Molecular Pharmacology Fast Forward. Published on May 3, 2006 as doi:10.1124/mol.106.024505

MOL24505

THE 3-SUBSTITUTED INDOLINONE CDK2 INHIBITOR SU-9516 KILLS HUMAN LEUKEMIA CELLS VIA DOWNREGULATION OF MCL-1 THROUGH A TRANSCRIPTIONAL MECHANISM

Ning Gao, Lora Kramer, Paul Dent, and Steven Grant¶

Department of Medicine (N.G., S.G.), Biochemistry (S.G., P.D.), and Pharmacology (S.G.)

Virginia Commonwealth University and Massey Cancer Center, Richmond VA, 23298

Molecular Pharmacology Fast Forward. Published on May 3, 2006 as DOI: 10.1124/mol.106.024505
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MOL24505

Running Title: SU9516 and Mcl-1 down-regulation

¶To whom correspondence should be addressed as follows:

Dr. Steven Grant

Division of Hematology/Oncology

MCV Station Box 230

Viriginia Commonwealth University/Medical College of Viriginia

Richmond, VA 23298

Phone: 804-828-5211

Fax: 804-828-8079

Email: stgrant@hsc.vcu.edu

Text pages: 39

Figures: 7

References: 41

Abstract: 239 words

Introduction: 688 words

Discussion: 1433 words

Abbreviations: CDK, cyclin-dependent kinase; CDT, carboxy-terminal domain; RT-PCR, reverse transcription-polymerase chain reaction; ROS, reactive oxygen species; pRb, retinoblastoma protein; SU9516, (3-[1-(3H-Imidazol-4-yl)-meth-(Z)-ylidene]-5-methoxy-1,3-dihydro-indol-2-one); FP, flavopiridol; PARP, poly(ADP-ribose)polymerase; FBS, fetal bovine serum; PI, propidium iodide; MFI, mean fluorescence intensity; DTT, dithiothreitol; NAC, N-acetylcysteine; SOD, superoxide dismutase; TBAP, tetrakis(4-benzoic acid)porphyrin chloride; pTEF-b, positive transcription elongation factor-b; NF-κB, nuclear factor-κB; IKK, IκB kinase; MKPs' MAP kinase phosphatases; JNK, c-jun NH_2-terminal kinase; TNFα, tumor necrosis factor α.

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ABSTRACT

Mechanisms of lethality of the 3-substituted indolinone and putatively selective CDK2 inhibitor SU9516 were examined in human leukemia cells. Exposure of U937 and other leukemia cells to SU9516 concentrations ≥ 5 μM rapidly (i.e., within 4 hr) induced cytochrome c release, Bax mitochondrial translocation, and apoptosis in association with pronounced down-regulation of the anti-apoptotic protein Mcl-1. These effects were associated with inhibition of phosphorylation of the carboxy-terminal domain (CTD) of RNA Pol II on serine 2 but not serine 5. RT-PCR analysis revealed pronounced down-regulation of Mcl-1 mRNA levels in SU9516-treated cells. Similar results were obtained in Jurkat and HL-60 leukemia cells. Furthermore, co-treatement with the proteasome inhibitor MG-132 blocked SU9516-mediated Mcl-1 down-regulation, implicating proteasomal degradation in diminished expression of this protein. Ectopic expression of Mcl-1 largely blocked SU9516-induced cytochrome c release, Bax translocation, and apoptosis, whereas knockdown of Mcl-1 by siRNA potentiated SU9516 lethality, confirming the functional contribution of Mcl-1 down-regulation to SU9516-induced cell death. Notably, SU9516 treatment resulted in a marked increase in ROS production, which was diminished, along with cell death, by the free radical scavenger NAC. Unexpectedly, NAC blocked SU9516-mediated inhibition of RNA Pol II CTD phosphorylation on serine 2, reductions in Mcl-1 mRNA levels, and Mcl-1 down-regulation. Collectively, these findings suggest that SU9516 kills leukemic cells through inhibition of RNA Pol II CTD phosphorylation in association with oxidative damage and down-regulation of Mcl-1 at the transcriptional level, culminating in mitochondrial injury and cell death.

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INTRODUCTION

The orderly progression of cells through the cell cycle is regulated by a group of proteins including cyclins, cyclin-dependent kinases (CDKs), and various endogenous CDK inhibitors including $p21^{CIP1}$, $p27^{KIP1}$, and members of the INK4 families (Deshpande et al., 2005; Pei and Xiong, 2005). In the most general sense, interplay between these proteins determines the phosphorylation status of the retinoblastoma protein (pRb), the dephosphorylated form of which binds to and inactivates members of the E2F transcription factor family, which induce diverse proteins required for entry into and progression through S-phase (Cobrinik, 2005). Inhibition of CDK activity by various means results in pRb dephosphorylation, increased binding and inactivation of E2F, and interference with cell cycle traverse. Disordered cell cycle regulation is a cardinal characteristic of the neoplastic state, and members of the cell cycle machinery implicated in cell cycle arrest (e.g., pRb) are commonly viewed as tumor suppressor genes (Seville et al., 2005). Because disruption of the cell cycle machinery in transformed cells frequently culminates in apoptosis (Seville et al., 2005), the identification of pharmacologic CDK inhibitors represents a major focus of antineoplastic drug development. This initiative has led to multiple clinical candidates, including the rohitukine alkaloid pan-CDK inhibitor flavopiridol (Dai and Grant, 2004) and the purine derivative CYC202, an analog of R-roscovitine (Senderowicz, 2003, MacCallum et al., 2005). While it is generally assumed that such agents kill neoplastic cells through cell cycle-related mechanisms, this is by no means certain, and attention has recently focused on cell cycle-independent actions (Senderowicz, 2003).

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CDK2 and its binding partners cyclin A and cyclin E play critical roles in S-phase progression; moreover, dysregulation of CDK2/cyclin E complexes have been implicated in carcinogenesis (Woo and Poon, 2003). These considerations have prompted the search for more effective and potentially selective CDK2 inhibitors. The 3-substituted indolinone compound SU 9516 (3-[1-(3H-Imidazol-4-yl)-meth-(Z)-ylidene]-5-methoxy-1,3-dihydro-indol-2-one) was selected by screening compounds based upon their ability to bind to and inhibit the activity of CDK2 (Li et al., 2003), and therefore represents a prototype of such agents. It is approximately twice as potent an inhibitor of CDK2 compared to CDK1, and more than 20-fold more potent against CDK2 than CDK4 (Lane et al., 2001). In RKO, SW480, and other colon carcinoma cell lines, SU9516 selectively inhibited CDK2 activity and potently induced apoptosis in association with pRb dephosphorylation and cell cycle arrest in G_1 or G_2M (Lane et al., 2001). These events were also associated with sequestration of E2F complexes with pRb and other pocket proteins (e.g., p107, p130) (Yu et al., 2002). However, recent studies employing genetic approaches suggested that CDK2 is dispensable for transformed cell survival and proliferation, and raised the possibility that CDK2 may not be an optimal target for anticancer drug development (Martin et al., 2005). While this may in fact be the case, it leaves open the question of how agents like SU 9516 induce apoptosis in transformed cells. In this context, CDKs are also involved in the regulation of transcription via phosphorylation of the carboxy-terminal domain (CTD) of RNA pol II, and certain less specific inhibitors such as flavopiridol and the R-roscovitine analog CYC202 (Seliciclib) have been shown to induce cell death in malignant hematopoietic cells via modulation of

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the expression of apoptotic regulatory proteins (Chen et al., 2005; MacCallum et al., 2005).

To address this question, we have investigated mechanisms by which SU 9516 triggers cell death in human leukemia cells (e.g., U937, HL-60, and Jurkat). Here we report that SU 9516 potently induces mitochondrial injury (i.e., cytochrome c release and Bax translocation), inhibition of phosphorylation on serine 2 of the CTD of RNA pol II, and the pronounced down-regulation of Mcl-1 through transcriptional repression combined with proteasomal degradation. Furthermore, ectopic expression of Mcl-1 substantially reverses SU9516-mediated lethality in these cells, and transient transfection with Mcl-1 siRNA significantly enhances SU9516-mediated lethality. Unexpectedly, the present results demonstrate that SU9516-mediated Mcl-1 transcriptional repression and lethality involve induction of oxidative damage. Together, these findings indicate that in human leukemia cells, the lethal effects of SU9516 stem in large part from inhibition of the CDK9/cyclin T transcriptional regulatory complex and induction of oxidative injury, resulting in down-regulation of the anti-apoptotic protein Mcl-1.

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MATERIALS AND METHODS

Reagents. SU9516 [3-[1-(3*H*-Imidazol-4-yl)-meth-(Z)-ylidene]-5-methoxy-1,3-dihydro-indol-2-one] was purchased from Alexis (San Diego, CA). Flavopiridol was provided by Dr. Dimitrios Colevas, Cancer Treatment and Evaluation Program, NCI. The pan-caspase inhibitor, Z-VAD-FMK, was purchased from Enzyme System Products (Livermore, CA). Actinomycin D, MG132, TBAP, and cycloheximide were from EMD Biosciences (La Jolla, CA). N-Acetyl-L-cysteine (NAC) was from Sigma (St. Louis, MO). CM-H_2DCFDA was obtained from Molecular Probes (Eugene, OR). Antibodies against cytochrome c and β-actin were from Santa Cruz (Santa Cruz, CA), cleaved caspase-3, and ubiquitin were from Cell Signaling (Beverly, MA), Mcl-1 and Bax were from PharMingen (San Diego, CA), PARP was from Biomol (Plymouth Meeting, PA), caspase-8 was from Alexis (San Diego, CA), cytochrome c oxidase was from Molecular Probes (Eugene, OR). Antibodies for total RNA polymerase II (8WG16), phosphorylated CTD at Ser2 (H5) or Ser5 (H14) were purchased from Covance Research Products (Berkeley, CA).

Cells. U937, HL-60, and Jurkat human leukemia cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 medium supplemented with sodium pyruvate, MEM, essential vitamins, L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (FBS). U937 cells stably overexpressing Mcl-1 were kindly provided by Dr. Ruth Craig (Dartmouth Medical School, Hanover). These cells, which have previously been described in detail (Rahmani et al., 2005), were obtained by transfecting U937 cells with a pCEP-Mcl-1 construct that encodes for the 40-

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kDa Mcl-1 protein. Stable single cell clones were selected in the presence of 400 μg/ml hygromycin, Thereafter, cells were analyzed for Mcl-1 protein expression by Western blot, and two clones, designated C14 and C16, which displayed the greatest overexpression of Mcl-1 compared to empty vector controls (pCEP) were used in all experiments. .

Assessment of Apoptosis. For Annexin V/PI assays, cells were stained with Annexin V-FITC and propidium iodide (PI), and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD PharMingen, San Diego, CA). Briefly, 1×10^6 cells were washed twice with cold PBS, and stained with 5 μl of Annexin V-FITC and 10 μl of PI (5 μg/ml) in 1× binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$) for 15 min at room temperature in the dark. The apoptotic cells were determined using a Becton Dickinson FACScan cytoflurometer (Mansfield, MA). Both early apoptotic (annexin V-positive, PI-negative) and late (annexin V-positive and PI-positive) apoptotic cells were included in cell death determinations.

Detection of intracellular ROS. Intracellular production of ROS was measured using CM-H_2DCFDA. To determine ROS production, control and drug-treated cells were incubated with CM-H_2DCFDA (5 μM) for 30 min, washed twice with cold PBS and analyzed within 1 h using a Becton-Dickinson FACScan flow cytometer (Hialeah, FL). For each condition, Mean Fluorescence Intensity (MFI) for each condition was determined as described previously (Rosato et al., 2005), and values expressed as the

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percentage increase of MFI for treated cells relative to controls, with values for untreated cells arbitrarily set at 1.0.

Quantitative Real Time PCR. U937 cells were untreated or treated with various concentrations of SU as indicated for 2 h, and 10 μM SU for the indicated time period. After treatment, cells were lysed and total RNA was extracted using the RNeasy mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. Quantitative real time PCR analysis was carried out on the ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) using the TaqMan® One Step PCR Master Mix Reagents Kit (P/N: 4309169) as recommended by the fabricant. The cycling conditions were: 48 °C/30 min; 95 °C/10 min; and 40 cycles of 95 °C/15 sec and 60 °C/1 min. The cycle threshold was determined to provide the optimal standard curve values (0.98 to 1.0). The probes (5'-TCAAGTGTTTAGCCACAAAGGCACCAAAAG-3') and Mcl-1 specific primers (forward, GGGCAGGATTGTGACTCTCATT; reverse 5'-gatgcagctttcttggtttatgg-3') were designed using the Primer Express® 2.0 version. Ribosomal RNA (18S rRNA) was used as internal control. Each sample was tested in triplicate, and Mcl-1 mRNA level was normalized to that of 18 S rRNA.

Western Blot Analysis. Western blot analysis was performed using the NuPAGE Bis-Tris electrophoresis system (Invitrogen, Carlsbad, CA). The total cellular samples were washed twice with cold PBS and lysed in 1× NuPAGE LDS sample buffer supplemented with 50 mM dithiothreitol (DTT, Fisher Biotech, Pittsburgh, PA). The protein concentration was determined using Coomassie Protein Assay Reagent (Pierce,

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Rockford, IL). The total cellular protein extracts were separated by SDS-PAGE, and transferred to nitrocellulose membrane in 20 mM Tris-HCl (pH 8.0) containing 150 mM glycine and 20% (v/v) methanol. Membranes were blocked with 5% nonfat dry milk in 1× TBS containing 0.05% Tween 20 and incubated with antibodies described in Materials. Protein bands were detected by incubating with horseradish peroxidase-conjugated antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and visualized with enhanced chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA).

Analysis of Cytosolic Cytochrome C, Mcl-1 and Bax, and Mitochondrial Mcl-1 and Bax. After treatment , cells were collected and washed twice in cold PBS. The cell pellet was resuspended in 5 times the volume of Buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 1 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol, 2 mM pepstatin, and 250 mM sucrose. The resuspended cell pellet was incubated on ice for 15 min before the cells were broken by passing them through a 22-gauge needle 25 times. The resulting broken cell mixture was centrifuged in three sequential steps: 1000g, 10,000g, and 100,000g. The 10,000g pellet was considered the “mitochondrial” fraction and the 100,000g supernatant (S100) the cytosol. The protein concentration was determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). 30 μg of cytosolic and mitochondrial extracts were separated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with antibodies against cytochrome C, Mcl-1, and Bax.

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siRNA Studies. U937 cells (2×10^6) were transfected with 100 nM control siRNA and Mcl-1 siRNA (Dharmacon, Lafayettee, CO) respectively using the Amaxa nucleofector™ (Koeln, Germany) as recommended by the manufacturer. After incubation at 37°C for 24 h, transfected cells were treated with various concentrations of SU9516, and subjected to apoptosis and Western blot analysis as described above.

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RESULTS

SU9516 potently induces mitochondrial injury, caspase activation, and apoptosis in U937 cells

A dose-response study in U937 cells revealed a moderate increase in apoptosis at 6 hr following exposure to an SU9516 concentrations of 7.5 μM and very extensive apoptosis at concentrations ≥ 10 μM (Figure 1A). Time course analysis of cells exposed to 10 μM SU9516 demonstrated a significant increase in apoptosis as early as 4 hr after drug administration, and over 50% apoptosis at 6 hr (Figure 1B). Induction of apoptosis by 10 μM SU9516 (6 hr) was equally effective in inducing apoptosis in Jurkat lymphoblastic leukemia and HL-60 promyelocytic leukemia cells (Figure 1C). Western blot analysis revealed that exposure of U937 cells to 7.5 μM SU9516 for 6 hr resulted in a marked increase in caspase-3 and -8 cleavage, and release of cytochrome c into the cytosolic S-100 fraction (Figure 1D), which were apparent as early as 4 hr after drug exposure (Figure 1E). Thus, SU9516 rapidly and potently induced mitochondrial injury and apoptosis in diverse human leukemia cell types.

SU9516 lethality is associated with the caspase-independent down-regulation of the anti-apoptotic protein Mcl-1

The effects of SU9516 on the expression of various anti-apoptotic proteins was examined in U937 cells. A dose-dependent study demonstrated that exposure of cells to varying concentrations of SU9516 did not discernibly modify the expression of Bcl-2, Bcl-X_L, XIAP, Bid, or Bax (Figure 2A). A time-course study also demonstrated that exposure of cells to 10 μM SU9516 for various intervals did not appreciably modify the

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expression of these proteins (Figure 2B). However, in marked contrast, SU9516 strikingly reduced expression of Mcl-1 in dose- and time-dependent manners (Figure 2A and 2B), in parallel with the extent of apoptosis induction. Down-regulation of Mcl-1 by 10 μM SU9516 occurred to an equivalent extent in the mitochondrial and cytosolic fractions, and was accompanied by the translocation of Bax from the cytosolic to the mitochondrial compartment (Figure 2C). Down-regulation of Mcl-1 in Jurkat and HL-60 cells by SU9516 was essentially identical (Figure 2D). To assess the caspase-dependence of these events, the pan-caspase inhibitor Z-VAD-fmk was employed. Z-VAD-fmk blocked SU9516-mediated caspase-3 and -8 activation, but had no effect on cytochrome c release (Figure 3A). ZVAD-fmk also failed to prevent down-regulation of Mcl-1 in the total cellular, cytosolic or mitochondrial compartments (Figure 3B). Together, these findings indicate that SU9516-mediated cytochrome c release and Mcl-1 down-regulation represent primary rather than caspase-dependent events, suggesting that they may be involved in SU9516-mediated lethality.

SU9516 down-regulates Mcl-1 through a transcriptional mechanism in association with inhibition of phosphorylaton of RNA Pol II CTD at serine 2

To elucidate the mechanism underlying Mcl-1 down-regulation by SU9516, RT-PCR analysis was employed. As shown in Figure 4A, exposure of U937 cells to 10 μM SU9516 for 1 hr resulted in a significant decline in Mcl-1 mRNA levels ($P < 0.01$), and down-regulation was very extensive by 2 hr and 4 hr. Reductions in mRNA levels were observed at SU9516 concentrations as low as 5 μM, and roughly paralleled the extent of protein down-regulation (Figure 4B). In view of evidence that other CDK inhibitors such

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as the pan-CDK inhibitor flavopiridol inhibit the cyclinT/CDK9 complex (Chen et al., 2005), phosphorylation of the carboxy-terminal domain of RNA Pol II by SU9516 was examined. As shown in Figure 4C, within 30 min, 10 μM SU9516 robustly blocked phosphorylation of RNA PolII at serine 2, consistent with inhibition of CDK9, but had little effect on phosphorylation at serine 5, a target of CDK7 (Ramanathan et al., 2001). Total PolII levels did not change with drug treatment. Inhibition of transcription by co-exposure of cells to actinomycin D (4 μg/ml) failed to modify the rate or extent of SU9516-mediated Mcl-1 down-regulation (Figure 4D), consistent with the concept that SU9516 acts primarily through a similar mechanism. Lastly, SU9516 exerted essentially identical effects on Mcl-1 mRNA levels (Figure 4E) and inhibition of RNA Pol II phosphorylation on serine 2, but not on serine 5, in Jurkat lymphoblastic and HL-60 promyelocytic leukemia cells (Figure 4F). Together, these findings suggest that in human leukemia cells, SU9516 blocks RNA Pol II CTD phosphorylation to repress Mcl-1 transcription elongation.

Reduction of Mcl-1 protein levels in SU9516-treated cells proceeds via a post-translational, proteasomal-dependent mechanism

To gain further insights into the mechanism by which SU9516 diminishes Mcl-1 expression in human leukemia cells, U937 cells were exposed to 10 μM SU9516 for various intervals in the presence or absence of the proteasome inhibitor MG132 (10 μM). As shown in Figure 5A, MG-132 essentially blocked the down-regulation of total cellular Mcl-1 as well as Mcl-1 expression in the mitochondrial and cytosolic compartments. Co-adminstration of the protein synthesis inhibitor cyclohexamide (20 μM) accelerated

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the rate of Mcl-1 down-regulation in SU9516-treated cells (Figure 5B), consistent with a separate (i.e., transcriptional rather than translational) inhibitory mode of action of this agent.

Ectopic expression of Mcl-1 markedly reduces SU9516-mediated mitochondrial injury and apoptosis in human leukemia cells

Attempts were then made to assess the functional significance of Mcl-1 down-regulation in SU9516-mediated lethality. To this end, two separate U937 clones ectopically expressing Mcl-1, designated C14 and C16, were employed, as previously described (Rahmani et al., 2005). As shown in the inset, Figure 6A, both C14 and C16 displayed a pronounced increase in Mcl-1 expression compared to empty vector (pCEP) controls. Importantly, ectopic expression of Mcl-1 markedly reduced the lethality of SU9516 (6 hr) over a range of concentrations (Figure 6A). This protective effect was first discernible after 4 hr of drug exposure, and pronounced after 6 hr (Figure 6B). Although a slight reduction in expression of ectopic Mcl-1 was observed in transfectants exposed to 10 μM SU9516, levels remained at least as high as in untreated empty vector control cells (Figure 6C). In addition, ectopic expression of Mcl-1 blocked SU9516-mediated mitochondrial translocation of Bax (Figure 6C). Ectopic Mcl-1 expression largely abrogated SU9516-mediated caspase-3 and -8 activation, PARP degradation, and cytochrome c cytosolic release (Figure 6D).

To further evaluate the functional significance of Mcl-1 down-regulation in SU9516-mediated lethality, parallel studies were performed using U937 cells transiently transfected with Mcl-1 siRNA. As shown the top panel of Figure 6E, transfection with

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Mcl-1 siRNA (24 hr) reduced levels of total Mcl-1 compared to control cells. Furthermore, exposure of U937 cells transfected with Mcl-1 siRNA to SU9516 resulted in a significant reduction in Mcl-1 expression at each SU9516 concentration evaluated compared to cells treated with control siRNA. Notably, treatment with Mcl-1 siRNA significantly increased SU9516-induced apoptosis compared to cells exposed to control siRNA ($P < 0.01$ in each case). Together, these findings, along with results obtained with cells ectopically expressing Mcl-1, support the notion that Mcl-1 down-regulation plays a significant functional role in SU9516 lethality.

SU9516-mediated lethality in human leukemia cells, but not that induced by flavopiridol, involves oxidative injury

The lethal actions of several novel targeted agents have been related to induction of oxidative injury (Engel and Evens, 2006). In addition, reactive oxygen species (ROS) have been implicated in regulating signaling events accompanying environmental stress. Consequently, the role of ROS generation in SU9516 lethality and Mcl-1 down-regulation was investigated. As shown in Figure 7A (upper panel), exposure of U937 cells to 10 μM SU9516 for 30 min resulted in a marked increase in ROS levels, compared to controls ($P < 0.01$). Furthermore, ROS generation was significantly reduced by the free radical scavenger N-acetylcysteine (NAC) as well as by the cell permeable superoxide dismutase (SOD)-mimetic TBAP (200 uM; data not shown). Notably, NAC (and TBAP; data not shown) significantly diminished SU9516-mediated lethality ($P < 0.01$; Figure 7A, lower panel). These findings implicate oxidative damage in SU9516-induced cytotoxicity.

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SU9516-induced inhibition of Mcl-1 transcription and phosphorylation of RNA polII on serine2 is ROS-dependent

Studies were then undertaken to determine what effect, if any, SU9516-induced oxidative injury might have on regulation of Mcl-1 transcription and protein expression. Contrary to expectations, co-administration of NAC (or TBAP; data not shown) largely abrogated SU9516-mediated reductions in Mcl-1 mRNA levels, as determined by RT-PCR (Figure 7B; lower panel). In contrast, NAC or TBAP failed to prevent flavopiridol-mediated declines in Mcl-1 mRNA levels. Consistent with these findings, NAC blocked SU9516-induced inhibition of serine 2 phosphorylation of RNA PolII (Figure 7B; upper panel), but not that induced by flavopiridol. These findings indicate that SU9516-mediated inhibition of phosphorylation of the CTD of RNA Pol II, and accompanying transcriptional repression of Mcl-1, are associated with ROS generation. They also suggest that SU9516-mediated oxidative damage occurs upstream of, and may be responsible for, at least in part, the observed transcriptional repression of Mcl-1.

In accord with previous results (i.e., Figure 7A), NAC (and TBAP; data not shown) diminished SU9516-induced caspase-3 and –8 cleavage, PARP degradation, and cytochrome c release in U937 cells (Figure 7C). Consistent with RT-PCR findings, NAC also blocked SU9516-mediated down-regulation of Mcl-1 protein levels. On the other hand, NAC had little effect on flavopiridol-induced Mcl-1 down-regulation or cytochrome c release (data not shown). Finally, NAC also blocked SU9516-induced inhibition of phosphorylation of RNA Pol II CTD on serine 2 as well as Mcl-1 down-regulation in Jurkat and HL-60 cells (Figure 7D) but did not modify the effects of

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flavopiridol (data not shown). Collectively, these findings support a model in which SU9516-induced oxidative injury plays a key role in blocking Mcl-1 transcription, resulting in diminished expression of this protein.

SU9516-mediated ROS generation occurs upstream of perturbations in Mcl-1 expression

To confirm the hierarchy of events associated with SU9516-induced ROS generation and Mcl-1 down-regulation, the ability of SU9516 to trigger increases in ROS production was examined in U937 cells ectopically expressing Mcl-1, and in which SU9516-induced lethality was largely abrogated (as demonstrated in Figure 6A and 6B). Our reasoning was that if Mcl-1 down-regulation was responsible for oxidative injury, cells ectopically expressing Mcl-1 should show diminished ROS generation in response to SU9516. Conversely, if ROS generation operated upstream of Mcl-1 transcriptional repression and reductions in Mcl-1 protein levels, no differences in ROS levels would be observed in the two cell lines following SU9516 exposure. Notably, the increase in ROS production induced by SU9516 was essentially equivalent in empty-vector controls and the two Mcl-1-expressing clones (C14 and C16; Figure 7E). These findings, which are entirely consistent with the ability of the free radical scavenger NAC to block SU9516-mediated inhibition of Mcl-1 transcription (Figure 7B, lower panel), effectively rules out the possibility that SU9516-induced oxidative damage stems from mitochondrial injury accompanying Mcl-1 down-regulation. Instead, these findings argue strongly that SU9516-mediated ROS generation acts upstream of, and is very likely responsible for, the observed reduction in Mcl-1 expression.

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DISCUSSION

Cell cycle dysregulation is one of the most characteristic perturbations in transformed cells, and is frequently associated with altered expression or activation of components of the cell cycle machinery. For example, increased cyclinA/CDK2 activity, which governs cell cycle progression through S-phase (Frouin et al., 2002), has been observed in human lung and colorectal carcinoma (Li et al., 2002), and increased cyclin E/CDK2 expression/activation has been described in human lung and colorectal carcinomas. Such observations have prompted the development of specific CDK2 inhibitors. The novel 3-substituted indolinone SU9516 was identified through high-throughput screening against CDK2, and has been shown to be a potent inducer of cell cycle arrest and apoptosis in colon carcinoma cells (Lane et al., 2001). These events were associated with inhibition of pRB phosphorylation and sequestration of E2F in complexes with pRb as well as the pocket proteins p130 and p107 (Yu et al., 2002). However, the mechanism by which SU9516 induces apoptosis in these or other cell types has not been clearly delineated, and it is uncertain whether this capacity depends upon or is even related to cell cycle dysregulation. Recently, the specific contribution of CDK2 inhibition to growth arrest or apoptosis has been called into question by studies in human colon cancer cells in which CDK2 expression/activity was ablated by antisense or dominant-negative constructs (Tetsu and McCormick, 2003). Notably, these cells proliferated normally and did not display an increase in cell death, indicating that at least in some cell types, CDK2 activity is dispensable for growth and survival. Consistent with these observations, embryonic fibroblasts lacking CDK2 proliferated normally, as did cells from most tissues in CDK2 knockout mice (Ortega et al., 2003). If such findings can be

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generalized, they suggest that SU9516 and possibly other compound identified through CDK inhibitor screens in all likelihood kill neoplastic cells through alternative mechanism.

The present findings indicate that in human leukemia cells, SU9516 potently and rapidly induces mitochondrial damage, caspase activation, and apoptosis, and that these events in all likelihood stem from down-regulation of Mcl-1. Mcl-1 is an anti-apoptotic member of the Bcl-2 family that acts to prevent mitochondrial injury by antagonizing the actions of pro-apoptotic, BH3-only members such as Bim and Bax (Kuwana et al., 2005). There is abundant evidence that Mcl-1 expression plays a critical role in the survival of transformed cells (Song et al., 2005), particularly those of hematopoietic origin (Opferman et al., 2005). Although debate exists, several studies have demonstrated that down-regulation of Mcl-1 by itself is sufficient to induce cell death. For example, in multiple myeloma and lymphoma cells, down-regulation of Mcl-1 e.g., by antisense oligonucleotides or by siRNA strategies potently induced apoptosis (Opferman et al., 2005; Nencioni et al., 2005). Similar findings have been described in human non-small cell lung carcinoma cells (Ma et al., 2003). Because Mcl-1 mRNA and protein have very short half-lives (e.g., 1 to 2 h for mRNA, and 30 min to 1 h for protein), interruption of Mcl-1 synthesis results in rapid proteasomal degradation (Zhong et al., 2005), culminating in early protein elimination. For these reasons, interference with Mcl-1 synthesis represents an attractive therapeutic strategy in hematopoietic malignancies.

A role for Mcl-1 down-regulation by SU9516 in leukemic cell lethality is supported by several lines of evidence, including the close correlation between dose- and time-dependent reduction in Mcl-1 expression and apoptosis, the demonstration that

Molecular Pharmacology Fast Forward. Published on May 3, 2006 as DOI: 10.1124/mol.106.024505
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SU9516 potently inhibited Mcl-1 transcription, and the finding that ectopic expression of Mcl-1 significantly attenuated SU9516-induced mitochondrial injury and cell death. In this regard, the actions of SU9516, the design of which was specifically directed against CDK2 (Li et al., 2003), resemble those of less specific CDK inhibitors such as flavopiridol and the R-roscovitine analog CYC202. Nevertheless, certain differences exist. While it was initially assumed that the pan-CDK inhibitor flavopiridol (Dai and Grant, 2004) killed cells by disrupting cell cycle traverse, it was subsequently shown that this agent was an effective inhibitor of phosphorylation of the CTD of the CDK9/cyclin T transcription elongation complex (pTEFb) (Chen et al., 2005) as well as CDK7 (Serizawa et al., 1995). Specifically, flavopiridol potently inhibits CDK9 and in so doing, blocks phosphorylation of the CTD on the serine 2 residue, thereby interfering with transcription elongation (Chen et al., 2005). Although one might anticipate that this would exert global effects on protein expression, its major actions, at least over relatively short intervals, involve down-regulation of short-lived proteins such as Mcl-1. In fact, flavopiridol has been shown to down-regulate Mcl-1 in various malignant hematopoietic cells (Gojo et al., 2002), which in the case of multiple myeloma, may represent the primary mechanism of lethality. However, in human leukemia cells, attempts to attribute flavopiridol cytotoxicity solely or primarily to Mcl-1 down-regulation are complicated by other lethal actions of this agent i.e., down-regulation of the short-lived proteins XIAP or $p21^{CIP1}$ (Wittmann et al., 2003; Rosato et al., 2002). In addition, flavopiridol is an inhibitor of IKK, and as a consequence, the anti-apoptotic NF-κB pathway (Gao et al., 2004). The inability of SU9516 to inhibit CDK7 and the association of Mcl-1 transcriptional

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repression with ROS generation distinguish the actions of this agent from those of flavopiridol.

Recently, several studies have also suggested a role for Mcl-1 down-regulation in the lethal actions of the roscovitine analog CYC202 in multiple myeloma cell death (MacCallum et al., 2005; Raje et al., 2005). Although roscovitine is somewhat more selective in its CDK inhibitors actions than the pan-CDK inhibitor flavopiridol, acting primarily against CDKs 1, 2, and 5 (Meijer et al., 1997), CYC202 has also been shown to inhibit phosphorylation of RNA PolII CTD via inhibition of CDK9, and by extension, to act as a transcriptional repressor of proteins such as Mcl-1 (Opferman et al., 2005). While it is tempting to speculate that this agent, as well as SU9516, block transcription by inhibiting CDK9, it should be noted that CDK2 has recently been implicated in phosphorylation of RNA Pol II CTD (on serine 2) in TAT-mediated HIV transcription (Deng et al., 2002). Consequently, the possibility that CDK2 inhibition by SU9516 may play a role in Mcl-1 down-regulation cannot be completely excluded. On the other hand, the failure of SU9516, in contrast to CYC202 (MacCallum et al., 2005), to inhibit CDK7 argues against a role for disruption of transcription initiation in Mcl-1 down-regulation by the former agent. Taken together, these findings suggest that CDK inhibitors of disparate classes, and which exhibit varying degrees of specificity for individual CDKs, may exert their lethality, at least in part, through a common mechanism involving transcriptional repression of Mcl-1.

It is noteworthy that SU9516-mediated inhibition of phosphorylation of the CTD of RNA Pol II, transcriptional repression of Mcl-1 expression, and induction of mitochondrial damage were associated with ROS generation. Recently, considerable

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attention has focused on the role of ROS in regulating various signal transduction pathways, and resulting effects on cell survival. For example, in inflammatory responses to cytokines such as TNFα, ROS inhibit MAP kinase phosphatases (MKPs), leading to increased activity of stress-related kinases such as JNK, culminating in cell death (Kamata et al., 2005). Additionally, oxidant compounds such as arsenite have been shown to inhibit IKKβ by modifying cysteine residues in the catalytic site (Kapahi, et al., 2000). However, while expression of the endogenous CDK inhibitor $p21^{CIP1}$ has been associated with ROS generation (Macip et al., 2002), the precise relationship between CDK inhibition and ROS induction has otherwise not been well defined. In this context, inhibition of transcription by oxidative damage has previously been described (Chen et al., 2005; Hildeman et al., 2003) though the basis for this phenomenon remains to be elucidated. Interestingly, Inukai et al. recently reported that in 786-O renal carcinoma cells, ROS, and more specifically, hydrogen peroxide, triggered ubiquintination of the large component of RNA PolII, leading to its degradation (Inukai et al., 2004). However, in contrast to this phenomenon, SU9516-mediated ROS generation had relatively little effect on total RNA PolII levels, but instead specifically inhibited serine 2 phosphorylation of the CTD, indicating a disparate mode of action. Lastly, the findings that ectopic expression of Mcl-1 protected cells from SU9516 lethality without diminishing ROS production, while antioxidants attenuated SU9516-induced transcriptional repression of Mcl-1, argue strongly that SU9516-mediated oxidative injury occurs upstream of and is causally related to Mcl-1 down-regulation. Additional studies will be required to determine whether SU9516-induced transcriptional repression of Mcl-1 is uniquely associated with oxidative injury, and if so, what the underlying

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mechanism might be. In any case, the present findings could have implications for the further development of SU9516 and potentially other candidate antineoplasic agents thought to act as CDK2 inhibitors, as well as their rational integration into combination regimens.

Molecular Pharmacology Fast Forward. Published on May 3, 2006 as DOI: 10.1124/mol.106.024505
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Molecular Pharmacology Fast Forward. Published on May 3, 2006 as DOI: 10.1124/mol.106.024505
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This work was supported by awards CA63753, CA 93738, and CA100866 from the National Cancer Institute, award 6045-03 from the Leukemia and Lymphoma Society of America, a Translational Research award from the V Foundation, and an award from the Department of Defense (DAMD-17-03-1-0209).

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FIGURE LEGENDS

Fig. 1. SU9516 induces mitochondrial damage, caspase activation, and apoptosis in U937 cells in dose- and time-dependent manners. A, U937 cells were treated with various concentrations of SU9516 (SU) as indicated for 6 hr. B, U937 cell were treated with 10 μM SU for 0, 1, 2, 4, 6, 12, and 24 hr. C, U937, Jurkat, and HL-60 cells were untreated or treated with 10 μM SU for 6 hr. After treatment, cells were stained with annexin V/PI, and apoptosis was determined using flow cytometry as described in Methods. The values obtained from annexin V/PI assays represent the mean ± SD for three separate experiments. In figures A, B, and C, ** = values for cells treated with SU are significantly increased compared to those for control cells by the Student t-test; $p < 0.01$. D, U937 cells were untreated or treated with various concentrations of SU as indicated for 6 hr. E, U937 cell were treated with 10 μM SU for 0, 1, 2, 4, 6 hr. After treatment, total cellular extracts and cytosolic S-100 fractions were prepared and subjected to Western blot assay using antibodies against PARP, cleaved-caspase-3, caspase-8, and cytochrome c as described in Methods. Each lane was loaded with 30 μg protein. Blots were subsequently stripped and reprobed with antibody against β-actin to ensure equivalent loading and transfer. Two additional studies yielded equivalent results.

Fig.2. SU9516 induces downregulation of Mcl-1 and the translocation of Bax.

A, U937 cells were untreated or treated with various concentrations of SU as indicated for 6 hr. B, U937 cells were untreated or treated with 10 μM SU9516 for various time period as indicated. After treatment, total cellular extracts were prepared and subjected to Western blot assay using antibodies against XIAP, Mcl-1, Bcl-2, Bcl-X_L, Bid, Bax,

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and β-actin. C, U937 cells were untreated or treated with 10 μM SU for various time points as indicated, after which total cell lysate, mitochondria (Mit), and cytosol (S100) fractions were prepared and subjected to Western blot assay using antibodies against Mcl-1, Bax, β-actin, and cytochrome c oxidase (Cyt c OX). D, U937, Jurkat, and HL-60 cells were also untreated or treated with 10 μM SU for 4 hr (Mcl-1) or 6 hr (PARP). After treatment, total cellular extracts were prepared and subjected to Western blot assay using antibodies against Mcl-1, PARP, and β-actin. Each lane was loaded with 30 μg protein. Two additional studies yielded equivalent results.

Fig. 3. SU9516 induces downregulation of Mcl-1 via caspase-independent pathway. A, U937 cells were pretreated with the pan-caspase inhibitor, Z-VAD-FMK (30 μM), followed by treatment with 10 μM SU for different time points as indicated. After treatment, the total cellular and cytosolic extracts were prepared and subjected to Western blot assay using antibodies against cleaved-caspase-3, caspase-8, cytochrome c, and β-actin. B, U937 cells were pretreated with the pan-caspase inhibitor, Z-VAD-FMK (30 μM), followed by treatment with 10 μM SU for different time points as indicated. After treatment, total cell lysates, mitochondrial, and cytosolic extracts were prepared and subjected to Western blot assay using antibodies against Mcl-1, β-actin, and cytochrome c oxidase. Each lane was loaded with 30 μg protein. Two additional studies yielded equivalent results.

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Fig.4. SU9516 substantially diminishes Mcl-1 mRNA levels and inhibits the phosphorylation of RNA polymerase II CTD. A, U937 cells were untreated or treated with 10 μM SU for different exposure intervals as indicated. B, U937 cells were untreated or treated with various concentrations of SU as indicated for 2 hr. After treatment, total RNA was isolated and Mcl-1 mRNA was determined using Real-time PCR as described in Methods. The values represent the means ± S.D. for three separate experiments performed in triplicate. In figures A and B, * or ** = values for cells treated with SU are significantly decreased compared to those for control cells by the Student t-test; $p < 0.05$ or $p < 0.01$, respectively. C, U937 cells were untreated or treated with 10 μM SU for different time points as indicated, after which the total cellular extracts were prepared and subjected to Western blot assay using antibodies specific for CTD phosphoserine 2, CTD phosphoserine 5, total RNA polymerase II, as well as β-actin. D, U937 cells were treated with 4 μg/ml actinomycin D (ActD) in the absence or presence of SU (10 μM) for indicated time period, after which the total cellular extracts were prepared and subjected to Western blot assay using antibodies against Mcl-1 and β-actin. E, U937, Jurkat, and HL-60 cells were untreated or treated with 10 μM SU for 2 hr, after which total RNA was isolated and Mcl-1 mRNA were determined using Real-time PCR as described in Methods. F, U937, Jurkat, and HL-60 cells were untreated or treated with 10 μM SU for 30 min, after which the total cellular extracts were prepared and subjected to Western blot assay using antibodies specific for CTD phosphoserine 2, CTD phosphoserine 5, total RNA polymerase II, as well as β-actin. Each lane was loaded with 30 μg protein. Two additional studies yielded equivalent results.

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Fig. 5. SU9516 induces degradation of Mcl-1 via the ubiquitin-proteasome pathway. A, U937 cells were pretreated with the proteasome inhibitor MG132 (10 μM) for 30 min, followed by treatment with 10 μM SU for different exposure intervals as indicated. After treatment, total cell lysates, as well as mitochondrial (Mit) and cytosolic (S100) fractions were prepared and subjected to Western blot assay using antibodies against Mcl-1, β-actin, and cytochrome c oxidase. B. U937 cells were pretreated with 20 μM cycloheximide (CHX) for 30 min, followed by treatment with 10 μM SU for varying intervals as indicated. After treatment, total cellular extracts were prepared and subjected to Western blot assay using antibodies against Mcl-1 and β-actin. Each lane was loaded with 30 μg protein.

Fig. 6. Enforced expression of Mcl-1 blocks SU-mediated apoptosis, and diminished expression of Mcl-1 enhances SU-mediated apoptosis in U937 cells. A, Total cellular extracts were prepared from an empty vector (pCEP) and two clones (Mcl-1[C14] and Mcl-1[C16]) of U937 cells ectopically expressing Mcl-1, and subjected to Western blot assay using antibodies against Mcl-1. Mcl-1(C14), Mcl-1(C16), and empty vector pCEP control cells were treated with or without various concentrations of SU as indicated for 6 h. B, Mcl-1(C14), Mcl-1(C16), and pCEP cells were untreated or treated with 10 μM SU for varying exposure intervals as indicated. After treatment, cells were stained with annexin V/PI, and apoptosis was determined using flow cytometry as described in Methods. The values obtained from annexin V/PI assays represent the mean ± SD for three separate experiments. In figures A and B, ** = values for Mcl-1(C14) and Mcl-

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1(C16) cells treated with SU were significantly decreased compared to those for the empty vector pCEP cells by the Student t-test; $p<0.01$. C, Mcl-1(C14), Mcl-1(C16), and pCEP cells were untreated or treated with 10 μM SU for varying exposure intervals as indicated, after which total cellular and mitochondrial extracts were prepared and subjectd to Western blot assay using antibodies against Mcl-1, Bax, as well as β-actin. D, Mcl-1(C14), Mcl-1(C16), and pCEP cells were untreated or treated with 10 μM SU for varying intervals as indicated, after which total cellular and cytosolic extracts were prepared and subjected to Western blot assay using antibodies against PARP, cleaved-caspase-3, caspase-8, cytochrome c, and β-actin. E, U937 cells were transiently transfected with control siRNA or Mcl-1 siRNA for 24 hr as described in detail in Methods, followed by treatment with various concentrations of SU9516 as indicated. After treatment, total cellular extracts were prepared and subjected to Western blot assay using antibodies against Mcl-1 and β-actin. Cells were stained with annexin V/PI, and apoptosis was determined using flow cytometry as described in Methods. The values obtained from annexin V/PI assays represent the mean ± SD for three separate experiments. ** = values for Mcl-1 siRNA-transfected cells treated with SU were significantly increased compared to those for the control siRNA-transfected cells by the Student t-test; $p<0.01$.

Fig.7. Role of ROS generation in SU9516-mediated Mcl-1 transcriptional repression, protein expression, and apoptosis. A, U937 cells were pretreated with the antioxidant NAC (10 mM) followed by the addition of SU (10 μM) for 30 min (ROS, upper panel) or 6 hr (apoptosis, lower panel). Cells were then labeled with the oxidative-sensitive dye

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CM-H_2DCFDA and ROS production was analyzed by flow cytometry as described in Methods. The increase in cells displaying enhanced ROS production was reflected by an increase in Mean Fluorescence Intensity (MFI). Values are expressed as the fold-increase in MFI for treated cells relative to untreated controls, which are arbitrarily assigned a value of 1.0. For analysis of apoptosis, cells were stained with annexin V/PI, and apoptosis was determined using flow cytometry as described in Methods. The values obtained from annexin V/PI assays represent the mean ± SD for three separate experiments. ** = significantly decreased compared to values obtained with SU treatment alone by the Student t-test; $p < 0.01$. B, U937 cells were pretreated with 10 mM NAC followed by the addition of SU (10 μM) for 30 min (SU) or 4 hr (flavopiridol; 150 nM) for Western blot analysis (upper panel) or 2 hr (SU) or 4 hr (flavopiridol; 150 nM) for mRNA determinations (lower panel). After treatment, the total cellular extracts were prepared and subjected to Western blot assay using antibodies specific for CTD phosphoserine 2, CTD phosphoserine 5, total RNA polymerase II, as well as β-actin. Total RNA was also isolated and Mcl-1 mRNA was determined using Real-time PCR as described in Methods. The values represent the means ± S.D. for three separate experiments performed in triplicate. ** = significantly increased compared to values obtained with SU treatment alone by the Student t-test; $p < 0.01$. C, U937 cells were pretreated with the antioxidant NAC (10 mM), followed by the addition of SU (10 μM) for 4 hr (Mcl-1) or 6 hr (caspase and PARP). Total cellular or cytosolic extracts were prepared and subjected to Western blot assay using antibodies against Mcl-1, PARP, cleaved caspase-3, procaspase-8, cytochrome c, and β-actin. D, Jurkat and HL-60 cells were pretreated with 10 mM NAC, followed by the addition of SU (10 μM) for 30 min or

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4 hr (Mcl-1). After treatment, total cellular extracts were prepared and subjected to Western blot assay using antibodies specific for CTD phosphoserine 2, CTD phosphoserine 5, total RNA polymerase II, Mcl-1, as well as β-actin. E, Mcl-1(C14), Mcl-1(C16), and empty vector pCEP control cells were treated without or with 10 μM SU for 30 min, after which ROS production was determined using CM-H_2DCFDA labeling and flow cytometry analysis as described in Methods. Numbers in parentheses represent the fold increase in the percentage of cells exhibiting an increase in ROS generation compared to untreated controls. Two additional experiments yielded equivalent results.

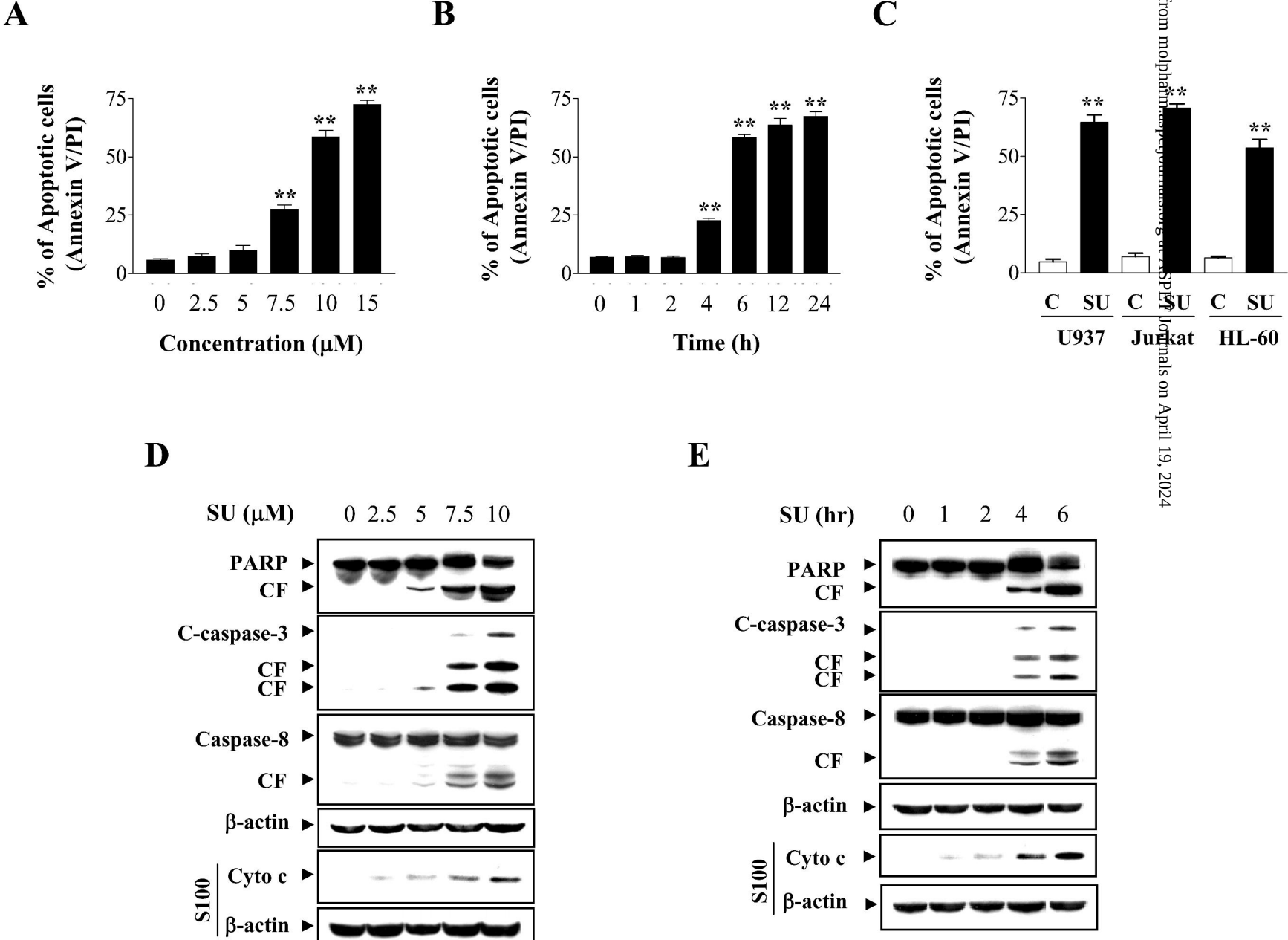
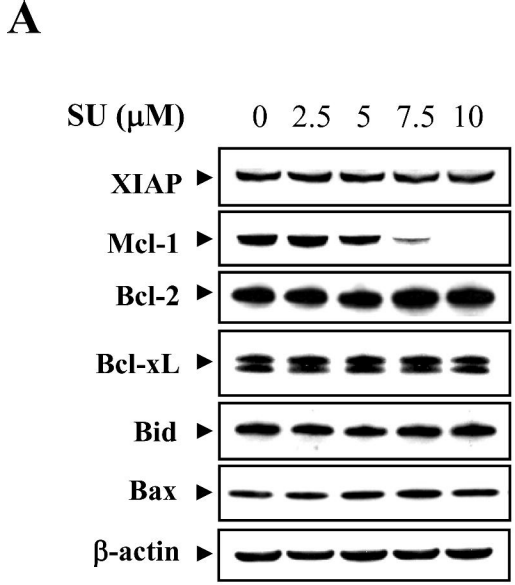
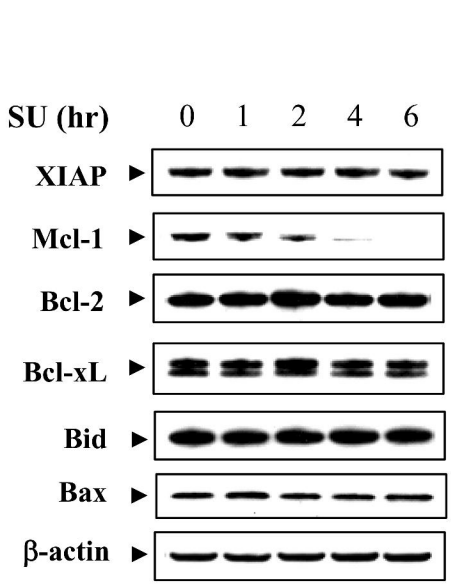
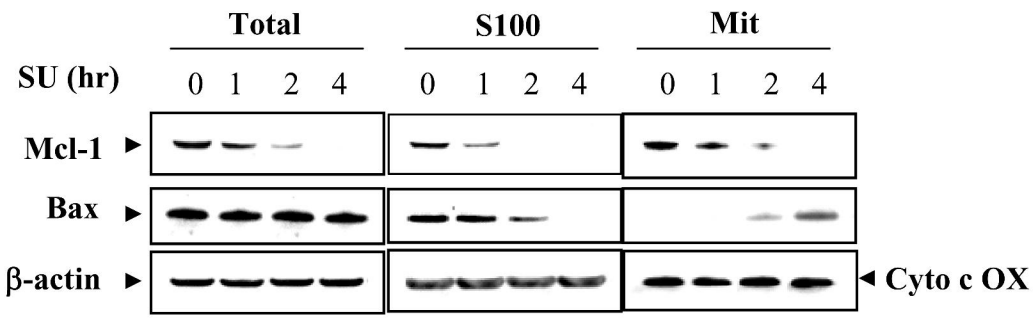


Figure 1







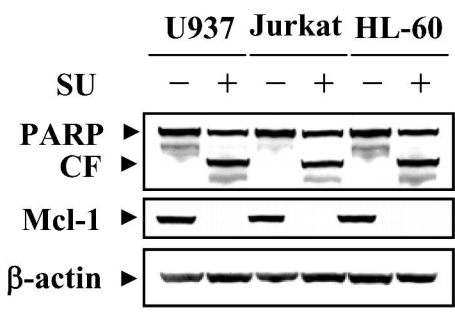


Figure 2

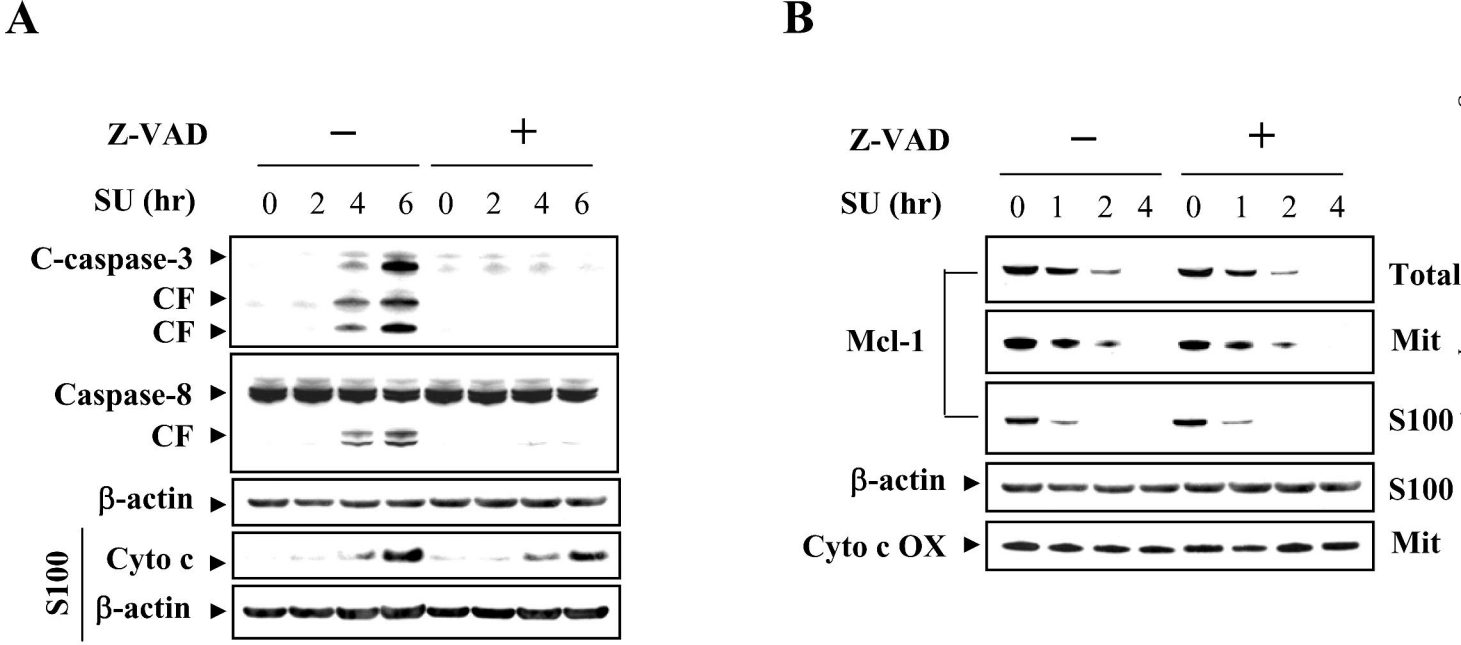

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Figure 3

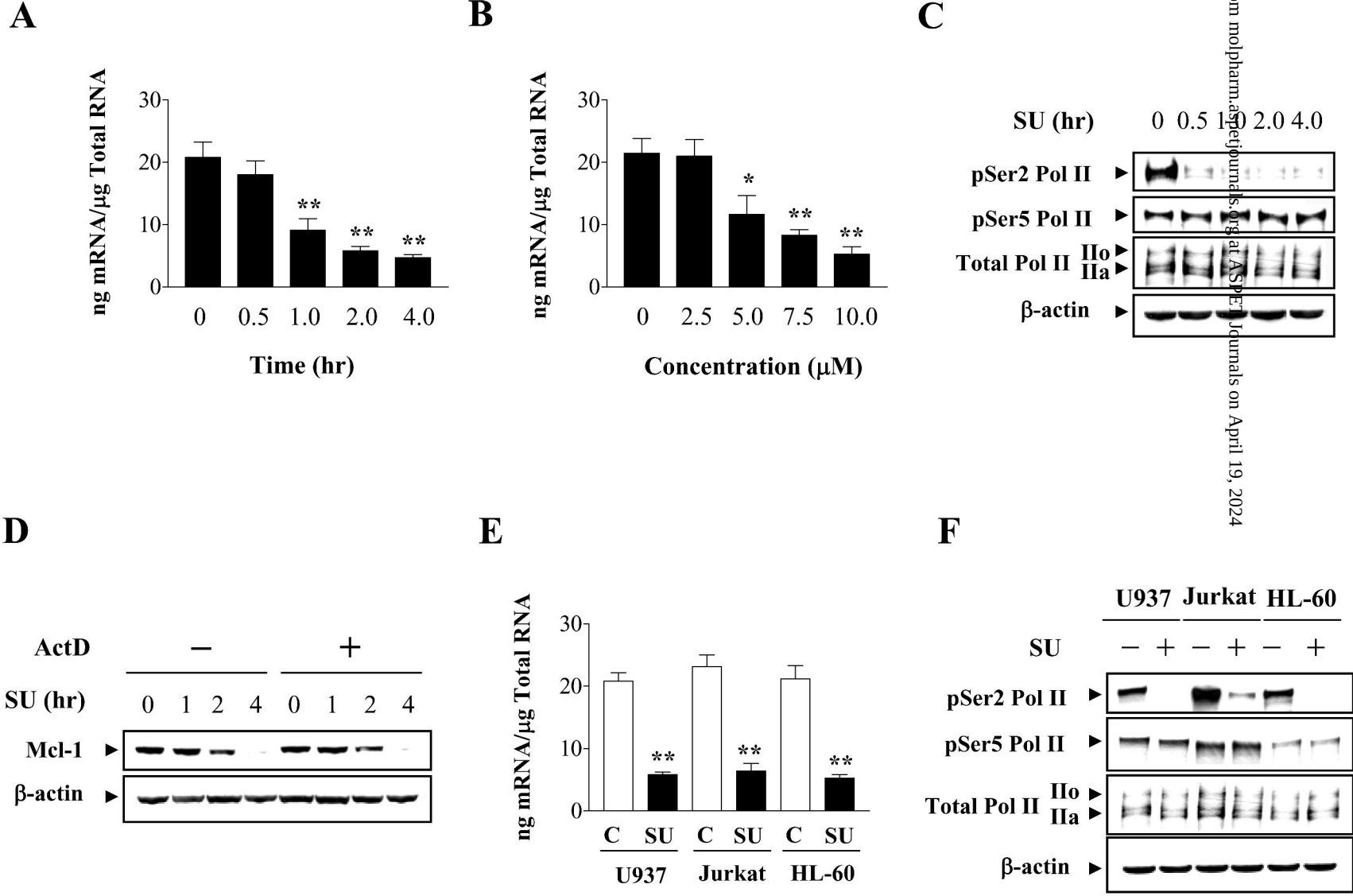

Figure 4

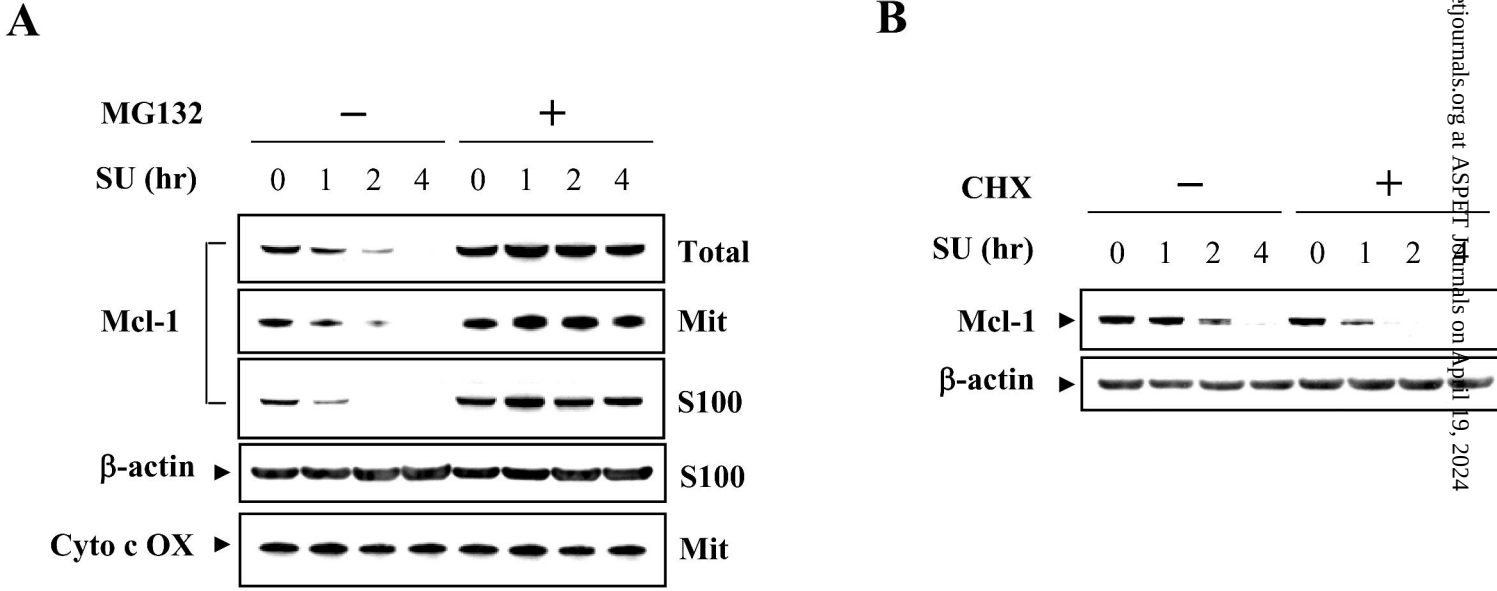

Figure 5

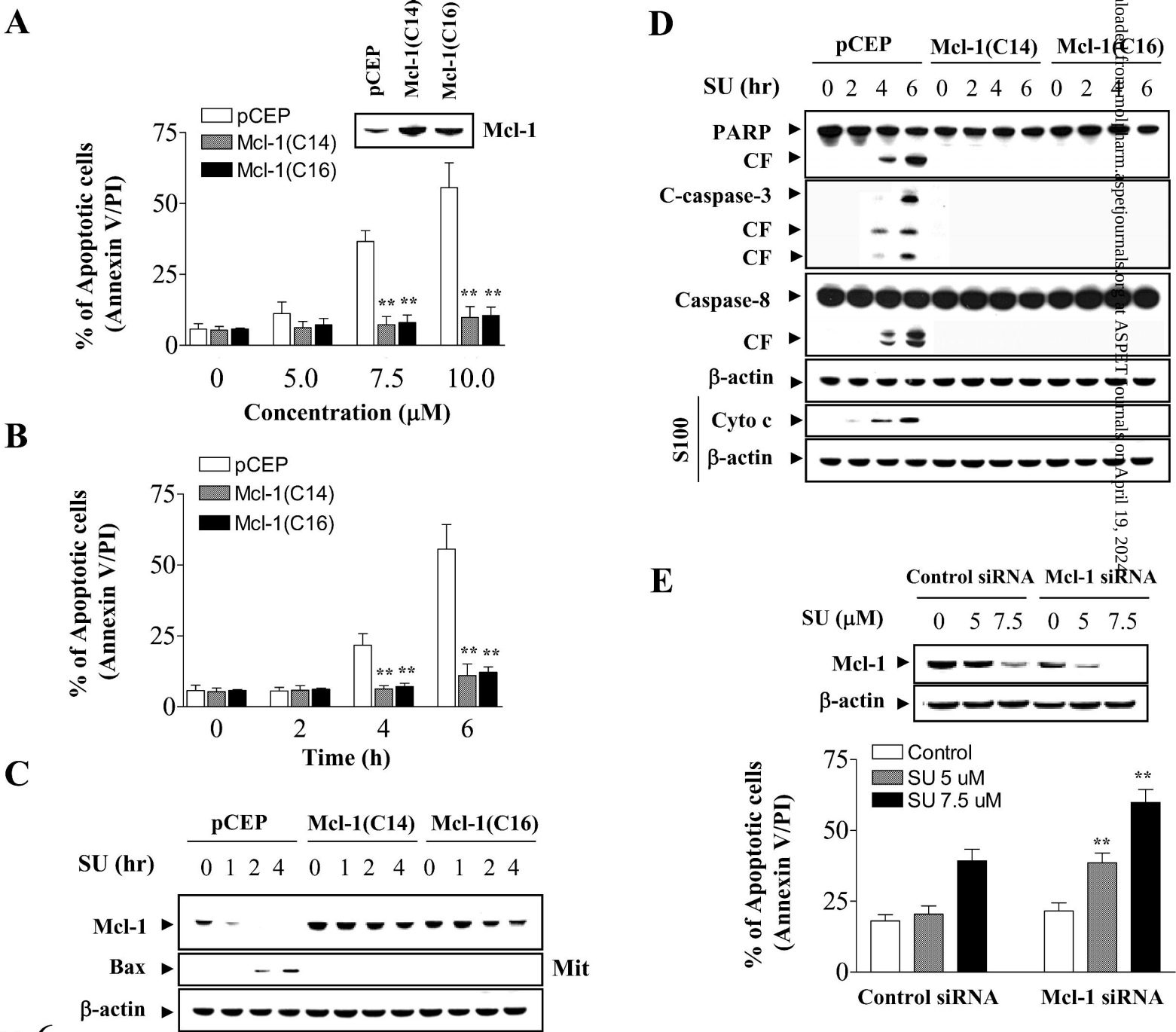

Figure 6

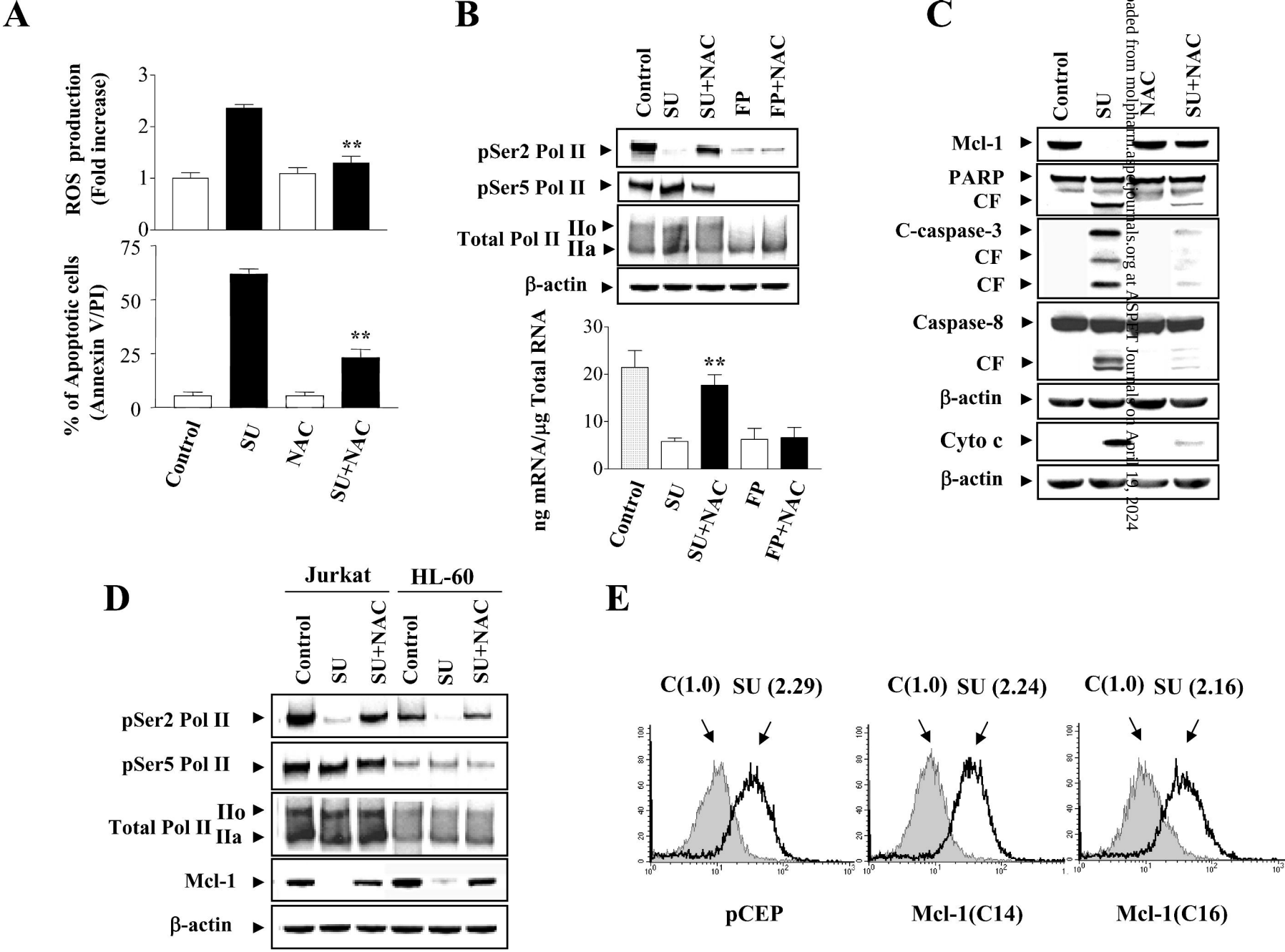

Figure 7