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Inhibition of Protein Kinase C Activator-Mediated Induction of $p21^{CIP1}$ and $p27^{KIP1}$ by Deoxycytidine Analogs in Human Leukemia Cells

RELATIONSHIP TO APOPTOSIS AND DIFFERENTIATION

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ABSTRACT. Events accompanying sequential exposure of U937 leukemic cells to the deoxycytidine (dCyd) analogs 1-[β-D-arabinofuranosyl]cytosine (ara-C) or 2′,2′-difluorodeoxycytidine (gemcitabine; dFdC) followed by two protein kinase C (PKC) activators [bryostatin 1 (BRY) or phorbol 12′-myristate 13′-acetate (PMA)] exhibiting disparate differentiation-inducing abilities were characterized. A 24-hr exposure to 10 nM BRY or PMA after a 6-hr incubation with 1 μM ara-C or 100 nM dFdC resulted in equivalent increases in apoptosis, caspase-3 activation, and polyADP-ribose polymerase degradation, as well as identical DNA cleavage patterns. BRY and PMA did not modify retention of the lethal ara-C metabolite ara-CTP or alter ara-CTP/dCTP ratios. Unexpectedly, pretreatment of cells with ara-C or dFdC opposed BRY- and PMA-related induction of the cyclin-dependent kinase inhibitors (CDKIs) $p21^{CIP1}$ and/or $p27^{KIP1}$. These effects were not mimicked by the DNA polymerase inhibitor aphidicolin or by VP-16, a potent inducer of apoptosis. Inhibition of PKC activator-induced CDKI expression by ara-C and dFdC did not lead to redistribution of proliferating cell nuclear antigen but was accompanied by sub-additive or antagonistic effects on leukemic cell differentiation. Sequential exposure of cells to ara-C followed by BRY or PMA led to substantial reductions in clonogenicity that could not be attributed solely to apoptosis. Finally, pretreatment of cells with ara-C attenuated PMA- and BRY-mediated activation of mitogen-activated protein kinase, an enzyme implicated in CDKI induction. Collectively, these findings suggest that pretreatment of leukemic cells with certain dCyd analogs interferes with CDKI induction by the PKC activators PMA and BRY, and that this action may contribute to modulation of apoptosis and differentiation in cells exposed sequentially to these agents. BIOCHEM PHARMACOL **58**;1:121–131, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. ara-C; gemcitabine; bryostatin; PMA; apoptosis; differentiation; $p21^{CIP1}$; $p27^{KIP1}$; U937 cells

In hematopoietic cell systems, a complex relationship exists between apoptosis and differentiation. For example, leukemic cells undergoing terminal differentiation in response to retinoic acid [1] or PMA¶ [2] ultimately undergo an apoptotic form of cell death. In contrast, differentiation induction has been shown to block apoptosis in response to diverse stimuli, including cytokines (transforming growth factor β) [3], proteasome inhibitors (lactacystin) [4], and cytotoxic drugs (e.g. etoposide) [5]. Moreover, leukemic cells displaying dysregulation of the Ca^{2+}- and lipid-dependent serine-threonine kinase PKC undergo apoptosis rather than maturation when exposed to PMA [6]. Collectively, such findings raise the possibility that apoptosis represents an alternative fate for cells unable to proceed along a normal differentiation pathway [7].

There is evidence that under appropriate conditions, induction of cellular maturation potentiates apoptosis in leukemic cells previously exposed to cytotoxic agents. For example, differentiating compounds such as vitamin D_3, DMSO, hexamethylamine bisacetamide, all-*trans* retinoic acid, and *n*-butyrate have been shown to enhance leukemic cell apoptosis following exposure to DNA-damaging drugs including ara-C, 5-fluorouracil, and camptothecin, among others [8–11]. Although the mechanism underlying this phenomenon is unknown, it has been hypothesized that induction of maturation interferes with DNA repair events that accompany genotoxic insults [12]. An alternative possibility, consistent with the findings cited above, is that drug-mediated DNA damage leads to a disruption of normal differentiation events, signaling the cell to proceed along an apoptotic pathway.

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¶ *Abbreviations:* PMA, phorbol 12′-myristate 13′-acetate; PKC, protein kinase C; ara-C, 1-[β-D-arabinofuranosyl]cytosine; dFdC, (gemcitabine), 2′,2′-difluorodeoxycytidine; BRY, bryostatin 1; CDKIs, cyclin-dependent kinase inhibitors; dCyd, deoxycytidine; PCNA, proliferating cell nuclear antigen; MAPK, mitogen-activated protein kinase; PARP, polyADP-ribose polymerase; PCR, polymerase chain reaction; and APC, aphidicolin.

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BRY is a non-tumor promoting PKC activator that exerts variable effects on leukemic cell differentiation [13] and exhibits antitumor activity in non-hematological malignancies as well [14]. It represents one of a group of natural products targeted by the National Cancer Institute for clinical development in humans [15]. In human monocytic leukemic cells (U937), BRY, administered at high concentrations (e.g. 200 nM), inhibits cell growth while inducing dephosphorylation of the cyclin-dependent kinase CDK2 [16], although several studies have shown that its capacity to inhibit proliferation in these cells is considerably weaker than that of PMA [17–19]. Recently, our laboratory undertook a direct comparison of BRY and PMA (10 nM each) with respect to their effects on cell cycle regulatory proteins and induction of differentiation in U937 cells [20]. This study demonstrated that BRY is considerably less potent than PMA in inducing $p21^{CIP1}$, inhibiting CDK2 activity, and triggering cell cycle arrest and growth inhibition, providing a possible mechanism for its limited differentiation-inducing capacity in this cell line.

In a previous paper, we reported that administration of BRY before, but not after ara-C in differentiation-unresponsive human leukemia cells (HL-60) leads to an increase in apoptosis accompanied by synergistic inhibition of clonogenicity [21]. In contrast, in weakly differentiation-responsive U937 leukemic cells, apoptosis is only potentiated when BRY follows ara-C [19]. Thus, in this system, BRY may function like a differentiation inducer in promoting apoptosis in cells previously exposed to a DNA-damaging agent [8–11]. Currently, virtually no information exists concerning the mechanism by which maturation-inducing compounds potentiate apoptosis in cells previously exposed to cytotoxic agents, nor have the events accompanying this phenomenon been well characterized. The purpose of the present study was to compare two PKC activators with disparate differentiation-inducing capacities (i.e. BRY and PMA) with respect to their effects on drug-induced apoptosis in U937 cells, and to identify factors that might be involved in modulating apoptosis and maturation in cells pre-exposed to two cytotoxic dCyd analogs, ara-C and dFdC. The potential relevance of this comparison is highlighted by a very recent report demonstrating the feasibility of administering PMA to patients with leukemia, as well as possible beneficial *in vivo* interactions of this agent with ara-C [22]. Our findings indicate that BRY and PMA exerted qualitatively and quantitatively similar effects on dCyd analog-mediated apoptosis; moreover, pretreatment of cells with ara-C or dFdC blocked PKC activator-associated induction of the (CDKIs) $p21^{CIP1}$ and/or $p27^{KIP1}$, a phenomenon associated with disruption of cellular maturation. These observations raise the possibility that dysregulation of the cell cycle arrest machinery may contribute to apoptotic and differentiation-related responses observed in cells sequentially exposed to ara-C or dFdC followed by PKC-activating agents.

MATERIALS AND METHODS

Cells

The human monocytic leukemic cell line U937 was derived from a cell line as previously reported [23]. Cells were cultured to logarithmic growth phase in RPMI 1640 medium supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (HyClone). Cells were checked routinely and determined to be mycoplasma-free using the Gen-Probe Kit (Gen-Probe Inc.).

Drug Treatment

Logarithmically growing cells were exposed to ara-C (free base), APC, etposide (Sigma), or dFdC (provided by Dr. M. H. Niedenthal, Eli Lilly) for 6 hr, after which the cells were washed three times in serum-free medium to remove drug, and resuspended in medium containing BRY (provided by Dr. A. J. Murgo, CTEP/DCT) or PMA (Sigma) for an additional 24 or 72 hr. Cell number was determined by hemacytometer and normalized prior to the studies of apoptosis, differentiation, and protein expression, as described below. Vehicle controls of water and DMSO ($\leq$0.01%) were found consistently to be equivalent to drug-free controls with respect to gene expression and apoptosis.

Differentiation and Apoptosis Studies

CD11b expression was evaluated after 72 hr of treatment as previously described [20, 24]. Briefly, treated cells were pelleted at 500 *g* and resuspended in cold PBS at 5×10^5 cells/mL. The cell suspension was mixed with fluorescein isothiocyanate-labeled antibody (CD11b or IgG2a control, Becton-Dickinson) and placed on ice for 20 min. Cells were diluted in cold PBS and analyzed with a Becton-Dickinson FACScan flow cytometer and CyCLOPS 2000 Version 4.0 software. Cell morphology and apoptosis were measured by cytocentrifuge preparations stained with the Diff-Quik stain set (Dade Diagnostics) and viewed by light microscopy. Features of cellular differentiation, as well as apoptosis (i.e. cell shrinkage, nuclear condensation, formation of apoptotic bodies) were evaluated as previously described [21]. The percentage of apoptotic cells was determined by evaluating $\geq$1000 cells per condition in three separate experiments.

Analysis of DNA Damage

To assess DNA fragmentation, pelleted cells (3×10^6 cells/pellet in triplicate) were resuspended in 0.5 mL of PBS and lysed by the addition of 5 mM Tris-HCl, 30 mM EGTA, 30 mM EDTA, 0.1% Triton X-100 (fully reduced), with gentle agitation. The lysates were centrifuged at 20,000 *g* at 4° for 40 min, the pellets were discarded, and the presence of DNA fragments in the supernatant was determined. Following treatment with RNase (50 μg/mL) for 2 hr at 37°, DNA from 3×10^5 cells was electropho-

resed on a 2.5% low-melting point agarose gel. Bands corresponding to mononucleosomal and dinucleosomal fragments were removed from the gel, and the DNA was recovered by β-agarase digestion (Epicentre Technologies) followed by ethanol precipitation. These DNA samples, as well as samples of genomic DNA that had been either sonicated and similarly gel-fractionated, or cut with *Alu*I, *Mbo*I, *Nla*III, *Tsp509*I, or *Sma*I, were Klenow treated to produce blunt ends and phosphorylated with polynucleotide kinase [25]. The DNA was ligated to the partial duplex GCGGTGACCCGGGAGATCTGAATTC-GAATTCAGATC [26], and amplified by anchored PCR, using the same 25-mer primer and primer CTCTGTCIC-CCAGGCTGGAGTGCA, corresponding to bases 268–245 of the minus strand of the *Alu* consensus sequence [27], for 25 cycles of 30 sec at 94°, 30 sec at 55° and 30 sec at 72° [28]. Following removal of primers by precipitation, the PCR products were subjected to runoff polymerization with the 5′-end-labeled, nested *Alu* primer CCCAGGCTG-GAGTGCAITGG (minus strand bases 260–241) using an Epicenter Epicycle sequencing kit but substituting a mixture containing 0.25 mM of each dNTP in place of the termination mixtures. Samples were electrophoresed for 4 hr on a 7% polyacrylamide gel and subjected to autoradiography. Cleavage positions were assigned to each band by reference to the restriction enzyme-treated samples, which yielded the expected prominent bands corresponding to site-specific cleavage of the *Alu* sequence.

Determination of ara-CTP Levels by HPLC

After treatment, cells were counted and equal numbers (20×10^6) were washed in cold PBS, lysed in 0.6 N trichloroacetic acid, and extracted in 1:3.5 trioctylamine: 1,1,2-trichlorotrifluoroethane (Sigma-Aldrich). The aqueous phase was stored at −80° until analysis. Immediately prior to column addition, the samples were thawed and extracted in succession with 0.5 M sodium periodate, 4 M methylamine, and 1 M rhamnose to convert the NTPs to their respective bases [29]. Extracts were run on a Waters radial-pak 10 μm SAX cartridge, monitored at 280 nm on a Beckman 160 detector, and analyzed with a Bio-Rad model 700 Chromatography Workstation (Version 3.63 software). Samples were run at 3 mL/min for 22 min in 25% ammonium phosphate (0.75 M, pH 3.7)–75% ammonium phosphate (5 mM, pH 2.8), which then was increased to 100% of the 0.75 M ammonium phosphate over the next 40 min [30]. Peaks were identified by relative retention time compared with authentic ara-C triphosphate and deoxycytidine triphosphate (Sigma).

Western Analysis

Cells were washed two times with PBS, resuspended in 100 μL PBS, lysed by the addition of 100 μL of 2× Laemmli buffer [60 mM Tris (pH 6.8), 4% SDS, 5.76 mM β-mercaptoethanol, 10% glycerol] and briefly sonicated. Lysates were quantified using Coomassie protein assay reagent (Pierce). Extracts (25 μg) were boiled for 10 min, fractionated by SDS–PAGE, transferred electrophoretically to Optitran nitrocellulose filters (Schleicher & Schuell), and probed with antibodies for $p21^{CIP1}$ and $p27^{KIP1}$ (1:500 dilution; Transduction Laboratories, Lexington). Detergent-insoluble and -soluble cell extracts for determination of PCNA were prepared exactly as described [31]. PCNA antibody was used at 1:500 (Upstate Biotechnology). After blocking in PBS-Tween (PBS-T; 0.05%) and 5% milk (Carnation) for 1 hr at room temperature, the membranes were incubated in fresh blocking solution with primary antibody for 4 hr at room temperature or overnight at 4°. Blots were washed subsequently three times for 10 min each in PBS-T, incubated for 1 hr with horseradish peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories) in blocking buffer, and washed three times for 10 min each in PBS-T. All blots were developed with the enhanced chemiluminescence method (Amersham). Equal gel loading and transfer were confirmed by staining the membrane with amido black after transfer and reprobing the blots with either anti-actin (Sigma) or anti-α-tubulin antibodies (Calbiochem).

Clonogenic Assay

Following drug treatment, cell number was determined by hemacytometer counting, and cells were washed three times in drug-free medium. Their ability to form colonies in soft agar was determined by a previously described technique [32]. Colonies, consisting of groups of ≥50 cells, were scored at day 10 utilizing an Olympus (Melville) model CK inverted microscope.

Determination of MAPK Activities

MAPK activity was determined as described previously [33]. Briefly, pelleted cells were washed in cold PBS, repelleted, and flash-frozen. MAPK was immunoprecipitated from clarified lysates with protein A/agarose-conjugated antibody/antisera (anti-ERK2, sc-154AC). Activity assay mixtures consisted of immunoprecipitated enzyme, substrate [myelin basic protein (Sigma)], and [γ-^{32}P]ATP (5000 Ci/pmol; NEN/DuPont), in 25 mM HEPES, pH 7.4, containing 15 mM $MgCl_2$, 100 mM trisodium orthovanadate, 0.01% β-mercaptoethanol, and 1 μM microcystin LR. Reactions were initiated by the addition of substrate and terminated by transfer to P81 filter paper. Filters were rinsed in 185 mM orthophosphoric acid, dehydrated in acetone, and then radioactivity was determined by liquid scintillometry.

Statistical Analysis

The significance of differences between experimental groups was determined utilizing Student's *t*-test for unpaired observations.

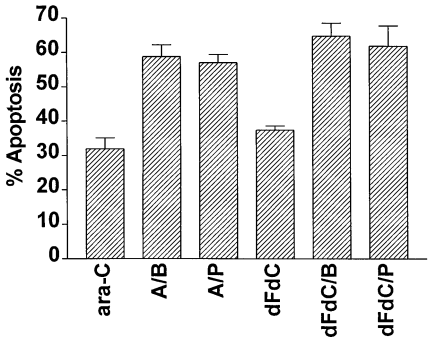

FIG. 1. Potentiation of ara-C- and dFdC-induced apoptosis by BRY or PMA. U937 cells at 4 × 10^5 cells/mL were pretreated with ara-C (1 μM) or dFdC (100 nM) for 6 hr, washed, and further incubated at 2 × 10^5 cells/mL with either medium, BRY (B) (10 nM), or PMA (P) (10 nM) for an additional 24 hr. The percentage of cells undergoing apoptosis was then assessed by monitoring Wright-Giemsa stained specimens for characteristic morphologic features as described in Materials and Methods. Values from triplicate experiments are expressed as the mean percent apoptosis ± SEM. Basal levels of apoptosis were less than 5%, and BRY and PMA treatment alone induced cell death in 8–10% of the cell population.

RESULTS

It has been shown in a previous report that exposure to BRY augments apoptosis in U937 cells previously treated with 10 or 100 μM ara-C [19]. However, the very extensive degree of cell destruction that resulted from such high ara-C concentrations, particularly in cells exposed subsequently to BRY or PMA, rendered analysis of accompanying events impractical. Consequently, for the purpose of the present study, a lower ara-C concentration (e.g. 1 μM) was employed. Whereas the extent of apoptosis (and its potentiation by BRY or PMA) was reduced thereby, this approach provided the major advantage of allowing associated events (e.g. expression of CDKIs and assessment of ara-C metabolism) to be monitored reliably.

The initial question to be addressed was whether the disparate abilities of PMA and BRY to induce U937 cell maturation [17, 18, 20] would have an impact on their capacity to modulate apoptosis in cells pre-exposed to ara-C (1 μM; 6 hr). Parallel studies were performed using equitoxic concentrations of the dCyd analog dFdC (e.g. 100 nM). In each case, drug concentrations were selected that induced apoptosis in ~35–40% of cells. Results are shown in Fig. 1. It can be appreciated that despite clear differences in their maturation-inducing ability, BRY and PMA produced equivalent increases in apoptosis in ara-C- and dFdC-pretreated cells (e.g. ~50–60% greater than that observed in cells exposed to dCyd analogs alone). It should be noted that a 24-hr exposure to BRY or PMA alone induced apoptosis to a limited degree (e.g. ~6–10%), and that in all cases the extent of apoptosis following sequential drug treatment was greater than additive ($P < 0.02$ in each case; not shown). Assessment of DNA fragmentation by bisbenzimide spectrofluorophotometry yielded equivalent results (not shown). Thus, despite its relatively weak differentiation-inducing capacity, BRY was as effective as PMA in triggering the characteristic morphologic features of apoptosis in ara-C- and dFdC-pretreated cells, suggesting that factors other than, or in addition to, differentiation *per se* were responsible for this phenomenon.

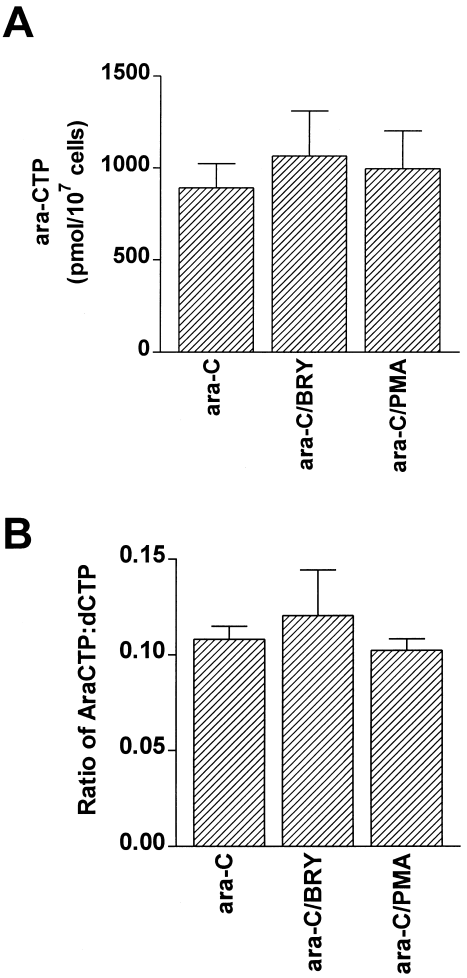

FIG. 2. Metabolism of ara-CTP in U937 cells following BRY or PMA treatment. Ara-CTP metabolism was determined by strong anion exchange chromatography from extracts prepared from cells exposed to ara-C (1 μM) for 6 hr, washed, and incubated in either medium, BRY (10 nM) or PMA (10 nM). Values represent the mean of triplicate determinations (± SEM) and are (A) expressed as retention of the ara-C derivative ara-CTP or (B) expressed as the ratio of ara-CTP:dCTP for each sample.

To determine whether BRY or PMA might act by modulating ara-C metabolism, the retention of the lethal ara-C derivative ara-CTP was compared in ara-C-pretreated cells subsequently incubated for 24 hr in either fresh medium or medium containing either 10 nM BRY or PMA (Fig. 2). It can be seen that subsequent exposure of cells to BRY or PMA did not increase ara-CTP retention significantly (Fig. 2A), nor did it alter the ratios of ara-CTP to its physiologic analog, dCTP ($P = 0.68$) (Fig. 2B). Consequently, potentiation of ara-C metabolism could not be

invoked to explain modulation of ara-C-mediated apoptosis by PKC activators. As we have reported previously, treatment of cells with PMA alone resulted in a modest arrest of cells in G_1 at 24 hr, whereas BRY has only a marginal effect [20]. When cells were exposed to ara-C or dFdC alone, an increase in the subdiploid fraction was observed, accompanied by a substantial reduction in the S-phase fraction (i.e. from ~40% in controls to 0.8 ± 0.4 and 8.0 ± 3.5% in the case of ara-C and dFdC, respectively; data not shown). When cells were exposed sequentially to the dCyd analogs followed by PMA or BRY, a further increase in the subdiploid fraction was noted, which occurred at the expense of the G_0/G_1, and, to a lesser extent, the G_2M fractions (data not shown). Reductions in the S-phase fractions under these conditions were equivalent to those seen in cells exposed to ara-C or dFdC alone. Thus, we were unable to attribute the observed increase in apoptosis in cells subsequently exposed to PMA or BRY to an expansion of the S-phase population.

To determine whether subsequent exposure of ara-C-pretreated cells to BRY or PMA produced qualitative differences in DNA fragmentation, mononucleosomal and dinucleosomal fragments were isolated from treated cells, and the positions of the double-strand breaks by which they were generated were mapped by ligation-mediated PCR in the highly repeated *Alu* sequence. In any defined DNA sequence, endonucleases typically exhibit very large site-to-site variation in cleavage frequency, resulting in a complex cleavage signature that is distinct for each nuclease (e.g. DNAse I [34], or the mammalian Ca^{2+}/Mg^{2+}-dependent nuclear endonuclease [35]). Thus, the finding that cleavage patterns in the *Alu* sequence were essentially identical for cells treated with ara-C either alone or followed by BRY or PMA (Fig. 3) suggests that the same endonuclease(s) was activated in each case. In separate studies, the extent of caspase-3 and PARP cleavage was equivalent in cells exposed to ara-C followed by either BRY or PMA (not shown). Moreover, caspase-3 activity increased to a similar extent following exposure to both sequences (e.g. 5.4 ± 0.2- vs 5.2 ± 0.2-fold over basal levels; $P > 0.5$). Collectively, these findings suggest that despite their disparate differentiation-inducing abilities, the PKC activators BRY and PMA do not exert qualitatively (or quantitatively) different effects on activation of the caspase cascade in cells previously exposed to ara-C, or on the characteristics of the DNA fragmentation that ensues.

To assess the biologic consequences of these actions, clonogenic survival studies were performed (Table 1). While induction of apoptosis has been shown to reduce clonogenic survival [21], several other studies have demonstrated a discordance between the extent of apoptosis and loss of self-renewal capacity [36, 37]. It can be seen that BRY alone had a relatively modest effect on self-renewal capacity, reducing clonogenicity to 49.1 ± 2.4% of control values, whereas PMA was considerably more inhibitory, reducing colony formation to 7.5 ± 4.7% of control levels. Cells treated with ara-C (1 μM) for 6 hr, washed, and incubated for an additional 24 hr in drug-free medium displayed a very substantial reduction in colony formation (e.g. 98%). In each case, inhibition of clonogenicity was considerably greater than the extent of apoptosis observed at the end of the drug exposure interval. Finally, when ara-C-pretreated cells were exposed subsequently to BRY or PMA, colony formation declined by an additional 75% in each case (e.g. to 0.5 ± 0.2 and 0.4 ± 0.3% of control

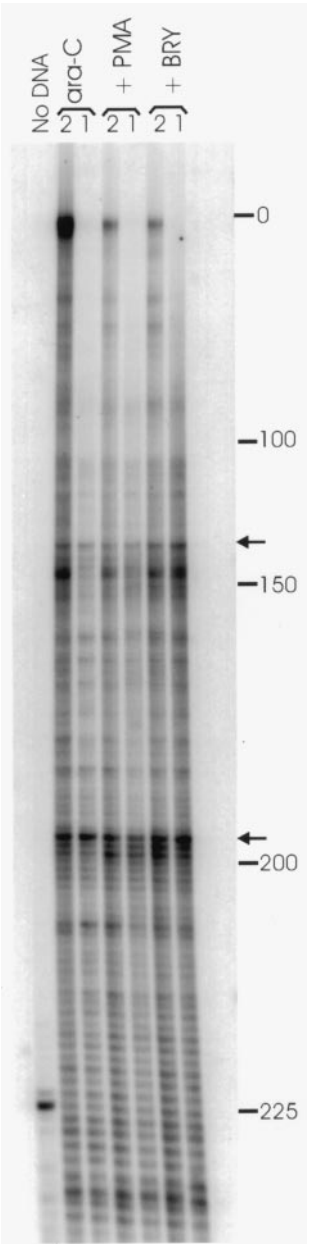

FIG. 3. Cleavage patterns for internucleosomal DNA fragmentation in the *Alu* sequence, as determined by ligation-mediated PCR. Cleavage in the + strand of the *Alu* sequence, for double-strand breaks producing dinucleosomal (lanes "2") and mononucleosomal (lanes "1") fragments, as determined by ligation-mediated PCR is shown. Numbers show *Alu* consensus sequence positions and arrows indicate cleavage hotspots at bases 197–198 (TC ↓ GC) and 142–143 (GG ↓ CG) of the *Alu* sequence, as assigned by reference to restriction enzyme-treated samples.

TABLE 1. Clonogenicity

Treatment	% of Control
BRY	49.1 ± 2.4
PMA	7.5 ± 4.7
ara-C	2.0 ± 0.3
ara-C → BRY	0.5 ± 0.2
ara-C → PMA	0.4 ± 0.3
APC	100.0 ± 9.4
APC → BRY	50.0 ± 8.1
APC → PMA	11.0 ± 1.0

Self-renewal capacity in U937 cells pretreated with either 1 μM ara-C or 1 μM APC (aphidicolin) for 6 hr (4×10^5 cells/mL), washed and further incubated with medium, 10 nM BRY or 10 nM PMA for an additional 24 hr (2×10^5 cells/mL). Values represent the means ± SEM of three separate experiments done in triplicate (N = 9).

values for ara-C/BRY and ara-C/PMA, respectively vs 2.0 ± 0.3% for ara-C alone; $P < 0.001$ in each case). The inhibitory effects of ara-C/BRY and ara-C/PMA did not, however, differ significantly ($P > 0.5$). Thus, while potentiation of ara-C-induced apoptosis by BRY or PMA was accompanied by a further reduction in leukemic cell self-renewal capacity, it appears likely that additional factors contributed to the very extensive loss of clonogenic potential after drug exposure.

Previous reports have shown that BRY and PMA induce expression of the CDKIs $p21^{CIP1}$ and $p27^{KIP1}$ [16, 20, 38, 39], both of which have been shown to influence the apoptotic response of neoplastic cells to various cytotoxic agents [40–42]. To determine what impact prior treatment with ara-C (or dFdC) might have on CDKI induction by PMA and BRY, $p21^{CIP1}$ and $p27^{KIP1}$ expression was monitored in cells exposed sequentially to these agents (Fig. 4). Consistent with the results of other groups [43] as well as our own [24], ara-C treatment alone failed to induce $p21^{CIP1}$ or $p27^{KIP1}$, nor did dFdC increase expression of either of these CDKIs. Unexpectedly, pretreatment of cells with either ara-C or dFdC markedly reduced induction of $p21^{CIP1}$ following a subsequent 24-hr exposure to PMA. A similar phenomenon was noted in the case of $p27^{KIP1}$, i.e. both PMA and BRY induced $p27^{KIP1}$, whereas ara-C and dFdC pretreatment inhibited this response. In view of evidence that interference with the function of CDKIs such as $p21^{CIP1}$ or $p27^{KIP1}$ may increase the susceptibility of malignant cells to drug-induced apoptosis [40, 41], and that enforced expression of $p21^{CIP1}$ may have the opposite effect [42], these findings raise the possibility, although indirectly, that antagonism of CDKI induction by nucleoside analogs may contribute to lethality in cells subsequently exposed to PKC activators.

Given the observation that pretreatment with deoxycytidine analogs opposed CDKI induction (Fig. 4), an attempt was made to determine what impact this action might have on leukemic cell maturation. To this end, cells were exposed to ara-C (1 μM) or dFdC (100 nM) for 6 hr, after which they were washed and incubated in either medium or

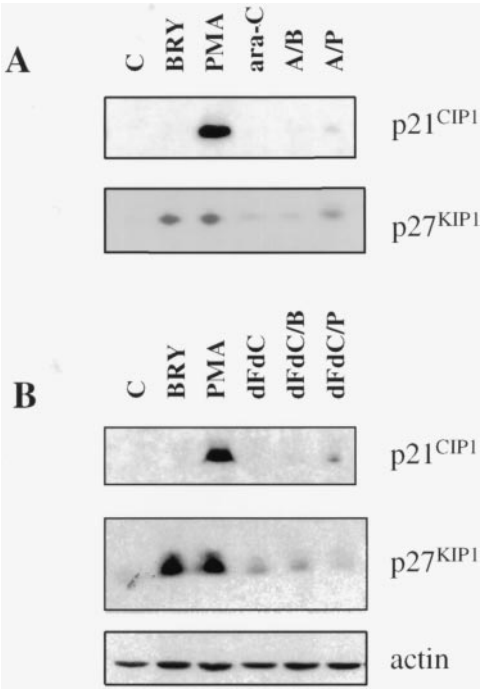

FIG. 4. Inhibition of CDKI expression by PKC activators, BRY or PMA, following pretreatment with dCyd analogs. Cell extracts were pretreated for 6 hr with either (A) ara-C (1 μM) or (B) dFdC (100 nM) followed by an additional 24-hr incubation with 10 nM BRY (B) or 10 nM PMA (P). Equal gel loading was confirmed by amido black staining of the membrane and reprobing with anti-actin antibody.

PMA (10 nM) for an additional 72 hr. At the end of this interval, expression of the myelomonocytic maturation marker CD11b was assayed. Exposure to PMA alone induced CD11b expression in 47% of cells, whereas treatment with ara-C or dFdC alone for 6 hr resulted in CD11b expression in 29.4 ± 3.5 or 20.8 ± 2.7% of cells, respectively (Fig. 5). The latter results are consistent with the established ability of nucleoside analogs such as ara-C to induce leukemic cell differentiation [44]. Interestingly,

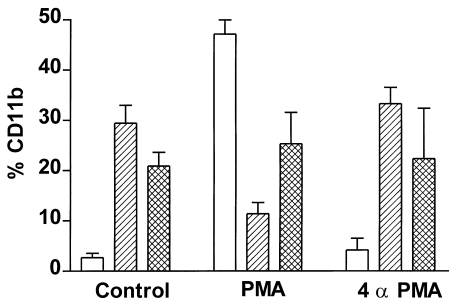

FIG. 5. Inhibition of PMA-induced maturation by pretreatment with dCyd analogs. Shown is CD11b expression in cells pretreated (6 hr) with ara-C (1 μM; right-hatched bars) or dFdC (100 nM; double-hatched bars), washed, and followed by an additional 72-hr incubation with 10 nM PMA or 10 nM 4α-PMA. U937 cells treated with vehicle, PMA or 4α-PMA for 72 hr are indicated by open bars. Values represent the means of triplicate determinations (± SEM).

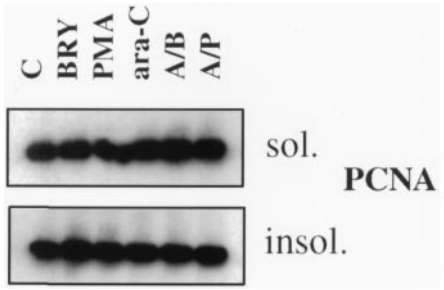

FIG. 6. PCNA distribution after exposure to ara-C. Shown are western blots of cell extracts from U937 cells exposed to ara-C (1 μM) for 6 hr, followed by an additional 24-hr incubation with medium, 10 nM BRY (A/B), or 10 nM PMA (A/P). Detergent-insoluble and -soluble cell fractions are indicated on the right.

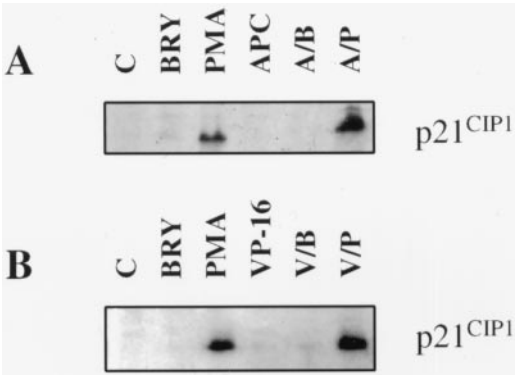

FIG. 7. Effect of pretreatment with APC or etoposide on induction of $p21^{CIP1}$ expression by BRY or PMA. (A) $p21^{CIP1}$ expression in cell extracts pretreated for 6 hr with APC (1 μM) followed by an additional 24-hr incubation with 10 nM BRY (A/B) or 10 nM PMA (A/P). (B) $p21^{CIP1}$ expression in cell extracts pretreated for 6 hr with VP-16 (10 μM) followed by an additional 24-hr incubation with 10 nM BRY (V/B) or 10 nM PMA (V/P).

sequential exposure of cells to ara-C or dFdC followed by PMA exerted clearly antagonistic effects on CD11b expression, resulting in positivity in only 11.4 ± 2.2 and 25.3 ± 6.2% of cells, respectively. In contrast, the inactive phorbol, 4α-PMA, exerted no effect on CD11b expression in drug-pretreated cells (Fig. 5). Results of parallel studies involving the weak differentiation-inducer BRY were less dramatic but also revealed subadditive effects on cell maturation (not shown). These data suggest that one of the consequences of inhibition of PKC activator-mediated CDKI induction by deoxycytidine analogs is interference with the normal maturation program.

$p21^{CIP1}$ is known to form quaternary complexes (also containing cyclins, PCNA, and DNA polymerase δ) [45–47] that are involved in the regulation of DNA synthesis [45, 48, 49]. Although the level of PCNA remains constant in replicating cells, it exists in two separate compartments, i.e. detergent-soluble and detergent-insoluble. The latter represents the functional form bound to DNA at the replication fork [46, 50]. Increases in DNA-bound PCNA have been implicated in potentiation of apoptosis by agents that inappropriately activate CDKs (e.g. UCN-01) [31]. To determine whether a similar event might be associated with ara-C- and dFdC-mediated inhibition of $p21^{CIP1}$ and $p27^{KIP1}$ induction, extracts from cells treated with ara-C followed by BRY or PMA were examined with respect to translocation of PCNA to the DNA-bound, detergent-insoluble fraction (Fig. 6). However, the cellular distribution of PCNA was not discernibly altered by ara-C or dFdC pretreatment, making it unlikely that this mechanism could be invoked to explain the observed effects on apoptosis.

It remained possible that ara-C and dFdC, whose triphosphate derivatives are potent inhibitors of DNA polymerase, might antagonize CDKI induction through a yet-to-be-identified feedback mechanism. To address this possibility, cells were pretreated with 1 μM APC for 6 hr, which, like ara-C and dFdC, inhibits DNA synthesis, but unlike these dCyd analogs, is not incorporated into elongating DNA strands [51]. Treatment with APC minimally induced apoptosis in these cells (e.g. 6.3 ± 0.5%); moreover, sequential exposure of cells to APC followed by either BRY or PMA was not associated with a significant increase in apoptosis (11.4 ± 0.8 and 14.2 ± 2.6%, respectively). Equivalent results were observed using 10 μM APC (not shown). In addition, exposure of cells to BRY or PMA following APC did not lead to enhanced DNA fragmentation (not shown), in contrast to results obtained in cells pretreated with ara-C. Consistent with these findings, APC treatment by itself (1 μM; 6 hr) did not inhibit clonogenicity, nor did pretreatment with APC significantly enhance the inhibitory effects of BRY or PMA (Table 1). Finally, in contrast to ara-C and dFdC, APC failed to block induction of $p21^{CIP1}$ by PMA (Fig. 7A) or $p27^{KIP1}$ by BRY or PMA (not shown). These findings raise the possibility that the observed effects of nucleoside analog pretreatment on apoptosis, loss of clonogenicity, and inhibition of CDKI induction in cells subsequently exposed to PKC activators may be related to analog incorporation into DNA, rather than to inhibition of DNA polymerase *per se*.

To test the possibility that inhibition of CDKI induction by ara-C and dFdC might represent a generalized consequence of drug-induced apoptosis, cells were treated with the topoisomerase II inhibitor VP-16 (5 or 10 μM; 6 hr) followed by a 24-hr exposure to 10 nM BRY or PMA. In contrast to ara-C or dFdC pretreatment, prior treatment with VP-16 failed to block PMA-mediated $p21^{CIP1}$ induction (Fig. 7B). Interestingly, subsequent exposure of VP-16-pretreated cells to BRY or PMA failed to increase apoptosis (i.e. 37.7 ± 1.8% for VP-16 alone vs 35.9 ± 2.6 and 34.8 ± 1.9% for the sequences VP-16 followed by BRY or PMA, respectively; $P > 0.05$). These results argue against the possibility that inhibition of CDKI expression represents a generic response to drug-induced apoptosis. They are also compatible with the hypothesis that interference with CDKI induction contributes to the increase in apoptosis observed when certain cytotoxic drugs are administered prior to PKC activators.

Finally, because induction of $p21^{CIP1}$ and $p27^{KIP1}$ expres-

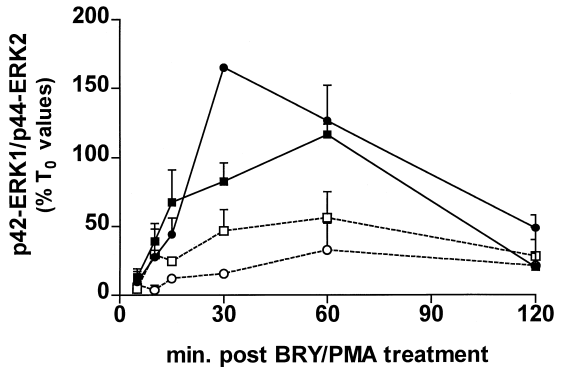

FIG. 8. Inhibition of MAPK activation by ara-C pretreatment. U937 cells were either untreated (control) or pretreated with ara-C (1 μM) for 6 hrs, washed, and further incubated with medium, or 10 nM BRY or PMA for an additional 2 hr. Activity of p42-ERK1/p44-ERK2 was determined by *in vitro* immune complex assay as described in Materials and Methods and expressed as the percent activity of either control or ara-C pretreated cells (time 0 = post-wash). Key: control/BRY (■), control/PMA (●), ara-C/BRY (□), and ara-C/PMA (○). Results represent the means ± range for duplicate experiments.

sion has been shown in response to MAPK [52, 53], the effects of ara-C preincubation on PMA- and BRY-associated activation of MAPK were investigated (Fig. 8). PMA and BRY induced 100–150% increases in basal MAPK activity that were maximal at 30 and 60 min, respectively. Significantly, preincubation of cells with ara-C reduced BRY- or PMA-stimulated increases in MAPK activity. These findings raise the possibility that interference with BRY and PMA-related MAPK activation may be responsible, at least in part, for the observed attenuation of $p21^{CIP1}$ and $p27^{KIP1}$ induction by deoxycytidine analogs.

DISCUSSION

The central goal of this study was to characterize the effects of two PKC activators (PMA and BRY) exhibiting disparate differentiation-inducing capacities in dCyd analog-pretreated leukemic cells. The results described herein indicate that despite the relatively weak maturation-inducing capacity of BRY [17–20], its effects on apoptosis in ara-C- and dFdC-pretreated cells are quantitatively and qualitatively similar to those of the potent differentiation-inducer PMA. Previous studies have shown that induction of leukemic cell maturation is associated with DNA strand breaks [54], and it has been proposed that subsequent differentiation induction may promote drug-induced DNA damage, perhaps by interfering with DNA repair [11]. An alternative possibility is that DNA damage triggered by differentiation-inducing agents, including PKC activators, differs in some fundamental manner from that initiated by cytotoxic agents. However, given the observation that treatment of cells with ara-C alone or ara-C followed by either BRY or PMA resulted in identical DNA cleavage patterns (Fig. 3), it appears that identical endonucleases are involved in each case. Moreover, subsequent exposure of ara-C-treated cells to BRY or PMA resulted in quantitatively (and qualitatively) equivalent effects on caspase-3 activation and degradation of downstream targets (e.g. PARP). Such findings are most compatible with the concept that each of these PKC activators increases the fraction of leukemic cells undergoing drug-induced apoptosis, but does not alter the nature of the cell death program itself. Furthermore, the equivalent effects of BRY and PMA on dCyd analog-mediated apoptosis suggest that differentiation induction *per se* is not primarily responsible for this phenomenon. Instead, they raise the possibility that early, precommitment events that in themselves are insufficient to initiate terminal differentiation account for the observed effects on cell death. Lastly, given evidence that induction of leukemic cell maturation may alter the activity of pyrimidine biosynthetic enzymes (e.g. deoxycytidine kinase; cytidine deaminase) involved in ara-C metabolism [55], it is important to note that neither BRY nor PMA enhanced retention of the lethal ara-C metabolite ara-CTP.

The ability of drugs such as ara-C and dFdC to oppose induction of CDKIs by BRY and PMA has not been described previously, and it seems plausible that this phenomenon may contribute, at least in part, to apoptosis observed in leukemic cells sequentially exposed to these agents. Treatment of leukemic cells with PMA [38, 43] or vitamin D_3 [8] results in induction of $p21^{CIP1}$ or $p27^{KIP1}$, inhibition of cyclin-dependent kinases, and ultimately dephosphorylation of the retinoblastoma protein, inactivation of the transcription factor E2F, and G_1 arrest [56]. The latter process has been shown to be required for normal maturation events to proceed [57]. Furthermore, there is considerable evidence that differentiation and apoptosis represent mutually exclusive cellular fates [7]. For example, U937 cells exhibiting dysregulation of the PKC isoform undergo apoptosis rather than maturation in response to PMA [6]. Conversely, several studies have demonstrated that dysregulation of the CDKIs $p21^{CIP1}$ and $p27^{KIP1}$ promotes drug-induced apoptosis, possibly by interfering with G_1 arrest and leading to uncoupling of S-phase and mitosis [41], or by antagonizing DNA repair [42]. It is tempting, therefore, to speculate that prevention of PKC activator-mediated CDKI induction by pretreatment with ara-C or dFdC contributes to cell death, possibly by disrupting cellular maturation. In this regard, we have reported recently that U937 cells exhibiting dysregulation of $p21^{WAF1/CIP1}$ (through stable expression of an antisense construct) are less susceptible to differentiation induction and correspondingly more susceptible to PKC activator-induced apoptosis, than their control counterparts [58]. In this way, CDKIs such as $p21^{WAF1}$ may direct cells along a differentiation-related pathway and thereby protect them from apoptosis [59]. Thus, the subadditive effects of dCyd analogs and PMA on U937 cell maturation (Fig. 5) and the accompanying potentiation of apoptosis may represent reciprocal consequences of CDKI dysregulation.

The mechanism by which dCyd analogs oppose CDKI

induction remains to be determined. It is has been shown that cell cycle regulation involves complex formation between CDKIs, CDKs, cyclins, and PCNA [45, 46, 48, 49]; moreover, PCNA interacts with DNA polymerase δ to regulate DNA replication and repair [46]. It therefore seemed plausible that inhibition of DNA polymerase by ara-CTP [60] might lead, through an as yet unidentified feedback mechanism, to CDKI down-regulation. However, the inability of APC, which is not incorporated into elongating DNA strands [51], to mimic the actions of ara-C or dFdC argues that nucleoside analog incorporation into DNA rather than inhibition of DNA polymerase, may be responsible for opposing CDKI induction. Furthermore, the failure of VP-16, a potent inducer of apoptosis [61], to exert the same effect suggests that this phenomenon does not merely represent a generalized consequence of cell death. Of possible mechanistic significance is the finding that pretreatment with ara-C or dFdC reduced PMA- or BRY-mediated activation of MAPK, which has been associated with cytoprotective effects [62]. In view of evidence that MAPK induces both $p21^{CIP1}$ and $p27^{KIP1}$ expression [52, 53], it is conceivable that perturbations in MAPK/ERK signaling by dCyd analogs contribute to antagonism of CDKI induction by BRY or PMA. One possibility is that initial activation of MAPK by analogs such as ara-C [63] may in some undetermined way attenuate subsequent activation by PKC activators. It is clear that additional studies will be required to resolve this issue.

It is important to note that while administration of PMA or BRY after ara-C led to a further reduction in U937 cell clonogenic survival, inhibition of colony formation following administration of drugs either individually or in sequence exceeded the extent of apoptosis. Several studies have demonstrated a discordance between apoptosis and clonogenic growth [36, 37], and these have led to speculation that an early commitment to cell death may occur in the absence of classic morphological and biochemical features of apoptosis [37]. Alternatively, inhibition of DNA synthesis (and the accompanying reduction in cell divisions) or induction of non-apoptotic forms of cell death could have the same net effect. In any event, it is unlikely to be coincidental that APC, which failed to (i) inhibit BRY- or PMA-related CDKI induction or (ii) lead to a significant increase in apoptosis in cells subsequently exposed to BRY or PMA, also failed to enhance the inhibitory effects of BRY and PMA on colony formation (Table 1). While these findings do not establish a causal relationship between interference with CDKI induction, promotion of apoptosis, and loss of clonogenicity, they are at least compatible with this concept.

In summary, the present study demonstrates that despite the limited ability of BRY to induce leukemic cell differentiation, it exerts effects that are comparable to those of the potent differentiation-inducer PMA with respect to induction of apoptosis, DNA fragmentation, and activation of the protease cascade in ara-C- and dFdC-pretreated U937 cells. This finding suggests that relatively early precommitment events, unable by themselves to engage a full differentiation program, underlie the observed interaction. Significantly, ara-C and dFdC pretreatment reduced induction of $p27^{KIP1}$ by BRY, and both $p21^{CIP1}$ and $p27^{KIP1}$ by PMA, raising the possibility that dysregulation of CDKIs by drug pretreatment contributes to apoptosis in cells exposed to these agents in sequence. The ability of dCyd analog pretreatment to interfere with PKC activator-mediated CDKI induction may also help to explain the sequence-dependent interactions of these agents on leukemic cell apoptosis [19]. To address this issue further, U937 cell variants stably expressing $p21^{CIP1}$ and $p27^{KIP1}$ antisense constructs have been isolated [58], and studies assessing their responses to the sequential administration of ara-C followed by PKC activators currently are underway. Finally, the present findings may have implication for the rational design of novel antileukemic strategies combining nucleoside analogs with agents acting through the PKC pathway. In this context, Phase I trials of BRY have been completed [64], and plans to combine this agent with ara-C in patients with leukemia currently are being implemented. Importantly, the recent introduction of PMA into clinical trials of humans [65], and its potentially favorable *in vivo* interactions with ara-C [22], suggest a possible role for this agent in the treatment of hematological malignancies. A better understanding of the mechanism(s) governing interactions of such PKC-activators with established antileukemic drugs may lead to more optimized treatment regimens.

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