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Bryostatin 1 and UCN-01 Potentiate 1-β-D-Arabinofuranosylcytosine-Induced Apoptosis in Human Myeloid Leukemia Cells through Disparate Mechanisms

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ABSTRACT

The effects of the PKC activator and down-regulator bryostatin 1 and the PKC and Chk1 inhibitor 7-hydroxystaurosporine (UCN-01) were compared with respect to potentiation of 1-β-D-arabinofuranosylcytosine (ara-C)-induced apoptosis in human myelomonocytic leukemia cells (U937). Whereas bryostatin 1 and UCN-01 both markedly enhanced ara-C–induced mitochondrial injury (e.g., cytochrome *c* and Smac/DIABLO release, loss of mitochondrial membrane potential), caspase activation, and apoptosis, ectopic expression of an N-terminal loop-deleted Bcl-2 mutant protein protected cells from ara-C/UCN-01– but not ara-C/bryostatin 1-mediated lethality. Conversely, ectopic expression of CrmA or dominant-negative caspase-8 abrogated potentiation of ara-C–mediated apoptosis by bryostatin 1 but not by UCN-01. Exposure of cells to ara-C and bryostatin 1 (but not UCN-01) resulted in sustained release of tumor necrosis factor (TNF) α; moreover, potentiation of ara-C lethality by bryostatin 1 (but not by UCN-01) was reversed by coadministration of TNF soluble receptors or the selective PKC inhibitor bisindolylmaleimide (1 μM). Finally, similar events were observed in the human promyelocytic leukemia cell line HL-60. Together, these findings suggest that potentiation of ara-C lethality in human myeloid leukemia cells by bryostatin 1 but not UCN-01 involves activation of the extrinsic, receptor-mediated apoptotic pathway, and represents a consequence of bryostatin 1-mediated release of TNF-α. They also argue that the mechanism by which bryostatin 1 promotes ara-C–induced mitochondrial injury, caspase activation, and apoptosis involves factors other than or in addition to PKC down-regulation or modulation of Bcl-2 phosphorylation status.

The macrocyclic lactone bryostatin 1 is a protein kinase C (PKC) activator derived from the marine bryozoan *Bugula neritina* (Pettit et al., 1981). It is active against a variety of human tumor cells, including those of hematopoietic origin (Jones et al., 1990), and is currently in clinical development (Varterasian et al., 2000). Bryostatin 1 exhibits a distinctly different spectrum of activity from that of tumor-promoting phorbols such as phorbol 12-myristate 13-acetate (PMA) and blocks those PMA-related actions that it does not itself possess (Kraft et al., 1986). Although the basis for the unique activity spectrum of bryostatin 1 is unclear, attention has focused on its capacity to induce extensive PKC down-regulation (Jarvis et al., 1994; Lee et al., 1997). Although bryostatin 1 inhibits the growth of human leukemia cells (Asiedu et al., 1995), its effects when administered alone are modest (Kraft et al., 1989). However, bryostatin 1 lowers the threshold for apoptosis induced by various cytotoxic agents, including paclitaxel (Wang et al., 1998,1999a), the nucleoside analogs fludarabine (Vrana et al., 1999a), and 1-β-D-arabinofuranosylcytosine (ara-C) (Jarvis et al., 1994). Given evidence that PKC activation opposes cell death (Lotem et al., 1991) and that pharmacologic PKC inhibitors induce apoptosis and promote ara-C lethality (Grant, 1997; Tang et al., 2000), it is tempting to relate bryostatin 1 actions to PKC down-regulation (Wang et al., 1997).

Bryostatin 1 also reverses, at least in part, the blockade to

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ABBREVIATIONS: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; UCN-01, 7-hydroxystaurosporine; ara-C, 1-β-D-arabinofuranosylcytosine; $DiOC_6$, 3,3-dihexlyoxacarbocyanine; GFX, bisindolylmaleimide (GF 109203X); TNFSR, tumor necrosis factor soluble receptor; pNA, *p*-nitroanalide; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; COX II, cytochrome oxidase unit II; $\Delta\Psi_m$, mitochondrial membrane potential; TNF, tumor necrosis factor; BRY, bryostatin 1; UCN, UCN-01; Chk1, checkpoint kinase 1; CrmA, cytokine response modifier A; ELISA, enzyme-linked immunosorbent assay; Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI.

apoptosis conferred by ectopic expression of antiapoptotic proteins such as Bcl-2 (Wang et al., 1997). Moreover, recent studies indicate that bryostatin 1 increases ara-C–related mitochondrial injury (e.g., loss of mitochondrial membrane potential and cytosolic release of cytochrome *c*) in myelomonocytic leukemia cells that ectopically express Bcl-x_L (Wang et al., 2002). Although early findings suggested that these events might stem from alterations in Bcl-2 phosphorylation status (Wang et al., 1997), the relationship between Bcl-2 phosphorylation and susceptibility of cells to apoptosis remains controversial. For example, in some systems (i.e., murine 32D cells), bryostatin 1-mediated phosphorylation of Bcl-2 seems to exert an anti-apoptotic rather than pro-apoptotic effect (Deng et al., 1998). Consequently, the capacity of bryostatin 1 to circumvent Bcl-2- or Bcl-x_L–mediated resistance to ara-C in leukemic cells is likely to involve factors other than or in addition to perturbations in Bcl-2 phosphorylation status.

Two major pathways have been implicated in apoptosis activation. In the intrinsic pathway, stresses such as chemotherapeutic drugs trigger mitochondrial damage (i.e., loss of mitochondrial membrane potential) (Petit et al., 1998) and release of apoptogenic proteins, such as cytochrome *c*, Smac/DIABLO, and apoptosis-inducing factor into the cytosol, where they promote activation of multiple caspases (Zou et al., 1999; Joza et al., 2001; Srinivasula et al., 2001). In the extrinsic pathway, events are triggered by members of the TNF receptor family [e.g., Fas(CD95)], which activates the death effector protein Fadd/Mort1, thereby recruiting and cleaving procaspase 8. Caspase 8 then activates the effector caspase procaspase-3 (Wajant, 2002). Although the intrinsic and extrinsic apoptotic pathways are in many respects distinct, they are also interrelated. For example, caspase 8 cleaves the BH3-only domain Bcl-2 family member Bid, which translocates to the mitochondria and triggers release of cytochrome *c* (Gross et al., 1999). Thus, activation of the extrinsic pathway can amplify the apoptotic cascade initiated by mitochondrial damage, including that induced by cytotoxic drugs (Sun et al., 1999; Cartee et al., 2002).

Previously, we reported that in addition to promoting ara-C–induced apoptosis, bryostatin 1 and the checkpoint abrogator and PKC inhibitor UCN-01 (7-hydroxystaurosporine) (Graves et al., 2000) attenuate ara-C resistance conferred by overexpression of antiapoptotic proteins such as Bcl-x_L or Bcl-2 (Wang et al., 1997, 2002; Tang et al., 2000). Recently, we observed that bryostatin 1 opposes the ability of Bcl-x_L to block ara-C–mediated cytochrome *c* release, thereby promoting activation of the intrinsic apoptotic cascade (Wang et al., 2002). However, the observation that interrupting the extrinsic apoptotic pathway (e.g., by CrmA) partially attenuated apoptosis raised the possibility that receptor-related events may contribute to synergistic interactions between bryostatin 1 and ara-C. The purpose of the present study was to compare contributions of the extrinsic pathway with potentiation of ara-C–induced lethality by bryostatin 1 versus UCN-01 in parental as well as in highly ara-C–resistant cells ectopically expressing a phosphorylation loop-deleted Bcl-2 protein (Tang et al., 2000). Our results indicate that in these cells, synergism between ara-C and bryostatin 1, in contrast to UCN-01, involves the PKC-dependent release of TNF-α, resulting in activation of the extrinsic apoptotic cascade.

Materials and Methods

Cells. U937 and HL-60 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium supplemented with sodium pyruvate, minimal essential vitamins, L-glutamate, penicillin and streptomycin, and 10% heat-inactivated fetal calf serum. They were maintained in a 37°C, 5% CO_2, fully humidified incubator, passed twice weekly, and prepared for experimental procedures when in log-phase growth.

For the generation of stable transfectants, U937 cells were transfected by electroporation as described previously (Wang et al., 1999b). Stable U937 transfectants expressing full-length Bcl-2, Bcl-2Δ (deletion of the loop region of Bcl-2, Δ32–80) (Chang et al., 1997), CrmA, and caspase-8 dominant-negative (C377A) expressing cell lines were used as described previously (Cartee et al., 2002). For all studies, cells containing empty vectors (pcDNA3.1 or pSFFV) were used as control cells.

Drugs and Chemicals. ara-C was purchased from Sigma-Aldrich (St. Louis, MO) and maintained as a dry powder at −20°C. It was reformulated in PBS before use. Bryostatin 1 was provided by the Cancer Treatment and Evaluation Program, National Institutes of Health (Bethesda, MD), and stored desiccated at −20°C. It was formulated in sterile dimethyl sulfoxide and subsequently diluted in RPMI 1640 medium so that the final concentration of dimethyl sulfoxide was in all cases <0.05%. UCN-01 was kindly provided by Dr. Edward Sausville (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD). It was stored frozen as a 1 mM stock solution in dimethyl sulfoxide in light-protected microcentrifuge tubes at −20°C and was subsequently diluted in sterile phosphate-buffered saline before each experiment. 3,3-Dihexyloxacarbocyanine ($DiOC_6$) was purchased from Molecular Probes (Eugene, OR). Recombinant human TNF-α was from Calbiochem (San Diego, CA); recombinant human soluble TNF sRI/Fc chimera (TNFRSF1A) was from R&D Systems (Minneapolis, MN); bisindoylmaleimide (GFX) was purchased from Sigma, formulated in dimethyl sulfoxide, and stored frozen in light-protected vials before use.

Experimental Format. Logarithmically growing cells (approximately 2×10^5 cells/ml) were placed in 25- or 75-cm^2 T-flasks (Greiner Labortechnik, Frickenhausen, Germany) and incubated simultaneously with 0.5 μM ara-C and 10 nM bryostatin 1 or 100 nM UCN-01 for 24 h. In experiments involving kinase inhibitors and TNF soluble receptor (TNFSR), cells were pretreated with each inhibitor 30 min before the addition of ara-C and bryostatin 1 or UCN-01.

Assessment of Apoptosis. After drug exposures, cytocentrifuge preparations were stained with Wright-Giemsa stain and viewed under light microscopy to evaluate features of cellular differentiation as well as apoptosis as described previously (Jarvis et al., 1994). For Annexin-V assay, 1×10^6 cells were double-stained with fluorescein isothiocyanate-conjugated Annexin-V and propidium iodide using the apoptosis kit according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). The percentage of apoptotic (annexin-V- and propidium iodide-positive) cells was determined by flow cytometric analysis.

Caspase Activity. The activities of caspase-3 and -8 were determined using commercially available kits (Bio Vision, Mountain View, CA) according to the manufacturer's specifications. The caspase-3 and -8 kits employ a colorimetric assay to monitor cleavages of DEVD-pNA and IETD-pNA substrates, respectively. Liberated pNA is monitored colorimetrically by absorbance at 405 nm. By comparing the fluorescence of apoptotic samples versus untreated control, caspase activity can be quantified.

Subcellular Fractionation. Both cytosolic and mitochondrial fractions were isolated at 4°C using a previous protocol (Gross et al., 1998) with some modifications. At each time point, cells were washed once in the phosphate-buffered saline (PBS), resuspended in isotonic buffer A (200 mM mannitol, 70 mM sucose, 1 mM EGTA, and 10 mM

HEPES, pH 7.5) supplemented with protease inhibitors (1 mM phenylmethysulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml soybean trypsin inhibitor, and 10 μg/ml aprotonin), and incubated on ice for 10 min. The cells were then homogenized by 20 passes through a 26-gauge, 0.5-inch needle fitted onto a 1-ml syringe. Nuclei and unbroken cells were separated at 120*g* for 5 min as the low speed pellet. The supernatant was collected and centrifuged at 2,000*g* for 10 min. The resulting supernatant was centrifuged at 14,000*g* for 20 min to yield the mitochondria fraction. The supernatant was further centrifuged at 100,000*g* for 30 min, and the resulting supernatant was collected as the cytosol fraction.

Western Analysis. Western blot analysis was performed essentially as described previously (Wang et al., 1999a). In brief, for each sample, 30 μg of protein per lane was separated by 4 to 20% SDS-PAGE (Invitrogen, Carlsbad, CA) and electroblotted to nitrocellulose (Schleicher and Schuell, Keene, NH). Subsequently, after incubation in PBS-Tween 20 (0.05%) supplemented with 5% nonfat dry milk for 1 h at 22°C, the blots were incubated for 2 h at 22°C in fresh blocking solution with an appropriate dilution of primary antibodies as follows: cytochrome *c,* 1:1000, procaspase-3 and -8, 1:1000 (BD Pharmingen); Smac/DIABLO, 1:500 (BIOMOL Research Laboratories, Plymouth Meeting, PA); and monoclonal anti-cytochrome oxidase unit II (COX II) antibody, 1:500 (Molecular Probes, Eugene, OR). Blots were washed three times for 5 min in PBS-Tween 20 and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at 22°C. Blots were again washed three times for 5 min in PBS-Tween 20 and then developed by enhanced chemiluminescence (Amersham Biosciences, Braunschweig, Germany).

Assessment of Mitochondrial Membrane Potential. Mitochondrial membrane potential ($\Delta\Psi_m$) was monitored by flow cytometry using $DiOC_6$ as previously described in detail (Wang et al., 2002).

Enzyme-Linked Immunosorbent Assay. U937 cells (4×10^6) were exposed to drug treatment at various time intervals (0, 5, and 10 h). Cell culture supernatants were collected by centrifugation and concentrated 10-fold with Macrosep centrifugal devices (Pall Life Sciences, Ann Arbor, MI). TNF-α protein was quantified by the ELISA OptEIA kit (BD Pharmingen) according to the manufacturer's instruction. TNF-α levels were normalized to untreated controls.

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

Results

Bryostatin 1 and UCN-01 Promote ara-C–Induced Cytochrome *c* and Smac/DIABLO Release in U937 Cells. To compare the effects of bryostatin 1 and UCN-01 on ara-C–induced mitochondrial injury, empty vector control U937 cells were exposed to 0.5 μM ara-C ± 10 nM bryostatin

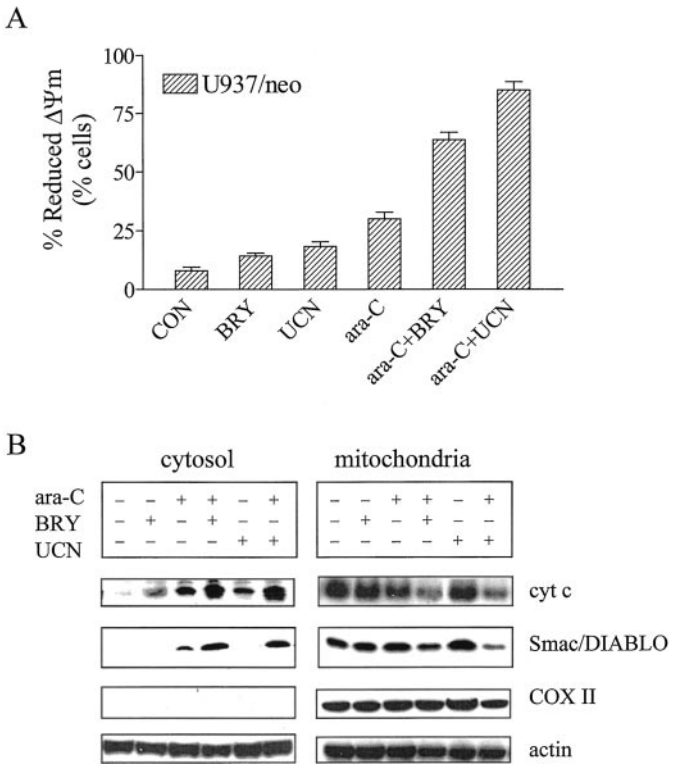

Fig. 1. Bryostatin 1 and UCN-01 promote ara-C–induced mitochondrial injury in U937 cells. A, U937/neo cells were exposed to bryostatin 1 (BRY; 10 nM) or UCN-01 (UCN; 100 nM) ± ara-C (0.5 μM) for 24 h, after which the loss of $\Delta\Psi_m$ was determined by flow cytometric analysis of cellular uptake of $DiOC_6$ as described under *Materials and Methods*. Values represent the means ± S.D. for three separate experiments performed in triplicate. B, cells were exposed to BRY (10 nM) or UCN (100 nM) ± ara-C (0.5 μM) for 24 h, after which cells were suspended in isotonic buffer, homogenized, and separated into cytosolic and mitochondrial fractions by differential centrifugation as described under *Materials and Methods*. The fractions were analyzed by Western blot using antibodies directed against cytochrome *c* (cyt c), Smac/DIABLO, and COX II. Each lane was loaded with 30 μg of protein; blots were stripped and reprobed for actin to ensure equivalent loading and transfer. For B, two additional studies yielded equivalent results

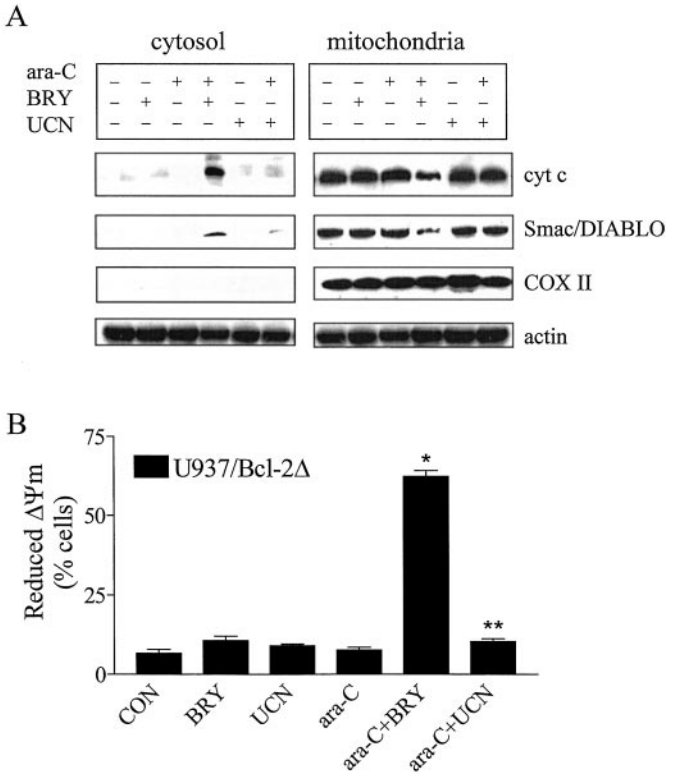

Fig. 2. Bryostatin 1 but not UCN-01 promotes ara-C–induced mitochondrial injury in U937 cells ectopically expressing an N-terminal phosphorylation loop-deleted mutant Bcl-2 protein. A, U937/Bcl-2Δ cells were exposed to BRY (10 nM) or UCN (100 nM) in the presence or absence of ara-C (0.5 μM) for 24 h, after which levels of cytochrome *c* (cyt c), Smac/DIABLO, or COX II in the cytosolic or mitochondrial fractions were determined by Western blot analysis as described under *Materials and Methods*. Each lane was loaded with 30 μg of protein; blots were subsequently stripped and reprobed for expression of actin to ensure equivalent loading and transfer of protein. Two additional studies yielded similar results. B, U937/Bcl-2Δ cells were exposed to BRY (10 nM) or UCN (100 nM) ± ara-C (0.5 μM) for 24 h, after which loss of $\Delta\Psi_m$ was determined by flow cytometry as above. Values represent the means ± S.D. for three separate experiments performed in triplicate. *, $P < 0.001$, significantly greater than values obtained for ara-C alone; **, $P > 0.05$, values not significantly greater than values obtained for ara-C alone; .

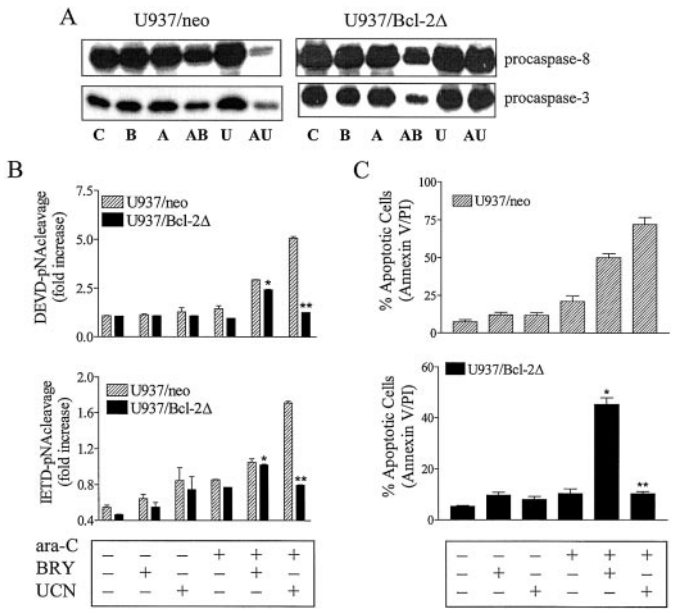

Fig. 3. Effects of bryostatin 1 or UCN-01 on ara-C–induced apoptosis and caspase-3 and -8 activation in U937/neo and U937/Bcl-2Δ cells. A, U937/neo and U937/Bcl-2Δ cells were exposed to BRY (B; 10 nM) or UCN (U; 100 nM) in the presence or absence of ara-C (A; 0.5 μM) for 24 h. At the end of this period, cells were lysed, the proteins separated by SDS-PAGE, each lane was loaded with 30 μg of protein; blots were probed with antibodies directed against procaspase-3 or -8 as described under *Materials and Methods*. Two additional studies yielded similar results. B, cells were treated as above, after which caspase-3 and caspase-8 activities, reflected by DEVD-pNA or IETD-pNA cleavage, respectively, were determined as described under *Materials and Methods*. C, cells were treated as above, after which cells were stained with Annexin V and propidium iodide, and the extent of apoptosis was determined by flow cytometric analysis as described under *Materials and Methods*. Values for B and C represent the means ± S.D. for three separate experiments performed in triplicate. *, $P > 0.05$, not significantly less than values for Bcl-2/neo cells; **, $P < 0.02$; significantly less than values for Bcl-2/neo cells.

1 or 100 nM UCN-01 for 24 h, after which loss of mitochondrial membrane potential was determined by flow cytometry, and cytochrome *c* and Smac/DIABLO release into the cytosolic fraction was monitored by Western blot analysis. To rule out contamination by mitochondrial protein, parallel studies monitoring the expression of the mitochondrial-specific protein COX II were performed. As shown in Fig. 1A, both bryostatin 1 and UCN-01 increased ara-C–mediated reductions in $\Delta\Psi_m$, although UCN-01 was slightly more effective in this regard. Moreover, ara-C alone induced a small amount of cytochrome *c* and Smac/DIABLO release into the cytosol, whereas coadministration of bryostatin 1 or UCN-01, which were minimally toxic by themselves, resulted in a substantial increase in cytochrome *c* and Smac/DIABLO redistribution (Fig. 1B). Thus, bryostatin 1 and UCN-01 both potentiate the ability of ara-C to induce mitochondrial injury in U937 cells.

Bryostatin 1 but Not UCN-01 Promotes ara-C–Induced Mitochondrial Injury in U937 Cells Ectopically Expressing an N-Terminal Phosphorylation Loop-Deleted Mutant Bcl-2 Protein. Previous studies indicated that UCN-01 promotes ara-C–induced apoptosis in U937 cells ectopically expressing full-length Bcl-2 protein but is significantly less effective in potentiating ara-C lethality in cells (U937/Bcl-2Δ) expressing a mutant protein lacking the N-terminal phosphorylation loop (Tang et al., 2000). The latter has been shown to confer a high degree of resistance to a variety of cytotoxic agents, including taxanes (Wang et al., 1999b) and flavopiridol (Decker et al., 2002), as well as growth factor depravity (Chang et al., 1997). In addition, we have also observed that potentiation of ara-C–induced apoptosis by bryostatin 1 or UCN-01 is associated with perturbations in Bcl-2 phosphorylation (Wang et al., 1997). Attempts were therefore made to compare the effects of bryostatin 1 and UCN-01 on ara-C–induced apoptosis in resistant U937/Bcl-2Δ cells. As shown in Fig. 2A, combined exposure of U937/Bcl-2Δ cells to ara-C and bryostatin 1 for 24 h resulted in a marked increase in cytosolic release of cytochrome *c* and Smac/DIABLO, and corresponding declines in the mitochondrial fractions. In contrast, UCN-01 was relatively ineffective in promoting ara-C–induced mitochondrial injury in these cells, consistent with results of our earlier study (Tang et al., 2000). Parallel results were obtained when reductions in $\Delta\Psi_m$ were monitored (Fig. 2B).

Bryostatin 1 but Not UCN-01 Promotes ara-C–Induced Caspase Activation and Apoptosis in U937/Bcl-2Δ Cells. Consistent with the preceding results, exposure of empty vector control cells to ara-C + either bryostatin 1 or UCN-01 resulted in clear reduction in levels of full-length procaspase-8 and procaspase-3 (Fig. 3A). In marked contrast, U937/Bcl-2Δ cells exhibited a decline in full-length procaspase-8 and -3 levels only when exposed to ara-C + bryostatin 1, but not to ara-C + UCN-01. In addition, ectopic expression of U937/Bcl-2Δ substantially attenuated caspase-3 and caspase-8 activation by the combination of ara-C and UCN-01 but did not significantly modify activation of these caspases in ara-C/bryostatin 1-treated cells (Fig. 3B). Lastly, consistent with these findings, coadministration of bryostatin 1 restored the sensitivity of U937/Bcl-2Δ to ara-C–induced apoptosis, whereas UCN-01 was largely ineffective in this regard (Fig. 3C). Together, these and the preceding findings indicate that bryostatin 1, but not UCN-01, is effective in promoting ara-C–induced mitochondrial injury, caspase activation, and apoptosis in highly drug-resistant U937 cells ectopically expressing a phosphorylation loop-deleted Bcl-2 protein.

Activation of the Extrinsic Apoptotic Cascade Is Implicated in Apoptosis Induced by ara-C + bryostatin 1, but Not by ara-C + UCN-01. Recent findings suggest that the ability of bryostatin 1 to promote ara-C–induced apoptosis may involve activation of both the intrinsic, mitochondria-associated and extrinsic, receptor-related cell death pathways (Wang et al., 2002). To determine whether the extrinsic pathway might be differentially involved in potentiation of ara-C lethality by bryostatin 1 versus UCN-01, studies were conducted in U937 cells ectopically expressing CrmA, which inhibits caspase-8 activation, or a dominant-negative caspase-8 (Fig. 4). As shown in Fig. 4A, ectopic expression of CrmA or DN-caspase-8 significantly protected cells from loss of $\Delta\Psi_m$ induced by ara-C and bryostatin 1 but not from that triggered by ara-C and UCN-01. Concordant results were obtained when apoptosis was monitored by annexin V/propidium iodide staining (Fig. 4B). These findings indicate that potentiation of ara-C lethality by bryostatin 1, but not by UCN-01, involves activation of the extrinsic, receptor-mediated cell death pathway.

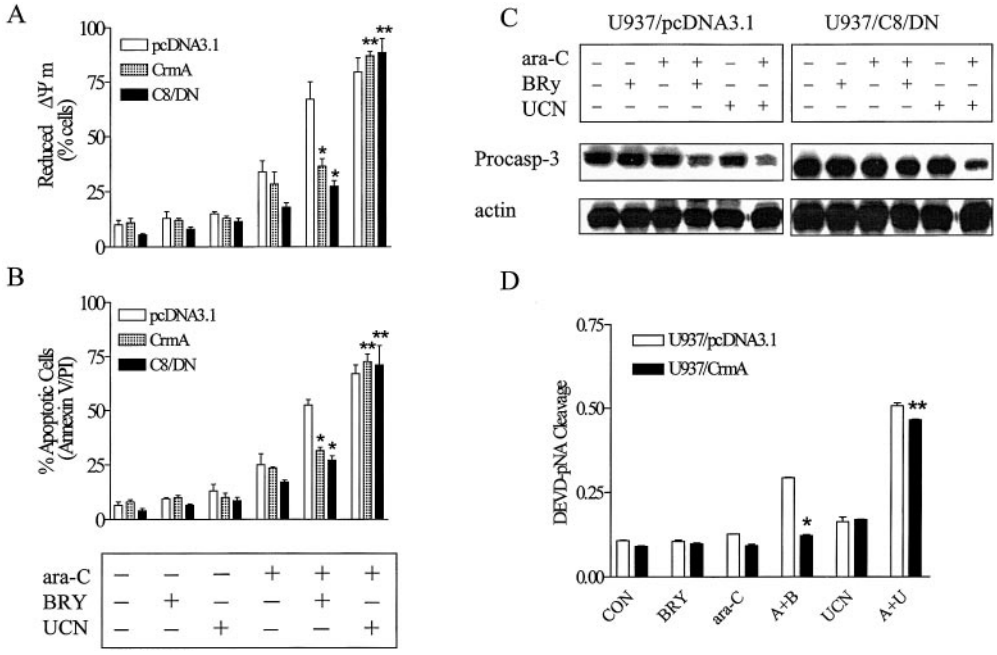

Fig. 4. U937 ectopically expressing CrmA or dominant-negative caspase-8 are protected from loss of $\Delta\Psi_m$, caspase-3 activation, and apoptosis induced by ara-C and bryostatin 1, but not by ara-C and UCN-01. A, U937/pcDNA3.1, U937/CrmA, and U937/C8/DN cells were exposed to BRY (10 nM) or UCN (100 nM) in the presence or absence of ara-C (0.5 μM) for 24 h. At the end of this period, the loss of $\Delta\Psi_m$ was determined by flow cytometry as above. B, cells were treated as above, after which they were stained with Annexin V and propidium iodide, and the extent of apoptosis was determined by flow cytometric analysis as above. Values for A and B represent the means ± S.D. for three separate experiments performed in triplicate. *, $P < 0.05$, significantly less than values for empty vector controls; **, $P > 0.05$, not significantly different from values obtained in empty vector controls. C, U937/pcDNA3.1 and U937/C8/DN cells were exposed to BRY (10 nM) or UCN (100 nM) in the presence or absence of ara-C (0.5 μM) for 24 h. At the end of this period, cells were lysed and the proteins were separated by SDS-PAGE and probed with antibodies directed against full-length procaspase-3 as described under *Materials and Methods*. Each lane was loaded with 30 μg of protein; blots were subsequently stripped and reprobed for expression of actin to ensure equivalent loading and transfer of protein. Two additional studies yielded similar results. D, U937/pcDNA3.1 and U937/CrmA cells were treated as above, after which caspase-3 activity, as reflected by DEVD-pNA cleavage, was determined as described under *Materials and Methods*. Values represent the means ± S.D. for three separate experiments performed in triplicate. *, $P < 0.01$, significantly less than values for empty vector controls; **, $P > 0.05$, not significantly different from values obtained in empty vector controls.

Activation of the Extrinsic Apoptotic Pathway Is Involved in Potentiation of ara-C–Mediated Procaspase-3 Degradation and Activation by Bryostatin 1 but Not by UCN-01. To characterize further the role of the extrinsic pathway in potentiation of ara-C–mediated lethality, procaspase-3 degradation and activity were monitored in CrmA or DN-caspase-8-expressing cells (Fig. 4C). Ectopic expression of DN-caspase-8 diminished the degradation of full-length procaspase-3 in cells exposed to ara-C and bryostatin 1 but minimally attenuated that induced by ara-C and UCN-01 (Fig. 4C). Similarly, ectopic expression of CrmA significantly reduced caspase-3 activity in cells exposed to ara-C and bryostatin 1 but had only a minor effect on activity in ara-C/UCN-01–treated cells (Fig. 4D). These findings support the notion that potentiation of ara-C lethality by bryostatin 1, but not by UCN-01, involves activation of the extrinsic apoptotic pathway.

TNF-α Plays a Role in Potentiation of ara-C–Induced Apoptosis by Bryostatin 1 but Not by UCN-01. In view of the ability of bryostatin 1 but not UCN-01 to promote ara-C–induced apoptosis in U937/Bcl-2Δ cells and evidence linking ara-C/bryostatin 1-induced lethality to activation of the extrinsic apoptotic pathway, the role of TNF-α in these events was investigated. As shown in Fig. 5A, coadministration of TNF soluble receptors, which inhibit TNF-related apoptosis (Aggarwal and Natarajan 1996), exerted no effect on ara-C–induced apoptosis in either U937/neo or U937/Bcl-2Δ cells. In marked contrast, administration of TNFSRs essentially abrogated the potentiation of ara-C–induced apoptosis by bryostatin 1 in both the parental and mutant cell lines. Also, in contrast to these results, coadministration of TNFSRs failed to block potentiation of ara-C lethality by UCN-01 (Fig. 5A). Examination of TNF-α release by ELISA revealed that bryostatin 1 by itself induced a marked increase in TNF-α levels by 5 h, followed by a slight decline at 10 h (Fig. 5B). Exposure of cells to ara-C alone did not induce TNF-α release. However, combined exposure of cells to ara-C and bryostatin 1 resulted in a sustained increase in TNF-α release, which persisted at 10 h. In separate studies, coadministration of UCN-01 with ara-C did not increase TNF-α levels over those observed in cells exposed to ara-C alone (data not shown). Finally, coadministration of ara-C and exogenous TNF-α induced a marked increase in apoptosis, an effect that was not attenuated by ectopic expression of the Bcl-2Δ protein, similar to results obtained with bryostatin 1 (Fig. 5C). Together, these findings implicate TNF-α in enhancement of ara-C lethality by bryostatin 1, as well as circumvention of resistance conferred by ectopic expression of a Bcl-2 phosphorylation loop-deleted protein.

PKC Activation Is Required for Potentiation of ara-C–Induced Apoptosis by Bryostatin 1. To examine the role of PKC activation in these events, the effects of the PKC activator PMA and the specific PKC inhibitor GFX were compared with those of bryostatin 1 in U937/neo and U937/Bcl-2Δ cells (Table 1 and Fig. 6). As reported previously (Vrana et al., 1999b), PMA modestly but significantly in-

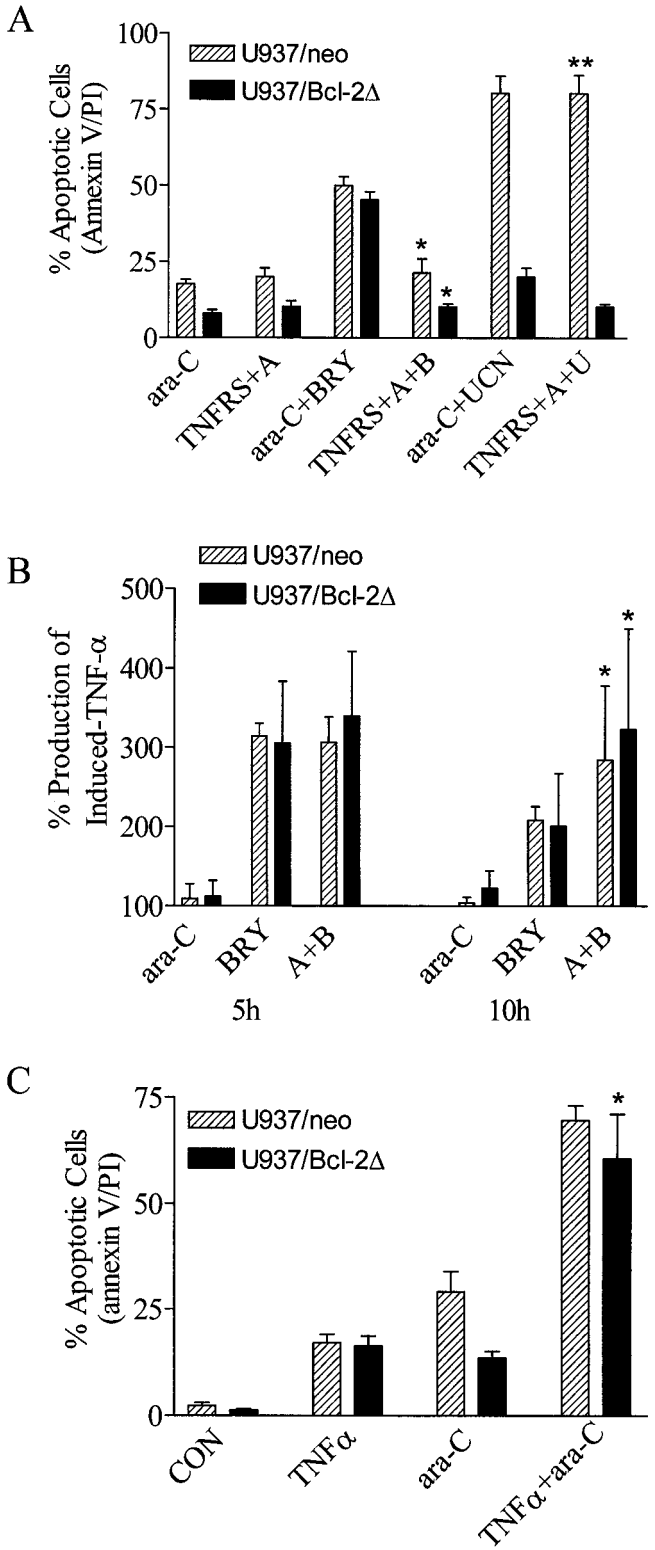

Fig. 5. TNF-α plays a role in potentiation of ara-C–induced apoptosis by bryostatin 1 but not by UCN-01. A, U937/neo and U937/Bcl-2Δ cells were exposed to BRY (10 nM) or UCN (100 nM) ± ara-C (0.5 μM) in the presence or absence of TNFSR (100 ng/ml) for 24 h, after which cells were stained with Annexin V and propidium iodide. The extent of apoptosis was determined by flow cytometric analysis as described above. *, $P < 0.05$, significantly less than values for for ara-C and BRY; **, $P > 0.05$, not significantly different from values for ara-C+UCN-01. Exposure to TNFSR alone exerted minimal toxicity (i.e., <5% apoptotic cells; data not shown). B, ELISA was employed to quantify the amount of TNF-α protein released into the medium after 5 or 10 h of drug treatment as described under *Materials and Methods*. U937/neo and U937/Bcl-2Δ cells were exposed to BRY (10 nM) ± ara-C (0.5 μM). TNF-α protein secretion is expressed as the percentage increase in TNF-α, levels relative to values for untreated control cells. Values represent the means ± S.D. for three separate experiments performed in triplicate. *, $P < 0.02$; significantly greater than values for ara-C alone. C, U937/neo and U937/Bcl-2Δ cells were exposed to ara-C (0.5 μM) ± TNF-α (0.25 ng/ml) for 24 h, after which apoptotic cells were quantified as described under *Materials and Methods*. *, $P > 0.05$; not significantly different from values for U937/neo.

creased ara-C–induced apoptosis in U937/neo cells (Table 1). Moreover, the relative increase was even greater in the resistant U937/Bcl-2Δ cell line. Approximately equivalent results were obtained when $\Delta\Psi_m$ was monitored (Table 1). In contrast, the inactive PMA derivative 4αPMA was ineffective in promoting ara-C–induced apoptosis and mitochondrial damage. These findings suggest that potentiation of ara-C–mediated apoptosis in U937 cells by PMA requires PKC activation. Furthermore, because the Bcl-2Δ protein lacks the major Bcl-2 phosphorylation domains, they argue against the possibility that this phenomenon involves PKC-dependent phosphorylation of Bcl-2.

Consistent results were obtained when the PKC inhibitor GFX was combined with bryostatin 1 (Fig. 6). Although GFX by itself did not enhance ara-C–mediated lethality in empty-vector or U937/Bcl-2Δ cells, coadministration of GFX with bryostatin 1 effectively abrogated potentiation of ara-C–induced mitochondrial injury (Fig. 6A) or apoptosis (Fig. 6B) in both cell lines. These findings suggest that as in the case of PMA, PKC activation by bryostatin 1 is required for potentiation of ara-C–induced cell death. Furthermore, they argue against the possibility that PKC down-regulation by bryostatin 1 is solely or primarily responsible for enhanced ara-C lethality in these cells.

TNFSR and GFX Block Potentiation of ara-C–Induced Mitochondrial Injury and Apoptosis by Bryostatin 1 in HL-60 Cells. To determine whether similar events might occur in human leukemia cell lines other than U937, parallel studies were performed in HL-60 cells. Consistent with earlier reports (Jarvis et al., 1994), bryostatin 1 increased ara-C–mediated mitochondrial damage and apoptosis in this line (Fig. 7). However, coadministration of either TNFSR or GFX effectively abrogated the bryostatin 1-mediated increase in ara-C lethality. These findings are consistent with the notion that as in the case of U937 cells, bryostatin 1 promotes ara-C mitochondrial damage and apoptosis through the PKC-dependent induction of TNF-α.

TNFSR and GFX Fail to Block UCN-01–Mediated Potentiation of ara-C–Mediated Lethality in U937 Cells. Lastly, the effects of TNFSR and the PKC inhibitor GFX on UCN-01–mediated potentiation of ara-C lethality were compared with those of bryostatin 1 (Fig. 8). In marked contrast to the results involving bryostatin 1, both TNFSR and GFX failed to attenuate apoptosis (Fig. 8A) or mitochondrial injury (Fig. 8B) in U937 cells. These findings further indicate that bryostatin 1 and UCN-01 enhance ara-C lethality through fundamentally different mechanisms. They also suggest that, as in the case of bryostatin, the ability of UCN-01 to potentiate ara-C–related cytotoxicity involves factors other than inhibition of PKC.

TABLE 1

The PKC activator PMA, but not the inactive PMA derivative 4αPMA, promote apoptosis in ara-C–treated cells

U937/neo and U937/Bcl-2Δ cells were exposed to ara-C (0.5 μM) with or without PMA (10 nM) or an inactive phorbol control (4α-PMA; 10 nM) for 24 hours. Values represent the means for triplicate experiments (± S.D.) and are expressed as the percentage of apoptotic cells determined by Annexin V and propidium iodide, or the percentage of cells exhibiting low levels of $DiOC_6$ uptake, reflecting loss of $\Delta \Psi_m$.

	Annexin V/PI		Reduced $\Delta\Psi_m$	
	U937/neo	U937/Bcl-2Δ	U937/neo	U937/Bcl-2Δ
	% apoptotic cells		*% cells*	
Control	2.5 ± 0.2	2.0 ± 0.1	8.5 ± 0.2	5.4 ± 0.2
PMA	8.5 ± 0.2	5.0 ± 0.6	23.4 ± 2.2	14.2 ± 0.6
ara-C	30.4 ± 1.2	9.5 ± 0.2	36.5 ± 1.2	9.5 ± 0.9
ara-C+PMA	59.5 ± 1.6*	44.6 ± 0.8**	63.1 ± 2.0*	34.7 ± 0.8**
4αPMA	7.5 ± 0.9	4.3 ± 0.6	12.4 ± 0.5	7.0 ± 0.6
ara-C+4αPMA	29.7 ± 1.1	8.6 ± 0.4	32.3 ± 0.8	13.5 ± 1.1

*, $P < 0.05$, significantly greater than values for ara-C alone.
**, $P > 0.05$, not significantly different from values for ara-C alone in empty-vector controls.

Discussion

The results of the present study indicate that although bryostatin 1 and UCN-01 share the capacity to interrupt the PKC signal transduction pathway and to promote ara-C–induced apoptosis, the mechanisms by which these two agents act differ fundamentally. There is abundant evidence that in hematopoietic cells, PKC exerts a cytoprotective function. For example, PKC activators such as PMA protect hematopoietic cells from growth factor deprivation-induced cell death (Lotem et al., 1991); in addition, pharmacologic PKC inhibitors are highly potent inducers of apoptosis in hematopoietic and nonhematopoietic cells (Jarvis et al., 1996). Moreover, PKC inhibitors have been shown to potentiate apoptosis induced by various cytotoxic drugs, including ara-C (Jarvis et al., 1994). Although bryostatin 1 acutely activates PKC, on long-term exposure it down-regulates the enzyme, a phenomenon that involves proteasomal degradation (Lee et al., 1997). Thus, under these circumstances, bryostatin 1 functions as a PKC antagonist. UCN-01, a derivative of the relatively nonspecific PKC and tyrosine kinase inhibitor staurosporine, was originally developed as a more selective PKC inhibitor (Mizuno et al., 1995). UCN-01 also promotes apoptosis induced by nucleoside analogs (Shi et al., 2001), although the recent observation that UCN-01 is a potent inhibitor of Chk1 (Graves et al., 2000) raises the possibility that interference with checkpoint control is primarily responsible for synergistic interactions with DNA-damaging drugs (Bunch and Eastman, 1996; Shi et al., 2001). Nevertheless, given the observations that bryostatin 1 and UCN-01 share the capacity to potentiate ara-C lethality in human leukemia cells, and to overcome, at least in part, ara-C resistance conferred by ectopic expression of antiapoptotic proteins such as Bcl-2 and Bcl-xL, (Wang et al., 1997; Tang et al., 2000), it is tempting to speculate that interference with PKC cytoprotective signaling pathways represents a common mechanism underlying for these events.

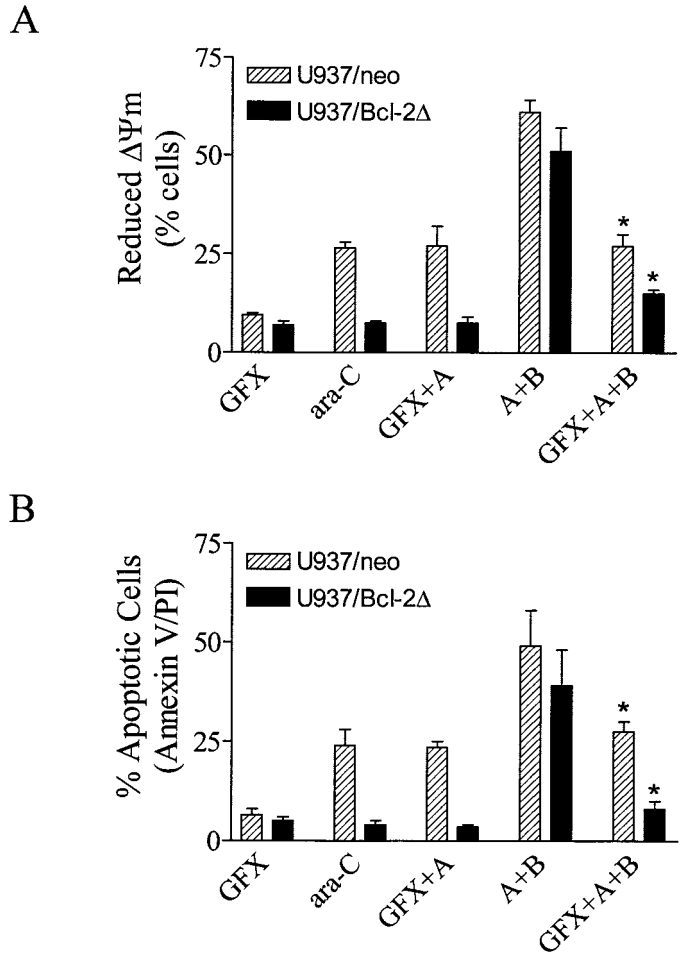

Fig. 6. PKC activation is required for potentiation of ara-C–induced apoptosis by bryostatin 1. A and B, U937/neo and U937/Bcl-2Δ cells were pretreated with the specific PKC inhibitor GFX (1 μM) for 20 min, after which the cells were treated with BRY (10 nM) ± ara-C (0.5 μM) for 24 h. The loss of $\Delta\Psi_m$ (A) and the extent of apoptosis (B) were determined by flow cytometry as above. *, $P > 0.05$, not significantly different from values obtained for cells exposed to ara-C alone. Values represent the means for triplicate experiments (± S.D.).

However, the present findings suggest that blockade of the PKC pathway plays little if any role in potentiation of ara-C–induced apoptosis by either bryostatin 1 or UCN-01. Moreover, in the case of bryostatin 1, enhanced ara-C lethality seems to be related to activation of the extrinsic apoptotic pathway via a PKC- and TNF-α–dependent process. Although most chemotherapeutic agents act primarily by triggering the intrinsic, mitochondria-dependent cascade (Sun et al., 1999), secondary engagement of the extrinsic pathway, resulting in caspase-8 cleavage and Bid activation, can amplify mitochondrial injury (e.g., cytochrome *c* release) and apoptosis (Cartee et al., 2002). However, interruption of the extrinsic pathway (e.g., by ectopic expression of CrmA or dominant-negative caspase-8) does not significantly diminish ara-C lethality, indicating that the lethal effects of this agent, when administered alone, do not involve this amplifi-

cation process. In marked contrast, potentiation of ara-C cytotoxicity was essentially abrogated by these interventions. Furthermore, U937 cells exposed to the combination of bryostatin 1 and ara-C exhibited a sustained increase in TNF-α, whereas coadministration of TNFSRs, which are known to antagonize TNF-α–related lethality (Aggarwal and Natarajan 1996), blocked the capacity of bryostatin 1 to potentiate ara-C lethality (Fig. 5A). Together, these findings are most consistent with a model in which bryostatin 1 triggers TNF-α release in ara-C–treated cells, resulting in a marked increase in mitochondrial injury, caspase activation, and apoptosis. It is noteworthy that the selective PKC inhibitor GFX blocked bryostatin 1-mediated potentiation of ara-C lethality, and that administration of PMA, which, like bryostatin 1 (Steube and Drexler, 1995), has been shown to induce TNF-α release in leukemic cells (Takada et al., 1999; Cartee et al., 2002), also resulted in enhanced ara-C killing. Together, these findings argue strongly against the possibility that down-regulation of PKC by bryostatin 1 is involved in the observed potentiation of ara-C lethality. Instead, they suggest that the initial activation of PKC by bryostatin 1, resulting in TNF-α release and engagement of the extrinsic pathway, is responsible for synergistic interactions between these agents.

In contrast to results obtained with bryostatin 1, potentiation of ara-C lethality by UCN-01 proceeded independently of the extrinsic apoptotic pathway. Thus, ara-C/UCN-01–mediated apoptosis, unlike that triggered by bryostatin 1, was unaffected by ectopic expression of CrmA or dominant-negative caspase-8, or by coadministration of TNFSRs. Although the possibility that UCN-01–mediated inhibition of PKC contributed to potentiation of ara-C–induced mitochondrial injury and apoptosis cannot be ruled out, the finding that the selective PKC inhibitor GFX failed to potentiate ara-C lethality argues against such a mechanism. Analogously, the inability of GFX to mimic UCN-01 in triggering apoptosis in human lymphoid leukemia cells (i.e., CCRF-

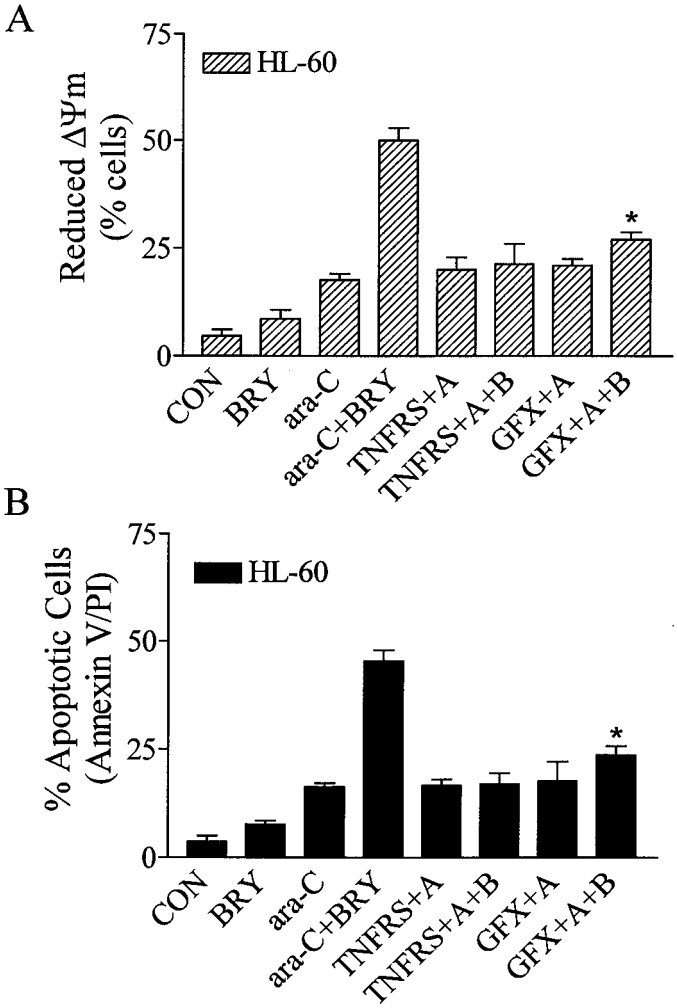

Fig. 7. Potentiation of ara-C–induced apoptosis by bryostatin 1 in HL-60 cells proceeds through a TNF-α- and PKC-dependent process. A, HL-60 cells were exposed to ara-C (0.5 μM) ± BRY (10 nM) in the presence or absence TNFSR (100 ng/ml) or GFX (1 μM) for 24 h. At the end of this period, the loss of $\Delta\Psi_m$ was determined by flow cytometry as above. B, cells were treated as above, after which they were stained with Annexin V and propidium iodide, and the extent of apoptosis was determined by flow cytometric analysis as above. Values for A and B represent the means ± S.D. for three separate experiments performed in triplicate. *, $P > 0.05$, not significantly different from values for ara-C alone.

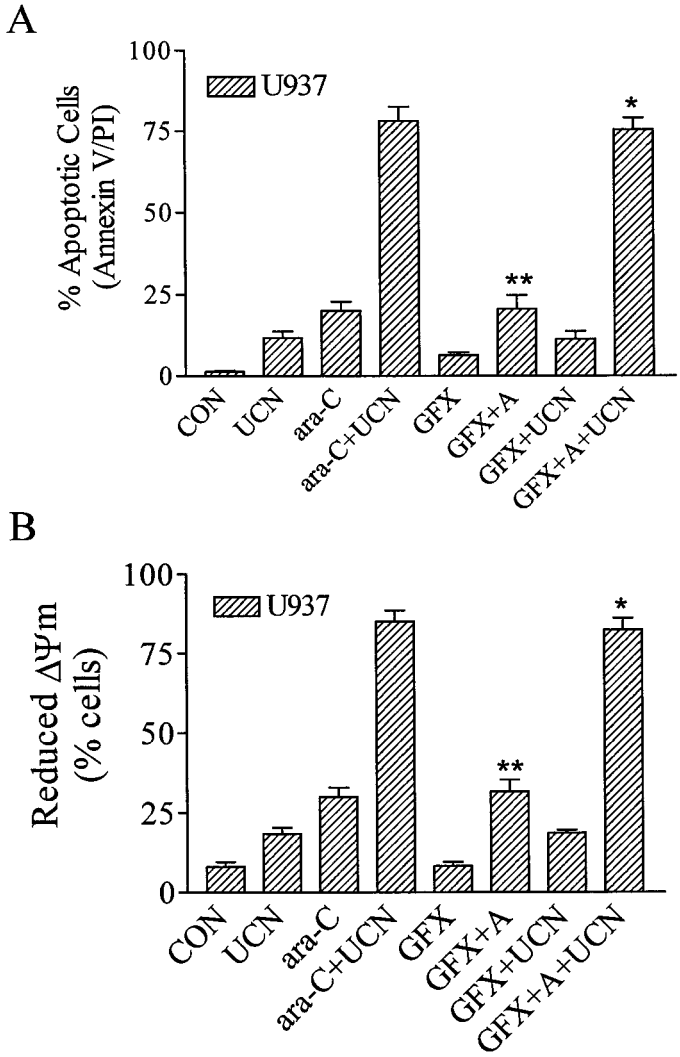

Fig. 8. GFX fails to block UCN-01–mediated potentiation of ara-C–mediated lethality in U937 cells. U937 cells were exposed to UCN-01 (100 nM) ± ara-C (0.5 μM) in the presence or absence of GFX (1 μM) for 24 h. Values represent the means for triplicate experiments (± S.D.) and are expressed as the percentage of apoptotic cells determined by Annexin V and propidium iodide (A), or the percentage of cells exhibiting low levels of $DiOC_6$, reflecting loss of mitochondrial membrane potential (B). *, $P > 0.05$; not significantly different from values obtained for cells exposed to ara-C and UCN-01. **, $P > 0.05$, not significantly different from values obtained for ara-C alone.

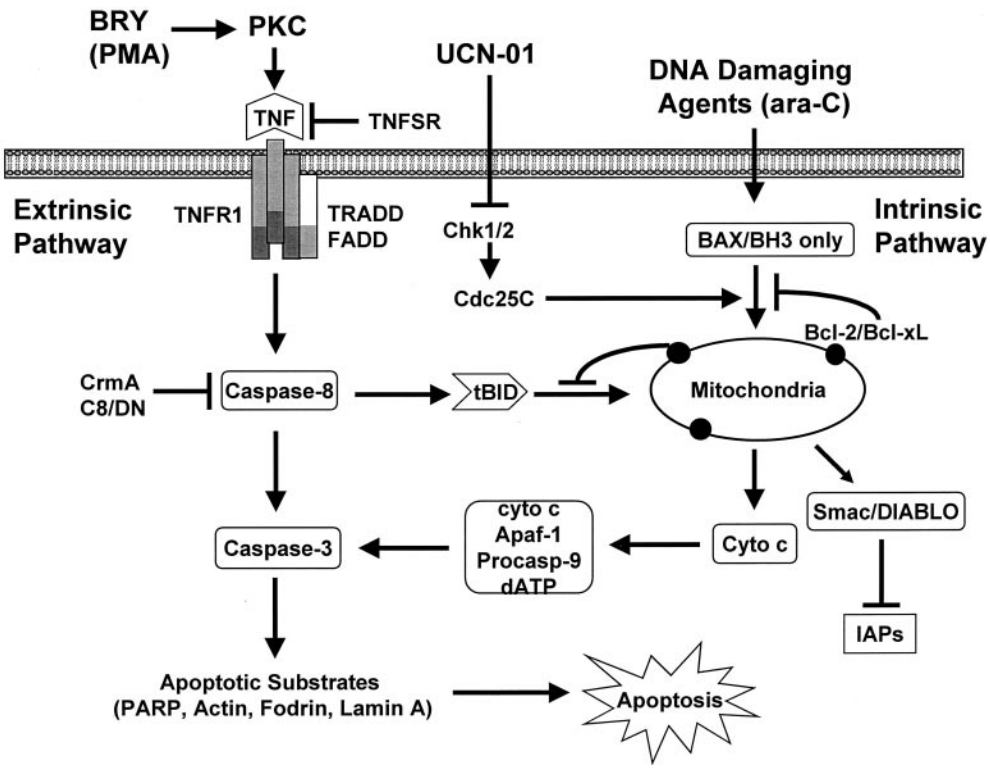

Fig. 9. Model of potentiation of ara-C–induced apoptosis by bryostatin 1 versus UCN-01. In this model, DNA-damaging agents such as ara-C trigger, by mechanisms yet to be elucidated, activation of proapoptotic proteins that induce mitochondrial damage, including release of cytochrome *c* and Smac/DIABLO, which block the actions of inhibitor of apoptosis proteins (IAPs). Cytochrome *c* forms a complex with Apaf-1 and dATP, and thereby activates procaspase-9, which in turn activates the effector caspase-3. This results in degradation of diverse cellular substrates that characterize the apoptotic process. Bryostatin 1 (and PMA) induce, through a PKC-dependent process, TNF-α, which triggers death receptor, such as tumor necrosis factor receptor 1 (TNFR1) and sequentially induces clustering and trimerization of the receptor, receptor engagement then leads to the recruitment of death domain-containing adaptor proteins such as TNF receptor-associated death domain (TRADD), or Fas-associated death domain (FADD), which interact with the prodomains of procaspase-8. This, in turn, results in cleavage and activation of procaspase-8, which induces cleavage and translocation of the BH3-only domain Bcl-2 protein tBID to the mitochondria, resulting in enhanced release of cytochrome *c*, as well as direct activation of procaspase-3. In this way, activation of the receptor-related extrinsic pathway by PKC activators amplifies the lethal effects of ara-C acting through the mitochondrial intrinsic apoptotic pathway. Such effects are inhibitable by TNFRs, acting at the level of TNF, or by ectopic expression of CrmA or dominant-negative caspase-8, which antagonize procaspase-8 activation. They are not, however, inhibited by ectopic expression of Bcl-2 or Bcl-x_L, which act primarily to limit mitochondrial injury. In contrast, UCN-01 inhibits Chk1, activates Cdc25C, and promotes ara-C–mediated activation of the intrinsic, mitochondrial pathway. In contrast to bryostatin 1, UCN-01 does not induce TNF-α, and its ability to potentiate ara-C lethality is not antagonized by interruption of the extrinsic, receptor-related pathway.

CEM) (Dai et al., 2001) suggests that the direct cytotoxic actions of the latter agent are also unrelated to PKC inhibition. Instead, the ability of submicromolar concentrations of UCN-01 to inhibit Chk1, and in so doing, to abrogate the G_1 checkpoint in leukemic cells exposed to S-phase agents (Graves et al., 2000; Shi et al., 2001) represents a more likely explanation for the observed potentiation of ara-C–related apoptosis.

The disparate mechanisms of action of bryostatin 1 and UCN-01 were further highlighted by the finding that bryostatin 1, but not UCN-01, potentiated ara-C lethality in leukemic cells ectopically expressing a Bcl-2 mutant protein lacking the N-terminal phosphorylation loop region (Chang et al., 1997). The impact of Bcl-2 phosphorylation on the antiapoptotic actions of this protein seems to be cell type- and stimulus-dependent. For example, the bulk of evidence suggests that in the case of taxanes, the phosphorylation of Bcl-2 contributes to induction of cell death (Wang et al., 1999b). Moreover, potentiation of ara-C–induced apoptosis by both bryostatin 1 and UCN-01 in Bcl-2–overexpressing human leukemia cells was associated with enhanced phosphorylation of Bcl-2 (Wang et al., 1997). On the other hand, in murine hematopoietic cells, Bcl-2 phosphorylation (i.e., by bryostatin 1) antagonizes cell death, at least in the setting of growth factor deprivation (Deng et al., 1998). In any case, the observation that loss of the phosphorylation loop domain enhances the antiapoptotic actions of this protein against diverse stimuli, including taxanes, ara-C, and growth factor deprivation (Chang et al., 1997; Wang et al., 1999a; Tang et al., 2000) are most consistent with a proapoptotic role for Bcl-2 phosphorylation. However, in view of evidence that Bcl-2 acts by interfering with interactions between BH3-only domain proteins and the pro-apoptotic proteins Bax and Bak (Cheng et al., 2001), the possibility exists that deletion of the N-terminal loop region induces conformational changes in the protein that enhance such actions. Whichever mechanism was responsible for the enhanced antiapoptotic properties of the Bcl-2Δ protein, it was unable to prevent bryostatin 1/ara-C–induced mitochondrial damage, caspase activation, and apoptosis, whereas that induced by UCN-01/ara-C was substantially attenuated. Interestingly, the ability of bryostatin 1 to potentiate ara-C lethality in Bcl-2Δ–expressing cells was mimicked by PMA and largely abrogated by the PKC inhibitor GFX or administration of TNFSRs. Given evidence that PMA and bryostatin 1 both trigger TNF-α release (Takada et al., 1999; Cartee et al., 2002), such find-

ings argue that these agents potentiate ara-C lethality in otherwise resistant Bcl-2Δ–expressing cells by engaging the TNF-related extrinsic pathway, against which the loop-deleted protein is unable to act. The observation that ectopic Bcl-2Δ expression effectively blocked apoptosis induced by UCN-01/ara-C, a phenomenon that was unaffected by TNF-SRs and presumably TNF-α–independent, is consistent with this model.

In summary, the present findings indicate that despite their shared capacity to interrupt the PKC cytoprotective pathway, bryostatin 1 and UCN-01 promote ara-C–induced lethality through fundamentally different mechanisms. More specifically, they suggest that potentiation of ara-C–related apoptosis by bryostatin 1 in human myeloid leukemia cells does not stem from PKC down-regulation but instead represents a consequence of the PKC-dependent release of TNF-α and resulting activation of the extrinsic apoptotic cascade. Moreover, the inability of the specific PKC inhibitor GFX to mimic the actions of UCN-01 argues that potentiation of ara-C lethality by the latter agent involves actions other than PKC inhibition (e.g., checkpoint abrogation) (Graves et al., 2000; Shi et al., 2001). Lastly, the capacity of PKC activators such as bryostatin 1 and PMA, which induce TNF-α release, to enhance ara-C toxicity in cells ectopically expressing an N-terminal loop-deleted Bcl-2 protein suggests that this phenomenon primarily results from engagement of the extrinsic apoptotic cascade rather than from perturbations in Bcl-2 phosphorylation status. A model system delineating the disparate apoptotic pathways involved in potentiation of ara-C–induced apoptosis by bryostatin 1 versus UCN-01 is shown in Fig. 9. Taken together, these findings underscore the importance of examining alternative mechanisms through which known PKC inhibitors/down-regulators promote drug-induced mitochondrial injury and apoptosis in leukemic and possibly other malignant cell types. In this regard, the results from a recent clinical trial demonstrated that in vivo administration of bryostatin 1 exerted highly variable effects on PKC down-regulation in leukemic blasts as well as ex vivo responses of these cells to ara-C (Cragg et al., 2002). Such findings raise the possibility that bryostatin 1-mediated potentiation of ara-C sensitivity in primary human leukemic cells may also depend upon the induction of TNF-α. Accordingly, studies designed to test this hypothesis are currently underway.

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