Biochemical Pharmacology, Vol. 56, pp. 635–644, 1998.
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ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00188-9

Effect of Bryostatin 1 on Taxol-induced Apoptosis and Cytotoxicity in Human Leukemia Cells (U937)*

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ABSTRACT. We have examined the effects of the macrocyclic lactone protein kinase C (PKC) activator bryostatin 1 on taxol-induced apoptosis and inhibition of clonogenicity in the human monocytic leukemia cell line U937. Exposure of cells to bryostatin 1 (10 nM; 15 hr) after (but not before) a 6-hr incubation with 0.5 μM taxol significantly increased apoptosis and resulted in an approximately 3 log reduction in clonogenicity. Cell cycle analysis revealed that the increase in apoptotic cells following bryostatin 1 treatment occurred primarily in the population undergoing taxol-mediated G_2M arrest. The actions of bryostatin 1 were not attributable to potentiation of taxol-induced tubulin stabilization or to a reduction in the intracellular retention of taxol. Following exposure of cells to taxol, the Bcl-2 protein displayed an alteration in mobility that was not modified appreciably by bryostatin 1 treatment. The mobility shift in Bcl-2 protein from cells exposed to taxol followed by bryostatin 1 was eliminated by treatment of lysates with the protein phosphatase 2A (PP2A); the latter effect was blocked by okadaic acid. Treatment of cells with taxol followed by bryostatin 1 did not increase the amount of total Bax (compared with treatment with taxol alone), but did increase the amount of free Bax in the supernatant fraction. Finally, the ability of bryostatin 1 to potentiate taxol-induced apoptosis in U937 cells was mimicked closely by 2′-amino-3′-methoxyflavone (PD98059), a specific inhibitor of the mitogen-activated protein kinase (MAPK) kinase (MEK). Collectively, these findings indicate that bryostatin 1 increases the susceptibility of U937 cells to taxol-induced apoptosis and inhibition of clonogenicity. They also raise the possibility that this phenomenon may involve functional alterations in Bcl-2 and/or other proteins involved in regulation of the cell death pathway. BIOCHEM PHARMACOL **56**;5:635–644, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. taxol; bryostatin 1; apoptosis; leukemic cells; Bcl-2

The antineoplastic agent taxol exhibits activity against a wide variety of human cancers [1, 2]. In contrast to Vinca alkaloids (i.e. vincristine), which disrupt microtubule assembly, taxol promotes tubulin polymerization, leading to microtubule stabilization and tubulin bundling [3]. Cells exposed to taxol undergo G_2 and mitotic arrest [4]. Death ensues via apoptosis [5], a process that displays certain similarities to a phenomenon referred to as the "mitotic catastrophe" [6]. Taxol-induced apoptosis has been associated recently with alterations in the mobility of the anti-apoptotic protein Bcl-2 on SDS–PAGE gels, an event attributed to hyperphosphorylation [7]. The Bcl-2 mobility shift following exposure to taxol has been reported to be mediated through a Raf-1-related pathway [8]. The resulting post-translational modification of Bcl-2 has been postulated to antagonize its anti-apoptotic function, possibly by preventing homodimerization with other family members such as Bax [9]. However, other studies have suggested that the altered mobility of the Bcl-2 protein following exposure to microtubule-active agents is related more to cell cycle events than to apoptosis *per se* [10].

The macrocyclic lactone bryostatin 1 is an activator of the serine-threonine kinase PKC,** an enzyme involved in diverse cellular processes including cell proliferation, differentiation, and stimulus–response coupling [11]. Although bryostatin 1 mimics some of the actions of tumor-promoting phorboids such as PMA, it exhibits a distinct spectrum of activity, and, in fact, blocks those phorbol-associated actions that it does not itself induce [12]. Divergencies between the actions of bryostatin 1 and tumor-promoting phorboids may stem from differential stimulation and/or down-regulation of individual PKC

* Portions of this work were presented in preliminary form at a meeting of the American Society of Hematology, San Diego, CA, December 5–9, 1997.

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Received 24 March 1998; accepted 21 May 1998.

** *Abbreviations:* ara-C, 1-β-arabinofuranosylcytosine; ERK, extracellular receptor kinase; FBS, fetal bovine serum; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; OA, okadaic acid; PBS-T, phosphate-buffered saline-Tween; PKC, protein kinase C; PMA, 12-phorbol 13-myristate acetate; PP2A, protein phosphatase 2A; and SAPK, stress-activated protein kinase.

isoforms [13] or unique patterns of isoform translocation [14]. In preclinical studies, bryostatin 1 has shown activity against diverse tumor types *in vitro* and *in vivo* [15, 16], and Phase I trials of this compound have been completed recently in humans [17].

Previous studies from this laboratory have shown that in human leukemic cells (HL-60) unresponsive to the differentiation-inducing actions of bryostatin 1, pretreatment with this agent increases susceptibility to ara-C-induced apoptosis and results in synergistic antileukemic effects for the combination [18]. Moreover, potentiation of ara-C-induced apoptosis in HL-60 cells by bryostatin 1 is temporally associated with down-regulation of PKC activity [19]. In contrast, bryostatin 1 induces a weak differentiation response in monocytic leukemia cells (U937), and fails to increase their susceptibility to subsequently administered ara-C [20]. On the other hand, exposure of U937 cells to ara-C followed by bryostatin 1 results in a dramatic potentiation of apoptosis [20]. These findings may be analogous to earlier observations demonstrating that differentiation-inducing agents potentiate the apoptotic response to previously administered DNA damaging drugs [21–23]. The present studies were prompted by two considerations. The first stemmed from a desire to determine whether bryostatin 1 could facilitate leukemic cell apoptosis by an agent that did not primarily act through induction of DNA damage. Second, bryostatin 1 has been shown to induce serine phosphorylation of Bcl-2 in murine hematopoietic cells (32D) [24], and, more recently, we have reported that bryostatin 1 overcomes, at least in part, leukemic cell resistance to ara-C-mediated apoptosis conferred by overexpression of Bcl-2 [25]. Moreover, this process is temporally associated with Bcl-2 phosphorylation [25]. Consequently, an ancillary goal was to determine what effect bryostatin 1 might have on taxol-induced modifications in the Bcl-2 protein.

MATERIALS AND METHODS

Cells

The human monocytic leukemic cell line U937 was isolated from the peripheral blood of a patient with diffuse histiocytic lymphoma as described earlier [25]. Cells were cultured in RPMI 1640 medium supplemented with sodium pyruvate, Modified Eagle's Medium essential vitamins, L-glutamate, penicillin and streptomycin, and 10% heat-inactivated FBS (HyClone). They were maintained in a 37°, 5% CO_2, fully humidified incubator, passed twice weekly, and prepared for experimental procedures when in log phase growth (cell density $\leq 4 \times 10^5$ cells/mL).

Drugs and Chemicals

Taxol was purchased from Sigma, stored in light-protected containers at −20°, and dissolved in sterile DMSO (Sigma) before use. Bryostatin 1 was provided by the Cancer Treatment and Evaluation Program, National Institutes of Health, and stored desiccated at −20°. It was formulated in sterile DMSO, and subsequently diluted in RPMI medium so that the final concentration of DMSO was in all cases ≤0.05%. Okadaic acid (Sigma) was stored at −20° and diluted in sterile water. PP2A was purchased from Upstate Biotechnology and dissolved in dilution buffer before use according to the manufacturer's instructions.

Assessment of Apoptosis

Following drug exposures, cytocentrifuge preparations were stained with Wright-Giemsa and viewed by light microscopy to evaluate features of cellular differentiation as well as apoptosis (e.g. cell shrinkage, nuclear condensation, and formation of apoptotic bodies) as previously described [18, 19]. For the latter studies, the percentage of apoptotic cells was determined by evaluating ≥1000 cells/condition in triplicate. We have reported previously that the incidence of apoptosis as determined by these morphological criteria correlates very closely with the degree of low molecular weight DNA fragmentation assayed quantitatively by spectrofluorometry, and qualitatively with the amount of internucleosomal DNA fragmentation determined by agarose gel electrophoresis [18, 19].

Agarose Gel Electrophoresis

For qualitative assessment of internucleosomal DNA fragmentation, DNA was extracted from cell lysates following appropriate drug treatment and subjected to agarose gel electrophoresis using a technique previously described in detail [19].

Western Analysis

A minor modification of a previously described method was employed [25]. Following treatment, whole cell pellets (1×10^7 cells/condition) were washed twice in PBS, resuspended in 50 μL of PBS, lysed by the addition of 50 μL of 2× Laemmli buffer [1× = 30 mM Tris-base (pH 6.8), 2% SDS, 2.88 mM β-mercaptoethanol, 10% glycerol], and briefly sonicated. Homogenates were quantified using Coomassie protein assay reagent (Pierce). Equal amounts of protein (20 μg) were boiled for 10 min, separated by SDS–PAGE (5% stacker and 10% resolving), and electroblotted to nitrocellulose. The blots were stained in 0.1% amido black and destained in 5% acetic acid to ensure transfer and equal loading. After blocking in PBS-T (0.05%) and 5% milk for 1 hr at 22°, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody (Bcl-2, 1:1000; Bax, 1:500; Santa Cruz Biotechnology; α-tubulin, 1:1000; Calbiochem) for 4 hr at 22°. Blots were washed three times for 5 min each time in PBS-T and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories) for 1 hr at 22°. Blots were again washed three times for 5 min

each time in PBS-T and then developed by Enhanced Chemiluminescence (Pierce).

Determination of Free Bax Levels

A minor modification of a previously described technique [26] was employed to quantify free Bax levels in supernatants immunodepleted of Bcl-2- and Bcl-xL-Bax heterodimers. Following treatment, cells were washed twice with cold PBS and lysed in 0.2% NP-40 isotonic lysis buffer containing protease inhibitors [142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.2), 1 mM EGTA, 0.2% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.7 μg/mL of leupeptin] by incubation for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation at 15,000 *g* for 10 min. Two hundred micrograms of supernatant protein per condition was incubated with 1 μg monoclonal Bcl-2 antibody (Dako A/S) and polyclonal Bcl-xL antibodies (Santa Cruz Biotechnology) for 4 hr at 4°. Immunoprecipitates were then captured utilizing Dynabeads (Dynal A.S.) for another 4 hr according to the manufacturer's instructions. The heterodimer-depleted supernatants were then exposed to anti-Bax polyclonal antibody (1 μg; Santa Cruz), and the resulting immunoprecipitates containing free Bax were washed three times in 0.2% NP-40 lysis buffer. Following solubilization in SDS–PAGE sample buffer, electrophoresis of samples was carried out on 12% SDS–PAGE gels as described above. Free Bax levels were detected by immunoblotting with primary Bax antibody, and quantification of Enhanced Chemiluminescence-developed blots was performed utilizing a PhosphorImager (Molecular Dynamics).

Phosphatase Treatment

Following treatment with taxol followed by bryostatin 1, cells were lysed with 1% Triton X-100 lysis buffer (25 mM Tris base, 0.1% β-mercaptoethanol, 1% Triton X-100) and centrifuged at 12,000 *g* for 10 min at 4°. Then the supernatants were incubated in lysis buffer for 4 hr at 37° in the presence or absence of PP2A (50 units/μg protein final concentration) ± the specific protein serine/threonine phosphatase inhibitor okadaic acid (1.0 μM). Alternatively, lysates were treated with 1.5 units/mL of alkaline phosphatase (Sigma). Then SDS–PAGE immunoblot analysis was performed utilizing anti-Bcl-2 monoclonal antibody and Enhanced Chemiluminescence as described above.

Cell-Cycle Analysis

After treatment, cells were pelleted at 500 *g* and resuspended in 70% ethanol. The cell pellets were incubated on ice for 1 hr, and resuspended in 1 mL of cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/mL of RNase A, and 0.01 mg/mL of propidium iodide (all Sigma) at a concentration of 10^6 cells/mL. Samples were stored in the dark before analysis at 4° (usually within 24 hr), and analyzed on a Becton–Dickinson FACScan flow cytometer, utilizing a commercially available software program (ModFit LT 2.0; Verity Software).

Cloning Studies

A previously described method was employed [18]. Briefly, following drug treatment, cells were washed three times in drug-free RPMI medium containing 5% FBS, and cell counts were determined using a model ZBI Coulter counter. After normalization, cells were plated in 18-mm 12-well plates (Costar). Each well contained 1 mL of supplemented RPMI 1640 medium, 20% FBS, 0.3% Bacto agar (Difco), and 4×10^2 cells/condition. The plates were placed in a fully humidified, 37°, 5% CO_2 incubator for 12 days, after which colonies, consisting of groups of ≥50 cells, were scored with the aid of an Olympus model CK inverted microscope. The total number of colonies for each condition was calculated by multiplying the cell number at the end of the initial incubation period by the cloning efficiency. Values for each condition were expressed as a percentage relative to untreated controls.

[^{3}H]Taxol Uptake

Logarithmically growing U937 cells were exposed to 0.5 μM [^{3}H]taxol (Moravek Biochemicals; 10 μCi/condition) for 6 hr, after which they were washed three times in fresh medium to remove radiolabeled drug. Then the cells were resuspended in fresh medium containing 10% FBS and incubated at 37° for an additional 12 or 24 hr. At the designated interval, cells were washed with PBS, lysed in 4% SDS, and aliquots removed for protein assay and scintillation counting. Cellular accumulation of radiolabeled drug was normalized to total protein content.

Isolation of Polymerized Microtubules

To analyze the effects of bryostatin 1 on taxol-mediated tubulin polymerization, polymerized tubulin was separated from tubulin dimers as previously described [27]. Briefly, after treatment, cells were washed with PBS twice and lysed with 0.5% NP-40 buffer [20 mM Tris base (pH 6.8), 1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40]. The lysates were then centrifuged at 12,000 *g* for 10 min at 4°. The resulting pellets, containing polymerized tubulin, were resuspended in 1× Laemmli buffer, and protein was separated on 10% SDS–PAGE gels as described above.

Statistical Analysis

The significance of differences between experimental conditions was determined utilizing the two-tailed Student's *t*-test. Analysis of drug synergism and antagonism was determined by Median Dose Analysis utilizing a commer-

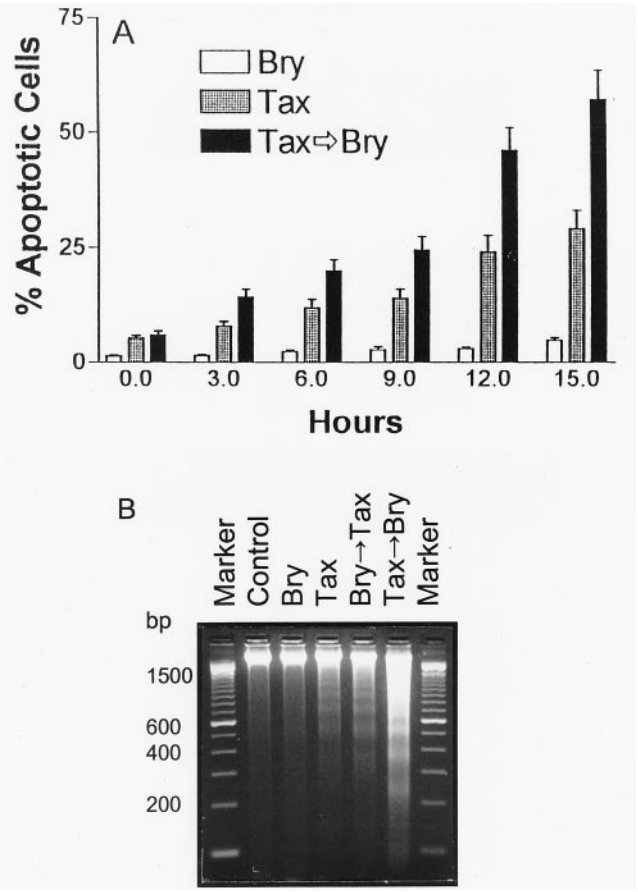

FIG. 1. Effects of bryostatin 1 on taxol-induced apoptosis in U937 cells. (A) Following a 6-hr exposure to 0.5 μM taxol, U937 cells were washed and resuspended in either drug-free medium or medium containing 10 nM bryostatin 1. At the indicated intervals, cytospin preparations were obtained, and the percentage of apoptotic cells was determined, as described in Materials and Methods. Values represent the means ± SD for three separate experiments performed in triplicate. (B) After a 6-hr exposure to 0.5 μM taxol, cells were resuspended in either drug-free medium or medium containing 10 nM bryostatin 1 for an additional 15 hr. Then the cells were pelleted, and DNA obtained from lysates was subjected to agarose gel electrophoresis. To assess sequence-dependent effects, DNA obtained from cells exposed to the sequence bryostatin 1 → taxol (lane 5) was compared with DNA isolated from cells exposed to taxol → bryostatin 1 (lane 6). The results of a representative study are shown; two additional experiments yielded similar results.

cially available software program (Biosoft), as previously described [28].

RESULTS

The effects of sequential exposure of cells to taxol followed by bryostatin 1 on apoptosis are shown in Fig. 1. In these studies, cells were exposed to 0.5 μM taxol for 6 hr, washed, and then incubated in the presence or absence of 10 nM bryostatin 1 for various intervals prior to evaluation. A progressive increase in the percentage of cells exhibiting apoptotic features was observed over time in cells treated with taxol alone, which reached ~30% after 15 hr of incubation. Bryostatin 1 treatment alone exerted minor effects at this and earlier intervals (e.g. ~5% apoptotic cells at 15 hr). However, when taxol-treated cells were incubated subsequently with bryostatin 1, the percentage of apoptotic cells increased markedly, and exhibited nearly a two-fold increase in apoptosis compared with cells exposed to taxol alone. Interestingly, when cells were preincubated with bryostatin 1 (10 nM; 15 hr) prior to exposure to taxol (0.5 μM; 6 hr), a significant potentiation of apoptosis was not observed (i.e. 12.2 ± 2.1 vs 11.0 ± 1.7; $P \geq 0.05$; data not shown). Parallel results were obtained when internucleosomal DNA fragmentation was monitored by agarose gel electrophoresis (Fig. 1B). It can be seen that the extent of internucleosomal fragmentation was considerably greater for the sequence taxol → bryostatin 1 (lane 6) compared with the sequence bryostatin 1 → taxol (lane 5).

TABLE 1. Effect of taxol with or without bryostatin 1 on cell-cycle distribution

	Percentage of cells			
Condition	Ap (sub-G_0G_1)	G_0G_1	S	G_2M
Control	1.3 ± 1.0	41.2 ± 3.5	47.8 ± 4.1	8.9 ± 2.1
Bry	3.2 ± 1.4	44.3 ± 4.9	42.4 ± 6.2	9.7 ± 2.8
Taxol	28.6 ± 5.9	19.1 ± 4.1	10.7 ± 3.9	44.6 ± 6.8
Tax → Bry	47.8 ± 6.3	25.6 ± 4.6	9.7 ± 2.8	18.9 ± 3.1

Cells were exposed to (a) taxol (0.5 μM; 6 hr), washed free of drug, and suspended in drug-free medium for an additional 15 hr; (b) bryostatin 1 (10 nM) for 15 hr; or (c) the sequence taxol (6 hr) followed by bryostatin 1 (15 hr). At the end of the incubation period, cells were fixed in ethanol and stained with propidium iodide, as described in Materials and Methods. Cell-cycle analysis was performed by flow cytometry utilizing a commercially available software program (ModFit). Values for the percentage of cells in the indicated phase of the cell cycle represent the means ± SD for three replicate experiments.

The cell cycle characteristics of cells exposed to taxol and bryostatin 1 are shown in Table 1. Cells incubated with 10 nM bryostatin 1 for 15 hr displayed a small decrease in the S-phase and a minor increase in the G_0/G_1 fraction, accompanied by the appearance of a small sub-G_0/G_1, apoptotic fraction (Ap). Taxol-treated cells exhibited a marked G_2M arrest, as has been reported previously [5], as well as a modest increase in the subdiploid apoptotic peak. When cells were exposed sequentially to taxol followed by bryostatin 1, a substantial increase in the hypodiploid, apoptotic component was noted, accompanied by a marked decrease in the G_2M fraction. This indicates that bryostatin 1 promotes apoptosis in cells arrested in G_2M following taxol treatment.

Bryostatin 1 and taxol concentration–response characteristics are shown in Fig. 2. A modest increase in taxol-mediated apoptosis was observed at a bryostatin 1 concentration of 1 nM, and a major increase at 10 nM (Fig. 2A). Increasing the bryostatin 1 concentration to 100 nM resulted in a very small further augmentation of taxol-induced cell death. These effects are qualitatively similar to those seen in the case of ara-C [19]. Potentiation of taxol-mediated cell death was noted over a wide range of

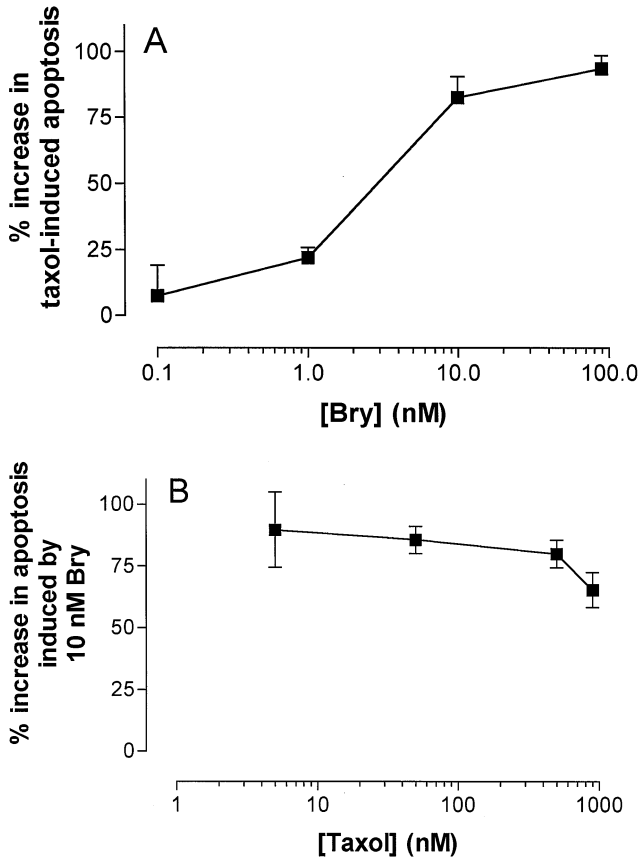

FIG. 2. Bryostatin 1 and taxol concentration–response characteristics. (A) Logarithmically growing U937 cells were exposed to 0.5 μM taxol for 6 hr, washed, and resuspended in medium containing the designated concentration of bryostatin 1 for 15 hr, after which the percentage of apoptotic cells was determined as described in the text. Values are expressed as a percent increase in apoptosis compared with cells incubated in bryostatin 1-free medium, and represent the means ± SD for three separate experiments performed in duplicate. (B) Cells were exposed to the indicated concentration of taxol for 6 hr, washed, and resuspended in medium containing 10 nM bryostatin 1 for 15 hr, after which the percentage of apoptotic cells was determined as above. Values are expressed as the percent increase in apoptosis in relation to cells exposed to the same concentration of taxol without bryostatin 1 post-treatment. Values represent the means ± SD for three separate experiments performed in duplicate.

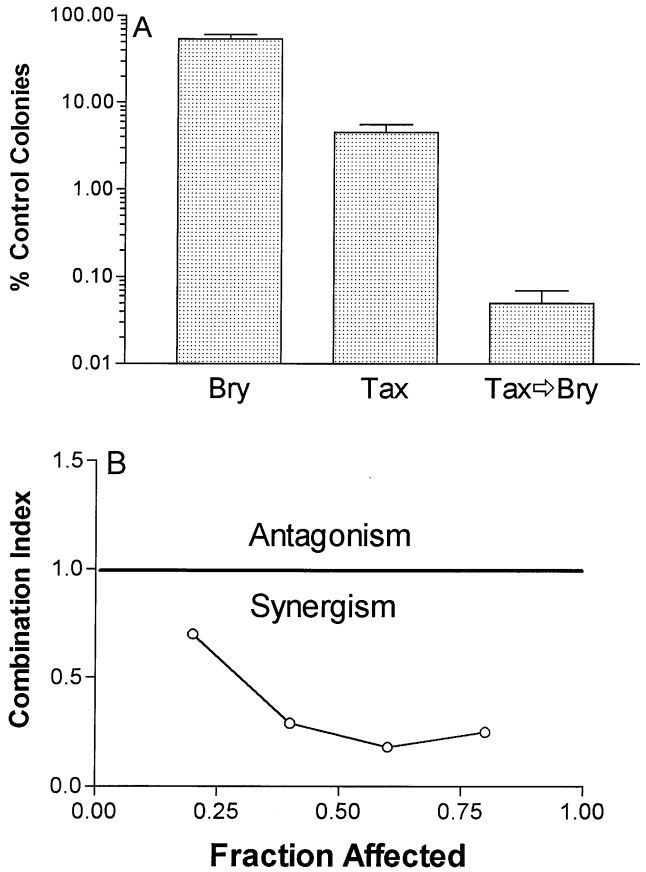

FIG. 3. Effects of taxol and bryostatin 1 on the clonogenicity of U937 cells. (A) Cells were exposed to taxol (0.5 μM; 6 hr), bryostatin 1 (10 nM; 15 hr), or the sequence taxol → bryostatin 1, washed free of drugs, and plated in soft agar as described in the text. Colonies, consisting of groups of ≥50 cells, were scored after 12 days. Values, expressed as a percentage of control cell colony formation, represent the means ± SD for three separate experiments performed in triplicate. (B) Isobologram analysis of the bryostatin 1/taxol interaction. Cells were exposed to taxol (0.05 to 10 μM) and bryostatin 1 (0.5 to 100 nM) alone and in fixed rations, cloned in soft agar, and Median Dose Analysis was performed as described in the text. Combination Index values <1 denote a synergistic interaction.

taxol concentrations, although a slight decline was observed at the highest concentration (1 μM) (Fig. 2B).

The biological consequences of exposure to these agents was assessed utilizing clonogenic assays (Fig. 3A). Bryostatin 1 alone (10 nM, 15 hr) resulted in a modest decrease in clonogenicity (~50%). When cells were exposed to 0.5 μM taxol for 6 hr, washed, and incubated in drug-free medium for an additional 15 hr, the reduction in clonogenicity was >1 log. However, when cells were exposed to taxol followed by bryostatin 1, colony formation was reduced by over 3 logs (e.g. to ~0.06%). In contrast, pre-exposure of cells to bryostatin 1 before taxol reduced colony formation by only ~50% (compared with taxol alone), and the survival fraction was significantly less than that observed for cells exposed to the sequence taxol followed by bryostatin 1 ($P \leq 0.002$; data not shown). Then Median Dose Analysis was conducted to characterize the interaction between these agents over a range of concentrations (taxol: 0.05 to 10 μM; bryostatin 1: 0.5 to 100 nM; Fig. 3B). Combination index (CI) values for the sequential administration of taxol followed by bryostatin 1 were consistently less than 1.0, denoting a synergistic interaction [28].

To determine whether bryostatin 1 might act by enhancing taxol-mediated microtubular polymerization, α-tubulin was examined by Western analysis following treatment of cells with taxol ± bryostatin 1 (Fig. 4A). Protein isolated from cell pellets, which contain polymerized microtubules, displayed clear evidence of microtubular stabilization following taxol exposure. Bryostatin 1 alone failed to modify tubulin polymerization, nor did it enhance the microtu-

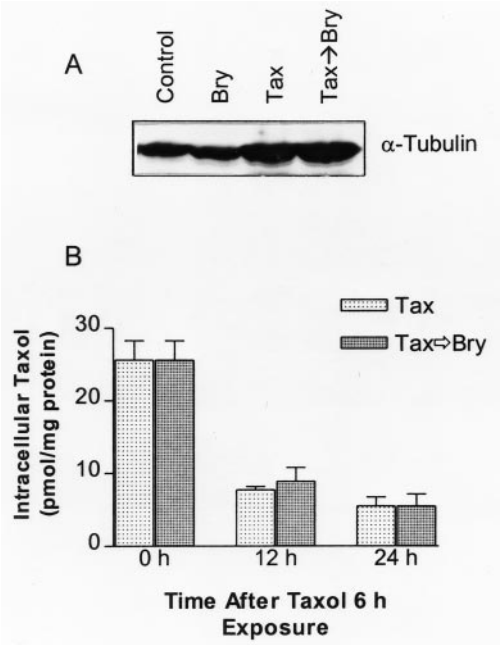

FIG. 4. Effect of bryostatin 1 on taxol-mediated tubulin polymerization and intracellular taxol retention. (A) Following treatment with taxol and bryostatin 1 as above, polymerized microtubules from the cell pellet were obtained as described in Materials and Methods. Proteins (25 μg/condition) were separated on SDS–PAGE gels and probed with anti-tubulin antibody; the blots were developed by Enhanced Chemiluminescence. The results of a representative study are shown; two additional experiments yielded equivalent results. (B) Cells were exposed to [^{3}H]taxol (10 μM; 6 hr), after which they were washed free of radiolabeled drug and resuspended in either drug-free medium or medium containing 10 nM bryostatin 1. At the designated intervals, aliquots of the cell suspension were removed, the cells were lysed, and radioactivity was determined by scintillography. Results represent the means ± SD for three separate experiments performed in duplicate.

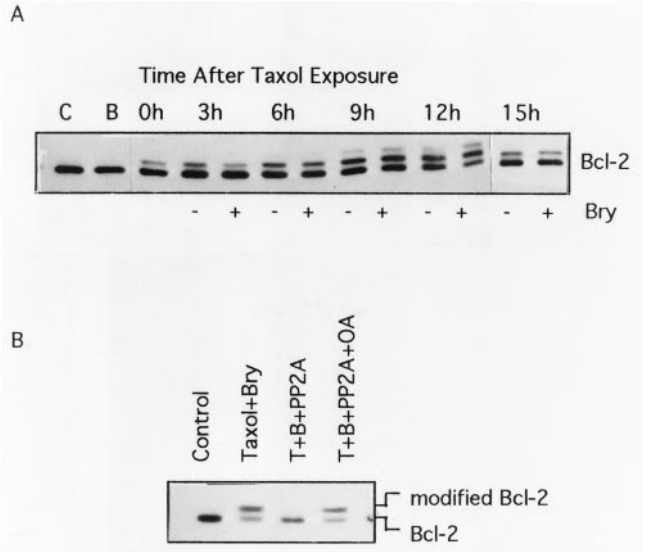

FIG. 5. Effect of bryostatin 1 on taxol-mediated alterations in Bcl-2 mobility. (A) Following treatment with taxol (0.5 μM; 6 hr), cells were washed free of drug and resuspended in either fresh medium (−) or medium containing 10 nM bryostatin 1 (+) for the designated interval. At the indicated time period, cells were lysed, and the protein was extracted separated on SDS–PAGE gels, and probed with anti-Bcl-2 antibody as described in the text. For comparison, blots corresponding to untreated cells (designated C) and to cells exposed to bryostatin 1 (designated B) (each at the 15-hr interval) are shown in the first two lanes. Two replicate experiments yielded equivalent results. (B) Following treatment of cells with taxol followed by bryostatin 1, extracts were exposed to the PP2A ± the phosphatase inhibitor okadaic acid (OA) as described in the text. Then western analysis was performed to monitor expression/mobility of the Bcl-2 protein. The result shown is representative of two additional experiments.

bule-stabilizing actions of taxol. Bryostatin 1 also did not modify the appearance of tubulin dimers isolated from the supernatant fraction (data not shown). In view of reports that bryostatin 1 induces phosphorylation of the P-glycoprotein [29], the effect of bryostatin 1 on intracellular [^{3}H]taxol retention was also examined (Fig. 4B). The results of these studies demonstrated that bryostatin 1 did not increase significantly the intracellular accumulation of taxol 12 or 24 hr after a 6-hr exposure to this agent. Together, these findings indicate that potentiation of taxol-induced cell death by bryostatin 1 involves factors other than enhancement of microtubular stabilization or potentiation of intracellular drug retention.

Several previous studies have demonstrated that treatment of cells with taxol, as well as various agents that interfere with microtubular assembly/disassembly, leads to alterations in the mobility of the Bcl-2 protein [8], an event that has been postulated to reflect phosphorylation [7]. Because bryostatin 1 has been reported to induce phosphorylation of the Bcl-2 protein in normal and neoplastic cells [24, 25], Western analysis was employed to examine the combined effect of these agents on Bcl-2 mobility (Fig. 5A). Bryostatin 1 (10 nM; 24 hr) by itself did not have a marked effect on mobility of the Bcl-2 protein after 15 hr of incubation (Fig. 5A; lane 2) or at earlier intervals (not shown). When cells were exposed to 0.5 μM taxol for 6 hr, washed, and incubated in drug-free medium for intervals of up to 15 hr, one or more slowly migrating species were noted, which were most pronounced at the 12-hr time point. When taxol-treated cells were exposed subsequently to bryostatin 1, changes in mobility of the Bcl-2 band were modest or absent. Treatment of extracts from cells exposed to the sequence taxol followed by bryostatin 1 with PP2A (or alkaline phosphatase; not shown) eliminated the slowly migrating Bcl-2 species, and the former effect was abrogated by the specific serine/threonine phosphatase inhibitor okadaic acid (Fig. 5B). Next, the functional consequences of drug treatment were examined with respect to effects on the pro-apoptotic protein Bax (Fig. 6). Previous studies have shown that taxol-mediated phosphorylation of Bcl-2 is associated with decreased Bax heterodimerization [9]. Treatment of cells with taxol, bryostatin 1, or the sequence taxol followed by bryostatin 1 did not result in changes in levels of total Bax (Fig. 6A). However, when extracts were

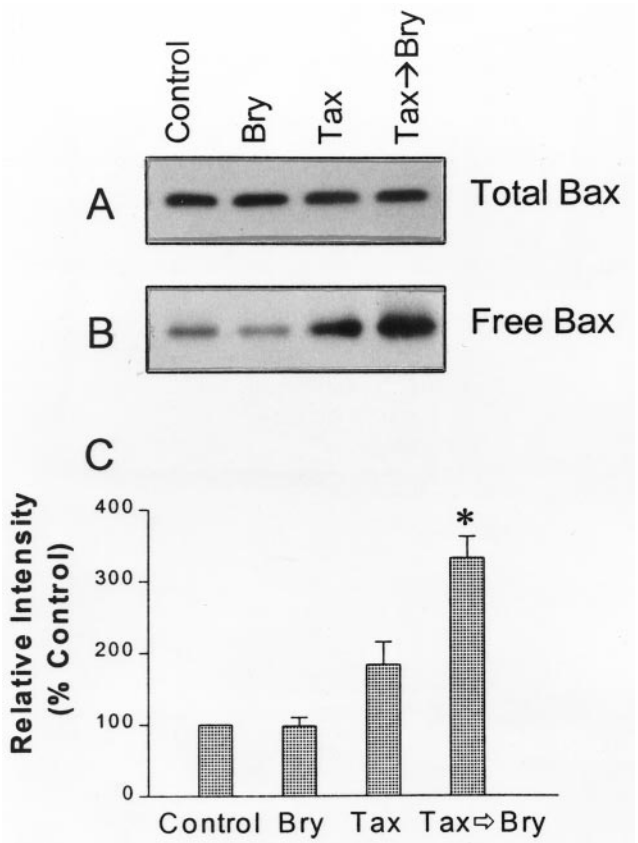

FIG. 6. Effect of bryostatin 1 and taxol on total and free Bax levels in U937 cells. (A) Following treatment with taxol (10 μM; 6 hr), bryostatin 1 (10 nM; 15 hr), or the sequence taxol → bryostatin 1, western analysis was employed to monitor expression of total Bax as described in the text. Each lane contains 20 μg of protein. Two additional experiments yielded equivalent results. (B) Following the same treatment, lysates were immunodepleted of Bax/Bcl-2 and Bax/Bcl-xL heterodimers, and immunoprecipitated free Bax was subjected to western analysis as described in Materials and Methods. Each lane contains material immunoprecipitated from supernatant containing 200 μg of protein. Free Bax levels were quantified using a PhosphorImager, and the mean intensity for each condition ± SD, representing three separate experiments, is shown in (C). *Significantly greater than the value for taxol alone, $P \leq 0.05$.

immunodepleted of Bcl-2- and Bcl-xL-Bax heterodimers, levels of free Bax were significantly higher following exposure to taxol and bryostatin 1 compared with those observed in cells treated with taxol alone (Fig. 6, B and C).

Lastly, the effects of the MEK (and MAPK) inhibitor 2′-amino-3′-methoxyflavone (PD98059) [30] were examined with respect to modulation of taxol-mediated apoptosis. Exposure to PD98059 (10 μM) for 15 hr induced apoptosis in a small percentage of cells (~6–7%) but increased taxol-mediated cell death substantially (e.g. from ~27 to ~58%) (Fig. 7). This increase was comparable to that associated with bryostatin 1 administration. Moreover, the addition of PD98059 to bryostatin 1 resulted in further potentiation of cell death in cells sequentially exposed to taxol followed by bryostatin 1 (e.g. from 56 to 80%; $P \leq$

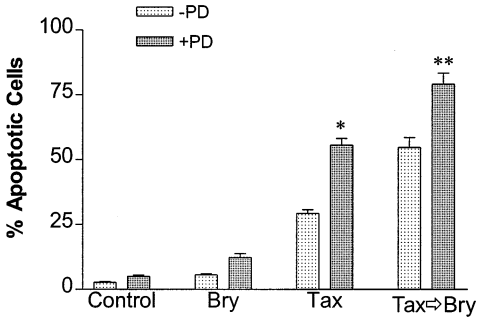

FIG. 7. Effect of the MEK inhibitor PD98059 on taxol and bryostatin 1-mediated apoptosis. Cells were exposed to taxol (0.5 μM; 6 hr), bryostatin 1 (10 nM; 15 hr), or the sequence taxol → bryostatin 1 in the presence or absence of 10 μM PD98059 (PD). At the end of the incubation period, the percentage of apoptotic cells was determined as described in Materials and Methods. Results represent the means ± SD for three separate experiments. Values marked with an asterisk(s) were significantly greater than values obtained for cells incubated in medium lacking PD: *$P \leq 0.002$ and **$P \leq 0.02$.

0.05). In separate studies, administration of PD98059 failed to modify the mobility shift in the Bcl-2 protein observed in cells exposed to taxol and bryostatin 1 (data not shown). Thus, pharmacologic interruption of the MAPK pathway mimicked the actions of the PKC down-regulator bryostatin 1 in lowering the threshold for taxol-mediated leukemic cell death.

DISCUSSION

Previous studies have shown that exposure of leukemic cells to taxol induces apoptosis in a concentration- and schedule-dependent manner, and that this process is inhibitable by Bcl-2 [5, 26]. While it is presumed that the lethal actions of taxol stem from microtubular stabilization and induction of G_2M arrest, the specific events that trigger the cell death process remain unknown. In a recent article, we reported that in the U937 monocytic leukemia cell line, sequential exposure of cells to the antimetabolite ara-C followed by bryostatin 1 led to potentiation of apoptosis [20]. Such results are consistent with previous observations that exposure of leukemic cells to DNA-damaging agents followed by a differentiation stimulus leads to promotion of cell death [21–23]. The present results now indicate that bryostatin 1, which has been reported to induce maturation in some human leukemia cells, including the U937 cell line [31], similarly enhances the lethal actions of taxol, an agent that does not directly induce DNA fragmentation. However, it has been demonstrated recently that at the concentrations employed in the current study, bryostatin 1 is a very weak inducer of leukemic cell differentiation, and is, in fact, impaired in its ability to induce G_1 arrest-associated events, including induction of $p21^{WAF1/CIP1}$, inhibition of cdk2, and dephosphorylation of the retinoblastoma protein (pRB) [32]. Therefore, it is unclear whether potentiation of taxol-induced apoptosis by bryostatin 1 stems from its

(limited) capacity to promote maturation (a phenomenon that might conceivably be extended to other, more effective differentiation inducers), or instead results from other bryostatin 1-associated actions (e.g. PKC down-regulation). Studies designed to address this issue are currently underway.

The finding that potentiation of taxol-induced apoptosis by bryostatin 1 is schedule dependent bears resemblance to previous results from this and other laboratories in which DNA-damaging agents were examined. For example, it has been shown that preincubation of human leukemia cells (HL-60) with the PKC activator PMA may not enhance, and may, in fact, inhibit, apoptosis induced by certain chemotherapeutic drugs (e.g. topoisomerase inhibitors) [33]. Analogously, we have reported that pretreatment of U937 cells with bryostatin 1 (in contrast to post-treatment) does not increase their susceptibility to ara-C-mediated apoptosis [20]. This would suggest that in the U937 cell line, prior initiation of cell damage (e.g. DNA fragmentation in the case of ara-C, or stabilization of microtubules in the case of taxol) is a prerequisite for lowering of the apoptotic threshold by bryostatin 1. It is also noteworthy that the reduction in clonogenicity induced by sequential administration of taxol followed by bryostatin 1 (i.e. $\geq$3 logs) exceeded the extent of apoptosis, at least at the 24-hr time interval. A lack of concordance between the degree of apoptosis and clonogenic survival has been described previously in CHO cells treated with aphidicolin [34]; alternatively, this phenomenon could reflect the contribution of nonapoptotic forms of cell death (e.g. multinucleated giant cell formation), as has been reported in cells exposed to topoisomerase inhibitors [35]. Furthermore, since the formation of colonies consisting of $\geq$50 cells requires at least six cell divisions, it is conceivable that slowing of the cell cycle traverse by taxol and bryostatin 1 could reduce clonogenicity in an apoptosis-independent manner. Lastly, preferential sensitivity of self-renewing, clonogenic cells to the taxol/bryostatin 1 combination could account for the dramatic reduction in clonogenicity following exposure to these agents.

It is important to distinguish between direct effects of bryostatin 1 on taxol action versus indirect effects related to modulation of the cell death threshold. The ability of taxol to induce tubulin stabilization is well documented [3]; it is theoretically possible that bryostatin 1 might act by enhancing this capacity. However, the results shown in Fig. 4 render this mechanism unlikely. Bryostatin 1 has also been shown to induce phosphorylation of the P-glycoprotein efflux pump (of which taxol is a substrate [36]), although it has not been found to be capable of reversing the multidrug-resistant phenotype [29]. The failure of bryostatin 1 to enhance the intracellular retention of taxol rules out the possibility that decreased efflux underlies the observed potentiation of apoptosis. Collectively, these observations are most consistent with the concept that augmentation of taxol-related apoptosis by bryostatin 1 stems from a lowering of the cell death threshold.

The protein product of the p26 Bcl-2 proto-oncogene (Bcl-2) has been shown to protect cells from the lethal actions of diverse antineoplastic agents, including taxol [36]. One of the mechanisms by which Bcl-2 opposes cell death may involve heterodimerization to the pro-apoptotic protein Bax [37], potentially antagonizing early apoptotic events such as loss of mitochondrial membrane potential and/or cytoplasmic release of cytochrome *c* [38]. Recently, several studies have demonstrated that taxol and other microtubule-active agents lead to altered mobility of the Bcl-2 protein [8, 39], which has been attributed by some to protein phosphorylation [7]. In at least one study, putatively phosphorylated Bcl-2 exhibited decreased heterodimerization with Bax [9]. On the other hand, alterations in Bcl-2 mobility following exposure of cells to taxol or other microtubule-active agents have been related by others to mitotic arrest rather than to a specific loss of Bcl-2 function [10]. Interestingly, while bryostatin 1 has been shown to induce Bcl-2 phosphorylation in normal murine hematopoietic cells, this action was associated with enhanced, rather than impaired, survival [24]. Using mutant murine NSE/N1.H7 cells expressing a Bcl-2 construct lacking the serine 70 phosphorylation site, this same group demonstrated that Bcl-2 phosphorylation was required for bryostatin 1 to antagonize interleukin-3 deprivation-induced apoptosis [40]. Interestingly, Bcl-2 phosphorylation was also implicated in protecting cells against VP-16-induced cell death. The differential actions of bryostatin 1 in that study [40] and in the present study may reflect cell-type-specific responses, or may reflect processes unrelated to Bcl-2 phosphorylation status. In either case, it is clear that bryostatin 1 does not protect U937 cells from the lethal actions of taxol; instead, it exerts the opposite effect. It is important to note that in a very recent report by our group, bryostatin 1 pretreatment restored, at least in part, the ability of Bcl-2-overexpressing HL-60 cells to activate the apoptotic protease cascade in response to ara-C, and that this phenomenon was accompanied by Bcl-2 phosphorylation [25]. These results differ in some respects from those of the present study in that subsequent exposure of taxol-treated U937 cells with bryostatin 1 did not exert a major effect on the mobility of the Bcl-2 protein. This difference may stem from cell-type-specific factors, or, alternatively, differential responses of ectopic versus endogenous Bcl-2 protein. The possibility that bryostatin 1 induced other, qualitative changes in Bcl-2 function that antagonized its protective effects also cannot be excluded. In this context, okadaic acid has been shown to induce post-translational modifications in the Bcl-2 protein that are unassociated with altered mobility [41]. Lastly, while subsequent exposure of taxol-treated cells to bryostatin 1 did not alter total levels of the Bax protein, it did lead to an increase in free Bax levels (relative to cells exposed to taxol alone). This raises the possibility that one of the putative modulatory effects of bryostatin 1 on the Bcl-2 protein involves interference with Bax heterodimerization, although the notion that increased levels of free Bax repre-

sent a consequence, rather than a cause, of enhanced cell death cannot be excluded at this time.

There is considerable evidence that PKC activity has the net effect of antagonizing drug-induced apoptosis. In the case of the antimetabolite ara-C, the PKC inhibitor staurosporine has been shown to promote leukemic cell death [42]. Chronic exposure to bryostatin 1, which is associated with significant PKC down-regulation [43], exerts a similar effect [19]. Conversely, acute exposure to bryostatin 1 or to the synthetic diacylglycerol DiC_8 (both of which increase PKC activity) protects cells from ara-C-mediated apoptosis [19]. Analogously, taxol-mediated apoptosis has been shown to be potentiated in HL-60 cells by staurosporine and inhibited by the PKC activator phorbol dibutyrate [44]. It is therefore tempting to postulate that interruption of a cytoprotective PKC-related pathway (e.g. by PKC inhibition, in the case of staurosporine, or down-regulation, in the case of bryostatin 1) may be responsible for the observed potentiation of taxol-induced apoptosis. However, the downstream targets of PKC that permit cells to escape taxol-induced apoptosis remain to be elucidated. In this regard, considerable attention has focused on the opposing roles of stress (e.g. SAPK/JNK) versus survival (e.g. MAPK/ERK) pathways in the regulation of cell death [45]. We have reported previously that the relative outputs of the SAPK versus the MAPK pathway may play a major role in determining cell survival following ceramide or sphingosine exposure [46]. More recent findings indicate that bryostatin 1-mediated inhibition of PKC and the resultant prevention of MAPK activation by ara-C may represent a critical determinant of leukemic cell death (Jarvis WD, Dent P and Grant S, unpublished observations). Thus, interruption of the PKC cascade, and down-regulation of one of its downstream targets, MAPK [47], may lower the threshold for leukemic cell death in response to cytotoxic agents such as ara-C and taxol. In support of this concept, treatment of U937 cells with PD98059, a highly specific inhibitor of the MAPK kinase, MEK [30], mimicked the actions of bryostatin 1 in promoting taxol-induced apoptosis. While definitive proof that bryostatin 1 acts by blocking the cytoprotective MAPK pathway is currently lacking, the present observations are potentially consistent with this notion. If such is indeed the case, it will be important to identify distal downstream targets of MAPK that might be involved more directly in cytoprotective actions. In this regard, the transcription factor NF-κB, which has been shown to inhibit cytotoxic drug-induced apoptosis [48], represents a plausible candidate. Studies addressing this issue are currently underway.

In summary, the present findings demonstrated that bryostatin 1 potentiates taxol-induced apoptosis in human leukemia cells in a schedule-dependent manner, and that the sequence taxol followed by bryostatin 1 results in a striking reduction in leukemic cell clonogenicity. They also demonstrated that incubation of taxol-treated cells with bryostatin 1 is associated with increases in free Bax levels but not with alterations in Bcl-2 mobility, and that the potentiation of taxol-mediated cell death by bryostatin 1 is mimicked by an agent (PD98059) known to inhibit MEK/MAPK activity. In view of the recent introduction of bryostatin 1 into clinical trials in humans [17], additional preclinical evaluation of this drug combination appears warranted.

This work was supported by awards CA 63753 and 66990 from the National Cancer Institute and a Translational Research Award from the Leukemia Society of America (6405-97). P. D. is supported by a "V" Foundation Award, and an institutional American Cancer Society Award (IN-105V).

References

1. Rowinsky EK, Update on the antitumor activity of paclitaxel in clinical trials. *Ann Pharmacother* **28:** S18–S22, 1993.
2. Rowinski EK, Onetto N, Canetta RM and Arbuck SG, Taxol: The first of the taxanes, an important new class of antitumor agents. *Semin Oncol* **18:** 646–662, 1992.
3. Kumar N, Taxol-induced polymerization of purified tubulin. Mechanism of action. *J Biol Chem* **256:** 10435–10441, 1981.
4. Manfredi JJ and Horwitz SB, Taxol: An antimitotic agent with a new mechanism of action. *Pharmacol Ther* **25:** 83–125, 1984.
5. Bhalla K, Ibrado AM, Tourkina E, Tang CQ, Mahoney ME and Huang Y, Taxol induces internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia cells. *Leukemia* **7:** 563–568, 1993.
6. Shi L, Nishioka WK, Th'ng J, Bradbury EM, Lichfield DW and Greenberg AH, Premature $p34^{cdc2}$ activation required for apoptosis. *Science* **263:** 1143–1145, 1994.
7. Haldar S, Nilamani J and Croce CM, Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci USA* **92:** 4507–4511, 1995.
8. Blagosklonny MV, Giannakakou P, El-Deiry WS, Kingston DGI, Higgs PI, Neckers L and Fojo T, Raf-1/bcl-2 phosphorylation: A step from microtubule damage to cell death. *Cancer Res* **57:** 130–135, 1997.
9. Haldar S, Chintapalli J and Croce CM, Taxol induces *bcl-2* phosphorylation and death of prostate cancer cells. *Cancer Res* **56:** 1253–1255, 1996.
10. Schandl CA, Priest DG and Willingham MC, Phosphorylation of BCL-2 is a cell cycle-regulated event. *Cell Pharmacol* **3:** 367–372, 1996.
11. Nishizuka Y, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258:** 607–614, 1992.
12. 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 cells HL-60. *Proc Natl Acad Sci USA* **83:** 1334–1338, 1986.
13. Szallasi Z, Smith CB, Pettit GR and Blumberg PM, Differential regulation of protein kinase C isozymes by bryostatin 1 and phorbol 12-myristate 13-acetate in NIH 3T3 fibroblasts. *J Biol Chem* **269:** 2118–2124, 1994.
14. Hocevar BA and Fields AP, Selective translocation of β_{II}-protein kinase C to the nucleus of human promyelocytic (HL-60) leukemia cells. *J Biol Chem* **266:** 28–33, 1991.
15. Lilly M, Brown C, Pettit G and Kraft A, Bryostatin 1: A potential anti-leukemic agent for chronic myelomonocytic leukemia. *Leukemia* **5:** 283–287, 1991.
16. Hornung RL, Pearson JW, Beckwith M and Longo DL, Preclinical evaluation of bryostatin as an anticancer agent

against several murine tumor cell lines: *In vitro* versus *in vivo* activity. *Cancer Res* **52:** 101–107, 1992.
17. Scheid C, Prendiville J, Jayson G, Crowther D, Fox B, Pettit GR and Stern PL, Immunomodulation in patients receiving intravenous bryostatin 1 in a phase I clinical study: Comparison with effects of bryostatin 1 on lymphocyte function *in vitro*. *Cancer Immunol Immunother* **39:** 223–230, 1994.
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 DNA. *Cancer Res* **52:** 6270–6278, 1992.
19. Jarvis WD, Gewirtz D, Povirk L, Turner A, Traylor R, Pettit GR and Grant S, Effect of bryostatin 1 and other activators of protein kinase C on 1-β-D-arabinofuranosylcytosine-induced apoptosis in HL-60 human promyelocytic leukemia cells. *Biochem Pharmacol* **47:** 839–852, 1994.
20. Grant S, Turner A, Freemerman A, Wang J, Kramer L and Jarvis WD, Modulation of PKC activity and calcium-sensitive isoform expression in human myeloid leukemia cells: Relationship to differentiation and ara-C-induced apoptosis. *Exp Cell Res* **228:** 65–75, 1996.
21. Huang Y and Waxman S, Enhanced apoptosis in leukemia cells following treatment with combination fluoropyrimidines and differentiation inducers. *Mol Cell Differ* **2:** 83–100, 1994.
22. Studzinski GP, Reddy KB, Hill HZ and Bhandal AK, Potentiation of 1-β-D arabinofuranosylcytosine cytotoxicity to HL-60 cells by 1,25-dihydroxyvitamin D_3 correlates with the rate of maturation of DNA replication intermediates. *Cancer Res* **51:** 3451–3455, 1991.
23. Bhatia U, Traganos F and Darzynkciewicz Z, Induction of cell differentiation potentiates apoptosis triggered by prior exposure to DNA-damaging agents. *Cell Growth Differ* **6:** 937–944, 1995.
24. May WS, Tyler PG, Ito T, Armstrong DK, Qatsha KA and Davidson NE, Interleukin-3 and bryostatin-1 mediate hyperphosphorylation of BCL-2α in association with apoptosis. *J Biol Chem* **269:** 26865–26870, 1994.
25. Wang S, Vrana JA, Bartimole T, Freemerman AJ, Jarvis WD, Kramer LB, Krystal G, Dent P and Grant S, Agents that down-regulate or inhibit protein kinase C circumvent resistance to 1-β-D-arabinofuranosylcytosine-induced apoptosis in human leukemia cells overexpressing Bcl-2. *Mol Pharmacol* **52:** 1000–1009, 1997.
26. Ibrado AM, Liu L and Bhalla K, Bcl-xL overexpression inhibits progression of molecular events leading to paclitaxel-induced apoptosis of human acute myeloid leukemia. *Cancer Res* **52:** 1109–1115, 1997.
27. Blagosklonny MV, Schulte TW, Nguyen P, Mimnaugh EG, Trepel J and Neckers L, Taxol induction of $p21^{WAF1}$ and p53 requires c-*raf*-1. *Cancer Res* **55:** 4623–4626, 1995.
28. 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.
29. Scala S, Dickstein B, Regis J, Szallasi Z, Blumberg PM and Bates SE, Bryostatin 1 affect P-glycoprotein phosphorylation but not function in multidrug-resistant human breast cancer cells. *Clin Cancer Res* **1:** 1581–1587, 1995.
30. Pumiglia KM and Decker SJ, Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. *Proc Natl Acad Sci USA* **94:** 448–452, 1997.
31. Asiedu C, Biggs J, Lilly M and Kraft AS, Inhibition of leukemic cell growth by the protein kinase C activator bryostatin 1 correlates with the dephosphorylation of cyclin-dependent kinase 2. *Cancer Res* **55:** 3716–3720, 1995.
32. Vrana J, Saunders AM, Chellapan SP and Grant S, Divergent effects of bryostatin 1 and PMA on cell cycle arrest and maturation in human myelomonocytic leukemia cells (U937). *Differentiation* **63:** 33–42, 1998.
33. Solary E, Bertrand R and Pommier Y, Differential induction of apoptosis in differentiating HL-60 cells to apoptosis by DNA topoisomerase inhibitors. *Blood* **81:** 1359–1368, 1993.
34. Yin DX and Schimke RT, *BCL-2* expression delays drug-induced apoptosis but does not increase clonogenic survival after drug treatment in HeLa cells. *Cancer Res* **55:** 4922–4928, 1995.
35. Lock RB and Stribinskience L, Dual modes of cell death induced by etoposide in human epithelial tumor cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. *Cancer Res* **56:** 4006–4012, 1996.
36. Huang Y, Ibrado AM, Reed JC, Bullock G, Ray S, Tang C and Bhalla K, Co-expression of several molecular mechanisms of multidrug resistance and their significance for paclitaxel cytotoxicity in human AML HL-60 cells. *Leukemia* **11:** 253–257, 1997.
37. Oltavi ZN, Milliman C and Korsmeyer S, Bcl-2 heterodimerized *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* **74:** 609–619, 1993.
38. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng T-I, Jones DP and Wang X, Prevention of apoptosis by Bcl-2: Release of cytochrome *c* from mitochondria blocked. *Science* **275:** 1129–1132, 1997.
39. Blagosklonny MV, Schulte T, Nguyen P, Trepel J and Neckers LM, Taxol-induced apoptosis and phosphorylation of Bcl-2 protein involves c-Raf-1 and represents a novel c-Raf-1 signal transduction pathway. *Cancer Res* **56:** 1851–1854, 1996.
40. Ito T, Deng X, Carr B and May WS, Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* **272:** 11671–11673, 1997.
41. Sheikh MS, Garcia M, Zhan Q, Liu Y and Fornace AJ Jr, Cell cycle-independent regulation of $p21^{Waf1/Cip1}$ and retinoblastoma protein during okadaic acid-induced apoptosis is coupled with induction of Bax protein in human breast carcinoma cells. *Cell Growth Differ* **7:** 1599–1607, 1996.
42. Grant S, Turner A, Bartimole TM, Nelms P, Joe V and Jarvis WD, Modulation of 1-β-D-arabinofuranosylcytosine-induced apoptosis in human promyelocytic leukemia cells by staurosporine and other inhibitors of protein kinase C. *Oncol Res* **6:** 87–99, 1994.
43. Lee H-W, Smith L, Pettit GR, Vinitsky A and Smith JB, Ubiquitination of protein kinase C-α and degradation by the proteasome. *J Biol Chem* **271:** 20973–20976, 1996.
44. Ponnathpur V, Ibrado AM, Reed JC, Ray S, Huang Y, Self S, Bullock G, Nawabi A and Bhalla K, Effects of modulators of protein kinases on taxol-induced apoptosis of human leukemic cells possessing disparate levels of p26BCL-2 protein. *Clin Cancer Res* **1:** 1399–1406, 1995.
45. Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME, Opposing effects of ERK and JNK p38 MAP kinases on apoptosis. *Science* **270:** 1326–1331, 1995.
46. Jarvis WD, Fornari FA, Auer KL, Freemerman AJ, Szabo E, Birrer MJ, Johnson CR, Barbour SE, Dent P and Grant S, Coordinate regulation of stress- and mitogen-activated protein kinases in the apoptotic actions of ceramide and sphingosine. *Mol Pharmacol* **52:** 935–947, 1997.
47. Cai H, Smola U, Wixler V, Eisenmann-Tappe I, Diaz-Meco MT, Moscat J, Rapp U and Cooper GM, Role of diglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase. *Mol Cell Biol* **17:** 732–741, 1997.
48. Wang C-Y, Mayo MW and Baldwin AS Jr, TNF- and cancer therapy-induced apoptosis: Potentiation by NF-κB. *Science* **274:** 784–787, 1996.