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Potentiation of ara-C-Induced Apoptosis by the Protein Kinase C Activator Bryostatin 1 in Human Leukemia Cells (HL-60) Involves a Process Dependent upon c-Myc

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ABSTRACT. The role of the nuclear phosphoprotein c-Myc has been examined with respect to the regulation of 1-β-D-arabinofuranosylcytosine (ara-C)-induced apoptosis in human leukemia cells exposed to bryostatin 1 and other pharmacologic protein kinase C (PKC) activators. Pretreatment of HL-60 cells for 24 hr with 10 nM bryostatin 1 significantly potentiated the ability of ara-C (10 μM; 6 hr) to induce apoptosis without reducing the expression of c-Myc protein. In contrast, equivalent exposure to the stage 2 tumor-promoting PKC activator mezerein (10 nM) in conjunction with ara-C reduced c-Myc levels by 87% and failed to potentiate apoptosis. Co-administration of bryostatin 1 with mezerein before ara-C prevented down-regulation of c-Myc and augmented cell death, whereas co-treatment with the calcium ionophore A23187 (250 nM) and bryostatin 1 reduced c-Myc levels by 80% and abrogated the increase in ara-C-induced apoptosis. When cells were exposed for 24 hr to a c-*myc* antisense oligonucleotide (AS-ODN;10 μM) but not to a scrambled sequence ODN (SS-ODN) prior to ara-C, c-Myc expression was reduced by 81%, and apoptosis and cell viability were unperturbed. However, AS-ODN (but not SS-0DN) reduced c-Myc protein in cells pre-exposed to bryostatin 1 by 74% and abrogated potentiation of ara-C-induced apoptosis. The actions of c-*myc* AS-ODN did not stem from proximal G_1 arrest/differentiation or biochemical events, since they were not associated with a reduction in the S-phase cell fraction, p21(WAF1/CIP1) induction, pRb hypophosphorylation, or alterations in ara-C metabolism. Together, these findings indicate that HL-60 cell apoptosis proceeds by both c-Myc-dependent and -independent pathways, and that only the former are involved in the potentiation of ara-C-mediated cell death by bryostatin 1. BIOCHEM PHARMACOL 54;5:563–573, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. c-*myc*; ara-C; apoptosis; bryostatin 1; antisense oligonucleotides

The proto-oncogene c-*myc* encodes a nuclear phosphoprotein (c-Myc) of the helix-loop-helix/leucine zipper family, which, when dimerized with its binding partner Max, binds to specific DNA sequences [1, 2]. Although its precise functions remain to be fully elucidated, c-*myc* is felt to play an important role in cell-cycle progression, as well as in cell differentiation and proliferation [3, 4]. For example, cells exposed to mitogenic stimuli regularly respond with an increase in c-*myc* expression [5]. Conversely, induction of differentiation (e.g. in the human leukemia cell line HL-60) is associated with c-*myc* down-regulation and G_1 arrest [6].

Several lines of evidence implicate c-Myc in a sequence of events referred to as apoptosis or programmed cell death. Apoptosis represents a genetically regulated process in which a cell commits itself to an organized program of self-destruction [7]. In fibroblasts lacking appropriate growth factor support, enforced expression of c-Myc results in extensive apoptosis [8]. This capacity has been postulated to result from conflicting signals that simultaneously promote growth arrest (growth factor deprivation) and cell cycle progression (increased c-Myc expression) [9]. Moreover, blockade of c-*myc* in T cell hybridomas by AS-ODN[‖] prevents apoptosis triggered by some stimuli (e.g. activation-induced) but not by others (e.g. dexamethasone) [10]. It has also been proposed that overexpression of the anti-apoptotic gene *bcl-2* permits the mitogenic activity of c-Myc to proceed unopposed, leading to cooperative oncogenic interactions [11].

Alterations in c-Myc expression may also be involved in

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‖ *Abbreviations:* AS-ODN, antisense oligonucleotides; SS-ODN, scrambled sequence oligonucleotides; ara-C, 1-β-D-arabinofuranosylcytosine; PDB, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; PBST, phosphate-buffered saline-Tween; MZN, mezerein; PKC, protein kinase C.

determining whether cells undergo differentiation or apoptosis in response to various stimuli, including antineoplastic agents. For example, tumor-promoting phorboids such as PMA, whose intracellular target is PKC, induce cellular maturation and c-*myc* down-regulation in human leukemia cells [12]. Differentiating agents such as PMA and DMSO also have been shown to antagonize leukemic cell apoptosis triggered by diverse cytotoxic agents including the topoisomerase II inhibitor VP-16 [13] and the antimetabolite 5-azacytidine [14]. In light of the findings cited above [8, 9], it appears plausible that continued (and inappropriate) expression of c-Myc may contribute to apoptosis in cells exposed to inhibitors of DNA synthesis. If this is the case, then c-Myc down-regulation could account, at least in part, for the ability of differentiating agents to oppose this process.

We have reported previously that bryostatin 1, a non-tumor-promoting PKC activator that variably induces leukemic cell maturation [15], potentiates ara-C-mediated apoptosis in a human leukemia cell line (HL-60) in which differentiation is not induced [16]. In contrast, the stage 2 tumor-promoting PKC activator MZN, a potent inducer of HL-60 cell maturation, lacks the capacity to facilitate ara-C-induced apoptosis [17]. These effects can occur independently of alterations in ara-C metabolism or cell cycle traverse [16–18]. It may be pertinent that bryostatin 1 does not down-regulate c-myc mRNA levels in leukemic cell sublines unresponsive to its differentiating actions [19]. Thus, the failure of this compound to down-regulate c-Myc expression may contribute to its potentiation of drug-induced apoptosis. Currently, little direct information is available concerning the possible role of c-Myc in the regulation of cytotoxic drug-induced cell death. The rationale for the present study was to address this issue by correlating the modulatory effects of bryostatin 1 (and other PKC activators) on ara-C-induced apoptosis with perturbations in c-Myc protein expression. A secondary goal was to employ an antisense strategy to assess the functional role of c-Myc in this process more rigorously, and to determine whether previous findings implicating c-Myc in activation-induced apoptosis in lymphoid cells [10] could be extrapolated to myeloid leukemia cells exposed to the antimetabolite ara-C. Our results support the notion that undiminished c-Myc expression in bryostatin 1-treated HL-60 cells plays an integral role in potentiation of ara-C-induced apoptosis.

MATERIALS AND METHODS

Cell Culture

The human leukemia cell line HL-60 was obtained from a patient with acute promyelocytic leukemia as previously described [20]. Cells were maintained in a 37°, 5% CO_2 atmosphere in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) containing 10% fetal bovine serum (Hyclone, Logan, UT), non-essential amino acids, sodium pyruvate, MEM vitamins, L-glutamine, penicillin, and streptomycin (all GIBCO), and passaged twice weekly. Logarithmically growing cells (cell density $\leq 5 \times 10^5$/mL) were used in all experiments. Cells were tested routinely for mycoplasma contamination using the Gen-Probe kit (Gen-Probe, La Jolla, CA) and consistently found to be negative.

Chemicals

PDB, PMA, and MZN were purchased from LC Laboratories (Woburn, MA), formulated in DMSO (Sigma Chemical Co., St. Louis, MO), and stored at −20° before use. Bryostatin 1 was provided as a lyophilized powder by Dr. James Pluda, Cancer Treatment and Evaluation Branch, NIH/NCI, and also dissolved in DMSO prior to storage at −20°. After appropriate dilutions, the final concentration of DMSO in all test conditions was ≤ 0.05%, a level found to have no discernible effect on apoptosis or c-*myc* expression. Calcium ionophore (A23187) was purchased from Sigma and diluted in sterile water before use.

Morphological Assessment of Apoptosis

Apoptosis was evaluated by morphological criteria as previously described [21]. Following treatment of cells, cytocentrifuge preparations were made utilizing a Shandon Cytocentrifuge. Slides were stained with 20% Wright-Giemsa, and viewed at 1000× magnification with the aid of an Olympus microscope. The percentage of apoptotic cells, defined as those exhibiting the characteristic features of cell shrinkage, nuclear condensation, and the formation of membrane-bound apoptotic bodies, was determined by evaluating at least 500 cells/condition in triplicate.

DNA Fragmentation

The degree of low molecular weight DNA fragmentation in cells exposed to various agents was determined by spectrofluorometry of bisbenzamide-treated samples as we have described previously in detail [17]. The results of this assay have been shown to correlate closely with the percentage of apoptotic cells monitored by morphological criteria [17].

Terminal dUTP End-Labeling (TUNEL) Assay

A minor modification of the technique of Gavrieli *et al.* [22] was employed for the TUNEL assay. This method is based upon the observation that the generation of DNA fragments bearing 3′-OH overhanging ends by endonucleases associated with apoptosis permits subsequent labeling of fragments with fluorescein isothiocyanate (FITC)-conjugated dUTP in the presence of terminal transferase. Cytospin samples were fixed in 4% formaldehyde in PBS for 10 min at room temperature followed by exposure to acetic acid:ethanol (1:2) for 5 min at −20°. After washing two times in PBS, cells were treated with 1 mg/mL bovine serum albumin in PBS for 10 min followed by two additional washes in PBS. The slides were treated with 20 μL of a

staining mixture [containing 5× reaction buffer (20 μL), 1 μL of terminal transferase (Boehringer Mannheim, Indianapolis, IN), 10 μL of 25 mM $CoCl_2$, 2 μL of fluorescein-12-dUTP (Boehringer Mannheim), 67 μL of distilled water] and incubated at 37° for 60 min in a humidified chamber. The cells were then counterstained with propidium iodide (0.5 μg/mL in 0.01% sodium citrate; Sigma) for 15 sec followed by two washes in PBS. Slides were then mounted in 1 mg/mL *p*-phenylenediamine in 70% glycerol/PBS and viewed with the aid of an Olympus model BH-40 fluorescence microscope. Photomicrographs of randomly selected fields were obtained at 600× magnification.

Western Analysis

Whole cell pellets (1×10^7 cells/condition) were washed twice in PBS and then sonicated on ice in a 50-mL volume (PBS, 0.05% SDS, 0.5% Triton X-100, 100 mM β-mercaptoethanol, and 1 μg/mL aprotinin) utilizing 2-sec pulses with the aid of a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) operating in a continuous mode. Homogenates were quantified using Coomassie protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (25 μg) were denatured in 2× Laemmli buffer, boiled for 10 min, separated by SDS–PAGE (5% stacker and 12% resolving), and electroblotted to nitrocellulose. The blots were stained in 0.1% amido black and destained in 5% acetic acid to ensure equivalent loading and transfer of protein. The blots were then blocked in PBST (0.05%) and 5% milk for 1 hr at 22°, after which they were incubated in fresh blocking solution containing a 1:500 dilution of c-Myc primary antibody (Oncogene Science, Uniondale, NY) for 4 hr at 22°. Blots were washed 3 × 5 min in PBST, and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA). Blots were again washed 3 × 5 min in PBST and then developed by enhanced chemiluminescence (ECL; Amersham Life Sciences, Arlington Heights, IL).

An identical procedure was employed in studies of p21 (WAF1/CIP1/MDA6) and pRb, except that primary antibodies directed against WAF1 (1:500; Transduction Laboratories, Lexington, KY) and pRb (1:500; Pharmingen, San Diego, CA) were employed. Resolution of pRb was carried out utilizing 6% gels. In each case, secondary antibodies were used at a dilution of 1:1000.

Quantitative Assessment of c-Myc Protein

Following ECL reactions, western blots were exposed to Fuji X-ray film, and bands of interest were scanned using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) in conjunction with Imagequant Software (Molecular Dynamics). For each experimental condition, 4–5 independent blots were scanned, and band intensity was expressed as a percentage relative to controls.

Northern Analysis

Total cellular RNA was isolated from 1×10^7 cells using RNA STAT-60 (Tel-Test "B," Inc., Friendswood, TX) per the manufacturer's instructions. Probes were nick-translated with [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA) using a kit and protocol from Life Technologies (GIBCO-BRL). Total RNA (15 μg) was then separated on a 1% agarose/formaldehyde gel as previously described [23]. The RNA was blotted onto nylon (Schleicher & Schuell, Keene, NH) by vacuum transfer for 4 hr, and then cross-linked to the nylon by baking at 80° for 2 hr. The blots were then hybridized with a c-*myc* probe, consisting of an *Eco*RI/*Cla*I fragment of pmC41 3RC containing the third exon of the human c-*myc* gene, provided by Dr. Eric Westin, Medical College of Virginia. To ensure equal loading, the blots were hybridized simultaneously with a GAPDH cDNA probe. The blots were then washed extensively in 0.2× SSC (1× = 150 mM NaCl, 15 mM sodium citrate)/0.1% SDS at 65°, placed on Fuji film with intensifying screens, and exposed at −90°.

c-myc AS-ODN

Phosphorothioate AS-ODN to c-*myc* were purchased from Oligos, Etc. (Wilsonville, OR). The sequence of the 21-mer c-*myc* AS-ODN was 5′-GAAGCTAACGTT GAGGGGCT-A3′ (AS) complementary to the initiation start codon. A corresponding scrambled sequence 21-mer ODN containing a GGGG repeat was used as a control (5′-AAGGCTCAAGTTGAGGGGTCA-3′, SS). Cells were exposed sequentially to bryostatin 1 and ara-C as described above in the presence or absence of either SS- or AS-ODN (10 μM). Following isolation of cell pellets, c-Myc protein was determined by western analysis as detailed above. Parallel studies were performed to evaluate the effects of ODNs on apoptosis or cell viability (detailed below).

Cell Cycle Analysis

Flow cytometry was used to monitor the cell cycle distribution of HL-60 cells following exposure to bryostatin 1 and AS-ODN [18]. Briefly, cells (10^6/condition) were pelleted at 400 *g* for 5 min at 4° and resuspended in 1.5 mL of PBS followed by 3 mL of 100% ethanol for 1 hr on ice. Cells were then repelleted at 300 × *g* for 5 min and resuspended in 1.0 mL of a mixture containing 3.8×10^{-3} M sodium citrate, 0.5 mg/mL RNase A (Sigma), and 0.01 mg/mL propidium iodide. After incubation of cells on ice for 3 hr, cells were repelleted at 1000 × *g* and resuspended in 1 mL of PBS before analysis. Cell cycle analysis was performed utilizing a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) in conjunction with a commercially available software program (RFIT; Verity Software, Topsham, ME).

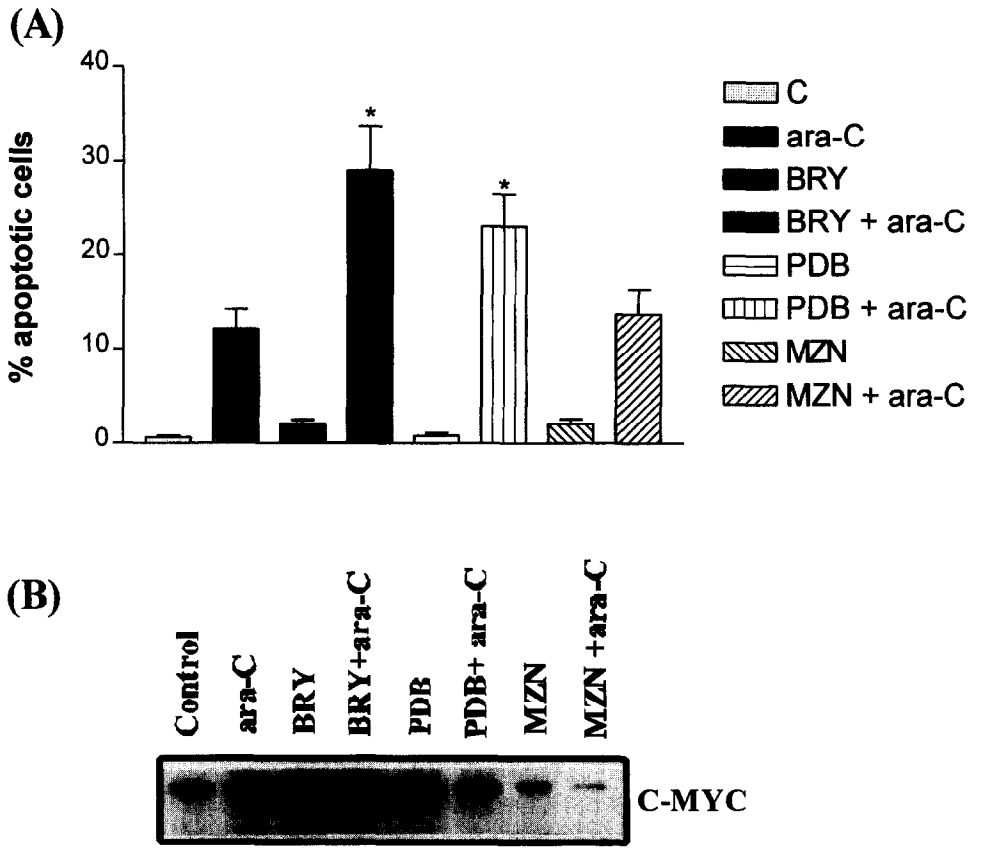

FIG. 1. Effect of PKC activators on ara-C-induced apoptosis and c-Myc protein levels. (A) Logarithmically growing HL-60 cells were exposed for 24 hr to 10 nM bryostatin 1 (BRY), phorbol dibutyrate (PDB), or mezerein (MZN), followed by a 6-hr exposure to 10 μM ara-C. The percentage of apoptotic cells was determined by viewing Wright Giemsa-stained preparations under light microscopy and scoring 500 cells/condition. Values represent the means for three to four separate experiments performed in triplicate ± 1 SD. Key: (*) significantly greater than ara-C alone, $P < 0.05$. (B) Alternatively, cells were sonicated, and the lysates (25 μg/condition) were separated by SDS–PAGE, followed by transfer to nitrocellulose, as described in Materials and Methods. After staining with amido black to ensure equivalent loading and transfer, levels of c-Myc protein were determined as described. The results of a representative study are shown; c-Myc protein levels for each condition were quantified densitometrically, and mean values (relative to controls) for five separate experiments are displayed in Table 1.

Viability Studies

Following treatment of cells with bryostatin 1 and ara-C in the presence or absence of AS- or SS-ODN, viability was monitored by combining aliquots of cells with an equal volume of a 0.4% trypan blue solution (Sigma) and assessing the percentage of cells excluding dye. Alternatively, the effects of various regimens on HL-60 cell clonogenicity were determined utilizing a previously described soft agar cloning assay [16]. Briefly, following treatment, cells were washed three times in fresh medium to remove drug and ODNs, and following normalization of cell counts, plated in 18-mm 12-well tissue culture plates (Corning, Corning, NY). Each well contained 1 mL of supplemented RPMI 1640 medium, 15% fetal bovine serum, 0.3% Bacto agar (Difco, Detroit, MI), and 400 cells/condition. Plates were placed in a 37°, 5% CO_2, fully humidified incubator for 12 days, after which colonies, consisting of groups of ≥50 cells, were scored with the aid of an Olympus model CK inverted microscope.

Statistical Analysis

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

RESULTS

The effects of 10 nM bryostatin 1, PDB, or MZN preexposure (24 hr) on HL-60 cell apoptosis following treatment with 10 μM ara-C for 6 hr are shown in Fig. 1A. As noted previously [17], pretreatment with both bryostatin 1 and, to a lesser extent, PDB resulted in potentiation of the percentage of cells displaying apoptotic features. In contrast, preincubation of cells with MZN did not have a significant effect on the fraction of apoptotic cells. Comparable results were obtained when DNA fragmentation was monitored. For example, levels of low molecular weight fragments in ara-C-treated cells increased from 532 ± 103 ng DNA/10^6 cells to 1246 ± 174 and 987 ± 134 ng DNA/10^6 cells following exposure to bryostatin 1 or PDB, respectively ($P \leq 0.02$), whereas levels were 597 ± 104 in cells incubated with MZN ($P \geq 0.05$; data not shown). Cells exposed to ara-C, whether or not they were pretreated with bryostatin 1 or PDB, did not display decreased expression of c-Myc protein (Fig. 1B; Table 1). Moreover, cells exposed to bryostatin 1 alone exhibited a slight increase in c-Myc expression which did not achieve statistical significance (e.g. 129.0 ± 31.6% of controls; $P \geq 0.05$). In contrast, expression of c-Myc was reduced following treatment with MZN (i.e. to 27.6 ± 9.2% relative to controls; $P \leq 0.02$), and to an even greater extent when combined with ara-C (mean value relative to controls = 12.5 ± 5.5%;

TABLE 1. Quantification of c-Myc in cells exposed to PKC activators ± ODNs

Condition	% Control c-Myc expression
(1) Control	100
+ AS	26.3 ± 6.5
+ SS	100.6 ± 9.9
(2) ara-C	109.3 ± 19.3
+ AS	18.9 ± 4.7
+ SS	99.2 ± 6.7
(3) BRY	129.0 ± 31.6
+ AS	25.7 ± 4.7
+ SS	106.5 ± 18.2
(4) BRY + ara-C	118.4 ± 18.8
+ AS	24.0 ± 8.2
+ SS	100.6 ± 12.3
(5) PDB	101.0 ± 11.0
(6) PDB + ara-C	89.2 ± 9.6
(7) MZN	27.6 ± 9.2
(8) MZN + ara-C	12.5 ± 5.5
(9) BRY + A23	43.4 ± 9.2
(10) BRY + A23 + ara-C	20.3 ± 7.2
(11) BRY + MZN	109.5 ± 14.6
(12) BRY + MZN + ara-C	106.4 ± 11.3

Cells were incubated for 24 hr with the indicated agents prior to a 6-hr exposure to 10 μM ara-C in the presence or absence of c-*myc* antisense or scrambled sequence oligonucleotides. At the end of this period, c-Myc protein was monitored by western analysis and quantified as described in the text. Values are expressed as a percentage of control c-Myc levels and represent the means ± 1 SD for 4–5 separate determinations. Abbreviations: BRY, bryostatin 1; PDB, phorbol dibutyrate; MZN, mezerein (all 10 nM); A23 = 250 nM A23187; AS, antisense oligonucleotide (10 μM); and SS, scrambled sequence oligonucleotide (10 μM).

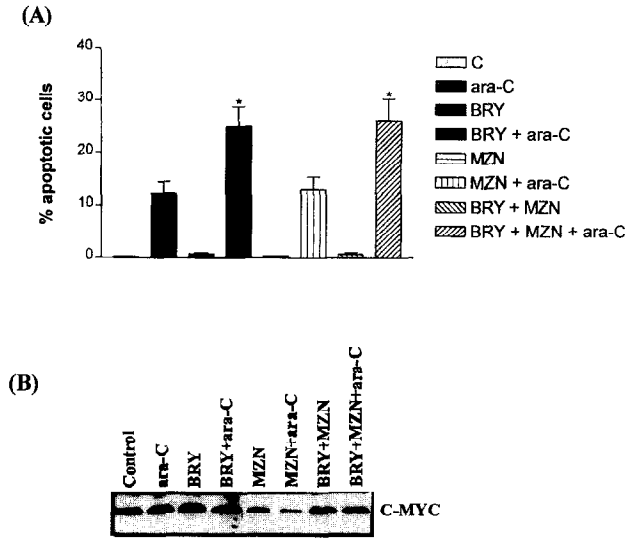

FIG. 2. Combined effect of bryostatin 1 and MZN on ara-C-induced apoptosis and c-Myc expression. (A) Cells were incubated for 24 hr with 10 nM bryostatin 1 or MZN, alone or in combination, prior to a 6-hr exposure to 10 μM ara-C, after which the percentage of apoptotic cells was determined as described previously. Values represent the means for three separate experiments performed in triplicate ± 1 SD. Key: (*) significantly greater than ara-C alone, $P < 0.05$. (B) After identical exposures, levels of c-Myc protein were monitored by western analysis, as described in Materials and Methods. A representative study is shown; three additional studies yielded similar results.

$P \leq 0.001$). Thus, bryostatin 1 and PDB, which potentiated ara-C-induced apoptosis, permitted undiminished c-Myc expression following ara-C exposure; conversely, MZN, which failed to augment ara-C-mediated apoptosis, resulted in substantial c-Myc down-regulation.

Bryostatin 1 has been shown previously to block certain phorboid-associated actions that it does not possess itself, including induction of HL-60 cell differentiation [24]. To determine whether an analogous phenomenon might occur in the present system, HL-60 cells were exposed simultaneously to 10 nM bryostatin 1 and MZN for 24 hr prior to a 6-hr incubation with 10 μM ara-C, after which effects on apoptosis were monitored (Fig. 2). The actions of bryostatin 1 were dominant to those of MZN in that potentiation of ara-C-induced apoptosis persisted in cells exposed to both agents (Fig. 2A). Parallel results were obtained when DNA fragmentation was monitored (not shown). Similarly, bryostatin 1 prevented down-regulation of c-Myc following treatment with MZN and ara-C, resulting in levels equal to 106.4 ± 11.3% of controls (Fig. 2B; Table 1). Parallel results were obtained when c-myc mRNA levels were monitored (Fig. 3). Thus, c-*myc* expression was unperturbed following exposure of cells to bryostatin 1, but reduced by MZN, and to an even greater extent by the combination of MZN and ara-C. Co-administration of bryostatin 1 prevented MZN from down-regulating c-myc mRNA levels in cells subsequently exposed to ara-C. Consequently, the ability of bryostatin 1 to prevent MZN-induced down-regulation of c-Myc protein (and mRNA) was accompanied by continuing potentiation of ara-C-induced apoptosis.

It has been reported recently that the calcium ionophore A23187 partially restores the differentiating capacity of HL-60 cells exposed to a low concentration of bryostatin 1 (0.5 nM), while antagonizing potentiation of ara-C-related

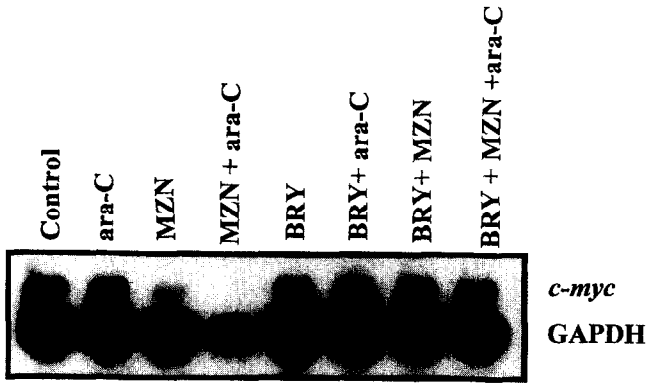

FIG. 3. Effect of bryostatin 1 and MZN on c-myc mRNA levels. Following a 24-hr incubation with 10 nM bryostatin 1 ± MZN, cells were exposed to 10 μM ara-C for 6 hr, after which total cellular mRNA was extracted and subjected to northern analysis as described in Materials and Methods. Levels of c-myc mRNA are displayed in relation to expression of the housekeeping gene GAPDH. A representative study is shown; two additional experiments yielded equivalent results.

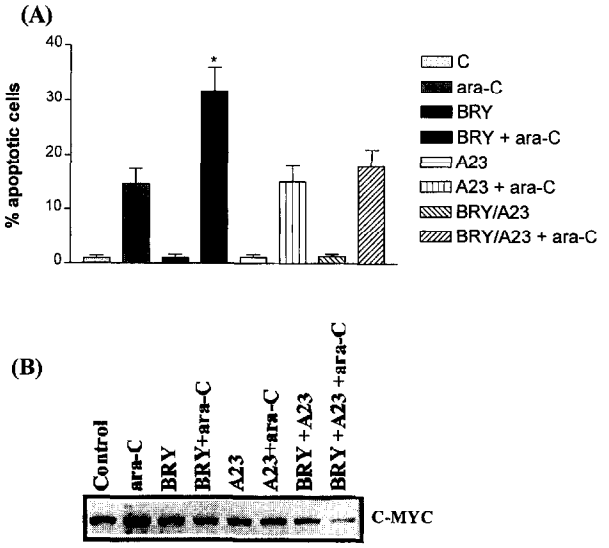

FIG. 4. Effect of bryostatin 1 and A23187 on ara-C-induced apoptosis and c-Myc levels. (A) Cells were incubated for 24 hr with bryostatin 1 (10 nM) ± A23187 (A23; 250 nM) prior to a 6-hr exposure to 10 μM ara-C. The percentage of apoptotic cells was determined as described previously. Values represent the means for three separate experiments performed in triplicate ± 1 SD. Key: (*) significantly greater than ara-C alone, $P < 0.05$. (B) Following identical drug exposures, cells were lysed, and c-Myc protein levels were determined by western analysis as described above. A representative study is shown; three additional experiments yielded similar results.

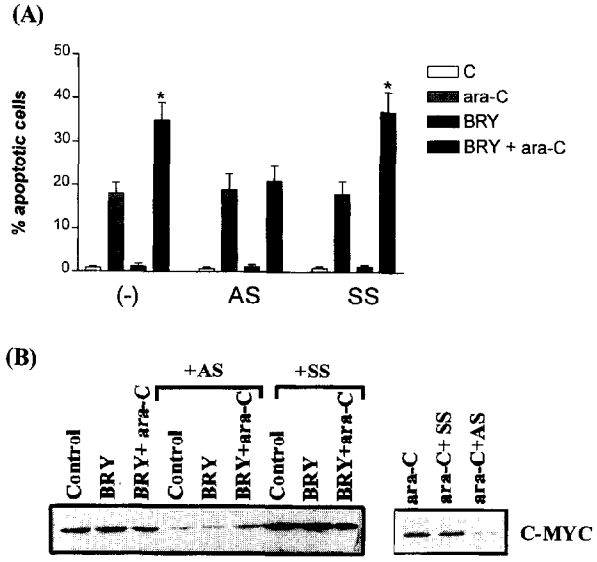

FIG. 5. Effect of c-*myc* AS-ODN on bryostatin 1- and ara-C-induced apoptosis and c-Myc expression. (A) Cells were incubated with 10 nM bryostatin 1 for 24 hr prior to a 6-hr exposure to 10 μM ara-C in the presence and absence of 10 μM c-*myc* antisense oligonucleotides (AS) or scrambled sequence oligonucleotides (SS), after which the percentage of apoptotic cells was determined as described previously. Values represent the means for three separate experiments performed in triplicate ± 1 SD. Key: (*) significantly greater than ara-C alone, $P < 0.05$. (B) In parallel studies, levels of c-Myc protein were determined by western analysis and quantified as described in Materials and Methods. The results of a representative study are shown; mean values (relative to controls) for four separate studies are listed in Table 1.

cell death [25]. Similarly, A23187 markedly reduced the capacity of 10 nM bryostatin 1 to potentiate ara-C-mediated apoptosis (Fig. 4A). Moreover, whereas preincubation of cells with A23187 or bryostatin 1 alone preceding ara-C treatment did not lead to a reduction in c-Myc expression, combined exposure to these agents reduced c-Myc levels to 43.4 ± 9.2% of control values ($P \leq 0.01$) (Fig. 4B; Table 1). In addition, sequential exposure to the combination of bryostatin 1 and A23187 followed by ara-C resulted in further c-Myc down-regulation (e.g. to 20.3 ± 7.2% of control levels; $P \leq 0.001$). Thus, an intervention that restored the ability of bryostatin 1 to down-regulate c-Myc expression was accompanied by loss of the capacity to augment ara-C-related apoptosis.

Collectively, these findings suggest a correlation between undiminished c-Myc expression and the ability of bryostatin 1 to potentiate ara-C-related apoptosis. To address this issue from a functional perspective, AS-ODNs directed against c-*myc* were employed. As reported by other investigators [26], an AS-ODN to c-*myc* (10 μM; 24-hr exposure) substantially reduced c-Myc protein expression (to 26.3 ± 6.5% of control values; $P \leq 0.002$), whereas the SS-ODN exerted no discernible effect (Fig. 5B; Table 1). Similarly, co-administration of c-*myc* AS-ODN with bryostatin 1, ara-C, or both, led to significant reductions in c-Myc expression (e.g. to 24.0 ± 8.2% of control levels in cells exposed to bryostatin 1 and ara-C; $P \leq 0.002$). In each case, the scrambled sequence failed to down-regulate c-Myc protein. Despite significantly reducing expression of c-Myc in ara-C-treated cells (e.g. by ~80%), c-*myc* AS-ODN did *not* antagonize the capacity of this agent to induce apoptosis (Fig. 5A). However, exposure of cells to AS-ODN abrogated the ability of bryostatin 1 to potentiate ara-C-induced cell death, in that it reduced the percentage of apoptotic cells to levels observed with ara-C alone. As above, reversal of the actions of bryostatin 1 was not observed with the SS-ODN.

The ability of c-*myc* AS-ODN to prevent bryostatin 1 from potentiating ara-C-induced apoptosis was accompanied by a reduction in cell lethality, as determined by trypan blue exclusion and clonogenicity (Fig. 6). In the upper panel, it can be seen that when compared with untreated controls (A), the number of TUNEL-positive cells increased after ara-C treatment (B), and this effect was undiminished by prior exposure to AS-ODN (C). Administration of bryostatin 1 increased the percentage of TUNEL-positive cells (D), an effect that was prevented by c-*myc* AS-ODN (E) but not by SS-ODN (F). Consistent with these results, administration of AS-ODN had no effect on ara-C-mediated reductions in trypan blue exclusion or clonogenicity (Fig. 6, lower panel). However, AS-ODN (but not SS-ODN) abrogated potentiation of ara-C lethality by bryostatin 1, resulting in survival values equivalent to those observed in cells exposed to ara-C alone. Because ara-C is an S-phase-specific agent [27], it was necessary to

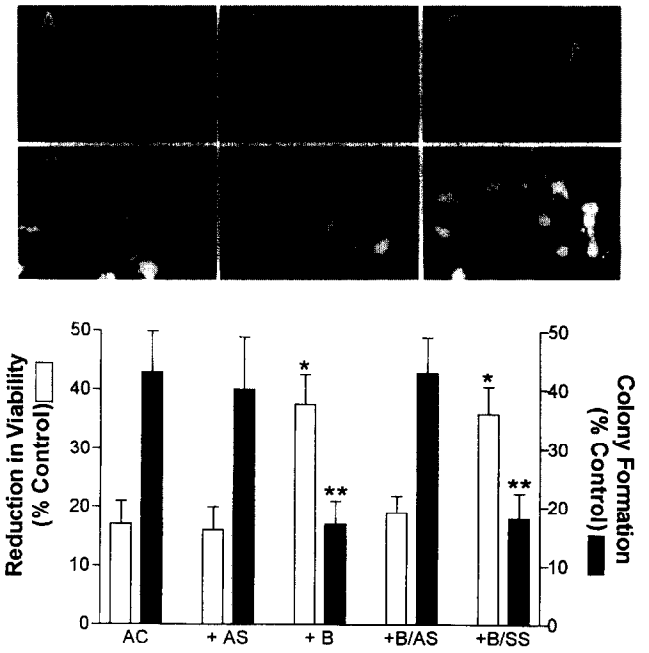

FIG. 6. Effect of c-*myc* AS-ODNs on TUNEL reactivity and clonogenicity of HL-60 cells exposed to bryostatin 1 and ara-C. Upper panel: Cells were incubated sequentially with 10 nM bryostatin 1 (24 hr) and 10 μM ara-C (6 hr) in the presence or absence of AS- or SS-ODN (10 μM each). The TUNEL assay was then employed to monitor apoptotic cells. (A) control; (B) ara-C; (C) ara-C + AS-ODN; (D) bryostatin 1 + ara-C; (E) bryostatin 1 + ara-C + AS-ODN; and (F) bryostatin 1 + ara-C + SS-ODN. Lower panel: Cells were exposed to the same agents, after which the reduction in trypan blue-excluding cells (open bars) or clonogenicity under drug-free conditions (solid bars) was determined as described in Materials and Methods. For trypan blue exclusion assays, cells were seeded at 2×10^5 cells/mL; for clonogenic assays, 4×10^2 cells/well were initially plated and colonies scored at day 12. Column labels: AC = ara-C; +AS = ara-C + AS-ODN; + B = ara-C + bryostatin 1; +B/AS = ara-C + bryostatin 1 + AS-ODN; +B/SS = ara-C + bryostatin 1 + SS-ODN. Exposure of cells to bryostatin 1, AS-ODN, or SS-ODN alone did not alter significantly trypan blue exclusion or clonogenicity (not shown). Values represent the means for three separate experiments performed in triplicate ± 1 SD. Key: (*) significantly greater than ara-C alone, $P < 0.05$; and (**) significantly less than ara-C alone, $P < 0.05$.

rule out the possibility that c-*myc* AS-ODN acted simply by inducing cell cycle arrest. Previous studies involving the HL-60 line have shown that such AS-ODN do not reduce the percentage of S-phase cells, at least over a comparable time frame (e.g. 12 hr) [28]. Similarly, exposure of these cells to c-*myc* AS-ODN for 24 hr, either in the presence or absence of bryostatin 1, did not reduce significantly the S-phase fraction (Table 2), despite the observed down-regulation of c-Myc expression (Table 1). In parallel studies, co-administration of c-*myc* AS-ODN did not lead to alterations in ara-CTP formation or ara-C DNA incorporation in cells treated with bryostatin 1 and ara-C (data not shown). Thus, the ability of c-*myc* AS-ODN to prevent potentiation of apoptosis by bryostatin 1 could not be

TABLE 2. Cell cycle traverse of cells exposed to bryostatin 1 ± AS-ODN

	G_0G_1	G_2M	S
Control	47.5 ± 5.8	8.9 ± 3.2	42.7 ± 7.3
AS	47.7 ± 6.1	10.5 ± 3.5	41.4 ± 5.4
BRY	47.8 ± 5.9	8.2 ± 2.6	44.6 ± 6.8
AS + BRY	45.5 ± 5.6	11.2 ± 4.4	40.5 ± 7.7

Logarithmically growing cells were incubated for 24 hr in the presence of 10 nM bryostatin 1 (BRY) or 10 μM c-*myc* AS-ODN (AS), alone and in combination, after which cell cycle analysis was performed as described in the text. Values represent the means for three separate experiments ± 1 SD.

attributed to a reduction in the susceptible S-phase cell fraction or to perturbations in ara-C metabolism.

Finally, an attempt was made to determine whether the effects of c-*myc* AS-ODN might stem from actions related to more proximal events involved in G_1 arrest. To this end, we examined expression of the cyclin-dependent kinase inhibitor p21(WAF1/CIP1/MDA6), activation of which is associated with hypophosphorylation of pRb [29]. Hypophosphorylated pRb binds to the transcription factor E2F, interfering with activation of cell cycle progression-related genes, including c-*myc* [30]. Exposure of HL-60 cells to 30 nM PMA, which induces differentiation and G_1 arrest in this line, resulted in p21 induction and pRb dephosphorylation (Fig. 7). However, these phenomena did not occur in cells treated with bryostatin 1 ± ara-C, whether or not they were co-exposed to c-*myc* AS-ODN. This suggests that the ability of c-*myc* AS-ODN to antagonize the actions of bryostatin 1 reflects consequences of c-Myc down-regulation, rather than more upstream cell cycle arrest-related events.

DISCUSSION

The results described herein suggest that in HL-60 leukemia cells the ability of bryostatin 1 to permit undiminished expression of c-Myc contributes to its capacity to potentiate ara-C-induced apoptosis. While c-Myc has been shown to be essential for apoptosis under certain conditions (e.g. activation-induced apoptosis in lymphoid cells) [10], its role in drug-induced cell death is less clear. In fact, there is evidence that certain agents (e.g. *N*-methylformamide; NMF) induce apoptosis in human leukemia cells despite reducing c-Myc expression [31], a phenomenon that has been extended to other classes of drugs, such as etoposide [32]. Additionally, increased expression of c-Myc has been associated with resistance to anticancer agents in NIH3T3 cells [33]. Collectively, these findings indicate that c-Myc-dependent and -independent apoptotic programs exist, and that at least certain forms of drug-induced cell death proceed along the latter pathway. The present results are compatible with this model, inasmuch as reduction in c-Myc expression by AS-ODN failed to antagonize either ara-C-mediated apoptosis or lethality. However, *potentiation* of ara-C-induced apoptosis appeared to be c-Myc depen-

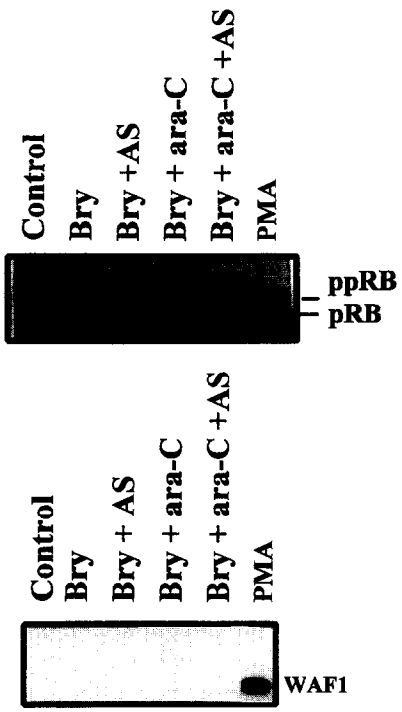

FIG. 7. Effect of bryostatin 1 and ara-C ± c-*myc* AS-ODN on pRB and WAF1 expression in HL-60 cells. Cells were incubated with 10 nM bryostatin 1 for 24 hr ± c-*myc* AS-ODN (10 μM) followed by 10 μM ara-C for 6 hr, after which pRB phosphorylation status and WAF1 levels were determined by western analysis as described in Materials and Methods. Parallel studies were performed in cells exposed to PMA (10 nM) for 24 hr. Upper panel: Phosphorylated RB (ppRB) corresponds to the 115 kDa species; hypophosphorylated RB (pRB) corresponds to the 110 kDa species. Lower panel: Induction of the 21 kDa WAF1 protein by PMA but not by other experimental treatments. A representative study is shown; two additional experiments yielded equivalent results.

dent, in that it failed to occur under conditions in which c-Myc expression was reduced, e.g. following exposure of cells to MZN, the combination of A23187 and bryostatin 1, or the combination of bryostatin 1 and c-*myc* AS-ODN. Thus, in contrast to the direct cytotoxic actions of ara-C, augmentation of ara-C-mediated apoptosis by bryostatin 1 involves an alternative, c-Myc-dependent pathway.

Since cellular maturation has been shown to oppose drug-induced apoptosis in leukemic cells [13], the question arises whether reduced expression of c-Myc directly inhibits apoptosis, or instead acts indirectly through differentiation-associated events. A related (and still unresolved) issue is whether down-regulation c-*myc*/c-Myc represents a cause or a consequence of cellular maturation. Evidence for a causal relationship stems from findings that interruption of c-*myc* function by AS-ODN exposure triggers a differentiation program in several cell lines, including F9 teratocarcinoma [34] and HL-60 leukemia [35–37] cells. However, in these studies, maturation was incomplete and, in general, appeared as a relatively late event. Moreover, evidence that drugs such as suramin and NMF down-regulate c-Myc expression in HL-60 cells without necessarily inducing differentiation [31, 38] further argues against a causal relationship. An alternative possibility is that c-*myc*/c-Myc down-regulation and the accompanying G_1 arrest represent necessary prerequisites for cellular maturation, but are not, by themselves, sufficient to induce this process. In this regard, Kimura and co-workers recently examined the effect of c-*myc* AS-ODN on HL-60 cell apoptosis and differentiation and observed that exposure of cells for 12 hr to a 15-mer AS-ODN (10 μM) did not induce G_1 arrest or cellular differentiation, and induced a minimal degree of apoptosis (e.g. ~3%) [28], results that are consistent with the present findings. Interestingly, subsequent culture of cells in AS-ODN-free medium led to a substantial increase in apoptosis, possibly the result of the ensuing c-Myc up-regulation. Finally, the inability of c-*myc* AS-ODN to induce p21 expression of pRb hypophosphorylation in treated cells, events associated with differentiation-associated G_1 arrest [39, 40], indicates a mechanism of action distinct from that of cellular maturation. Collectively, these findings suggest that the ability of c-*myc* AS-ODN to antagonize the actions of bryostatin 1 does not result from induction of differentiation, but instead represents a more direct consequence of reduced c-Myc expression. They are also consistent with the view that the net level of c-Myc activity may contribute to a cell's decision to engage an apoptotic versus a differentiation program.

Although the basis for the unique spectrum of activity of bryostatin 1 remains unknown, it is possible that dysregulation of c-Myc may be involved in at least some of its actions. Like phorboids, bryostatin 1 activates [41] and down-regulates [42] PKC. However, bryostatin 1 blocks phorboid-mediated maturation in HL-60 leukemic cells unresponsive to its differentiating effects [24]. Furthermore, in differentiation-responsive leukemic cells, bryostatin 1 down-regulates c-*myc* message [15, 43]; conversely, bryostatin 1 fails to reduce expression of c-*myc* message [15, 19] or c-Myc protein (this report) in HL-60 cells resistant to its differentiating actions. It is worth noting that bryostatin 1 induces p21 and inhibits the activity of the cyclin-dependent kinase, cdk-2, in a differentiation-responsive leukemic cell line (U937) [44]. In addition, it has been shown recently that the organotellurium compound AS101 partially restores the differentiating capacity of bryostatin 1 in HL-60 cells and, in so doing, leads to reduced expression of c-Myc and p21 induction [45]. Since predifferentiation events in G_1 are required for cells to engage in a normal differentiation program [46], the ability of bryostatin 1 to prevent down-regulation of c-Myc (e.g. following administration of MZN) may contribute to its capacity to disrupt leukemic cell maturation. In view of accumulating evidence that differentiation and programmed cell death represent divergent and, to an extent, mutually exclusive processes [47], it is tempting to speculate that interference with cellular maturation (and c-Myc down-regulation) by bryostatin 1 contributes to facilitation of apoptosis.

Currently, two major mechanistic models have been invoked to explain the apparently paradoxical ability of

c-Myc to promote both cell proliferation and death. In the "conflict" model, enforced expression of c-Myc provides a positive signal by triggering S-phase entry and cell proliferation [48]. In contrast, negative growth arrest signals are induced by certain adverse conditions (e.g. low serum concentrations or the absence of appropriate survival factors such as IGF-I) [8]. When these signals occur together, the cell is unable to reconcile them, and embarks upon a program of self-destruction. In the "dual signal" model, apoptosis and cell proliferation represent alternative, physiologic consequences of c-Myc function [49]. Under conditions of c-Myc activation, the relative availability of survival factors determines which of the two outcomes prevails. While there is clear evidence that growth factor deprivation in the face of enforced c-Myc expression is a potent inducer of apoptosis, it is presently unclear whether this model can be extended to include cytotoxic drugs. In fact, evidence that certain agents induce apoptosis in conjunction with c-*myc*/c-Myc down-regulation [31, 32] would argue against this possibility. In the present study, apoptosis induced by ara-C alone was not antagonized by interventions that reduced c-Myc expression (e.g. administration of MZN or the combination of bryostatin 1 and A23187) or by c-*myc* AS-ODN. These findings are consistent with previous reports indicating that continued c-Myc expression is not a prerequisite for drug-induced apoptosis [32]. In contrast, potentiation of ara-C-mediated apoptosis by bryostatin 1 was essentially abrogated by c-Myc down-regulation, indicating that there exists in these cells a latent, c-Myc-dependent apoptotic pathway that can be triggered by the appropriate stimulus. In this regard, it is known that PKC activation inhibits leukemic cell apoptosis [50]; moreover, reductions in PKC activity (e.g. by phorbol ester-mediated down-regulation or by PKC inhibitors) have been associated with reduced expression of c-*myc* [51]. It is therefore possible that chronic treatment with bryostatin 1, a potent down-regulator of PKC [42], presents the cell with two conflicting signals (i.e. reduced PKC activity and continued c-Myc expression) that are difficult to reconcile. While such conflicting signals appear to be insufficient by themselves to induce cell death, they may lower the threshold for ara-C-induced apoptosis in a sub-population of susceptible cells.

Issues remaining to be resolved include (a) identification of the downstream targets of c-Myc that might contribute to its capacity to modulate cell death, and (b) the significance of the absence of functional p53 [52] in the susceptibility of HL-60 cells to c-Myc-associated apoptosis. With regard to the first point, ornithine decarboxylase has been suggested as a target possibly involved in c-Myc-related cell death [48, 53], and recently the cell-cycle phosphatase cdc25 has been implicated in this process [54]. With respect to the second issue, wild-type *p53* has been reported to be required for c-Myc-mediated apoptosis in certain cell types (e.g. quiescent murine fibroblasts) [55]. However, a requirement for functional *p53* in c-Myc-dependent apoptosis has not been established in human myeloid leukemia cells. It also remains to be determined whether the current findings are unique to cells, which, like the HL-60 cell line, express amplified c-Myc [56]. Lastly, in view of the documented relationship between c-Myc and Bcl-2 in the regulation of apoptosis [10], and the recently established role of Bcl-2 and Bcl-x_L in antagonizing activation of the apoptotic protease cascade following ara-C exposure [57], it is conceivable that c-Myc may act, at least in part, through interactions with anti-apoptotic proteins.

The present findings could have implications for future attempts to enhance the efficacy of ara-C and perhaps other antileukemic drugs through the use of agents that modulate c-Myc expression. For example, bryostatin 1 elicits a differentiation response in and inhibits the proliferative capacity of certain leukemic cell lines [43] and a subset of primary AML specimens [15]. If the inability of bryostatin 1 to induce differentiation in other sublines or primary samples reflects a failure to trigger G_1 arrest and c-Myc down-regulation, then this defect might be exploited to potentiate the sensitivity of these cells to apoptosis induced by cytotoxic agents such as ara-C. A corollary of this hypothesis is that leukemic cells able to escape the effects of differentiation agents, including c-Myc down-regulation, might exhibit collateral sensitivity to subsequently administered DNA-damaging agents. Studies designed to test these hypotheses are currently in progress.

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