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IN VITRO EFFECTS OF BRYOSTATIN 1 ON THE METABOLISM AND CYTOTOXICITY OF 1-β-D-ARABINOFURANOSYLCYTOSINE IN HUMAN LEUKEMIA CELLS*

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Abstract—Bryostatin 1 is a macrocyclic lactone protein kinase C (PK-C) activator which has demonstrated promising antileukemic activity in preclinical studies. We have examined the effect of this agent on the metabolism and cytotoxicity of 1-β-D-arabinofuranosylcytosine (ara-C) in both log phase and high-density human promyelocytic leukemia cells (HL-60). Exposure of low-density cells to 12.5 nM bryostatin 1 for 24 hr prior to a 4-hr incubation with 1 or 10 μM ara-C resulted in nearly a 2-fold increase in ara-CTP formation. When cells were maintained under high-cell density conditions (e.g. 5×10^6 cells/mL) for 24 hr prior to ara-C exposure, a 90% reduction in ara-CTP formation and ara-C DNA incorporation was observed. However, coincubation of high-density cells with bryostatin 1 for 24 hr increased ara-CTP formation 6- to 8-fold, yielding levels essentially equivalent to those achieved in low-density cells. Smaller (but still significant) increases in ara-C DNA incorporation were also noted. Enhancement of ara-CTP formation by bryostatin 1 occurred over a broad ara-C concentration range (0.1 to 100 μM), involved a temperature-dependent process, could not be mimicked by addition of hematopoietic growth factors, and was not related to neutralization of toxic or inhibitory substances in high-density medium. Exposure of cells to bryostatin 1 did not lead to morphologic or functional evidence of HL-60 cell maturation or an increase in cell viability, but did produce a decline in cellular proliferative activity as determined by thymidine and bromodeoxyuridine incorporation and cytofluorometric analysis. Bryostatin 1 did not exert its effects in high-density cells by inhibiting ara-C deamination or by interfering with ara-CTP dephosphorylation, but instead appeared to act by enhancing ara-C phosphorylation. Although cell-free extracts obtained from high-density cells exposed to bryostatin 1 exhibited levels of deoxycytidine kinase activity compared to controls, treated cells did display a significant decline in intracellular dCTP levels (e.g. 0.7 vs 1.3 pmol/10^6), and nearly a 2-fold increase in ATP and UTP concentrations. Ara-CTP formation was also increased substantially by other PK-C activators including phorbol dibutyrate and mezerein (10–100 nM); this process was inhibited more than 70% by the PK-C inhibitor H-7 (50 μM), but not by the PK-C inhibitors staurosporine, tamoxifen, and HA1004. Finally, coadministration of ara-C and bryostatin 1 resulted in greater than expected inhibitory effects toward HL-60 cell clonogenic growth. These findings suggest that the novel agent bryostatin 1 induces biochemical perturbations in leukemic cells that favor ara-C activation, particularly in high-density cells exhibiting impaired ara-C nucleotide formation. They also raise the possibility that pharmacologic agents acting through second messenger pathways may modulate the metabolism of ara-C, and potentially other nucleoside analogs.

Bryostatin 1 is a macrocyclic lactone derived from the marine bryozoan, *Bugula neritina*, which initially displayed activity against a variety of murine hematopoietic neoplasms in preclinical screening studies [1]. Like the phorbol esters, bryostatin 1 is a potent activator of the enzyme protein kinase C (PK-C¶), but unlike these agents, it does not exhibit tumor promoting activity [2]. It has been shown to support the growth of normal multipotent hematopoietic progenitors *in vitro* [3], presumably through an indirect, accessory cell-mediated mechanism [4], while inducing maturation in some HL-60 sublines [5] and in myeloblasts obtained from some patients with acute non-lymphocytic leukemia [6]. More recently, this agent has been found to inhibit the *in vitro* clonogenic growth of human leukemic myeloblasts when administered at concentrations that promote colony for-

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¶ Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; dCyd, 2′-deoxycytidine; dCMP, 2′-deoxycytidine 5′-monophosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dThd, thymidine; dTTP, thymidine 5′-triphosphate; dUrd, 2′-deoxyuridine; BrdUrd, 5′-bromo-2′-deoxyuridine; dCK, deoxycytidine kinase, TCA, trichloroacetic acid; PBS, phosphate-buffered saline; THU, tetrahydrouridine; dTHU, deoxytetrahydrouridine; rGM-CSF, recombinant granulocyte macrophage-colony stimulating factor; rIL-3, recombinant interleukin 3; PDBu, phorbol dibutyrate; and PK-C, protein kinase C.

mation by their normal hematopoietic counterparts [7]. The ability of bryostatin 1 to stimulate normal hematopoietic cell growth while inhibiting leukemic cell proliferation and inducing maturation suggests a possible role for this agent in leukemia therapy.

1-β-D-Arabinofuranosylcytosine (ara-C) is a nucleoside analog which is widely used in the treatment of acute non-lymphocytic leukemia in humans [8]. It is converted to its nucleotide derivative, ara-CMP, by the pyrimidine salvage pathway enzyme, deoxycytidine kinase (dCK) [9]; this process represents the rate-limiting step in ara-C metabolism, particularly at extracellular ara-C concentrations greater than 10 μM [10]. Ara-C may also be converted to its inactive derivative, ara-U, by the degradative enzyme cytidine deaminase, which is present in the plasma and liver, and within leukemic cells [11]. Ara-C is ultimately metabolized to its lethal triphosphate derivative, ara-CTP, which (weakly) inhibits DNA polymerase α [12], and is incorporated into elongating DNA strands [13]. The latter process has been shown to correlate closely with ara-C-mediated lethality in leukemic cells [14]. In addition to these biochemical considerations, ara-C is an S-phase specific agent, exerting its lethal effects only towards cells actively engaged in DNA synthesis [15]. The cytotoxic effects of ara-C toward leukemic cells therefore depend upon the complex interplay between multiple pharmacologic, intracellular biochemical, and cytokinetic factors.

Several recent observations provide a theoretical basis for the combined use of bryostatin 1 and ara-C in an antileukemic regimen. First, it has been shown that hematopoietic growth factors such as rGM-CSF, rIL-3, and rG-CSF can sensitize clonogenic leukemic cells to the actions of ara-C, either by increasing the susceptible S-phase fraction [16, 17], or by augmenting ara-CTP formation and retention [18]. Since bryostatin 1 exhibits certain growth factor-like actions [3], it might also be capable of potentiating ara-C-mediated lethal effects. Second, in contrast to the hematopoietic growth factors, which increase the survival and stimulate the proliferation of leukemic cells [19], bryostatin 1 displays intrinsic antileukemic activity [7]. Finally, it has been established that certain agents including interferon [20], tumor necrosis factor [21], and ara-C [22] are effective in inhibiting the secondary cloning efficiency, or self-renewal capacity of leukemic cells, an *in vitro* characteristic which correlates closely with clinical outcome [23]. Recent findings now indicate that bryostatin 1 is also inhibitory to leukemic cell self-renewal capacity [24], raising the possibility that the combined use of two such agents may be associated with improved antileukemic efficacy. There is currently no information available concerning the effect of bryostatin 1 on the cytokinetic or biochemical determinants of ara-C-mediated cytotoxicity in leukemic cells. The present studies were undertaken in order to characterize the interaction between these agents in the human promyelocytic leukemia cell line HL-60, both in cells maintained under standard log phase conditions, as well as in high-density cells whose behavior and biochemical characteristics may mimic those of human leukemia cells *in vivo*. Our results indicate that administration of bryostatin 1 is associated with biochemical changes leading to a marked potentiation of ara-C metabolism, particularly in high-density cells displaying a reduced capacity for ara-C phosphorylation.

MATERIALS AND METHODS

Drugs and chemicals

Chemicals. Ara-C hydrochloride, dCyd hydrochloride, thymidine (dThd), 2′-deoxyuridine (dUrd), 5′-bromo-2′-deoxyuridine (BrdUrd), dCMP, ara-CTP, and 2′-deoxycytidine 5′-triphosphate (dCTP) were purchased from the Sigma Chemical Co., St. Louis, MO. They were maintained as dry powders, stored in dessicator jars at −80°, and formulated in distilled water or sterile medium prior to use. [^{3}H]ara-C (21 Ci/mmol), [^{3}H]dCyd (23 Ci/mmol), and [^{3}H]dThd (18 Ci/mmol) were purchased from Amersham Radiochemicals, Arlington Heights, IL.

Growth factors. rGM-CSF (batch 20026-133) was provided by Dr. Paul Trotta, Schering Corp., Bloomfield, NJ. rIL-3 was furnished by Dr. Jay Stoudemire, Genetics Institute, Cambridge, MA. These were aliquoted into vials containing sterile medium and 1% bovine serum albumin (BSA), and stored frozen at −80° prior to use.

PK-C activators. Bryostatin 1 was extracted and purified from *Bugula neritina* as previously described [21]. It was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and diluted in complete medium prior to use. The final concentration of DMSO in all experiments was less than 0.02%. Phorbol dibutyrate (PDBu) and mezerein were purchased from L. C. Biochemicals (Woburn, MA). They were formulated and diluted exactly as described above for bryostatin 1.

PK-C inhibitors. Staurosporine and tamoxifen were purchased from Sigma, dissolved in sterile water and DMSO, respectively, under light-protected conditions, and diluted in fresh medium prior to use. H-7 and HA1004 were purchased from Seikagaku America, Inc. (St. Petersburg, FL), dissolved in sterile water in the dark, and diluted as described above prior to use.

Deaminase inhibitors. Tetrahydrouridine (THU) and deoxytetrahydrouridine (dTHU) were provided by Dr. David Johns, Drug Development Branch, NCI. They were maintained in air-tight, desiccant containers at −80°, and reformulated in sterile water prior to use.

Cells

HL-60 cells were derived from the line originally isolated by Gallegher *et al.* [25]. Cells below passage 75 were used during the course of these experiments. The cells were maintained in RPMI medium containing 1% sodium pyruvate, 1% non-essential amino acids, 10,000 units/mL penicillin and streptomycin, and 15% fetal bovine serum (FBS; Hyclone, Logan, UT). Passage was performed twice weekly, and cultures were examined routinely for mycoplasma contamination utilizing the Gen-Probe assay (Gen-Probe, San Diego, CA). The doubling time for cells maintained in this manner was approximately 30 hr.

Biochemical studies

Ara-CTP formation. Ara-CTP formation was

assessed in both log phase and high-density HL-60 cells. In the former studies, logarithmically growing cells (cell density $<5 \times 10^5$/mL) were centrifuged at room temperature at 400 *g* for 6 min. The cell pellets were resuspended in fresh medium containing 15% FBS at a concentration of 5×10^6 cells/mL. Aliquots (4 mL) of the cell suspension (20×10^6 cells/condition) were placed in 15-mL polystyrene conical centrifuge tubes (Nunc, Napierville, IL), along with either 1 or 10 μM ara-C with or without the designated concentration of bryostatin 1. In some studies, low-density cells were incubated with bryostatin 1 for 24 hr prior to the ara-C exposure. The tubes were capped loosely and placed in the incubator at a 30° horizontal angle for 4 hr. At hourly intervals, the tubes were agitated gently to reduce cell settling. At the end of the incubation period, the cells were centrifuged at 400 *g* at 4°, and the medium was discarded. The cells were then washed once with ice-cold phosphate-buffered saline (PBS), and the cell pellet was precipitated with 100 μL of ice-cold 0.6 M trichloroacetic acid (TCA). The TCA was extracted with freon–octylamine as previously described [26], and ribonucleotides were eliminated utilizing the periodate method of Garrett and Santi [27]. The neutralized extract was then subjected to high pressure liquid chromatographic analysis employing the gradient system described by Plunkett *et al.* [28]. A Bio-Rad model 700 system was employed in conjunction with a Beckman model 260 UV detector and a Waters Radial-Pak SAX column. Absorbance was recorded at 280 nm; peak areas were integrated automatically and quantitated by comparison with values for known standards. Ara-CTP levels were expressed as picomoles per 10^6 cells.

For high-density cell studies, cells were suspended in medium containing 15% FEBS at a density of 5×10^6 cells/mL, and 4-mL aliquots were placed in the conical centrifuge tubes as described above. After addition of bryostatin 1 (and/or other agents), the tubes were placed in the incubator for 24 hr. At the end of this period, 1 or 10 μM ara-C was added to the tubes, the cell suspension was mixed thoroughly, and the cells were returned to the incubator for an additional 4 hr as described above. In control studies, centrifugation of cells and resuspension of the pellet in fresh medium prior to ara-C exposure yielded equivalent results. Following the 4-hr incubation interval, cell extracts were obtained and ara-CTP levels quantitated as described above. In some studies, high-density cells were exposed to bryostatin 1 for only 4 hr in conjunction with ara-C.

Ara-CTP dephosphorylation. Ara-CTP degradation in HL-60 cells was assayed utilizing a minor modification of the techniques of Abe *et al.* [29]. Briefly, following a 4-hr exposure to ara-C, 100 μM dCyd was added to the tubes to block further ara-C phosphorylation. At time 0 and at various intervals thereafter, aliquots of the cell suspension were removed, a cell pellet was obtained, and ara-CTP levels were determined by HPLC. The retention of ara-CTP at each time point was expressed as a percentage relative to the time 0 control value, and the intracellular ara-CTP half-life was determined as previously described [30].

Deoxyribonucleoside and ribonucleoside triphosphate levels. Deoxyribonucleoside triphosphate (dCTP, dTTP, dATP, and dGTP) levels were assayed in cell extracts utilizing the HPLC method described above for ara-CTP formation. The retention times for dCTP, dTTP, dATP and dGTP using this system were 26.7, 35.6, 41.3 and 50.7 min, respectively. Levels were quantitated by comparing peak areas to those of known standards, and values expressed as picomoles triphosphate per 10^6 cells.

Ribonucleoside triphosphates were assayed utilizing the same method except that extracts were not subjected to periodation prior to HPLC analysis. The retention times of CTP, UTP, ATP, and GTP were 22.3, 31.9, 44.3, and 53.2 min, respectively. Peak areas were quantitated, compared to values for known standards, and intracellular levels expressed at picomoles NTP/per 10^6 cells.

Ara-C DNA incorporation. Logarithmically growing HL-60 cells (cell density $<5 \times 10^5$ cells/mL) were centrifuged and resuspended in fresh medium at a cell density of 5×10^6 cells/mL, and 2-mL aliquots were placed in slanted 15-mL conical centrifuge tubes as described above. The cells were then exposed to either 1 or 10 μM [^{3}H]ara-C (±bryostatin 1) for 4 hr with periodic agitation, centrifuged at 4°, and washed twice with ice-cold PBS prior to DNA extraction. In some studies, logarithmically growing HL-60 cells were exposed to bryostatin 1 for 24 hr prior to incubation with ara-C. For high-density cell studies, cells were incubated at a concentration of 5×10^6 cells/mL for 24 hr (with or without bryostatin 1 or other agents) prior to exposure to [^{3}H]ara-C. The DNA was then isolated by RNAase and proteinase digestion, phenol extraction, and ethanol precipitation as previously described [31]. DNA was quantitated spectrophotometrically, and ara-C incorporation expressed as picomoles ara-C per microgram DNA.

Enzyme assays

Cytidine deaminase. Cytidine deaminase activity was assessed in HL-60 cells utilizing a minor modification of the method of Steuart and Burke [11]. Briefly, cell extracts were obtained by sonication, clarified by centrifugation at 20,000 *g* for 30 min at 4°, and assayed for cytidine deaminase activity by measuring the conversion of [^{3}H]dCyd to [^{3}H]dUrd. The reaction mixture (total volume 200 μL) contained 0.05 M Tris (pH 8.0), 0.001 M EDTA, 0.002 M dithiothreitol, 50 μg protein, and 0.1 μmol [^{3}H]dCyd. After a 15-min incubation at 37°, the reaction was terminated by the addition of TCA which was then extracted with freon–octylamine as described previously. The neutralized extract was then separated by HPLC employing a Waters Bondapak C18 ODS column and an isocratic 0.005 M, pH 3.2, NH_4PO_4 buffer system with a flow rate of 1 mL/min. Fractions were collected at 30-sec intervals, and radioactivity was quantitated in a liquid scintillation counter. Retention times for dCyd and dUrd utilizing this sytem were 11.5 and 18.5 min, respectively. A unit of cytidine deaminase activity was defined as 1 nmol dUrd formed/mg protein/hr.

Deoxycytidine kinase. The assay system for dCK involved a minor modification of the method originally described by Ives and Durham [9] and measured the conversion of [^{3}H]dCyd to [^{3}H]dCMP. The reac-

tion mixture (total volume 200 μL) contained 10 mM ATP, 10 mM $MgCl_2$, 50 mM Tris (pH 8.0), 15 mM NaF, 1 mM THU, 50 μg protein, and 0.028 mM [^{3}H] dCyd. The mixture was incubated for 15 min at 37°, the reaction was terminated by the addition of TCA, and a neutralized extract was obtained as described above. The reaction product (dCMP) was quantitated utilizing the same HPLC system employed in the cytidine deaminase assay. With this method, dCMP and dCyd exhibited retention times of 6.5 and 11.5 min, respectively, and were clearly separable from each other. Thirty-second fractions were collected and radioactivity was determined by scintillation counting. A unit of dCK activity was defined as 1 nmol dCMP formed/mg protein/hr.

Cloning studies

HL-60 cells. The clonogenic growth of HL-60 cells in response to ara-C and bryostatin 1 was assessed by a minor modification of a previously reported technique [32]. Briefly, after exposure to drugs, cells were washed three times, resuspended in fresh medium, and plated in 35 mm^2 tissue culture dishes. Each dish contained 1 mL RPMI medium, 15% fetal bovine serum, 0.3% Bacto agar (Difco, Detroit, MI), and 400 cells/plate. In continuous exposure studies, agents were directly incorporated into the agar prior to addition of cells. The plates were then placed in a 37°, 5% CO_2, fully humidified incubator for 8 days, after which colonies, consisting of groups of 50 or more cells, were scored with an inverted microscope.

Cytokinetic studies

Thymidine incorporation. HL-60 cells were suspended in fresh medium at a density of 5×10^6 cells/mL and 5-mL aliquots were transferred to 15-mL centrifuge tubes. Drug was then added to the suspension, and the tubes were placed in the 37°, 5% CO_2, fully humidifed incubator for 24 hr. For the final 4 hr, 10^4 cells in a total volume of 0.1 mL were transferred to each well of a 96-well tissue culture plate. Tritiated thymidine (0.5 μCi/well) was then added to the cell suspensions, and the plates were returned to the incubator for the final 4 hr of incubation. Cells were then harvested with a Skatron cell harvester onto glass microfiber filters, and incorporated [^{3}H]dThd was quantitated by scintillation counting.

Cell cycle analysis. After 24 hr of drug treatment (as described above), 10^6 cells were pelleted, washed with 1.5 mL of PBS, centrifuged, and resuspended in 3 mL of 100% ethanol. The cells were fixed by allowing them to stand overnight at 4°, and then were resuspended in 1 mL of propidium iodide staining solution (3.8×10^{-3} M sodium citrate, 0.5 mL RNAase A, and 0.01 mg/mL propidium iodide (all Sigma). Following a 1-hr incubation on ice, the cells were removed from the staining solution and resuspended in PBS. Analysis of the DNA content was then performed on a FACSCAN flow cytometer (Becton-Dickinson), and the data were analyzed with the Cellfit (Becton-Dickinson) program.

BrdUrd incorporation. BrdUrd incorporation in HL-60 cells was determined by the method of Dolbeare *et al.* [33]. After exposure to drugs as in the previous sections, cells were washed and suspended in fresh medium containing 10% FBS; 2×10^6 cells/condition were then exposed to 10 μM BrdUrd for 30 min at 37° in the 5% CO_2 incubator, washed twice with cold PBS, and fixed with ice-cold ethanol. Following exposure to 4 N HCl for 30 min to denature double-stranded DNA, cells were washed twice with 0.1 sodium borate (pH 8.4) and resuspended in cold ethanol overnight at −20°. The cells were then washed and suspended in 0.5% Tween/PBS solution containing 10 μL monoclonal anti-BrdUrd Ab (Becton-Dickinson)/10^6 cells. After a 30-min incubation, the cells were washed and resuspended in Tween/PBS containing 10 μL goat anti-mouse IgG fluorescein isothiocyanate (Sigma) for 30 min. After two additional washings in PBS, the cells were suspended in PBS containing 20 μg/mL propidium iodide and 30 μg/mL RNAase (Sigma) for 2 hr and stored at −20° prior to analysis. Analysis was performed utilizing a Coulter Epics IV cytofluorometer and excitation elicited by a 448 nm argon laser. Bivariate analysis of BrdUrd labeling (green emissions monitored at 515–530 nm) and DNA content (red emissions monitored at >630 nm) were performed by constructing bitmaps for control and treated samples (>10,000 cells/condition). The percentage of cells in S-phase for each condition was then determined as previously described [30].

Statistical analysis

The statistical significance of differences between experimental values was determined utilizing the two-tailed Student's *t*-test for paired observations.

RESULTS

The effects of bryostatin 1 and other agents on ara-CTP formation in both low- and high-density HL-60 cells are shown in Table 1. Exposure of low-density HL-60 cells to bryostatin 1 for 24 hr led to a 2-fold increase in ara-CTP formation when 1 μM ara-C was administered, and a smaller (but still significant; $P < 0.05$) increase when 10 μM ara-C was present. Administration of bryostatin 1 for only the 4-hr ara-C exposure interval had no effect on ara-CTP formation in these cells. A considerably more dramatic response was observed in cells maintained at a high density (e.g. 5×10^6 cells/mL) for 24 hr prior to ara-C exposure. These cells exhibited approximately a 90% reduction in ara-CTP formation compared to their low-density counterparts at both 1 and 10 μM ara-C concentrations. Coadministration of bryostatin 1 during the 24-hr preincubation period produced 6- to 8-fold increases in ara-CTP formation at both ara-C concentrations, and resulted in values that were essentially equivalent to those of low-density cells. In contrast to results obtained with their low-density counterparts, incubation of high-density cells with bryostatin 1 for only the 4-hr ara-C exposure interval led to a significant (2-fold) increase in ara-CTP formation. Because hematopoietic growth factors such as rGM-CSF have been shown to potentiate ara-CTP formation in leukemic cells [18], parallel studies were performed utilizing rGM-CSF, rIL-3 and 10% conditioned medium from a human bladder cell tumor line (5637-CM) [34]. A 24-hr preincubation of high-density cells with each of these factors did not enhance ara-CTP formation, suggesting that bryostatin 1

Table 1. Effect of bryostatin 1 on ara-CTP formation and ara-C DNA incorporation in low- and high-density HL-60 cells

	ara-CTP formation (pmol/10^6 cells)								
	Low density			High density					
[ara-C]	Control	Bryo 1 (4 hr)	Bryo 1 (24 hr)	Control	Bryo 1 (4 hr)	Bryo 1 (24 hr)	rGM-CSF	rIL-3	5637-CM
1 μM	6.3 ± 0.9	6.6 ± 1.2	12.3 ± 1.7*	1.1 ± 0.4	ND	6.9 ± 0.9*	1.0 ± 0.3	1.0 ± 0.4	1.1 ± 0.2
10 μM	27.6 ± 4.6	27.8 ± 3.8	37.6 ± 2.6*	3.7 ± 1.2	6.5 ± 1.9*	29.6 ± 3.2*	4.1 ± 0.7	4.2 ± 0.8	4.8 ± 0.3
	ara-C DNA incorporation (pmol/μg DNA)								
	Low density			High density					
[ara-C]	Control	Bryo 1 (4 hr)	Bryo 1 (24 hr)	Control	Bryo 1 (4 hr)	Bryo 1 (24 hr)	rGM-CSF	rIL-3	5637-CM
1 μM	0.034 ± 0.008	ND	0.048 ± 0.006*	0.006 ± 0.001	ND	0.027 ± 0.008*	0.005 ± 0.001	0.006 ± 0.002	0.006 ± 0.002
10 μM	0.110 ± 0.020	0.118 ± 0.024	0.148 ± 0.022*	0.021 ± 0.006	0.036 ± 0.006*	0.092 ± 0.021*	0.018 ± 0.004	0.017 ± 0.003	0.024 ± 0.007

Logarithmic phase (cell density $<5 \times 10^5$ cells/mL) or high-density (cell density 5×10^6 cells/mL) HL-60 cells were incubated with 12.5 nM bryostatin 1 for 24 hr prior to, or for 4 hr in conjunction with, 1 or 10 μM labeled or unlabeled ara-C. High-density cells were also preincubated for 24 hr with 50 ng/mL rGM-CSF, 50 ng/mL rIL-3, or 10% 5637 conditioned medium prior to the 4-hr ara-C exposure. Intracellular ara-CTP formation was determined by HPLC; ara-C DNA incorporation was determined utilizing RNAase and proteinase digestion, phenol extraction, and ethanol precipitation. Values are the means of three separate experiments performed in triplicate ± 1 SD. ND = not done.

* Significantly greater than control ($P < 0.05$).

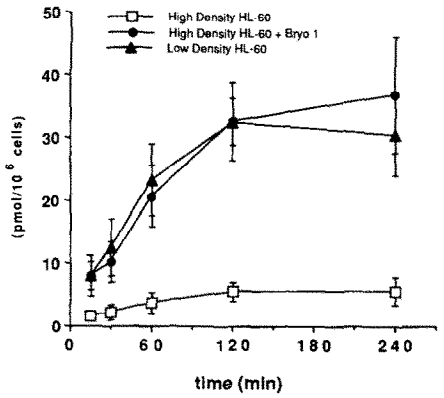


Fig. 1. Effect of bryostatin 1 on the 4-hr time course of ara-CTP formation in high-density HL-60 cells. High-density cells were incubated in the presence (●) or absence (□) of 12.5 nM bryostatin 1 for 24 hr prior to a 4-hr exposure to 10 μM ara-C. At various intervals following addition of ara-C to the medium, aliquots of the cell suspension were removed and intracellular ara-CTP levels were determined by HPLC as described in the text. Values for low-density cells (▲) are presented for comparison. Each point is the mean of three separate experiments performed in triplicate ± SD.

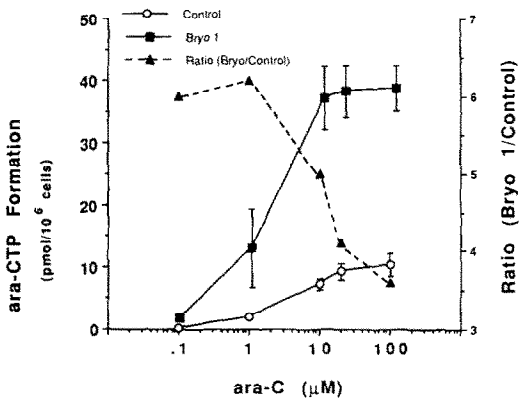


Fig. 2. Effect of bryostatin 1 on the 4-hr formation of ara-CTP by high-density HL-60 cells exposed to increasing concentrations of ara-C. High-density cells were incubated with complete medium alone (○) or medium containing 12.5 nM bryostatin 1 (■) for 24 hr, followed by a 4-hr incubation with 0.1 to 100 μM ara-C. At the end of the incubation period, ara-CTP levels (left ordinate) were determined as described in the text. Values are the means of two separate experiments performed in triplicate ± 1 SD. The relative values (▲) for ara-CTP formation in bryostatin 1 treated and control cells are expressed as a ratio (right ordinate).

exerts its effects on ara-C metabolism through a separate mechanism of action.

Bryostatin 1 also produced an increase in the incorporation of ara-C into HL-60 cell DNA, although the magnitude of this effect was less than that observed for ara-CTP formation. For example, exposure of low-density cells to bryostatin 1 for 24 hr led to a modest (e.g. 30–40%) but statistically significant increase in ara-C DNA incorporation compared to untreated controls. In contrast, a 4-hr bryostatin 1 exposure interval had no discernible effect. As in the case of ara-CTP formation, cells maintained at a high-cell density for 24 hr prior to incubation with 1 or 10 μM ara-C exhibited 85–90% reductions in ara-C DNA incorporation compared to low-density controls. Coadministration of bryostatin 1 partially restored these levels to within 70% of values obtained in low-density cells. Co-incubation of high-density cells with bryostatin 1 and ara-C for only 4 hr was also associated with a significant increase in ara-C DNA incorporation, in contrast to results obtained in low-density cells. Finally, preincubation of high-density cells with rGM-CSF, rIL-3, or 5637-CM had no discernible effect on ara-C DNA incorporation.

The effect of bryostatin 1 on the time course of ara-CTP formation in high-density HL-60 cells is shown in Fig. 1; values for low-density controls are included for comparison. A plateau in ara-CTP formation was observed after a 2-hr incubation with ara-C for all conditions. This plateau level was approximately 7-fold greater for low-density and bryostatin 1-treated high-density cells than for their untreated high-density counterparts. However, comparable differences were noted as early as 15 min following addition of ara-C to the medium. Calculation of the rate of ara-CTP formation from the linear portions of the curves [30] revealed the following values (nmol/10^9 cells/min): low density (0.420); high-density, bryostatin 1-treated (0.360); high-density, untreated (0.084).

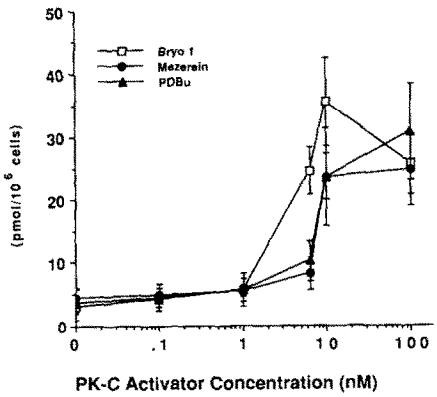


Fig. 3. Four-hour ara-CTP formation by high-density HL-60 cells exposed to various concentrations of PK-C activators. High-density cells were incubated for 24 hr with 0.1 to 100 nM bryostatin 1, PDBu, or mezerein prior to a 4-hr exposure to 10 μM ara-C. Ara-CTP levels were determined as described previously. Values are the means of triplicate determinations for experiments performed on 2–3 separate occasions ± 1 SD.

The effect of bryostatin 1 on ara-CTP formation was also assessed in high-density HL-60 cells exposed to ara-C administered over a broad concentration range (e.g. 10^{-7}–10^{-4} M) (Fig. 2). Concentrations of ara-C were included which exceed the K_m value for dCK employing ara-C for a substrate (e.g. 20 μM) [35]. Bryostatin 1 produced substantial increases in ara-CTP formation throughout the ara-C concentration range, although the degree of enhancement declined somewhat at higher ara-C concentrations. This decline resulted from the ability of untreated high-density cells to increase ara-CTP formation slightly as extracellular ara-C concentrations exceeded 10 μM.

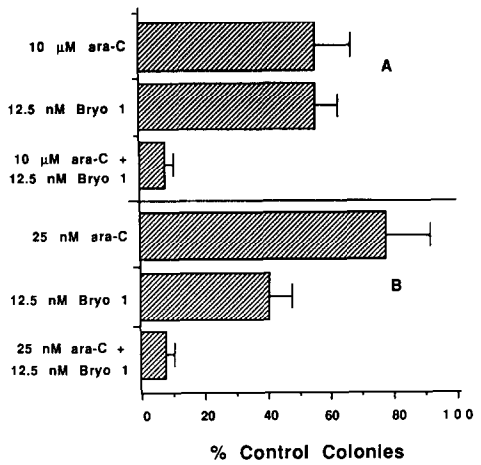


Fig. 4. Effects of sequential and simultaneous exposure to bryostatin 1 and ara-C on HL-60 cell colony formation. High-density cells were exposed to the designated concentrations of bryostatin 1 and ara-C for 24 hr, washed, and cloned in soft agar as described in the text (panel A). Alternatively, cells were cloned in the continuous presence of ara-C and bryostatin 1 (panel B). Colonies, consisting of groups of 50 or more cells, were scored after 8 days of incubation. Values are the means of at least three separate experiments performed in triplicate ± 1 SD.

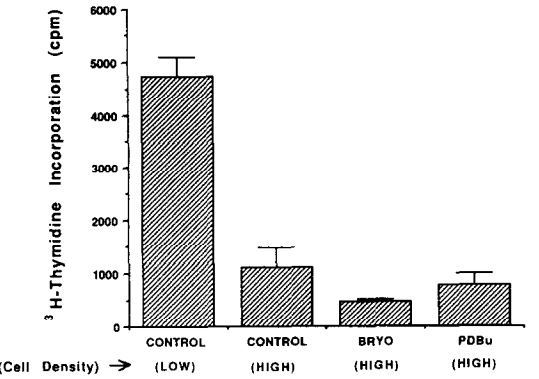


Fig. 5. Effect of bryostatin 1 and PDBu on dThd incorporation by HL-60 cells. High-density HL-60 cells (5×10^6 cells/mL) were exposed to either 12.5 nM bryostatin 1 or PDBu for 24 hr and [^{3}H]dThd for the final 4 hr of incubation. A logarithmic phase control is included for comparison. Incorporation of dThd into retained material was determined as described in the text and is expressed as cpm/10^4 cells. Values are the means of triplicate determinations ± 1 SD. Additional experiments yielded essentially identical results.

The concentration-dependence of effects on ara-CTP formation was determined for bryostatin 1 as well as for the tumor promoting PK-C activators phorbol dibutyrate (PDBu) and merzerein (Fig. 3). A steep concentration–response relationship was observed for each of these agents; no increases in ara-CTP formation were noted at drug concentrations of 1 nM or less. For bryostatin 1, peak ara-CTP formation occurred at 10 nM and declined when the concentration was increased to 100 nM. Mezerein also exerted maximal effects at 10 nM, but these were less than those observed for bryostatin 1. However, an increase in the mezerein concentration to 100 nM was not associated with a reduction in ara-CTP formation. PDBu exhibited approximately half-maximal effects on ara-CTP formation at 10 nM concentrations, and maximal effects at 100 nM. The latter were essentially equivalent to those obtained with 10 nM bryostatin 1.

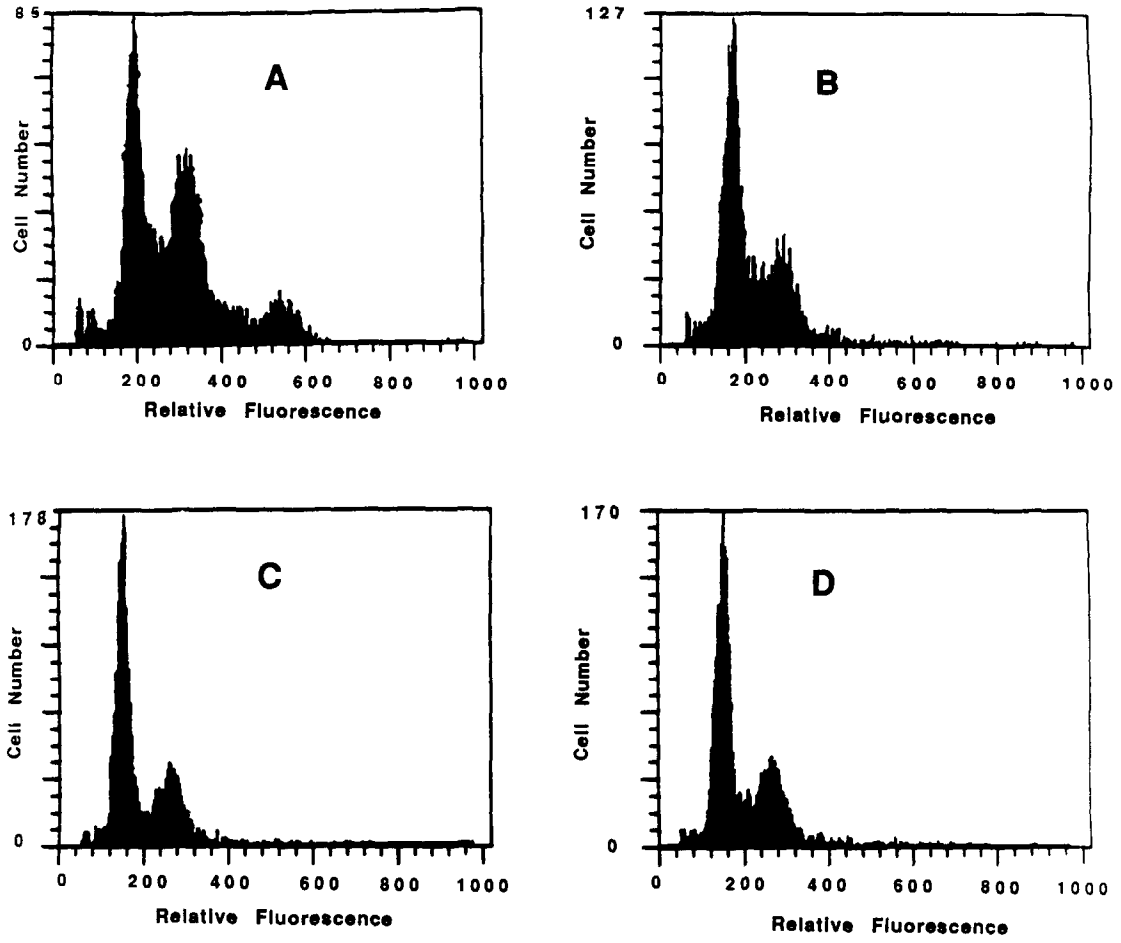
To determine whether bryostatin 1 induced perturbations in ara-C metabolism might have biologic consequences, the effects of combinations of these agents were assessed with respect to the clonogenic growth of HL-60 cells (Fig. 4). A 24-hr exposure of high-density cells to 10 μM ara-C or 12.5 nM bryostatin 1 resulted in survival fractions of 54 and 52%, respectively (panel A). Simultaneous exposure to both agents led to a survival fraction of 7% versus an expected value of 28% (representing the product of the individual values). In continuous exposure studies (panel B), a low concentration of ara-C (25 nM) and 12.5 nM bryostatin 1 reduced colony formation by 20 and 60%, respectively; combined exposure to both agents reduced colony formation by 94%.

To establish whether bryostatin 1 mediated enhancement of ara-C metabolism might be related to effects on cell proliferation, [^{3}H]dThd incorporation and cytofluorometric studies were performed (Figs. 5 and 6). High-density cells maintained for 24 hr exhibited a 75% reduction in dThd incorporation compared to log phase controls. However, coincubation with 12.5 nM bryostatin 1 did not affect dThd incorporation. A similar effect on dThd uptake was observed in cells exposed to 12.5 nM PDBu. Cytofluorometric analysis (Fig. 6) revealed that high-density cells incubated with bryostatin 1 exhibited a decrease in the percentage of S-phase cells (19 vs 32%) compared to high-density controls. These findings suggest that the ability of bryostatin 1 to potentiate ara-C metabolism in high-density cells does not involve recruitment of cells into cycle and is not associated with an enhanced proliferative capacity. Instead, the increase in ara-C nucleotide formation occurs despite a net decline in the S-phase cell fraction.

The effects of bryostatin 1 on several biologic and biochemical characteristics of high-density HL-60 cells are listed in Table 2. Cells exposed to bryostatin 1 for 24 hr did not differ from untreated controls with respect to morphology, viability, ANAE staining, or cytidine deaminase activity. Bryostatin 1 treated cells also did not exhibit a change in BrdUrd incorporation or dCK activity compared to their untreated counterparts. Treated cells also displayed slightly greater plastic adherence than controls, but this difference was marginal.

In separate studies, the effect of temperature on bryostatin 1 induced alterations in ara-C metabolism was assessed by exposing treated cells to ara-C for 4 hr at both 4 and 37° (data not shown). The enhancement in ara-CTP formation noted at 37° was abrogated when the reaction took place at 4°, suggesting a temperature-dependent process underlying bryostatin 1 mediated effects.

To determine whether potentiation of ara-CTP formation by bryostatin 1 might result from interference with ara-CTP dephosphorylation, the 4-hr retention of ara-CTP was assessed in low- and high-density HL-



treatment	% total cells		
	G_1/G_0	S	G_2/M
A. low density control	31.1	37.2	31.7
B. high density control	53.0	31.8	15.2
C. high density + bryostatin 1	61.1	19.2	19.7
D. high density + PDBu	58.2	23.2	18.6

Fig. 6. Cytofluorometric analysis of HL-60 cells exposed to bryostatin 1 and PDBu. Cells were treated with agents as described in Fig. 1, and subsequently exposed to propidium iodide as detailed in the text. Cytofluorometric analyses were then performed and the percentage of cells in G_1, S, and G_2M was determined. A = low-density controls; B = high-density controls; C = high-density cells + 12.5 nM bryostatin 1; and D = high-density cells + 12.5 nM PDBu. A representative experiment is shown; additional studies yielded equivalent results.

60 cells following exposure to 10 μM ara-C (Fig. 7). Because the peak level of ara-CTP may influence the rate of dephosphorylation [29], low-density cells were also exposed to 1 μM ara-C in order to achieve an intracellular ara-CTP level equivalent to that of high-density cells exposed to 10 μM ara-C. High-density HL-60 cells exposed to bryostatin 1 did not exhibit a difference in the 30, 60, and 120 min retention of ara-CTP compared to their untreated counterparts. However, the 4-hr ara-CTP retention for untreated cells was significantly greater than the value for cells exposed to bryostatin 1 (24.5 ± 4.3 vs 8.9 ± 3.8;

Table 2. Effect of a 24-hr exposure to bryostatin 1 on various characteristics of HL-60 cells

	Control	Bryostatin 1
1. Viability (%)	88.7 ± 6.3	85.5 ± 4.2
2. BrdUrd incorporation (%)	18.3 ± 3.2	15.8 ± 2.6
3. Deoxycytidine kinase (units)*	0.52 ± 0.11	0.48 ± 0.08
4. Cytidine deaminase (units)†	79.6 ± 8.2	83.4 ± 11.9
5. Macrophage morphology	0	0
6. Adherence (0–4+)	0	1+
7. ANAE (0–4+)	0	0

HL-60 cells were maintained at a cell density of 5×10^6 cells/mL for 24 hr in the presence or absence of 12.5 nM bryostatin 1, and the designated studies were performed as described in the text. Morphology, adherence, and α-naphthyl acetate esterase (ANAE) staining were assessed as previously described [6]. Values are the means of at least three separate experiments performed in triplicate ± 1 SD.

* One unit of deoxycytidine kinase activity = 1 nmol dCMP formed/mg protein/hr.

† One unit of cytidine deaminase activity = 1 nmol dUrd formed/mg protein/hr.

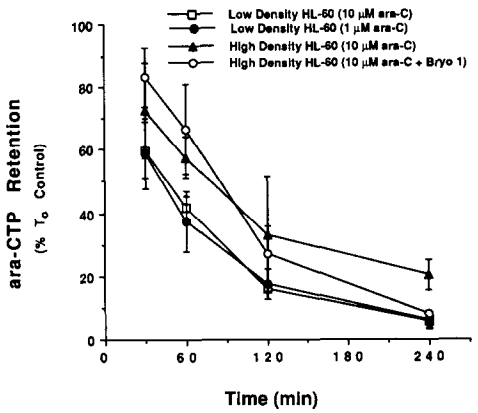


Fig. 7. Effect of bryostatin 1 on ara-CTP dephosphorylation by HL-60 cells. The degree of ara-CTP dephosphorylation was determined by incubating high-density cells previously exposed to bryostatin 1 for 24 hr with 10 μM ara-C for 4 hr. At the end of this incubation interval, 100 μM dCyd was added to the tubes to prevent further ara-C phosphorylation, and cell extracts were analyzed at various time intervals to ascertain the retention of ara-CTP. Values for low-density cells exposed to 1 μM and 10 μM ara-C are included for comparison. The ordinate represents the percentage retention of the T_0 ara-CTP level (28.7 pmol/10^6 cells). Values are the means of two separate experiments performed in triplicate ± 1 SD.

$P < 0.05$). Low-density cells exposed to 1 or 10 μM ara-C exhibited decreased ara-CTP retention (compared to treated or untreated high-density cells) at time intervals up to 120 min, and a value equivalent to that of bryostatin 1 treated high-density cells at the 4-hr interval. These findings demonstrate that a reduced capacity to dephosphorylate ara-CTP cannot account for the enhanced accumulation of ara-CTP in high-density cells exposed to bryostatin 1.

Although the activity of cytidine deaminase was equivalent in crude cell extracts obtained from treated and untreated high-density cells, it is possible that regulatory factors operative in intact cells may affect ara-C deamination and thereby influence bryostatin 1 mediated actions. To address this issue, cells preincubated with bryostatin 1 were exposed to ara-C in conjunction with either THU, a potent inhibitor of cytidine deaminase [36], or dTHU, an inhibitor or dCMP deaminase [37], and ara-CTP levels were determined (Fig. 8). Coadministration of THU or dTHU with ara-C did not increase significantly ara-CTP formation in untreated cells. Bryostatin 1 treated cells exposed to the deaminase inhibitors did not display reductions in ara-CTP formation. These findings suggest that the effect of bryostatin 1 on ara-CTP formation in high-density cells is not mediated through alterations in the activity of cytidine or dCMP deaminase.

The effect of a 24-hr exposure to bryostatin 1 on ribonucleoside and deoxyribonucleoside triphosphate levels in high-density HL-60 cells was assessed, and the results are shown in Table 3. Bryostatin 1 treated cells displayed a significant increase in both UTP and ATP pool sizes compared to untreated controls; CTP and GTP levels were unchanged. It should be noted that the ribonucleoside triphosphate levels in high-density HL-60 cells are somewhat lower than values previously reported for low-density murine leukemia cell lines [38]. Exposure of cells to bryostatin 1 was associated with a significant decline in dCTP (e.g. 0.7 vs 1.3 pmol/10^6) and an increase in dATP and dGTP pool sizes. No change in dTTP levels was observed.

To determine whether exposure to bryostatin 1 might alter the allosteric regulation of dCK, enzyme activity was assayed in extracts from control and bryostatin 1-treated high-density cells in the presence of 0.1 and 1.0 mM concentrations of dCTP (Table 4). No differences could be detected in the inhibitory effects of dCTP on the activity of dCK obtained from treated or untreated cells. In separate studies, enzyme activity was assayed in cell-free extracts containing ribonucleoside and deoxyribonucleoside triphosphate levels approximating those present in intact cells (Table 3). Under these conditions, nearly 2-fold

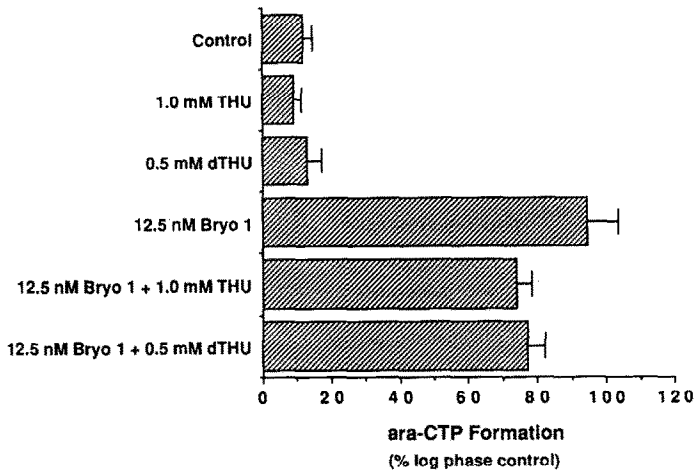


Fig. 8. Effects of THU and dTHU on ara-CTP formation in HL-60 cells exposed to bryostatin 1. High-density cells were exposed to 12.5 nM bryostatin 1 for 24 hr prior to a 4-hr exposure to 10 μM ara-C in conjunction with either 1 mM THU or 0.5 mM dTHU (lowermost three bar graphs). High-density cell controls were exposed to the same agents for comparison (uppermost three bar graphs). At the end of the incubation period, ara-CTP levels were determined by HPLC as described in the text. Values are expressed as a percentage relative to the 4-hr formation of ara-CTP in low-density cells incubated with ara-C alone (32.8 pmol/10^6 cells). Each value is the mean of triplicate determinations performed on at least two separate occasions ± 1 SD.

Table 3. NTP and dNTP levels in high-density HL-60 cells

NTP (dNTP)	Intracellular level (pmol/10^6 cells) Control	Bryostatin 1
CTP	82.3 ± 11.2	108.5 ± 15.68
UTP	291.6 ± 48.5	499.8 ± 66.3*
ATP	529.5 ± 91.7	942.8 ± 119.7*
GTP	124.7 ± 15.8	158.2 ± 22.6
dCTP	1.3 ± 0.2	0.7 ± 0.1†
dTTP	3.9 ± 1.0	4.8 ± 1.2
dATP	5.3 ± 1.8	10.6 ± 3.2*
dGTP	2.4 ± 1.3	4.9 ± 1.4*

High-density HL-60 cells were exposed to 12.5 nM bryostatin 1 for 24 hr, neutralized cell extracts were obtained, and intracellular NTP and dNTP levels were determined as described in the text. Values are the means ± 1 SD for 2–5 experiments performed in triplicate.

* Significantly greater than control ($P < 0.05$).

† Significantly less than control ($P < 0.05$).

Table 4. Effects of dCTP levels on dCK activity

dCTP (mM)	dCK activity (% control) Control	Bryostatin 1
0.1	55.5 ± 7.6	54.5 ± 7.0*
1.0	14.0 ± 4.3	16.7 ± 3.3*

Enzyme was obtained from control and bryostatin 1 treated cells, and dCK activity was assayed in the presence of the designated concentrations of dCTP. Values are expressed as a percentage of control activity (obtained in the absence of dCTP) and are the means of three experiments performed in duplicate ± 1 SD. Control activities were 0.48 and 0.53 units of enzyme obtained from untreated and bryostatin 1 treated cells, respectively.

* Not significantly different from control ($P > 0.05$).

increases in dCK were noted when triphosphate concentrations mimicked those present in cells exposed to bryostatin 1 compared to values obtained when triphosphate concentrations approximated those of control cells (data not shown). However, increases in enzyme activity comparable to the 5-fold or greater potentiation of ara-CTP formation were not observed.

Because of the theoretical possibility that accumulation of toxic substances in the medium and/or exhaustion of critical nutrients might contribute to decreased ara-CTP formation in high-density cells, medium cross-over studies were carried out to address this issue. High- and low-density cells were pre-incubated for 24 hr in the presence or absence of bryostatin 1, and then exposed to ara-C for 4 hr in either (a) fresh medium (±bryostatin 1) or (b) high-density cell medium (±bryostatin 1). The major finding was that low-density cells incubated with "exhausted" medium (i.e. medium previously exposed to high-density cells for 24 hr) exhibited no decline in ara-CTP formation (data not shown). Furthermore, a 24-hr coincubation with bryostatin 1 was required to restore ara-CTP formation in high-density cells to control values, regardless of whether exposure to ara-C took place in fresh or high-density medium. In separate studies, low-density cells incubated in serum-free medium for 24 hr did not exhibit a significant decline in ara-CTP formation compared to cells maintained in complete medium (data not shown). Together these observations suggest that bryostatin 1 does not exert its effects by blocking the release of inhibitory substances or by preventing the exhaustion of critical nutrients necessary for cellular metabolism.

Because individual PK-C inhibitors may selectively

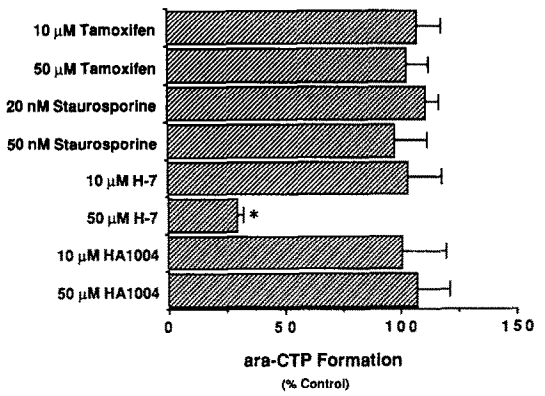


Fig. 9. Effects of PK-C inhibitors on bryostatin 1 mediated potentiation of ara-CTP formation in HL-60 cells. Following a 24-hr exposure to 12.5 nM bryostatin 1 ± the designated concentration of the PK-C inhibitors H-7, staurosporine, tamoxifen, and HA1004, high-density cells were exposed to 10 μM ara-C for 4 hr. At the end of the incubation period, ara-CTP formation was determined as previously described. Values are expressed as a percentage relative to levels achieved in bryostatin 1 treated cells exposed to ara-C in the absence of an inhibitor (31.6 pmol/10^6 cells). Values are the mean of triplicate determinations for experiments performed on 2–3 separate occasions ± 1 SD, Key: (*) significantly less than control ($P < 0.01$).

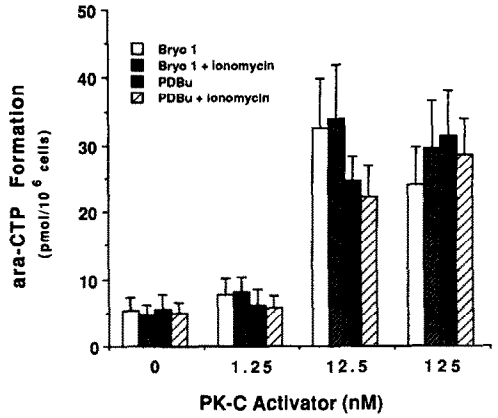


Fig. 10. Effect of Ca^{2+} ionophore on ara-CTP formation in high-density HL-60 cells exposed to bryostatin 1. High-density cells were incubated with the designated concentration of PDBu or bryostatin 1 for 24 hr in the presence or absence of 500 nM Ca^{2+} ionophore. Following a 4-hr exposure to 10 μM ara-C, neutralized cell extracts were obtained and ara-CTP formation was determined by HPLC as previously described. Values for ara-CTP formation are the means of triplicate determinations for experiments performed on two separate occasions ± 1 SD.

block specific events induced by PK-C activation [39], the effects of the PK-C inhibitors tamoxifen, staurosporine, and H-7 on bryostatin 1 mediated actions were assessed (Fig. 9). The concentrations utilized have been shown previously to inhibit PK-C activity in several cell systems, including human hematopoietic cells [40, 41]. Administration of 10–50 μM tamoxifen, 20–50 nM staurosporine, or 10 μM H-7 did not prevent the potentiation of ara-CTP formation by bryostatin 1. However, 50 μM H-7 reduced ara-CTP formation to a significant degree (e.g. 70%: $P < 0.01$). As a control, the agent HA1004, which is a considerably more potent inhibitor of cyclic-AMP-dependent protein kinase than PK-C [42], had a negligible effect on ara-CTP formation. This suggests that bryostatin 1 does not potentiate ara-C phosphorylation through a cyclic-AMP-dependent mechanism.

Finally, it has been shown previously that release of intracellular Ca^{2+} stores by Ca^{2+} ionophore may synergistically potentiate the actions of phorbol esters and other activators of PK-C [43]. Therefore, the effect of Ca^{2+} ionophore on the ability of bryostatin 1 and PDBu to potentiate ara-CTP formation was assessed (Fig. 10). Coadministration of 500 nM Ca^{2+} did not increase ara-CTP formation significantly at any of the bryostatin 1 or PDBu concentrations examined. Lower and higher concentrations of Ca^{2+} were also without effect (data not shown).

DISCUSSION

Bryostatin 1 has proven recently to be a valuable tool in probing the signal transduction process by which extracellular events trigger diverse cellular responses in a variety of cell systems [43]. The present studies now suggest that bryostatin 1, and potentially other pharmacologic agents acting through second messenger pathways, may have a significant impact on the intracellular metabolism of the nucleoside analog ara-C. While the basis for this interaction is not intuitively obvious, it may be relevant that activation of PK-C has been implicated in hematopoietic cell proliferation and differentiation [2, 5, 6]. Since these events have been shown by Nicander and Reichard [44] and others [45] to be associated with specific perturbations in deoxyribonucleotide biosynthesis, they may provide a mechanism by which activators of PK-C could alter ara-C metabolism. For example, Chiba *et al.* [46] have reported that the proliferative and maturational state of human leukemia cells may correlate with the activity of several enzymes involved in ara-C metabolism, including dCK, cytidine deaminase, and dCMP deaminase. Specifically, dCK activity is greatest in S-phase, phenotypically immature cells, whereas the reverse is true for cytidine deaminase [35, 46]. Bryostatin 1 has been shown to exert diverse effects on normal and leukemic hematopoietic cells *in vitro*, including stimulation of normal multipotent progenitors [3], inhibition [47] or promotion [5] of HL-60 cell differentiation, induction of differentiation in primary cultures of human leukemic myeloblasts [6], and inhibition of leukemic blast progenitor cell growth [7]. The nature of the effect of bryostatin 1 on pyrimidine biosynthesis (and consequently ara-C metabolism) in leukemia cells would therefore depend upon the specific response that this agent elicited.

The finding that bryostatin 1 primarily potentiated ara-C metabolism in high-density HL-60 cells displaying a reduced capacity to phosphorylate this agent was unanticipated, and has a number of implications. Although the factors responsible for the inhibition of

ara-C metabolism under high-density conditions are not well defined (e.g. cell–cell interactions, exhaustion of nutrients, accumulation of toxic or inhibitor substances), it has been established that several characteristics of such cells (e.g. deoxyribonucleotide profiles, low S-phase fraction and cloning efficiency) closely resemble those of primary cultures of human leukemic myeloblasts [48, 49]. Furthermore, the cell density of leukemic myeloblasts in their physiologic environment (e.g. bone marrow) may be 1–2 orders of magnitude higher than cells maintained under standard *in vitro* logarithmic phase conditions. The observation that serum-deprived conditions or exposure of low-density cells to high-density medium did not result in a decline in ara-C phosphorylation argues against the possibility that extracellular factors, such as inhibitory substances or nutrient deprivation, could be responsible for the observed effects. Instead, they suggest that intrinsic changes occur within cells in response to high-density conditions, and that these perturbations may be blocked by PK-C activating agents including bryostatin 1. In earlier studies, Snyder and Malick [50] examined the metabolism of ara-C and dCyd in logarithmic and stationary phase normal human fibroblasts, and found major differences between the two cell populations which they attributed to differential patterns of deamination. In contrast, we were unable to correlate alterations in ara-C metabolism in high-density HL-60 cells with an enhanced capacity for deamination. Nevertheless, it remains possible that HL-60 cells maintained under high-density conditions experience other perturbations in pyrimidine biosynthesis which inhibit ara-C metabolism, and that these may be reversed, at least in part, by bryostatin 1.

Although several potential mechanisms could account for the ability of bryostatin 1 to potentiate ara-CTP formation in high-density HL-60 cells, the available evidence is most consistent with a contributing role for enhanced phosphorylation of ara-C by dCK. As noted above, changes in ara-C metabolism mediated by bryostatin 1 could not be attributed to altered deamination, nor could they be related to decreased rates of ara-CTP dephosphorylation. The possibility that bryostatin 1 acts by potentiating ara-C transport is unlikely, in view of a previous report by White *et al.* [10] demonstrating that ara-C phosphorylation, rather than transport, represents the rate-limiting step for ara-CTP formation at extracellular ara-C concentrations in excess of 10 μM. Conversely, we noted substantial increases in ara-CTP formation in high-density cells exposed to bryostatin 1 and 100 μM ara-C. In separate studies, we were unable to demonstrate alterations in rates of ara-C transport in bryostatin 1 treated cells compared to their untreated counterparts (unpublished observations). The major remaining possibility is that bryostatin 1 increases the functional activity of dCK, either by a direct or indirect mechanism, resulting in enhanced ara-C nucleotide formation. Although we were unable to detect an increase in dCK activity in cell-free extracts obtained from bryostatin 1 treated cells, it has been shown by Liliemark and Plunkett [51] that such assays may not reflect accurately the consequences of intracellular biochemical perturbations occurring within intact cells. For example, the activity of dCK is under stringent negative and positive feedback regulatory control by intracellular levels of dCTP and dTTP, respectively [9]. It has been reported recently that dCTP exhibits a K_i of 5.9 μM with respect to dCK in leukemic cells, and that intracellular concentrations of this metabolite represent a major determinant of ara-C phosphorylation [51]. In our studies, bryostatin 1 treated cells exhibited a significant decrease (e.g. 50%) in intracellular dCTP levels and an increase in dATP and dGTP concentrations. The reduction in dCTP pool size and corresponding potentiation of ara-C metabolism were similar to that displayed by log phase murine and human leukemia cells exposed to pyrimidine antagonists such as hydroxyurea [52] and 3-deazauridine [53]. Moreover, high-density cells exposed to bryostatin 1 experienced a significant increase in intracellular ATP and UTP levels. Since both of these nucleotide triphosphates can serve as a phosphate donor for ara-C phosphorylation [9], and reduce the negative allosteric regulatory effects of dCTP on dCK, an increase in their concentrations may contribute to enhanced ara-CTP formation [54]. For example, Ives and Durham [9] demonstrated that ATP significantly reduces the inhibitory effects of dCTP on calf thymus enzyme. Similarly, White and Hines [54] have shown that a reduction in UTP concentrations substantially increases the inhibitory effects of dCTP on ara-C phosphorylation by dCK isolated from Ehrich ascites tumor cells. They also demonstrated that agents which deplete intracellular UTP pools (e.g. pyrazofuran) produce a net antagonism of ara-C phosphorylation despite a reduction in dCTP levels. In the present studies, we observed that enzyme obtained from bryostatin 1 treated cells did not exhibit reduced negative feedback regulatory control by dCTP, and therefore it is unlikely that this mechanism could account for enhanced functional activity. However, when cell-free extracts were assayed in the presence of nucleoside and deoxyribonucleoside triphosphate concentrations approximating those detected in intact cells exposed to bryostatin 1, a 2-fold increase in dCK activity was noted. Although this increase in activity was significant, it was considerably less than the 5-fold or greater increase in ara-CTP formation observed in bryostatin 1 treated cells. One possible explanation for this discrepancy is that the biochemical perturbations induced by bryostatin 1, which may contribute to the dramatic increases in ara-C phosphorylation in intact cells, may not exhibit the same capacity in broken cell preparations. It is also conceivable that other, as yet undefined, events occur in intact HL-60 exposed to bryostatin 1 that may influence the functional activity of dCK. While it is plausible that the biochemical perturbations induced by bryostatin 1 contribute to the potentiation of ara-C metabolism, it is apparent that additional studies will be required to identify alternative mechanisms by which this agent (and other PK-C activators) enhances ara-C phosphorylation in high-density HL-60 cells.

The observation that bryostatin 1 augmented ara-C phosphorylation in high-density cells despite inhibiting their proliferative capacity was also unanticipated, and may reflect the complex interplay between biochemical, cytokinetic, and differentiate-related events, as well as their indirect effects on ara-C metabolism. As noted previously, entry of cells into

S-phase has been associated with an increase in dCK activity in diverse cell systems [35, 46, 55], although this has not been a universal finding [56]. Conversely, more differentiated cells generally display a decreased S-phase fraction, reduced dCK activity, and increased cytidine deaminase activity [46]. In our system, bryostatin 1 did not induce morphological or functional evidence of HL-60 cell differentiation, an event ordinarily associated with a decline in ara-C metabolism. However, while exposure of high-density cells to bryostatin 1 resulted in a decrease in BrdUrd and dThd incorporation, a reduction in the S-phase fraction, and inhibition of clonogenicity, a significant potentiation of ara-CTP formation was observed. This suggests that administration of bryostatin 1 leads to a dissociation between certain biochemical processes (e.g. enhanced ara-C phosphorylation) and the cytokinetic events with which they are usually associated. In this regard, it is noteworthy that bryostatin 1, despite inhibiting DNA synthesis, partially restored ara-C DNA incorporation in high-density cells to control cell values, possibly as a result of the 10-fold increase in ara-CTP/dCTP ratios that it produced.

The observation that the PK-C activators PDBu and mezerein also potentiated ara-C metabolism in high-density cells supports the contention that signal transduction events may modulate pyrimidine biosynthesis in leukemic cells. It is noteworthy that whereas the PK-C inhibitor H-7 was able to block augmentation of ara-CTP formation, the inhibitors tamoxifen and staurosporine were not. The differential effect exerted by specific PK-C inhibitors has been described previously for inhibition of histamine release by mast cells [39], and has given rise to speculation that activation of different PK-C isoenzymes may be responsible for distinct cellular events [57]. A similar explanation might account for the selective effects of specific inhibitors on ara-C metabolism. Finally, in contrast to the results of earlier studies in which Ca^{2+} ionophore synergistically potentiated the ability of phorbol esters and bryostatin 1 to deliver proliferative signals to cells of lymphoid origin [43], this agent did not alter bryostatin 1 or PDBu-mediated enhancement of ara-C metabolism in HL-60 cells.

In summary, the present studies demonstrate that bryostatin 1 leads to biochemical perturbations favoring ara-C phosphorylation in HL-60 cells, particularly when the latter are maintained under high-density conditions and exhibit impaired nucleoside metabolism. Furthermore, potentiation of ara-C nucleotide formation occurs despite a net reduction in cellular proliferative capacity, a unique association that may provide a possible rationale for the combined use of these agents in an antileukemic regimen. Although it is not always possible to extrapolate results obtained from immortalized cell lines to primary human tumors, preliminary evidence from our laboratory suggests that bryostatin 1 substantially augments ara-C metabolism in at least some patient-derived human leukemic myeloblasts *in vitro*, and that a combined exposure to these agents is highly inhibitory to leukemic cell self-renewal capacity [58]. Apart from these considerations, the present studies raise the possibility that pharmacologic agents acting through second messenger pathways may modulate the metabolism of nucleoside analogs in leukemic cells, and potentially in their normal counterparts. A better understanding of these events may provide the basis for employing a novel class of PK-C activating agents to improve the antileukemic efficacy of ara-C, and perhaps other purine and pyrimidine antagonists.

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