Abrogation of p21 Expression by Flavopiridol Enhances Depsipeptide-Mediated Apoptosis in Malignant Pleural Mesothelioma Cells

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ABSTRACT

Purpose: Recent insights regarding the pathogenesis of malignant pleural mesothelioma (MPM) provide new opportunities for targeted molecular therapies for this highly lethal disease. The present study was undertaken to examine the effects of the histone deacetylase inhibitor, Depsipeptide (DP) FK228, in conjunction with the cyclin-dependent kinase inhibitor, Flavopiridol (FLA), in cultured MPM cells.

Experimental Design: Proliferation and apoptosis in drug-treated, virally transduced, or control cells were assessed by -D-ribofuranosyl-benzimidazole, or adenoviral p21 transduction. Western blot and ELISA techniques were used to examine signal transduction and cell cycle-related protein levels in MPM cells exposed to DP and/or FLA in the presence or absence of calphostin, phorbol-12,13-dibutyrate, 5,6-dichloro-1-\beta\-D-ribofuranosyl-benzimidazole, or adenoviral p21 transduction.

Results: DP (1–50 ng/ml × 6 h) or FLA (100–200 nM × 72 h) alone, mediated low-level, dose-dependent growth inhibition in MPM cells. In contrast, sequential DP/FLA treatment mediated marked growth inhibition and apoptosis in these cell lines. The cytotoxic effects of DP/FLA were considerably less pronounced in cultured normal cells. The proapoptotic effects of DP/FLA treatment coincided with inhibition of DP-mediated induction of p21 by FLA. Overexpression of p21 by adenoviral gene transfer techniques rendered MPM cells refractory to the cytotoxic effects of this treatment regimen. In p21 reporter assays, promoter activation by DP was antagonized by FLA. The magnitude of inhibition of DP-mediated p21 induction by FLA exceeded that observed with the pTEFb antagonist 5,6-dichloro-1-\beta\-D-ribofuranosyl-benzimidazole. Calphostin C abrogated p21 induction mediated by DP and enhanced DP-mediated apoptosis in a manner comparable with FLA in MPM cells; in contrast, phorbol-12,13-dibutyrate blocked FLA-mediated inhibition of p21 induction by DP and markedly protected these cells from the apoptotic effects of sequential DP/FLA.

Conclusions: FLA abrogates DP-mediated induction of p21 expression, in part, via inhibition of protein kinase C signaling and markedly potentiates the cytotoxic effects of DP in MPM cells.

INTRODUCTION

Malignant pleural mesothelioma (MPM) afflicts ~2500 Americans annually. The worldwide incidence of this disease has risen dramatically during the past 2 decades as a result of widespread exposure to asbestos. Recently, SV40 has been implicated in the pathogenesis of mesothelioma. Currently, the overall survival of mesothelioma patients ranges between 3 and 6%, despite aggressive multimodality intervention (1). These data highlight the need for a more comprehensive understanding of the molecular mechanisms contributing to mesothelioma and the development of innovative therapies for this disease.

Recently, considerable insight has been achieved regarding the molecular pathogenesis of mesothelioma. Crocidolite asbestos induces activation of epidermal growth factor receptor in cultured cells, resulting in activation of the transcription factor activator protein-1 and extracellular signal-regulated kinase (ERK)-dependent FRA-1 expression (2, 3). Although asbestos alone is insufficient to induce transformation of mesothelial cells, crocidolite markedly enhances transformation mediated by SV40 (4). Nearly 60% of mesotheliomas contain SV40 DNA sequences (5); despite low-level expression, SV40 oncoproteins contribute significantly to the malignant phenotype of pleural mesothelioma cells (6). SV40Tt antigen-positive mesothelioma cells exhibit aberrant methylation of the RASSF1A tumor suppressor gene (7), and SV40 T antigen induces expression of Notch-1, insulin-like growth factor-1, and HCF-MET, which mediate proliferation via ras-related pathways (8–10). Furthermore, SV40 T antigen sequesters retinoblastoma (Rb) as well as p53 (11), and SV40 T antigen enhances expression of cyclin D1 and phosphorylation of mitogen-activated protein kinases (12). These events, which typically occur in conjunction with allelic deletions that simultaneously inactivate p16\(^{n\kappaA}\) and p14\/^ARF\(^{(11)}\), result in complete abrogation of the Rb and p53 tumor suppressor pathways in mesothelial cells.

Previously, we reported that the synthetic cyclin-dependent kinase (cdk) inhibitor Flavopiridol (FLA) mediates cell cycle arrest and apoptosis in cultured mesothelioma cells, irrespective of SV40 Tt antigen expression (13). Growth inhibition in cul-
tured mesothelioma cells after FLA exposure coincides with diminished expression of phosphorylated Rb, cyclin D1, and Bcl-2 protein levels. Recently, we reported that the histone deacetylase (HDAC) inhibitor Depsipeptide (DP) FK228 mediates growth inhibition in cancer cells via acetylation of wild-type p53 and induction of p21 expression, with depletion of mutant p53, erbB1, erbB2, and RAF-1 protein levels, and diminished Erk1/2 signal transduction (14). These data extend studies by Sandor et al. (15), indicating that DP diminishes cyclin D1 and phosphorylated Rb protein levels in cancer cells.

In light of the fact that FLA and DP potentially target multiple, interrelated molecular defects typically observed in thoracic malignancies (13, 16, 17), we speculated that when administered together, these agents might exhibit additive, if not synergistic, cytotoxicity in mesothelioma cells. Herein, we report that under conditions potentially achievable in clinical settings, sequential DP/FLA exposure mediates profound cytotoxicity in mesothelioma cell lines but not cultured normal cells. These data support further analysis of the mechanisms regulating cell cycle arrest and apoptosis in cancer cells after sequential DP/FLA exposure and the clinical evaluation of FLA in conjunction with HDAC inhibitors for the treatment of thoracic malignancies.

**MATERIALS AND METHODS**

**Cells and Reagents.** MPM cells H513, H28, H2373, H2052, and REN were obtained from tissue culture banks at the National Cancer Institute (Bethesda, MD). Data concerning SV40, p53, p16, and p14/ARF expression in these cells are available on request. These cell lines were maintained in RPMI supplemented with glutamine (2 mM), antibiotics available on request. These cell lines were maintained in RPMI SV40, p53, p16, and p14/ARF expression in these cells are National Cancer Institute (Bethesda, MD). Data concerning malignancies.

DP/FLA exposure and the clinical evaluation of FLA in conjunction with HDAC inhibitors for the treatment of thoracic malignancies.

**Cytotoxicity, Caspase 3, and Apoptosis Assays.** Cells were seeded into 96-well microtiter plates and incubated overnight before drug treatment. For dose-dependent cytotoxicity assays, cells were treated with DP (1–50 ng/ml) for 6 h; after drug removal and one wash with drug-free complete RPMI, cells were further treated with FLA (50 or 100 nM) for 66 h. Viable cells were quantitated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. Combination index at 50% growth inhibition by DP and FLA was calculated as described previously (18). For time course experiments, cells were similarly treated with DP, FLA, or sequential DP/FLA; cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays at 24, 48, and 72 h after the onset of drug exposure. Sensitivity of tumor cells to DP (after DP treatment alone or sequential DP/FLA exposure) was determined by estimating the DP IC50 (DP doses that mediated 50% growth inhibition) from the dose-response curves. Appropriate corrections were made to account for the mild growth inhibitory effect of FLA when calculating DP IC50s of cells treated with the drug combination. Caspase 3 activity in MPM cells exposed to normal media, DP, FLA, or DP/FLA was determined by colorimetric techniques using protocols and reagents provided by R&D Systems.

Drug-induced apoptosis was assessed by Apo-bromodeoxyuridine techniques using reagents and protocols provided by Pharmigen (Torrence, CA). Briefly, cells were seeded in six-well plates at 2–3 × 104 cells/well and allowed to adhere overnight. Cells were then either treated with DP (50 ng/ml × 6 h), FLA (150 nM × 42 h), or sequential DP/FLA. Forty-eight h after initiation of drug treatment, cells were fixed with 1% formaldehyde and stored in 70% ethanol at −20°C until assayed for apoptosis.

**Quantitation of p21 Protein Expression.** p21 protein levels in untreated and drug-exposed MPM cells were evaluated using protocols and reagents contained in the p21 ELISA kit from Oncogene Science (Boston, MA). Briefly, cells were harvested 24 h after the onset of drug exposure (treatment conditions are detailed in respective figure legends); p21 protein levels were quantitated by ELISA, normalized for protein content of cell lysates, and expressed as units per milligram cellular protein.

**Adenovirus-Mediated p21 Gene Transfer.** The adenoviral vector [Ad-cytomegalovirus (CMV) p21] was purchased from the Keck Institute for Gene Therapy (Houston, TX). An adenoviral vector expressing green fluorescence protein (Ad-CMV-green fluorescence protein) was used to determine transduction efficiency of adenovirus in MPM cells. The adenoviral vector Ad-CMV-LacZ was used instead of Ad-CMV-green fluorescence protein for Ado-bromodeoxyuridine experiments to avoid interference of fluorescent green fluorescence protein-positive cells with FITC-labeled apoptotic cells during flow cytometry. Adenoviral vectors were titered by plaque-forming assays. For gene transfer experiments, MPM cells were grown in six-well tissue culture plates to 60% confluence, washed once with HBSS, and infected with adenoviral vectors at a multiplicity of infection of 10 or 30 plaque-forming units/cell in 1 ml of HBSS for 2 h at 37°C. Thereafter, HBSS was replaced with complete RPMI, and cells were incubated for ~48 h before being used in subsequent experiments.

**Western Blot Analysis of Protein Expression and Erk1/2 Kinase Activity.** Expression levels of a variety of signal transduction and cell cycle-related proteins were evaluated by Western blot techniques as described previously (14).
Murine monoclonal antibodies recognizing Rb, cyclin A, cyclin B, and cyclin E, as well as the rat monoclonal antibody recognizing cdk 4, were obtained from PharMingen. The rabbit polyclonal antiserum recognizing cyclin D was purchased from Upstate Biotechnology (Lake Placid, NY). Murine monoclonal antibodies recognizing cdk1, cdk2, and raf-1 were purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal antisera recognizing erbB1 and erbB2 were obtained from Cell Signaling Technology (Beverly, MA). Erk1/2 kinase activity in drug-treated and control mesothelioma cells was evaluated using the p44/42 mitogen-activated protein kinase assay kit and phosphorylation-specific rabbit antisera recognizing phospho-p44/42 and total p44/42 (Cell Signaling Technology).

Transfection Experiments Using p21 Reporter Constructs. The p21P SmaΔ2 (full-length p21 promoter from which the region −62 to −113 was deleted) and p21P 93-S (0 to −93) were described previously (19). REN cells were transfected with the p21 promoter-luciferase reporter constructs and pRL-TK Renilla luciferase vector (Promega, Madison, WI) using LipofectAMINE (Invitrogen, Carlsbad, CA). A full day after transfection, cells were exposed to normal media or DP (25 ng/ml) for 6 h. The medium was exchanged, and the cells were incubated for an additional 18 h in the presence or absence of FLA. Luciferase activity was assayed using a Packard luminometer and normalized to Renilla luciferase activity using the Dual-Luciferase Assay System (Promega).

Data Analysis. Data are presented as means ± SD. Statistical analysis when indicated was performed using Student’s t test, with P < 0.05 considered significant.

RESULTS
Synergistic Enhancement of DP-Mediated Cytotoxicity by FLA. Preliminary studies were undertaken to ascertain the effects of DP and FLA administered by various treatment regimens potentially achievable in clinical settings. This analysis revealed that cultured MPM cells exhibited a wide range of sensitivity to the cytotoxic effects of DP. Brief exposure of these cells to DP (1–50 ng/ml for 6 h) resulted in a dose-dependent inhibition of growth, with DP IC_{50}s ranging from 10 ng/ml (REN cells) to >1000 ng/ml (H2373 cells; Fig. 1A). Subsequent exposure of DP-treated MPM cells to FLA (50–200 nm) greatly enhanced the magnitude of growth inhibition in all of the mesothelioma cell lines, with the exception of H2052. The growth inhibitory effects of sequential DP/FLA were synergistic in REN, H513, H2373, and H28 cells, as evidenced by the fact that the degree of cytotoxicity observed in cells treated with the drug combination far exceeded the growth retardation mediated by either DP or FLA alone (Fig. 1A). Combination indices at 50% growth inhibition confirmed a significant, synergistic cytotoxic effect of sequential DP/FLA in these four cell lines (Fig. 1B). In DP-sensitive H2052 cells, the enhanced growth inhibition observed after DP/FLA 50 nm exposure relative to treatment with DP alone appeared totally attributable to FLA effect. In this cell line, higher doses of FLA appeared to antagonize DP-mediated cytotoxicity in H2052 cells.

The cytotoxic effects of the aforementioned sequential DP/FLA exposure were compared with several other DP and FLA treatment regimens. In general, the combined effects of 72-h continuous FLA exposure with concurrent 6-h exposure at the start of FLA treatment were inconsistent and less impressive than those observed after sequential DP/FLA administration. In addition, FLA did not enhance DP-mediated cytotoxicity when administered for 24 or 66 h before 6-h DP treatment (data not shown).

Additional experiments were undertaken to further examine the effects of sequential DP/FLA, which appeared to be the most consistent cytotoxic regimen in cultured MPM cells. Time course analysis indicated that although tumor cells treated with either DP or FLA alone exhibited varying degrees of growth retardation (Fig. 1C), cell death occurred in MPM cells treated with sequential DP/FLA, as evidenced by the rapid reduction of viable cells to levels well below those present at the beginning of the experiments.

To determine whether sequential DP/FLA would be toxic to normal cells, similar experiments were performed using semi-confluent normal human dermal fibroblasts and human umbilical vein endothelial cells, as well as peripheral blood mononuclear cells. Consistent with data reported previously regarding sensitivity of endothelial cells to HDAC inhibitors (20), this analysis revealed moderate growth inhibitory effects of DP in human umbilical vein endothelial cells; the addition of FLA subsequent to DP exposure resulted in additive, but not synergistic, growth inhibition in these endothelial cells. Sequential DP/FLA treatment mediated minimal cytotoxicity in normal fibroblasts and peripheral blood mononuclear cells (Fig. 1D).

Additional experiments were undertaken to further characterize the growth inhibitory effects of DP/FLA in MPM cells. Apo-bromodeoxyuridine analysis (Fig. 2) revealed that DP alone (50 ng/ml × 6 h) induced low-level apoptosis in two (H28 and H2373) of four cultured mesothelioma lines. Under exposure conditions used in the present study, FLA treatment alone (150 nM for 66 h) did not mediate discernable apoptosis in these cells. In contrast, sequential DP/FLA treatment induced profound apoptosis within 48 h of initiation of drug exposure in all four MPM lines. A clear, synergistic proapoptotic effect was observed in these cell lines when DP and FLA were administered in a sequential manner.

Modulation of Signal Transduction and Cell Cycle-Related Proteins by Sequential DP/FLA. To examine potential mechanisms by which sequential DP/FLA exposure mediates apoptosis in mesothelioma cells, Western blot techniques were used to evaluate expression levels of a variety of cell signaling and cell cycle-related proteins in REN and H513 cells after DP, FLA, or sequential DP/FLA exposure. As shown in Fig. 3, sequential DP/FLA treatment diminished erbB1, erbB2, and raf-1 protein levels in REN as well as H513 cells; these effects, which appeared attributable primarily to DP, coincided with decreased levels of phosphorylated (activated) ERK1/2 in these cells. In addition, sequential DP/FLA treatment diminished phosphorylated Rb, as well as cyclin A and B protein levels, in these mesothelioma lines. Modulation of phosphorylated Rb and cyclin A expression in MPM cells appeared related to DP exposure, whereas the dramatic decrease in cyclin B levels appeared attributable to DP as well as FLA. Sequential DP/FLA treatment diminished cyclin D and E levels in H513...
cells; these effects, which appeared attributable to FLA, were not readily apparent in REN cells.

Similar to other HDAC inhibitors, DP is a potent inducer of p21 expression in cancer cells (15, 21). Because FLA inhibits induction of p21 mediated by the phorbol ester phorbol 12-myristate 13-acetate, as well as the HDAC inhibitors suberoylanilide hydroxamic acid and sodium butyrate (22–24), additional experiments were performed to analyze p21 protein expression in MPM cells after DP, FLA, or sequential DP/FLA treatment. Preliminary Western blot analysis indicated that p21 levels in these cells rose within 6–12 h and peaked within 18–24 h after 6-h DP exposure (50 ng/ml; data not shown). Subsequent ELISA experiments revealed that DP (25 or 50 mg/ml × 6 h) mediated a dose-dependent, 5–20-fold increase in p21 protein levels in mesothelioma cells 24 h after the onset of drug exposure (Fig. 4). Interestingly, in DP-sensitive H2052 cells, DP mediated minimal (1.2–2.5-fold) induction of p21 protein expression. DP-mediated induction of p21 expression in

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**Fig. 1 A**, dose-dependent enhancement of Depsipeptide (DP)-mediated cytotoxicity of cultured malignant pleural mesothelioma cells H513, H28, H2373, H2052, and REN by Flavopiridol (FLA; 50, 100 nM). Tumor cells seeded in 96-well microtiter plates and exposed to various concentrations of DP (1–50 ng/ml for 6 h). After removal of DP, cells were treated with FLA for an additional 66 h. Cell viability was quantitated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays upon drug treatment. Data expressed as means ± SD of four independent experiments. **B**, combination indices of DP/FLA in malignant pleural mesothelioma cells. The sequential treatment regimen was synergistic in four of five malignant pleural mesothelioma cell lines. **C**, time course of the synergistic cytotoxic effect of sequential DP/FLA in H513, H28, H2373, and REN malignant pleural mesothelioma cells. Although DP or FLA alone decreased cell proliferation, significant cell death was observed in cells treated with the drug combination. Cell viability was quantitated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, and data are depicted as means ± SD of three independent experiments. **D**, effects of DP, FLA, or sequential DP/FLA in cultured normal cells. Although human umbilical vascular endothelial cells (HUVEC) were moderately sensitive to the cytotoxic effect of DP, this was not enhanced by subsequent exposure to FLA. The data are depicted as means ± SD of three independent experiments.
MPM cells was abrogated by FLA (150 nM) administered continuously for 18 h after DP exposure. Collectively, these data indicate that FLA markedly abrogates p21 induction by DP in mesothelioma cells.

**Modulation of Apoptosis in MPM Cells by p21.** Several recent studies have indicated that abrogation of p21 expression enhances sensitivity of cancer cells to the cytotoxic effects of HDAC inhibitors (15, 23). To ascertain the role of p21 in modulating apoptosis induced by DP and sequential DP/FLA, mesothelioma cells were transduced with an adenoviral vector expressing p21 (Ad-CMV- p21) or a control vector (Ad-CMV-LacZ) 24 h before treatment with DP, FLA, or sequential DP/FLA. Preliminary experiments using the Ad-CMV-green fluorescence protein vector demonstrated reporter gene expression in 100% of MPM cells within 24 h after adenoviral infection at multiplicity of infections of 10 or 30 plaque-forming units/cell (data not shown). Western blot analysis of cell lysates harvested 48 h after adenoviral infection revealed that, at comparable multiplicities of infection, Ad-CMV-p21 mediated robust p21 expression in all of the mesothelioma cell lines. In contrast, the Ad-CMV-LacZ control vector did not affect p21 expression in these cells (representative data for H2373 and H28 cells are shown in Fig. 1).
REN cells depicted in Fig. 5A). ELISAs indicated that expression of p21 in transduced cells was unaffected by subsequent treatment with DP, FLA, or sequential DP/FLA (data not shown). Overexpression of p21 totally abrogated the profound proapoptotic effects of sequential DP/FLA in mesothelioma cells (Fig. 5B). In contrast, Ad-CMV-LacZ could not rescue mesothelioma cells from apoptosis mediated by DP/FLA. Collectively, these data indicate that p21 expression markedly affects sensitivity of mesothelioma cells to sequential DP/FLA treatment.

Mechanisms Regulating p21 Expression in MPM Cells Exposed to DP or Sequential DP/FLA. In light of the fact that p21 expression regulated sensitivity of mesothelioma cells to DP/FLA, additional studies were performed to examine the mechanisms by which FLA inhibited DP-mediated induction of p21. Recent studies from our lab have indicated that DP/FLA induces apoptosis in lung and esophageal cancer cells via caspase-dependent mechanisms (25). Hence, experiments were performed to examine whether FLA-associated inhibition of DP-mediated induction of p21 was secondary to caspase activation in MPM cells, because caspase 3 is known to target p21 during apoptosis (26). Preliminary experiments confirmed that sequential DP/FLA treatment markedly enhanced caspase 3 activity in MPM cells (Fig. 6A). Subsequent experiments (Fig. 6B) demonstrated that neither the general caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone (40 \( \mu \)M) nor the selective caspase 3 inhibitor (z-DEVD-fmk; 40 \( \mu \)M) abrogated FLA-associated inhibition of DP-mediated induction of p21 expression in REN and H513 mesothelioma cells. These results indicated that FLA diminished p21 levels in DP-treated MPM cells via caspase-independent mechanisms.

Previous studies have indicated that the HDAC inhibitor, apicidin, enhances p21 expression in cancer cells by transcriptional mechanisms mediated via SP1 (27). To determine whether the changes in p21 expression mediated by DP or FLA in MPM cells were attributable to transcriptional mechanisms, REN cells were transiently transfected with the p21 promoter reporter plasmids 93S or Smaa2 24 h before DP, FLA, or sequential DP/FLA treatment. As shown in Fig. 7A, DP medi-
ated a 4-fold activation of the 93S p21 promoter sequence. Although FLA did not appear to diminish basal expression of the reporter construct, this agent completely abrogated DP-mediated activation of the p21 promoter, results which were consistent with the p21 ELISA data discussed previously. Deletion of SP1-binding sites within the 93S promoter region diminished p21 promoter activation by DP. Collectively, these data indicated that DP and FLA modulate p21 expression via transcriptional mechanisms in MPM cells.

Because recent data have indicated that FLA is a potent antagonist of pTEFb (28), additional experiments were performed to determine whether abrogation of DP-mediated induction of p21 expression by FLA was attributable simply to global transcriptional inhibition. After 6-h DP exposure, REN cells were treated for 18 h with DRB or FLA at doses demonstrated previously to be comparable with respect to inhibition of pTEFb activity in cancer cells (28). Under these exposure conditions, DRB alone exhibited no significant inhibition of basal p21 protein levels; however, this pTEFb antagonist mediated a 40–50% reduction in p21 protein levels in DP-treated cells (Fig. 7B). In contrast, FLA inhibited basal expression of p21 by 16–44% and diminished DP-mediated p21 protein expression by 50–75% relative to untreated controls. The magnitude of inhibition of p21 expression by FLA was ~2-fold higher than that mediated by DRB across concentration ranges shown previously to inhibit pTEFb activity in cancer cells (28, 29). These data raised the possibility that abrogation of DP-mediated p21 expression by FLA was not attributable solely to inhibition of pTEFb activity in mesothelioma cells.

Because the HDAC inhibitor, apicidin, induces p21 expression by PKC pathways mediated via SP1 (27), additional experiments were performed to examine whether modulation of
PKC signaling influenced DP-mediated induction of p21 expression in MPM cells. REN and H513 cells were treated for 1 h with either the PKC inhibitor calphostin C (5 μM) or PKC agonist PDBu (100 or 200 nm) before DP exposure (50 ng/ml × 6 h); after removal of DP, cells were incubated in complete RPMI with calphostin or PDBu for an additional 18 h. Concurrent experiments using sequential DP/FLA were performed as well. As shown in Fig. 7C, calphostin inhibited DP-mediated induction of p21 to an extent comparable with or exceeding that observed after FLA treatment. In contrast, PDBu alone markedly induced p21 protein expression (albeit to a lesser extent than DP) and abrogated the inhibitory effects of FLA on p21 expression in DP-treated MPM cells. These data, in conjunction with the results of the promoter reporter experiments, strongly suggested that DP induces p21 expression in MPM cells via PKC signal pathways and that FLA-mediated abrogation of p21 induction by DP may be attributable, in part, to inhibition of PKC signaling.

In light of the fact that calphostin and PDBu markedly influenced p21 expression in DP-treated MPM cells, additional experiments were undertaken to ascertain the role of PKC signaling in modulating DP-mediated cytotoxicity in these cells (data pertaining to REN are depicted in Fig. 8). Interestingly, the magnitude of apoptosis observed in mesothelioma cells treated with DP/calphostin was comparable with that seen after exposure to sequential DP/FLA. DRB as well as the PKC inhibitor GF-109203X (2 μM) also potentiated DP-mediated cytotoxicity in MPM cells. In contrast, PDBu protected MPM cells from apoptosis induced by sequential DP/FLA. The differential proapoptotic effects of calphostin and PDBu were consistent with the effects of these agents on p21 expression in DP-treated cells. Collectively, these data strongly suggest that abrogation of PKC signaling contributes to the dramatic enhancement of DP-mediated cytotoxicity by FLA in mesothelioma cells.

DISCUSSION

MPMs pose considerable challenges for clinical oncologists because of their insidious nature and refractoriness to conventional multidisciplinary treatment regimens. Although the global impact of MPM will increase substantially during the next two decades, recent insights into the mechanisms contributing to the pathogenesis of MPM provide new opportunities for the rational development of targeted molecular therapies for this disease (13).

In the present study, we examined the effects of the HDAC inhibitor, DP, in conjunction with the cdk inhibitor, FLA, in a panel of well-characterized MPM lines; these experiments were initiated because these two novel anticancer agents potentially target multiple molecular defects induced by asbestos and SV40 cocarcinogens implicated in the pathogenesis of MPM (4, 30). Our analysis revealed that under exposure conditions potentially achievable in clinical settings, FLA markedly potentiated the cytotoxic effects of DP in cultured MPM cells irrespective of SV40 T/antigen, p53, p14/ARF, and p16 expression status. The proapoptotic effects of sequential DP/FLA appeared attributable to abrogation of DP-mediated p21 expression by FLA, as evidenced by the fact that overexpression of p21 by an adenoviral vector protected these cells from cytotoxicity mediated by sequential DP/FLA treatment.

Although abrogation of p21 expression by FLA coincided with marked enhancement of DP-mediated cytotoxicity in MPM cells, the precise mechanisms responsible for this phenomenon remain unclear. p21 modulates the activity of multiple intracellular proteins, and accumulating evidence indicates that p21 is a major negative regulator of p53-dependent as well as p53-independent apoptosis (31). p21 exists in a quaternary complex with proliferating cell nuclear antigen, cyclin D, and cdkks in normal cells (32). Interaction of p21 with proliferating cell nuclear antigen results in inhibition of DNA synthesis by DNA polymerase δ (33). By mediating cell cycle arrest, p21 may function to antagonize the cytotoxic effects of conventional chemotherapeutic agents, as well as a variety of other novel agents, including FLA and HDAC inhibitors (15, 34, 35). p21 inhibits apoptosis in p53-deficient cancer cells after transduction with a recombinant adenovirus expressing p53 (36). These observations may be particularly relevant regarding the role of p21 in modulating apoptosis in mesothelioma cells after DP exposure. The majority of mesothelioma cells express wild-type p53,
Fig. 7 A, promoter activity of transiently transfected smaΔ2 and 93S p21 reporter constructs in REN cells after exposure to normal media, Depsipeptide (DP), Flavopiridol (FP), or sequential DP/FP. Data are representative of two independent experiments. B, p21 protein levels in REN cells after exposure to DP, Flavopiridol (FLA), DP/FLA, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB), or DP/DRB, as determined by ELISA techniques. The magnitude of inhibition of DP-mediated p21 expression by FLA exceeded that of the prototypic pTEFb inhibitor, DRB. C, p21 protein levels in REN cells after exposure to DP, FLA, DP/FLA, calphostin, DP/calphostin, phorbol-12,13-dibutyrate (PDBu), or PDBu/DP/FLA. DP-mediated induction of p21 was clearly abrogated by the protein kinase C inhibitor calphostin. Conversely, the PKC agonist PDBu blocked FLA-mediated inhibition of p21 protein expression in DP-treated cells.
which is frequently sequestered by SV40 T antigen (37). Our previous studies have demonstrated that DP mediates phosphorylation and acetylation of p53; these post-translational modifications are known to enhance the stability and DNA-binding activities of this tumor suppressor protein (14, 38, 39). In addition, p21 suppresses activation of procaspase 3 by masking its serine protease cleavage site (40). Survivin, a member of the inhibitors of apoptosis protein family, initiates p21/procaspase 3 complex formation, which occurs in the mitochondria and requires phosphorylation of p21 by protein kinase A (41, 42); phosphorylation of p21 within the procaspase 3/p21 complex renders cancer cells resistant to Fas-mediated apoptosis (43). Because FLA inhibits protein kinase A signaling under exposure conditions used in our experiments (44), it is conceivable that abrogation of phosphorylation of p21 by protein kinase A signaling under exposure conditions used in our experiments (44), it is conceivable that abrogation of phosphorylation of p21 alters its ability to sequester procaspase 3 in the mitochondria. In addition, FLA inhibits phosphorylation of survivin, which is required to maintain expression of this antiapoptotic protein in cancer cells (45); inhibition of p34cdc2 kinase activity decreases survivin levels and dramatically enhances chemotherapy-induced cytotoxicity in vivo (45). Furthermore, apoptosis mediated by the novel HDAC inhibitor, chlamydacin, coincides with proteosome-mediated degradation of survivin in cancer cells (46). Although not formally demonstrated in our experiments, it is quite likely that disruption of p21/procaspase 3 complex formation contributes to potentiation of DP-mediated cytotoxicity by FLA in mesothelioma cells.

In additional studies, we have observed that sequential DP/FLA treatment decreases expression of a variety of cell signaling and cell cycle-related proteins in MPM cells. Although some of these effects may be attributable to caspase activation, the precise mechanisms underlying these alterations have not been fully defined and are the focus of ongoing experiments in our laboratory. Rosato et al. (24) noted that sodium butyrate/FLA treatment diminished levels of phosphorylated Rb and cyclins A, D, and E in human leukemia cells. In our study, we noted similar changes in MPM cells after sequential DP/FLA exposure; in addition, we observed significant decreases in cyclin B expression levels. These effects, in conjunction with FLA-mediated inhibition of cdks (28), would be expected to significantly disrupt cell cycle progression and p21/procaspase 3 complex formation via multiple mechanisms in cancer cells. In addition, we observed that sequential DP/FLA treatment diminished erbB-1, erbB-2, and ras-1 protein levels, and decreased activation of Erk1/2 in MPM cells. These findings may be particularly relevant with regard to the clinical management of MPM patients. Human mesotheliomas express epidermal growth factor receptor, which is known to mediate asbestos-induced proto-oncogene expression (2, 47); inhibition of epidermal growth factor receptor by ZD1835 inhibits mitogen-activated protein kinase signaling, proliferation, and clonogenicity of cultured MPM cells (48). These observations clearly establish the relevance of targeting HDAC inhibitors in the treatment of mesothelioma.

Experiments presented in this study indicate that in addition to inhibiting p21 expression, sequential DP/FLA treatment decreases expression of a variety of cell signaling and cell cycle-related proteins in MPM cells. Although some of these effects may be attributable to caspase activation, the precise mechanisms underlying these alterations have not been fully defined and are the focus of ongoing experiments in our laboratory. Rosato et al. (24) observed that FLA inhibits p21 induction, and markedly potentiates cytotoxicity mediated by sodium butyrate in leukemia cells; apoptosis coincides with diminished Bcl-2, Mcl-1, and X-linked inhibitor of apoptosis protein levels after combination drug treatment. Collectively, these data indicate that depletion of a variety of antiapoptotic proteins and disruption of mitochondrial integrity contribute to FLA-mediated potentiation of cytotoxicity by HDAC inhibitors in cancer cells.

Fig. 8 Apo-bromodeoxyuridine analysis of apoptosis in REN mesothelioma cells after exposure to normal media, Depsipeptide (DP), Flavopiridol (FLA), or sequential DP/FLA with or without calphostin or phorbol-12,13-dibutyrate (PDBu). Data clearly demonstrate that calphostin potentiates DP-mediated cytotoxicity in malignant pleural mesothelioma cells. Conversely, PDBu completely protected REN cells from apoptosis after sequential DP/FLA treatment. Results are representative of two independent experiments. p21 protein levels depicted in Fig. 7B were simultaneously analyzed in these treated cells.
vance of epidermal growth factor receptor signaling in MPM and highlight the potential impact of targeting this pathway in this disease. ErbB1 and erbB2 mediate signal transduction via Erk as well as AKT pathways, which are known to induce proliferation and resistance to genotoxic stress in cancer cells; as such, it is possible that depletion of these oncoproteins also contributes to growth inhibition and apoptosis in MPM cells mediated by sequential DP/FLA treatment (14, 49).

Although several publications have demonstrated that p21 expression coincides with diminished cytotoxicity mediated by HDAC inhibitors in cancer cells (15, 23), the mechanisms by which FLA modulates p21 expression have not been fully defined. Our experiments clearly indicate that FLA diminishes p21 via caspase-independent mechanisms; these results are consistent with data reported by Almenara et al. (23) and Cartee et al. (22) regarding FLA-mediated inhibition of p21 induction in human leukemia cells after exposure to suberylanilide hydroxamic acid or phorbol 12-myristate 13-acetate, respectively. FLA is a potent inhibitor of pTEFb, and the effects of this agent are particularly pronounced regarding expression of genes, such as p21, which encode transcripts with short half-lives (28, 29). Although pTEFb levels were not directly examined in our present experiments, our data indicate that the extent of p21 transcriptional inhibition by FLA in MPM cells exceeded that observed after exposure to the prototypic pTEFb inhibitor, DRB, across concentration ranges shown previously to be comparable with regard to inhibition of pTEFb activity (28); these data suggest that inhibition of pTEFb activity alone is insufficient to account for the effects of FLA on p21 expression in DP-treated cancer cells. Indeed, our analysis indicates that DP modulates p21 expression via PKC signaling mediated via S1p sites within the p21 promoter. These observations are consistent with data reported by Han et al. (27) demonstrating that apicidin mediates p21 induction via PKCe, and that calphostin inhibits apicidin-induced translocation of PKCe as well as p21 induction in HeLa cells. PKCe abrogates doxorubicin- or etoposide-induced apoptosis by inhibiting mitochondrial dependent caspase activation in lung cancer cells (50). Although expression levels of PKC isoforms in human MPM have not been systematically evaluated, inhibition of PKC signaling abrogates asbestos-induced proto-oncogene expression in cultured mesothelial cells (51). Interestingly, under the exposure conditions used for the present study, calphostin mediated no significant apoptotic effects in MPM cells, suggesting that abrogation of PKC signaling alone is insufficient to mediate growth arrest and apoptosis in these cells. On the other hand, calphostin enhanced DP-mediated apoptosis in these cells to an extent comparable with FLA administered at a concentration exceeding that which has been shown previously to inhibit PKC activity in cancer cells (44). Equally important, PDBu protected MPM cells from apoptosis after sequential DP/FLA exposure. Collectively, these data indicate that modulation of PKC signaling by FLA may contribute to potentiation of DP-mediated apoptosis in mesothelioma cells, and suggest that the utilization of PKC antagonists in conjunction with HDAC inhibitors may represent a novel treatment strategy for mesothelioma. Experiments are under way to further examine this issue.

DP and FLA are currently being evaluated in clinical trials. After administration as a 6-h infusion at the maximum tolerated dose, DP has exhibited limited activity in patients with solid tumors; however, plasma DP levels exceeding those used in our experiments have been readily achieved in these patients without significant systemic toxicities (52). When administered at a dose of 50 mg/m²/day × 3 days, FLA has exhibited minimal activity in patients with lung or renal cell carcinomas; steady-state plasma FLA concentrations ≥ 200 mT have been achieved in these individuals without significant hematological toxicities (53). Although data pertaining to the effects of sequential DP/FLA in animal tumor models have not been presented in this study, the pronounced and preferential cytotoxicity of this treatment regimen in cultured cancer cells and potential lack of overlapping clinical toxicities of DP and FLA provide compelling rationale for evaluation of sequential DP/FLA infusion in mesothelioma patients.

REFERENCES


Abrogation of p21 Expression by Flavopiridol Enhances Depsipeptide-Mediated Apoptosis in Malignant Pleural Mesothelioma Cells


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