Pergamon

Biochemical Pharmacology, Vol. 47, No. 5, pp. 839–852, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0060-K

EFFECTS OF BRYOSTATIN 1 AND OTHER PHARMACOLOGICAL ACTIVATORS OF PROTEIN KINASE C ON 1-[β-D-ARABINOFURANOSYL]CYTOSINE-INDUCED APOPTOSIS IN HL-60 HUMAN PROMYELOCYTIC LEUKEMIA CELLS

W. DAVID JARVIS,* LAWRENCE F. POVIRK,† AMY J. TURNER,* REBECCA S. TRAYLOR,* DAVID A. GEWIRTZ,† GEORGE R. PETTIT‡ and STEVEN GRANT*†§

Departments of *Medicine and †Pharmacology/Toxicology, Medical College of Virginia, Richmond, VA 23298-0230; and the ‡Cancer Research Institute, Arizona State University, Tempe, AZ 85287-1604, U.S.A.

(*Received* 23 *April* 1993; *accepted* 29 *September* 1993)

Abstract—We have demonstrated previously that bryostatin 1, a macrocyclic lactone with putative protein kinase C (PKC)-activating properties, synergistically augments the antileukemic actions of the deoxycytidine analog 1-[β-D-arabinofuranosyl]cytosine (ara-C) in HL-60 human promyelocytic leukemia cells (Grant *et al.*, *Biochem Pharmacol* **42**: 853–867, 1991), and that this effect appears to be related to sensitization to ara-C-induced apoptosis (Grant *et al.*, *Cancer Res* **52**: 6270–6278, 1992). In the present studies, we have assessed the extent of this damage by quantitative spectrofluorophotometry of small molecular weight, double-stranded DNA fragments in order to provide: (a) a more complete characterization of the interaction between ara-C and bryostatin 1, and (b) a direct comparison of the relative effects of bryostatin 1 treatment with other pharmacological manipulations known to modulate protein kinase C activity. Exposure of cells to ara-C (10^{-9} to 10^{-4} M; 1–24 hr) induced time- and concentration-related increases in the extent of DNA fragmentation. Treatment with bryostatin 1 (10^{-11} to 10^{-7} M; 1–24 hr) alone failed to induce DNA damage, but promoted substantial time- and concentration-related increases in the extent of fragmentation induced by a subsequent 6-hr exposure to ara-C. Maximal potentiation of fragmentation (e.g. 2- to 3-fold greater than that obtained with ara-C alone) was observed following a 24-hr pretreatment with 10^{-8} M or 10^{-7} M bryostatin 1, and correlated closely with enhanced inhibition of HL-60 cell clonogenicity. The stage-1 tumor-promoter phorbol dibutyrate potentiated the effects of ara-C in a biphasic manner, maximally augmenting the response at 2.5×10^{-8} M, but exerting no effect at 10^{-7} M, whereas the stage-2 tumor-promoter mezerein failed to augment ara-C-related DNA fragmentation at low concentrations, and antagonized ara-C action at high concentrations. In contrast, ara-C-related DNA fragmentation was attenuated or abolished either by continual preexposure to synthetic diglyceride or by pretreatment with exogenous phospholipase C at all concentrations tested. Increased DNA fragmentation was not specifically related to recruitment of cells into S-phase or enhancement of ara-C-related cellular differentiation. Finally, concentrations of bryostatin 1 that maximally potentiated ara-C-related DNA fragmentation were associated with virtually complete down-regulation of total cellular PKC activity, whereas diglyceride and phospholipase C, which suppressed the response to ara-C, moderately increased total PKC activity. Taken together, these findings provide a quantitative basis for assessing the ability of bryostatin 1 and related compounds to potentiate ara-C-induced damage to DNA, and underscore the complex and pleiotropic effects that modulation of the PKC family of isoenzymes may exert on ara-C-related apoptosis in human leukemia cells.

Key words: bryostatin 1; protein kinase C; ara-C; apoptosis; leukemia; PKC activators

The deoxycytidine analog ara-C‖ is widely recognized as an effective antimetabolite in the treatment of human leukemia [1]. Multiple biochemical processes appear to contribute to the mechanism of ara-C-related lethality. The best understood of these processes involves the intracellular conversion of ara-C to its lethal derivative ara-CTP, and subsequent incorporation of this metabolite into DNA [2], resulting in premature chain termination and/or interference with chain elongation [3]. Other biochemical processes postulated to contribute to the antileukemic properties of ara-C include impaired replicon initiation [4], fragmentation of nascent DNA [5], and induction of chromosomal reduplication [6].

Recently, the actions of ara-C [7] and several other antineoplastic agents such as etoposide (VP-16) [8], Adriamycin® analogs [9], and *cis*-diamminedichloroplatinum(II) (*cis*-DDP) [10] have

§ Corresponding author: Dr. Steven Grant, Department of Medicine, Division of Hematology/Oncology, Medical College of Virginia, MCV Station Box 230, Richmond, VA 23298-0230. Tel. 804-371-5211; FAX 804-371-8079.

‖ Abbreviations: ara-C, 1-[β-D-arabinofuranosyl]cytosine; ara-CTP, 1-[β-D-arabinofuranosyl]cytosine 5′-triphosphate; BrdUrd, bromodeoxyuridine; diC_8, 1,2-dioctanoyl-*sn*-glycerol (dicaproyloin); dThd, thymidine; MZN, mezerein; PDB, phorbol 12,13-dibutyrate; PKC, protein kinase C; PL'ase C, phospholipase C; PMA, phorbol 12-myristate,13-acetate; and PtdCho, phosphatidylcholine.

been related to induction of programmed cell death, or *apoptosis*, in human leukemic cell lines. Apoptosis is an active, energy-dependent mode of cell death characterized by cell shrinkage and the expression of stereotypical morphologic changes, such as nucleoplasmic and cytoplasmic condensation, and the formation of extensive membrane blebs and novel membranous structures known as apoptotic bodies [11]. Although the events underlying the initiation of apoptosis are not well understood, this process appears to be closely associated with activation of one or more species of Ca^{2+}/Mg^{2+}-dependent endonucleases which introduce double-stranded breaks at internucleosomal regions of mature DNA, resulting in the appearance of "laddered" electrophoretic patterns of oligonucleosomal DNA fragments on conventional agarose gels [12]. Apoptosis thus appears to represent a specific and highly regulated program mediating self-destruction at the cellular level. This process is distinct from *necrosis*, which represents a passive, energy-independent response to various noxious stimuli, and is characterized by gross cell swelling, intracellular dispersion of lysosomal enzymes, and random degradation of DNA [13]. Necrosis is believed to arise from loss of membrane integrity, ultimately leading to osmoregulatory failure.

Although the factors regulating apoptosis remain obscure, involvement of PKC has been implied by numerous, albeit conflicting, lines of evidence. For example, tumor-promoting phorbol diesters have been reported both to promote [14, 15] as well as to inhibit [16] the induction of apoptosis in hematopoietic cells by diverse stimuli. In a recent paper, we described the synergistic potentiation of ara-C action by the macrocyclic lactone bryostatin 1 in the human promyelocytic cell line HL-60 [17], and subsequently demonstrated that preincubation of these cells with bryostatin 1 confers enhanced susceptibility to ara-C-induced apoptosis [18]. Because bryostatin 1 has been reported to possess PKC-activating properties [19], these findings suggest that PKC-dependent signaling processes may, in some instances, enhance the induction of apoptotic cell death by ara-C.

Currently, there is little available information regarding the effects of PKC activators on ara-C-induced apoptotic cell death in myeloid leukemia cells. In the present report, quantitation of small molecular double-stranded DNA fragments by direct spectrofluorophotometry was used to monitor DNA damage in HL-60 cells, with the intent of characterizing more completely the time–course and concentration–response relationships of ara-C-induced apoptosis and its potentiation by bryostatin 1. Parallel studies were conducted with both physiologic and non-physiologic activators of PKC. Our findings demonstrate that, as previously reported with regard to promotion of murine skin tumors [20] and induction of leukemic cell differentiation [21], bryostatin 1 exhibits a unique pattern of potentiation of ara-C-related apoptosis. In addition, these studies indicate that the pleiotropic effects of PKC activators on the induction of apoptotic cell death and DNA fragmentation in myeloid leukemic cells are highly dependent upon schedule and concentration, and suggest that the modulatory role of the PKC isoenzyme family in these events may be more complex than previously appreciated.

MATERIALS AND METHODS

Drugs and reagents

Crystalline preparations of 1-[β-D-arabinofuranosyl]cytosine (cytosine arabinoside) hydrochloride (ara-C; Sigma Chemical Co., St. Louis, MO) were stored desiccated at 4°, and dissolved in sterile PBS immediately before use. Bryostatin 1 was isolated from *Bugula neritina* as previously described [22], and maintained either desiccated or dissolved in sterile DMSO. The tumor-promoters phorbol dibutyrate (PDB), phorbol myristate acetate (PMA), and mezerein (MZN; all from Sigma) were dissolved in sterile DMSO; synthetic 1,2-dioctanoyl-*sn*-glycerol (diC_8; Sigma) was dissolved in 100% ethanol, and partially purified Type V phospholipase C isolated from *Clostridium perfrigens* (PL'ase C; Sigma) was dissolved in sterile water. Unless noted otherwise, stock preparations of all reagents were stored at −20°. All reagents were diluted to appropriate final concentrations in medium. Final concentrations of DMSO or ethanol never exceeded 0.01% (levels known to be without effect on the response of HL-60 cells to ara-C).

Cell culture

The human leukemic cell line HL-60, originally derived from cells obtained from a patient with acute promyelocytic leukemia as originally described [23], was obtained from Dr. R. E. Gallegher (Albert Einstein College of Medicine, Bronx, NY); cells from passage No. 74 and below were used during the course of these studies. HL-60 cells were grown in RPMI medium (phenol red-free formulation) supplemented with 1.0% sodium pyruvate, non-essential amino acids, L-glutamine, penicillin and streptomycin (all from GIBCO, Grand Island, NY) and 10% heat-activated fetal bovine serum (Hyclone, Ogden, UT); all cultures were maintained under a fully humidified atmosphere of 95% room air/5% CO_2 at 37°. These cultures were passed twice weekly and exhibited a doubling time of approximately 24 hr. HL-60 cell cultures were routinely screened for Mycoplasma contamination by a rapid hybridization assay selective for Mycoplasma ribosomal RNA (Gen-Probe, San Diego, CA), and were consistently found to be free of contamination. Where appropriate, cell densities were determined by a Coulter Counter (Coulter Electronics, Hialeah, FL), and the number of viable cells was assessed by hemacytometer and trypan blue exclusion.

Test exposures

Throughout these studies, HL-60 cells in log-phase growth were suspended at a density of 4.5×10^5 cells/mL in 25-cm^2 sterile polystyrene T-flasks (Corning Industries, Corning, NY) and maintained as described above; the cells were exposed to ara-C, bryostatin 1, and/or other agents for appropriate intervals in complete medium, and were then transferred to sterile centrifuge tubes and centrifuged at 400 *g* for 10 min. Loss of cells under these

conditions, due either to the washing procedure or to cell adherence, has consistently proven negligible (≤ 5%). Test incubations were terminated by gentle pelleting of the cells; following determination of cell density and viability, the cells were prepared variously for cloning, examination of cell morphology, spectrofluorophotometric assay for DNA fragmentation, or agarose gel electrophoresis as described below.

Analysis of DNA damage

Alkaline sucrose gradient sedimentation. HL-60 cell DNA fragment size was initially evaluated by alkaline sucrose gradient sedimentation as described previously [24]. Briefly, 36-mL 5–20% alkaline sucrose gradients containing 0.9 M NaCl and 2×10^{-2} M EDTA were prepared, and a lysis layer of 0.1% Nonidet P-40, 2×10^{-2} M EDTA, 0.2 M NaOH was poured onto each gradient. Cells were prelabeled with [^{3}H]Thd for 24 hr, washed extensively, and exposed to bryostatin 1 and/or ara-C as indicated. The cells were pelleted and resuspended in PBS at a density of 2×10^5 cells/mL; aliquots of the cell suspensions (500 μL) were added to the lysis layers and allowed to stand for 6 hr at 22°. DNA fragments in the cell lysates were then separated by ultracentrifugation of the gradients for 4 hr at 20°; 1.25-mL fractions were collected from each cushion, and the radioactivity in each fraction was determined by liquid scintillometry.

Spectrofluorophotometric quantitation of DNA fragments. Following exposure to test agents, cells in suspension (4.5×10^5 cells/mL; 5.0×10^6 cells total) were centrifuged at 800 *g* for 15 min at 4°. Aliquots of the supernatant were withdrawn for direct assay of released DNA fragments. The remainder of the medium was aspirated, and the pellets were resuspended in lysis buffer (0.05% hydrogenated Triton X-100, 5×10^{-3} M Trizma, 2×10^{-2} M EDTA, pH 8.0; 100 μL/10^6 cells); cell lysates were then centrifuged at 30,000 *g* for 40 min at 4° to separate fragmented and intact DNA. Aliquots of the supernatant were withdrawn for assay of DNA fragments. The presence of non-sedimenting small molecular weight DNA fragments in cell lysate and medium samples was determined by a miniaturized adaptation of quantitative bisbenzimide spectrofluorophotometry originally described by Cesarone *et al.* [25]; similar methods adapted to the measurement of apoptotic DNA fragments have been widely reported using either bisbenzimide [26] or diphenylamine [27]. In this assay, a 20-μL lysate sample was diluted in modified TNE buffer (3×10^{-3} M NaCl, 1×10^{-2} M Tris–HCl, 1×10^{-3} M EDTA, pH 7.4) containing 1.0 μg/mL bisbenzimide trihydrochloride (Hoechst No. 33258; Sigma). Fluorescence was then determined spectrofluorophotometrically with excitation at 365 nm (100-nm band width) and filtered emission at 460 nm (10-nm peak width). All DNA fragmentation values in HL-60 cell lysates were calculated relative to a highly purified calf thymus DNA calibration standard; values for all such responses are uniformly expressed in the text as ng DNA per 1×10^6 cells, and reflect the absolute amount of non-sedimenting, small (i.e. ≤ 3000 bp) fragments of DNA present in preparations of cell lysate and incubation medium.

Agarose gel electrophoresis. DNA fragmentation was analyzed by conventional agarose gel electrophoresis as reported previously [18]. Suspension cultures of HL-60 cells (2×10^7 cells/group) were treated with various agents as described above; following test incubations, the cells were washed several times in serum-free medium and the final pellet was resuspended in PBS. Cells were lysed by incubation in 1×10^{-2} M Tris–HCl (pH 7.4) containing 2×10^{-2} M EDTA and 0.1% Nonidet P-40 with brief mechanical agitation; the resulting lysate was then treated with 5×10^{-4} g/mL proteinase-K (Sigma) for 16 hr at 55°. The deproteinated extract was centrifuged at 30,000 *g* for 45 min at 4° to separate unfragmented bulk DNA from small nucleosomal DNA fragments; the resulting pellet was discarded and the supernatant was treated with 10^{-4} g/mL ribonuclease-A (Sigma) for 18 hr at 37°. Small molecular weight DNA residing in the final extract was resolved by electrophoresis at 85–115 V for 90–180 min on 2.0% agarose gels impregnated with ethidium bromide; routinely, multiple DNA molecular weight reference preparations (100-, 123-, and 1000-bp ladders; GIBCO-BRL) were run in parallel. DNA fragments were visualized under UV light.

Other studies

Clonogenicity. To assess cloning efficiency, HL-60 cells were plated in soft agar as described previously [17]. Briefly, cells were pelleted, washed twice, and resuspended in cold RPMI-1640 medium. After determining cell densities by Coulter Counter, 400 cells were seeded in 35 mm tissue culture plates containing 1 mL of RPMI medium, 20% fetal bovine serum, 10% conditioned medium from the human bladder carcinoma cell line 5637 (ATCC, Rockville, MD), and 0.3% Bacto agar. The plates were placed in a 37°, 5% CO_2, fully humidified incubator for 10–11 days, after which colonies, consisting of groups of 50 or more cells, were scored on an inverted microscope.

Cell morphology. For examination of altered morphology associated with contrasting modes of cell death (i.e. apoptosis vs necrosis), cells were washed twice with cold serum-free RPMI-1640 medium, cytocentrifuge slides were prepared and stained with 20% Wright-Giemsa stain, and morphologic assessments were performed. The mode of cell death in drug-treated populations was determined based on the expression of morphologic features characteristic either of apoptosis (e.g. cell shrinkage, nuclear condensation, extensive formation of membrane blebs and apoptotic bodies) or of necrosis (e.g. cell swelling, nuclear expansion, gross cytolysis); at least 500 cells were scored for each drug treatment.

Cell differentiation. Cells were washed twice with cold RPMI-1640 medium, cytocentrifuge slides were prepared and stained with α-naphthyl acetate esterase (Sigma Kit No. 91-A), and the percentage of esterase-positive cells was determined through enumeration of at least 200 cells per experimental condition as previously described [17].

Cytokinetic studies. Cell cycle characteristics of HL-60 cells were assessed by monitoring bromodeoxyuridine (BrdUrd) in ethanol-fixed cells according to the method of Dolbeare *et al.* [28], adapted as described previously [17]. Cytofluorometry was then performed with excitation by argon laser at 448 nm; bivariate analyses of BrdUrd labeling (green emission monitored at 515–530 nm) and DNA content (red emission monitored at ≥630 nm) were performed by comparing bitmaps constructed for control versus treated samples ($\geq 10^4$ cell/treatment) to determine the fraction of cells in S-phase for each condition.

Assay of total cellular PKC

Activity of the total cellular fraction of PKC was determined by an adaptation of the method of Yasuda *et al.* [29] using materials provided by GIBCO-BRL. Briefly, pelleted cells were homogenized in 2×10^{-2} M Tris, 5×10^{-4} M EDTA, 5×10^{-4} M EGTA, pH 7.5, containing 2.5 g/mL protease inhibitors (aprotinin, leupeptin) and 0.5% Triton X-100. The homogenate was incubated on ice for 30 min, centrifuged for 2 min at 1200 *g*, and maintained on ice pending assay. The homogenates were partially purified by passage over DEAE-cellulose with elution in homogenization buffer containing 10^{-2} M β-mercaptoethanol and 2×10^{-1} M NaCl; enzyme fractions were normalized for total protein content and added directly to an assay reaction mixture containing mixed micelles of phosphatidylserine and PMA in suspension. The reaction was initiated by addition of 2.5×10^{-5} Ci/mL [γ-^{32}P]ATP, 2×10^{-5} M non-isotopic ATP, and 5×10^{-5} M synthetic peptide substrate (acetylated myelin basic protein *N*-terminal peptide $AcMB_{4-14}$); after a 5-min incubation at 30°, aliquots of the reaction mixture were transferred to nitrocellulose filters, and the reaction was terminated by immersion of the discs in cold 1% (v/v) phosphoric acid. The discs were washed thoroughly, and radioactivity was quantitated by conventional liquid scintillometry.

Statistical analysis

The significance of differences between experimental values was determined by analysis of variance. Interactions between ara-C and bryostatin 1 were characterized by median dose–effect analysis as described by Chou and Talalay [30] and processed using commercially available software (*Dose–Effect Analysis*; Biosoft, Cambridge, U.K.). The combination index (CI) values for this drug combination were determined using either mutually exclusive or non-exclusive assumptions; a CI value lower than 1 denotes a synergistic interaction as described in the text.

RESULTS

Time–course of ara-C action

As shown in Fig. 1, agarose gel electrophoresis revealed that ara-C exposure induced internucleosomal breakage in a time-dependent manner, resulting in the stereotypical "laddered" profile of oligonucleosomal fragments ranging from 200 to 1400 bp. Such fragments were not observed under basal conditions, nor in response to a 24-hr treatment with bryostatin 1 alone. A pronounced pattern of oligonucleosomal DNA fragments was also noted following a 6-hr treatment with VP-16 (7.5×10^{-5} M).

In both untreated and bryostatin 1-pretreated cells, the laddered pattern of DNA fragments observed following ara-C exposure was transient. Oligonucleosomal fragments appeared within 3 hr, were most clearly discernible at 6 hr, and disappeared within 18 hr. The loss of these bands was associated with the appearance of a continuous streak or smear of DNA fragments that was first manifested at 6 hr and became progressively more evident with time, an aspect of drug-induced apoptosis that we [18] and others [31, 32] have noted previously. The appearance of both discrete bands and streaks of DNA was qualitatively more pronounced in cells pretreated with bryostatin 1.

Corresponding quantitative spectrofluorophotometric assessment of the formation of DNA fragments and their release into the medium is shown in Fig. 2. Exposure to ara-C alone resulted in the time-dependent accumulation of cellular DNA fragments that was detectable within 3 hr and increased progressively with time, reaching a stable level at 18 hr (Fig. 2A). Treatment with bryostatin 1 alone did not induce DNA fragmentation, but substantially enhanced the magnitude of ara-C-induced DNA damage. The release of DNA into the medium became evident only after approximately 6 hr of exposure to ara-C, but progressed linearly with time throughout the remainder of the exposure interval (Fig. 2B). In all instances, pretreatment with bryostatin 1 augmented both the extent of ara-C-induced DNA fragmentation and the release of DNA into the medium, a finding consistent with the increased intensity of DNA bands observed on agarose gels. In addition, bryostatin 1 altered the time–course of ara-C action in that (a) the onset of the response was accelerated, becoming detectable within only 1 hr of ara-C addition, and (b) the rate of a response remained linear throughout the 24-hr ara-C exposure interval and did not reach a stable level.

Together, these findings suggest that the latency of the onset of DNA release into the medium reflects the physical dissolution of cells undergoing apoptosis and, similarly, that gradual disappearance of oligonucleosomal DNA fragments (as indicated by agarose gel profiles) results from the continuous loss of DNA and other debris during cellular disintegration. For these reasons, all subsequent ara-C exposures were restricted to 6-hr intervals to coincide with the most pronounced appearance of the classical laddered electrophoretic patterns of DNA fragments.

Alkaline sucrose gradient sedimentation profile of DNA fragments

Alkaline sucrose gradient sedimentation analysis was performed to characterize the size distribution of fragments induced by ara-C and by the combination of bryostatin 1 and ara-C (Fig. 3). DNA fragments present in HL-60 cells were comprised of two components: (a) very small fragments that eluted in

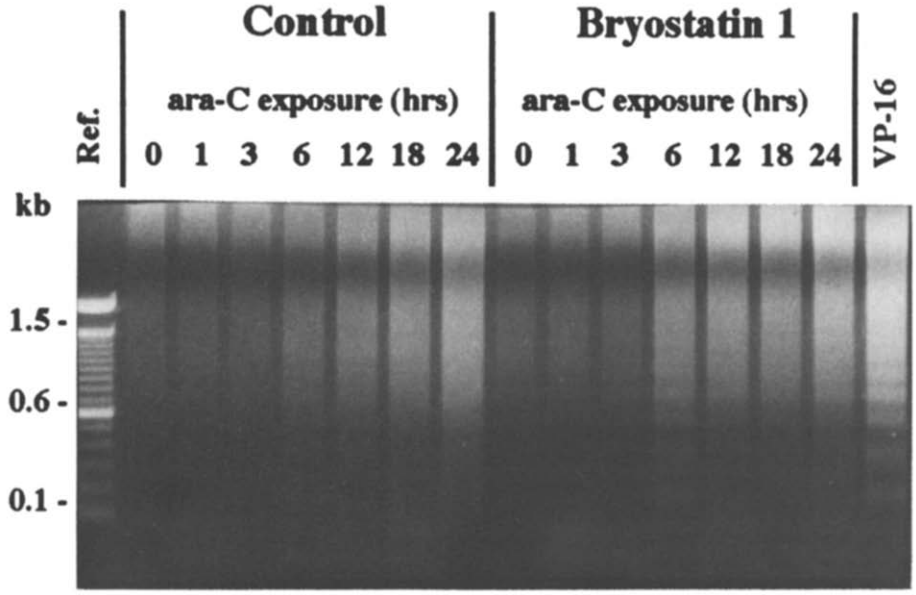

Fig. 1. Time–course of ara-C-induced DNA fragmentation: agarose gel electrophoresis. HL-60 cells were pretreated for 24 hr with or without bryostatin 1 (10^{-8} M) and then exposed to ara-C (10^{-5} M) for varied intervals (0–24 hr). The cells were pelleted and prepared for agarose gel electrophoresis. Data are from a representative study, repeated eight times with comparable results.

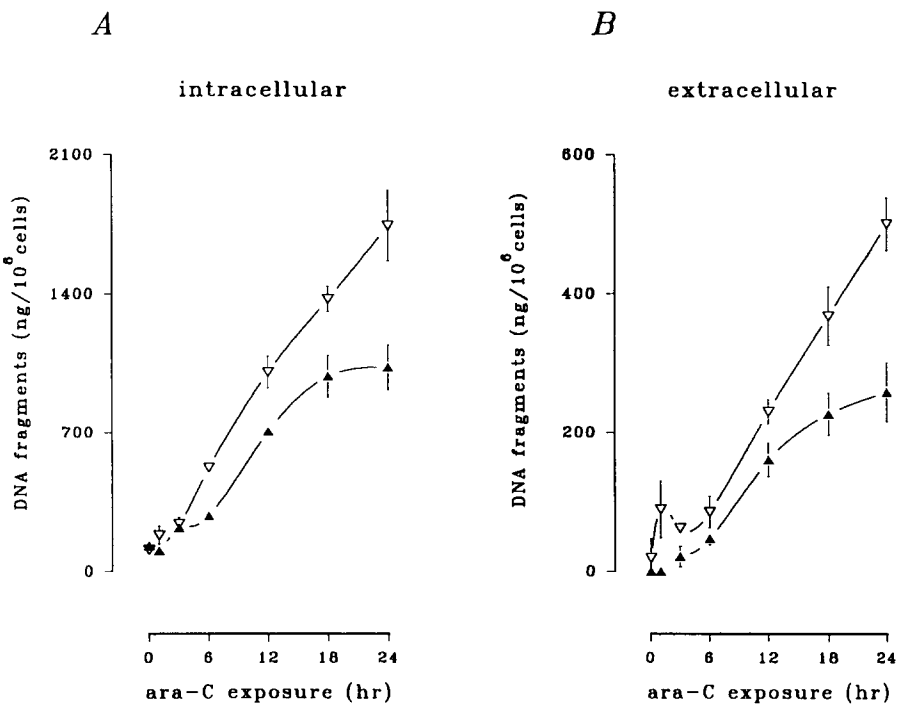

Fig. 2. Time–course of ara-C-induced DNA fragmentation: spectrofluorometry. HL-60 cells were preincubated for 24 hr in the absence (▲) or presence (▽) of bryostatin 1 (10^{-8} M) and then exposed to ara-C (10^{-5} M) for varied intervals (0–24 hr). The cells were pelleted, and high-speed supernatants of cell lysates were prepared. The accumulation of double-stranded DNA fragments in both intracellular (A) and extracellular (B) compartments was monitored by quantitative spectrofluorophotometry. Values are the means ± SEM of quadruplicate determinations. Data are from a representative study, repeated six times with comparable results.

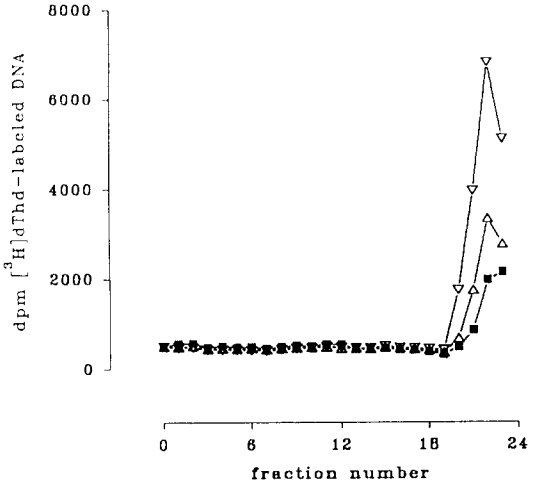

Fig. 3. Characterization of ara-C-induced DNA fragmentation by alkaline sucrose gradient sedimentation. HL-60 cells were prelabeled for 24 hr with [^{3}H]Thd (0.5 μCi/10^6 cells). The medium was then replaced and the cells were incubated for 24 hr without drug treatment (■), exposed to ara-C alone for 6 hr (10^{-5} M; △), or exposed to ara-C for 6 hr following 24-hr pretreatment with bryostatin 1 (10^{-8} M; ▽); the cells were then pelleted and lysed. DNA fragments in the resulting lysates were sedimented by ultracentrifugation on alkaline sucrose gradients. Fractions were collected from each gradient, and the radioactivity in each fraction was determined by liquid scintillometry; fractions 19 to 23 correspond to low molecular weight fragments (i.e. <10 kb). Data are from a representative experiment, repeated four times with comparable results.

fractions 19 through 23, and (b) larger sedimenting species that eluted in fractions 1 through 18. A 6-hr exposure of HL-60 cells to ara-C resulted in degradation of a portion of DNA to fragments ≤ 10 kb, whereas breakage in the residual DNA appeared to be minimal within the limits of this procedure (i.e. approximately 1 break per 500 kb). Pretreatment with bryostatin 1 increased the proportion and absolute amount of extensively degraded DNA (relative to that produced by ara-C alone), as indicated by the presence of increased radioactivity in the earlier, low molecular weight fractions. This enhanced generation of low molecular weight DNA fragments presumably resulted from increased internucleosomal cleavage (evident at the 6-hr interval in Fig. 1), as well as random DNA degradation (observed at subsequent time points).

Concentration–response characteristics of ara-C action: inhibition of clonogenicity and induction of DNA fragmentation

As shown in Fig. 4A, exposure to ara-C for 6 hr resulted in a concentration-related inhibition of clonogenicity, a response that was potentiated markedly by 24-hr pretreatment with subinhibitory concentrations of bryostatin 1. Exposure to bryostatin 1 in combination with the highest concentration of ara-C (e.g. 10^{-4} M) produced a 75% reduction in clonogenicity. Isoeffect analysis of the cytotoxic interaction between ara-C and bryostatin 1 (depicted in the inset Fa–CI plot) confirmed that the CI values for this interaction were consistently < 1.0 over a broad range of ara-C and bryostatin 1 concentrations, indicating a high degree of synergy. As shown in Fig. 4B, under comparable experimental conditions, bryostatin 1 pretreatment increased ara-C-related DNA fragmentation in a parallel fashion. In each instance, the median EC_{50} values for ara-C actions were reduced by slightly more than one order of magnitude following pretreatment with bryostatin 1.

Time–course and concentration–response characteristics of bryostatin 1 action

Exposure of HL-60 cells to ara-C alone (10^{-5} M) for 6 hr increased DNA fragmentation over basal levels by 167.5% (Fig. 5A), whereas treatment with bryostatin 1 alone (10^{-8} M) for 1–24 hr was without effect (not shown). Pretreatment with bryostatin 1 caused a distinctly time-dependent enhancement of ara-C action, such that brief (i.e. 1- to 3-hr) exposure modestly attenuated ara-C-related DNA fragmentation, whereas a more prolonged (i.e. 6- to 24-hr) exposure produced a linear increase in the response to ara-C over time, resulting in a 2-fold increase in ara-C action at 24 hr. Pretreatment with bryostatin 1 for longer intervals (e.g. 48–72 hr) did not further potentiate ara-C action, however (not shown). Exposure to ara-C alone (10^{-5} M) for 6 hr resulted in a 159.3% increase in basal DNA fragmentation (Fig. 5B). As noted above, treatment of HL-60 cells with bryostatin 1 alone over a wide range of concentrations for 24 hr was without effect on DNA fragmentation, but produced a concentration-related increase in the response to subsequent exposure to ara-C. This potentiation was clearly evident at 10^{-9} M (33% over ara-C alone), and maximal at 10^{-7} M (94% over ara-C alone).

Effects of other pharmacological activators of PKC on ara-C-induced DNA fragmentation

Other pharmacological manipulations known to augment PKC activity were evaluated with respect to their capacity to potentiate ara-C action. In these experiments, cells were incubated for 6 hr with ara-C (10^{-5} M) following a 24-hr pretreatment with the potent stage-1 tumor-promoter PDB, the stage-2 tumor-promoter MZN, diC_8, or PL'aseC. Results are shown in Fig. 6. As observed in the preceding studies of bryostatin 1, there was no apparent increase in DNA fragmentation following 24-hr treatment with any of these agents alone; subsequent responses to ara-C were surprisingly varied following pretreatment with these agents, however. PDB (Fig. 6A) produced a biphasic, concentration-related potentiation of ara-C-induced fragmentation, increasing the effects of ara-C primarily at higher concentrations (i.e ≥ 10^{-8} M). Maximal potentiation was observed at 2.5×10^{-8} M, but, unlike bryostatin 1, PDB did not increase ara-C-induced fragmentation at the highest concentration tested (10^{-7} M). Similar results were obtained with PMA (not shown). In contrast, MZN (Fig. 6B) failed to augment ara-C-induced fragmentation at any concentration and, in fact, reduced the response to ara-C at higher concentrations. Moreover, manipulations known to

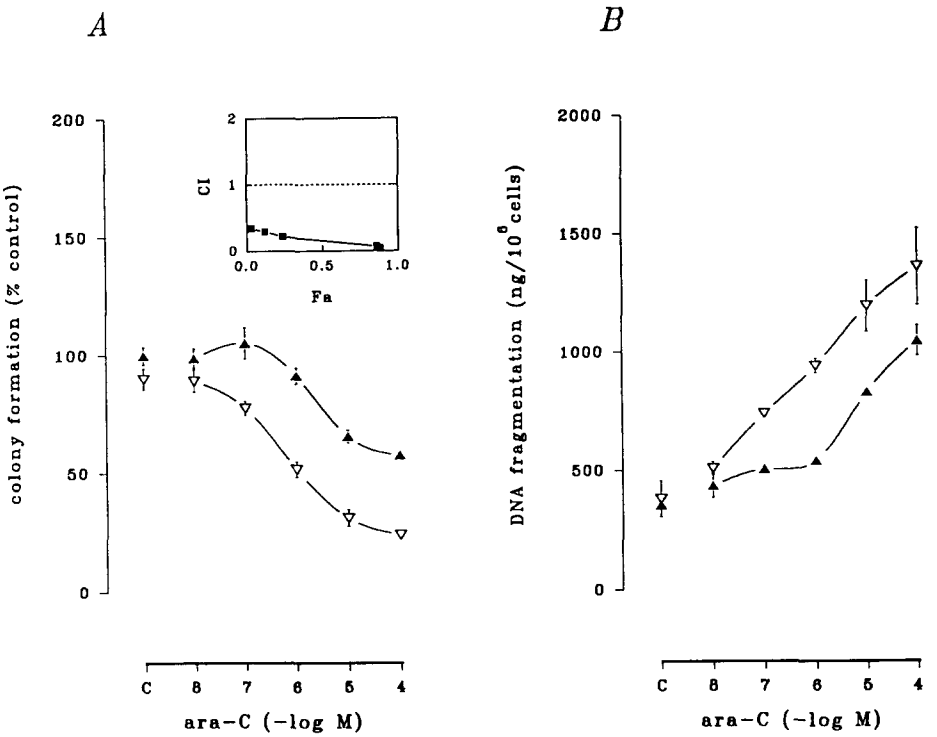

Fig. 4. Concentration–response characteristics of ara-C action: Effects on clonogenicity and fragmentation following exposure to bryostatin 1. HL-60 cells were preincubated for 24 hr in the absence (▲) or presence (▽) of bryostatin 1 (10^{-8} M), and then exposed to ara-C (10^{-8} to 10^{-4} M) for 6 hr; the cells were then harvested and prepared for assays of clonogenicity and DNA fragmentation as described. For assessment of cloning efficiency (A), cells were harvested, washed extensively, and seeded into soft agar; *in vitro* colony formation was scored after 10 days of incubation. Inset: isoeffect plot depicting cytotoxic interactions between ara-C and bryostatin 1 with respect to clonogenicity as change in the combination index (CI) as a function of the fraction affected (Fa). The broken line indicates the threshold for synergy; the region above represents antagonistic interactions, whereas the region below represents agonistic (i.e. synergistic) interactions. For assessment of DNA damage, (B), cells were pelleted, resuspended in PBS, and lysed in detergent; high-speed supernatant from the lysate was then subjected to quantitative spectrofluorophotometric assay of DNA fragments. Values are the means ± SEM of quadruplicate determinations. Data are from a representative study, repeated four times with comparable results.

promote chronic elevation of cellular diglyceride, such as continual pre-exposure to diC_8 (Fig. 6C) or pretreatment with PL'ase C (Fig. 6D), resulted in unambiguous reductions of the extent of DNA fragmentation induced by ara-C.

Qualitative assessment of the effects of these agents on ara-C-induced internucleosomal DNA fragmentation by agarose gel electrophoresis yielded results consistent with the quantitative studies described above (Fig. 7). Fragmentation induced by ara-C was clearly increased following pretreatment with bryostatin 1 (10^{-8} M) or PDB (2.5×10^{-8} M); in contrast, ara-C-related internucleosomal cleavage was reduced substantially following exposure of cells to MZN (10^{-7} M), diC_8 (10^{-5} M), or PL'ase C (10^{-2} U/mL).

Other studies

The ability of these agents, when present at various concentrations, to modulate ara-C-induced DNA fragmentation was compared with their capacity to induce apoptotic cell death (Table 1). Induction of DNA fragmentation by ara-C was closely associated with an increase in the fraction of cells undergoing apoptosis, and both responses were potentiated dramatically following pretreatment with bryostatin 1 (10^{-8} M); in contrast, the fraction of necrotic cells was not altered significantly by these treatments. In studies of tumor-promoting agents, PDB (10^{-8} M) significantly augmented the induction of DNA fragmentation and apoptosis by ara-C, whereas MZN (10^{-7} M) nearly abolished ara-C-induced DNA fragmentation and substantially reduced the fraction of cells undergoing apoptosis. Parallel reductions in both induction of DNA fragmentation and the occurrence of apoptosis were observed in cells pretreated with either diC_8 or PL'ase C. None of these treatments significantly increased the extent of ara-C-induced differentiation, with the exception of MZN, which slightly enhanced esterase positivity (32 ± 5 vs $22 \pm 4\%$ for ara-C-treated controls; $P < 0.05$); all other treatments were without

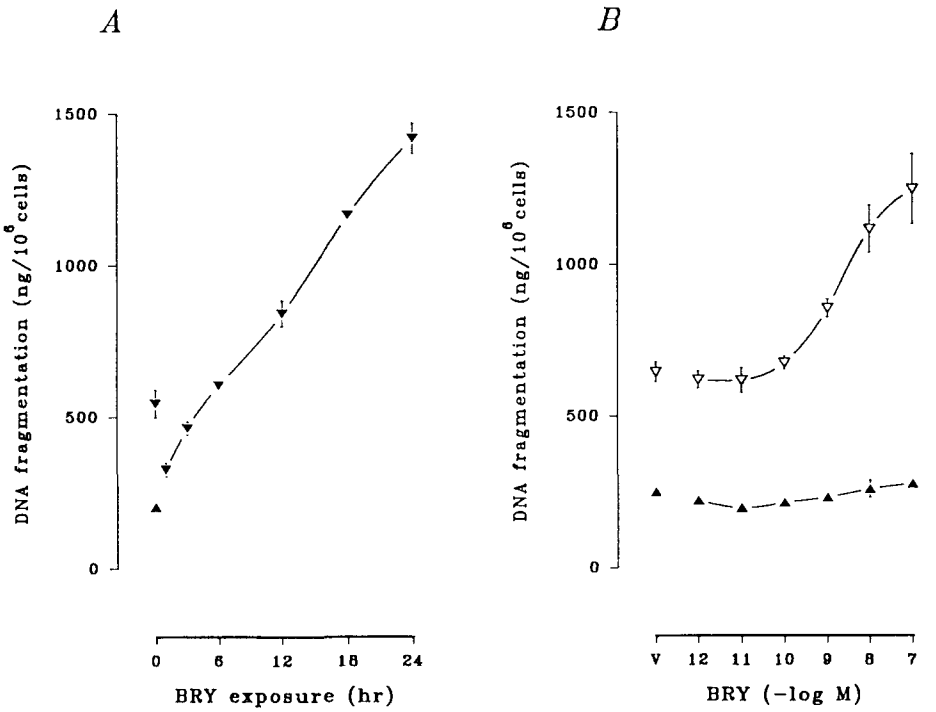

Fig. 5. Time–course and concentration–response characteristics of bryostatin 1 action. HL-60 cells were pretreated with bryostatin 1 (10^{-8} M) for varied intervals (0–24 hr) and then incubated in the absence (▲) or presence (▼) of ara-C (10^{-5} M) for 6 hr (A); alternatively, cells were pretreated with bryostatin 1 at varied concentrations (10^{-11} to 10^{-7} M) for 24 hr, and then incubated in the absence (▲) or presence (▽) of ara-C (10^{-5} M) for 6 hr (B). Fragmentation of DNA was measured by spectrofluorophotometry as before. Values are the means ± SEM of quadruplicate determinations. Data are from a representative study, repeated five times with comparable results.

significant effect on the response to ara-C (not shown). Similarly, none of these agents significantly increased the S-phase fraction of cells (range 32 to 54%, control 46%; not shown), including those which enhanced ara-C-related fragmentation (e.g. bryostatin 1, PDB; not shown).

Modulation of total cellular PKC activity

Finally, total cellular PKC activity was monitored in cells following a 24-hr exposure to pharmacological PKC activators (Fig. 8). Incubation with 10^{-8} M bryostatin 1, a concentration that significantly potentiated ara-C-related apoptosis, resulted in the loss of essentially all cellular PKC activity ($P < 0.001$); similar results were obtained with 10^{-7} M bryostatin 1 (not shown). Incubation with PDB (2.5×10^{-8} M), which also resulted in enhanced ara-C-related fragmentation, produced a much smaller decline in total activity (approximately 20%; $P < 0.05$). In contrast, values were not altered in cells exposed to MZN at a concentration that did not enhance ara-C actions (i.e. 10^{-8} M; $P > 0.05$). Interestingly, cells preincubated with diC_8 and PL'ase C concentrations that antagonized ara-C-induced apoptosis exhibited modest but significant *increases* in total cellular PKC activity ($P < 0.05$ for each). Finally, virtually complete enzyme down-regulation was also observed in cells exposed sequentially to bryostatin 1 (10^{-8} M) for 24 hr and ara-C (10^{-5} M) for 6 hr ($P < 0.001$).

DISCUSSION

Previous studies demonstrating potentiation of ara-C-induced apoptosis in HL-60 cells by bryostatin 1 [18] were restricted to single concentrations of ara-C (10^{-5} M) and bryostatin 1 (10^{-8} M) as well as to fixed drug exposure intervals (24 hr). Quantitative assessment of DNA fragmentation has now permitted us to (a) establish a threshold concentration for the effects of bryostatin 1 (i.e. 10^{-9} M), (b) fully characterize the relative time courses of ara-C and bryostatin 1 action, and (c) demonstrate the potentiation of ara-C-induced DNA fragmentation over a broad range of therapeutically relevant ara-C concentrations (i.e. 10^{-7} to 10^{-4} M). It is important to note that the degree of DNA fragmentation closely paralleled the inhibition of clonogenicity and morphological evidence of apoptosis, and that these responses were observed following exposure of HL-60 cells to ara-C for 6 hr, an interval associated with optimal expression of the "laddered" pattern of DNA fragments typical of apoptosis, but relatively free of the DNA smearing noted after longer incubations [18, 31, 32]. These findings support the position that the cytotoxic interaction between bryostatin 1 and ara-C is manifested, at least in part, through induction of apoptotic DNA damage, although the contribution of other DNA degradative processes cannot be excluded completely.

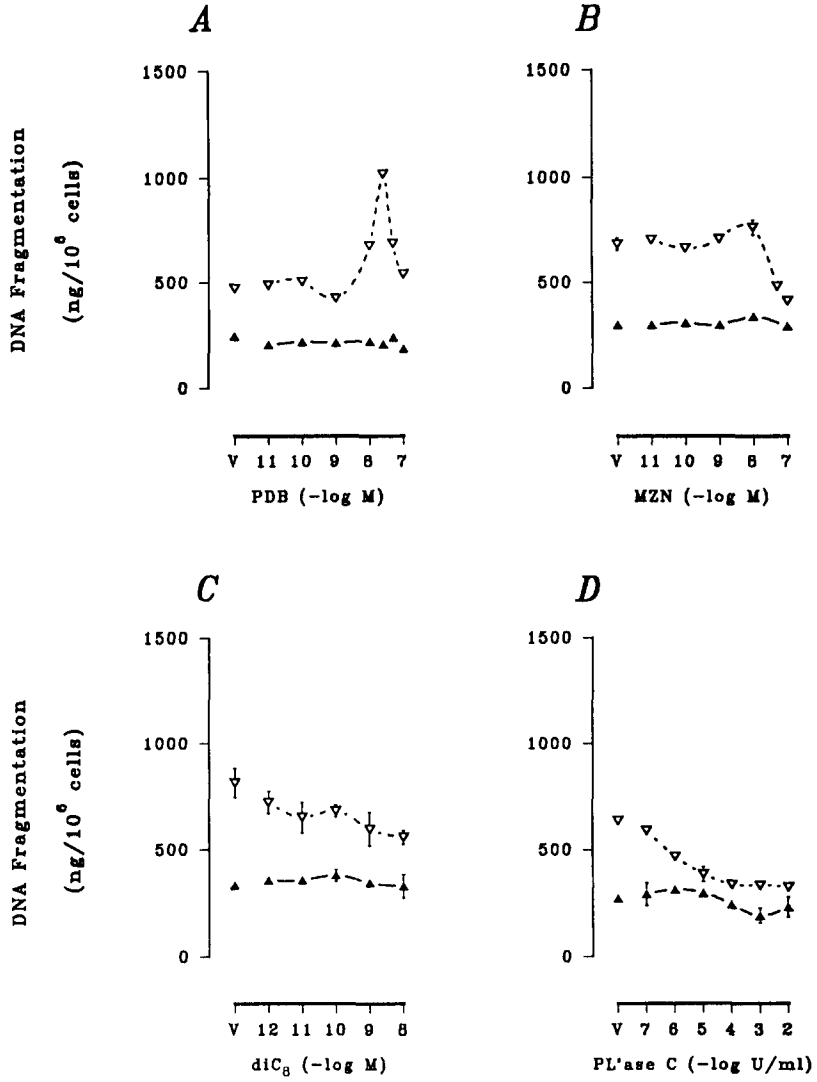

Fig. 6. Modulation of ara-C-induced DNA fragmentation by other pharmacological manipulations associated with increased PKC activity. HL-60 cells were incubated for 6 hr in the absence (▲) or presence (▽) of ara-C (10^{-5} M) following 24 hr of pretreatment with pharmacological agents known to enhance activity of PKC, including the stage-1 tumor-promoter phorbol dibutyrate (PDB; 10^{-11} to 10^{-7} M; panel A), the stage-2 tumor-promoter mezerein (MZN; 10^{-11} to 10^{-7} M; panel B), 1,2-dioctanoyl-*sn*-glycerol (diC_8; 10^{-12} to 10^{-8} M, repleted every 4 hr; panel C), or phospholipase C isolated from *C. perfrigens* (PL'ase C; 10^{-7} to 10^{-2} U/mL; panel D). Fragmentation of genomic DNA was evaluated as before. Values are the means ± SEM of quadruplicate determinations. Data are from representative parallel studies, each represented four times with comparable results.

Attempts to define a role for PKC in the regulation of apoptotic events have been impaired by numerous conflicting reports. For example, an inhibitory (or protective) influence is supported by the findings that PMA prevents apoptosis in murine thymocytes following glucocorticoid exposure [16], in normal hematopoietic progenitors following growth factor deprivation [33], or in CLL lymphocytes following exposure to 2-chlorodeoxyadenosine (CDA) and fludarabine (F-ara-A) [34]. Conversely, a stimulatory role is suggested by the reported ability of PMA (in conjunction with Ca^{2+} ionophore) to induce apoptosis in T-cell hybridoma cells [14], and of staurosporine, a pharmacological inhibitor of PKC, to antagonize the formation of apoptotic bodies in HL-60 cells [35]. The observation that bryostatin 1 increases PKC activity [19] and potentiates ara-C-induced apoptosis [18] appears to be most consistent with the latter findings. In this regard, it is worth noting that the influence of bryostatin 1 on ara-C-induced apoptosis differed substantially from that of other experimental manipulations known to activate PKC, and that these effects were highly dependent upon concentration. Whereas bryostatin 1 exerted maximal potentiating effects at 10^{-8} to 10^{-7} M, PDB and PMA were most effective at 2.5×10^{-8} M, but

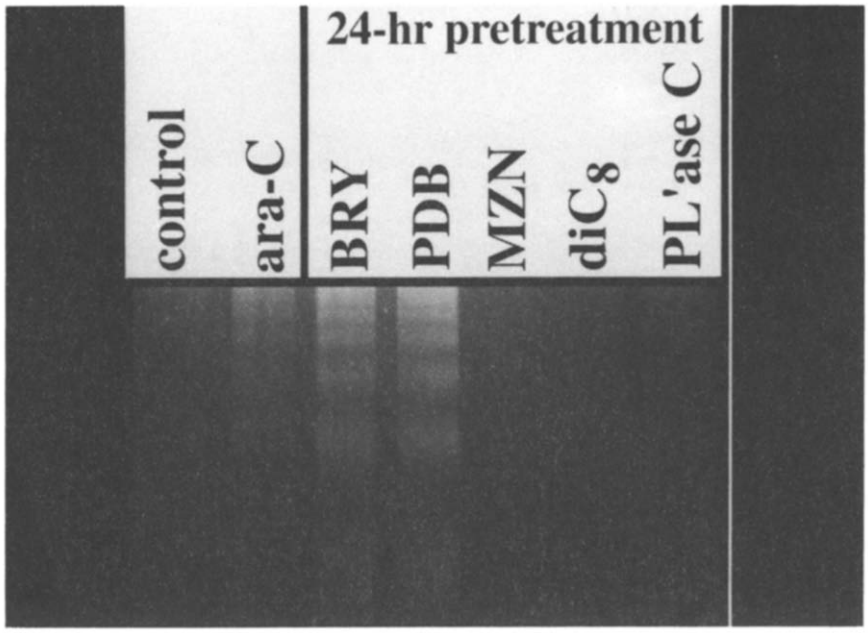

Fig. 7. Modulation of oligonucleosomal fragmentation by PKC activators. HL-60 cells were exposed to ara-C (10^{-5} M) for 6 hr following pretreatment with bryostatin 1 (BRY, 10^{-8} M), PDB (2.5 ± 10^{-8} M), MZN (10^{-7} M), diC_8 (10^{-5} M), or PL'ase C (10^{-2} U/mL) for 24 hr. DNA fragments were then resolved by agarose gel electrophoresis as before. The results shown are from a representative study, performed four times with comparable results.

Table 1. Association of DNA fragmentation with mode of cell death: relative effects of bryostatin 1 and pharmacological manipulations associated with activation of protein kinase C

Pretreatment		DNA fragmentation ($ng/10^6$ cells)	Mode and extent of cell death	
			% Apoptosis	% Necrosis
None	Control	293.3 ± 31.9	4.3 ± 1.9	2.3 ± 0.7
	ara-C	756.7 ± 65.7*	19.2 ± 1.8*	3.6 ± 0.9
Bryostatin 1	Control	310.0 ± 28.3	3.0 ± 1.3	1.0 ± 0.5
(10^{-8} M)	ara-C	1330.0 ± 12.2†	39.4 ± 3.4†	1.8 ± 0.7
Phorbol dibutyrate	Control	240.8 ± 12.9	3.2 ± 0.5	2.4 ± 1.2
(10^{-8} M)	ara-C	1283.0 ± 44.9†	27.8 ± 0.9†	3.6 ± 1.2
Mezerein	Control	343.3 ± 40.2	3.8 ± 1.6	2.8 ± 0.8
(10^{-7} M)	ara-C	383.3 ± 38.9‡	9.0 ± 1.5‡	0.8 ± 0.2
Dioctanoylglycerol	Control	353.3 ± 28.6	2.0 ± 0.5	1.5 ± 0.6
(10^{-5} M)	ara-C	523.7 ± 24.8‡	14.4 ± 1.4‡	2.0 ± 0.4
Phospholipase C	Control	363.3 ± 52.1	2.0 ± 1.0	0.4 ± 0.4§
(10^{-2} U/mL)	ara-C	473.3 ± 63.4‡	8.6 ± 1.0‡	0.5 ± 0.5‡

Suspension cultures of HL-60 cells were incubated for 6 hr in the absence or presence of ara-C (10^{-5} M) following a 24-hr preincubation with bryostatin 1 (10^{-8} M), phorbol dibutyrate (10^{-8} M), mezerein (10^{-7} M), 1,2-dioctanoyl-*sn*-glycerol (10^{-5} M, re-added every 4 hr), or phospholipase C (10^{-2} U/mL). Spectrofluorophotometry of fragmented DNA was then performed as before. Values are the means ± SEM of triplicate determinations. Alternatively, the cells were prepared for microscopic examination to determine the mode of cell death; 5 fields of 100 cells each were scored for each slide. Values are the mean percentage ± SEM of apoptotic or necrotic cells identified per treatment group.

* Increased vs untreated control ($P < 0.01$).

† Increased vs ara-C ($P < 0.01$).

‡ Decreased vs ara-C ($P < 0.01$).

§ Decreased vs untreated control ($P < 0.01$).

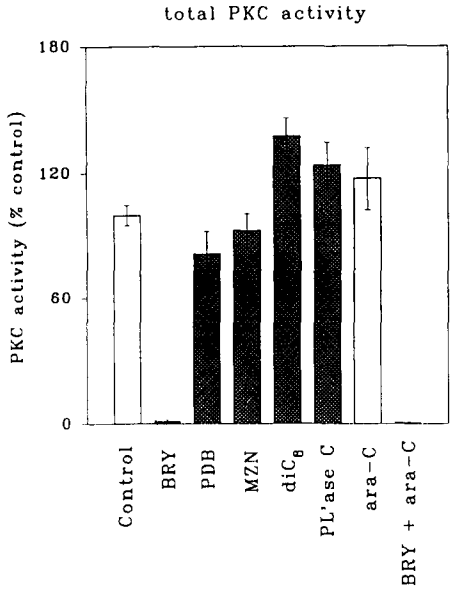

Fig. 8. Modulation of total cellular PKC activity. HL-60 cells were treated with BRY (10^{-8} M), PDB (2.5×10^{-8} M/), MZN (10^{-8} M), diC_8 (10^{-5} M), or PL'ase C (10^{-2} U/mL) for 24 hr; alternatively, cells were exposed to ara-C (10^{-5} M) without or with BRY pretreatment for 24 hr. The total cellular fraction of PKC activity was then assayed as described in the text. Values are the means ± SD for three to four separate experiments, and are expressed as a percentage of PKC activity in untreated controls. Control values were 9.0 ± 0.1 pmol phosphate incorporated into peptide/10^6 cells/min.

ineffective at 10^{-7} M. In contrast, the stage-2 tumor-promoter MZN did not promote DNA damage, but instead inhibited fragmentation at higher concentrations. These results are consistent with previous findings from this laboratory that high concentrations of bryostatin 1 potentiate, whereas PDB and MZN inhibit, the proliferative response of normal bone marrow progenitors to interleukin-3 [36]. Interestingly, diC_8 moderately suppressed ara-C-induced DNA damage, while PL'ase C uniquely reduced basal fragmentation, and virtually abolished the response to ara-C. These findings indicate that, at least as far as induction of apoptosis is concerned, the effects of pharmacologic modulators of PKC such as bryostatin 1 and tumor-promoting phorboids differ markedly from each other, as well as from more physiological effectors.

Several mechanisms may underlie the disparate actions of bryostatin 1, tumor-promoting phorboids, and diglycerides on ara-C action. For example, contrasting effects of bryostatin 1 and phorboids on promotion of murine skin tumors [20] and induction of leukemic cell differentiation [21] have been postulated to arise from the phosphorylation of different protein substrates; a similar mechanism may underlie the differing effects of bryostatin 1 and phorboids on drug-induced apoptosis. In addition, at least nine isoforms of PKC, divided into two distinct functional subfamilies, are normally expressed in mammalian systems [37]. It is possible that modulation in the activity of a particular isoform, or pattern of isoforms, specifically renders HL-60 cells more susceptible to drug-induced apoptotic cell death. For example, a differential effect of bryostatin 1 and PMA on PKCα has been postulated to explain the disparate actions of these agents on proliferation of the MCF-7 breast cancer cell line [38], and has also been demonstrated in HeLa cells [39]; a similar phenomenon may underlie the divergent effects of these agents on ara-C-related DNA fragmentation in HL-60 cells.

The present findings support the alternative possibility that the ability of bryostatin 1 to potentiate ara-C-induced apoptosis is mediated by down-regulation of one or more isoforms of PKC. Numerous reports provide evidence consistent with this model. For example, chronic stimulation of cells by phorboids initially activates, then down-modulates PKC activity in 3T3 cells [40]; similarly, chronic exposure to bryostatin 1 exerts comparable effects in several cell types, including HL-60 cells [19, 38, 39]. In the present studies, 24-hr pretreatment of HL-60 cells with bryostatin 1 at a concentration that substantially potentiated the effects of ara-C (10^{-8} M) totally depleted cellular PKC activity, suggesting a protective role for basal activity of the enzyme in the regulation of apoptosis. The ability of diC_8 and PL'ase C to suppress ara-C-induced apoptosis and increase total PKC activity supports this hypothesis and, in addition, is consistent with a recent report that v-*abl*-mediated antagonism of apoptosis in hematopoietic cells is associated with parallel increases in the intracellular availability of diglyceride and the up-regulation of PKC [41].

This model could also potentially explain the schedule-dependent effects of PKC activators on drug-induced apoptosis. For example, the suppression of ara-C action by brief (i.e. 1- to 3-hr) pretreatment of HL-60 cells with bryostatin 1, and the previously described antagonism of 2′-chlorodeoxyadenosine- and fludarabine-induced DNA damage by brief exposure of CLL cells to PMA [34], may reflect the protective influence of initial PKC activation; conversely, chronic stimulation and enzyme down-regulation may underlie the ability of prolonged pretreatment with bryostatin 1 to potentiate this process. Also consistent with this hypothesis, we* and others [42] have noted the induction of apoptosis in HL-60 cells by pharmacological inhibitors of PKC. Although PDB, which significantly potentiated ara-C-induced fragmentation, was clearly less effective than bryostatin 1 in the down-regulation of PKC, it should be noted that only total cellular activity was monitored in these studies. One possibility, currently under investigation, is that augmentation of ara-C-related apoptosis results from down-regulation of a specific isoform that confers resistance to this process, an occurrence that may not be detectable

* Jarvis WD, Turner AJ, Traylor RS and Grant S, Induction of apoptotic DNA fragmentation in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. Manuscript submitted for publication.

in assays of total enzyme activity. Lastly, it is worth noting that treatment of human leukemia cells with ara-C has been associated with activation of PKC [43], an event that may result from generation of diglyceride and ara-CDP-Cho through reversal of PtdCho synthase activity [44]. Since increased intracellular availability of free diglyceride was found in the present studies to inhibit ara-C-induced apoptosis, the antileukemic property of ara-C may be self-limiting. Furthermore, this property theoretically could be antagonized by prolonged exposure to bryostatin 1 (as a consequence of enzyme down-regulation), but potentiated by brief exposures. Studies designed to test these hypotheses more rigorously are currently in progress.

The relationship between leukemic cell differentiation and apoptosis also deserves comment in view of the association between these processes in U937 [45] and HL-60 cells [46], and a recent report that phorboids (i.e. PMA) antagonize topoisomerase II-related apoptosis in HL-60 cells [47]. It is also noteworthy that (a) bryostatin 1, which does not induce cell differentiation in our HL-60 subline [17], sharply potentiated apoptosis, and that (b) MZN, which was the only agent to increase esterase positivity in ara-C-treated cells, suppressed the apoptotic response to ara-C. Together, these observations suggest that potentiation of ara-C-related apoptosis and cellular differentiation are not specifically related. The discrepancy between our results and those of other investigators [47], at least with respect to phorboids, may reflect differences in the behavior of individual HL-60 cell sublines or, alternatively, in the form of apoptosis elicited by ara-C and inhibitors of topoisomerase II. Lastly, it is significant that exposure to ara-C at high concentrations substantially augments the availability of cellular free diglyceride by increasing degradation of PtdCho [44], and that the actions of other antineoplastic agents, including *cis*DDP and Adriamycin, have also been associated with enhanced glycerophospholipid catabolism and concomitantly increased cellular diglyceride levels [48, 49]. In addition, the differential effects of bryostatin 1 and tumor-promoting phorboids on the catabolism of PtdCho and other glycerophospholipids (e.g. phosphatidylethanolamine) have been invoked to explain the divergent actions of these agents in leukemic cells [50–53]. It is therefore conceivable that the unique pattern of bryostatin 1-mediated potentiation of ara-C-induced apoptosis results from specific alterations in phospholipid metabolism. Further investigations are underway to resolve this issue.

In summary, the present studies demonstrated that bryostatin 1 augments ara-C-induced DNA fragmentation and apoptosis in HL-60 cells over a wide range of pharmacologically relevant concentrations, and that the magnitude of this effect is highly dependent upon both schedule and concentration. These observations also provide evidence that diverse activators of PKC may exert unique—and potentially opposing—effects on ara-C-induced apoptotic cell death and DNA fragmentation in human myeloid leukemia cells, and that pharmacodynamic considerations appear to play a major role in determining the nature of these responses. Lastly, the findings that (a) increases in the availability of diglyceride, and consequently in PKC activity, inhibited ara-C-induced apoptosis, and (b) treatment with bryostatin 1, which potentiates the response to ara-C, led to PKC down-modulation, suggest a possible basis for defining the role of this enzyme and lipid second messenger systems in regulating ara-C action. A better understanding of the signal transduction events involved in the capacity of bryostatin 1 and related agents to augment ara-C-induced apoptosis in human leukemia cells may therefore permit the rational design of novel and potentially more effective combination regimens in the treatment of human myeloid leukemias.

Acknowledgements—This work was supported by Grant CH-523 from the American Cancer Society (S.G.), and Grant CA-40615 (L.F.P.) and Cancer Center Support Core Grant CA-16059 from the National Cancer Institute. W.D.J. is a recipient of National Research Service Award CA-09380, and G.R.P. is a recipient of Outstanding Investigator Award CA 44344-03A1. Additional funding was provided by the Medical College of Virginia Grants-in-Aid Program and the A.D. Williams Foundation, the Fannie E. Rippel Foundation, the Robert B. Dalton Endowment Fund, the Thomas F. Jeffres and Kate Miller Memorial Trust, and the Bone Marrow Transplantation Core Research Laboratory of the Medical College of Virginia.

REFERENCES

1. Ellison RR, Holland JF, Weill M, Jacquillat C, Boiron M, Bernard J, Savitsky A, Rosner E, Sussoff B, Silvers RT, Karanas A, Cuttner J, Spurr C, Hayes DM, Blom J, Leone LA, Haurani F, Kyle R, Hutchison J, Forcier RJ and Moon JH, Arabinofuranosylcytosine: A useful agent in the treatment of acute leukemia in adults. *Blood* **32**: 507–533, 1968.
2. Kufe D, Beardsley GP, Major P and Egan EM, Correlation of cytotoxicity with incorporation of ara-C into DNA. *J Biol Chem* **255**: 8997–9000, 1980.
3. Mikita T and Beardsley GP, Functional consequences of the arabinofuranosylcytosine structural lesion in DNA. *Biochemistry* **27**: 4698–4705, 1988.
4. Fridland A, Effect of cytosine arabinoside on replicon initiation in human lymphoblasts. *Biochem Biophys Res Commun* **74**: 72–78, 1977.
5. Ross DD, Chen S-R and Cuddy DP, Effects of 1-β-D-arabinofuranosylcytosine on DNA replicative intermediates monitored by pH-step alkaline elution. *Cancer Res* **50**: 2658–2666, 1990.
6. Woodcock DM, Fox RM and Cooper IA, Evidence for a new mechanism of cytotoxicity of 1-β-D-arabinofuranosylcytosine. *Cancer Res* **39**: 1418–1424, 1979.
7. Gunji H, Kharbanda S and Kufe D, Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. *Cancer Res* **51**: 71–74, 1991.
8. Kaufmann SH, Induction by endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: A cautionary note. *Cancer Res* **49**: 5870–5878, 1989.
9. Zwelling LA, Altschuler E, Cherif A and Farquhar D, *N*-(5,5-Diacetoxypentyl)doxorubicin: A novel anthracycline producing DNA interstrand cross-linking and rapid endonucleolytic cleavage in human leukemic cells. *Cancer Res* **51**: 6704–6707, 1991.

10. Barry MA, Behnke CA and Eastman A, Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem Pharmacol* **40**: 2353–2362, 1990.
11. Wyllie AH, The significance of apoptosis. *Int Rev Cytol* **68**: 251–306, 1980.
12. Wyllie AH, Glucocorticoid-induced thymocyte apoptosis is associated with endonuclease activation. *Nature* **284**: 555–556, 1980.
13. Gerschenson LE and Rotello RJ, Apoptosis: A different type of cell death. *FASEB J* **6**: 2450–2455, 1992.
14. Merćep M, Noguchi PD and Ashwell JD, The cell cycle block and lysis of an activated T cell hybridoma are distinct processes with different Ca^{2+} requirements and sensitivity to cyclosporine A. *J Immunol* **142**: 4085–4092, 1989.
15. Kizaki H, Tadekuma T, Odaka C, Muramatsu J and Ishimura Y, Activation of a suicide process in thymocytes through DNA fragmentation by calcium ionophores and phorbol diesters. *J Immunol* **143**: 1790–1794, 1989.
16. McConkey DJ, Hartzell P, Jondal M and Arrenius S, Inhibition by DNA fragmentation in thymocytes and isolated thymocyte nuclei by agents that stimulate protein kinase C. *J Biol Chem* **264**: 13399–13402, 1989.
17. Grant S, Boise L, Westin E, Howe C, Pettit GR, Turner A and McCrady C, *In vitro* effects of bryostatin 1 on the metabolism and cytotoxicity of 1-β-D-arabinofuranosylcytosine in human leukemia cells. *Biochem Pharmacol* **42**: 853–867, 1991.
18. Grant S, Jarvis WD, Swerdlow PS, Turner AJ, Traylor RS, Wallace HJ, Lin P-S, Pettit GR and Gewirtz DA, Potentiation of the activity of 1-β-D-arabinofuranosylcytosine by the protein kinase C activator bryostatin-1 in HL-60 cells: Association with enhanced fragmentation of mature genomic DNA. *Cancer Res* **52**: 6270–6278, 1992.
19. Kraft AS, Baker VV and May WS, Bryostatin 1 induces changes in protein kinase C location and activity without altering c-*myc* gene expression in human promyelocytic leukemia cells (HL-60). *Oncogene* **1**: 111–118, 1987.
20. Hennings H, Blumberg PM, Pettit GR, Herald CL, Shores R and Yuspa SH, Bryostatin 1, an activator of protein kinase C, inhibits tumor promotion by phorbol esters in SENCAR mouse skin. *Carcinogenesis* **8**: 1343–1346, 1987.
21. Kraft AS, Smith JB and Berkow RL, Bryostatin, an activator of the calcium phospholipid-dependent protein kinase, blocks phorbol ester-induced differentiation of human promyelocytic leukemia cell HL-60. *Proc Natl Acad Sci USA* **83**: 1334–1338, 1986.
22. Pettit GR, Day JF, Hartwell JL and Wood HB, Antineoplastic components of marine animals. *Nature* **227**: 962–963, 1970.
23. Gallegher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F and Gallo R, Characterization of the continuous differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. *Blood* **54**: 713–733, 1979.
24. Clarkson JM and Painter RB, Repair of X-ray damage in aging WI-38 cells. *Mutat Res* **23**: 107–112, 1974.
25. Cesarone C, Bolognesi C and Santi L, Improved microfluorometric DNA determination in biological material using Hoechst 33258. *Anal Biochem* **100**: 188–197, 1979.
26. Heinemann V, Murray D, Walters R, Meyn RE, and Plunkett W, Mitoxantrone-induced DNA damage in leukemia cells is enhanced by treatment with high-dose arabinosylcytosine. *Cancer Chemother Pharmacol* **22**: 205–210, 1988.
27. McConkey DJ, Orrenius S and Jondal S, Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. *J Immunol* **145**: 1227–1230, 1990.
28. Dolbeare F, Gratzner H, Pallavicini MG and Gray JW, Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. *Proc Natl Acad Sci USA* **80**: 5573–5577, 1983.
29. Yasuda I, Kishimoto A, Tanaga S, Tominaga M, Sakurai A and Nishizuka Y, Assay of protein kinase C *in vitro* using synthetic peptide substrates. *Biochem Biophys Res Commun* **166**: 1220–1224, 1990.
30. Chou TC and Talalay P, Quantitative analysis of dose–effect relationships: The combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22**: 27–55, 1984.
31. Sorenson CM, Barry MA and Eastman A, Analysis of events associated with cell cycle arrest at G_2 phase and cell death by cisplatin. *J Natl Cancer Inst* **82**: 749–755, 1990.
32. O'Connor PM, Wasermann K, Sarang M, Magrath I, Bohr V and Kohn KW, Relationship between DNA cross-links, cell cycle and apoptosis in Burkitt's lymphoma cell lines differing in sensitivity to nitrogen mustard. *Cancer Res* **51**: 6550–6557, 1991.
33. Lotem J, Cragoe EJ and Sachs L, Rescue from programmed cell death in leukemia and normal cells. *Blood* **78**: 953–960, 1991.
34. Robertson LE, Chubb S, Meyn RE, Story M, Ford R, Hittleman WN and Plunkett W, Induction of apoptotic cell death in chronic lymphoma leukemia by 2-chloro-2′-deoxyadenosine and 9-β-D-arabinofuranosyl-2-fluoroadenine. *Blood* **81**: 143–150, 1993.
35. Cotter TG, Lennon SV, Glynn JM and Green DR. Microfilament-disrupting agents present the formation of apoptotic bodies in tumor cells undergoing apoptosis. *Cancer Res* **52**: 997–1005, 1992.
36. Li F, Grant S, Pettit G and McCrady CW, Bryostatin 1 modulates the proliferation and lineage commitment of human myeloid progenitor cells exposed to recombinant interleukin-3 and recombinant granulocyte-macrophage colony-stimulating factor. *Blood* **80**: 2495–2502, 1992.
37. Nishizuka Y, The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334**: 661–665, 1988.
38. Kennedy MJ, Prestigiacomo LJ, Tyler G, May WS and Davidson NE, Differential effects of bryostatin 1 and phorbol diester on human breast cancer cell lines. *Cancer Res* **52**: 1278–1283, 1992.
39. Basu A and Lazo JS, Sensitization of human cervical carcinoma cells to *cis*-diamminedichloroplatinum(II) by bryostatin 1. *Cancer Res* **52**: 3119–3124, 1992.
40. Rodriguez-Pena A and Prozengurt E, Disappearance of Ca^{2+}-sensitive phospholipid-dependent protein kinase activity in phorbol diester-treated 3T3 cells. *Biochem Biophys Res Commun* **120**: 621–623, 1983.
41. Dive C, Evans CA, Lynch PJ, Musk P and Whetton AD, The role of protein kinase C in the suppression of apoptosis by V-*abl* in hematopoietic cells. *Proc Am Assoc Cancer Res* **34**: 516, 1993.
42. Gorczyca W, Gong J, Ardelt B, Traganos F and Darzynkiewicz Z, The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res* **53**: 3186–3192, 1993.
43. Kharbanda S, Datta R and Kufe D, 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**: 7947–7952, 1991.
44. Kucera GL and Capizzi RL, 1-β-D-Arabinofuranosylcytosine-diphosphate-choline is formed by the

reversal of cholinephosphotransferase and not via cytidylyltransferase. *Cancer Res* **52**: 3886–3891, 1992.
45. Gunji H, Hass R and Kufe D, Internucleosomal DNA fragmentation during phorbol ester-induced monocytic differentiation and G_0/G_1 arrest. *J Clin Invest* **89**: 954–960, 1992.
46. Martin SJ, Bradley JG and Cotter TG, HL-60 cells indiced to differentiate towards neutrophils subsequently die via apoptosis. *Clin Exp Immunol* **79**: 448–453, 1990.
47. Solary E, Bertrand R, Kohn KW and Pommier Y, Differential induction of apoptosis in undifferentiated and differentiated HL-60 cells by topoisomerase I and II inhibitors. *Blood* **81**: 1359–1368, 1993.
48. Nishio N, Sugimoto Y, Fujiwara Y, Ohmori T, Morikage T, Takeda Y, Ohata M and Saijo N, Phospholipase C-mediated hydrolysis of phosphatidylcholine is activated by *cis*-diamminedichloroplatinum(II). *J Clin Invest* **89**: 1622–1628, 1992.
49. Posada J, Vichi P and Tritton TR, Protein kinase C in adriamycin action and resistance in mouse sarcoma 180 cells. *Cancer Res* **49**: 6634–6639, 1989.
50. Besterman JM, Duronio V and Cuatrecasas P, Rapid formation of diacylglycerol from phosphatidylcholine: A novel pathway for generation of a second messenger. *Proc Natl Acad Sci USA* **83**: 6785–6789, 1986.
51. Kiss Z and Anderson WB, Phorbol ester stimulates the hydrolysis of phosphatidylethanolamine in leukemic HL-60, NIH 3T3, and baby hamster kidney cells. *J Biol Chem* **264**: 1483–1487, 1989.
52. Kiss Z, Chattopadhyay J and Pettit GR, Stimulation of phosphatidylcholine synthesis is dissociable from increased phospholipid hydrolysis. *Biochem J* **273**: 189–194, 1991.
53. Kiss Z, Rapp UR, Pettit GR and Anderson WB, Phorbol ester and bryostatin differentially regulate the hydrolysis of phosphatidylethanolamine in Ha-*ras*- and *raf*-oncogene-transformed NIH 3T3 cells. *Biochem J* **276**: 505–509, 1991.