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ORIGINAL ARTICLE

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Gemcitabine induces programmed cell death and activates protein kinase C in BG-1 human ovarian cancer cells

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Abstract *Purpose*: Cytosine arabinoside induces apoptosis and this cell death process is influenced by protein kinase C signaling events in leukemic cells. We present findings that extend these observations to include another deoxycytidine analog, gemcitabine, which is more potent in solid tumors. *Methods and results*: Gemcitabine induced programmed cell death in BG-1 human ovarian cancer cells based on biochemical and morphologic analyses. The DNA was fragmented in BG-1 cells exposed to gemcitabine (0.5 μ*M*, 1.0 μ*M* and 10 μ*M*) for 8 h, but gemcitabine treatment did not induce internucleosomal DNA degradation. Scanning and transmission electron microscopy of BG-1 cells showed morphologic changes associated with apoptosis in response to gemcitabine: membrane blebbing, the formation of apoptotic bodies and chromatin condensation. Thus, BG-1 cells undergo programmed cell death in response to gemcitabine treatment without internucleosomal DNA fragmentation. Furthermore, gemcitabine (10 μ*M*) activated protein kinase C in BG-1 cells and the phosphorylation of the endogenous protein kinase C substrate, myristoylated alanine-rich C kinase substrate, was increased following exposure of BG-1 cells to gemcitabine for up to 6 h. Clonogenicity studies with gemcitabine in combination with various protein kinase C-modulating agents demonstrated that gemcitabine cytotoxicity was influenced by protein kinase C signaling events in BG-1 cells. Short-term (1 h) exposure to TPA (1 or 10 n*M*) followed by gemcitabine (0.5 μ*M* for 4 h) did not alter the response to gemcitabine. However, a 24-h exposure to TPA followed by gemcitabine resulted in synergistic cytotoxicity, while coincubation of TPA with a PKC inhibitor (e.g. bisindolylmaleimide or calphostin-C) in this regimen abrogated the synergistic response. *Conclusions*: Based on our findings, it is plausible that gemcitabine therapy could be improved by modulating PKC signaling events linked to drug-induced apoptosis/cytotoxicity.

Key words Gemcitabine · Programmed cell death · Protein kinase C · Ovarian cancer

Abbreviations *ara-C* 1-β-D-arabinofuranosylcytosine · *bisM* bisindolylmaleimide · *cal-C* calphostin-C · *CI* combination index · *dCK* deoxycytidine kinase · *dFdC* 2′,2′-difluorodeoxycytidine · *DG* diglyceride · *DMEM* Dulbecco's modified Eagle's medium · *FBS* fetal bovine serum · *MARCKS* myristoylated alanine-rich C kinase substrates · *NaF* sodium fluoride · *PCD* programmed cell death · *PKC* protein kinase C · *PMSF* phenylmethylsulfonyl fluoride · *SDS* sodium dodecyl sulfate · *SEM* scanning electron microscopy · *TBS* Tris-buffered saline · *TEM* transmission electron microscopy · *TPA* 12-*O*-tetradecanoylphorbol-13-acetate

Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine; dFdC) is a novel antimetabolite in clinical development that exhibits a broad spectrum of activity against leukemic cell lines and solid tumors (e.g. ovarian, small-cell lung, squamous carcinoma of the head and neck, breast, pancreatic and colorectal) in murine models and human xenografts [5]. The drug is a deoxycytidine analog with two fluorine atoms substituted for the two hydrogen atoms at the 2′-position of the deoxyribose sugar moiety [35]. Although its mechanism of action is not completely understood, dFdC is a prodrug that must be activated by deoxycytidine kinase (dCK) ultimately leading to the formation of the active metabolite, dFdCTP. The cytotoxicity of dFdCTP is attributed to its ability to inhibit DNA synthesis by competing with dCTP for incorporation into the growing DNA strand [29]. Furthermore,

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dFdC is inactivated by cytidine deaminase which catalyzes the conversion to dFdU [4]. The activation and deamination of dFdC are saturable enzymatic processes. The initial phosphorylation of dFdC by dCK is rate-limiting and cells deficient in dCK, or those expressing high levels of cytidine deaminase, are resistant to dFdC. DFdC inhibits deoxycytidine monophosphate deaminase and ribonucleotide reductase to ultimately reduce dNTP concentrations (particularly dCTP) [17, 18]. These properties are self-potentiating with respect to the mechanism of action for dFdC. Studies have demonstrated that the cytotoxicity of the drug is strongly correlated with the amount of dFdC incorporated into the cellular DNA up to the maximum achievable intracellular concentration of 20 μ*M* [29, 35]. Furthermore, analog incorporation has been shown to be a critical event preceding the induction of apoptosis by dFdC in a human T lymphoblastoid cell line [22].

DFdC has structural and metabolic similarities to cytosine arabinoside (1-β-D-arabinofuranosylcytosine; ara-C) [15]. Both dFdC and ara-C require intracellular phosphorylation to form their cytotoxic triphosphate, dFdCTP or ara-CTP, respectively. Studies in our laboratory with mouse L5178Y leukemia cells have indicated that dFdC activates protein kinase C (PKC) via reversal of the cholinephosphotransferase pathway in a manner similar to ara-C [28]. When dFdC and ara-C reverse the forward reaction of cholinephosphotransferase, diglyceride (DG) and dFdCDP-choline or ara-CDP-choline are formed. The DG generated by this pathway activates PKC, which then phosphorylates dCK to increase the V_{max} of this enzyme [39]. The rate-limiting enzyme for the conversion of ara-C and dFdC to their monophosphate forms is dCK. Thus, reversal of the cholinephosphotransferase pathway is self-potentiating for the activation of dFdC and ara-C. In fact, the apoptosis induced by ara-C is preceded by the activation of PKC [16, 27], and growth factors (e.g. granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor and interleukin-3) or other agents (e.g. bryostatin 1, phorbol esters) which modulate PKC activity can enhance ara-C cytotoxicity in leukemic cells [1–3, 9, 21]. The effects of these agents on ara-C cytotoxicity are dose- and schedule-dependent.

We postulated that PKC signaling would influence dFdC cytotoxicity based on observations that PKC signaling modulates apoptotic cell death signaling in many cell lines [30] and previous studies with ara-C [1–3, 9, 21]. The PKC mechanisms associated with ara-C cytotoxicity have been characterized in leukemia cells. The goal of this investigation was to determine whether PKC-associated phenomena would apply to dFdC-induced cytotoxicity in an ovarian tumor cell line because understanding this association could improve dFdC efficacy in the clinical setting. We selected the BG-1 human ovarian cancer cell line [12] for these studies because BG-1 cells produce the ovarian tumor antigen CA 125, express estrogen and progesterone receptors, and exhibit karyotypic features often associated with ovarian carcinoma. We established that dFdC induces a cell death program in BG-1 ovarian cancer cells that exhibits some, but not all of the classical features of apoptosis. We also demonstrated that dFdC activates PKC in BG-1 cells and that dFdC efficacy is increased when combined with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent PKC-activating agent, under appropriate scheduling conditions. These novel findings suggest that the efficacy of dFdC may be improved by agents that modulate PKC signal transduction in the clinical setting.

Materials and methods

Chemicals and reagents

The nucleoside analog dFdC was supplied by Lilly Research Laboratories (Indianapolis, Ind.) and ara-C was purchased from the Upjohn Company (Kalamazoo, Mich.). The PKC inhibitors bisindolylmaleimide (bisM) and calphostin C (cal-C) were purchased from Calbiochem (Cambridge, Mass.). Proteinase K, RNase (DNase-free), high molecular weight protein standards, agarose and TPA were purchased from Sigma Chemical Co. (St Louis, Mo.). The DNA standards were supplied by Gibco BRL (Melville, N.Y.). Carrier-free $[^{32}P]_i$ was obtained from ICN Radiochemicals (Irvine, Calif.). Phenol was supplied by Ambion (Austin, Tx.), and other organic solvents were purchased from Fisher Scientific (Norcross, Ga.). All other chemicals were purchased from Sigma Chemical Company.

Cell culture

BG-1 human ovarian cancer cells and HL-60 cells were maintained in RPMI-1640 (Gibco BRL, Grand Island, N.Y.) supplemented with 15% or 10% heat-inactivated fetal bovine serum (FBS), respectively. The cells were kept in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Cell doubling time was approximately 24 h under these culture conditions and the cells tested negative for mycoplasma contamination. Cell numbers were measured with a Coulter counter (Coulter Electronics, Hialeah, Fl.). All studies were performed with cells growing in log phase.

DNA fragmentation assay

To detect internucleosomal DNA fragmentation, DNA was purified from control and drug-treated BG-1 or HL-60 cells (3×10^6 cells/sample). Cells were washed with phosphate-buffered saline and lysed in 400 μl buffer (0.1% NP-40, 20 m*M* EDTA, 10 m*M* Tris, pH 7.4) containing 0.1 mg/ml ribonuclease A. The cell lysates were incubated at 37 °C for 30 min and, following the addition of 0.5 mg/ml of proteinase K, incubated for an additional 12 h at 55 °C. DNA was isolated from proteins by phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) extraction and the aqueous layer reextracted with chloroform/isoamyl alcohol (24:1 v/v). The aqueous phase was transferred to a microcentrifuge tube, 0.3 *M* sodium acetate and 10 m*M* $MgCl_2$ added, and the DNA precipitated in two volumes of ethanol for 24 h at −70 °C. Samples were centrifuged at 12 000 *g* for 30 min at 4 °C, the supernatant removed, and the DNA pellets dried in a Speedvac evaporator (Savant Instruments, Farmingdale, N.Y.). The pellets were washed with 70% ethanol and the DNA pellet was resuspended in 20 μl TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8.0) and quantified by spectrofluorometry. Sample buffer (0.25% bromophenol blue, 100 m*M* EDTA, 30% glycerol, 0.1% SDS) was added and 1 μg DNA per sample was loaded onto a 2% agarose gel stained with ethidium bromide. The DNA was fractionated at a constant

voltage of 5 V/cm and visualized under UV illumination using an IS-500 Digital Imaging System manufactured by Alpha Innotech Corporation (Torrence, Calif.).

Electron microscopy

Control and drug-treated samples of BG-1 cells (5×10^5/ml) were designated for either scanning electron microscopy (SEM) or transmission electron microscopy (TEM). Both TEM and SEM samples were fixed in 2.5% glutaraldehyde and 0.1 *M* phosphate buffer, rinsed, and dehydrated through an ethanol series (25% to 100%). Samples designated for SEM were dried from CO_2 by the critical point method, mounted on aluminum stubs, and sputter coated using a gold/palladium target. SEM samples were viewed using a Philips SEM 515 at 15 keV. Samples designated for TEM were postfixed in osmium tetroxide and embedded in epoxy resin. Thin sections (80 nm) of the embedded material were viewed using a Philips EM400 at 80 keV.

Phosphorylation of endogenous PKC substrate

To determine whether dFdC triggers intracellular signaling events associated with PKC activation, phosphorylation of the 87-kDa myristoylated alanine-rich C kinase substrate (MARCKS) by PKC was assessed according to a method described by Chepenik and Grunwald [7]. The method was modified for the time course studies as described below. Briefly, BG-1 cells (2×10^6 cells/60-mm^2 culture dish) were labeled with carrier-free $[^{32}P]_i$ (0.15 μCi/ml) in DMEM containing 10% FBS for 1 h. After 1 h, the medium containing $[^{32}P]_i$ was removed and replaced with fresh medium. BG-1 cells were then treated with dFdC (1 or 10 μ*M*), TPA (100 n*M*) and/or bisM (5.0 μ*M*) for various times (0.5–6 h). Following drug treatment, the cells were scraped into 2 ml ice-cold Tris-buffered saline (TBS) containing 1 m*M* PMSF and 50 m*M* NaF (TBS/PMSF/NaF). The samples were centrifuged (2000 rpm for 5 min, 4 °C), the supernatant removed and the cell pellet resuspended in 200 μl cold TBS/PMSF/NaF. An equal volume of 2 × sample buffer was added and the samples boiled immediately. An equal volume of sample proteins was then loaded onto a 7.5% SDS polyacrylamide gel and separated at a constant current of 15 mA. The $[^{32}P]_i$-labeled MARCKS protein (MW 87 kDa) was visualized and the amount of radioactivity quantified using a Phosphorimaging System Model 445 Si, Molecular Dynamics (Sunnyvale, Calif.).

PKC activity assay

PKC was partially purified from BG-1 cells as previously described [27]. Briefly, BG-1 cells (about 5×10^6) were exposed to 10 μ*M* dFdC or 1, 10 and 100 n*M* TPA for various times. Cells were then scraped from the plate (100 × 20 mm) into ice-cold TEM buffer (20 μ*M* Tris-HCl, pH 7.5, 0.5 m*M* EDTA, 0.5 m*M* EGTA and 10 m*M* β-mercaptoethanol) containing 0.5% Triton X-100 and 25 μg/ml aprotinin and leupeptin. The cells were sonicated and incubated on ice for 30 min, and then spun in a microcentrifuge for 2 min. The supernatant was then applied to DEAE-cellulose ion-exchange columns (Whatman DE52; 0.25 g/column). Total PKC activity eluted from the DEAE column (2 ml 0.2 *M* NaCl in TEM) was determined by measuring phosphorylation of acetylated myelin basic protein (the protein kinase C assay system; Gibco BRL, Grand Island, N.Y.), a PKC substrate peptide originally described by Yasuda et al. [40].

Clonogenicity assay

Clonogenic survival assays were used to assess the cytotoxicity of dFdC, TPA, bisM, and cal-C, or various combinations of these agents in BG-1 human ovarian cancer cells. BG-1 cells (2000 cells in 3 ml RPMI-1640 medium supplemented with 15% FBS) were plated in 9.6-cm^2 culture dishes. Cells were exposed 18 h after plating to dFdC (0.1, 0.5 or 1.0 μ*M*), TPA (1–100 n*M*), bisM (1.0 or 5.0 μ*M*) or cal-C (250 n*M*) alone, or in combination under different scheduling conditions. Colony formation was assessed 7–10 days later by fixing and staining the cells in methanol containing 0.16% methylene blue. The percentage colony growth inhibition was calculated for the various drug treatment regimens and compared with untreated controls. The significance of differences between treatment regimens was evaluated using Student's *t*-test. A combination index (CI) was calculated using a dose effect analysis program (Elsevier Biosoft, Cambridge, UK) to determine positive or negative drug interactions. A CI value equal to 1 indicates additivity, higher than 1 indicates antagonism and less than 1 indicates synergism.

Results

The ability of dFdC or ara-C to induce internucleosomal DNA fragmentation characteristic of apoptosis was evaluated in log phase BG-1 or HL-60 cells by agarose gel electrophoresis (Fig. 1). HL-60 cells were included as a positive control since it had been previously established that 10 μ*M* ara-C induces internucleosomal DNA fragmentation associated with apoptosis in these cells [33]. An earlier study determined that BG-1 cells exposed to 10 μ*M* dFdC for 8, 16 or 24 h exhibit DNA fragmentation that is maximal at 8 h (data not shown). Thus, DNA was isolated from BG-1 cells that were untreated or exposed to different concentrations of dFdC (0.5, 1.0, 10 and 50 μ*M*) for 8 h and fractionated on a 2% agarose gel. DNA fragmentation occurred in BG-1 cells exposed to 0.5, 1.0 or 10 μ*M* dFdC. The degree of fragmentation increased with dose, but the "laddering" pattern associated with internucleosomal DNA fragmentation was not observed in BG-1 cells exposed to dFdC (Fig. 1, lanes 3–5). BG-1 cells exposed to 50 μ*M* dFdC for 8 h died by necrosis (Fig. 1, lane 6);

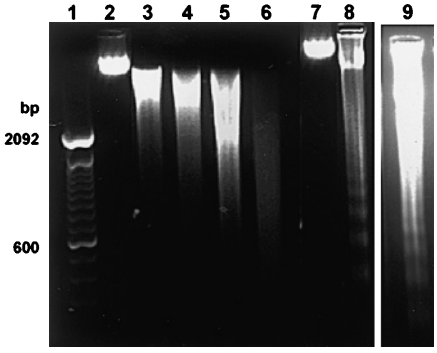

Fig. 1 DNA fragmentation in BG-1 ovarian cancer cells exposed to different concentrations of dFdC for 8 h compared with a positive control (HL-60 cells treated with 10 μ*M* ara-C for 8 h). In addition, BG-1 cells were treated with 1.0 μ*M* staurosporine for 6 h. DNA was extracted from drug-treated and untreated BG-1 or HL-60 cells and separated on a 2% agarose gel. The DNA was visualized with ethidium bromide (*lane 1* molecular weight marker, *lane 2* untreated control BG-1 cells, *lane 3* 0.5 μ*M* dFdC in BG-1 cells, *lane 4* 1.0 μ*M* dFdC in BG-1 cells, *lane 5* 10 μ*M* dFdC in BG-1 cells, *lane 6* 50 μ*M* dFdC in BG-1 cells, *lane 7* untreated control HL-60 cells, *Lane 8* 10 μ*M* ara-C in HL-60 cells, *lane 9* 1.0 μ*M* staurosporine in BG-1 cells)

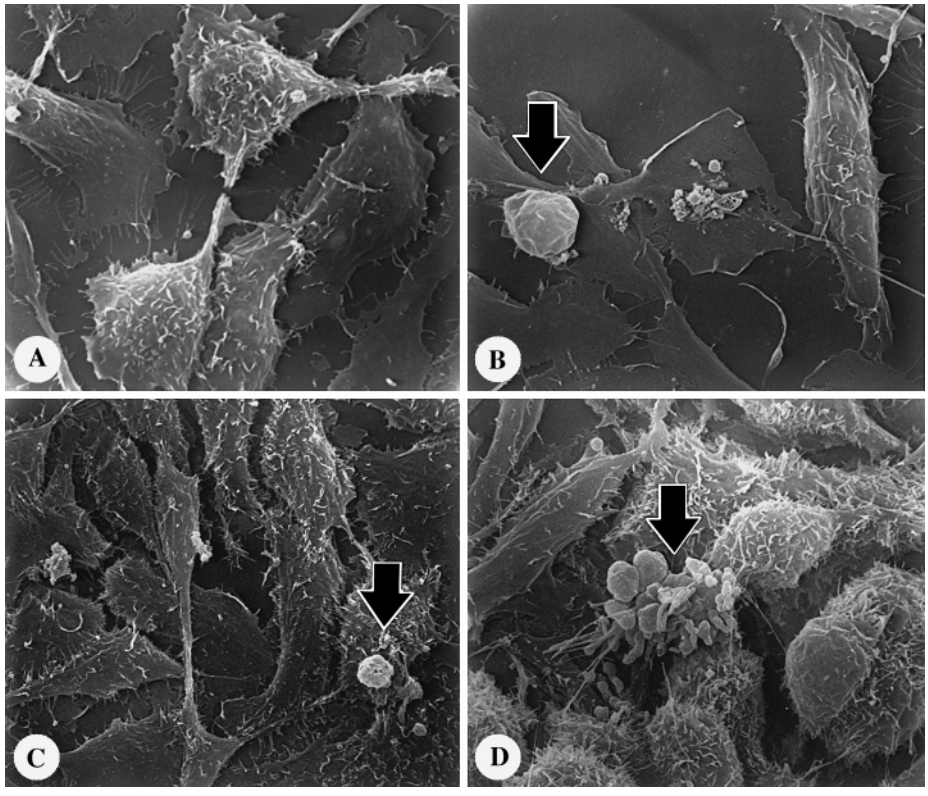

Fig. 2A–D Scanning electron microscopy of BG-1 cells exposed to 10 μ*M* dFdC for up to 24 h. Following drug treatment, BG-1 cells exhibited morphologic changes associated with apoptosis such as membrane blebbing and apoptotic body formation. (**A** untreated control BG-1 cells, **B** BG-1 cells-exposed to 10 μ*M* dFdC for 8 h, **C** 10 μ*M* dFdC for 16 h, **D** 10 μ*M* dFdC for 24 h)

the DNA disintegrated into small pieces and appeared as a smear in the agarose gel [8]. In contrast, internucleosomal DNA fragmentation was observed in HL-60 cells exposed to 10 μ*M* ara-C for 8 h (Fig. 1, lane 8).

In order to determine whether BG-1 cells contain the endonucleases (Ca^{++}/Mg^{++}-dependent) responsible for DNA cleavage between the nucleosomes [36], we exposed BG-1 cells to staurosporine, a drug that generally induces apoptosis. Exposure to 1.0 μ*M* staurosporine for 6 h triggered internucleosomal fragmentation of the DNA in BG-1 cells (Fig. 1, lane 9). The inability of dFdC to trigger internucleosomal DNA fragmentation in BG-1 cells, however, did not preclude the possibility that BG-1 cells undergo programmed cell death (PCD) in response to drug treatment. This type of DNA degradation is not essential for apoptosis [31, 32]. Furthermore, Huang and Plunkett [23] have shown that dFdC induces morphologic changes associated with apoptosis in CEM leukemia cells in the absence of internucleosomal DNA fragmentation.

Therefore, SEM and TEM studies were conducted to characterize the morphologic changes associated with the cell death induced by 10 μ*M* dFdC in BG-1 ovarian cancer cells. Cells undergoing apoptosis in response to chemotherapy exhibit a characteristic morphology. These changes include: (1) condensation of the chromatin which eventually circumscribes the inner leaflet of the nuclear membrane, (2) condensation of the cytoplasm, (3) convolution of the nuclear membrane, (4) blebbing of the cytoplasmic membrane, and (4) the formation of apoptotic bodies varying in size and composition [26]. BG-1 cells were exposed to 10 μ*M* dFdC for 8, 16 or 24 h for the SEM and TEM studies. Cell surface changes that occurred in response to 10 μ*M* dFdC treatment were visualized by SEM (Fig. 2) and chromatin changes were observed by TEM (Fig. 3).

Untreated BG-1 cells as viewed by SEM are shown in Fig. 2A. These cells were epithelial in origin, ovoid in shape and had extensive microvilli on their surface. Following drug exposure, membrane blebs were prominent on the surface of BG-1 cells exposed to 10 μ*M* dFdC for 8 or 16 h (Fig. 2B,C). Apoptotic body formation was also observed by SEM (Fig. 2D) following a 24-h exposure to 10 μ*M* dFdC. The SEM studies indicated that BG-1 cells exposed to 10 μ*M* dFdC over the course of 24 h exhibited morphological changes characteristic of apoptosis (e.g. membrane blebbing and the formation of apoptotic bodies) as originally described by Kerr and colleagues [25].

The TEM studies indicated alterations in the chromatin of BG-1 cells exposed to 10 μ*M* dFdC for 8, 16 or 24 h (Fig. 3). As shown in Fig. 3B–D, the chromatin in BG-1 cells condensed in response to 10 μ*M* dFdC treatment, but chromatin circumscribing the inner leaflet of the nuclear membrane was not observed. Rather, the chromatin eventually formed a crossbridge that linked one side of the nuclear membrane with the other. These crossbridges were prominent in the nuclei of BG-1 cells exposed to 10 μ*M* dFdC for 16 or 24 h (Fig. 3C,D) and were a unique response to drug treatment. Thus, BG-1 cells exposed to 10 μ*M* dFdC exhibited many, but not all, of the classic features of apoptosis.

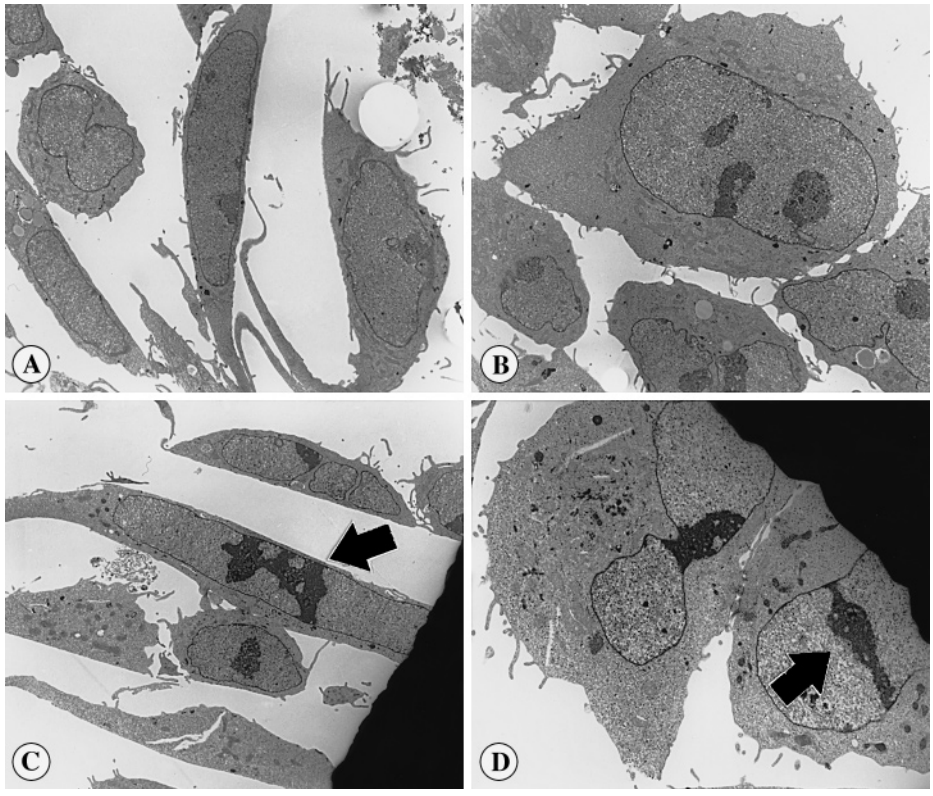

Fig. 3A–D Transmission electron microscopy of BG-1 cells exposed to 10 μ*M* dFdC for up to 24 h (**A** BG-1 control cells). BG-1 cells exposed to 10 μ*M* dFdC for 8 h (**B**) or 10 μ*M* dFdC for 16 h (**C**) had condensed chromatin that abutted against the nuclear membrane. Chromatin crossbridges formed in the nuclei of BG-1 cells exposed to 10 μ*M* dFdC for 24 h (**D**)

To determine whether dFdC activates PKC, we monitored MARCKS protein phosphorylation in BG-1 cells exposed to dFdC over time, since the MARCKS protein is an endogenous PKC substrate and its phosphorylation is a good measure of cellular PKC activity [6]. Phosphorylation of the 87-kDa MARCKS protein [19] was assessed, following exposure of BG-1 cells to dFdC (1.0 or 10 μ*M*) for 0.5, 1, 3 or 6 h. BG-1 cells exposed to 100 n*M* TPA for 1 h were included as a positive control. TPA, a potent activator of PKC, produced a maximal response within 1 h. Untreated controls were included at each timepoint to account for time-dependent changes in $[^{32}P]_i$ phosphate pools and cell cycle influences on MARCKS activity [20]. Following drug exposures, BG-1 cells (2×10^6) were lysed and an equal volume of cell lysate loaded onto a 7.5% SDS-polyacrylamide gel for electrophoretic separation. The extent of 87-kDa MARCKS protein phosphorylation was determined by phosphorimaging. The 87-kDa protein band in the SDS polyacrylamide gel is shown in Fig. 4A and is a representative example from five separate experiments. The MARCKS protein band was identified based on its migration compared with molecular weight marker standards and the ability of the specific PKC inhibitor, bisM, to diminish the phosphorylation of MARCKS.

Figure 4B shows the time course of 87-kDa MARCKS protein phosphorylation in BG-1 cells following treatment with 100 n*M* TPA or dFdC (1.0 or 10 μ*M*). Values representing $[^{32}P]_i$-labeling of the 87-kDa MARCKS protein in drug-treated samples are expressed as a percentage of the untreated control and averaged over five separate experiments. A one-sided *t*-test was used to determine whether 87-kDa MARCKS protein phosphorylation in drug-treated samples was significantly greater than control values at the doses and timepoints tested. Exposure to 100 n*M* TPA for 1 h increased phosphorylation of the 87-kDa MARCKS protein 1.5-fold ($P < 0.001$). In the presence of 10 μ*M* dFdC, 87-kDa MARCKS phosphorylation increased 1.3-fold ($P = 0.036$) at 1 h, 1.5-fold at 3 h ($P = 0.051$), and 1.3-fold at 6 h ($P = 0.009$). Following treatment with 1.0 μ*M* dFdC, MARCKS phosphorylation increased up to 1.4-fold at 3 h, but only the increase at 30 min was statistically significant (1.2-fold; $P = 0.047$). Thus, the increases in MARCKS phosphorylation were dose-related since 10 μ*M* dFdC had a greater effect on PKC-associated phosphorylation events than 1.0 μ*M* dFdC. In addition, coincubation of 100 n*M* TPA or 10 μ*M* dFdC with the PKC inhibitor bisM (5.0 μ*M*) for 1 h significantly diminished 87-kDa MARCKS phosphorylation compared with TPA or dFdC alone ($P < 0.001$; Table 1). This finding confirmed that 87-kDa MARCKS phosphorylation resulted from PKC activation.

To complement the MARCKS studies, total PKC activity was determined in BG-1 cells exposed to TPA (10 or 100 n*M*) or dFdC (10 μ*M*) over the course of 1 h according to the method of Yasuda et al. [40]. Total PKC activity increased two- to threefold in BG-1 cells exposed to 10 μ*M* dFdC over 1 h reaching levels comparable to those induced by 10 or 100 n*M* TPA at

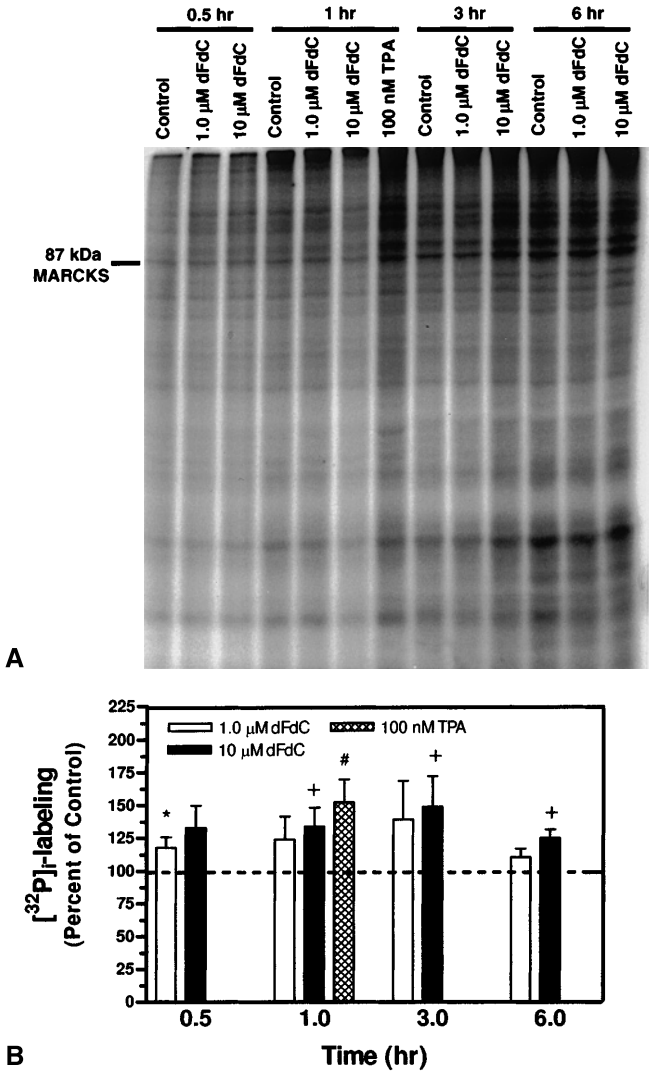

Fig. 4A,B The time course of 87-kDa MARCKS protein phosphorylation following exposure of BG-1 cells to 1.0 or 10 μ*M* dFdC for 0.5, 1, 3 and 6 h or 100 n*M* TPA for 1 h. **A** An SDS-polyacrylamide gel representative of five separate experiments. Treatment with 100 n*M* TPA served as the positive control for PKC activation and subsequent 87-kDa MARCKS protein phosphorylation. The amount of $[^{32}P]_i$ incorporated by the 87-kDa MARCKS protein was determined by phosphorimaging analysis. **B** The $[^{32}P]_i$ incorporated into the 87-kDa MARCKS protein following drug treatments, expressed as a percentage of the untreated control for each timepoint tested. The means ± standard error of five experiments are shown. The symbols *, + and # denote timepoints where 87-kDa MARCKS phosphorylation was significantly greater ($P < 0.05$) than control for 1.0 μ*M* dFdC, 10 μ*M* dFdC and 100 n*M* TPA, respectively

Table 1 The inhibition of 87-kDa MARCKS phosphorylation by coincubation of 100 n*M* TPA or 10 μ*M* dFdC with the PKC inhibitor bisM (5.0 μ*M*) for 1 h. The inhibitor was added 15 min prior to TPA or dFdC addition. The results presented are representative of four independent experiments. The amount of $^{32}[P]_i$ labeling is expressed as a percentage of the untreated control. The *P*-values were calculated using a two-sided *t*-test

Treatment	$[^{32}P]_i$ labeling of MARCKS (% of control)	*t*-test (two-tailed)
TPA (100 n*M*)	142	
bisM (5.0 μ*M*)/ TPA (100 n*M*)	63	$P < 0.001$
dFdC (10 μ*M*)	155	
bisM (5.0 μ*M*)/ dFdC (10 μ*M*)	73	$P < 0.001$

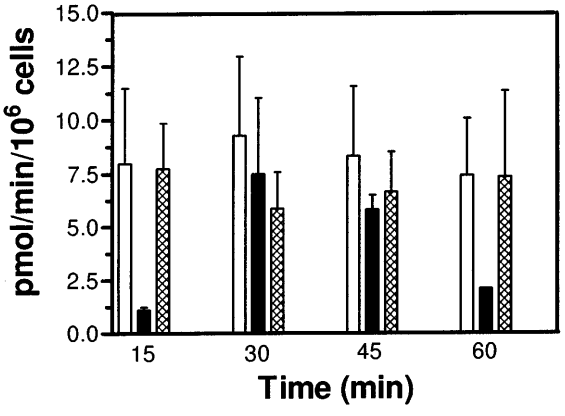

Fig. 5 Total PKC activity in BG-1 ovarian cancer cells exposed to TPA (10 or 100 n*M*) or 10 μ*M* dFdC over the course of 1 h. The *individual bars* represent the means ± standard error of two or more independent experiments for each timepoint done in triplicate. The basal level of PKC activity was 2.5 pmol/min per 10^6 BG-1 cells (□ 100 n*M* TPA, ■ 10 μ*M* dFdC, ▨ 10 n*M* TPA)

30 min (Fig. 5). The increase in total PKC activity preceded the increases in MARCKS protein phosphorylation following treatment with 10 μ*M* dFdC. Taken together, these experiments showed that dFdC activated PKC in BG-1 cells and that PKC-associated phosphorylation of the 87-kDa MARCKS protein was maintained in the presence of 10 μ*M* dFdC for 6 h.

To test our hypothesis that PKC modulation would influence dFdC cytotoxicity in BG-1 cells, clonogenicity studies were performed to assess the cytotoxicity of dFdC, TPA, bisM and cal-C administered alone or in combination regimens. Analysis of cloning data determined an IC_{50} of 0.53 μ*M* for dFdC in BG-1 cells exposed to the drug for 4 h. The IC_{50} value decreased tenfold to 0.05 μ*M* when BG-1 cells were exposed to the drug for 8 h. In addition, cloning assays determined that BG-1 cells responded differently to various concentrations of TPA (0.1–100 n*M*), depending on the dose of TPA and schedule of administration. In general, 24-h exposures to TPA were cytotoxic, while 1-h exposures induced slight cellular proliferation or had minimal to no effect. The only exception was 100 n*M* TPA, which was cytotoxic to BG-1 cells in both the 1-h and 24-h exposure regimens (data not shown).

Combination regimens with TPA (1.0 or 10 n*M*) and dFdC (0.1, 0.5 or 1.0 μ*M*) were evaluated in clonogenicity assays of BG-1 cells (Table 2). We used 4-h exposures to dFdC in our drug combination clonogenicity studies because this condition was most suitable for evaluating drug interactions in vitro. The regimen

Table 2 The clonogenic survival of BG-1 cells treated with 1.0 or 10 n*M* doses of TPA and/or 0.1, 0.5 and 1.0 μ*M* doses of dFdC. BG-1 cells (2000 cells in 3 ml RPMI-1640 medium with 15% FBS) were plated in 60-mm culture dishes. The cells were treated 18 h after plating with dFdC for 4 h, with TPA for 1 or 24 h, or with TPA for 1 or 24 h followed by dFdC for 4 h. After treatment, cells were incubated in drug-free medium. The colonies were stained and counted 7–10 days later. Values are the mean percentages of control ± standard error of three or more independent experiments with each treatment performed in triplicate. A CI value was calculated using the dose effect analysis program (Elsevier Biosoft; Cambridge, UK) to determine positive or negative drug interactions (*NS* not significant)

Treatment	Colony formation (% of control) (mean ± std error)	*t*-test *P*-value[a]	CI value[b]
dFdC (0.1 μ*M*) × 4 h	68.0 ± 1.0		
dFdC (0.5 μ*M*) × 4 h	53.7 ± 5.1		
dFdC (1.0 μ*M*) × 4 h	30.0 ± 2.0		
TPA (1 n*M*) × 1 h	94.2 ± 4.3		
TPA (1 n*M*) × 24 h	90.7 ± 2.9		
TPA (10 n*M*) × 1 h	92.4 ± 6.6		
TPA (10 n*M*) × 24 h	36.3 ± 5.9		
TPA (1 n*M*) × 1 h then dFdC (0.5 μ*M*) × 4 h	50.3 ± 2.6	N.S.	> 1
TPA (1 n*M*) × 24 h then dFdC (0.5 μ*M*) × 4 h	26.3 ± 1.3	< 0.01	< 0.1
TPA (10 n*M*) × 1 h then dFdC (0.5 μ*M*) × 4 h	60.4 ± 1.3	N.S.	> 1
TPA (10 n*M*) × 24 h then dFdC (0.5 μ*M*) × 4 h	7.6 ± 0.5	< 0.01	< 0.1
TPA (1 n*M*) × 24 h then dFdC (0.1 μ*M*) × 4 h	27.5 ± 0.7	< 0.01	< 0.1
TPA (1 n*M*) × 24 h then dFdC (1.0 μ*M*) × 4 h	8.2 ± 0.8	< 0.01	< 0.1
TPA (10 n*M*) × 24 h then dFdC (0.1 μ*M*) × 4 h	11.7 ± 0.3	< 0.01	< 0.1
TPA (10 n*M*) × 24 h then dFdC (1.0 μ*M*) × 4 h	3.0 ± 0.3	< 0.01	< 0.1

[a] Compared to dFdC alone
[b] Combination index (CI): < 1 synergistic; = 1 additive; > 1 antagonistic

exposing BG-1 cells to 1.0 n*M* TPA for 1 h, followed by 0.50 μ*M* dFdC for an additional 4 h did not significantly affect dFdC cytotoxicity in BG-1 cells. In contrast, prior exposure to 1.0 n*M* TPA for 24 h followed by 0.5 μ*M* dFdC for an additional 4 h was synergistic (CI < 0.1) and significantly different from dFdC alone ($P < 0.01$). Pretreatments with 1.0 n*M* TPA for 24 h followed by exposure to 0.1 or 1.0 μ*M* dFdC were also synergistic. A similar profile was observed when dFdC was studied in combination with 10 n*M* TPA (Table 2). Prior exposure to 10 n*M* TPA for 24 h followed by 0.50 μ*M* dFdC for another 4 h was synergistic (CI < 0.1) and significantly different from 0.50 μ*M* dFdC alone ($P < 0.01$). This synergism was also observed with 0.1 and 1.0 μ*M* dFdC. However, short-term (1 h) exposure to 10 n*M* TPA did not enhance dFdC (0.5 μ*M*) cytotoxicity. In addition, coincubation of 10 n*M* TPA with bisM (1.0 μ*M*) or cal-C (250 n*M*) for 24 h, prior to the addition of dFdC (0.50 μ*M* for 4 h), eliminated the synergism of dFdC with TPA ($P < 0.001$; Fig. 6). The ability of PKC inhibitors to interfere with the synergism of TPA and dFdC in cloning assays indicated that the activation and subsequent downregulation of PKC influenced dFdC-induced cytotoxicity. Thus, dFdC efficacy may be improved by agents that modulate PKC signal transduction pathways.

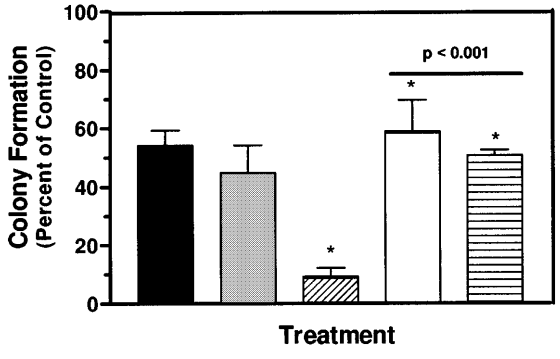

Fig. 6 Clonogenic assays assessing the cytotoxicity of dFdC, TPA and bisM or cal-C in combination. The PKC inhibitors bisM (1.0 μ*M*) or cal-C (250 n*M*) were coincubated with 10 n*M* TPA for 24 h followed by 0.5 μ*M* dFdC for an additional 4 h. Treatments were performed in triplicate. The *bars* represent the means ± standard error of two or more independent experiments. The PKC inhibitors antagonized the synergism observed in the TPA (10 n*M* for 24 h) followed by dFdC (0.5 μ*M* for 4 h) regimen ($P < 0.001$). (■ dFdC 4 h, ▒ TPA 24 h, ▨ TPA 24 h/dFdC 4 h, □ bisM + TPA 24 h/dFdC 4 h, ▤ cal-C + TPA 24 h/dFdC 4 h)

Discussion

These studies demonstrate that dFdC activates PKC and provide evidence that dFdC-induced cytotoxicity is influenced by PKC signaling mechanisms in BG-1 human ovarian cancer cells. The ability of dFdC to activate PKC was determined by measuring MARCKS protein phosphorylation following treatment of BG-1 cells with dFdC, because MARCKS proteins are intracellular substrates of PKC and their phosphorylation is directly linked to PKC activation [6]. Increases in 87-kDa MARCKS phosphorylation were observed at 1 h and were sustained for 6 h in the presence of 10 μ*M* dFdC,

and in the presence of 1.0 μ*M* dFdC a significant difference from baseline was produced after 30 min of drug exposure, but this was not sustained. Furthermore, total PKC activity increased two- to threefold in BG-1 cells exposed to 10 μ*M* dFdC for 30 min and this increase occurred prior to the increase in MARCKS protein phosphorylation. Additionally, increases in 87-kDa MARCKS phosphorylation generated by 100 n*M* TPA or 10 μ*M* dFdC were inhibited by coincubation with the PKC inhibitor bisM (5.0 μ*M*), further confirming that dFdC activates PKC signaling in BG-1 cells.

Our results suggest that dFdC activates PKC in BG-1 cells in a dose-dependent manner since 10 μ*M* dFdC had a more profound effect on MARCKS protein phosphorylation than 1.0 μ*M* dFdC. We believe that the ability of dFdC to activate PKC in BG-1 cells results from drug influences on intracellular phospholipid metabolism. Previous studies in our laboratory have demonstrated that dFdC and ara-C are anabolized by reversal of the cholinephosphotransferase pathway generating dFdCDP-choline or araCDP-choline and DG formation in mouse L5178Y cells [28]. It is presumed the DG generated from this pathway activates PKC. Thus, the ability of dFdC to reverse cholinephosphotransferase is a dose-dependent phenomenon that requires extracellular drug concentrations greater than 1.0 μ*M* [28].

The results of clonogenic survival experiments in BG-1 cells suggested that PKC modulation influences cellular responses to the drug. Our rationale for evaluating acute and chronic regimens with TPA are based on studies by Grant and others of ara-C-induced cytotoxicity in leukemic cells [14, 24]. Acute exposure to TPA increases total PKC activity, while chronic exposure downregulates total PKC activity. Chronic exposure (24 h) to 1 or 10 n*M* TPA prior to dFdC enhanced drug cytotoxicity in BG-1 cells, while short-term exposure regimens (1 h) with TPA had no significant effect on drug efficacy in these assays. We determined that total PKC activity decreased 1.5- to 2-fold in BG-1 cells exposed to 1 or 10 n*M* of TPA for 24 h compared with untreated controls (data not shown), demonstrating that the TPA concentrations used in the cloning assays were able to downregulate total PKC activity in BG-1 cells after 24 h. Coincubations with PKC inhibitors were able to negate the synergy of TPA with dFdC in the chronic exposure regimens. These observations imply that both PKC activation and its subsequent downregulation are critical to dFdC-induced cytotoxicity in BG-1 ovarian cancer cells based on the ability of PKC inhibitors to negate the synergy of TPA with dFdC in our chronic exposure regimens.

We postulate that chronic treatment with TPA in BG-1 cells alters the distribution and expression of PKC isoforms in a manner that favors cell death, and herein lies the explanation for the synergy of TPA with dFdC in our cloning assays with BG-1 cells. The activation and subsequent downregulation of PKC isoforms may trigger cellular and nuclear alterations that sensitize BG-1 cells for apoptosis in response to DNA-damaging agents. Our observations coincide with other studies demonstrating the ability of TPA to induce cell death via its effects on PKC signaling. For example, Vente et al. [37] have shown that the ability of TPA to induce death in U937 cells depends on the activation of specific PKC isoforms and such alterations are critical to phorbol ester-induced apoptosis in these cells. Similar observations were made in MCF-7 breast cancer cells engineered to overexpress the PKC-α isoform [38]. TPA treatment induces death in MCF-7 cells overexpressing the PKC-α isoform and cytostasis in parental MCF-7 cells. Thus, we are currently evaluating PKC isozyme expression in BG-1 cells following dFdC or TPA treatment to further elucidate the role of PKC in dFdC-induced cytotoxicity.

We also determined that BG-1 cells undergo PCD in response to dFdC treatment and this PCD exhibited many, but not all, of the biochemical and morphologic features associated with classic apoptotic cell death. Specifically, BG-1 cells exposed to 0.5, 1.0 and 10 μ*M* dFdC had fragmented DNA, but the fragmentation was not internucleosomal. The degree of fragmentation increased with dose and was highest in BG-1 cells exposed to 10 μ*M* dFdC, while a concentration of 50 μ*M* induced necrotic cell death after 8 h of exposure. We also determined that BG-1 cells contain the Ca^{++}/Mg^{++}-dependent endonucleases responsible for internucleosomal DNA fragmentation, since exposing these cells to 1.0 μ*M* staurosporine, a general inducer of apoptosis, for 6 h triggered internucleosomal DNA cleavage.

Since cells can exhibit morphologic changes associated with apoptosis in the absence of internucleosomal DNA fragmentation, we used electron microscopy to further characterize the cell death induced by dFdC in BG-1 cells. SEM studies of BG-1 cells exposed to 10 μ*M* dFdC over a 24-h period indicated that BG-1 cells exhibited morphologic changes characteristic of apoptosis: membrane blebs and apoptotic bodies formed in response to drug treatment. In addition, TEM studies demonstrated that the chromatin condensed and formed crossbridges in the nuclei of BG-1 cells exposed to 10 μ*M* dFdC for 16 or 24 h. Chromatin crossbridge formation has not been previously reported in cells undergoing apoptosis. However, Schwartz et al. [34] have shown that not all cell deaths occur via apoptosis in the classical sense. The dFdC-induced chromatin changes observed by TEM along with the data from SEM and agarose gel electrophoresis studies indicated that BG-1 cells undergo PCD in response to dFdC treatment. The PCD induced by dFdC in BG-1 cells exhibited some, but not all, of the classic features of apoptosis [25].

It is possible that dFdC treatment in BG-1 cells elicits cellular responses similar to those described by Emoto et al. in leukemia cells [10, 11], in which PKC-δ was cleaved and activated by a CPP32-like protease in response to ara-C and other DNA damaging agents. The proteolytic activation of PKC-δ was selective, since there was no detectable cleavage of other PKC isoforms, and cleavage of PKC-δ contributed to the phenotypic

changes associated with apoptosis [13]. The activation of cysteine proteases [41] is a seminal event in apoptosis. Therefore, evaluation of cysteine proteases and PKC-δ cleavage in dFdC-induced PCD/cytotoxicity in BG-1 ovarian cancer cells is warranted.

In conclusion, we established that dFdC induced PCD in BG-1 cells by biochemical and morphologic analyses. We determined that dFdC activated PKC in BG-1 cells and demonstrated that TPA enhanced the cytotoxicity of dFdC in clonogenicity studies of BG-1 cells exposed to both agents. The effects of TPA on dFdC-induced cytotoxicity were dose- and schedule-dependent and were inhibited by coincubation with a PKC inhibitor. Thus, alterations within the PKC pathway were critical to dFdC-induced cytotoxicity in BG-1 ovarian cancer cells. Our results indicate that dFdC efficacy may be improved by agents that modulate PKC signal transduction in the clinical setting.

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References

1. Bhalla K, Holladay C, Arlin Z, Grant S, Ibrado AM, Jasiok M (1991) Treatment with interleukin-3 plus granulocyte-macrophage colony-stimulating factors improves the selectivity of Ara-C in vitro against acute myeloid leukemia blasts. Blood 78: 2674
2. Bhalla K, Tang C, Ibrado AM, Grant S, Tourkina E, Holladay C, Hughes M, Mahoney ME, Huang Y (1992) Granulocyte-macrophage colony-stimulating factor/interleukin-3 fusion protein (pIXY 321) enhances high-dose Ara-C-induced programmed cell death or apoptosis in human myeloid leukemia cells. Blood 80: 2883
3. Bhalla K, Tourkina E, Huang Y, Tang C, Mahoney ME, Ibrado AM (1993) Effect of hemopoietic growth factors G-CSF and pIXY 321 on the activity of high dose Ara-C in human myeloid leukemia cells. Leuk Lymphoma 10 [Suppl]: 123
4. Bouffard DY, Momparler RL (1994) Comparison of cytosine arabinoside and 2′,2′-difluorodeoxycytidine (Gemcitabine) on the induction of apoptotic cell death in HL-60 myeloid leukemic cells. Proc Am Assoc Cancer Res 35: 317
5. Boven E, Schipper H, Erkelens CA, Hatty SA, Pinedo HM (1993) The influence of the schedule and the dose of gemcitabine on the anti-tumour efficacy in experimental human cancer. Br J Cancer 68: 52
6. Carballo E, Colomer D, Vives Corrons JL, Blackshear PJ, Gil J (1995) 2-Phosphorylation of the MARCKS family of protein kinase C substrates in human B chronic lymphocytic leukemia cells. Leukemia 9: 834
7. Chepenik KP, Grunwald GB (1988) Effects of epidermal growth factor and phorbol 12-myristate 13-acetate on protein phosphorylation in mouse embryo palate mesenchyme cells, in vitro. J Craniofacial Genet Dev Biol 8: 147
8. Columbano A (1995) Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes in vivo. J Cell Biochem 58: 181
9. Curtis BM, Williams DE, Broxmeyer HE, Dunn J, Farrah T, Jeffrey E, Clevenger W, DeRoos P, Martin U, Friend D, Craig V, Gayle R, Price V, Cosman D, March CJ, Park LS (1991) Enhanced hematopoietic activity of a human granulocyte/macrophage colony-stimulating factor-interleukin 3 fusion protein. Proc Natl Acad Sci USA 88: 5809
10. Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichselbaum R, et al. (1995) Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. EMBO J 14: 6148
11. Emoto Y, Kisaki H, Manome Y, Kharbanda S, Kufe D (1996) Activation of protein kinase C delta in human myeloid leukemia cells treated with 1-β-D-abinofuranosyl-cytosine. Blood 87: 1990
12. Geisinger KR, Kute TE, Pettenati MJ, Welander CE, Dennard Y, Collins LA, Berens ME (1989) Characterization of a human ovarian carcinoma cell line with estrogen and progesterone receptors. Cancer 63: 280
13. Ghayur T, Hugunin M, Talanian RV, Ratnofsky S, Quinlan C, Emoto Y, Pandey P, Datta R, Huang YY, Kharbanda S, Allen H, Kamen R, Wong W, Kufe D (1996) Proteolytic activation of protein kinase C delta by an ICE/CED 3-like protease induces characteristics of apoptosis. J Exp Med 184: 2399
14. Grant S, Turner A, Jarvis D (1994) Regulation of 1-β-D-arabinofuranosylcytosine-induced apoptosis in myeloid leukemia cells by pharmacologic and physiologic activators of PKC: evidence that potentiation of this process is related to enzyme down-regulation. Proc Am Assoc Cancer Res 35: 312
15. Guchelaar H, Richel DJ, Knapen A van (1996) Clinical, toxicological, and pharmacological aspects of gemcitabine. Cancer Treat Rev 22: 15
16. Gunji H, Kharbanda S, Kufe D (1991) Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. Cancer Res 51: 741
17. Heinemann V, Hertel LW, Grindey GB, Plunkett W (1988) Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. Cancer Res 48: 4024
18. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, Plunkett W (1990) Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2′,2′-difluorodeoxycytidine. Mol Pharmacol 38: 567
19. Herget T, Brooks SF, Broad S, Rozengurt E (1992) Relationship between the major protein kinase C substrates acidic 80-kDa protein-kinase-C substrate (80 K) and myristoylated alanine-rich C-kinase substrate (MARCKS). Members of a gene family or equivalent genes in different species. Eur J Biochem 209: 7
20. Herget T, Oehrlein SA, Pappin DJ, Rozengurt E, Parker PJ (1995) The myristoylated alanine-rich C-kinase substrate (MARCKS) is sequentially phosphorylated by conventional, novel and atypical isotypes of protein kinase C. Eur J Biochem 233: 448
21. Hiddemann W, Kiehl M, Zuhlsdorf M, Busemann C, Schleyer E, Wormann B, Buchner T (1992) Granulocyte-macrophage colony-stimulating factor and interleukin-3 enhance the incorporation of cytosine arabinoside into the DNA of leukemic blasts and the cytotoxic effect on clonogenic cells from patients with acute myeloid leukemia. Semin Oncol 19: 31
22. Huang P, Plunkett W (1995) Fludarabine and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event. Cancer Chemother Pharmacol 36: 181
23. Huang P, Plunkett W (1995) Induction of apoptosis by gemcitabine. Semin Oncol 22: 19
24. Jarvis WD, Povirk LF, Turner AJ, Traylor RS, Gewirtz DA, Pettit GR, Grant S (1994) Effects of bryostatin 1 and other pharmacological activators of protein kinase C on 1-[β-D-ar-

abinofuranosyl]cytosine-induced apoptosis in HL-60 human promyelocytic leukemia cells. Biochem Pharmacol 47: 839
25. Kerr JFR, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239
26. Kerr JF, Gobe GC, Winterford CM, Harmon BV (1995) Anatomical methods in cell death. Methods Cell Biol 46: 1
29. Kharbanda S, Datta R, Kufe D (1991) Regulation of c-*jun* gene expression in HL-60 leukemia cells by 1-β-D-arabinofuranosylcytosine. Potential involvement of a protein kinase C dependent mechanism. Biochemistry 30: 7947
28. Kucera GL, Capizzi RL (1992) 1-β-D-Arabinofuranosylcytosine-diphosphate-choline is formed by the reversal of cholinephosphotransferase and not via cytidylyltransferase. Cancer Res 52: 3886
29. Lund B, Kristjansen PEG, Hansen HH (1993) Clinical and preclinical activity of 2′,2′-difluorodeoxycytidine (gemcitabine). Cancer Treat Rev 19: 45
30. Obeid LM, Hannun YA (1995) Ceramide: a stress signal and mediator of growth suppression and apoptosis. J Cell Biochem 58: 191
31. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 12: 3679
32. Ormerod MG, O'Neill CF, Robertson D, Harrap KR (1994) Cisplatin induces apoptosis in a human ovarian carcinoma cell line without concomitant internucleosomal degradation of DNA. Exp Cell Res 211: 231
33. Ray S, Ponnathpur V, Huang Y, Tang C, Mahoney ME, Ibrado AM, Bullock G, Bhalla K (1994) 1-beta-D-arabinofuranosylcytosine-, mitoxantrone-, and paclitaxel-induced apoptosis in HL-60 cells: improved method for detection of internucleosomal DNA fragmentation. Cancer Chemother Pharmacol 34: 365
34. Schwartz LM, Smith SW, Jones ME, Osborne BA (1993) Do all programmed cell deaths occur via apoptosis? Proc Natl Acad Sci USA 90: 980
35. Schy WE, Hertel LW, Kroin KS, Bloom LB, Goodman MF, Richardson FC (1993) Effect of a template-located 2′,2′,-difluorodeoxycytidine on the kinetics and fidelity of base insertion by klenow (3′-5′ exonuclease) fragment. Cancer Res 53: 4582
36. Stewart BW (1994) Mechanisms of apoptosis: integration of genetic, biochemical, and cellular indicators. J Nat Cancer Inst 86: 1286
37. Vente J de, Kiley S, Garris T, Bryant W, Hooker J, Posekany K, Parker P, Cook P, Fletcher D, Ways DK (1995) Phorbol ester treatment of U937 cells with altered protein kinase C content and distribution induces cell death rather than differentiation. Cell Growth Differ 6: 371
38. Vente JE de, Kukoly CA, Bryant WO, Posekany KJ, Chen J, Fletcher DJ, Parker PJ, Pettit GJ, Lozano G, Cook PP, et al. (1995) Phorbol esters induce death in MCF-7 breast cancer cells with altered expression of protein kinase C isoforms. Role for p53-independent induction of gadd-45 in initiating death. J Clin Invest 96: 1874
39. Wang LM, Kucera GL (1994) Deoxycytidine kinase is phosphorylated in vitro by protein kinase C alpha. Biochim Biophys Acta 1224: 161
40. Yasuda I, Kishimoto I, Tanaka S, Tominga M, Sakura A, Nishizuka Y (1990) A synthetic peptide substrate for selective assay of protein kinase C. Biochem Biophys Res Commun 166: 1220
41. Zhivotovsky B, Burgess DH, Vanags DM, Orrenius S (1997) Involvement of cellular proteolytic machinery in apoptosis. Biochem Biophys Res Commun 230: 481