expression of p53 was not affected by DFMO until day 6, at which time it was actually decreased. DFMO also caused a dramatic increase in p15, but not p16, expression. In addition, as shown in Fig. 3A, on day 6, DFMO induced a decrease in cdk-2 expression and, to a greater degree, in the expression of cdk-2 phosphorylated on Thr160, the phosphorylation of which leads to activation of cdk-2. All these effects of DFMO were reversed by the MEK inhibitor PD98059. Administration of PD98059 alone induced a marked increase in p27 expression, which was already maximal on day 1 (Fig. 2). This effect was not influenced by DFMO. In agreement with our previous report (15), DFMO treatment increased ERK phosphorylation in a time-dependent fashion (Fig. 3A). As expected, this effect was completely blocked by administration of PD98059 (Fig. 3A).

Next, we tested the effect of DFMO and/or PD98059 on the expression of cyclin E, which positively regulates the activity of cdk-2. Our MDA-MB-435 cells expressed both the full-length and low molecular weight forms of cyclin E, which are biochemically hyperactive when complexed with cdk-2 (ref. 19; Fig. 3B). As can be seen in Fig. 3B, DFMO markedly suppressed both isoforms of cyclin E. Of interest, PD98059 by itself reduced cyclin E expression and did not reverse the effect of DFMO (Fig. 3B).

**Reversibility of the effects of α-difluoromethylornithine by putrescine and the mitogen-activated protein kinase/extra-cellular signal-regulated kinase kinase inhibitor U0126.** To show the specific involvement of the polyamine pathway in mediating the cell cycle effects of DFMO, we tested their reversibility with exogenous putrescine administration as well as the effects of the treatments on cellular polyamine levels. As can be seen in Fig. 4A, all the cell cycle effects of DFMO as well as its induction of ERK phosphorylation were completely reversed by the addition of putrescine. In addition, as shown in Fig. 4B, treatment with DFMO markedly suppressed the cellular levels of all

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**Figure 3.** Effect of DFMO and/or PD98059 treatment on cdk-2, cyclin E, and ERK. A, cdk-2 and ERK proteins and phosphorylation. MDA-MB-435 cells were incubated in the presence or absence of 1 mmol/L DFMO and/or 50 μmol/L PD98059 for 1, 2, 3, 4, and 6 days. One hundred micrograms of cell extract proteins were fractionated by 4% to 20% gradient SDS-PAGE gel electrophoresis and then transferred to Immobilon membranes, which were probed with antibodies against Thr160-phospho-cdk-2, cdk-2, phospho-ERK-1/-2, ERK-1/-2, and β-actin. Three replicate experiments were done with similar results. B, cyclin E. MDA-MB-435 cells were incubated in the presence or absence of 1 mmol/L DFMO and/or 50 μmol/L PD98059 for 6 days. One hundred micrograms of cell extract proteins were fractionated by 6% SDS-PAGE gel electrophoresis and then transferred to Immobilon membranes, which were probed with antibodies against cyclin E and β-actin. Three replicate experiments were done with similar results.
polyamines, which were replenished well above control level by the addition of putrescine. In these experiments, we further tested the role of the MAPK pathway in these cell cycle events by assessing the effects of another MEK inhibitor, U0126, given alone or in combination with DFMO. As can be seen in Fig. 4A, the effects of U0126 were virtually identical to those of PD98059 (compared with the corresponding conditions in Figs. 2 and 3), thus confirming and further strengthening the involvement of the MAPK pathway in these cell cycle perturbations. Of interest, treatment with U0126 alone also caused a modest suppression of polyamine levels.

Effect of α-difluoromethylornithine and/or PD98059 treatment on cyclin-dependent kinase-2 and cyclin-dependent kinase-4 activities. Following the results shown above, we were interested in testing the effects of DFMO and PD98059 on the activities of cdk-2 and cdk-4, their phosphorylation status, and their association with cdk inhibitors. Figure 5A shows the results on cdk-2. Following a 6-day incubation with the indicated treatments, the protein extracts were immunoprecipitated with the anti-cdk-2 antibody and separate aliquots of the same immunoprecipitates were used either for the kinase assay or Western blots. To compensate for the expected reduction in cdk-2 protein induced by DFMO (based on our previous experiments shown in Fig. 3A), we increased by 5-fold the amount of proteins from DFMO-treated cells in the immunoprecipitation reaction (lane b). By equalizing the amount of cdk-2 protein in the kinase assay, we were able to test specifically the treatment effects on cdk-2 activity. However, we were also interested in testing the overall effect of DFMO on cdk-2 activity, including the contribution from reduction in the protein. Therefore, we included a lane where no compensation for the decrease in protein was made (lane e). As can be seen in Fig. 5A, DFMO treatment markedly reduced cdk-2 protein (lane a versus lane e). Both DFMO and PD98059 markedly decreased cdk-2 activity, with the effect of DFMO being slightly superior (lane e versus lane d). The effect of DFMO was largely due to a decrease in cdk-2 protein because the specific effect on cdk-2 activity was relatively modest (lane a versus lane b). In contrast, the effect of PD98059 was entirely due to a decrease in activity because the level of cdk-2 protein was not affected (lane a versus lane d). Figure 5A also shows that DFMO and PD98059 induced association of cdk-2 with p21 and p27 respectively. Finally, both treatments reduced phosphorylation of cdk-2 on Thr160, an effect that would be expected to result in reduced activity. Cdk-4 activity, on the other hand, was not significantly affected by DFMO and/or PD98059 (Fig. 5B).

Effects of α-difluoromethylornithine and/or PD98059 on retinoblastoma phosphorylation and protein. As can be seen in Fig. 5C, DFMO treatment markedly reduced the expression and phosphorylation of Rb and increased the underphosphorylated Rb. Both effects, shown here on day 6, were already present after 3 days of treatment (data not shown). PD98059, on the other hand, increased the underphosphorylated Rb and reduced Rb phosphorylation but did not decrease the level of the protein. Furthermore, PD98059 reversed the effect of DFMO on Rb protein level but not on Rb phosphorylation.

Discussion

The polyamines are actively involved in promoting cell cycle progression. Both ODC and S-adenosylmethionine decarboxylase are activated in a biphasic manner during the cell cycle with a first peak in late G1 and a second peak at the S-G2 transition (20). Inhibition of polyamine biosynthesis has been shown consistently...

Figure 4. A, reversibility of the DFMO effects on cell cycle events by exogenous putrescine (2.5 mM) and U0126 (30 μmol/L). MDA-MB-435 cells were incubated in the absence and presence of the indicated treatment for 6 days. One hundred micrograms of cell extracts were fractionated by 4% to 20% SDS-PAGE gel electrophoresis and then transferred to Immobilon membranes, which were probed with antibodies against the indicated peptides. B, treatment effects on cellular polyamine levels.
to induce a G1-S arrest in both normal and transformed cells (21–23). In this report, we show that the polyamine biosynthetic inhibitor DFMO caused a G1-S arrest and a G2-M block also in MDA-MB-435 breast cancer cells.

Our results indicate that the cell cycle arrest induced by DFMO in MDA-MB-435 cells was due to a multifactorial decrease in cdk-2 activity leading to Rb hypophosphorylation. We observed that the major determinant to the reduction in cdk-2 activity was the decrease in cdk-2 protein induced by DFMO whereas specific inhibition of kinase activation contributed to a lesser extent. A decrease in cdk-2 activity and protein following DFMO administration has also been reported in the normal intestinal cell line IEC-6 (24). However, the relative contribution of these two factors to the overall decrease in cdk-2 activity was not assessed.

DFMO administration exerted several effects on cell cycle–related proteins involved in G1-S transition which would be expected to reduce cdk-2 activity. In agreement with previous reports in other experimental systems (22, 23), DFMO caused an early and striking increase in p21 expression. Furthermore, we show here that the drug induced p21 association with cdk-2. We believe that the increase in p21 is responsible for the DFMO-induced G2-M block because p21 has been shown to cause G2 arrest by blocking the interaction between CDC25C and the proliferating nuclear antigen (25–27). Induction of p21 has been shown to be both p53 dependent and independent (28). Our data are consistent with the p53-independent effect because DFMO did not increase p53 expression in our MDA-MB-435 cells. In other experimental systems, DFMO treatment has been shown to induce a parallel increase in p53 and p21 expressions (23). However, when the cause-effect relationship of these effects was tested, under conditions of polyamine depletion, p53 stimulation was not found to mediate the increase in p21 (22, 29).

Signal transducer and activator of transcription (STAT) proteins can recognize the sis-inducible element in the promoter of p21 and can increase its transcription (30). Nemoto et al. (22) reported that induction of p21 expression by DFMO in MKN45 human gastric cancer cells was mediated by increased phosphorylation and binding of STAT1 to the promoter region of p21 followed by activation of transcription. Of interest, we have observed that DFMO treatment of MDA-MB-435 cells caused a marked increase in tyrosine phosphorylation of STAT1 (31), thus raising the possibility that this may be at least one of the mechanisms by which DFMO increases p21 expression in our system.

**Figure 5.** Effect of DFMO and/or PD98059 treatment on cdk-2 and cdk-4 activities and Rb protein and phosphorylation. A, cdk-2 activity. MDA-MB-435 cells were incubated in the presence or absence of 1 mmol/L DFMO and/or 50 μmol/L PD98059 for 6 days and harvested. Five hundred micrograms of protein extract were immunoprecipitated with anti-cdk-2 antibodies. The pellets of all samples were divided evenly in two parts. One aliquot was resuspended in sample loading buffer and separated by electrophoresis on 4% to 12% gradient SDS-PAGE gel. After transfer, the blots were subjected to Western blot analysis using anti–phospho-cdk-2, anti-cdk-2, anti-p21, and anti-p27 antibodies. For the cdk-2-dependent kinase activity, the kinase activity in the other aliquot was assayed as detailed in Materials and Methods by using GST-Rb as a substrate. Reaction mixtures were separated onto 4% to 12% gradient SDS-PAGE gel and the gel was dried and autoradiographed. A replicate experiment gave similar results. B, cdk-4 activity. MDA-MB-435 cells were incubated in the presence or absence of 1 mmol/L DFMO and/or 50 μmol/L PD98059 for 6 days and harvested. Two milligrams of protein extract were immunoprecipitated with anti-cdk-4 antibodies. The pellets were processed as described above for either Western blot analysis using an anti-cdk-4 antibody or cdk-4 activity analysis using GST-Rb as a substrate. A replicate experiment gave similar results. C, Rb protein and phosphorylation. MDA-MB-435 cells were incubated in the presence or absence of 1 mmol/L DFMO and/or 50 μmol/L PD98059 for 6 days. One hundred micrograms of cell extract proteins were fractionated by 6% SDS-PAGE gel electrophoresis and then transferred to Immobilon membranes, which were probed with antibodies against Rb and β-actin, pRb, phosphorylated and highly phosphorylated Rb, pRb, underphosphorylated Rb.
Additional effects of DFMO observed in our system, which are expected to reduce cdk-2 activity, include inhibition of phosphorylation at Thr160 (a residue of which phosphorylation leads to activation of cdk-2) and down-regulation of cyclin E. Neither of these effects has been previously reported with DFMO treatment in any experimental systems. This latter effect is of particular translational significance because overexpression of cyclin E and its low molecular weight forms is an adverse prognostic factor for breast cancer patients (32, 33). The low molecular weight cyclin E forms, which are derived by posttranslational modification of the full-length cyclin E by the elastase class of serine proteases (34), are biologically hyperactive in breast cancer and induce resistance to p21, p27, and antiestrogens (35). Hence, it is noteworthy that DFMO suppressed the expression of these low molecular weight forms in addition to that of the full-length cyclin E.

We were unable to show any consistent effect of DFMO on cdk-4 activity despite a striking increase in the expression of p15, which would be expected to result in decreased activation of cdk-4. We are not aware of any reports of the effect of DFMO on cdk-4 activity in any system. Broaddus et al. (36) showed that DFMO treatment of uterine leiomyomas increased p16 (not affected by DFMO in our system), which would also be expected to reduce cdk-4 activity. However, the effect of DFMO on cdk-4 activity was not reported. Because cdk-4 activity was not affected by DFMO in our system, we did not pursue additional investigation of the possible effects of DFMO on the multiple events known to regulate the activity of this kinase.

A major goal of our experiments was to test the role of the MAPK pathway in mediating the effect of DFMO on cell cycle in breast cancer cells. Our interest in these experiments originates from our observation that DFMO markedly increases ERK phosphorylation in our system and that this effect mediates, at least in part, the anti-invasive effect of the drug (15). We confirm here the induction of ERK phosphorylation by DFMO and show the time dependency of this effect (Fig. 3A). We report here that inhibition of the MAPK pathway with the MEK inhibitors PD98059 and U0126 reversed some of the effects of DFMO on cell cycle while having other effects of its own. Most noticeably, both compounds blocked the induction of p21 by DFMO. This finding is in agreement with previous reports indicating that in certain circumstances, stimulation of the MAPK pathway can induce p21 and lead to cell cycle arrest (23, 29). Of particular relevance is the report by Bauer et al. (37) who also observed an inhibition of DFMO-induced p21 up-regulation with the MEK inhibitors PD98059 and U0126 in rat aortic smooth muscle cells. At variance with our findings, however, these authors observed a reversal of the antiproliferative effects of DFMO with these two compounds. However, because p21 expression was the only cell cycle event examined in that report (37), it is not possible to identify the potential differences in other cell cycle perturbations which may account for this discrepancy. We believe that, in our system, inhibition of p21 is responsible for the reversal by PD98059 of the DFMO-induced G2-M block because p21 is known to induce a G2 arrest. PD98059 and U0126, on the other hand, markedly up-regulated p27 and its association with cdk-2, contributing to the decrease in cdk-2 activity and the increase in underphosphorylated level of the Rb protein. A similar effect of PD98059 has been reported in fibrosarcoma and renal cell carcinoma cell lines (38), thus pointing to the critical role of the MAPK pathway in the regulation of p27 expression. In addition to up-regulating p27, both MEK inhibitors markedly down-regulated cyclin E and decreased cdk-2 phosphorylation at Thr160. In the latter effect, it is of interest that PD98059 and U0126, while having an inhibitory effect of their own, partially reversed the suppressive effect of DFMO on cdk-2 phosphorylation. In contrast to DFMO, neither PD98059 nor U0126 affected the level of cdk-2 protein and both reversed the down-regulation of cdk-2 induced by DFMO. Therefore, the reduction in cdk-2 activity induced by inhibition of MEK was entirely due to a decrease in kinase activity resulting from the increase in p27, decrease in cyclin E, and reduction in cdk-2 phosphorylation at Thr160. It is noteworthy that PD98059 reversed the down-regulation of Rb protein induced by DFMO. This finding is in line with the observation that induction of p21 has been shown to sharply down-regulate Rb protein (39). Therefore, it would be expected that PD98059 would restore Rb protein levels by blocking the DFMO-induced increase in p21. PD98059, on the other hand, did not reverse Rb dephosphorylation caused by DFMO because of its suppressive effect on cdk-2 activity. As a result, the G1-S block induced by DFMO was not reversed by PD98059, which caused a similar cell cycle arrest when given alone.

Our results point to the complex interaction between polyamines and the MAPK pathway in the regulation of cell cycle in breast cancer cells. Activation of the MAPK pathway by DFMO accounts for some of the major effects of DFMO such as induction of p21 and down-regulation of the cdk-2 protein, which are largely responsible for the suppression of cdk-2 activity. Inhibition of the MAPK pathway, however, does not reverse the antiproliferative effects of DFMO because of activation of alternative mechanisms that lead to suppression of cdk-2 activity such as p27 up-regulation.

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