The Lethal Effects of Pharmacological Cyclin-dependent Kinase Inhibitors in Human Leukemia Cells Proceed through a Phosphatidylinositol 3-Kinase/Akt-dependent Process

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INTRODUCTION

Disregulation of the cell cycle traverse represents one of the hallmarks of malignant transformation (1). Cell cycle progression is regulated by a highly complex network of proteins that include the cyclins, levels of which fluctuate throughout the cell cycle, as well as the CDKs, e.g., CDKs 1, 2, 5, 4/6, 7, and 9 (2). CDKs 2 and 4/6, in association with cyclins D, E, and A, are primarily involved through sequential phosphorylation of the retinoblastoma protein, in governing the G1-S phase transition as well as progression through S phase (3). In contrast, CDK1, in association with cyclins B and A, regulates progression of cells through G2-M (4). The central importance of cell cycle dysregulation in the malignant phenotype has prompted intensive efforts to develop small-molecule inhibitors of various cell cycle regulatory components. The first of these to enter clinical trials has been the semisynthetic flavonoid FP (NSC 649890), which binds to the CDK ATP binding site, resulting in the inhibition of catalytic activity (5). FP is a broadly acting CDK antagonist and inhibits, to various degrees, the activities of CDKs 1, 2, 4/6, and 7 (6). More recently, FP has been shown to interfere with the CDK9/T1 complex and, thereby, to function as a transcriptional repressor (7). Other pharmacological CDK inhibitors in preclinical development include olomoucine, roscovitine, butyrolactone, the paullones, and CGP74514A, among others (8, 9).

In addition to their capacity to induce cell cycle arrest, CDK inhibitors are also lethal to tumor cells, a phenomenon that may reflect the close relationship that exists between the apoptotic and cell cycle machinery (10). For example, FP triggers apoptosis in lung cancer cells (11), and is a particularly potent inducer of apoptosis in malignant cells of hematopoietic origin (12). The induction of apoptosis by other CDK inhibitors has also been widely reported (13, 14). In the case of FP, the induction of cell death has been linked to perturbations in cell cycle proteins (i.e., down-regulation of cyclin D1; Ref. 15), diminished expression of certain antiapoptotic proteins (i.e., Mcl-1 and XIAP; Ref. 16), or induction of mitochondrial injury (17). However, the mechanism by which such agents activate the apoptotic cascade remains, for the most part, unclear.

In addition to a continuously expanding family of pro- and antiapoptotic proteins (18), apoptosis is also regulated by multiple signal transduction cascades, of which the MAPK pathways have received considerable attention. The MAPK pathways represent three parallel serine-threonine kinase modules that are triggered by various mitogen- and stress-related stimuli (19). The MAPK pathways consist of

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3 The abbreviations used are: CDK, cyclin-dependent kinase; PI3K, phosphatidylinositol 3-kinase; FP, flavopiridol; LY, LY294002; AIF, apoptosis-initiating factor; IAP, inhibitor of apoptosis; Smac/DIABLO, second mitochondria-derived activator of caspases/direct inhibitor of apoptosis; MEK, MAP/ERK kinase; PARP, poly(ADP-ribose) polymerase; AML, acute myeloblastic leukemia; PI, propidium iodide.
the ERK, JNK, and p38 MAPK kinase modules (20). Although exceptions exist, ERK activation is generally associated with cell survival, whereas JNK and p38 MAPK have been linked to proapoptotic events (21). Recently, however, the Ras/P13K/Akt signal transduction pathway has become the focus of intense interest as a critical regulator of cell survival/death decisions (22). Activation of P13K results in the generation of the lipid second messenger PIP3 from its precursor, phosphatidylinositol-4,5-biphosphate (PIP2; Ref. 23). PIP3 recruits to the plasma membrane through interactions with a pleckstrin homology domain, the serine/threonine kinase Akt, which is then phosphorylated by phosphoinositide-dependent kinase-1 (24). Phosphorylation of Akt leads to the modulation of the expression/function of multiple downstream targets involved in apoptosis regulation, which include, but are not restricted to, Bad, procaspase-9, FKHRs, m-TOR, p70S6K, nuclear factor κB, CREB, and p21CIP1, among others (25–28). Attempts to elucidate the role of the P13K pathway in apoptosis regulation have been facilitated by the availability of agents such as wortmannin, a P13K antagonist that also inhibits DNA PK (29), as well as LY (30).

Currently, little information exists concerning the signaling events responsible for apoptosis induction by FP or other pharmacological CDK inhibitors. Moreover, essentially nothing is known of the role of the P13K pathway in regulating apoptosis in cells subjected to CDK inhibition. To address these issues, the effects of established P13K inhibitors have been examined in relation to the response of human leukemia cells to FP and other pharmacological CDK inhibitors. Here we report that exposure of such cells to CDK inhibitors, in conjunction with P13K antagonists such as LY, diminishes Akt phosphorylation and triggers a very early (e.g., within 2 h) and striking increase in mitochondrial damage, caspase activation, and apoptosis. In contrast, blockade of the PKC, ERK, p38 MAPK, PKA, or m-TOR cascades exerts minimal effects on apoptosis induction by CDK inhibitors. Moreover, inducible expression of a constitutively active, myristoylated Akt mutant significantly, albeit partially, attenuates the lethal effects of FP as well as the FP/LY regimen. Taken together, these findings indicate that P13K inhibitors dramatically increase the lethality of pharmacological CDK inhibitors in human leukemia cells and suggest that this phenomenon involves, at least in part, interference with Akt-related cytoprotective functions.

MATERIALS AND METHODS

Cells. U937, Jurkat, CCRF-HL60, and NB4 human leukemia cells were purchased from the American Type Culture Collection, Manassas, VA. All of the cells were cultured in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, t-glutamate, penicillin, streptomycin, and 10% heat-inactivated FCS (Hyclone, Logan, UT). They were maintained in a 37°C, 5% CO2, fully humidified incubator, passed twice weekly, and prepared for experimental procedures when in log-phase growth (cell density ≤5 × 105 cells/ml).

U937 cells, stably transfected with constructs expressing Bcl-2, a phosphorelay loop-deleted Bcl-2 mutant, or dominant-negative caspase-8, were generated and maintained as described previously in detail (17, 31).

Reagents. FP (L86 8275; NCS 649890) was kindly provided by Dr. Edward Sausville (Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD), formulated in DMSO as a 10° M solution, and was stored at −20°C. L-Carnitine, phosphatidylcholine, d-tocopherol, and wortmannin were purchased from Sigma (St. Louis, MO), were dissolved in DMSO, and were stored at −20°C. Rapamycin was purchased from Cell Signaling Technology (Beverly, MA); SB202190, H-89, SP600125, U0126, and SP600125 were purchased from BioMol Research Laboratories (Plymouth, MA); The PKC inhibitor bisindoylmaleimide I (GF1109203X; Calbiochem, San Diego, CA) was formulated in DMSO and was added 0.5 h before the addition of FP. The pan-caspase inhibitor BOC-D-fmk was purchased from Enzyme System Products (Livermore, CA) and dissolved in DMSO.

Experimental Format. Logarithmically growing cells were transferred to sterile plastic T-flasks (Corning, Corning, NY) to which were added the designated drugs, after which the flasks were replaced in the incubator for various intervals. At the end of the incubation period, cells were transferred to sterile centrifuge tubes, pelleted by centrifugation at 400 × g for 10 min at room temperature, and prepared for analysis as described below.

Assessment of Apoptosis. After drug treatment, cyto centrifuge preparations were stained with Wright-Giemsa and viewed by light microscopy to evaluate the extent of apoptosis (i.e., cell shrinkage, nuclear condensation, formation of apoptotic bodies) as described previously (32). For these studies, the percentage of apoptotic cells was determined by evaluating ≥500 cells/condition in triplicate. To confirm the results of morphological analysis, Annexin V/PI staining was used. Annexin V/PI (BD Pharmingen, San Diego, CA) analysis of cell death was carried out as per the manufacturer’s instructions. For these experiments, 1–2 × 10° cells per condition were harvested. Analysis was carried out using a Becton-Dickinson FACScan cytofluorometer (Becton Dickinson, MA).

Determination of Loss of MMP (ΔΨm). Loss of MMP (ΔΨm) was monitored using the fluorochrome DiOC6 as previously described (33). For each condition, 4 × 105 cells were incubated for 15 min at 37°C in 1 ml of 40 mM DIOCs (Calbiochem) and subsequently analyzed using a Becton Dickinson FACScan cytofluorometer with excitation and emission settings of 488 and 525 nm, respectively. Control experiments demonstrating the loss of ΔΨm were performed by exposing cells to 5 μM of carbamoyl cyanide m-chlorophenylhydrazone (Sigma Chemical Co.; 15 min, 37°C), an uncoupling agent that abolishes the MMP.

Preparation of S-100 Fractions for Assessment of Cytochrome c, Smac/DIABLO, and AIF Release. U937 cells were harvested after drug treatment as described previously (33) by centrifugation at 600 × g for 10 min at 4°C and washed in PBS. Cells (4 × 10°) were lysed by incubating them for 3 min in 100 μl of lysis buffer containing 75 mM NaCl, 8 mM Na2HPO4, 1 mM NaH2PO4, 1 mM EDTA, and 350 μg/ml digitonin. The lysates were centrifuged at 12,000 × g for 5 min, and the supernatant was collected and added to an equal volume of 2× Laemmli buffer. The protein samples were quantified and separated by 15% SDS-PAGE. Expression of the mitochondrial proteins cytochrome c, Smac/DIABLO, and AIF was assessed by Western blot analysis as described below.

Immunoblot Analysis. Immunoblotting was performed as described previously (33). In brief, after drug treatment, cells were pelleted by centrifugation and were lysed immediately in Laemmli buffer [1% = 30 mM Tris-base (pH 6.8), 2% SDS, 2.88 mM β-mercaptoethanol, and 10% glycerol], and were briefly sonicated. Homogenates were quantified using Coomassie protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were boiled for 10 min, separated by SDS-PAGE (5% stacking and 10% resolving gel), and electrophoretically transferred to nitrocellulose membrane. After blocking in TBS-T (0.05%) and 5% milk for 1 h at 22°C, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody for 4 h at 22°C. Sources of primary antibodies were as follows: Bcl-2 and Bcl-xL, rabbit polyclonal (Santa Cruz Biotechnology); XIAP, rabbit polyclonal (R&D Systems, Minneapolis, MN); Mcl-1, mouse monoclonal (PharMingen, San Diego, CA); cyclins A, E, D1, p21CIP1, p27KIP1, mouse monoclonal (PharMingen); ERK 1/2, rabbit polyclonal (Cell Signaling Technology, Beverly, MA); phospho-ERK 1/2 (Thr202/Tyr204), rabbit polyclonal (Cell Signaling Technology); JNK, rabbit polyclonal (Santa Cruz Biotechnology); phospho-JNK, mouse monoclonal (Santa Cruz Biotechnology); phospho-p38 MAPK, rabbit polyclonal (Cell Signaling Technology); phospho-cdc2 and phospho-p70S6K (Thr389), rabbit polyclonal (Cell Signaling Technology); phospho-Akt (Ser473), rabbit polyclonal (Cell Signaling Technology); phospho-GSK (Cell Signaling Technology); phospho-FKHR, rabbit polyclonal (Cell Signaling Technology); phospho-p70S6K, rabbit polyclonal (Cell Signaling Technology); phospho-CREB, rabbit polyclonal (Upstate Biotechnology, Lake Placid, NY); phospho-Bad and total Bad, rabbit polyclonal (Cell Signaling Technology); pRB, mouse monoclonal (PharMingen); under-phosphorylated pRb, mouse monoclonal (PharMingen); pBlp/TB21 CDK2 phosphospecific site, rabbit polyclonal (BioSource); pBlp/TB24/25 phosphospecific site, rabbit polyclonal (BioSource); caspase 3, mouse monoclonal (Transduction Laboratories, Lexington, KY); PARP C2-10, mouse monoclonal (BioMoR Research Laboratories, Plymouth, MA); cytochrome c, mouse monoclonal; caspase 8, rabbit polyclonal (PharMingen); and α-tubulin.
CDK INHIBITORS AND THE PI3K/Akt PATHWAY IN LEUKEMIA CELLS

(AcriBiochem). Blots were washed three times for 15 min each time in TBS-T and then were incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) for 1 h at 22°C. Blots were again washed three times for 15 min in TBS-T and then were developed by enhanced chemiluminescence (Pierce, Rockford, IL).

Tet-On Inducible Jurkat Cell Lines. A stable Jurkat lymphoblastic leukemia cell line inducibly expressing a myristoylated Akt (myr-Akt), which is constitutively active, was generated as follows. Myc-tagged myr-AKT (Upstate Biotechnology, Lake Placid, NY) was subcloned into the pTRE2-hygro expression vector (Clontech) according to standard techniques. Jurkat “Tet-On” cells that stably express an rtTA (reverse tet transactivator) regulator protein (Clontech) were transfected with Myc-tagged myr-AKT-pTRE2-hygro by electroporation (600 V, 60 ms) using 0.4 μg cuvettes. Stable clones derived from single cells were selected by limiting dilution in RPMI 1640 supplemented with 10% of Tet-System-approved FBS (Clontech) in the presence of 400 μg/ml hygromycin. To test for induced expression of the myr-AKT, stable clones were left untreated or treated for 24 h with 2 μg/ml doxycycline, after which they were harvested and analyzed for myc-AKT and phosphorylated (activated) Akt expression by Western blot as described above.

AML Patient-derived Cells and Normal Peripheral Blood Mononuclear Cells. Leukemic cells were obtained with informed consent and as described previously (34) from the peripheral blood of a patient with AML (subtype M2) who relapsed after induction therapy. These studies have been sanctioned by the Investigational Review Board of Virginia Commonwealth University/Medical College of Virginia. For these studies, the percentage of blasts in the peripheral blood was >90%. Blood was collected into sterile tubes containing preservative-free heparin, layered over Ficoll-Hypaque (Sigma; specific gravity, 1.077–1.081) in a sterile 50-cc plastic centrifuge tube. The diluted cells were layered over a cushion of Ficoll-Hypaque (Sigma) and centrifuged at 400 × g for 38 min at room temperature, after which, an interface layer, consisting of normal peripheral blood mononuclear cells, was extracted, washed, and exposed to drugs as outlined above in the case of leukemic blasts. The percentage of apoptotic cells for each condition was determined as described above.

Statistical Analysis. The significance of differences between experimental conditions was determined using the two-tailed Student t test. Analysis of synergy was carried out using Median Dose Effect analysis (35) with the aid of a commercially available software program (CalcuSyn; Biosoft, Ferguson, MO).

RESULTS

To assess the effects of interruption of the PI3K pathway on the lethal actions of CDK inhibitors, U937 cells were exposed to a sublethal concentration of FP (i.e., 75 nM) for 6 h in the presence of various concentrations of LY, after which apoptosis was assessed. As shown in Fig. 1A, coadministration of LY at concentrations ≥10 μM, which were minimally toxic alone, resulted in apoptosis in the majority of FP-treated cells. A FP dose-response curve revealed that when cells were exposed to a fixed concentration of LY (15 μM) in conjunction with FP concentrations ≥50 nM, a marked increase in apoptosis was observed (Fig. 1B). Very similar patterns were noted when LY was administered in combination with the CDK inhibitors CGP74514A and roscovitine (Fig. 1, C and D). Thus, disruption of the PI3K pathway resulted in an early and striking increase in apoptosis in U937 cells exposed to several CDK inhibitors. These results were confirmed using annexin/PI analysis, which distinguishes between early apoptosis (in which cells stain positively for annexin) and late apoptosis (in which cells stain positively for both annexin and PI; Fig. 2A). It is noteworthy that even at an early exposure interval (e.g., 6 h), a modest increase in late apoptosis was observed in LY/CDK inhibitor-treated cells. In accord with these results, coadministration of each of the CDK inhibitors with FP for 6 h

Fig. 1. A, logarithmically growing U937 cells were exposed for 6 h to the designated concentration of LY ± 75 μM FP (FP), after which cytospin preparations were stained with Wright-Giemsa, and cells were evaluated for the morphological features of apoptosis under light microscopy as described in “Materials and Methods.” B, cells were treated as above with various concentrations of FP ± 15 μM LY, after which the percentage of apoptotic cells was determined. C and D, cells were exposed for 6 h to 15 μM LY ± the designated concentration of CGP74514A (C, CGP) or roscovitine (D, ROS), after which apoptosis was monitored as described above. For A–D, values represent the means ± SD for three separate experiments.
resulted in a marked loss of MMP \( \Delta \Psi_m \); Fig. 2B), a hallmark of mitochondrial injury. A time course study of U937 cells exposed to 75 nM FP, 1.5 \( \mu \)M CGP74514A (CGP), or roscovitine (Ros) for 6 h, after which the extent of apoptosis was assessed by Annexin V/PI staining as described in “Materials and Methods.” Early apoptotic cells (Annexin V only), late apoptotic cells (Annexin VPI”), B, cells were treated with LY \( \pm \) FP, CGP74514A (CGP), or roscovitine (Ros) as above for 6 h, after which the percentage of cells exhibiting loss of \( \Delta \Psi_m \), reflected by “low” uptake of the fluorochrome DiOC \(_6\), was determined by flow cytometry as described in “Materials and Methods.” C, cells were exposed to the designated concentrations of FP \( \pm \) LY for the indicated intervals, after which the extent of apoptosis was determined as above. For A–C, values represent the means \( \pm SD\) for three separate experiments. D, cells were exposed for 24 h to various concentrations of LY (10–25 \( \mu \)M) \( \pm \) FP (50–125 nM) at a fixed ratio (200:1), after which the percentage of apoptotic cells was determined as above. Median Dose Effect analysis was used to determine the C.I. for each F.A. C.I. values <1.0 correspond to synergistic interactions. Values for a representative experiment are shown; two others yielded equivalent results. con, control. 

As shown in Fig. 3A, treatment of U937 cells with LY (15 \( \mu \)M) in combination with roscovitine (10 \( \mu \)M) or with CGP (1.5 \( \mu \)M; data not shown). Finally, Median Dose Effect analysis of apoptosis induction in U937 cells exposed to a range of LY and FP concentrations for 6 h yielded C.I. values considerably less than 1.0, corresponding to a highly synergistic interaction (Fig. 2D).
Various human leukemia cell lines were exposed to LY ± FP for intervals ranging from 12 to 24 h, after which the percentage of apoptotic cells was determined by examining Wright-Giemsa-stained cytospin preparations under light microscopy as described in “Materials and Methods.” Values represent the means for three separate experiments ± SD.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control</th>
<th>LY</th>
<th>FP</th>
<th>FP + LY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jurkat</td>
<td>0.6 ± 0.3</td>
<td>1.2 ± 0.8</td>
<td>2.6 ± 1.2</td>
<td>74.3 ± 6.3</td>
</tr>
<tr>
<td>CCRF</td>
<td>0.8 ± 0.4</td>
<td>2.1 ± 0.7</td>
<td>4.5 ± 1.4</td>
<td>78.8 ± 4.7</td>
</tr>
<tr>
<td>NB4</td>
<td>1.2 ± 0.5</td>
<td>2.2 ± 1.2</td>
<td>4.7 ± 1.7</td>
<td>52.1 ± 2.9</td>
</tr>
<tr>
<td>HL-60</td>
<td>2.1 ± 0.6</td>
<td>3.3 ± 1.4</td>
<td>3.8 ± 1.3</td>
<td>57.5 ± 3.5</td>
</tr>
</tbody>
</table>

**Table 2.** Induction of apoptosis by FP and LY in leukemia cells ectopically expressing full-length and loop-deleted Bcl-2.

<table>
<thead>
<tr>
<th>Time</th>
<th>pCEP4</th>
<th>D9(Bcl-2)</th>
<th>pSSFV</th>
<th>ΔBcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 h</td>
<td>2.3 ± 0.8</td>
<td>0.8 ± 0.6</td>
<td>1.7 ± 0.9</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>FF + LY</td>
<td>74 ± 5.4</td>
<td>1.8 ± 0.7</td>
<td>69.4 ± 4.4</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>24 h</td>
<td>2.6 ± 0.6</td>
<td>0.9 ± 0.7</td>
<td>1.8 ± 0.7</td>
<td>0.8 ± 0.6</td>
</tr>
<tr>
<td>FF + LY</td>
<td>87.2 ± 4.6</td>
<td>14.2 ± 3.3</td>
<td>81.6 ± 3.9</td>
<td>1.3 ± 0.7</td>
</tr>
</tbody>
</table>

U937 cells ectopically expressing full-length Bcl-2 (D9(Bcl-2)), a NH2-terminal phosphorylation loop deleted mutant (ΔBcl-2), and their respective empty-vector controls (pCEP4, pSSFV) were exposed to 75 nM FP ± 15 μM LY for 6 and 24 h, after which the percentage of apoptotic cells was determined by examining Wright-Giemsa-stained cytospin preparations under light microscopy as described in “Materials and Methods.” Values represent the means for three separate experiments ± SD.

Fig. 4. A, U937 cells were exposed to 75 nM FP ± 15 μM LY for the indicated interval, after which S-100 cytosolic fractions were isolated and cytochrome c (cyt c) expression was monitored by Western blot as described in “Materials and Methods.” B, cells were exposed to 15 μM LY ± 75 nM FP, 1.5 μM CP75417A (CPG), or 10 μM roscovitine (Ros) for 1–3 h (B) or 6 h (C), after which cytochrome c release into the S-100 cytosolic fraction was determined. For A–C, each lane contained 25 μg protein; blots were subsequently stripped and reprobed with antibodies to tubulin to ensure equivalent loading.

Fig. 5. Various human leukemia cell lines were exposed to LY for intervals ranging from 12 to 24 h, after which the percentage of apoptotic cells was determined by examining Wright-Giemsa-stained cytospin preparations under light microscopy as described in “Materials and Methods.” Values represent the means for three separate experiments ± SD.
Interactions between CDK inhibitors and LY were then examined in relation to effects on several cell cycle-related proteins, with an emphasis on pRb, the phosphorylation status of which is known to be regulated by cyclin D in association with various CDKs. As shown in Fig. 6, coexposure of cells to each of the CDK inhibitors in conjunction with LY for 3 h resulted in a marked diminution in pRb phosphorylation on CDK2 and CDK4 phosphorylation sites. Western analysis of total pRb protein revealed an increase in the abundance of the slowly migrating hypophosphorylated form in LY/CDK inhibitor-treated cells, as well as evidence of pRb cleavage. These findings were confirmed in studies using an antibody specific for the underphosphorylated form of pRb. Combined treatment with LY and CDK inhibitors resulted in a marked diminution in expression of cyclin D1, but no changes in expression of cyclins A or E. In separate studies, no differences could be detected between levels of activity of CDK2/cyclin E versus CDK2/cyclin A, or in the amount of cyclin A versus cyclin E coimmunoprecipitating with CDK2 (data not shown). Finally, coexposure of cells to LY and CDK inhibitors was associated with a modest diminution in p34\textsuperscript{cdk2} phosphorylation. Thus, the exposure of cells to CDK inhibitors in combination with a PI3K inhibitor resulted in multiple perturbations in cell cycle-related proteins, including pRb dephosphorylation and cleavage, p34\textsuperscript{cdk2} activation, and cyclin D1 down-regulation.

An attempt was then made to characterize the effects of LY and CDK inhibitors, alone and in combination, on the status of various signaling pathways in U937 cells. Treatment with LY alone (3 h) modestly reduced phosphorylation of Akt on the Ser473 site (Fig. 7A), whereas the CDK inhibitors produced little effect. However, the coadministration of LY with each of the CDK inhibitors essentially abrogated expression of the phosphorylated Akt species. Total Akt expression was unchanged by any of these treatments. Combined treatment with LY and CDK inhibitors also resulted in a marked reduction in phosphorylation of GSK and FKHR, both well-established downstream targets of Akt (25). Coadministration of LY and CDK inhibitors also induced cleavage and diminished expression of phosphorylated p70\textsuperscript{S6K}, another Akt downstream target (26).

As shown in Fig. 7B, the cotreatment of cells with LY \pm each of the CDK inhibitors (3 h) induced a slight reduction in levels of phosphorylated (activated) MAPK and minor effects on JNK phosphorylation. In addition, exposure of cells to LY alone resulted in a very marked increase in p38 MAPK phosphorylation/activation, a response that was not modified by coadministration of CDK inhibitors. Combined treatment of cells with LY/CDK inhibitors also did not alter the expression of other potential PI3K downstream targets, including IκB-α, IκB-β, p21\textsuperscript{cip1}, or p27\textsuperscript{kip1}.

To evaluate the functional significance of some of these events, and to determine whether the potentiation of FP-mediated lethality was restricted to interruption of the PI3K pathway, interactions between FP and a variety of other pharmacological inhibitors were examined in U937 cells (Table 3). Cotreatment of cells for 6 h with FP (75 nM) in combination with the PI3K inhibitor wortmannin (300 nM) also resulted in a marked increase in apoptosis. Similar results were observed in Jurkat cells. For example, in this line, exposure to 300 nM wortmannin or 100 nM FP for 12 h induced apoptosis in 3.2 \pm 1.2 and 2.8 \pm 1.6% of cells, respectively, whereas combined exposure resulted in apoptosis in 52.3 \pm 4.7% of cells (data not shown). In contrast, potentiation of apoptosis was minimal or absent in cells exposed to the combination of FP and the MEK1/2 inhibitor U0126.

Fig. 5. A, U937 cells were exposed to 15 μM LY \pm 75 nM FP, 1.5 μM CGP74514A (CGP), or 10 μM roscovitine (Ros) for 3 h, after which cells were lysed, cytosolic S-100 fractions isolated, and the proteins separated by SDS-PAGE. The blots were then probed with antibodies directed against the mitochondrial proteins cytochrome c (cyto c), Smac/DIABLO (SMAC), and AIF. B, Alternatively, whole cell lysates were subjected to Western analysis as above, using antibodies directed against Bcl-2, Bcl-x\textsubscript{L}, Mcl-1, XIAP, Bad, or phospho-Bad. All of the exposure intervals were for 3 h. Each lane contained 25 μg protein; blots were subsequently stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer of protein. The results of a representative study are shown; two additional experiments yielded equivalent results.

Fig. 6. U937 cells were exposed to 15 μM LY \pm 75 nM FP, 1.5 μM CGP74514A (CGP), or 10 μM roscovitine (Ros) for 3 h, after which cells were lysed and the proteins separated by SDS-PAGE. The blots were then probed with antibodies directed against pRb phosphorylated at the CDK2- and CDK4-specific sites (T621 and T252, respectively), total pRb (ppRb, pRb), underphosphorylated pRb (under-p-RB), cyclin D1, -A, -E, and -p34\textsuperscript{cdk2} (p-cdc2). Each lane contained 25 μg protein; blots were subsequently stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer of protein. The results of a representative study are shown; two additional experiments yielded equivalent results.
(38), the p38 MAPK inhibitor SB202190 (39), the m-TOR inhibitor rapamycin (40), the PKA inhibitor H-89 (41), or the PKC inhibitor GFX (42). In addition, the lethal effects of FP were not enhanced by coadministration of caffeine, arguing against the possibility that the effects of LY or wortmannin involve the ATM/Chk1 axis. Interestingly, coadministration of FP with the JNK inhibitor SP600125 (25 μM; Ref. 43) did lead to a slight increase in apoptosis (e.g., ~12%), but it was significantly less than that observed in the case of LY. Together, these findings support the notion that early potentiation of FP lethality is specifically linked to the disruption of the PI3K/Akt pathway.

To characterize the functional role of perturbations in Akt in the apoptotic response of leukemia cell to FP, a Jurkat cell line that inducibly expresses a constitutively active myristoylated Akt mutant in the presence of doxycycline was used. As shown in Fig. 9A, addition of doxycycline to the medium resulted in expression of the Myc tag and induction of activated (phosphorylated) Akt on the Ser473 site. Addition of doxycycline to the medium resulted in expression of the Myc tag and induction of activated (phosphorylated) Akt on the Ser473 site. Moreover, in the presence of doxycycline, marked Akt phosphorylation was noted in cells exposed to LY ± FP (12 h), in effect circumventing the blockade of Akt activation by LY (Fig. 9B). After exposure to 250 nM FP alone for 12 h, apoptosis was partially but significantly attenuated when cells were cultured in the presence of doxycycline, which resulted in a clear increase in Akt phosphorylation on CDK2- and CDK4-specific sites was only partially reversed by the administration of the caspase inhibitor (Fig. 8B). These findings indicate that FP/LY-mediated perturbations in the cell cycle and apoptotic regulatory events proceed through both caspase-dependent and -independent pathways.

To characterize the functional role of perturbations in Akt in the apoptotic response of leukemia cell to FP, a Jurkat cell line that inducibly expresses a constitutively active myristoylated Akt mutant in the presence of doxycycline was used. As shown in Fig. 9A, addition of doxycycline to the medium resulted in expression of the Myc tag and induction of activated (phosphorylated) Akt on the Ser473 site. Moreover, in the presence of doxycycline, marked Akt phosphorylation was noted in cells exposed to LY ± FP (12 h), in effect circumventing the blockade of Akt activation by LY (Fig. 9B). After exposure to 250 nM FP alone for 12 h, apoptosis was partially but significantly attenuated when cells were cultured in the presence of doxycycline, which resulted in a clear increase in Akt phosphorylation (P < 0.02; Fig. 9C). Moreover, an even greater protective effect was observed when cells were exposed to the combination of LY + FP (P < 0.002 versus cells exposed to drugs in the absence of doxycycline). Together, these findings suggest that FP-mediated lethality is regulated, at least in part, by Akt, and that interruption of the Akt pathway contributes to the potentiation of FP-induced apoptosis by LY.
Finally, to determine whether such events were restricted to continuously cultured cell lines, parallel studies were performed in primary leukemic blasts obtained from the peripheral blood of a patient with AML (M2 subtype). As shown by the histograms in Fig. 10A, 24-h exposure to 15 μM LY or 100 nM FP alone resulted in a modest degree of apoptosis, reflected by annexin V and annexin V/PI positivity (i.e., 3 and 6%, respectively). However, cotreatment of blasts with FP/LY induced apoptosis in the large majority of leukemic blasts (e.g., 80%; Fig. 10B). These findings indicate that the dramatic potentiation of cell death in leukemic cells that are simultaneously exposed to a CDK inhibitor in conjunction with a PI3K inhibitor occurs in at least some primary AML specimens. On the other hand, treatment of normal peripheral blood mononuclear cells with identical concentrations of FP/LY, either alone or in combination, induced little apoptosis at either the 6- or 24-h interval (Fig. 10C), raising the possibility that this strategy may offer the potential for therapeutic selectivity.

**DISCUSSION**

In view of evidence linking dysregulation of cell cycle progression to apoptosis (10), it is tempting to invoke this mechanism to explain the ability of FP and other pharmacological CDK inhibitors to induce cell death in leukemia and other neoplastic cell types. However, identification of the specific cell cycle events responsible for CDK inhibitor-mediated lethality remains an elusive goal. An alternative possibility is that FP and similar agents (e.g., UCN-01) act by modulating expression of certain antiapoptotic proteins (e.g., Mcl-1 and XIAP; Ref. 16). However, it is unlikely that such a mechanism could entirely explain the early induction of mitochondrial injury and apoptosis by FP and other CDK inhibitors (e.g., CGP74514A) that has been reported (9). A third possibility is that the induction of cell death by CDK inhibitors like FP is regulated by the activity of one or more signal transduction pathways, which are known to play critical roles in cell survival decisions (21). Of the various possibilities, activation of the MEK1/2/MAPK and the PI3K signaling pathways, both of which are intimately involved in promoting cell survival (21, 22), represent two plausible candidates. In this context, exposure of human leukemia cells to UCN-01, which inhibits CDKs in addition to PKC and Chk1 (44), has recently been shown to trigger an early and dramatic increase in MAPK activity (45). Moreover, interference with the latter process (e.g., by MEK1/2 inhibitors) results in a striking potentiation of UCN-01-mediated mitochondrial injury and apoptosis (45). However, in marked contrast to these findings, the CDK inhibitors FP, CGP74514A, and roscovitine failed to elicit a major MAPK response, nor did coadministration of MEK1/2 inhibitors enhance CDK-mediated lethality. Together, these findings suggest that the PI3K cytoprotective signaling pathway, rather than the MEK1/2/MAPK pathway, plays a potentially specific role in attenuating mitochondrial damage and apoptosis in leukemic cells subjected to dysregulated CDK function.

In contrast to the failure of the MEK1/2 inhibitor U0126 to promote FP-mediated lethality, LY and wortmannin, both of which inhibit
PI3K (29, 30), strikingly enhanced the lethal effects of each of the CDK inhibitors. These interactions were noteworthy for (a) the extent of synergism; (b) the very early induction of mitochondrial damage (i.e., within 2–3 h); and (c) the ability to extend this phenomenon to include multiple leukemic cell types. Whereas FP acts broadly to inhibit CDKs 1, 2, 4/6, 7, and 9 (5–7), roscovitine and CGP are somewhat more specific for CDK1 (46, 47). Whether inhibition of this CDK is required or sufficient for synergistic interactions with PI3K inhibitors remains to be determined. Although LY and wortmannin are viewed as PI3K inhibitors, they also inhibit several other enzymes involved in cell cycle regulation and DNA repair, including ATM/ATM and Rad3-related (48) and DNA-PK (49). However, the failure of the ATM inhibitor caffeine to potentiate FP-induced lethality argues against a role for dysregulation of ATM in the CDK/PI3K inhibitor interaction. Nevertheless, the possibility that the other actions of LY or wortmannin contribute to the potentiation of CDK inhibitor lethality cannot be ruled out. Lastly, the striking increase in p38 MAPK activation in LY-treated cells (Fig. 7) is consistent with previous reports in the literature (50). However, the inability of the p38 MAPK inhibitor SB202190 (39) to promote FP toxicity suggests

Fig. 9. A, Jurkat cells expressing a tetracycline-inducible constitutively active (myristolated) Akt construct containing a c-Myc tag were incubated for 24 h in the absence or presence of 1 μM doxycycline (DOX), after which the cells were lysed and the proteins separated by SDS-PAGE. The blots were then probed with antibodies directed against phospho-Akt and c-Myc. B, cells were incubated as above in the presence or absence of DOX, and then exposed to 75 nM FP ± 15 μM LY (LF) for an additional 12 h. Cells were then lysed, and the proteins were separated by SDS-PAGE and subjected to Western analysis as in A. Con, control. For both A and B, lanes contained 25 μg protein; blots were subsequently stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer of protein. The results of a representative study are shown; two additional experiments yielded equivalent results. C, cells were treated as in B, after which cytoplasmic preparations were obtained, stained with Wright-Giemsa, and the percentage of apoptotic cells determined as described in “Materials and Methods.” Values represent the means for three separate experiments ± SD. * significantly less than values obtained for cells exposed to drugs in the absence of DOX; P < 0.02. ** P < 0.002.

Fig. 10. A, leukemic blasts from the peripheral blood of a patient with acute myelogenous leukemia (subtype M2) were isolated as described in “Materials and Methods” and exposed to 15 μM LY ± 100 nM FP for 24 h, after which the extent of apoptosis was determined by flow cytometric analysis of Annexin V/PI-stained specimens. Cells in the lower right quadrant (Annexin−/PI−), early apoptotic cells; cells in the upper right quadrant (Annexin+/PI−), late apoptotic cells. B, blasts were treated as above, after which the percentage of total apoptotic cells was determined as in A. Values represent the means for three replicate determinations ± SD. C, normal peripheral blood mononuclear cells were exposed to FP (100 nM) ± LY (15 μM) for 6 or 24 h, after which the percentage of apoptotic cells was determined as above. Values represent the means for triplicate determinations ± SD.
that, in this setting, p38 MAPK activation represents a secondary response to lethal events, rather than a cause.

The finding that FP/LY-induced apoptosis was substantially blocked by ectopic expression of Bcl-2 or by an NH2-terminal phosphorilation loop-deleted protein is consistent with a mitochondrial basis for the lethality of this drug combination. Although the precise mechanism by which Bcl-2 and related proteins block activation of the apoptotic cascade remains the subject of some controversy, there is strong evidence that Bcl-2 and Bcl-xL interfere with interactions between BH3-only members of the Bcl-2 family and proapoptotic multidomain family members such as Bak and Bak, thereby preventing the release of proapoptotic mitochondrial proteins (51). The roles that such mitochondrial proteins play in triggering apoptosis may vary between cell types, and even within the same cell depending on the stimulus. For example, in multiple myeloma cells, dexamethasone and ionizing radiation exerted divergent effects on the release of cytochrome c and Smac/DIABLO (52), a pro-apoptotic protein that acts by antagonizing the actions of members of the XIAP family of caspase inhibitors (53). In contrast, Smac-DIABLO release has recently been shown to play a critical role in facilitating cytochrome c release in prostate carcinoma cells exposed to LY (54). In the present study, coadministration of FP with LY in U937 cells induced the early release of each of the major proapoptotic mitochondrial proteins, i.e., cytochrome c, Smac/DIABLO, and AIF. However, the observation that release of Smac/DIABLO, in contrast to the other mitochondrial proteins, was inhibitable by caspase inhibitors suggests that, in these cells, Smac/DIABLO redistribution plays a secondary role in the initiation of the apoptotic cascade. Lastly, the observation that ectopic expression of dominant-negative caspase-8 exerted only modest effects on LY/FP-mediated apoptosis, at least compared with that of Bcl-2, suggests that engagement of the intrinsic, mitochondrial cell death pathway is primarily involved in the lethality of this drug combination. Nevertheless, the possibility that activation of the extrinsic pathway plays a role in amplification of the apoptotic process, i.e., through activation of BID, as occurs in the case of certain cytotoxic drugs (55), cannot be excluded.

Because Akt represents a major downstream target of PI3K (21), and has been linked, through both indirect and direct mechanisms, to a wide variety of antiapoptotic functions (25, 26, 56), it is tempting to speculate that LY promotes the lethal effects of CDK inhibitors by blocking the activation of Akt and one or more of its downstream targets, thereby lowering the threshold for mitochondrial damage and apoptosis. For example, Akt has been implicated in posttranslational modification of Bad (57), regulation of the expression of antiapoptotic proteins including Bcl-2 and XIAPs (58), and modulation of diverse pathways governing cell survival decisions, including those associated with GSK (59), m-TOR/p70S6K (60), and nuclear factor-κB (61), among numerous others (23–25). In this regard, the finding that rapamycin, an inhibitor of m-TOR, and by extension, p70S6K, failed to enhance FP lethality argues against a critical role for this pathway in the regulation of apoptosis. FP/LY did not substantially modify expression of the antiapoptotic proteins Bcl-2 or Bcl-xL, although cells exposed to both agents did exhibit marked down-regulation of XIAP and Mcl-1. Such actions are similar to those described in chronic lymphocytic leukemia and multiple myeloma cells exposed to FP alone (16, 62) and raise the possibility that the interruption of the PI3K pathway may increase the ability of FP to down-regulate expression of certain antiapoptotic proteins. Coadministration of CDK inhibitors and LY also led to diminished Bad phosphorylation, an event that spares Bad from proteasomal degradation and permits it to associate with and antagonize the ability of antiapoptotic Bcl-2 family members such as Bcl-xL to protect cells from mitochondrial injury (63). It is, therefore, conceivable that reduced phosphorylation of Bad contributed to the potentiation of CDK inhibitor-mediated apoptosis by LY.

The observation that enforced expression of Akt attenuated, at least in part, LY/FP-mediated lethality is consistent with reports that the interruption of the PI3K pathway can potentiate the response of tumor cells to various conventional cytotoxic agents. For example, LY has been shown to enhance the activity of multiple conventional cytotoxic agents (e.g., paclitaxel, gemcitabine, VP-16, doxorubcin) in diverse neoplastic cell types (e.g., ovarian, pancreatic, leukemia, non-small cell lung cancer; Refs. 64–67) However, to the best of our knowledge, enforced activation of Akt has not previously been shown to protect cells from the lethal actions of agents that specifically target the cell cycle, i.e., pharmacological CDK inhibitors. In addition, the striking ability of minimally toxic concentrations of LY to induce mitochondrial injury and apoptosis in cells exposed to subtoxic or nontoxic concentrations of FP may have particular significance. It is noteworthy that, whereas the induction of Akt exerted a very significant protective effect against LY/FP-related lethality, protection was not complete, raising the possibility that factors other than, or in addition to, Akt inhibition may be involved in this phenomenon. In this context, other examples of PI3K-dependent survival functions that operate independently of Akt have been described. For example, the ability of glucagons-like peptide 2 to circumvent LY-mediated lethality in baby hamster kidney cells transfected with the glucagons-like peptide 2 receptor was associated with Bad phosphorylation but occurred independently of Akt activation (68). Interestingly, in this system, survival events were blocked by the PKA inhibitor H89, whereas in the present studies, H89 failed to mimic the potentiation of CDK inhibitor lethality by LY. Together, these findings suggest that signaling cascades that regulate the lethal response to PI3K interruption vary with cell type and/or the inciting stimulus. Other recently described PI3K-dependent, Akt-independent downstream targets include the small GTP-binding proteins CDC42 and RAC1 as well as the serum and glucocorticoid-inducible kinases (SGK), including the cytokine-independent survival kinase (CISK; Refs. 69–71). Whether any of these plays a role in the potentiation of CDK inhibitor-mediated lethality by PI3K interruption will require further investigation.

In summary, the present findings indicate that the pharmacological interruption of the PI3K pathway dramatically lowers the threshold for the early initiation of mitochondrial injury, caspase activation, and apoptosis in multiple leukemia cell types in response to a variety of pharmacological CDK inhibitors. Such findings suggest that PI3K, and one or more of its downstream targets, play a particularly important role in preventing apoptosis in leukemic cells experiencing CDK dysregulation. In addition, these and earlier studies focusing on interactions between the Chk1 inhibitor UCN-01 and MEK1/2 inhibitors (45) provide further evidence that leukemic cells are particularly vulnerable to a strategy in which cell cycle and survival signaling events are simultaneously disrupted. In this regard, it may not be coincidental that both the UCN-01/MEK inhibitor and FP/LY regimens induced dephosphorylation of p34^cdc2, unscheduled activation of which has been associated with enhanced lethality (72). The frequency with which dysregulation of the PI3K pathway is associated with malignant transformation (22, 25, 26), as well as evidence that PI3K inhibitors can enhance the lethality of conventional chemotherapeutic drugs (64–67) have prompted the search for clinically relevant agents that interrupt the PI3K/Akt pathway (73). In this context, the dramatic potentiation of CDK inhibitor-mediated cytotoxicity by pharmacological PI3K antagonists, and the striking synergism of this regimen against multiple leukemia cell types, suggest that efforts to interrupt PI3K/Akt signaling cascades may be particularly effective when combined with novel, clinically relevant inhibitors of cell cycle...
progression. Given these considerations, attempts to identify the specific PI3K targets responsible for such actions are clearly warranted. Accordingly, such studies are currently underway.

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