

Research Article

Vinblastine Induces Acute, Cell Cycle Phase–Independent Apoptosis in Some Leukemias and Lymphomas and Can Induce Acute Apoptosis in Others when Mcl-1 Is Suppressed

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Abstract

Chemotherapeutic agents modify intracellular signaling that culminates in the inhibition of Bcl-2 family members and initiates apoptosis. Inhibition of the extracellular signal-regulated kinase by PD98059 dramatically accelerates vinblastine-mediated apoptosis in ML-1 leukemia with cells dying in 4 hours from all phases of the cell cycle. Inhibition of protein synthesis by cycloheximide also markedly accelerated vinblastine-induced apoptosis, showing that the proteins required for this acute apoptosis are constitutively expressed. Vinblastine induced the rapid induction of Mcl-1 that was inhibited by PD98059 and cycloheximide. No change in Bcl-2 or Bcl-X was observed. We hypothesize that ML-1 cells use Mcl-1 for protection from the rapid vinblastine-induced apoptosis. This was confirmed by targeting Mcl-1 with short hairpin RNA. We also investigated the response of 13 other leukemia and lymphoma cell lines and cells from seven chronic lymphocytic leukemia patients. Four cell lines and all chronic lymphocytic leukemia cells were killed in 6 hours by vinblastine alone. Two additional cell lines were sensitized to vinblastine by PD98059, which suppressed Mcl-1. This acute apoptosis either alone or in combination with PD98059 required vinblastine-mediated activation of c-Jun-NH₂-terminal kinase. PD98059 did not suppress Mcl-1 in other cell lines whereas sorafenib did, but this did not sensitize the cells to vinblastine, suggesting that the acute apoptosis varies depending on which Bcl-2 protein mediates protection. Most of the cell lines were sensitized to vinblastine by cycloheximide, suggesting that inhibition of a short-lived protein in addition to Mcl-1 can acutely sensitize cells. These results suggest several clinical strategies that might provide an effective therapy for selected patients. *Mol Cancer Ther*; 9(4): 791–802. ©2010 AACR.

Introduction

Apoptosis can be triggered by numerous types of chemotherapeutic agents culminating in the activation of caspases and nucleases that degrade protein and genomic DNA within the dying cells. Cancer cells frequently express elevated levels of antiapoptotic proteins of the Bcl-2 family, including Mcl-1 and Bcl-X, which make them resistant to chemotherapy. Elevated expression of these proteins is often a consequence of enhanced signal transduction that results from mutations that cause the oncogenic phenotype. The inhibition of signal transduction pathways and downregulation of antiapoptotic Bcl-2

proteins have been shown to induce apoptosis through the intrinsic apoptotic pathway (1).

Microtubule-interfering agents are clinically important chemotherapeutic agents. Vinca alkaloids such as vinblastine bind to the ends of microtubules, suppressing their dynamic instability and causing their depolymerization (2, 3). In contrast, paclitaxel stabilizes microtubules by preventing their depolymerization. Biological consequences of interfering with microtubule dynamics include G₂-M phase arrest, inhibition of cell proliferation, and induction of apoptosis (4).

In a previous study, we examined the effect of vinblastine on mitogen-activated protein kinase (MAPK) activity and apoptosis in myeloid leukemia ML-1 cells (5). Vinblastine alone induced apoptosis in only 45% of the cells within 48 hours. When the mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor PD98059 was combined with vinblastine, there was a dramatic enhancement in apoptosis with 70% of cells undergoing apoptosis in 4 hours. In a related work, we showed that vinblastine rapidly induced Mcl-1 in ML-1 cells and this was dependent on the MEK-ERK pathway (6). Accordingly, we hypothesized that the acute sensitization induced by

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PD98059 was due to the suppression of Mcl-1. This was confirmed in the current studies. In addition, we have examined a panel of leukemia and lymphoma cell lines, and found only two others that are sensitive to this drug combination. Suppression of Mcl-1 did not sensitize other cell lines to vinblastine. Four cell lines, as well as freshly isolated chronic lymphocytic leukemia cells (CLL) were acutely sensitive to vinblastine alone. These results suggest that various leukemias and lymphomas may exhibit marked differences in response to *Vinca* alkaloids and that inhibition of Mcl-1 may be an effective means to enhance vinblastine-mediated cell killing. However, many hematopoietic tumors likely rely on other antiapoptotic Bcl-2 family members to resist this drug combination. We believe that *Vinca* alkaloids alone or in novel combinations deserve further consideration as therapy for selected patients.

Materials and Methods

Materials

Vinblastine and paclitaxel were purchased from Sigma. PD98059, c-Jun-NH₂-terminal kinase (JNK) inhibitor VIII, and SP600125 were purchased from Calbiochem. Sorafenib was purchased from LC Laboratories. Hoechst 33342 was purchased from Molecular Probes. The caspase inhibitor zVAD-fmk was purchased from Enzyme Systems Products, Inc. Protease/phosphatase inhibitor was purchased from Roche. Unless otherwise indicated, all other reagents were purchased from Sigma.

Antibodies used were as follows: phospho-ERK1/2 (9101), c-Jun (9165), phospho-c-Jun (Ser-63; 9261), phospho-JNK1/2 (9255), JNK1/2 (9252), and poly ADP ribose polymerase (PARP; 9542; Cell Signaling); Bim (sc11425), Bad (sc8044), Bax (sc493), and Puma (Santa Cruz Biotechnology; sc19187); Noxa (OP180) and actin (EMD Biosciences; JLA20); Bak (06-536) and ERK1/2 (Upstate Biotechnology; 06-182); Bid (R&D Systems; AF860); Bcl-X (Zymed; 33-6300); Bcl-2 (DAKO; M0887); and Mcl-1 (BD Biosciences; 559027). Antiserum raised in rabbit against a glutathione S-transferase fusion protein of full-length human Bfl-1 was a gift from Jannie Borst (The Netherlands Cancer Institute, Amsterdam, the Netherlands). Secondary antibodies were purchased from Bio-Rad.

Cell culture

Human myeloid leukemia ML-1 cells were obtained from Ruth Craig (Dartmouth Medical School, Hanover, NH) and NB4 cells were obtained from Ethan Dmitrovsky (Dartmouth Medical School, Hanover, NH) with permission from M. Lanotte (Institut National de la Sante et de la Recherche Medicale, Paris, France). THP-1, U937, and Jurkat cell lines were obtained from the American Type Culture Collection. HL60-neo and HL60-Bcl-X cells were kindly provided by Kapil Bhalla (Medical College of Georgia, Augusta, GA). OCI-AML1, OCI-AML3, and OCI-AML4 cells were provided by Mark Minden (Ontario

Cancer Institute, Toronto, Canada). Many of the cell lines are derived from acute myeloid leukemia: ML-1, OCI-AML1, OCI-AML3, and OCI-AML4 are all classified as AML subtype M4; HL60 and NB4 are subtype M3; and THP-1 cells are subtype M5. Jurkat cells are T-cell leukemia and U937 cells were derived from a histiocytic lymphoma. The cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS). HL60-neo and HL60-Bcl-X cells were maintained in media containing 500 µg/mL G418. OCI-AML cell lines were maintained in α -MEM containing 10% FBS, 5% L-glutamine, to which 10 ng/mL granulocyte macrophage colony-stimulating factor was added in the case of OCI-AML1 and 4. Mantle cell lymphoma lines were provided by William Plunkett (M.D. Anderson Cancer Center, Houston, TX) and were maintained as follows: Granta519 DMEM/high glucose plus 20% FBS; Jeko1: RPMI 1640 plus 10% FBS; Mino and SP-53: RPMI 1640 plus 20% FBS.

CLL cells were obtained from consented patients at the Norris Cotton Cancer Center. Cells from 10 mL of blood were diluted in PBS and purified by centrifugation in Ficoll-Paque PLUS. Lymphocytes were collected washed thrice in PBS + 2 mmol/L EDTA and plated in RPMI 1640 plus 10% serum at 1×10^6 cells/mL. Cells were either incubated immediately or after 24 h with drugs. In several experiments, the CLL cells were also incubated over a monolayer of murine bone marrow stromal cells (M210b4, American Type Culture Collection).

Unless otherwise stated, vinblastine was used at 2.2 µmol/L (2 µg/mL) based on previous studies. Although cells incubated with a 10-fold lower concentration were also sensitized by PD98059 (5), this lower concentration is somewhat more borderline for response so we have consistently used the higher concentration. However, peak plasma concentrations within the range of 0.22 to 2.2 µmol/L have been observed in patients (7). PD98059, JNK inhibitor VIII, SP600125, or the appropriate vehicle control were added to cells (1×10^6 /mL) 30 min before the addition of vinblastine and were left in the medium for the duration of the experiment. DMSO concentrations in the media never exceeded 0.2%. To assess chromatin condensation, cells were incubated with 2 µg/mL Hoechst 33342 for 20 min at 37°C and visualized with a fluorescent microscope. At least 200 cells were scored from each sample and data were expressed as the percentage of cells with condensed chromatin (5). To assess cell cycle distribution, cells were fixed in 70% ethanol overnight, stained with propidium iodide, and analyzed on a Becton Dickinson FACScan flow cytometer. Data were analyzed using CellQuest and ModFit programs.

Immunoblot analysis

Cells were lysed in urea sample buffer [4 mol/L urea, 10% β -mercaptoethanol, 6% SDS, 125 mmol/L Tris (pH 6.8), 0.01% bromphenol blue, and protease/phosphatase inhibitor cocktail] and boiled for 5 min. Proteins were subsequently separated by SDS-PAGE (6%, 8%, or 12%) and transferred to polyvinylidene difluoride

membrane (Millipore). Membranes were blocked with Zymed blocking solution or 5% nonfat milk in TBS and 0.05% Tween 20, and were probed with the appropriate primary antibody overnight. Subsequently, membranes were washed in TBS and 0.05% Tween 20, and then incubated with secondary antibody conjugated to horseradish peroxidase. Proteins were visualized by enhanced chemiluminescence (Amersham). ERK, MEK, Bcl-2, or actin were used as loading controls in Western blots as these proteins did not seem to change under the experimental conditions used here. When comparing different cell lines, lysates from a constant number of cells were loaded on each gel.

Lentiviral infection

Lentiviral shRNA constructs were purchased from Open Biosystems. Lentiviral vector particles were produced in 293FT cells as previously described (8). Cells were transiently infected for 48 h with viral media containing one of four Mcl-1 shRNA constructs. Following the 48-h incubation time, cells were washed twice with PBS and plated in fresh medium overnight before treatment with vinblastine.

Results

Vinblastine-mediated apoptosis in ML-1 cells

We have previously shown that ML-1 cells are dramatically sensitized to vinblastine-induced apoptosis by co-incubation with the MEK inhibitor PD98059 (5). Whereas vinblastine alone induced little apoptosis during the first 12 hours of incubation, the combination with PD98059 induced apoptosis in 70% of the cells within 4 hours (Fig. 1A). Concurrent with chromatin condensation, we also observed cleavage of the caspase substrate PARP (Fig. 1B). We refer to this rapid onset of apoptosis as "acute apoptosis" to distinguish it from the delayed apoptosis induced by vinblastine alone. Vinblastine strongly activates JNK as assessed by its phosphorylation as well as increased expression and phosphorylation of c-Jun. We have previously suggested that the activation of JNK is important for this acute apoptosis, although those studies used a JNK-inhibiting peptide that could only partially suppress JNK signaling (5). We have now used the JNK inhibitor SP600125 and show that it can completely suppress acute apoptosis, although it only partially reverses the apoptosis induced by vinblastine alone at 24 hours (Fig. 1A).

It is important to discriminate two different responses of cells to vinblastine. When used alone, vinblastine is thought to induce cells to accumulate in mitosis before the induction of apoptosis and this results in apoptosis occurring over a long period. Cell cycle analysis shows that vinblastine induces both G₁ and G₂-M arrest in ML-1 cells with a marked decrease in the S-phase population, but little apparent apoptosis over 24 hours (Fig. 2). This arrest in G₁ has been attributed to vinblastine-mediated induction of p21^{waf1} that occurs in some cells (9). The combination of vinblastine and PD98059

induced significant apoptosis as assessed by sub-G₁ DNA content and, consistent with previous results (5), this seemed to occur from all phases of the cell cycle. It should be noted that the appearance of a sub-G₁ population in Fig. 2 reflects cells with extensive DNA fragmentation and is delayed compared with the initial onset of chromatin condensation and PARP cleavage observed in Fig. 1. Importantly, inhibition of JNK prevented the appearance of apoptotic cells with the cells now accumulating in G₁ and G₂ as though they had been incubated with vinblastine alone (Fig. 2). We used two different JNK inhibitors in this experiment to confirm the role of JNK and it is evident that JNK inhibitor VIII is somewhat more effective at preventing apoptosis.

Role of Mcl-1 in response of ML-1 cells to vinblastine

Incubation with vinblastine induces a marked increase in Mcl-1, which is suppressed by PD98059 (Fig. 1B). In contrast, incubation with vinblastine did not alter the level of Bcl-2. Furthermore, these cells do not express a detectable level of Bcl-X (see below). This led to the hypothesis that the induction of Mcl-1 prevents vinblastine from inducing acute apoptosis. To further investigate the importance of Mcl-1, we incubated ML-1 cells with vinblastine for 6 hours to induce Mcl-1 and then added PD98059 to determine whether it would still induce apoptosis rapidly. Apoptosis began to occur within the following 2 hours and this correlated with the decrease in Mcl-1 expression (Fig. 1D). Hence, PD98059 is still able to suppress Mcl-1 and sensitize ML-1 cells to vinblastine even when added at a later time point.

Although incubation with PD98059 seemed to suppress Mcl-1, it is also possible that the loss of Mcl-1 is a consequence of apoptosis. Accordingly, we incubated cells with the pan-caspase inhibitor z-VAD-fmk. Analysis of both chromatin condensation (data not shown) and PARP cleavage (Fig. 1B) showed that pretreatment with z-VAD-fmk effectively prevented apoptosis induced by the combination of vinblastine and PD98059. However, z-VAD-fmk did not prevent the suppression of Mcl-1 caused by incubation with PD98059. This shows that the acute apoptosis is caspase dependent and that the loss of Mcl-1 is likely to be a cause and not a consequence of apoptosis. Again, this supports our hypothesis that Mcl-1 is protecting ML-1 cells from the acute vinblastine-induced apoptosis.

Apoptosis is frequently the result of the induction of proapoptotic genes and this could contribute to the acute apoptosis observed here. In an unpublished study, B.L. Salerno and A. Eastman did a microarray analysis to determine the spectrum of genes induced by vinblastine. In addition to Mcl-1, we observed a >3-fold induction of 272 genes following a 4-hour incubation with vinblastine. One of the genes induced was the proapoptotic BH3-only protein PUMA. To investigate the possible role of any of these genes in the induction of apoptosis, we incubated cells with cycloheximide to prevent their expression. The efficacy of cycloheximide at inhibiting

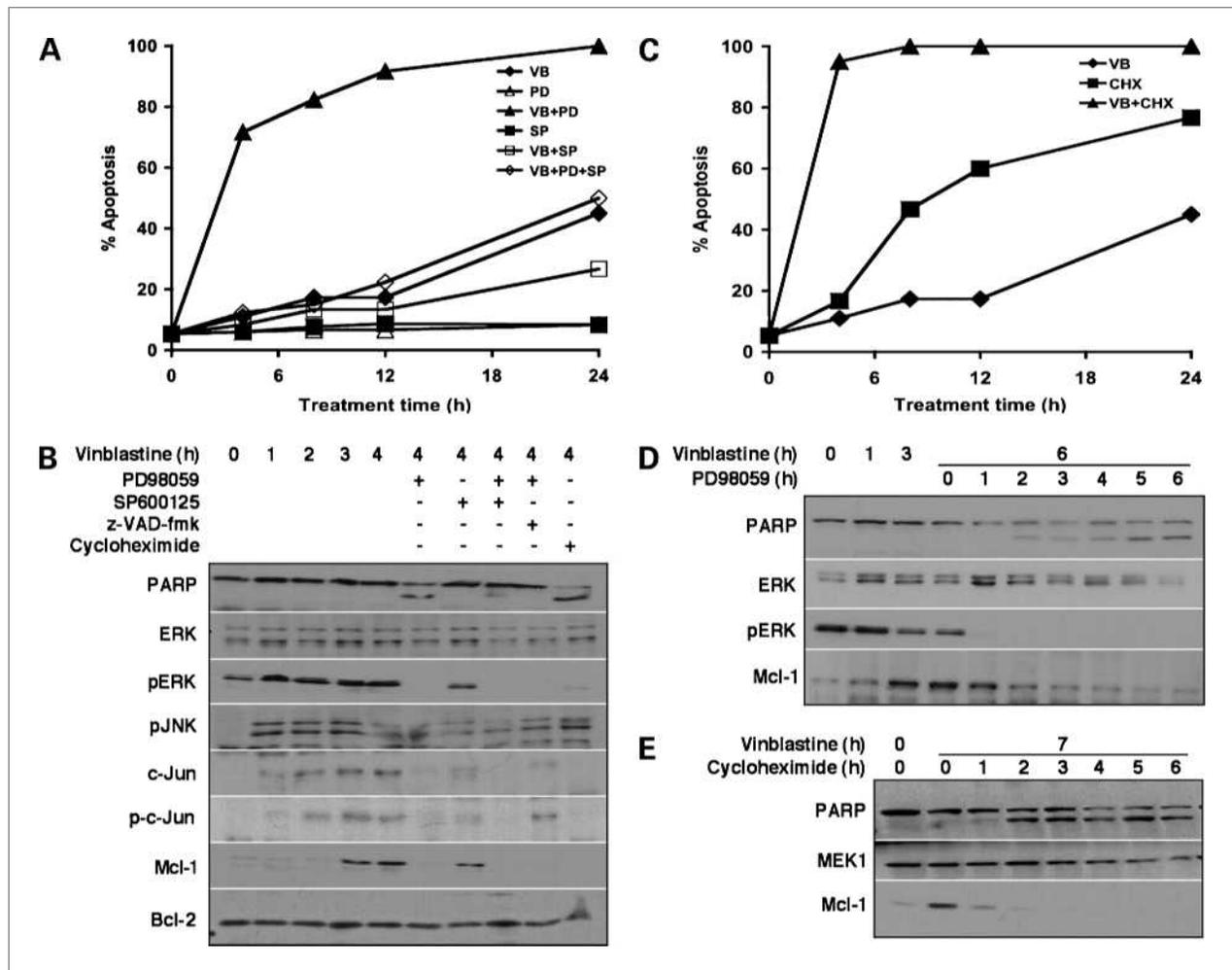


Figure 1. Effect of MAP kinase signaling and protein synthesis on apoptosis induced by vinblastine in ML-1 cells. **A**, ML-1 cells were incubated with 2.2 $\mu\text{mol/L}$ vinblastine (VB), 50 $\mu\text{mol/L}$ PD98059 (PD), 30 $\mu\text{mol/L}$ SP600125 (SP), or a combination of each for the indicated time, and apoptotic cells were scored by chromatin condensation. **B**, ML-1 cells were incubated with 2.2 $\mu\text{mol/L}$ vinblastine alone or in combination with 50 $\mu\text{mol/L}$ PD98059, 30 $\mu\text{mol/L}$ SP600125, 42 $\mu\text{mol/L}$ z-VAD-fmk, or 10 $\mu\text{g/mL}$ cycloheximide. Lysates were prepared at the indicated times and protein expression was analyzed by immunoblotting. **C**, ML-1 cells were incubated with 2.2 $\mu\text{mol/L}$ vinblastine, 10 $\mu\text{g/mL}$ cycloheximide (CHX) or the combination, and apoptotic cells were scored by chromatin condensation. **D**, ML-1 cells were incubated for 6 h with 2.2 $\mu\text{mol/L}$ vinblastine followed by 50 $\mu\text{mol/L}$ PD98059 for 1 to 6 h. Lysates were prepared and analyzed by immunoblotting for the indicated proteins. **E**, ML-1 cells were incubated for 7 h with 2.2 $\mu\text{mol/L}$ vinblastine followed by 10 $\mu\text{g/mL}$ cycloheximide for 1 to 6 h and lysates were analyzed for protein expression.

protein synthesis can be seen as complete and rapid suppression of Mcl-1 and c-Jun (Fig. 1B and E). Interestingly, coincubation of cycloheximide and vinblastine also induced an acute apoptosis, thereby ruling out the requirement for any induced genes in the induction of apoptosis (Fig. 1B and C). These results show that the necessary machinery for apoptosis is constitutively present in the cells. To further investigate the importance of Mcl-1, we incubated ML-1 cells with vinblastine for 7 hours to induce Mcl-1 and then added cycloheximide to determine whether it would still induce apoptosis rapidly. Apoptosis began to occur within 2 hours and this correlated with the decrease in Mcl-1 expression (Fig. 1E). Hence, cycloheximide is still able to sensitize ML-1 cells to vinblastine even when added at a later time point.

To directly test our hypothesis that the elevated Mcl-1 is protecting ML-1 cells from vinblastine, we used shRNA targeted against Mcl-1. Cells were infected with lentiviruses expressing four different shRNA constructs. Following the 48-hour infection period, the cells were incubated with or without vinblastine for 6 hours. Immunoblot analysis showed that vinblastine-mediated induction of Mcl-1 was significantly suppressed in all infected cells (Fig. 3). Bcl-2 levels were unaffected by the shRNA. This suppression of Mcl-1 resulted in marked elevation of vinblastine-induced apoptosis as assessed both by chromatin condensation and PARP cleavage. Infection with a variety of other unrelated shRNA constructs did not affect Mcl-1 levels or the sensitivity to vinblastine (data not shown). These experiments

confirmed that the induction of Mcl-1 is critical for the protection of ML-1 cells from acute apoptosis induced by vinblastine.

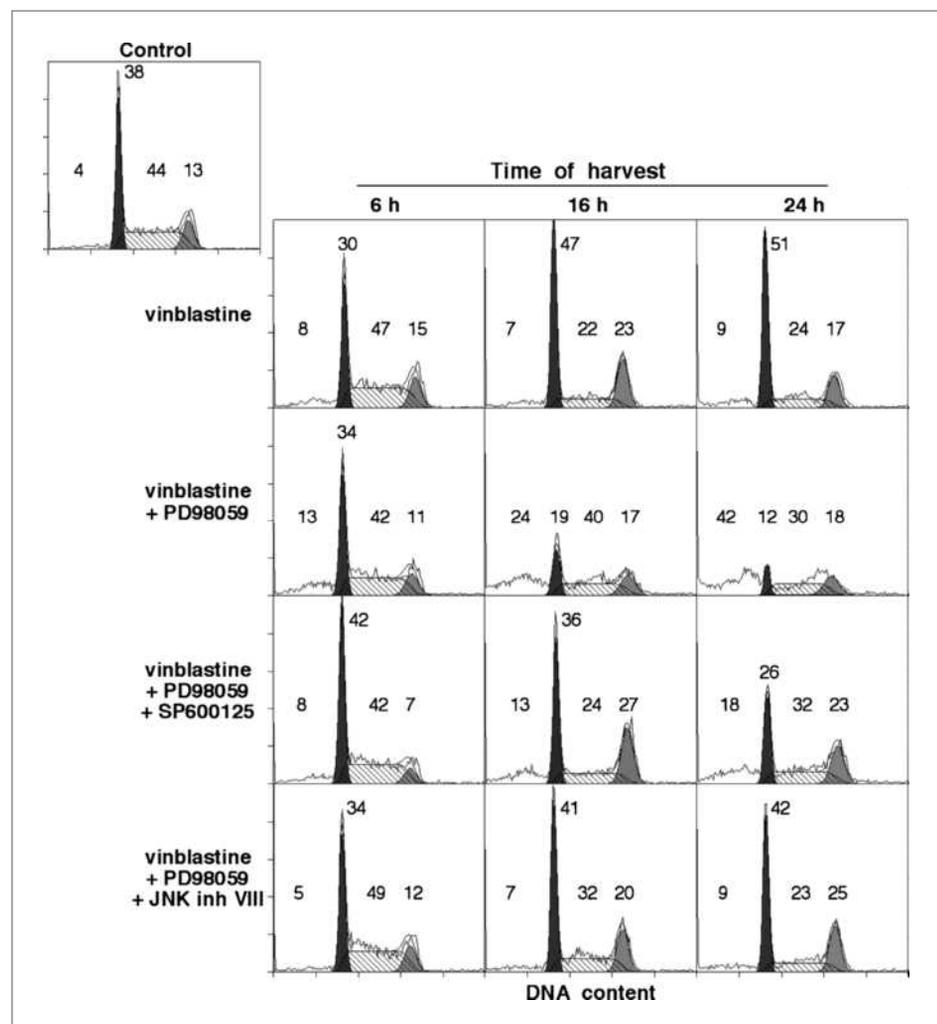
Vinblastine-mediated apoptosis in other cell lines

We next investigated a panel of eight other leukemia cell lines and one lymphoma cell line in search of another that could be sensitized to vinblastine by PD98059. The first unexpected phenotype was observed in OCI-AML1 cells that were acutely sensitive to vinblastine alone with 100% of the cells dying in 4 hours (Fig. 4A). This acute apoptosis was observed as early as 2 hours and was prevented by incubation with JNK inhibitor VIII (data not shown). This phenotype was also observed subsequently in three mantle cell lymphoma lines and CLL cells discussed in more detail below. Several other cell lines (NB4 and THP-1) showed more extensive apoptosis than ML-1 cells with ~50% of the cells dying within 12 hours. At the other extreme, OCI-AML3 and OCI-AML4 were almost totally resistant to vinblastine over this 24-hour time frame.

When vinblastine was combined with PD98059, only OCI-AML4 and HL60-neo cells showed acute apoptosis and this was slightly slower than that observed in ML-1 cells. Vinblastine did not induce Mcl-1 in either of these cell lines, although concurrent incubation with PD98059 did suppress the endogenous levels (Fig. 4A, inset for OCI-AML4 cells). To confirm that Mcl-1 was protecting OCI-AML4 cells from acute apoptosis induced by vinblastine, we used the same lentiviral shRNA constructs that were used in the ML-1 cells. Mcl-1 levels were successfully suppressed and the cells were acutely sensitive to vinblastine alone (Fig. 3). Construct 514 was less effective at suppressing Mcl-1 and also resulted in less apoptosis in response to vinblastine treatment. These results show that the sensitive cells are dependent on Mcl-1 for protection against vinblastine.

Mcl-1 is targeted at the transcriptional level and protein stability level by multiple pathways. The failure of PD98059 to sensitize other cell lines may be attributed to its failure to suppress Mcl-1. For example, we found that PD98059 failed to suppress Mcl-1 in THP-1, U937,

Figure 2. Protection from apoptosis by inhibition of JNK. ML1 cells were incubated with 2.2 $\mu\text{mol/L}$ vinblastine in combination with either PD98059, SP600125, or JNK inhibitor VIII as indicated. After 6 to 24 h, cells were harvested, stained, and analyzed by flow cytometry for cell cycle distribution. Numbers reflect the percentage of sub-G₁ cells and those in each cell cycle phase.



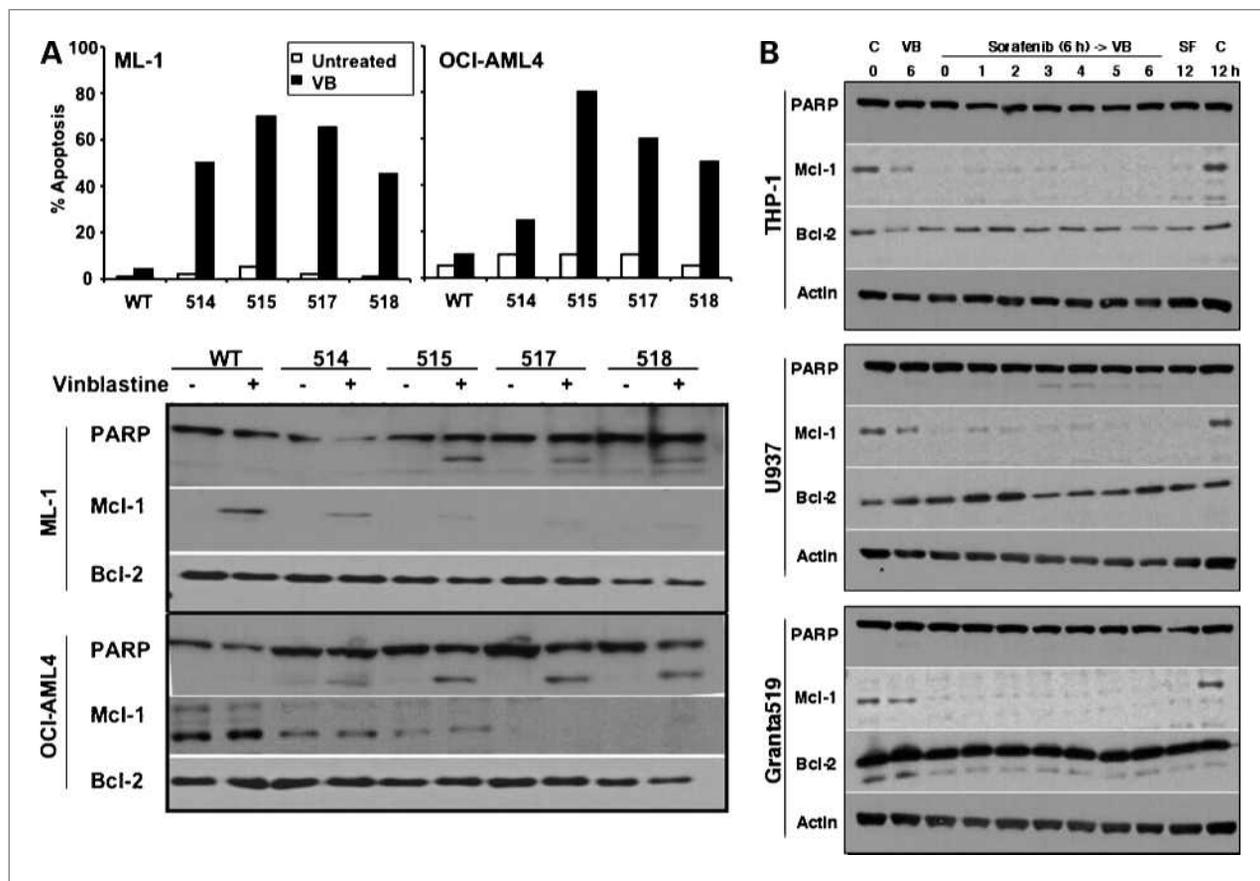


Figure 3. Suppression of Mcl-1 sensitizes ML-1 and OCI-AML4 cells to vinblastine. **A**, cells were infected with four different Mcl-1 shRNA lentivirus constructs. After 48 h, the cells were incubated with 2.2 $\mu\text{mol/L}$ vinblastine (VB) and scored 6 h later for chromatin condensation (top) and protein expression (bottom). WT, wild-type. **B**, U937, THP-1, and Granta519 cells were incubated with 20 $\mu\text{mol/L}$ sorafenib (SF) for 6 h to suppress Mcl-1, then with 2 $\mu\text{mol/L}$ vinblastine for a further 6 h. Lysates were analyzed by immunoblotting. The lack of cleavage of PARP shows the lack of dependence on Mcl-1 for protection of these cells from vinblastine.

and Granta519 cells. We therefore investigated sorafenib, a multikinase inhibitor that has been shown to suppress Mcl-1 (10). Sorafenib successfully suppressed Mcl-1 in these three cell lines but there was no enhanced apoptosis when it was combined with vinblastine (Fig. 3). These results suggest that the vinblastine-resistant cell lines likely rely on other members of the Bcl-2 family.

Mcl-1 has a very short half-life; hence, an alternative approach to suppress Mcl-1 is to inhibit protein synthesis. Accordingly, experiments were done to determine whether cycloheximide could sensitize the cell lines to vinblastine (Fig. 4B). Cycloheximide alone induced acute apoptosis in OCI-AML1 cells. Jurkat cells were also relatively sensitive to cycloheximide (>50% apoptosis in 8 hours), whereas the remainder of the cell lines did not attain 50% apoptosis until at least 12 hours. The addition of cycloheximide dramatically accelerated vinblastine-induced apoptosis in three of the cell lines, NB4, THP-1, and OCI-AML4, and also increased the rate in U937 and HL60-neo cells (Fig. 4B). OCI-AML3 cells were again very resistant to either cycloheximide alone or the combination with vinblastine. Although ML-1 and OCI-

AML4 cells were sensitized to vinblastine by both PD98059 and cycloheximide consistent with the contribution of Mcl-1 to protection, these results are particularly interesting because cycloheximide also sensitized a much broader range of cell lines, including those that were not sensitized when sorafenib was used to suppress Mcl-1. These results suggest that another short-lived protein may be contributing to cell survival.

We surmise that the differential sensitivity of each of the cell lines to vinblastine alone or in combination with either PD98059 or cycloheximide reflects their variable expression of Bcl-2 proteins. Accordingly, we analyzed both constitutive protein expression and possible changes occurring following a 4-hour incubation with 2.2 $\mu\text{mol/L}$ vinblastine (Fig. 5). As shown above, ML-1 cells have constitutive ERK activation as depicted by phosphorylation of ERK, whereas vinblastine induces the phosphorylation of JNK and elevation of Mcl-1; Bcl-2 levels do not change, whereas little or no Bcl-X or Bfl-1 is expressed. Most of the other cell lines showed constitutive activation of ERK albeit with some variation. NB4 were the only cells in which vinblastine led to the activation of ERK.

JNK activity in response to vinblastine treatment varied among the cell lines, with Jurkat cells showing no detectable activation even on longer exposures of the Western blot. The Bcl-2 family members were heterogeneously expressed. Only the ML-1 cells showed the strong induction of Mcl-1 upon incubation with vinblastine. Mcl-1 expression was detectable in most of the cell lines, although very low to undetectable levels were expressed in the NB4 and OCI-AML1 cells. The latter cells also express relatively low levels of Bcl-2 and little if any Bcl-X or Bfl-1 consistent with their acute activation of apoptosis when incubated with vinblastine alone. Bcl-2 and Bcl-X were present at variable levels throughout the panel but changed very little upon incubation with vinblastine. The ML-1 and NB4 cells showed no detectable Bcl-X. Bfl-1 was only detected in U937, THP-1, and Jurkat cells (although the later showed an additional larger band that

could reflect a different isoform). In summary, each cell line is very different with respect to activation of signal transduction pathways and their expression of antiapoptotic Bcl-2 family proteins and the observed levels could not be used to predict sensitivity to the combination of vinblastine plus PD98059.

The induction of apoptosis also depends on proapoptotic members of the Bcl-2 family. Bax and Bak were fairly consistently expressed across these cell lines with the exception that Bax is absent from Jurkat cells (Fig. 5). All but the OCI-AML1 cells expressed Bim, which did not change upon incubation with vinblastine. Bid, Bad, and Puma were variably expressed across these lines, but the only notable change upon incubation with vinblastine was the loss of Bid in OCI-AML1 cells consistent with its cleavage during the rapid onset of apoptosis. Whereas little Noxa was seen in most cells, it was elevated following vinblastine

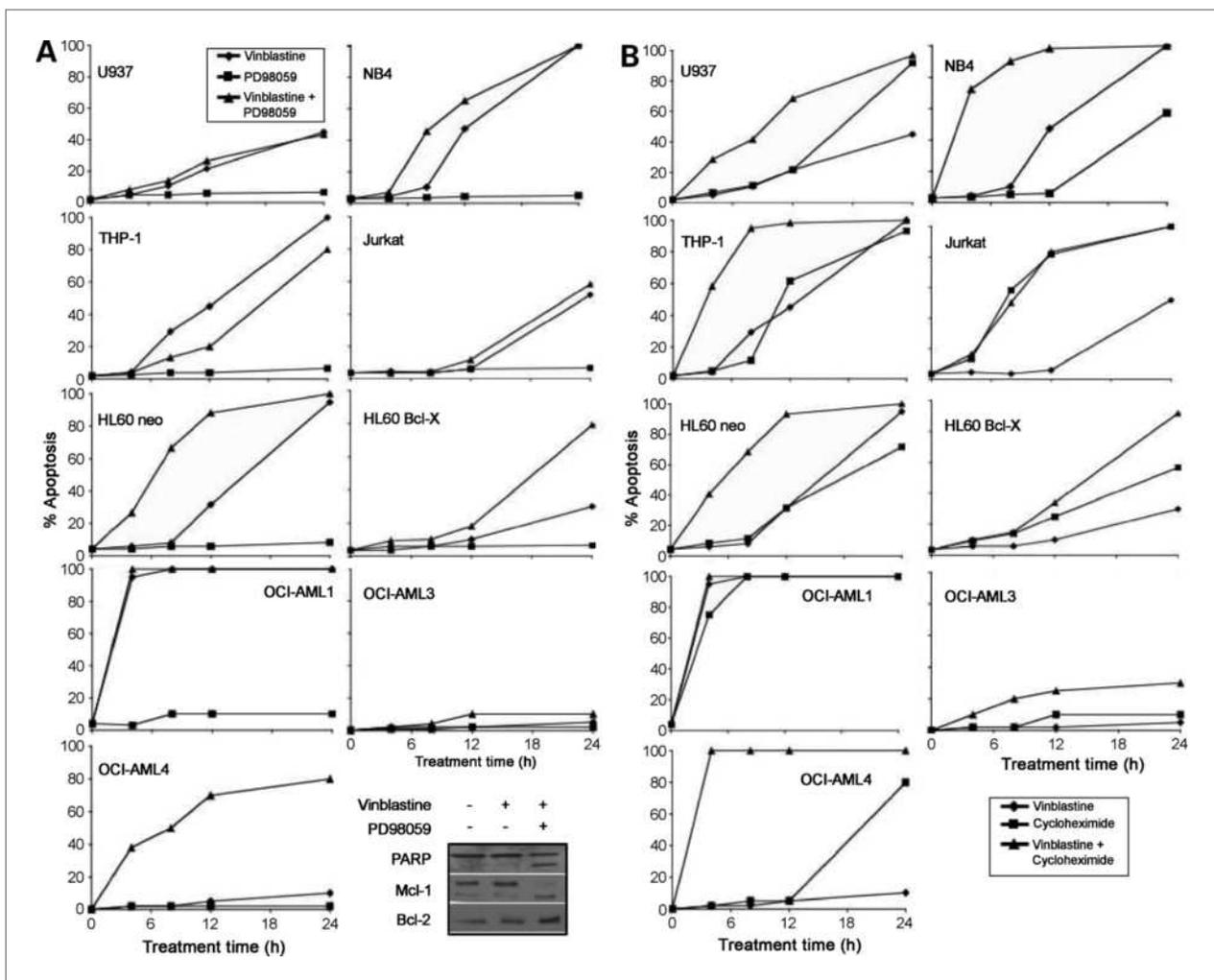


Figure 4. Effect of PD98059 and cycloheximide on apoptosis induced by vinblastine in a panel of cell lines. A, the indicated cell lines were incubated with 2.2 $\mu\text{mol/L}$ vinblastine, 50 $\mu\text{mol/L}$ PD98059, or a combination of each, and apoptotic cells were scored by chromatin condensation. Inset, protein expression in OCI-AML4 cells after a 6-h incubation as indicated. B, the same cell lines were incubated with 2.2 $\mu\text{mol/L}$ vinblastine, 10 $\mu\text{g/mL}$ cycloheximide, or a combination of each, and apoptotic cells were scored by chromatin condensation.

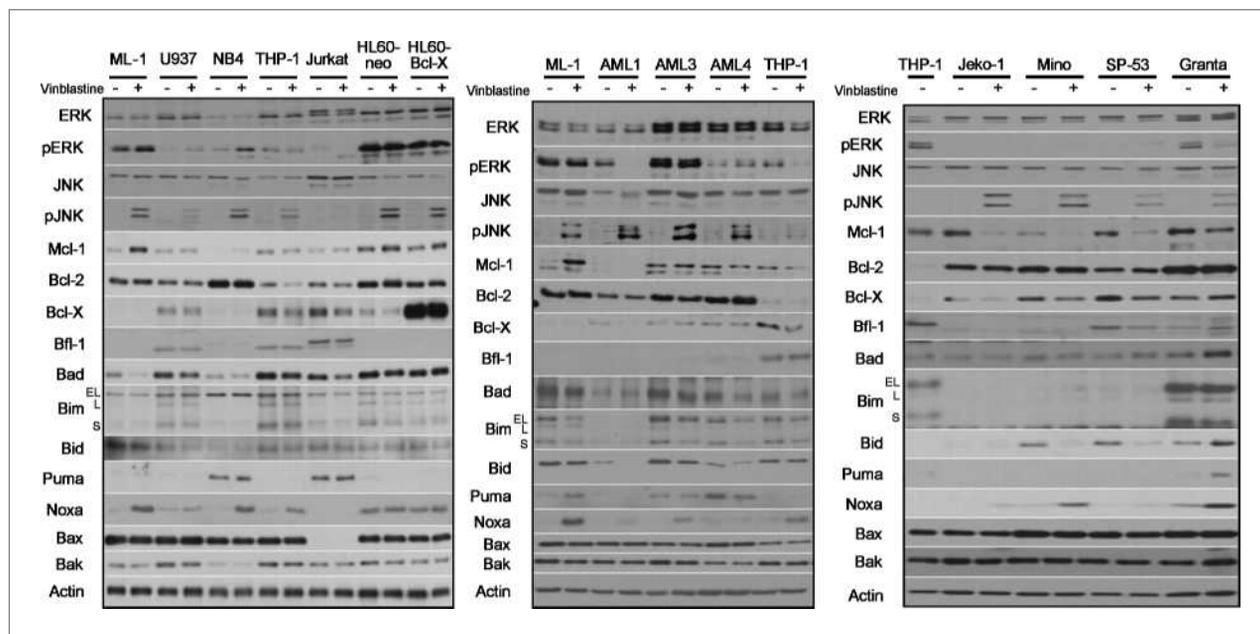


Figure 5. Expression of Bcl-2 family members and activation of MAP kinase proteins in the panel of cell lines. The indicated cell lines were either undamaged or incubated with 2.2 $\mu\text{mol/L}$ vinblastine for 4 h. Lysates were analyzed by immunoblotting for expression of the indicated proteins. Second and third panels, ML-1 and THP-1 cells were reanalyzed to facilitate comparison between the various blots.

in most cases. Notably, it was not expressed in Jurkat cells, which were not sensitized to vinblastine by either PD98059 or cycloheximide.

Given that Bim has been reported to bind microtubule motors, it seemed a likely candidate for the critical proapoptotic protein in these cell lines. We therefore investigated the effect of vinblastine in four mantle cell lymphoma lines, two of which are deleted for Bim (11). In addition to SP53 and Jeko-1, we found that Mino also expressed no detectable Bim (Fig. 5). Surprisingly, these three Bim-deficient lines all succumbed rapidly to incubation with vinblastine alone (Fig. 6), whereas the Bim-proficient Granta519 cells did not (data not shown). These hypersensitive mantle cell lymphoma lines showed little if any phosphorylated ERK but strong activation of JNK (Fig. 5); inhibition of JNK prevented this acute apoptosis (Fig. 6). Mcl-1 declined in the dying cells, but this was not a consequence of cell death as it was prevented by inclusion of a caspase inhibitor (data not shown). However, inhibition of JNK did rescue Mcl-1 expression (Fig. 6). These cells also showed marked accumulation of Noxa when incubated with vinblastine, which might contribute to this acute apoptosis, although Noxa was still elevated in cells protected by inhibition of either JNK or caspase.

Vinblastine-mediated apoptosis in CLL

As all of the above results were obtained in cell lines, we have begun to determine whether this acute apoptosis can also occur in freshly isolated tumor cells. Accordingly, we have assessed the effect of vinblastine on CLL cells collected from patients and immediately assessed for sensitivity to vinblastine. We have studied seven

patient samples to date, all but one of which was from patients who had not yet received any chemotherapy for their disease. Surprisingly, we found that all samples underwent apoptosis within 6 hours when incubated with vinblastine alone (Fig. 6B). This acute apoptosis was observed at 100 to 200 nmol/L, which is a concentration that can be achieved in human plasma upon administration of the drug (7). The acute apoptosis was suppressed by inclusion of a JNK inhibitor (Fig. 6). In contrast, nontumorigenic lymphocytes isolated from peripheral blood did not undergo acute apoptosis at 2 $\mu\text{mol/L}$ vinblastine showing selectivity for the tumor cells. The acute apoptosis observed in CLL cells precluded a study of vinblastine in combination with the MEK inhibitor or protein synthesis inhibitor.

The response of CLL cells *ex vivo* is affected by the stroma. For example, incubation on a monolayer of murine bone marrow stroma has been shown to rescue CLL cells from the spontaneous apoptosis that can occur in culture (12). We did a similar study in which we incubated CLL cells with the same bone marrow stroma cells. This had no effect on the acute apoptosis observed upon incubation with vinblastine (data not shown). We also looked at the expression levels of Bcl2 (high), Bcl-X (negligible), and Mcl-1 (negligible) in these patient samples (data not shown) and the results were similar to those previously reported (13).

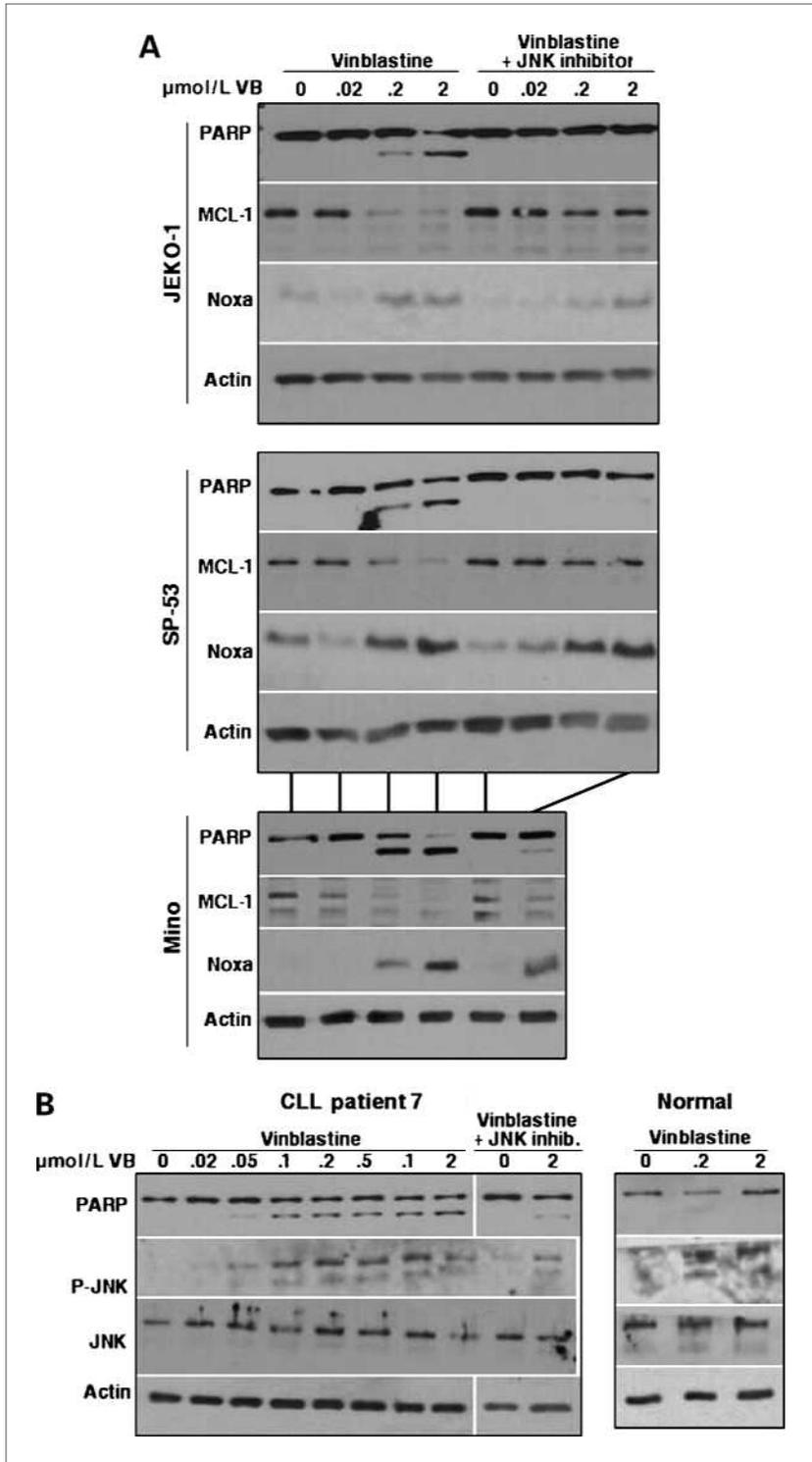
Discussion

Previously, we have shown that very little apoptosis is induced by vinblastine alone even after 24 hours in ML-1 leukemia cells (5). This is consistent with the expectation

that microtubule-interfering agents cause apoptosis by disrupting the progression of cells through the M phase of the cell cycle (2). In this report, we have examined a panel of leukemia and lymphoma cell lines and have discovered a very heterogeneous response to vinblastine.

In particular, the induction of apoptosis in 100% of the OCI-AML1 cells in 4 hours clearly implies that these cells can die from all phases of the cell cycle. This phenotype was also observed in three mantle cell lymphoma lines and freshly isolated CLL cells. This suggests an alternative

Figure 6. Acute apoptosis induced by vinblastine alone in mantle cell lymphoma and CLL cells. **A**, the three mantle cell lymphoma lines were incubated with 0 to 2 $\mu\text{mol/L}$ vinblastine for 6 h and then analyzed for expression of the indicated antigens. Parallel experiments included concurrent incubation with JNK inhibitor VIII. **B**, cells from a CLL patient and peripheral lymphocytes from a normal individual were incubated with 0 to 2 $\mu\text{mol/L}$ vinblastine for 6 h and then analyzed for expression of the indicated antigens. Cells from the CLL patient were also incubated concurrently with JNK inhibitor VIII. These results are similar to those observed in all seven CLL samples.



mechanism for vinblastine apart from its traditionally understood ability to arrest cells in mitosis and cause apoptosis. One surprising correlation was that these hypersensitive cells all exhibited little or no Bim; this was also true for the CLL cells (data not shown). This suggests that this proapoptotic Bcl-2 family member might elicit resistance to vinblastine, but how this might occur is not currently known. For some of the other cell lines, a similarly rapid, mitosis-independent onset of apoptosis can be induced by the combination of vinblastine with PD98059 or cycloheximide. In addition to ML-1 cells, we have now found that OCI-AML4 and HL60-neo cells are also acutely sensitive to the combination of PD98059 plus vinblastine.

The OCI cell lines were selected for this study because they are reportedly the same AML phenotype as ML-1 cells (M4; ref. 14). However, there is clearly a large difference in response between these lines, ranging from AML1, which are hypersensitive to vinblastine alone, AML4, which like ML-1 are sensitized by PD98059 and cycloheximide, to AML3 that seem resistant to all these drugs alone or in combination. This suggests the sensitive phenotype is not a reflection of this specific disease type.

We have confirmed the mechanism by which the ML-1 and OCI-AML4 cells can protect themselves from the acute induction of apoptosis. Although vinblastine elevates the levels of Mcl-1 in the ML-1 cells but not in the OCI-AML4 cells, PD98059 is effective at suppressing the expression of Mcl-1 in both cell lines and consequently they both succumb to vinblastine-induced apoptosis. Use of a lentiviral delivery system to direct shRNA against Mcl-1 in these cell lines revealed the importance of Mcl-1 to their protection; elimination of Mcl-1 from these cell lines allowed for acute sensitivity to vinblastine as a single agent. PD98059 did not suppress Mcl-1 in all the cell lines, yet this is unlikely to explain their resistance to vinblastine-mediated acute apoptosis. We found that the multikinase inhibitor sorafenib was able to more broadly suppress Mcl-1, yet this did not enhance sensitivity to vinblastine. These results suggest that the other cell lines rely on another Bcl-2 family protein for protection from vinblastine.

Mcl-1 is a very tightly regulated Bcl-2 family member; it is regulated transcriptionally, posttranscriptionally, and posttranslationally. In the ML-1 cells, vinblastine induces Mcl-1 expression in as little as 2 hours. The ERK signaling pathway is involved in the upregulation of Mcl-1 mRNA in response to several cytotoxic agents including vinblastine (6, 15). In addition, although Mcl-1 is normally rapidly turned over, ERK-mediated phosphorylation within its PEST region is associated with increased protein stability (16–18). There are several other pathways known to regulate Mcl-1 protein stability such as Mule/ARF-BP1, TCTP, and GSK-3 β / β -TrCP (19–21); whether vinblastine affects these pathways remains to be determined.

The proapoptotic protein Noxa also binds Mcl-1 and the Mcl-1/Noxa complex is then targeted for proteasome degradation (22). Noxa was undetectable in undamaged cells but upon incubation with vinblastine, Noxa was el-

evated in most of the cell lines. In the cell lines acutely sensitive to vinblastine alone, Noxa was more markedly elevated at shorter time points but had decreased again following apoptosis; this decrease could be prevented with a caspase inhibitor (data not shown). We believe that the elevated Noxa provides evidence that vinblastine is inducing some other BH3 protein, albeit not Bim, to bind Mcl-1, thereby displacing Noxa, which is observed because of its increased stability. However, the ability of cycloheximide to sensitize cells to vinblastine suggests that, at least in this case, Noxa is not required for apoptosis.

We have previously suggested that PD98059 sensitization of vinblastine-treated ML-1 cells may be JNK dependent and this was confirmed here (5). We also observed that the acute apoptosis induced by vinblastine alone was JNK dependent. There are several plausible hypotheses for why JNK activation may be crucial under these circumstances. Several reports have shown that JNK activation results in the phosphorylation and inactivation of Bcl-2 (23–26). This could contribute to apoptosis in ML-1 and OCI-AML4 cells when coincubated with vinblastine and PD98059 as they only express Bcl-2 and Mcl-1, and this would leave no Bcl-2 family members to protect the cells from vinblastine-induced toxicity. However, the NB4 cells that express Bcl-2 and negligible levels of Mcl-1 are not acutely sensitive to vinblastine, which suggests that the JNK-mediated inhibition of Bcl-2, if it occurs, is not sufficient to sensitize the cells. JNK activation in response to radiation has also been shown to phosphorylate and inactivate the antiapoptotic protein Bcl-X (27). Following exposure of cells to oxidative stress, JNK has been shown to phosphorylate and inactivate Mcl-1 (28). JNK-mediated phosphorylation of 14-3-3 ζ or σ has also been shown to dissociate Bax or release Bad, allowing translocation to the mitochondria where they both can activate the apoptotic cascade (29, 30). Finally, the JNK-mediated phosphorylation of Bim_{EL} on Ser-65 potentiates its proapoptotic function (31).

The ability of cycloheximide to sensitize cells to vinblastine is intriguing as it implies that a short-lived protein is responsible for protecting the cells under normal growth conditions. Although Mcl-1 is a likely candidate, this cannot explain why cycloheximide sensitizes most of the cell lines. For example, sorafenib was effective at suppressing Mcl-1 in THP-1 and U937 cells yet it did not sensitize the cells to vinblastine, whereas cycloheximide did. Furthermore, NB4 cells express negligible levels of Mcl-1 but are still sensitized by cycloheximide. These results suggest that many of these cells must rely on an alternate short-lived protein for survival.

Enhancement of apoptosis by the disruption of antiapoptotic Bcl-2 family members offers an attractive hypothesis for the rational design of drugs that could be used in conjunction with current chemotherapeutic agents. There is currently no available inhibitor specific for the antiapoptotic member Mcl-1. Various MEK inhibitors have been tested in clinical trials but the goal was to chronically suppress ERK activity (32). Our results

suggest that MEK inhibitors could be used acutely in combination with *Vinca* alkaloids as only transient inhibition of the pathway is required to enhance apoptosis. This might overcome the toxicity observed with chronic administration. In addition, there are several agents in clinical development that are inhibitors of the Bcl-2 family and these may provide an effective therapy when used as a bolus in combination with vinblastine (33–37).

We believe that the true therapeutic potential of *Vinca* alkaloids has never been realized because of the failure to recognize that acute apoptosis occurs. We have shown that vinblastine alone or in combination with an inhibitor of the Mcl-1-mediated protection induces rapid apoptosis in some cells. We hypothesize that the sensitivity to *Vinca* alkaloids varies significantly depending on whether cells die through this acute apoptosis or by mitotic death. In the latter case, we can markedly accelerate the rate of apoptosis using appropriate combinations. Whether this will improve therapeutic efficacy in patients remains to be established.

The acute apoptosis observed in CLL cells, but not in normal lymphocytes, requires further comment as the results suggest that vinblastine alone might be an effective therapy for this disease. Vincristine has been the primary *Vinca* alkaloid used for the treatment of CLL and this occurred because the combination of cytoxan, vincristine, and prednisone was found effective in other B-cell cancers; accordingly, this combination was extrapolated to CLL. However, the efficacy of vincristine has not been confirmed in CLL. At first glance, it seems unlikely that vincristine should have any effect in CLL because of its expected mitotic mechanism of lethality. We have found that vincristine and vinblastine are both effective at inducing acute apoptosis. However, vincristine rather than vinblastine is administered in the cytoxan, vincristine, and prednisone regimen because it lacks the bone marrow toxicity of the other two agents. The dose of vincristine in cytoxan, vincristine, and prednisone is limited by

neurotoxicity and it is possible this is inadequate to elicit the acute apoptosis. We believe studies with vinblastine administration should be investigated further.

A complexity in translating these observations into clinical trials relates to the need to predict which tumors will respond. Expression of Mcl-1 does not predict whether a tumor will respond. The presence of proapoptotic Bcl-2 proteins is also likely to contribute, although ongoing experiments have also failed to predict response based on their expression levels. Currently, we envision the need to perform *ex vivo* analysis of leukemias to determine which would be sensitive. A second question with respect to translation of these observations is the potential for toxicity to the patient. Given the selectivity of the combination of PD98059 and vinblastine for a limited set of leukemias, there is optimism that this may not be generally toxic. Furthermore, previous work from our laboratory and others (38, 39) have failed to acutely sensitize carcinoma cell lines, suggesting that sensitivity will only be observed in selected hematopoietic lineages. In summary, we believe these drug combinations may have value as a therapeutic strategy, and future research will investigate this possibility.

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No potential conflicts of interest were disclosed.

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Vinblastine Induces Acute, Cell Cycle Phase–Independent Apoptosis in Some Leukemias and Lymphomas and Can Induce Acute Apoptosis in Others when Mcl-1 Is Suppressed

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