Protein Kinase C-dependent Activation of the Tumor Necrosis Factor Receptor-mediated Extrinsic Cell Death Pathway Underlies Enhanced Apoptosis in Human Myeloid Leukemia Cells Exposed to Bryostatin 1 and Flavopiridol

Leanne Cartee, Sonia C. Maggio, Rebecca Smith, Heidi M. Sankala, Paul Dent, and Steven Grant

Abstract

Interactions between the protein kinase C activator bryostatin 1 and the cyclin-dependent kinase (CDK) inhibitor flavopiridol (FP) have been examined in human myeloid leukemia cells (U937 and HL-60). Previous studies have demonstrated synergistic induction of apoptosis in leukemic cells exposed to the potent differentiation-inducer phorbol 12-myristate 13-acetate (PMA) in conjunction with FP [L. Cartee et al., Cancer Res., 61: 2583–2591, 2001]. Although bryostatin 1 (10 nM) is a very weak inducer of differentiation compared with PMA in these cells, coadministration of a minimally toxic concentration of FP (100 nM) did not promote bryostatin 1-related maturation but instead caused a marked increase in mitochondrial damage (e.g., cytochrome c release; loss of ΔΨm), caspase activation, poly(ADP-ribose) polymerase cleavage, and apoptosis. Bryostatin 1/FP-induced apoptosis was significantly diminished in cells ectopically expressing dominant-negative Fas-associated death domain or by coadministration of tumor necrosis factor (TNF)-α soluble receptors, implicating the extrinsic pathway in bryostatin 1/FP actions. Enhanced apoptosis in bryostatin 1/FP-treated cells was accompanied by down-regulation of Mcl-1 and a sustained increase in TNF-α release. The selective protein kinase C inhibitor GFX blocked TNF-α and cytochrome c release in bryostatin 1/FP-treated cells and attenuated apoptosis.

Finally, coadministration of bryostatin 1 (or PMA) with FP induced a marked increase in apoptosis in U937 cells ectopically expressing an NH2-terminal phosphorylation loop-deleted Bcl-2 protein, which are otherwise highly resistant to FP-mediated lethality. Taken together, these findings suggest that synergistic induction of apoptosis by bryostatin 1 and FP does not stem from disruption of the leukemic cell maturation process but instead results from enhanced release of TNF-α and activation of the extrinsic apoptotic cascade, culminating in cell death.

Introduction

Apoptosis is a highly conserved process of cell suicide that involves the activation of a family of cysteine proteases referred to as caspases (1). Apoptosis proceeds via two distinct biochemical caspase cascades, designated as the intrinsic and extrinsic pathways (2, 3). The intrinsic or mitochondrial pathway is triggered by ionizing radiation or cytotoxic drugs and is initiated by cyt c release from the mitochondria (4). When cyt c is released into the cytoplasm, it forms a multimeric protein complex with apoptosis activating factor-1 and procaspase-9 referred to as the apoptosome. The apoptosome then cleaves and activates downstream effector caspases such as caspase-3, caspase-6, and caspase-7, which promote cell death by initiating DNA fragmentation and intracellular protein degradation (5). In contrast, the extrinsic apoptotic pathway is receptor mediated and involves the recruitment of procaspase-8 to the DISC of cell surface death receptors (6, 7). The DISC contains the FADD, an adapter protein with a death domain effector sequence that binds to a homologous sequence within procaspase-8. After ligand binding and recruitment by FADD, procaspase-8 oligomerization triggers its autoactivation by self-cleavage. Caspase-8 then activates the downstream effector caspases via type I or type II receptor-mediated pathways (8, 9).

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3 The abbreviations used are: cyt c, cytochrome c; BisM, bisindolylmaleimide; BO-FMK, BOC-Asp(Ome)-fluoromethylketone; CDK, cyclin-dependent kinase; CDK1, CDK inhibitor; CHX, cycloheximide; CI, combination index; DIOC6, 3,3-diethyloxacarbocyanine; DISC, death-inducing signaling complex; DN, dominant-negative; FADD, Fas-associated death domain; FP, flavopiridol; MEK, MAP/ERK kinase; PARP, poly(ADP-ribose)polymerase; PI, propidium iodide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TBS-T, Tris-buffered saline with 0.1% Tween 20; TNF-α, tumor necrosis factor α; TNFR, TNF receptor; TNFSF13, TNF soluble receptor; VP16, etoposide; pRb, retinoblastoma protein.
FP is a CDKI that interacts with the adenine-binding pocket of CDKs at concentrations ~100 nM for CDKs 1, 2, 4, and 6, and 300 nM for CDK 7, the CDK-activating kinase (10). FP has also been found to inhibit the CDK9/T1 transcription elongation factor b complex and thereby act as a transcriptional repressor (11). FP induces either G1 and/or G2-M cell cycle-arrest (12) and is an effective inducer of apoptosis in human leukemia cells (13). Recent findings indicate that in U937 human leukemia cells, FP triggers cell death through mitochondrial release of cyt c and independently of receptor-mediated procaspase-8 activation (14). Although FP by itself has been shown to induce maturation, e.g., in small cell lung cancer cells (15), it does not necessarily potentiate the actions of established differentiation-inducing agents. For example, when combined with PMA, a PKC activator and tumor promoter that potently induces terminal differentiation in human myeloid leukemia cells (e.g., HL-60 and U937; Refs. 16, 17), FP blocks rather than promotes maturation (18). Instead, it interferes with a variety of differentiation-related events, particularly induction of the CDKI p21cip1, which culminates in caspase activation and cell death. Similar interactions have been observed between FP and the histone deacetylase inhibitor sodium butyrate (19), which also induces leukemic cell maturation (20). Interestingly, whereas PMA potently induces leukemic cell maturation (16, 17), it has also been observed to induce apoptosis in U937 and K52 cells by triggering TNF-α production and release (21). In this context, we have recently reported that in human leukemia cells, PMA/FP-mediated apoptosis is characterized by early cyt c redistribution, followed by a subsequent PKC-dependent increase in TNF-α transcription and release, culminating in procaspase-8 activation and potentiation of cell death (22). Collectively, these findings suggest that FP disrupts PMA-mediated maturation in human leukemia cells, causing cells to engage an alternative TNF-α-related cell death program.

The macrocyclic lactone bryostatin 1, like PMA, also binds to and activates PKC (23). However, its spectrum of activity is distinctly different from that of the phorbol esters. This phenomenon may reflect differential effects of bryostatin 1 on PKC isoform activation (24), nuclear translocation (25), or PKC down-regulation (26). Bryostatin 1, unlike PMA, is a nontumor promoter (27) and is a considerably weaker inducer of leukemic cell differentiation than PMA (28, 29). In fact, in human promyelocytic leukemia cells (HL-60), bryostatin 1 has been shown to block PMA-related differentiation induction (30). The mechanism underlying the relative inability of bryostatin 1 to trigger leukemic cell differentiation is not known but is likely related to its failure to induce the CDKI p21cip1, dephosphorylation of pRb, and arrest of cells in G0-G1 (27).

As recently postulated (18), the synergistic induction of apoptosis in human leukemia cells by FP and PMA represents a consequence of disruption of the maturation process, then FP should not interact synergistically with bryostatin 1, which is poor differentiation-inducer. Furthermore, because arrest of cells in G0-G1 is essential to the maturation process (31), the possibility arises that a CDKI such as FP might restore the ability of bryostatin 1 to initiate leukemic cell differentiation. To test these possibilities, the effects of FP have been examined with respect to the response of human leukemia cells (U937 and HL-60) to bryostatin 1. Here, we report that coadministration of FP with bryostatin 1, as in the case of PMA, does not promote leukemic cell maturation. Instead, combined treatment with FP and bryostatin 1 is associated with increased mitochondrial damage (e.g., cyt c release) resulting from the PKC-dependent release of TNF-α, culminating in enhanced caspase activation and the synergistic induction of apoptosis. Moreover, administration of bryostatin 1 circumvents resistance to FP-mediated apoptosis conferred by ectopic expression of an NH2-terminal phosphorylation loop-deleted Bcl-2 protein, suggesting that perturbations in Bcl-2 phosphorylation status are unlikely to play a role in this phenomenon. Taken together, these findings argue strongly against the notion that FP promotes apoptosis in human myeloid leukemia cells exposed to PKC activators by disrupting the differentiation program but instead indicate that this phenomenon primarily results from enhanced release of TNF-α and subsequent engagement of the extrinsic apoptotic cascade.

Materials and Methods

Drugs and Reagents. Bryostatin 1 was supplied by the National Cancer Institute Cancer Treatment and Evaluation Program courtesy of Dr. Anthony Murgo. Bryostatin 1 was supplied as a sterile powder and was dissolved in DMSO as a 10–4 μ stock solution and stored at −20°C. PMA (Sigma Chemical Company, St. Louis, MO) was dissolved in DMSO and 10–3 μ stock solution and aliquots stored at −20°C. FP was kindly provided by Dr. Edward Sausville (Developmental Therapeutics Program, National Cancer Institute). FP was formulated in DMSO and 10−2 μ stock solutions stored at −20°C. BD-FMK was purchased from Enzyme Systems Products (Livermore, CA). The mitochondrial dye DiOC6 was purchased from Molecular Probes (Eugene, OR). The human TNF soluble receptor I/Fc chimera (R&D Systems, Inc., Minneapolis, MN) and human TNF-α (Calbiochem, San Diego, CA) were dissolved in sterile PBS containing 0.5% FBS and aliquots stored at −80°C. U0126 (Alexis Biochemicals, San Diego, CA) was dissolved in DMSO and a 10 μ stock solution was stored at −20°C. BisM (Calbiochem) was formulated in DMSO, and a 1-m stock solution was stored at −20°C. The pancaspase inhibitor BD-FMK was purchased from Enzyme Systems Products and prepared in DMSO according to instructions provided by the manufacturer. Geneticin and Hygromycin were obtained from Life Technologies, Inc. (Grand Island, NY). The primary antibodies for PARP, caspase 8, and pRb were purchased from Transduction Laboratories (Lexington, KY). The primary antibodies for PARP, caspase 8, and pRb were purchased from Biomol Research Laboratories (Plymouth Meeting, PA), Alexis Biochemicals, and Biosource Interna-

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England Nuclear (Boston, MA). Annexin V–FITC was purchased from BD PharMingen. All other chemicals or reagents were from Sigma Chemical Company.

**Cell Culture.** The myelomonocytic leukemia cell line U937 was obtained from American Type Culture Collection. HL-60 cells were derived from a patient with acute promyelocytic leukemia as described previously (32). All cells were cultured in suspension in phenol red-free RPMI 1640 (Life Technologies, Inc.) and 10% (v/v) FCS (Hyclone, Logan, UT) and maintained in a humidified atmosphere (95% air/5% CO₂) at 37°C. To obtain FADD-DN (33)-expressing cell lines, U937 cells were transfected by electroporation with pcDNA vector 3.1 (Invitrogen, Carlsbad, CA) containing the appropriate coding region sequences as described previously (34). These cells designated as U937/FADD-DN were maintained along with their empty vector counterparts (U937/pcDNA3.1) as described above in the presence of Geneticin (400 μg/ml). U937 cells ectopically expressing the NH₂-terminal phosphorylation loop-deleted (residues 32–80) Bcl-2 protein were generated along with their empty vector counterparts (U937/psFFV) as described previously (35). Transfectant cell lines were maintained in selection media (Hygromycin or Geneticin; 400 μg/ml) and transferred to selection-free media 24 h before experimentation. All experiments were performed on cells in logarithmic phase.

**Assessment of Drug Interactions.** Interactions between PMA and bryostatin 1 with FP were characterized as previously described by Chou and Talalay (36). The percentage of apoptotic cells was assessed after exposure to PMA or bryostatin 1 (1–10 μM) alone or in combination with FP (100 μM) treatment alone for 24 h using a nonconstant dose ratio design. The CI was determined using Median Dose Effect Analysis Software (Elsevier Biosoft, Cambridge, United Kingdom). CI values < 1 correspond to synergistic drug interactions.

**Western Analysis.** Equal quantities of protein (25 μg/condition) were separated by SDS-PAGE [PARP (8.0%) and procaspase 8 (12%)] and electroblotted onto nitrocellulose. Blots were blocked in TBS-T/5% milk, washed twice with TBS-T, and incubated overnight at 4°C with the appropriate primary antibody. The blots were incubated with a horseradish-peroxidase-conjugated secondary antibody diluted in TBS-T/5% milk. After incubation, blots were developed by enhanced chemiluminescence exposure to Kodak X-OMAT film and reprobed with antibodies directed against actin to control for equal loading of protein.

**Morphological Assessment of Apoptosis.** Leukemic cells were evaluated for apoptosis by morphological assessment of Wright and Giemsa-stained cytospin preparations. Cells were transferred to slides by cytocentrifugation, fixed, stained, and evaluated under light microscopy for treatment-induced apoptosis. Apoptotic cells were identified by classical morphological features (i.e., nuclear condensation, cell shrinkage, and formation of apoptotic bodies). Five or more randomly selected fields, encompassing a total of 500 cells/slide, were evaluated to determine the percentage of apoptotic cells for each treatment condition.

**Cyt c Release.** The assay for cyt c release was performed as previously described by Single et al. (37) with modifications. At designated times after drug treatment, 4 × 10⁶ cells were washed with PBS and resuspended in 50 μl of assay buffer (75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 1 mM EDTA, and 250 mM sucrose) containing digitonin (700 μg/ml). Three min after addition of digitonin assay buffer, cells were pelleted by centrifugation. The supernatants were transferred to tubes containing 5 × Laemmli buffer (50 μl) and boiled for 5 min. Proteins were separated by SDS-PAGE as described above.

**Flow Cytometric Analysis of Apoptosis in Annexin V/PI-treated Cells.** After drug treatments, cells (1 × 10⁶) were costained with annexin V conjugated to FITC and PI per instructions provided by the manufacturer (BD PharMingen). Samples were analyzed using a Becton Dickinson FACScan flow cytometer. The percentage of apoptotic cells was determined by flow cytometric analysis. This assay is based on the premise that phosphatidylserine externalization to the outer leaflet of the plasma membrane is an early event in apoptosis (38) and allows for discrimination between apoptotic (annexin V positive, A⁻) and necrotic (PI positive, PI⁺) cells.

**Assessment of Mitochondrial Membrane Potential.** At designated intervals, 1-ml aliquots of cells (2 × 10⁶) were harvested and incubated with 40 nM DiOC₆ for 15 min at 25°C as described previously (39). Samples were analyzed using a Becton Dickinson FACScan flow cytometer. Results were expressed as the percentage of total cells exhibiting loss of mitochondrial membrane potential (Δψₘ), manifested by a reduction in DiOC₆ uptake relative to untreated controls. Data acquisition and analysis was performed using CellQuest Software (Becton Dickinson, Mansfield, MA).

**ELISA.** U937 cells (2 × 10⁶) were exposed to drug treatment at early (2–9 h) and late (18 h) time points after which the cells were pelleted. Cell culture supernatants were collected and flash frozen for storage at −80°C. The supernatants were tested for the presence of TNF-α by the ELISA OptEIA kit (BD PharMingen) according to the protocol provided. Data were normalized to live cell number (2 × 10⁶) to reflect the toxicity of various treatments relative to untreated controls.

**Cell Adherence.** Differentiation was monitored by determining the percentage of U937 cells exhibiting plastic adhesion after drug treatment. The percentage of adherent cells was determined as previously described in detail (28).

**Statistical Analysis.** The significance of differences between experimental conditions was determined using the Student’s t test for unpaired observations.

**Results**

**Cotreatment with Bryostatin 1 and FP Results in the Synergistic Induction of Apoptosis in U937 Human Leukemia Cells.** To characterize interactions between bryostatin 1 and FP, induction of apoptosis was assessed in U937 cells exposed for 24 h to a range of bryostatin 1 concentrations (i.e., 0.5–50 nM) in the presence or absence 100 nM FP (Fig. 1). In each case, coadministration of FP with bryostatin 1, exposures that were minimally toxic by themselves, resulted in a marked increase in cell death. CI values for these combinations were <1 for each dose of bryostatin 1.
tested, indicating synergistic responses (Table 1). When identical concentrations of PMA (0.5–50 nM) were evaluated, the degree of synergism was somewhat greater than that observed in the case of bryostatin 1. In addition, when bryostatin 1 and PMA (10 nM each) were coadministered with 100 nM FP, the extent of apoptosis was comparable with that observed in the case of bryostatin 1. These findings indicate that administering FP with bryostatin 1 leads to a significant increase in apoptosis in human myeloid leukemia cells, results that are qualitatively similar to those previously reported in the case of PMA (18, 22). They also demonstrate that in contrast to its ability to block PMA-related maturation (30), bryostatin 1 does not interfere with potentiation of PMA-mediated apoptosis by FP.

**Potentiation of PKC Activator-associated Apoptosis by FP Occurs Independently of Differentiation Induction.**

Previous studies have shown that bryostatin 1 is a considerably weaker differentiation-inducing agent than PMA (28). As shown in Fig. 2A, prolonged exposure of U937 cells to PMA markedly increased cell adherence (e.g., to ~80% of cells). In contrast, bryostatin was an ineffective inducer of maturation, and in fact when coadministered, it blocked PMA-mediated apoptosis by FP. However, coadministration of FP resulted in a marked reduction of Mcl-1 expression in control as well as bryostatin 1-treated cells (Fig. 3). How- ever, coadministration of FP resulted in a marked reduction of Mcl-1 expression in control as well as bryostatin 1-treated cells. Moreover, down-regulation of Mcl-1 was also observed in the presence of the pan-caspase inhibitor BD-FMK.

![Fig. 1](image1.png)  
**Fig. 1.** Characterization of the interactions between bryostatin 1 (B) and FP. The percentage of apoptotic cells was monitored after a 24-h exposure of U937 cells to a range of (B) concentrations (0.5–50 nM) alone and in combination with 100 nM FP (B/FP). Apoptosis was determined by morphological assessment of cytospin preparations as described in “Materials and Methods.” Values represent the means for five or more separate experiments ± SE.

![Fig. 2](image2.png)  
**Fig. 2.** A, PMA and bryostatin 1 exert differential effects on the maturation response of U937 human leukemia cells. Maturation was assessed by monitoring the percentage of adherent cells and cells remaining in suspension after a 72-h exposure to 10 nM bryostatin 1 ± 10 nM PMA as described in “Materials and Methods.” Values represent the means for three separate experiments ± SE. B, FP blocks PMA-related maturation and fails to promote bryostatin 1-induced differentiation in the presence of caspase inhibition. U937 cells were exposed to 10 nM bryostatin 1 or PMA for 12 h ± FP (100 nM) in the presence or absence of the pan-caspase inhibitor BD-FMK (Inh; 25 μM). At the end of the incubation period, the percentage of adherent cells was determined as above. Values represent the means for more than or equal to four separate experiments ± SE.

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<th>Dose (nM)</th>
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* A nonconstant dose ratio design was used to assess interactions between varying concentrations of bryostatin (B) 1 or PMA (P) (0.5–50 nM each) and a fixed concentration of FP (100 nM) after a simultaneous 24-h drug exposure. At the end of the incubation period, the percentage of apoptotic cells was determined by examining Wright Giemsa-stained cytospin preparations. CI values were determined by median dose effect analysis as described in “Materials and Methods” (36). CI values < 1.0 correspond to synergistic drug interactions. Results are representative of more than or equal to three separate experiments.

actions (Fig. 2A), consistent with the results of earlier reports (30). Interestingly, FP blocked PMA-induced cell adherence in the presence of the pan-caspase inhibitor BD-FMK (Fig. 2B). Significantly, FP failed to potentiate bryostatin 1-mediated maturation, nor did administration of BD-FMK enhance cell adherence in cells exposed to bryostatin 1 alone or to the combination of bryostatin 1 and FP. Taken together, these findings indicate that the failure of U937 cells exposed to FP and either PMA or bryostatin 1 to undergo maturation does not simply represent a consequence of cell death. They also suggest that enhanced apoptosis in bryostatin 1/FP-treated cells does not stem from disruption of the differentiation process.

**Exposure of U937 Cells to Bryostatin 1 or PMA in Conjunction with FP Is Associated with Down-Regulation of the Antiapoptotic Protein Mcl-1.** Mcl-1 is an antiapoptotic protein which increases in abundance as leukemic cells undergo maturation (40). Consistent with its relative inability to induce differentiation in U937 cells, treatment with bryostatin 1 did not lead to increased Mcl-1 expression (Fig. 3). However, coadministration of FP resulted in a marked reduction in Mcl-1 expression in control as well as bryostatin 1-treated cells. Moreover, down-regulation of Mcl-1 was also observed in the presence of the pan-caspase inhibitor BD-FMK.
(Fig. 3), indicating that this process does not reflect caspase-mediated degradation. In separate studies, treatment with PMA resulted in up-regulation of Mcl-1, consistent with earlier reports (40), whereas coadministration of FP blocked this response (data not shown). These findings raise the possibility that down-regulation of Mcl-1 may contribute to enhanced apoptosis in cells exposed to FP in combination with bryostatin 1 or PMA.

**U937 Cells Ectopically Expressing FADD-DN Exhibit Resistance to Bryostatin 1/FP but not VP-16-induced Apoptosis.** To evaluate the possibility that receptor-related events might be implicated in bryostatin 1/FP-induced apoptosis, studies were conducted in U937 cells ectopically expressing FADD-DN, which interferes with caspase-8 activation at the level of the DISC. Consistent with earlier results (22), U937/FADD-DN cells exhibited marked resistance to TNF/CHX-induced apoptosis but remained fully sensitive to cell death induced by VP-16, which is primarily mediated through the intrinsic mitochondrial-dependent pathway (Fig. 4). Notably, bryostatin 1/FP-induced lethality was significantly attenuated in U937/FADD-DN cells compared with their empty vector counterparts based on both morphological features (i.e., 25 ± 1% versus 60 ± 2%; *P* < 0.001) as well as annexin V/PI positivity (*P* < 0.005 versus control). Taken together, these data suggest that receptor-mediated events, particularly FADD activation, are critical for bryostatin 1/FP-induced apoptosis in leukemic cells.

**Enhanced Apoptosis in Bryostatin 1/FP-treated Cells Is Associated with a Delayed PKC-dependent Increase in TNF-α Release.** In view of evidence that bryostatin 1 induces TNF-α release (41, 42), TNF-α protein levels were monitored by ELISA after treatment of U937 cells with bryostatin 1 or FP (Fig. 5A). Although FP by itself did not induce TNF-α, bryostatin 1 triggered an initial increase in TNF-α levels at 6 h (e.g., 7-fold greater than control values), which returned toward baseline values over the ensuing 6 h (e.g., to only ~2-fold greater than controls at 12 h). In contrast, the combination of bryostatin 1/FP triggered a more modest increase in TNF-α levels at 6 h (e.g., 3-fold greater than controls), but this increase was sustained (e.g., 5-fold greater than basal levels after 12 h). At the latter interval, TNF-α levels were significantly higher than those observed with bryostatin 1 alone (*P* < 0.04). Furthermore, the specific PKC inhibitor BisM (2 μM) completely abrogated TNF-α release triggered by the combination of FP and bryostatin 1, consistent with the results of previous studies linking TNF-α release to PKC activation (43, 44).

To assess the functional significance of these events, U937 cells were exposed to bryostatin 1/FP in the presence or absence of TNFSRs, which block TNF-mediated lethality (45). As anticipated, addition of TNFSRs essentially abrogated apoptosis induced by TNF-α + CHX (Fig. 5B). Moreover, TNFSRs significantly attenuated bryostatin 1/FP-mediated cell death i.e., from 62 ± 2% to 24 ± 4% at 24 h (*P* < 0.02). Western blot analysis confirmed that TNFSRs largely blocked caspase-8 and PARP cleavage in TNF/CHX-treated cells as well as in cells exposed to bryostatin 1/FP, although to a slightly lesser extent (Fig. 5C). Collectively, these findings argue strongly that synergistic induction of apoptosis by bryostatin 1 and FP proceeds, at least in part, through TNF receptor-mediated events.

**Interruption of PKC- and MEK1/2-related Signaling Events Markedly Attenuate Bryostatin 1/FP-mediated Apoptosis in U937 Cells.** In view of evidence that the enhanced release of TNF-α in bryostatin 1/FP-treated cells is PKC dependent (Fig. 6A), the functional significance of this event with respect to lethality was assessed using the selective PKC antagonist BisM. Parallel studies were performed using the MEK1/2 inhibitor U0126 (46). Administration of BisM (2 μM) or U0126 (20 μM) with bryostatin 1/FP resulted in a substantial reduction in cell death, reflected by morphological criteria (Fig. 6A) or diminished annexin V/PI positivity (data not shown; *P* < 0.001 in each case). Similar reductions were observed when loss of mitochondrial membrane potential (∆Ψm) and PARP degradation were monitored (Fig. 6, B and C). Collectively, these and the preceding find-
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The absence of TNFSRs as described above. Each lane contained 20 ng of protein; blots were reprobed with actin to ensure equivalent protein loading and transfer. An additional experiment yielded equivalent results.

**Fig. 6.** Coadministration of PKC (BisM) or MEK1 inhibitors (U0126) significantly attenuates bryostatin 1/FP-mediated lethality in U937 cells. In each case (A-C), cells were exposed for 24 h to 10 nm bryostatin (B), 100 nm FP, or 10 nm bryostatin + 100 nm FP (B/FP) in the presence or absence of BisM (2 μM) or U0126 (20 μM). A, the percentage of apoptotic cells was quantified by morphological assessment of Wright-Giemsa-stained cytospin preparations as described in "Materials and Methods." The symbol + denotes values significantly less than those obtained after combined treatment with bryostatin and FP; P < 0.001 in each case. B, loss of mitochondrial membrane potential (ΔΨm) was monitored in U937 cells after treatment as described above. Values represent the percentage of cells exhibiting low uptake of the fluorochrome DiOC6. The symbols + and ++ denote values significantly less than those obtained in cells exposed to the combination of bryostatin 1 and FP; + = P < 0.04; ++ = P < 0.01, respectively. For A and B, values represent the means for three separate experiments ± SE. C, Western blot analysis of PARP degradation after 24-h exposure of U937 cells to the agents as described above. CF corresponds to a M, 85,000 PARP cleavage fragment. Blots were reprobed with actin to ensure equivalent protein loading and transfer. An additional experiment yielded similar results.

**Fig. 5.** A, ELISA was used to quantify TNF-α protein released into the media after 6 or 12 h of drug treatment as described in "Materials and Methods." U937 cells (2 × 10⁶ cell/condition) were exposed to 10 nm bryostatin 1 (B), 100 nm FP, or bryostatin 1 + 100 nm FP (B/FP) in the presence or absence of TNFSRs (SR) 100 ng/ml for 24 h. As controls, cells were also exposed to TNF (10 ng/ml) + CHX (1 μM, T/C) ± TNFSRs (SR) 100 ng/ml for 24 h. Apoptotic cells were quantified by morphological analysis of Wright-Giemsa-stained cytospin preparations as described in "Materials and Methods." Values represent the means for five or more separate experiments ± SE. The symbol + denotes values that are significantly less than those obtained in the absence of TNFSRs (P < 0.02). C, Western analysis of procaspase-8 and PARP cleavage in U937 cells after 24-h exposure to bryostatin 1/FP or TNF/cycloheximide (TC) in the presence or absence of TNFSRs as described above. Each lane contained 20 μg of protein; blots were reprobed with actin to ensure equivalent loading and transfer of protein. An additional experiment yielded equivalent results.

Findings (Fig. 5) support the notion that the PKC/MEK1/2-dependent release of TNF-α plays a major role in the synergistic induction of apoptosis by bryostatin 1 and FP in U937 cells.

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bryostatin 1 and FP resulted in a marked increase in release of cyt c. This effect is qualitatively similar to that previously observed in cells exposed to FP and PMA (18, 22). However, coexposure of cells to bryostatin 1/FP and either TNFSRs or BisM largely abrogated cyt c release into the cytosol. These findings imply that the PKC-dependent induction of TNF-α is largely responsible for or contributes to cyt c release, and by extension, activation of the intrinsic apoptotic cascade.

**TGF Receptor-related Pathways Contribute to the Synergistic Induction of Apoptosis by Bryostatin 1 and FP in Promyelocytic Leukemia Cells (HL-60).** To determine whether PKC-dependent TNF receptor-related pathways played a role in bryostatin 1/FP-induced apoptosis in leukemia cells other than U937, parallel studies were conducted in human promyelocytic HL-60 cells (Fig. 8, A and B). As shown in Fig. 8A, TNFSRs significantly attenuated bryostatin 1/FP-induced apoptosis in HL-60 cells (i.e., from 59 ± 5% to 33 ± 4%; *P < 0.009), whereas BisM (2 μM) reduced lethality to 16 ± 5% (*P < 0.001). Equivalent results were obtained when annexin V/PI positivity was used to monitor apoptotic cell death (data not shown). Consistent with these results, TNFSRs or BisM diminished degradation of full-length PARP in HL-60 cells exposed to bryostatin 1/FP (Fig. 8B). These findings indicate that PKC-dependent TNF receptor-mediated events contribute significantly to the synergistic induction of apoptosis by bryostatin 1 and FP in leukemia cells other than U937.

A NH₂-Terminal Phosphorylation Loop-deleted Bcl-2 Protein Fails to Protect U937 Cells from Apoptosis Induced by Bryostatin 1/FP. In previous studies, FP (200 nM) effectively induced apoptosis in U937 cells that overexpress full-length Bcl-2, whereas U937 cells ectopically expressing a mutant protein lacking the NH₂-terminal Bcl-2 phosphorylation loop domain, i.e., U937/Bcl-2Δ, were highly resistant to this agent (47). Cells expressing this mutant protein are also resistant to apoptosis induced by growth factor deprivation and by a variety of other cytotoxic drugs (35, 48, 49). Therefore, attempts were made to determine whether ectopic expression of U937/Bcl-2Δ would modify the response of U937 cells to bryostatin 1/FP. As shown in Fig. 9, 100 nM FP, 10 nM bryostatin 1, or 10 nM PMA alone induced a very modest degree of apoptosis in parental or U937/Bcl-2Δ cells, although attenuation of PARP cleavage was noted in the mutant line (Fig. 9B). Interestingly, although U937/Bcl-2Δ cells were highly resistant to VP-16-induced apoptosis (P < 0.007), these cells were sensitive to cell death induced by FP + either bryostatin 1 or PMA, as well as by TNF/CHX. Parallel results were obtained when PARP degradation was monitored (Fig. 9B). In separate studies, ectopic expression of full-length Bcl-2 was ineffective in protecting cells from FP/ bryostatin 1-mediated apoptosis (data not shown) or to FP/ PMA-mediated lethality, as described previously (18). Taken together, these observations suggest that the capacity of the bryostatin 1/FP regimen to overcome resistance conferred by ectopic expression of a Bcl-2 phosphorylation loop-deleted protein stems from activation of the TNF-α pathway.

**Discussion**

The results of this study indicate that synergistic induction of apoptosis in human myeloid leukemia cells by bryostatin 1 and FP largely stems from the TNF-α-dependent activation of the extrinsic apoptotic pathway. Although in some cell types, FP-mediated apoptosis has been attributed to activation of the extrinsic pathway (14), in human leukemia cells such as U937, it appears to act primarily through release of cyt c from the mitochondria and independently of receptor-mediated procaspase-8 activation (14). On the other hand,
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The observation that bryostatin 1 and PMA interact in a similar manner with FP in human leukemia cells provides insights into the mechanism underlying synergism between these agents. For example, in leukemic cells, differentiation and apoptosis often represent alternative cellular fates (50), and dysregulation of the maturation process, particularly induction of the CDKI p21CIP1, is known to convert a maturation stimulus into an apoptotic one (51, 52). Moreover, FP has been shown to bind to duplex DNA (53) and to down-regulate expression of the antiapoptotic protein Mcl-1 (54).

The latter action may reflect the ability of FP to block transcription through inhibition of CDK-9 and the cyclin T complex (pTEFb; Ref. 11). In view of these observations, it was tempting to speculate that FP, by disrupting the normal differentiation process, most notably induction of p21CIP1, converted the response of leukemia cells from maturation to cell death. However, for several reasons, this model is highly unlikely to be operative in the case of bryostatin 1. First, bryostatin 1 is a relatively weak inducer of leukemic cell maturation (28) and, as noted previously, opposes the maturation-inducing actions of PMA (30). Moreover, FP interacted synergistically with the combination of bryostatin 1 and PMA, although coadministration of these agents abrogated differentiation (Fig. 3A). Thus, the positivity that FP potentiates bryostatin 1-mediated apoptosis in leukemia cells by disrupting the differentiation program appears remote. On the other hand, the ability of FP to promote sustained TNF-α release and activation of the extrinsic apoptotic pathway in cells exposed to either bryostatin 1 (this study) or PMA (22) represents a common feature that could plausibly account for synergistic antileukemic interactions between these agents. In this context, synergistic induction of apoptosis in leukemia cells by FP and the differentiation-inducers/histone deacetylase inhibitors sodium butyrate (19) and suberoylanilide hydroxamic acid (55) have recently been observed. However, in marked contrast to results involving PKC activators, e.g., bryostatin 1 (this study) and PMA (22), FP/suberoylanilide hydroxamic acid and FP/sodium butyrate-mediated lethality was not attenuated by TNFRs (55). Such findings suggest that activation of the extrinsic apoptotic pathway in cells exposed to FP in conjunction with maturation-inducing agents varies with the differentiation inducer.

The capacity of FP to down-regulate expression of the antiapoptotic protein Mcl-1 could also contribute to the enhanced lethality of the bryostatin 1/FP regimen. Mcl-1 has been shown to play an important role in the survival of malignant hematopoietic cells, including myeloma (56) as well as myeloid leukemia cells (40). In this regard, FP-induced apoptosis in myeloma (54) and chronic lymphocytic leukemia cells (57) has been related to Mcl-1 down-regulation. In accord with these findings, reduction of Mcl-1 expression (e.g., by an antisense strategy) has been shown to promote apoptosis in U937 cells undergoing maturation in response to PMA (40). Although bryostatin 1 is a poor inducer of differentiation in U937 cells and hence a relatively weak inducer of Mcl-1 expression (i.e., compared with PMA),

Fig. 9. Coadministration of FP with bryostatin 1 or PMA markedly potentiates apoptosis in U937 cells ectopically expressing a Bcl-2 NH2-terminal loop-deleted protein (i.e., U937/Bcl-2Δ). A, empty vector U937/psFFV and U937/Bcl-2Δ cells were exposed to 10 nM bryostatin 1 (B) or PMA (P) in the presence or absence of 100 nM FP for 24 h. For controls, cells were exposed to TNF (10 ng/ml) + CHX [1 μM, TNF/cycloheximide (T/C)] or VP16 (50 μM) for 4 h. The percentage of apoptotic cells was quantified by morphological analysis of Wright-Giemsa-stained cytospin preparations as described in “Materials and Methods.” Values represent the means for three or more separate experiments ± SE, * = significantly less than values obtained in empty vector control cells; P < 0.001. B, Western blot analysis of PARP degradation in U937/psFFV and U937/Bcl-2Δ cells after 24-h exposure to drugs as described above. CF corresponds to a M, 85,000 PARP cleavage fragment. Each lane contained 20 μg of protein; blots were reprobed for actin to ensure equivalent loading and transfer of protein. An additional experiment yielded identical results.

Both PMA and bryostatin 1 have been shown to induce TNF-α production and release (21, 41, 42), and in the case of PMA, these events have been specifically linked to apoptosis induction (21). Recently, we have reported that FP markedly enhances PMA-mediated apoptosis through activation of both the extrinsic, receptor-related as well as the intrinsic, mitochondrial-dependent apoptotic pathways (22). The findings that expression of dominant-negative FADD-DN, as well as coadministration of TNFRs (45) substantially attenuated the apoptotic response to bryostatin 1/FP argue strongly that activation of the TNF receptor pathway plays a major role in bryostatin 1/FP-induced lethality as well. It is important to note that induction of mitochondrial damage, including cyto c release, by certain cytotoxic drugs can be enhanced by procaspase-8 activation and Bid cleavage, thereby amplifying the cell death process (3). Indeed, coadministration of bryostatin 1 and FP resulted in the early release of cyto c into the cytosol. However, this release was substantially blocked by TNFRs and PKC inhibitors, indicating that in the case of bryostatin 1/FP-induced apoptosis, engagement of the extrinsic apoptotic cascade represents a primary rather than a secondary phenomenon, and operates upstream of cyto c redistribution.

The observation that bryostatin 1 and PMA interact in a similar manner with FP in human leukemia cells provides insights into the mechanism underlying synergism between

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R. Rosato and S. Grant, unpublished results.
it mimics the ability of PMA to trigger TNF-α release. Consequently, FP-mediated down-regulation of Mcl-1 expression could provide a common mechanism by which this agent lowers the threshold for bryostatin 1 and PMA-induced mitochondrial injury and apoptosis.

The observation that the PKC inhibitor BisM to blocked TNF-α secretion in bryostatin 1/FB-treated cells indicates that in this setting, TNF-α modulatory events depend upon PKC activation. This finding is consistent with previous reports suggesting that PKC activation is critical for the induction of multiple inflammatory cytokines such as TNF-α and interleukin 1β (43, 58, 59). In addition, activation of TNF-α-converting enzyme, which is responsible for cleaving pro-TNF-α (M, 26,000) to the secreted soluble M, 17,000 form (60), has been shown to be regulated by PKCδ (44). In view of these observations, as well as recent evidence that bryostatin 1 actions are MAP kinase-dependent (61), the capacity of PKC inhibitors such as BisM or the MEK1 inhibitor U0126 to block bryostatin 1/FP-mediated TNF-α induction are understandable. However, given recent findings linking cyt c release and apoptosis to mitochondrial translocation of specific PKC isoforms (62, 63), the possibility that BisM may directly attenuate bryostatin 1/FP-mediated mitochondrial injury cannot be excluded.

It is noteworthy that coadministration of bryostatin 1 (as well as PMA) with FP also circumvented resistance to apoptosis in U937 cells ectopically expressing a Bcl-2 NH2-terminal loop-deleted protein (U937/Bcl-2Δ). This cell line has been shown to be highly resistant to the apoptosis induced by FP (47), taxanes (35), and growth factor deprivation (49). In this context, phosphorylation of Bcl-2 e.g., by taxanes has been linked to promotion of apoptosis (64), as has cell death induced by PMA (65). On the other hand, bryostatin 1-mediated Bcl-2 phosphorylation has been associated with both pro- and antiapoptotic actions, depending upon the experimental context (66, 67). However, given the fact that the NH2-terminal Δ (32–80) region contains the major Bcl-2 phosphorylation sites (49), it seems likely that factors other than or in addition to bryostatin 1-mediated Bcl-2 phosphorylation account for synergistic interactions with FP in these mutant cells. An additional possibility is that loss of the phosphorylation loop enhances the ability of Bcl-2 to block interactions between proapoptotic BH3-only domain family members and multidomain proapoptotic proteins such as Bax and Bak (68). Whatever the mechanism underlying the enhanced antiapoptotic activity of the Bcl-2 Δ protein, it is important to note that although Bcl-2 effectively protects cells from noxious stimuli acting through the intrinsic, mitochondrial-related cascade, it is relatively ineffective in blocking activation of the extrinsic cell death pathway (9). Given the observation that ectopic expression of the Bcl-2 Δ protein failed to protect cells from TNF-α-relatated lethality (Fig. 9 A), it is tempting to postulate that enhanced activation of the extrinsic pathway by the combination of bryostatin 1 and FP is responsible for circumventing apoptosis resistance in these cells.

In summary, the present findings indicate that despite its limited capacity to induce maturation, bryostatin 1, like PMA, interacts in a highly synergistic manner with the CDK1 FP to trigger the apoptosis in human leukemia cells. This interaction does not appear to be related to disruption of the maturation program but instead results from enhanced TNF-α production and engagement of the extrinsic apoptotic pathway. Such actions may also help to explain the capacity of FP/PKC activator-containing regimens to circumvent resistance conferred by certain Bcl-2 family members. Given the recent introduction of bryostatin 1 and FP into clinical trials in humans (10, 69), the present findings could have implications for the development of novel antileukemic strategies. Finally, recent studies have shown that the lethal actions of other classes of differentiation-inducers (e.g., retinoids) proceed, at least in part, through activation of the TNF-related extrinsic pathway (70). In this regard, preliminary findings suggesting that FP does in fact interact synergistically with all trans-retinoic acid to induce apoptosis in promyelocytic leukemia cells may be relevant. It remains to be determined whether interactions between FP and disparate differentiation-inducing agents (i.e., PKC activators, histone deacetylase inhibitors, retinoids) share common mechanisms or are instead unique to each class of agents involved. Accordingly, efforts to address such issues, as well as attempts to extend these findings to primary human leukemia cells, are currently underway.

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Protein Kinase C-dependent Activation of the Tumor Necrosis Factor Receptor-mediated Extrinsic Cell Death Pathway Underlies Enhanced Apoptosis in Human Myeloid Leukemia Cells Exposed to Bryostatin 1 and Flavopiridol

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