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Potentiation of 1-β-D-Arabinofuranosylcytosine-Mediated Mitochondrial Damage and Apoptosis in Human Leukemia Cells (U937) Overexpressing Bcl-2 by the Kinase Inhibitor 7-Hydroxystaurosporine (UCN-01)*

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ABSTRACT. Antileukemic interactions between the nucleoside analog 1-β-D-arabinofuranosylcytosine (ara-C) and the kinase inhibitor 7-hydroxystaurosporine (UCN-01) have been examined in relation to Bcl-2 expression/phosphorylation, mitochondrial damage, caspase activation, and loss of clonogenic potential. Subsequent exposure of ara-C-pretreated U937 cells (1 μM; 6 hr) to UCN-01 (300 nM; 24 hr) resulted in marked potentiation of pro-caspase-3 and -9 cleavage/activation, poly(ADP-ribose)polymerase degradation, diminished mitochondrial membrane potential ($\Delta\psi_m$), enhanced cytochrome *c* release, reduction in the S-phase fraction, and induction of classic apoptotic morphologic features. Enforced expression of full-length Bcl-2 significantly protected cells (at 24 hr) from ara-C/UCN-01-induced caspase activation and apoptosis, but was ineffective in preventing loss of $\Delta\psi_m$ and cytochrome *c* release. Ectopic expression of a Bcl-2 N-terminal phosphorylation loop-deleted protein (Bcl-2Δ_{32-80}) was more potent than its full-length counterpart in blocking drug-induced loss of $\Delta\psi_m$, caspase activation, and apoptotic morphology, but not cytochrome *c* release. Examination of cells at later intervals revealed that ectopic expression of Bcl-2 or Bcl-2Δ_{32-80} could only delay, but not prevent, mitochondrial damage, caspase activation, and cell death induced by ara-C/UCN-01 treatment. Despite their initial ability to inhibit apoptosis, neither full-length nor truncated Bcl-2 protein restored clonogenic potential to drug-treated cells. These findings indicate that subsequent exposure of ara-C-pretreated human leukemia cells to UCN-01 potently triggers mitochondrial damage and apoptosis, and that these events are postponed but not prevented by ectopic expression of Bcl-2 or its phosphorylation loop-deleted counterpart. BIOCHEM PHARMACOL **60**;10:1445–1456, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. apoptosis; Bcl-2; ara-C; UCN-01; leukemia; PKC

The antimetabolite ara-C†† is among the most effective agents available for the treatment of AML. The ability of ara-C, as well as numerous other cytotoxic agents, to kill neoplastic cells has been linked to the induction of an organized program of cell suicide referred to as apoptosis [1]. In many model systems, apoptotic stimuli have been shown to act by triggering mitochondrial damage, including loss of the mitochondrial membrane potential ($\Delta\psi_m$) and/or release of cytochrome *c* into the cytosol [2]. These events ultimately culminate in cleavage/activation of apoptotic effector caspases, e.g. pro-caspase-3 (apopain; CPP32), which are directly responsible for cellular degradation and disassembly [3].

Bcl-2 and related family members represent a group of interacting pro- and anti-apoptotic proteins homologous to the products of the *Caenorhabditis elegans* death gene family. Of these, Bcl-2 and Bcl-x_L block apoptosis triggered by diverse noxious stimuli, including cytotoxic drugs [4]. The mechanism by which such anti-apoptotic proteins act remains uncertain, but may involve modulation of proton transport [5], antagonism of loss of $\Delta\psi_m$ and/or cytochrome *c* release [6], or interference with activation of pro-caspase-3 by caspase-9 [7], among other possibilities. The potential significance of these findings is underscored by studies demonstrating that the expression of Bcl-2 in

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†† *Abbreviations*: ara-C, 1-β-D-arabinofuranosylcytosine; AML, acute myelogenous leukemia; PKC, protein kinase C; CDKs, cyclin-dependent kinases; $DiOC_6$, 3,3-dihexyloxacarbocyanine iodide; FBS, fetal bovine serum; and PARP, poly(ADP-ribose)polymerase.

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leukemic blasts correlates inversely with responsiveness to chemotherapy [8], raising the possibility that defects in the distal cell death pathway may play an important role in determining clinical outcome, at least in AML.

7-Hydroxystaurosporine (UCN-01) is an inhibitor of PKC as well as other kinases, and currently is undergoing Phase I evaluation in humans [9]. This agent induces apoptosis in Jurkat lymphoblastic leukemia cells in association with dephosphorylation of the CDKs CDK1 ($p34^{cdc2}$) and CDK2 [10]. Since phosphorylation of CDKs (on thr_{14} or tyr_{15}) exerts a negative effect on the activity of cyclin/CDK complexes [11], UCN-01 can therefore act as a cell-cycle checkpoint inhibitor. In this regard, UCN-01 has been shown recently to abrogate G_2 arrest by inactivating the Wee1Hu kinase, thereby activating the Cdc25C phosphatase [12]. The resulting loss of checkpoint control may contribute to potentiation of the lethal actions of alkylating agents such as mitomycin C and *cis*-platinum, the S-phase-specific agent camptothecin, and ionizing radiation [13–15].

In a previous article [17], we reported that bryostatin 1, a PKC activator and down-regulator [16], promotes ara-C-induced apoptosis in human promyelocytic leukemia cells (HL-60) overexpressing the Bcl-2 protein, and that this phenomenon is related, at least temporally, to Bcl-2 phosphorylation. Moreover, these actions are mimicked by PKC inhibitors including staurosporine and UCN-01 [17]. Collectively, such findings raise the possibility that agents that interrupt kinase pathways may circumvent Bcl-2-mediated drug resistance through a mechanism involving Bcl-2 phosphorylation. Currently, little information is available concerning the functional consequences of these events on leukemic cell clonogenic survival, particularly in relation to induction of mitochondrial dysfunction. The purpose of the present study was to characterize further the biologic consequences of combined exposure of Bcl-2-overexpressing monocytic leukemia cells (U937) to ara-C and UCN-01, with the goal of defining the relationship between mitochondrial damage, apoptosis, and the loss of leukemic cell self-renewal capacity. A second aim was to employ a recently described Bcl-2 variant lacking the N-terminal phosphorylation loop domain ($Bcl\text{-}2\Delta_{32-80}$) [18] to define more rigorously the functional role of Bcl-2 phosphorylation in modulation of ara-C-mediated lethality by UCN-01.

MATERIALS AND METHODS

Drugs

UCN-01 was provided by Dr. Edward Sausville (Division of Clinical Sciences, National Cancer Institute). It was stored frozen as a 10 mM stock solution in DMSO in light-protected microcentrifuge tubes at −20°, and subsequently was diluted in sterile PBS (1x = 137 mM NaCl, 2.7 mM KCl, 10.2 mM Na_2HPO_4, 1.76 mM KH_2PO_4) prior to each experiment. Ara-C was purchased from the Sigma Chemical Co. and maintained as a dry powder at −20°. It was reconstituted in sterile PBS prior to use.

Cells and Culture Conditions

The human monocytic leukemic cell line U937 was obtained from the American Type Culture Collection and was cultured in logarithmic growth phase in RPMI 1640 medium (GIBCO) supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin (all from GIBCO), and 10% (v/v) heat-inactivated FBS (Hyclone). U937 cells were transfected by electroporation as previously described in detail [17] with plasmids containing either full-length *Bcl-2* cDNA (provided by Dr. M. Cleary, Stanford University) or an HA-tagged *Bcl-2* lacking residues 32–80 encoding the phosphorylation loop domain (provided by Dr. C. Thompson, University of Chicago) along with either a hygromycin or a neomycin selection marker. This loop encompasses the $serine_{70}$ phosphorylation site implicated in regulation of Bcl-2 function. Single cells were obtained by limiting dilution and expanded under selection pressure in medium containing either 400 μg/mL of hygromycin B (Boehringer-Mannheim Biochemicals) or 400 μg/mL of G418 (GIBCO). Transfectant cell lines are termed U937/pCEP4, U937/Bcl-2, U937/pSFFV, and U937/Bcl-2Δ. All cell lines were cultured in a 37°, 5% CO_2, fully humidified incubator, and cells were passaged twice weekly.

Experimental Format

Logarithmically growing cells (approximately 4×10^5 cells/mL) were placed in 25- or 75-cm^2 T-flasks (Greiner Labortechnik) and incubated with 1 μM ara-C for 6 hr, after which cells were washed three times with serum-free medium, and resuspended in fresh medium containing 10% (v/v) FBS at 2×10^5 cells/mL in the presence or absence of 300 nM UCN-01 for various intervals (generally 24 hr). At the end of the incubation period, cells were harvested and prepared for analysis as described below. In separate studies, we determined that this sequence of drug administration resulted in a greater extent of apoptosis than exposure of cells to ara-C and UCN-01 simultaneously, or to the sequence UCN-01→ara-C (data not shown). Consequently, this experimental design was utilized in all subsequent studies.

Assessment of Apoptosis

Following the indicated drug exposure, morphologic evidence of apoptosis was monitored by evaluating cytocentrifuge preparations stained with the Diff-Quik stain set (Dade Diagnostics) and viewed under light microscopy. The percentage of apoptotic cells was determined by scoring the number of cells exhibiting classic features of apoptosis (e.g. cell shrinkage, nuclear condensation, and extensive formation of membrane blebs and apoptotic

bodies) as previously described [17]. Five different fields were selected randomly, and at least 500 cells were scored for each drug treatment.

Clonogenic Assays

A modification of a previously described method was employed [19]. Briefly, following drug treatment, cells were washed three times with serum-free medium, cell numbers were normalized, and 500 cells/well were plated in 12-well plates (Costar) containing RPMI medium, 20% FBS, and 0.3% (w/v) agar (Sigma). The plates were incubated in a 37°, 5% CO_2, fully humidified incubator for 10–12 days, after which colonies, consisting of groups of ≥50 cells, were scored using an Olympus model CK inverted microscope (Olympus).

Ara-CTP formation

Following drug exposure, pelleted cells were rinsed in cold PBS, repelleted, lysed in 0.6 N trichloroacetic acid, and extracted in 1:3.5 trioctylamine:1,1,2-trichlorotrifluoroethane (Sigma). The aqueous phase was stored at −80° until analysis. Pyrimidine nucleotide extracts were prepared and analyzed as we described previously [19]. Extracts were separated on a Waters Radial-Pak 10 μ SAX cartridge (Waters, Millipore), and absorbance was monitored at 254 nm using a Beckman 160 detector (Beckman). Retention times for ara-CTP and dCTP were 32.8 and 28.2 min, respectively. Levels of ara-CTP and dCTP in cell samples were determined by comparing peak areas under the curve with those of known standards (Sigma). Values are expressed as picomoles ara-CTP/20 × 10^6 cells.

Western Analysis

After treatment, whole-cell pellets (1 × 10^7 cells/condition) were washed twice in PBS, resuspended in 50 μL PBS, lysed by the addition of 50 μL of 2x sample buffer [1x = 30 mM Tris (pH 6.8), 2% SDS, 2.88 mM β-mercaptoethanol, 10% (v/v) glycerol]. The lysates were sonicated, boiled for 5 min, and centrifuged at 12,800 *g* for 5 min, and protein was quantified using Coomassie protein assay reagent (Pierce). Equal amounts of protein (25 μg) were separated by SDS–PAGE, transferred electrophoretically to Optitran nitrocellulose filters (Schleicher & Schuell), and blocked in PBS–Tween (PBS–T; 0.05%)/5% (w/v) dry milk for 1 hr at 22°. The blots were probed for 4 hr at 22° or overnight at 4° with primary antibodies: Bcl-2 (1:2000, DAKO), Bcl-x_L (1:1000, Santa Cruz Biotechnology), Bax (1:1000, Santa Cruz Biotechnology), CPP32 (1:1000, Transduction Laboratories), and PARP (1:2000, BIOMOL Research Laboratories). Blots were subsequently washed three times for 5 min each in PBS–T and incubated with horseradish peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories) in PBS–T for 1 hr at 22°. The blots again were washed 3 × 5 min each in PBS–T and developed with the enhanced chemiluminescence method (Amersham). Equivalent loading and protein transfer were documented by reprobing blots with an anti-tubulin antibody (1:2000, Calbiochem).

In vivo *Labeling, Immunoprecipitation, and Western Analysis*

Briefly, U937/Bcl-2 and U937/Bcl-2Δ cell lines were washed twice and maintained in phosphate-free RPMI medium containing 10% (v/v) dialyzed FBS. Inorganic [^{32}P]orthophosphate was added to each culture (1 mCi/mL), and samples were incubated at 37°. After appropriate drug treatments, cells were pelleted, washed once with phosphate-free RPMI, repelleted, and snap-frozen in liquid N_2. Cell lysis and homogenization were carried out in 0.2 mL of ice-cold lysis buffer [1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 2 mM EDTA, 2 mM EGTA, 25 mM β-glycerolphosphate, 0.5 mM sodium pyrophosphate, 25 mM sodium fluoride, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate, 1 μg/mL of pepstatin A, 0.1% (w/v) aprotinin, 0.7 μg/mL of leupeptin], with gentle trituration using a P200 pipet to lyse the cells. Lysed samples were centrifuged at 15,000 *g* for 20 min to remove nuclei and cellular debris. Lysates (400 μg) were incubated with specific monoclonal anti-Bcl-2 and polyclonal anti-Bcl-2 antibodies (Santa Cruz Biotechnology, Inc.) overnight, and the immunoprecipitates were captured with goat anti-mouse and sheep anti-rabbit Dynabeads (Dynal). The immunocomplexes were washed with lysis buffer 3–5 times, eluted from the magnetic beads by boiling in 1 x SDS sample buffer for 10 min, and electrophoresed on 15% (v/v) SDS–polyacrylamide gels. The gels were dried and visualized with a PhosphorImager (Molecular Dynamics).

Preparation of S-100 Fraction and Western Analysis for Cytochrome C and Caspase 9

Following the designated treatment, 20 × 10^6 cells were harvested by centrifugation at 800 *g* for 10 min at 4°. The cell pellets were washed twice with ice-cold PBS and resuspended with 5 vol. of buffer A [20 mM HEPES–KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride] containing 250 mM sucrose. The cells were incubated on ice for 30 min, and homogenized with a 25-gauge needle. The homogenates were centrifuged at 100,000 *g* for 30 min at 4°. The supernatants (S-100 fraction) were collected, and the protein concentration of S-100 was quantified using the Coomassie protein assay reagent. Twenty-five micrograms of the S-100 fraction was used for western analysis of cytochrome *c* (1:1000) and caspase-9 (1:2000, Pharmingen) as described previously.

Assessment of Mitochondrial Membrane Potential ($\Delta\psi_m$)

Mitochondrial membrane potential was monitored using $DiOC_6$. For each condition, 4×10^5 cells were incubated for 15 min at 37° in 1 mL of 40 nM $DiOC_6$ and subsequently analyzed using a Becton-Dickinson FACScan cytofluorometer with excitation and emission settings of 488 and 525 nm, respectively. Values were expressed as the percentage of cells displaying reduced levels of $DiOC_6$ uptake relative to untreated control cells. Control experiments documenting the loss of $\Delta\psi_m$ were performed by exposing cells to 5 μM carbamoyl cyanide *m*-chlorophenylhydrazone (15 min, 37°), an uncoupling agent that abolishes the $\Delta\psi_m$.

Cell Cycle Analysis

Following treatment, cells (2×10^6) were collected by centrifugation at 400 *g* for 5 min at 4°, and resuspended in 1.5 mL of cold PBS. Cells were fixed by the addition of 3 mL of 100% (v/v) cold ethanol. After a 1-hr fixation on ice, cells were pelleted, resuspended in 1 mL of cell cycle buffer (3.8 mM sodium citrate, 0.5 mg/mL of RNAse A, and 0.01 mg/mL of propidium iodide), and incubated on ice for an additional 3 hr. Finally, cells were pelleted, resuspended in 1 mL PBS, and analyzed for cell cycle distribution using a Becton-Dickinson FACScan flow cytometer and ModFit LT 2.0 software (Verity).

Statistical Analysis

The significance of differences between experimental values was determined utilizing Student's *t*-test for paired or unpaired observations.

RESULTS

Western analysis revealed that levels of Bcl-2 expression were increased substantially in U937/Bcl-2 cells compared with either untransfected cells or their empty-vector counterparts (U937/pCEP4; Fig. 1A). In contrast, expression of Bcl-x_L and Bax was equivalent in the three lines. Following a 24-hr exposure to various concentrations of UCN-01, Bcl-2-overexpressing cells clearly were protected from UCN-01-mediated apoptosis; in fact, UCN-01 concentrations = 500 nM induced minimal apoptosis in U937/Bcl-2 cells (Fig. 1B). Similarly, Bcl-2 overexpression substantially protected U937 cells from apoptosis induced by a 6-hr exposure to various concentrations of ara-C, with the maximal extent of apoptosis (~20%) occurring at ara-C concentrations ≥ 10 μM (Fig. 1C). Similar results were obtained with a second Bcl-2-overexpressing cell line (U937/Bcl-2D9; data not shown). In these, as well as in other studies, morphologic assessment of apoptosis correlated closely with the results obtained utilizing a FITC–dUTP/terminal transferase (TUNEL) assay (data not shown). Thus, ectopic expression of Bcl-2 protected U937 cells from both UCN-01- and ara-C-mediated cell death.

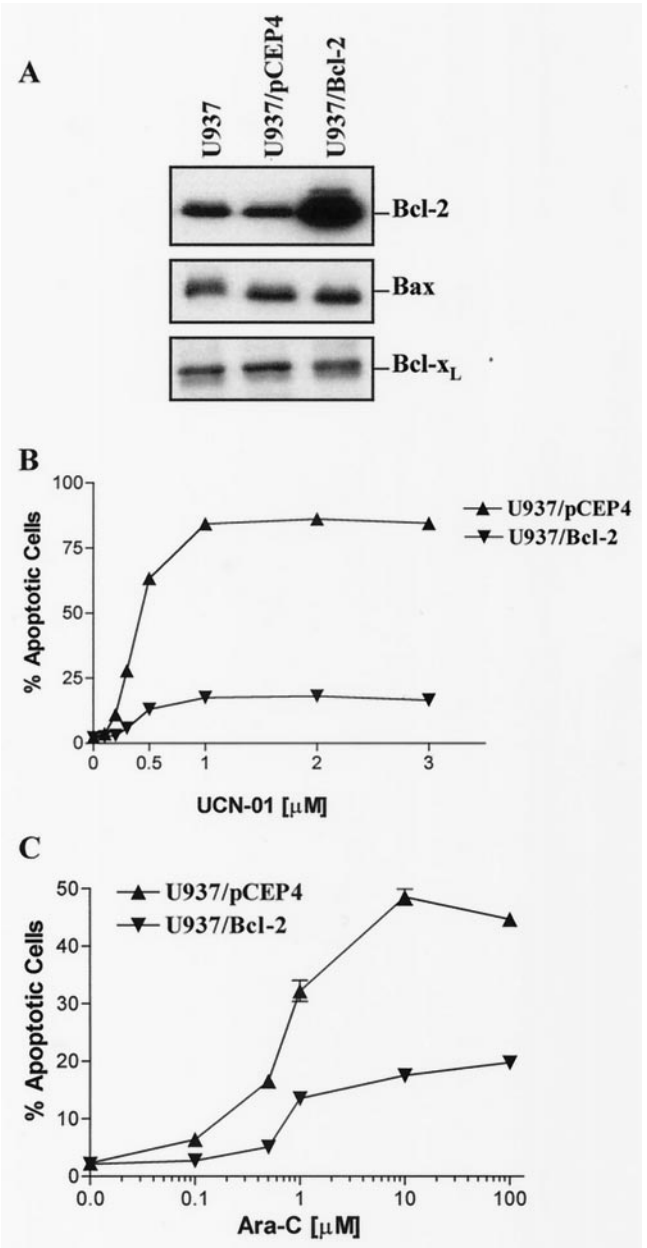

FIG. 1. Western analysis of Bcl-2, Bax, and Bcl-x_L expression and concentration–response relationship of UCN-01 and ara-C-mediated apoptosis. (A) U937 cells were transfected with empty vector (pCEP4) or a vector containing the human *Bcl-2* cDNA. Protein was extracted from parental U937, U937/pCEP4, and U937/Bcl-2 cells, and expression of Bcl-2, Bax, and Bcl-x_L (25 μg protein/lane) was assessed by western analysis as described in the text. U937/pCEP4 and U937/Bcl-2 cells were exposed to the indicated concentrations of either UCN-01 (24 hr) (B) or ara-C (6 hr) (C), washed, and subsequently incubated in drug-free medium for an additional 24 hr. The percentage of apoptotic cells was assessed by monitoring Diff-Quik-stained specimens for the characteristic morphologic features of apoptosis as described in the text. Values represent the means ± SD for three separate experiments.

To determine whether subsequent exposure of ara-C-

pretreated cells to UCN-01 could overcome the protective effects of ectopic Bcl-2 expression, U937/pCEP4 and U937/Bcl-2 cells were exposed to 1 μM ara-C for 6 hr, washed, and incubated in drug-free medium or medium containing 300 nM UCN-01 for an additional 24 hr, after which the percentage of apoptotic cells was determined (Fig. 2A). Several findings emerged from these studies. First, sequential exposure of empty vector controls to the sequence ara-C followed by UCN-01 induced apoptosis in the large majority of cells (e.g. ~90%). Second, ectopic expression of Bcl-2 clearly protected U937 cells from death induced by the combination of ara-C and UCN-01. Nevertheless, in U937/Bcl-2 cells, a marginally toxic concentration of UCN-01 (300 nM) increased apoptosis in ara-C-pretreated cells to levels approximating those observed in empty vector controls exposed to ara-C alone (e.g. ~36%; $P > 0.05$). In contrast, pretreatment of U937/Bcl-2 cells with UCN-01 (for 24 hr) did not enhance apoptosis induced by subsequently administered ara-C (data not shown). Consistent with these results, sequential exposure of Bcl-2-overexpressing cells to the sequence of ara-C followed by UCN-01 led to a small reduction in levels of full-length pro-caspase-3, a phenomenon that reflects enzyme cleavage and activation [20], although the extent of the reduction was clearly less than that observed in wild-type cells, in which the full-length caspase-3 was essentially completely cleaved (Fig. 2B). In addition, subsequent exposure of U937/Bcl-2 cells to UCN-01 increased the ability of ara-C to induce cleavage of the major pro-caspase-3 substrate PARP (Fig. 2B). Finally, subsequent exposure of ara-C-pretreated U937/Bcl-2 cells to UCN-01 resulted in cleavage of 48-kDa full-length pro-caspase-9 to its active 37-kDa fragment (Fig. 2C), although the extent to which this occurred was not as great as that observed in the empty-vector control line. Similar results were obtained in other Bcl-2-overexpressing cell lines (data not shown). Thus, analogous to results obtained in HL-60 cells *pretreated* with bryostatin 1 [17], subsequent exposure of U937 cells to UCN-01 was able to overcome, at least in part, inhibition of ara-C-mediated caspase activation and apoptosis conferred by Bcl-2 overexpression.

The cell cycle distribution of cells exposed to ara-C (6 hr) alone or followed by UCN-01 (24 hr) was then examined (Fig. 3). As might be predicted from the results shown in Fig. 2A, a significant fraction of U937/Bcl-2 cells, and the large majority of U937/pCEP4 cells, exhibited subdiploid quantities of DNA (data not shown). To simplify interpretation of results, the sub-G_1 apoptotic fraction was excluded from the analysis. The most striking finding was that in U937/pCEP4 cells exposed to the sequence ara-C→UCN-01, essentially all of the residual (non-apoptotic) cells were in G_0G_1, and the S-phase population was diminished substantially. In marked contrast, U937/Bcl-2 cells surviving exposure to ara-C→UCN-01 resided largely in S-phase (e.g. >50%), whereas the G_0G_1 population was reduced relative to untreated controls. A qualitatively similar pattern was observed in cells exposed to ara-C alone. These findings suggest, although indirectly, that ectopic expression of Bcl-2 protects S-phase U937 cells from the lethal actions of ara-C ± UCN-01.

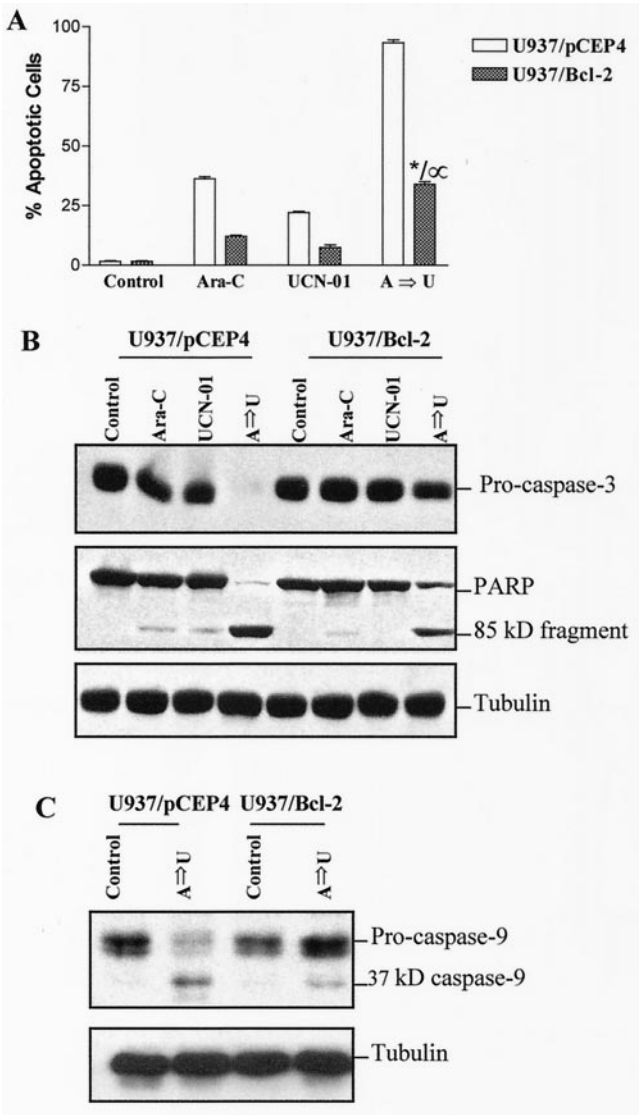

FIG. 2. Impact of Bcl-2 overexpression on apoptosis and caspase activation induced by ara-C→UCN-01. U937/pCEP4 and U937/Bcl-2 cells were exposed to 1 μM ara-C for 6 hr, washed, and then incubated with either drug-free medium or medium containing 300 nM UCN-01 for an additional 24 hr. (A) The percentage of apoptotic cells was determined by morphological assessment as described in the text. Values from triplicate experiments were expressed as means ± SD. Key: (*) significantly less than values for U937/pCEP4 cells exposed to the sequence ara-C→UCN-01 ($P = 0.01$); and (∝) not significantly different from values obtained for U937/pCEP4 cells exposed to ara-C alone ($P > 0.05$). Alternatively, extracted protein (25 μg/condition) from cells treated with ara-C, UCN-01, or the sequence ara-C→UCN-01 was subjected to western analysis using antibodies to pro-caspase-3 or PARP (B) or to pro-caspase-9 (C) as described in the text. Blots were stripped and re-probed with antibodies to tubulin to document equal loading and transfer.

To determine whether enhanced apoptosis in cells treated with ara-C and UCN-01 might stem from pharma-

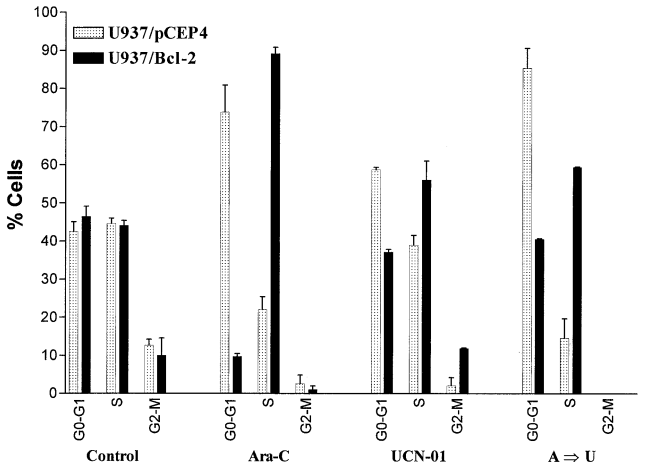

FIG. 3. U937/pCEP4 and U937/Bcl-2 cell cycle distribution. Logarithmically growing cells were exposed to ara-C (1 μM) for 6 hr, washed, and subsequently incubated in medium in the presence or absence of UCN-01 (300 nM) for an additional 24 hr. Cells then were stained with propidium iodide and subjected to cell cycle analysis as described in the text. Values, corresponding to the percentage of residual (non-apoptotic) cells in G_0-G_1, S, or G_2-M, represent the means ± SD for three separate experiments.

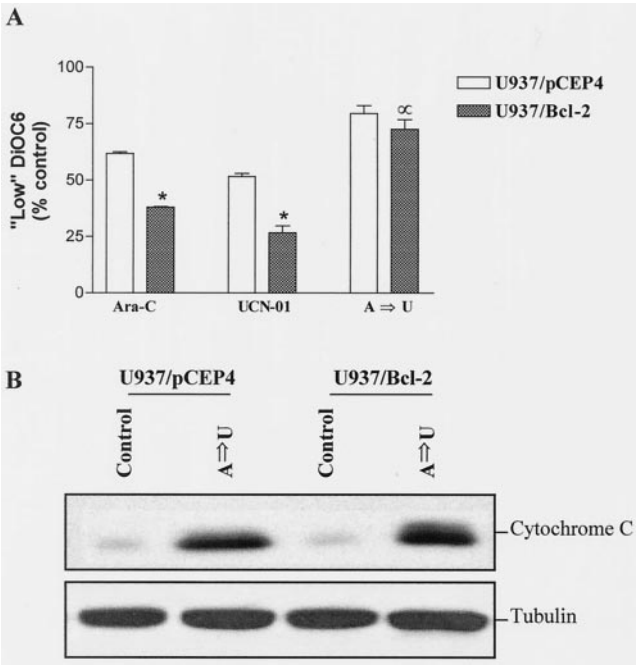

FIG. 4. Effects of drug treatment on mitochondrial dysfunction. Cells were exposed to ara-C (1 μM) for 6 hr, washed, and subsequently incubated in either drug-free medium or medium containing UCN-01 (300 nM) for an additional 24 hr. Then the cells were harvested and exposed to 40 nM $DiOC_6$, and the decrease in $\Delta\psi_m$ was analyzed by flow cytometry (A) as described in the text. Values, expressed as the percentage of cells exhibiting a decrease in $DiOC_6$ uptake relative to untreated controls, represent the means ± SD for three independent experiments. Key: (*) significantly less than values for U937/pCEP4 cells exposed to ara-C or UCN-01 alone ($P < 0.02$); and (∝) not significantly less than values for U937/pCEP4 cells exposed to ara-C→UCN-01 ($P > 0.05$). Alternatively, western analysis was employed (B) to assess the release of cytochrome c into the cytosolic S-100 fraction after treatment of cells with drugs as described above. Each lane was loaded with 25 μg protein. The results of a representative experiment are shown; two additional studies yielded equivalent results.

codynamic factors, levels of the lethal ara-C metabolite, ara-CTP, as well as the naturally occurring competing deoxyribonucleotide, dCTP, were examined. In both pCEP4 and Bcl-2-overexpressing cells, subsequent treatment with UCN-01 for 24 hr failed to increase ara-CTP retention. For example, in U937/Bcl-2 cells exposed to 1 μM ara-C for 6 hr followed by UCN-01, intracellular ara-CTP levels were 41.3 ± 11.6 versus 31.8 ± 8.6 pmol ara-CTP/10^7 cells for cells subsequently incubated in drug-free medium ($P > 0.05$; data not shown). Moreover, ratios of ara-CTP/dCTP were, if anything, less in cells exposed to ara-C and UCN-01 than in cells exposed to ara-C alone (data not shown). Thus, enhanced lethality of the combination of ara-C and UCN-01 was unlikely to reflect potentiation of ara-C metabolism.

In view of evidence linking apoptosis to mitochondrial dysfunction [21], loss of $\Delta\psi_m$ was monitored in cells exposed to ara-C and UCN-01 alone and in combination (Fig. 4A). For ara-C and UCN-01 administered individually, loss of $\Delta\psi_m$ at the 24-hr interval was attenuated significantly in cells overexpressing Bcl-2 ($P = 0.02$ in each case), although protection was incomplete. However, following sequential exposure of U937/pCEP4 and U937/Bcl-2 cells to ara-C and UCN-01, loss of $\Delta\psi_m$ was more pronounced and did not differ significantly between the two lines ($P = 0.05$). Consistent with effects on $\Delta\psi_m$, sequential exposure to ara-C followed by UCN-01 was equally effective in inducing release of cytochrome *c* into the S-100 cytosolic fraction at 24 hr in empty-vector control cells and cells ectopically expressing Bcl-2 (Fig. 4B). Thus, while Bcl-2 overexpression substantially blocked the morphologic features of apoptosis in cells exposed to ara-C and UCN-01 (Fig. 2A), it was relatively ineffective in preventing mitochondrial dysfunction induced by this drug combination, at least at the 24-hr time interval.

Studies from other laboratories [22], as well as our own [17], have shown that inhibition or down-regulation of PKC leads to potentiation of apoptosis, and that this phenomenon may be accompanied by Bcl-2 phosphorylation. However, the functional role of Bcl-2 phosphorylation in modulation of the apoptotic threshold remains unclear. To address this issue, U937 cells were stably transfected with an expression construct encoding a truncated Bcl-2 protein lacking the N-terminal phosphorylation loop domain (Bcl-$2\Delta_{32-80}$). This protein has been shown to be more effective than its full-length counterpart in protecting cells from certain noxious stimuli (e.g. growth factor deprivation) [18]. Western analysis of three stably transfected clones, designated 2B9, 2C9, and 2G10 (Fig. 5A), demonstrated the appearance of a rapidly migrating 19-kDa loop-deletant species, as well as a marked reduction in levels of the endogenous 26-kDa Bcl-2 protein, a phenom-

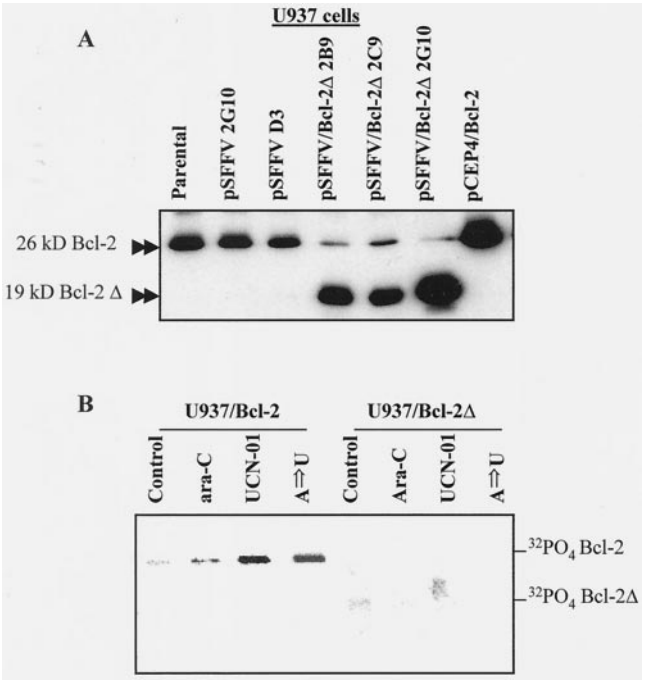

FIG. 5. Western analysis of Bcl-2 and Bcl-2Δ protein expression and *in vivo* $^{32}PO_4$ incorporation. (A) Protein (25 μg/condition) isolated from U937 cells transfected with either an empty vector (pSSFV), the cDNA encoding the loop deletant Bcl-2Δ_{32-80}, or full-length Bcl-2 was analyzed by western blot as described in Materials and Methods. Results for three separate loop-deletant clones (2B9, 2C9, and 2G10) are shown. (B) Cells were labeled with $^{32}PO_4$ as described in the text, and exposed to ara-C, UCN-01, or the sequence ara-C→UCN-01. Bcl-2 and Bcl-2Δ immunoprecipitates (400 μg protein/condition) then were separated by SDS–PAGE and monitored for incorporation of labeled $^{32}PO_4$ by autoradiography as described in the text.

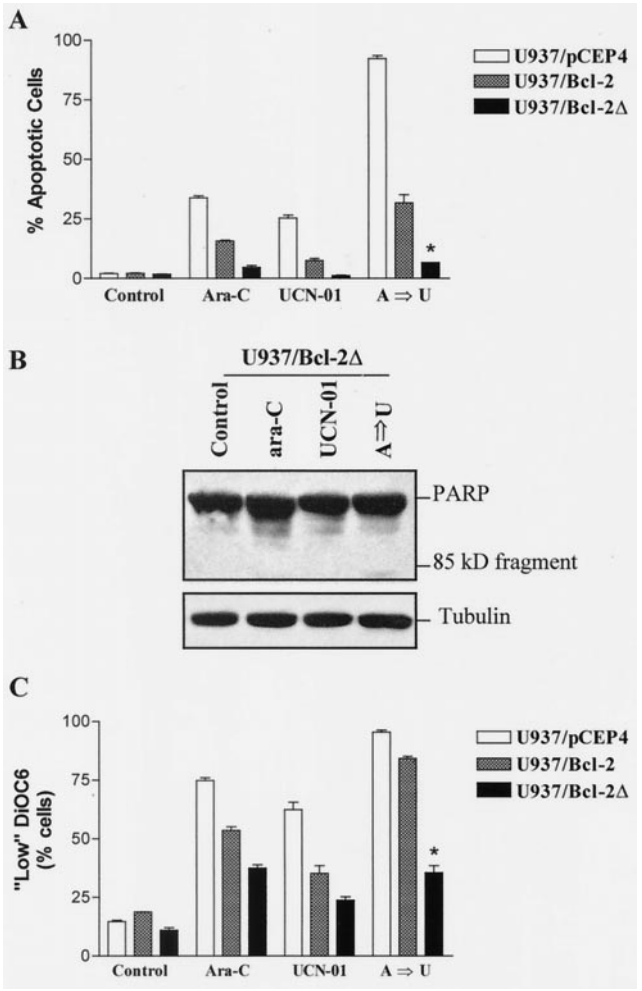

FIG. 6. Functional impact of ectopic expression of Bcl-2Δ (clone 2B9) on the response of U937 cells to ara-C and UCN-01. (A) Cells were exposed to ara-C (1 μM) for 6 hr, washed, and incubated with or without UCN-01 (300 nM) for an additional 24 hr. At the end of the treatment interval, cells were harvested, cytocentrifuge preparations were obtained, and the percentage of apoptotic cells was determined as previously described. (B) Alternatively, protein lysates (25 μg/lane) were obtained, and the extent of PARP degradation was determined by western analysis. (C) Following treatment, cells were incubated with 40 nM $DiOC_6$, and the percentage of cells exhibiting a decrease in $\Delta\psi_m$ was determined by flow cytometry as described in the text. For (A) and (C), values represent the means for three separate experiments ± SD. Key: (*) significantly less than the values for U937/Bcl-2 exposed to ara-C→UCN-01 ($P < 0.001$). Results obtained with other clones (e.g. 2C9 and 2G10) were similar.

enon that has been described previously [18]. ^{32}P-Labeling of immunoprecipitated Bcl-2 protein obtained from full-length Bcl-2 overexpressors revealed a clearly discernible increase in phosphorylation in cells exposed to UCN-01 or to the combination of UCN-01 and ara-C (Fig. 5B). This observation is in accord with previous findings involving HL-60 cells ectopically expressing Bcl-2 [17]. In contrast, a specific increase in labeling was not apparent in drug-treated cells expressing the Bcl-2Δ_{32-80} protein, either in the 26-kDa region (presumably due to the minimal expression of endogenous full-length Bcl-2 species in this line) or in the 19-kDa region (due to the absence of the N-terminal phosphorylation loop domain).

To assess the functional impact of loss of the Bcl-2 phosphorylation loop on the response of U937 cells to ara-C and UCN-01, studies utilizing empty-vector controls, U937/Bcl-2, and U937/Bcl-2Δ cells were performed in parallel (Fig. 6). Several striking differences were apparent when comparisons were made between results obtained in cells ectopically expressing full-length Bcl-2 versus its phosphorylation loop-deleted counterpart. First, overexpression of the loop-deleted Bcl-2 protein conferred significantly greater protection than its full-length counterpart against apoptosis induced by exposure of cells to ara-C, UCN-01, or the sequence ara-C→UCN-01 ($P = 0.001$; Fig. 6A). This was confirmed by the observation that the loop-deleted protein completely blocked PARP degradation induced by ara-C and UCN-01 (Fig. 6B), in contrast to the partial protection conferred by the full-length protein (Fig. 2A). Moreover, whereas full-length Bcl-2 was unable to prevent loss of $\Delta\psi_m$ induced (at 24 hr) by the sequence ara-C→UCN-01 (Fig. 4A), the loop-deleted protein was very effective in this regard (Fig. 6C). Thus, loss of the Bcl-2 phosphorylation loop increased the ability of this protein to inhibit the morphologic features of apoptosis and

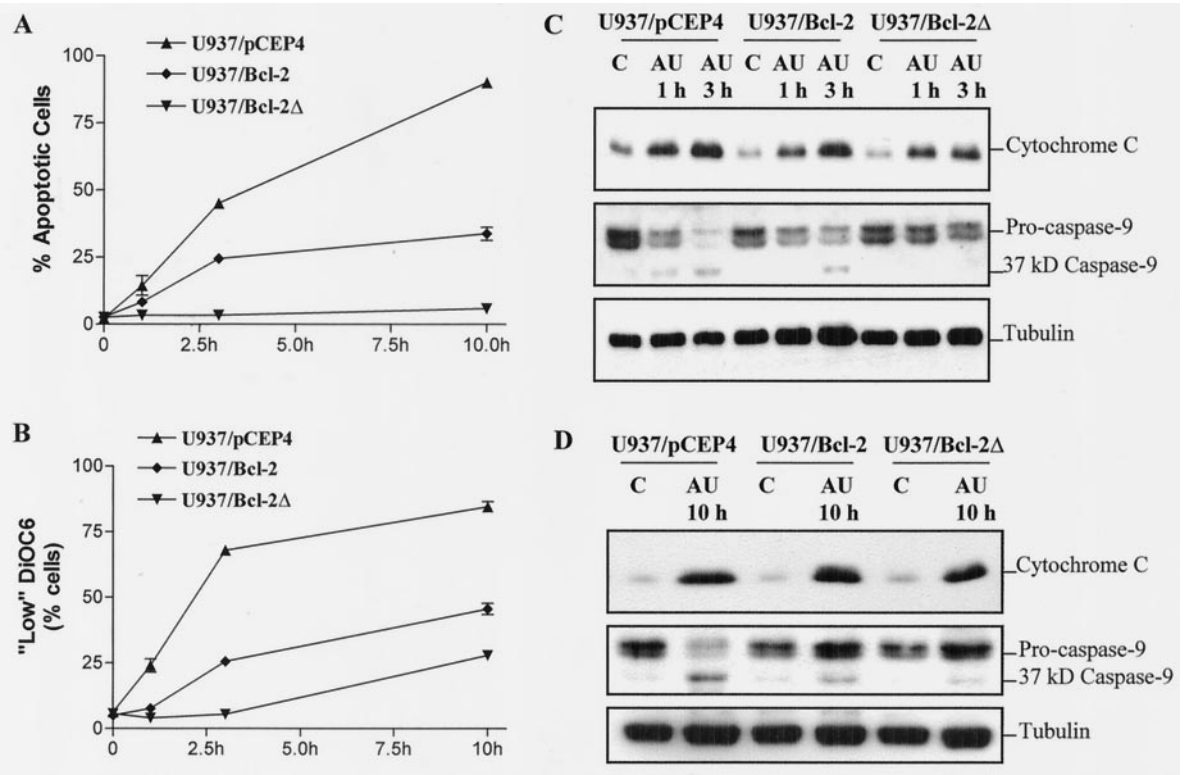

FIG. 7. Early effects of ara-C and UCN-01 on apoptosis, loss of mitochondrial membrane potential, cytochrome *c* release, and pro-caspase-9 activation. U937/pCEP4, U937/Bcl-2, and U937/Bcl-2Δ (clone 2B9) cells were exposed to 1 μM ara-C for 6 hr, washed, and incubated in the presence or absence of UCN-01 (300 nM) for 1, 3, or 10 hr. At the end of these intervals, cells were harvested and evaluated for morphological evidence of apoptosis (A) or loss of $\Delta\psi_m$ (B) as described above. Values represent the means for three separate experiments ± SD. Alternatively, S-100 fractions were obtained at the designated intervals as described in Materials and Methods, and release of cytochrome *c* or cleavage of pro-caspase-9 was determined by western blot analysis (C, D). Each lane was loaded with 25 μg protein. Results for a representative experiment are shown; two additional studies yielded equivalent results.

the reduction in $\Delta\psi_m$ observed in cells exposed to the sequence ara-C→UCN-01, at least at the 24-hr time interval.

To gain further insights into the temporal sequence of events occurring in each of the cell lines following exposure to the sequence ara-C→UCN-01, apoptosis, $\Delta\psi_m$, cytochrome *c* release, and pro-caspase-9 activation were monitored simultaneously at early time points (e.g. 1, 3, and 10 hr; Fig. 7). Differential induction of apoptosis at each of these early intervals was observed (Fig. 7A), the extent of which roughly paralleled findings at the 24-hr period (Figs. 2 and 6). At each interval, the percentage of cells exhibiting a reduction in $\Delta\psi_m$ was similar to, or slightly greater than, the fraction of morphologically apoptotic cells (Fig. 7B). However, at 1 and 3 hr, the extent of cytochrome *c* release in U937/Bcl-2Δ cells was only marginally less than that observed in samples obtained from the empty-vector and full-length Bcl-2-overexpressing cell line (Fig. 7C). Moreover, by 10 hr, release of cytochrome *c* was essentially equivalent in each of the cell lines (Fig. 7D). Furthermore, whereas cleavage of pro-caspase-9 to its active 37-kDa form was observed after 1 hr in empty vector controls, it was only noted after 3 hr in Bcl-2-overexpressors (Fig. 7C), and, although to a limited extent, after 10 hr in cells ectopically expressing Bcl-2Δ (Fig. 7D). Collectively, these findings suggest that ectopic expression of Bcl-2 and its phosphorylation loop-deletant counterpart is able to delay but not prevent cytochrome *c* release and pro-caspase-9 activation in cells exposed to ara-C followed by UCN-01.

Because induction of apoptosis does not necessarily correlate with loss of clonogenic survival [23], colony formation was assessed in various cell lines following exposure to ara-C ± UCN-01 (Fig. 8). The major findings were that whereas ectopic expression of full-length Bcl-2 protected clonogenic cells to some extent from the inhibitory effects of ara-C and UCN-01 administered individually, protection against the combination of ara-C and UCN-01 was minimal, with colony formation equal to 0.025 versus 0.055% of control values in U937/pCEP4 and U937/Bcl-2 cells, respectively (Fig. 8A). Moreover, despite conferring striking resistance to apoptosis at early time intervals, ectopic expression of the Bcl-2-loop deletant protein was essentially ineffective in protecting clonogenic U937 cells from the growth-inhibitory effects of ara-C and UCN-01 administered alone and in sequence. To relate these findings to induction of apoptosis and mitochondrial damage, more prolonged time–course studies were performed (Fig. 8, B and C). It can be seen that whereas

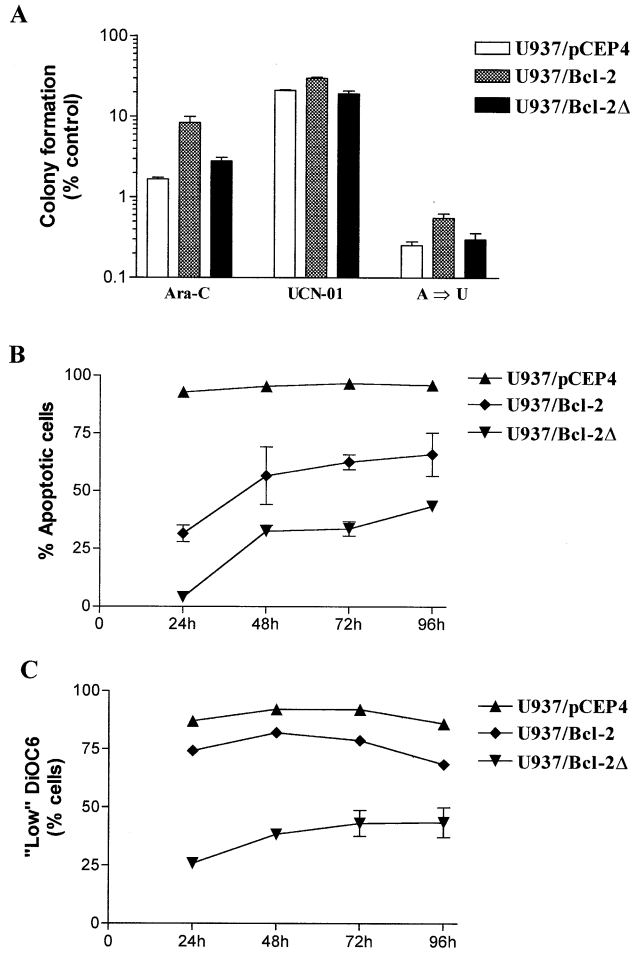

FIG. 8. Late effects of ara-C and UCN-01 on colony formation, apoptosis, and loss of mitochondrial potential. Cells were exposed to ara-C (1 μM; 6 hr), washed, and incubated with or without UCN-01 for an additional 24 hr. (A) Cells were washed thoroughly in fresh medium to remove all drugs, and plated in soft agar to assess colony formation as described in the text. Alternatively, cells were incubated in drug-free medium for the designated intervals, after which morphological assessment of apoptosis (B) or loss of $\Delta\psi_m$ by flow cytometry (C) was determined as described above. Values represent the means for three separate experiments ± SD.

protection from ara-C- and UCN-01-induced apoptosis was very apparent in full-length Bcl-2 overexpressors at the 24-hr interval, and even more pronounced in cells expressing the loop-deletant protein, the extent of differential protective effects declined over the ensuing 72 hr (Fig. 8B). A similar pattern was noted when loss of $\Delta\psi_m$ was examined (Fig. 8C). Taken in conjunction with the previous findings, these observations raise the possibility that Bcl-2 and its phosphorylation loop-deletant counterpart are able to delay but not prevent mitochondrial damage and subsequent apoptosis induced by ara-C and UCN-01. They also indicate that both the full-length and loop-deletant Bcl-2 proteins are relatively ineffective in protecting clonogenic U937 cells from the lethal effects of sequential exposure to ara-C and UCN-01.

DISCUSSION

The present results demonstrated that subsequent exposure of ara-C-pretreated U937 human leukemia cells to UCN-01 substantially increases mitochondrial damage and apoptosis, and that these effects may be delayed, but not ultimately prevented, by ectopic expression of the anti-apoptotic protein Bcl-2. Attempts to define the mechanism(s) by which UCN-01 potentiates the lethality of cytotoxic drugs are complicated by the pleiotropic effects that this agent exerts in different model systems. For example, in human epidermoid cancer cells (A431), UCN-01 induces dephosphorylation of CDK2, a phenomenon associated with inhibition of CDK2 activity and G_1 blockade [24, 25]. In contrast, in human leukemic T-cell lines, UCN-01-mediated dephosphorylation of CDK2 leads to increased kinase activity accompanied by apoptosis [10]. In view of evidence that the actions of UCN-01 vary with p53 status [13, 10], it is possible that responses to this agent differ in epithelial tumor cells exhibiting wild-type p53 versus continuously cultured human leukemia cells, which are generally p53-null [26]. These issues notwithstanding, UCN-01 has been shown to act as a checkpoint abrogator capable of potentiating the cytotoxicity of agents that induce G_2M arrest (e.g. cisplatin) [13], as well as those whose actions are S-phase-specific (e.g. camptothecin) [14]. The marked diminution in the residual S-phase fraction of cells exposed to the sequence ara-C→UCN-01 (Fig. 3) is consistent with the notion that UCN-01 acts, at least in part, by promoting entry of ara-C-treated cells into the vulnerable DNA-synthetic phase of the cell cycle. However, the alternative possibility that potentiation of ara-C-mediated lethality by UCN-01 stems from induction of conflicting cell cycle regulatory signals (e.g at the level of CDK2 activation) cannot be excluded at this time. Finally, whether similar interactions occur *in vivo* may ultimately depend upon pharmacokinetic considerations, particularly in view of the avid binding of UCN-01 to plasma α_1-acidic glycoprotein reported to occur in humans [9].

Whereas ectopic expression of Bcl-2 has been shown to block apoptosis by diverse stimuli, a number of studies have demonstrated a discordance between effects of this protein on cell death and clonogenic survival [27]. Moreover, distal blockade of the apoptotic caspase cascade (e.g. by caspase inhibitors such as zVADfmk) can prevent the apoptotic morphology without restoring self-renewal capacity [28]. Consistent with these findings, ectopic expression of Bcl-2 (partially) protected ara-C- and UCN-01-treated U937 cells from the morphologic features of apoptosis, at least when examined at the 24-hr interval, but was relatively ineffective in preserving clonogenic survival. In this context, the mechanism by which Bcl-2 and related proteins oppose apoptosis has not been firmly established, but appears to involve either prevention of mitochondrial damage (e.g. loss of $\Delta\psi_m$ and/or cytochrome *c* release) [4, 21] or disruption of the apaf-1/cytochrome *c*/apaf-3 complex [7]. It therefore is unlikely to be coincidental that

ectopic expression of Bcl-2 was relatively ineffective in preventing mitochondrial damage in cells exposed sequentially to ara-C and UCN-01, and that the degree of such damage increased over time, as did the extent of apoptosis. Interestingly, the delay in cytochrome *c* release conferred by Bcl-2 overexpression was considerably less pronounced than the delay in loss of $\Delta\psi_{m,}$ consistent with evidence that in some systems, cytochrome *c* release precedes the mitochondrial permeability transition [29]. Taken together, the present findings suggest that ectopic expression of Bcl-2 can delay, but not prevent, mitochondrial damage and subsequent induction of apoptosis in U937 cells treated with ara-C→UCN-01, and that these events may contribute to the ultimate failure of Bcl-2 overexpression to protect clonogenic cells from the lethal effects of this drug sequence. An alternative explanation for this finding is that clonogenic cells may be more sensitive to sequential administration of ara-C and UCN-01 than the population of leukemic cells as a whole.

Attempts to define the role of Bcl-2 phosphorylation in regulation of the cell death process have been complicated by conflicting reports in the literature. For example, Ras-induced apoptosis in Jurkat cells has been associated with Bcl-2 phosphorylation [30], as has been cell death accompanying phorbol myristate acetate-mediated PKC down-regulation [22]. Analogously, we have reported that direct inhibition of PKC (e.g. by staurosporine or UCN-01) or enzyme down-regulation (by the macrocyclic lactone bryostatin 1) promotes ara-C-mediated apoptosis in Bcl-2-overexpressing HL-60 cells, and that these events are associated, at least temporally, with phosphorylation of the Bcl-2 protein [17]. Similarly, paclitaxel-mediated apoptosis in Jurkat cells and human hormone-independent prostate cancer cells has been attributed to phosphorylation-mediated inactivation of Bcl-2 [31, 32]. Taken in conjunction with the observations that a truncated Bcl-2 protein lacking the N-terminal phosphorylation loop domain (Bcl-$2\Delta_{32-80}$) is more potent than its full-length counterpart in protecting murine cells from growth factor deprivation-induced apoptosis [18], and that a construct lacking residues 51–85 is more effective in blocking p53- and c-myc-induced apoptosis in B-lymphoma cells [33], these findings suggest that phosphorylation of Bcl-2, through a mechanism as yet to be identified, antagonizes the ability of this protein to block mitochondrial dysfunction and apoptosis. However, this notion is more difficult to reconcile with reports that Bcl-2 phosphorylation is necessary for the anti-apoptotic effects of PKC activators (including bryostatin 1) in murine interleukin-3-dependent cells deprived of growth factors [34] and in Jurkat cells exposed to cytotoxic drugs such as etoposide (VP-16) [35]. Moreover, HL-60 cells ectopically expressing the phosphorylation loop deletant protein employed in the present study were found to be more rather than less susceptible to paclitaxel-mediated apoptosis [36], although the opposite result was obtained in subsequent studies involving transfectant U937 leukemic cells [37] and MCF-7 breast cancer cells [38]. Aside from the possibility that point mutations in the Bcl-2 protein (e.g. ser_{70}→ala) [35] may have different functional consequences than enforced expression of a phosphorylation loop deletant [18], such divergent findings suggest that the impact of Bcl-2 phosphorylation very likely varies with the cell type and/or the inciting apoptotic stimulus. Furthermore, the fact that enforced expression of a Bcl-2 loop deletant construct conferred enhanced protection against ara-C/UCN-01-induced apoptosis is potentially consistent with a functional role for Bcl-2 phosphorylation in facilitation of cell death. It must be emphasized, however, that whereas the loop deletant was more effective than its full-length counterpart in opposing drug-induced apoptosis and loss of $\Delta\psi_m$ (at the 24-hr interval), it was only marginally capable of delaying cytochrome *c* release, and was essentially ineffective in preserving leukemic cell self-renewal capacity. This finding indicates that the gain of function associated with loss of the N-terminal Bcl-2 loop domain does not prevent early commitment to cell death, at least in those cells with clonogenic potential.

In summary, the present findings indicate that subsequent exposure of ara-C-treated U937 cells to the kinase inhibitor UCN-01 strikingly promotes mitochondrial dysfunction, caspase activation, and apoptosis. Moreover, these events are variably delayed by enforced expression of either full-length Bcl-2 or, to an even greater extent, a deletion mutant lacking the N-terminal domain encompassing the $serine_{70}$ phosphorylation site. Whereas the latter finding does not rule out the possibility of a functional role for Bcl-2 phosphorylation in modulation of apoptosis by UCN-01, it is important to note that neither the full-length or loop-deletant Bcl-2 construct was ultimately able to prevent mitochondrial damage and apoptosis or restore clonogenic potential to drug-treated cells. In view of evidence that increased expression of Bcl-2 by leukemic blasts correlates inversely with clinical response to chemotherapy [8], the present findings raise the possibility that combined treatment with ara-C and UCN-01 may prove effective in patients whose cells display high levels of this anti-apoptotic protein. They also suggest, as recently proposed [39], that responsiveness to such a strategy may correlate less with early evidence of apoptosis than with other indicators of commitment to cell death, including induction of mitochondrial dysfunction (e.g. cytochrome *c* release) or loss of clonogenic survival. Efforts to test this hypothesis in primary blast specimens are currently underway.

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