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The p210$^{bcr-abl}$ protein was shown to inhibit apoptosis induced by DNA damaging agents. Apoptotic DNA fragmentation is delayed in the *bcr-abl*$^+$ K562 and KCL-22 compared with the *bcr-abl*$^-$ U937 and HL-60 cell lines when treated with etoposide concentrations that induce similar DNA damage in the four cell lines. By the use of a cell-free system, we show that nuclei from untreated cells that express p210$^{bcr-abl}$ remain sensitive to apoptotic DNA fragmentation induced by triton-soluble extracts from p210$^{bcr-abl-}$ cells treated with etoposide. In the four tested cell lines, apoptotic DNA fragmentation is associated with a decreased expression of procaspase-3 (CPP32/Yama/apopain) and its cleavage into a p17 active fragment, whereas the long isoform of procaspase-2 (ICH-1L) remains unchanged and the poly(adenosine diphosphate–ribose)polymerase protein is cleaved. These events are delayed in *bcr-abl*$^+$ compared with *bcr-abl*$^-$ cell lines. The role of p210$^{bcr-abl}$ in this delay is confirmed by comparing the effect of etoposide on the granulocyte-macrophage colony-stimulating factor (GM-CSF)–dependent UT7 cells and the *bcr-abl*–transfected GM-CSF–independent UT7/9 clone. We conclude that the cytosolic pathway that leads to apoptotic DNA fragmentation in etoposide-treated leukemic cells is delayed upstream of procaspase-3–mediated events in *bcr-abl*$^+$ cell lines.

THE PHILADELPHIA chromosome is the cytogenetic hallmark of chronic myelogenous leukemia (CML).[1] Translocation of the c-*abl* gene, located on chromosome 9q, within the *bcr* gene, located on chromosome 22q, results in the formation of a chimeric protein, p210$^{bcr-abl}$.[2] This event is generally accepted to be the primary initiating event in the genesis of CML because mice transplanted with bone marrow cells infected with retroviral vectors encoding p210$^{bcr-abl}$ develop a CML-like disease.[3] The c-ABL protein is a nuclear protein with low intrinsic tyrosine kinase activity, whereas the BCR-ABL protein is a cytoplasmic, membrane-associated protein that contains a constitutively high level of tyrosine kinase activity. It was initially assumed that CML was caused by uncontrolled cell proliferation resulting in the clonal expansion evident in this disease.[4] Actually, CML progenitors have similar proliferation rates as their normal counterparts.[5] Recent studies have shown that both the v-*abl* transforming oncogene product and the p210$^{bcr-abl}$ prolonged hematopoietic cell survival by inhibition of apoptosis.[6-8] The BCR-ABL chimeric protein confers on hematopoietic cells the ability to survive treatments such as growth factor deprivation, cytotoxic drugs, protein tyrosine kinase inhibitors, and Fas-ligand.[9-12] Antisense oligonucleotides that downregulate BCR-ABL protein expression in CML cell lines either induce apoptotic cell death[13] or render the cells more susceptible to cell death induction by cytotoxic drugs and Fas-ligand.[9,10] The mechanisms by which the deregulated v-ABL and BCR-ABL tyrosine kinases delay apoptotic cell death remain poorly understood. Translocation of the protein kinase C βII,[14] increased glucose uptake,[15] delayed G2/M transition after DNA damage,[9] and activation of Ras functions[16] were proposed to mediate this inhibitory effect, whereas the MAP kinase kinase/MAP kinase pathway was shown not to be required for BCR-ABL–mediated suppression of apoptosis.[17]

Transduction of the apoptotic signal and execution of apoptosis require the coordinate action of several aspartate-specific cysteine proteases, the so-called caspases. The 10 human caspases identified so far can be divided into four subfamilies based on their structure and their homology to the human prototype interleukin-1β converting enzyme (ICE) and the nematode prototype CED-3.[18] These subfamilies are the ICE-like caspases, the CED-3–like caspases, the caspases that contain prodomains highly related to the death effector domain of the Fas/APO-1 receptor, and the NEDD2/ICH-1 caspases. All these enzymes are initially synthesized as single-chain inactive proenzymes that require cleavage after aspartate residues to obtain the active protease. Under physiological conditions, this process probably involves heterotypic protein-protein interactions and a cascade of caspases.[19] Although molecular ordering of this cascade is only partly known, it seems that several apoptotic pathways probably exist. For example, deletion of the ICE (caspase-1) gene inhibits Fas-induced apoptosis without modifying several other apoptotic pathways.[20] By contrast, activation of CED-3–like caspases could be common to most apoptotic pathways.[21] Distinct from ICE, these caspases cleave the cell death substrate poly(adenosine diphosphate [ADP]–ribose) polymerase (PARP) into signature apoptotic fragments.[22-24] Activation of CED-3–related caspases was shown recently to be controlled by the CED-9/Bcl-2 family of molecules. This control was suggested to take place at the mitochondrial membrane[25] and to involve the so-far unidentified mammalian homolog of the nematode protein CED-4.[26]

In the present study, we addressed the question of the relationship between BCR-ABL–mediated inhibition of apoptosis and the activation of CED-3–related caspases. As a model system, we used etoposide (VP-16)-induced apoptosis. This topoisomerase II-reactive agent produces double-strand breaks by stabilizing a transient intermediate of the topoisomerase reaction.[27] We have shown previously that etoposide induced similar levels of these DNA damage in *bcr-abl*$^+$ (K562, KCL-22) and *bcr-abl*$^-$ (HL-60, U937) cell lines.[28] However,

From the Department of Biology and Therapy of Cancer, INSERM CJF 94-08, Faculty of Medicine, Dijon, and INSERM U 362, IGR, Villejuif, France.

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Address reprint requests to Eric Solary, MD, INSERM CJF 94-08, Faculté de Médecine, 7, Blvd Jeanne d'Arc, 21033 DIJON Cedex, France.

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apoptosis was strongly delayed in *bcr-abl*$^+$ cells. Here, we show that this delay occurs upstream of procaspase-3 activation.

MATERIAL AND METHODS

Drug and chemicals. Etoposide was obtained from Sigma-Aldrich laboratories (St Quentin Fallavier, France). Stock solution (20 mmol/L) in dimethyl sulfoxide (DMSO) was conserved at −20°C for less than 1 month. Further dilutions were made in culture medium just before use. The final concentration of DMSO in culture did not exceed 1% (vol/vol), which was nontoxic to the cells. [2-^{14}C]thymidine (50 mCi/mmol) was obtained from Amersham (Les Ulis, France). All other chemicals were purchased from local sources.

Cell lines and culture. The four human leukemic cell lines, HL60 (promyelocytic), U937 (monocytic), K562 (CML in erythroblastic blast crisis), and KCL22 (CML in lymphoid blast crisis) were grown in suspension in RPMI 1640 medium (BioWhittaker, Fontenay-sous-bois, France) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and 2 mmol/L L-glutamine in an atmosphere of 95% air and 5% CO_2. The human pluripotent UT-7 line, established from a patient with megakaryocytic leukemia, was cultured in the presence of 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). Transfer of *bcr-abl* gene into UT-7 cells was accomplished using a defective amphotropic p210 retrovirus. The GM-CSF–independent UT7/9 clone was selected for its high expression of BCR-ABL mRNA as previously reported.[29] Cell viability was determined using the trypan blue exclusion test. Cells were resuspended at a density of 1.5×10^6/mL in fresh medium before treatment.

Reconstituted cell-free system. Triton-soluble extracts and nuclear fractions were prepared as previously described.[30] Briefly, cells were washed twice in 10 mL ice-cold phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, and once in a buffer containing 150 mmol/L NaCl, 1 mmol/L KH_2PO_4, 1 mmol/L EGTA, 1 mmol/L Na_3VO_4, 5 mmol/L $MgCl_2$, and 10% glycerol (pH 7.2). Then, the cells were incubated for 10 minutes on ice in the same buffer containing 0.3% Triton X-100 (Sigma, Aldrich, France) before centrifugation (2,000*g* for 10 minutes at 4°C) and the supernatants collected were considered as triton-soluble extracts. Protein concentration in the supernatants was determined using the bicinchoninic acid method.[31] Pellets (nuclei) were washed twice in the lysis buffer without Triton X-100. Triton-soluble extracts (500 µL) from untreated or treated cells were incubated for 30 minutes at 37°C in the presence of nuclei from 1.0×10^6 untreated cells and DNA fragmentation was measured as described below. Controls were performed by incubating nuclei from untreated cells with triton-soluble extracts from untreated cells, in the absence or in the presence of 100 µmol/L etoposide.

Quantification of DNA fragmentation. DNA fragmentation was measured using a previously reported filter elution assay.[32] Exponentially growing cells were prelabeled by adding 0.02 µCi/mL of [2-^{14}C]thymidine in the culture medium for 2 days. Approximately 1.0×10^6 [^{14}C]-labeled cells or [^{14}C]-labeled nuclei were loaded onto a protein absorbing filter (Polyvinylidene fluorure filters, 0.65 µM pore size, 25 mm diameter; Durapore membrane, Millipore, St Quentin, France). After washing, lysis was performed with 5 mL of LS10 buffer (0.2% sodium sarkosyl, 2 mol/L NaCl, 0.04 mol/L EDTA, pH 10.0). Filters were washed with 7 mL of 0.02 mol/L EDTA, pH 10. DNA was depurinated by adding 0.4 mL of 1N HCl at 65°C for 45 minutes, then released from the filters by adding 2.5 mL of 0.4 N NaOH for 45 minutes at room temperature. Radioactivity was counted by liquid scintillation spectrometry in each fraction (wash, lysis, EDTA wash, and filter). DNA fragmentation was measured as the fraction of disintegrations per minute in the lysis fraction plus EDTA wash relative to the total intracellular dpm.

Analysis of DNA fragmentation by agarose gel electrophoresis. Cellular DNA was extracted by a salting-out procedure as described previously.[33] Electrophoresis was performed in a 1.8% agarose gel in Tris-borate-EDTA buffer (pH 8) at 20 V for 15 hours. After electrophoresis, DNA was visualised by ethidium bromide staining.

Western blot analysis. After treatment, cells were washed twice in PBS, lysed in lysis buffer (150 mmol/L NaCl, 1 mmol/L KH_2PO_4, 1 mmol/L EGTA, 1 mmol/L Na_3VO_4, and 5 mmol/L $MgCl_2$; and 10% glycerol containing PmsF 0.1 mmol/L, Aprotinin 0.15 U/mL, and Pepstatin 1 µg/mL), and then centrifugated (15 minutes, 15000*g*). Fifty micrograms of proteins of supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using a 12% polyacrylamide gel and electroblotted to PVDF membrane (BioRad, Ivry sur Seine, France). After blocking nonspecific binding sites, the membrane was incubated for 2 hours at room temperature with anti-human CPP32 or Ich-1L monoclonal antibody (MoAb) (Transduction Laboratories, Lexington, KY), washed, and further incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA) for 30 minutes at room temperature. Immunoblot was revealed by using enhanced chemiluminescence detection kit (Amersham) by autoradiography.

For the PARP and CPP32 cleavage analyses, cells were lysed in SDS-PAGE sample buffer (125 mmol/L Tris-HCl, pH 6.8; 10% β-mercaptoethanol; 2% SDS; 20% glycerol; and 0.003% bromophenol blue) and cell lysates were subjected to SDS-PAGE on a 8% polyacrylamide gel. The 116-kD native PARP protein and its 85-kD cleavage product were detected by immunoblotting with anti-human PARP polyclonal antibody (Vic. 5; kindly given by Dr G. De Murcia, Ecole supérieure de biotechnologies, Strasbourg, France) and horseradish-peroxidase conjugated anti-rabbit antibody (Amersham) as described above. A rabbit polyclonal anti-apopain/CPP32-p17 antibody (kindly provided by Dr D. Nicholson, Merck Center for Therapeutic Research, Pointe Claire, Daval, Quebec, Canada) that recognizes both CPP32 proenzyme and its p19 and p17 subunits[34] was used to detect CPP32 activation in apoptotic U937 cells.

RESULTS

Etoposide-induced apoptotic DNA fragmentation is delayed in bcr-abl$^+$ *cell lines.* We used a filter elution assay[32] to measure apoptotic DNA fragmentation induced by continuous exposure to etoposide in the four human leukemic cell lines. In HL-60 and U937 cells, apoptotic DNA fragmentation appeared rapidly, beginning after 3 hours of exposure to 100 µmol/L etoposide, and increased dramatically because 80% to 100% of DNA was cleaved after 24 hours of drug exposure (Fig 1A). Conversely, in K562 and KCL-22 cells, apoptotic DNA fragmentation was barely detected 24 hours after the beginning of cells exposure to 100 µmol/L etoposide (Fig 1A). DNA fragmentation was confirmed to be internucleosomal by agarose gel electrophoresis (Fig 1B). We had previously shown that the levels of DNA double-strand breaks induced by this concentration of etoposide were similar in the four cell lines.[28] This suggested that the apoptotic pathway was delayed downstream of DNA double-strand breaks induction in the two *bcr-abl*$^+$ resistant cell lines.

Delayed apoptosis in bcr-abl$^+$ *cell lines is not related to Bcl-2 and Bax expression.* Bcl-2 overexpression was shown to delay drug-induced apoptosis in a variety of cell systems. Moreover, the Bcl-2 to Bax ratio was shown to determine the fate of tumor cell treatment with various cytotoxic drugs.[35,36] Therefore, we analyzed the expression of Bcl-2 and Bax proteins in the tested cell lines (Fig 2). By using Western blot analysis, Bcl-2 protein

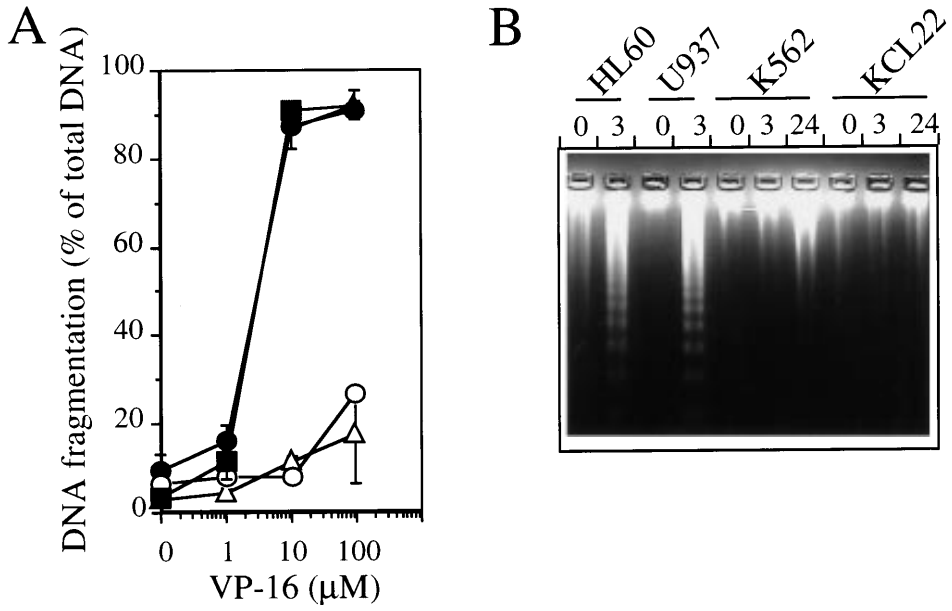

Fig 1. Etoposide-induced apoptotic DNA fragmentation and expression of Bcl-2/Bax proteins in four human leukemic cell lines. (A) DNA fragmentation was measured by a filter elution assay in HL60 (●), U937 (■), K562 (○), and KCL-22 (△) cell lines treated for 24 hours with indicated concentrations of etoposide (VP-16). (B) DNA fragmentation was identified as internucleosomal by agarose gel electrophoresis. Cells were treated for indicated times with 100 μmol/L etoposide.

was not detected in K562 cells, whereas its level was rather similar in the three other cell lines. Bax protein level was similar in the four cell lines (Fig 2). We also analyzed the expression of the Bcl-2–related protein Mcl-1, whose expression is prominent in hematopoietic cells. After 3 hours of treatment with etoposide, Bcl-2 and Mcl-1 expressions were slightly decreased in HL-60 and KCL-22 cells, whereas it was unchanged in U937 cells and Bax remained stable in the four cell lines (Fig 2B).

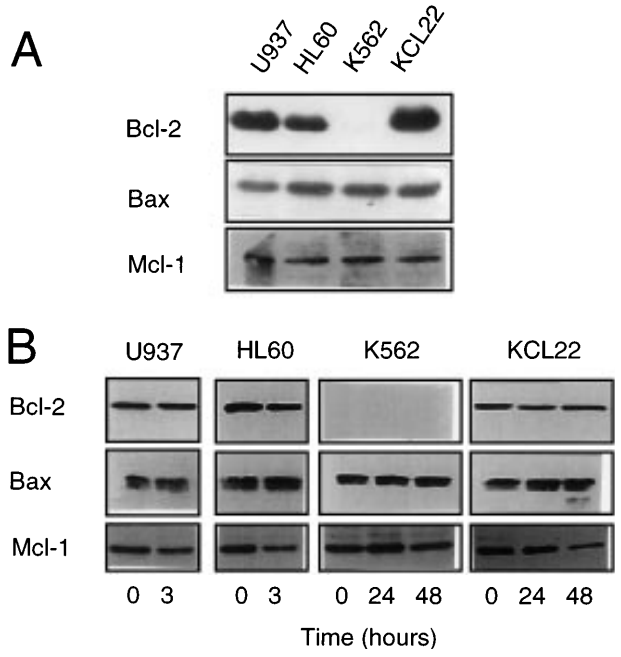

Fig 2. Expression of Bcl-2, Bax, and Mcl-1 in untreated and etoposide-treated leukemic cell lines. (A) Western blot analysis of Bcl-2, Bax, and Mcl-1 basal expression in the four studied cell lines. (B) Western blot analysis of Bcl-2, Bax, and Mcl-1 expression in the four studied cell lines treated for indicated times with 100 μmol/L etoposide.

These results indicated that the delay in etoposide-induced apoptosis in *bcr-abl*$^+$ compared with *bcr-abl*$^-$ cell lines could not be related to the Bcl-2 to Bax ratio, nor to Mcl-1 expression.

The nuclei of bcr-abl$^+$ *cell lines are sensitive to apoptotic DNA fragmentation.* To determine whether differences between *bcr-abl*$^+$ and *bcr-abl*$^-$ cell lines were related to differences in the ability of cell nuclei to undergo apoptotic DNA fragmentation, we used a cell-free system in which triton-soluble extracts from etoposide-treated cell lines were incubated with ^{14}C-labeled nuclei from untreated cell lines.[30,32] We observed that triton-soluble extracts from HL-60 and U937 cells treated for 3 hours with 100 μmol/L etoposide induced apoptotic DNA fragmentation in nuclei from *bcr-abl*$^+$ and *bcr-abl*$^-$ cell lines (Fig 3A through D). These results suggested that the delayed induction of apoptotic DNA fragmentation could not be related to nuclei changes in *bcr-abl*$^+$ cell lines. Apoptotic DNA fragmentation was lower in K562 nuclei compared with the three other cell lines. Agarose gel electrophoresis confirmed that DNA fragmentation measured in nuclei from the four studied cell lines treated with triton-soluble extracts from etoposide-treated HL-60 and U937 cells was internucleosomal DNA degradation (Fig 3E). Conversely, extracts from *bcr-abl*$^+$ cells treated with etoposide in the same conditions did not induce any DNA fragmentation in nuclei from either *bcr-abl*$^+$ or *bcr-abl*$^-$ cells (Fig 3). Thus, the apoptotic pathway that leads to apoptotic DNA fragmentation is blocked at the cytosolic level in *bcr-abl*$^+$ cells.

Cleavage of procaspase-3 is delayed in bcr-abl$^+$ *cell lines.* Using an MoAb that recognizes the 32-kD precursor form of the CED-3–related caspase-3 and immunoblot analysis, we investigated the effects of etoposide treatment on procaspase-3 expression in the four studied cell lines. A rabbit polyclonal anti–caspase-3–p17 antibody was used to identify the p19 intermediate cleavage form and the p17 active subunit of caspase-3. Expression of the long isoform of procaspase-2 (ICH-1L), a protease distinct from CED-3–related caspase, was studied simultaneously (Fig 4). Procaspase-3 basal expression

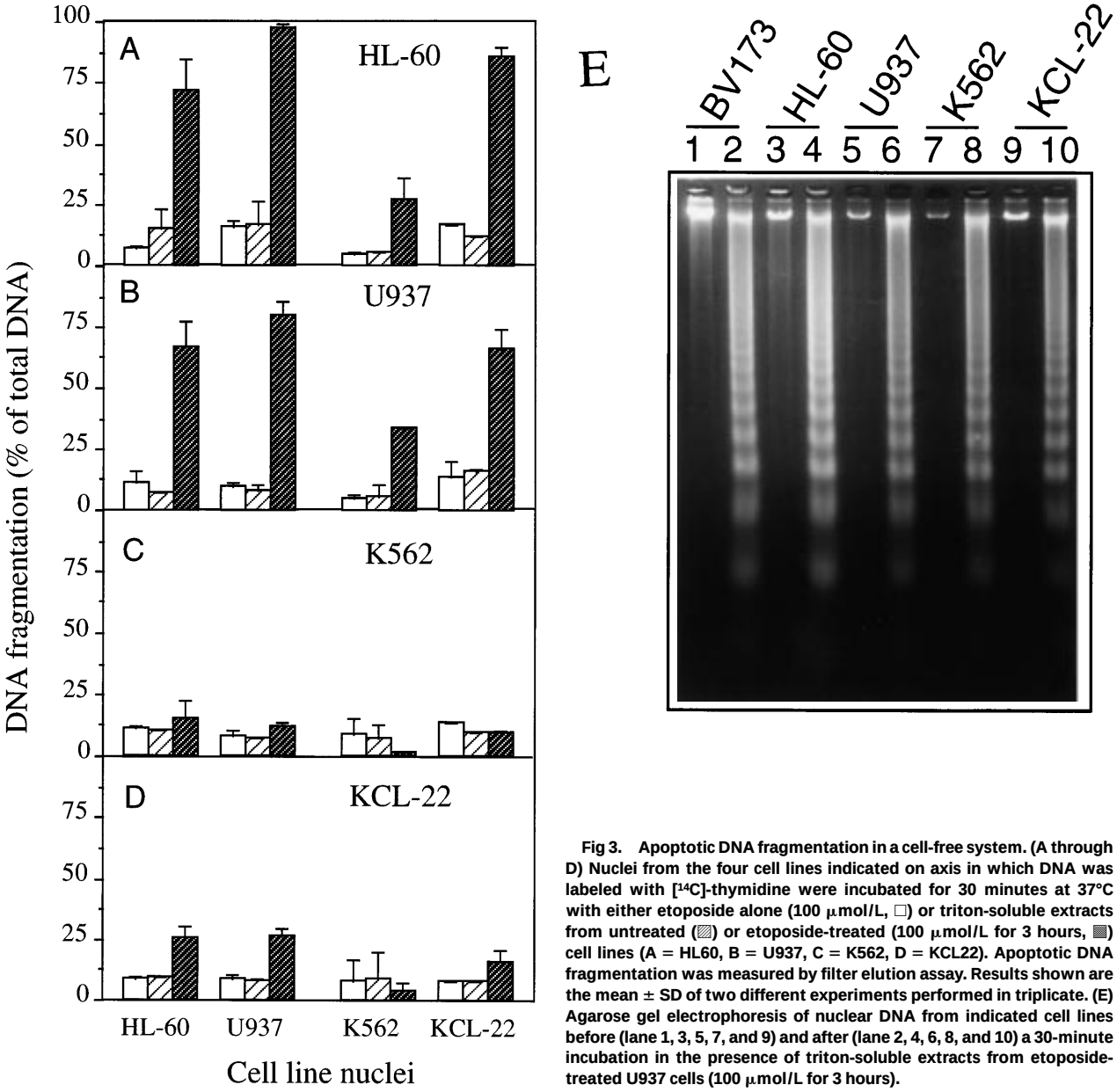

Fig 3. Apoptotic DNA fragmentation in a cell-free system. (A through D) Nuclei from the four cell lines indicated on axis in which DNA was labeled with [^{14}C]-thymidine were incubated for 30 minutes at 37°C with either etoposide alone (100 μmol/L, □) or triton-soluble extracts from untreated (▨) or etoposide-treated (100 μmol/L for 3 hours, ▩) cell lines (A = HL60, B = U937, C = K562, D = KCL22). Apoptotic DNA fragmentation was measured by filter elution assay. Results shown are the mean ± SD of two different experiments performed in triplicate. (E) Agarose gel electrophoresis of nuclear DNA from indicated cell lines before (lane 1, 3, 5, 7, and 9) and after (lane 2, 4, 6, 8, and 10) a 30-minute incubation in the presence of triton-soluble extracts from etoposide-treated U937 cells (100 μmol/L for 3 hours).

was observed to be lower in HL-60 cells than in other cell lines. When HL-60 and U937 cells were treated for 3 hours with 50 μmol/L etoposide, procaspase-3 expression decreased, the p19 and p17 subunits appeared, and the expression of the long isoform of procaspase-2 remained unchanged. Conversely, when *bcr-abl*$^{+}$ K562 and KCL-22 cells were treated with 100 μmol/L etoposide for 3 hours, the procaspase-3 was cleaved only 48 hours after the beginning of drug treatment (Fig 4).

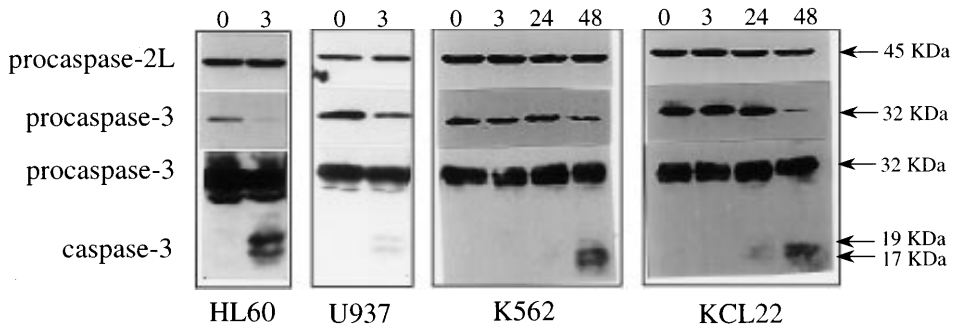

Fig 4. Activation of procaspase-3 in etoposide-treated leukemic cells. Western blot analysis of procaspase-2L (the 45-kD long isoform of ICH-1), procaspase-3 (the 32 kD proenzyme CPP32/Yama/Apopain) and active caspase-3 fragments (p19 and p17) in cell lines treated for indicated times with 100 μmol/L etoposide.

These data suggested that the apoptotic pathway was delayed upstream of procaspase-3 activation in the *bcr-abl*$^{+}$ cell lines.

bcr-abl *Overexpression in UT7 cells confirms that p210*$^{bcr\text{-}abl}$ *delays procaspase-3 activation and target protein cleavage.* Although the resistance of K562 cells to drug-induced apoptosis was previously shown to be related to BCR-ABL expression, we checked some of the previously related observations in the GM-CSF–dependent UT7 cells and a *bcr-abl*–transfected clone, UT7/9 (Fig 5). The sensitivity of UT7 cells to etoposide-induced apoptosis was lower than that of U937 and HL60 cells. Nevertheless, drug treatment clearly induced apoptotic DNA fragmentation that was delayed in *bcr-abl*–transfected UT7/9 cells. The proteolytic cleavage of the nuclear enzyme PARP is a common apoptosis-associated event that was related to the proteolytic activation of caspases.[37] Cleavage of both procaspase-3 and PARP was delayed in UT7/9 cells as compared with UT7 cells, whereas the level of the long isoform of procaspase-2 remained stable (Fig 5). These results confirmed that p210$^{bcr\text{-}abl}$-mediated inhibition of the apoptotic pathway occured upstream of procaspase-3 activation. By use of the cell-free system, we showed that triton-soluble extracts from U937 cells treated for 3 hours with 100 μmol/L etoposide induced apoptotic DNA fragmentation in nuclei from UT7/9 cells. This observation confirmed that *bcr-abl* expression did not inhibit the ability of nuclear DNA to undergo apoptotic fragmentation (Fig 6).

Untreated BCR-ABL–expressing cells do not contain an inhibitor of the apoptotic pathway triggered by etoposide. To further explore the mechanisms by which BCR-ABL delays etoposide-induced apoptosis in leukemic cells, we analyzed the influence of triton-soluble extracts from untreated *bcr-abl*$^{+}$ cells (K562, KCL22, and UT7/9) on the ability of extracts from etoposide-treated U937 cells to trigger apoptotic DNA fragmentation in the nuclei from untreated U937 cells (Fig 7). Extracts from untreated *bcr-abl*$^{-}$ cell lines (U937, HL60, and UT7) were used as control. A mixture of an equal volume of extracts from untreated cells and etoposide-treated U937 cells (100 μmol/L for 3 hours) was incubated for 30 minutes at 37°C with [^{14}C]-thymidine–labeled nuclei from untreated U937 cells. DNA fragmentation was measured by the filter elution assay. This experiment indicated that untreated *bcr-abl*$^{+}$ cells do not contain an inhibitor of the apoptotic pathway triggered by etoposide, further supporting the observation that the p210$^{bcr\text{-}abl}$ protein inhibits the caspase cascade at or before the cleavage of procaspase-3.

DISCUSSION

Decreased ability to undergo apoptosis in response to drug-induced cell damage is one of the mechanisms by which tumor cells can escape their cytotoxic activity.[27] Cells which overexpressed v-*abl* or p210$^{bcr\text{-}abl}$ receive all the measurable damage induced by cytotoxic drugs but are unable to couple this damage

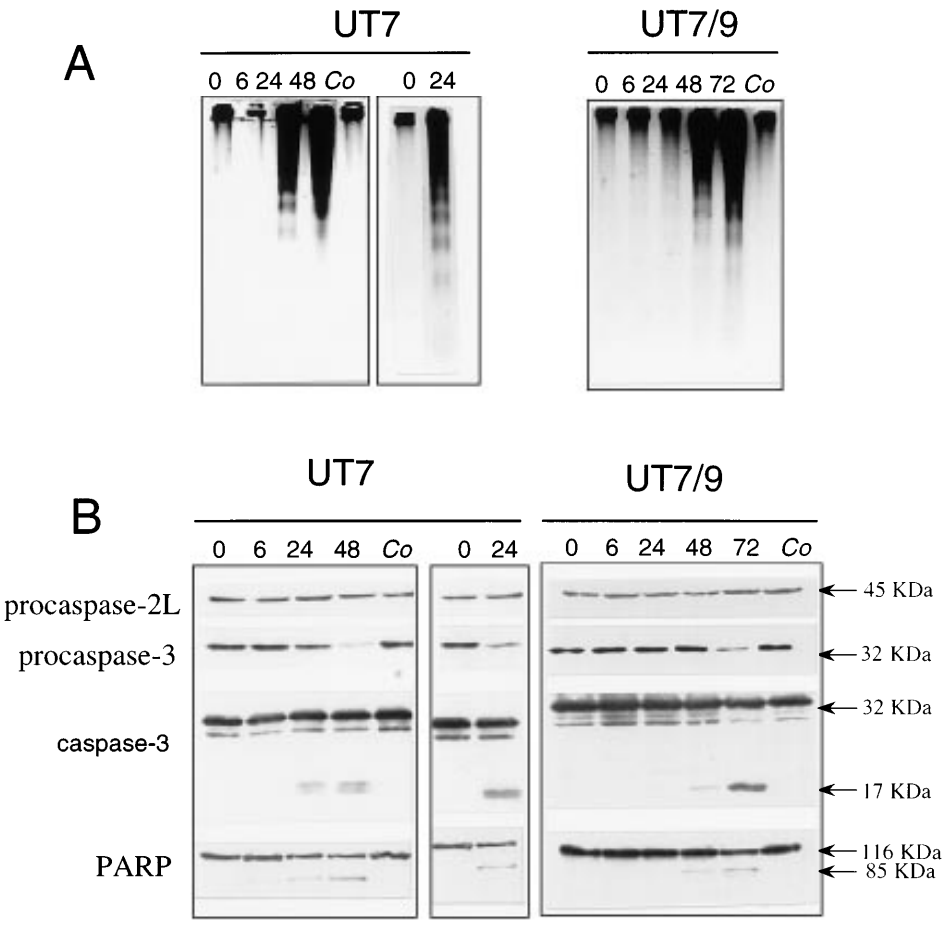

Fig 5. p210$^{bcr\text{-}abl}$ expression delays apoptosis upstream of procaspase-3 activation and PARP cleavage. (A) Agarose gel electrophoresis of DNA from UT7 and UT7/9 cells treated for indicated times with 100 μmol/L etoposide (UT7, left panel; UT7/9: whole panel) or deprived from GM-CSF for indicated times (UT7, right panel). (B) Western blot analysis of procaspase-2L (the 45-kD long isoform of ICH-1), procaspase-3 (the 32-kD proenzyme CPP32/Yama/Apopain), active caspase-3 fragments and PARP in cell lines treated for indicated times with 100 μmol/L etoposide (UT7, left panel; UT7/9: whole panel) or deprived from GM-CSF for indicated times (UT7, right panel). (Co = control untreated cells = time 0.)

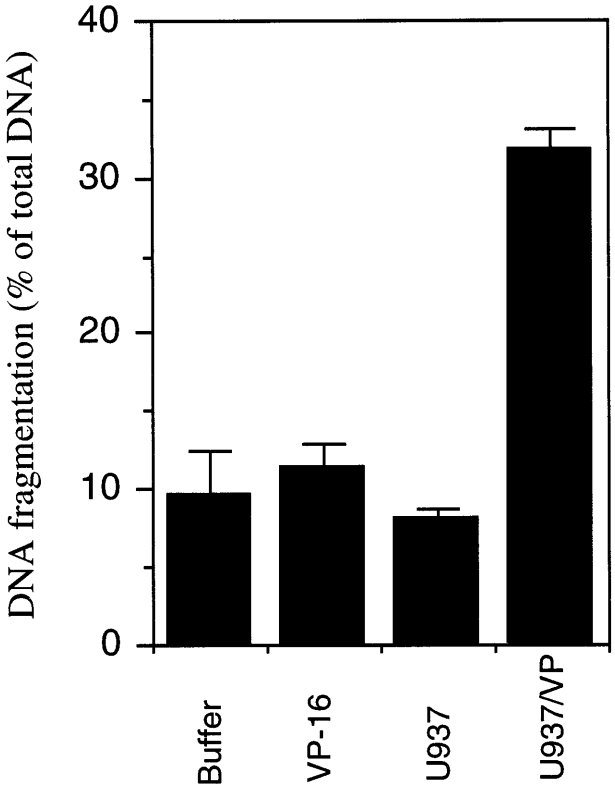

Fig 6. Nuclear DNA fragmentation in the p210$^{bcr-abl}$-expressing UT7/9 cells. Nuclei from UT7/9 cells in which DNA was labeled with [^{14}C]-thymidine were incubated for 30 minutes at 37°C with buffer alone (Buffer) or 100 μmol/L etoposide alone (VP-16) or triton-soluble extracts from either untreated (U937) or etoposide-treated (100 μmol/L for 3 hours; U937/VP) U937 cells. Apoptotic DNA fragmentation was then measured by filter elution assay. Results are the mean ± SD of two different experiments performed in duplicate.

to the apoptotic pathway.[7,28] Antisense oligonucleotides that downregulate p210$^{bcr-abl}$ expression render K562 cells more susceptible to cell death induction by a variety of cytotoxic drugs.[8,10] These oligonucleotide antisenses also render Fas-transfected K562 cells more sensitive to Fas-induced apoptosis.[12] Thus, v-*abl* and p210$^{bcr-abl}$ act downstream of the drug-target interaction to prevent the coupling of drug-induced DNA damage to the apoptotic pathways. This decreased sensitivity to apoptosis induction could contribute to the progression of CML if resistant cells that are genetically damaged subsequently proliferate. Therefore, it is essential to understand the molecular mechanisms by which p210$^{bcr-abl}$ delays the apoptotic pathway triggered by DNA damaging agents.

The *bcl-2* oncogene is a well-characterized inhibitor of drug-induced cell death. Apoptosis induction by DNA damaging agents is suppressed by transfection of cells with the oncogene *bcl-2* despite similar levels of drug-induced DNA damage in both parent and transfected cells.[27] The clinical syndromes produced by BCR-ABL expression (CML) and Bcl-2 deregulation (follicular lymphoma) both exhibit an initial indolent phase with uninterrupted differentiation and are both incurable with conventional doses of chemotherapy.[38] Therefore, p210$^{bcr-abl}$-mediated inhibition of cell death could have been mediated by overexpression of Bcl-2. Actually, we did not observe any relationship between Bcl-2 and Bax protein levels in both untreated (Fig 1) and treated (not shown) cell lines and their sensitivity to etoposide-induced cell death. Accordingly, overexpression of v-*abl* in a clone of HL-60 cells in which no Bcl-2 protein could be detected remains capable of inducing an antiapoptotic state.[39,40] Bcl-2 is now known to belong to a growing family of apoptosis-regulatory gene products which may either be death antagonists or death agonists and we cannot rule out a role for other Bcl-2 family members as mediators of p210$^{bcr-abl}$-induced inhibition of cell death.

We have shown previously that triton-soluble extracts from etoposide-treated leukemic cell lines such as HL-60 and U937 activated a nuclear endonuclease that triggered apoptotic DNA fragmentation at neutral pH in the presence of Mg^{2+}.[21] In the present study, experiments performed in this cell-free system show that the nuclei from untreated cells that express p210$^{bcr-abl}$ remain sensitive to triton-soluble extracts from p210$^{bcr-abl}$-negative cells treated with etoposide. These results indicate that the nuclease responsible for apoptotic DNA fragmentation in these cells is not inhibited by p210$^{bcr-abl}$.

Growth factors influence the sensitivity of myeloid cell lines to apoptosis induced by chemotherapeutic drugs.[41] The phosphorylation patterns induced by p210$^{bcr-abl}$ and growth factors show some similarities. Calphostin C, an inhibitor of protein kinases such as protein kinase C, restores drug sensitivity in cells with active v-*abl*, suggesting a role for protein kinases in the

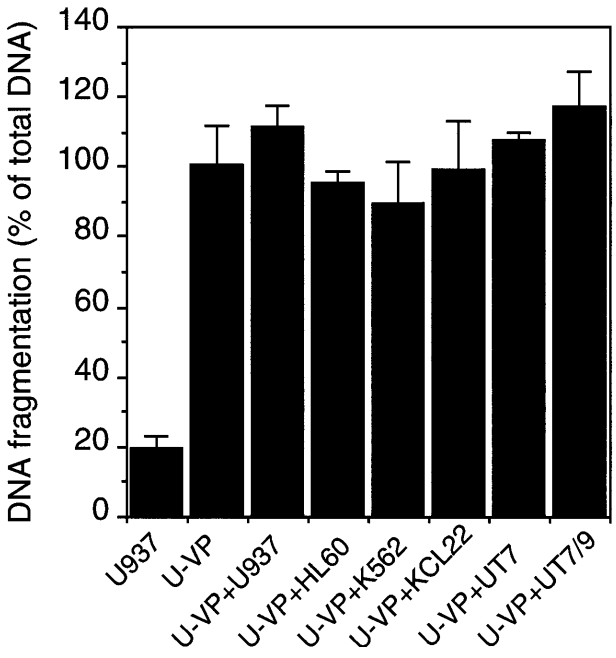

Fig 7. Lack of apoptosis inhibitory effect of triton-soluble extracts from untreated *bcr-abl*$^{+}$ cell lines. Nuclei from U937 cells in which DNA was labeled with [^{14}C]-thymidine were incubated for 30 minutes at 37°C with either triton-soluble extracts from untreated (U937) or etoposide-treated (100 μmol/L for 3 hours; U-VP) U937 cells or a mixture or an equal volume of triton-soluble extracts from etoposide-treated (100 μmol/L for 3 hours) U937 cells and triton-soluble extracts from untreated cell lines (U-VP + name of the untreated cell line). Results of a representative experiment are expressed as the mean ± SD of triplicate measurements standardized to the U-VP sample (100%).

suppression of drug-induced apoptosis by v-ABL and $p210^{bcr\text{-}abl}$.[7] However, drug treatment induces more rapid apoptosis in $p210^{bcr\text{-}abl-}$ UT7 cells cultured in the presence of GM-CSF compared with UT7/9 cells in the absence of GM-CSF, suggesting that $p210^{bcr\text{-}abl}$ imposes a survival advantage over that imparted by GM-CSF and that cellular components other than $p210^{bcr\text{-}abl}$ are probably involved in its antiapoptotic effect.[9,14-16]

Recent evidences indicate that activation of procaspases in apoptosis occurs via a proteolytic cascade.[26] For example, caspase-4 can activate procaspase-1 that, in turn, can process procaspase-3.[42] Under normal physiological conditions, procaspase-1 (pro-ICE) is activated in monocytes and cleaves pro–interleukin (IL)-1β to produce mature IL-1β cytokine. Other downstream procaspases such as procaspase-3 might also be activated by active caspase-1; nevertheless, the cells do not undergo apoptosis. This suggests that a threshold of tolerance of active caspases exists in different cells. The present study indicates that $p210^{bcr\text{-}abl}$-mediated delay in apoptosis induction is associated with a delayed activation of procaspase-3 in response to DNA damage and growth factor–deprivation rather than to an increased threshold of active caspase-3 tolerance. Human PARP is one of the specific targets of apoptosis-associated proteolysis.[36] Although most caspases cleave this protein in vitro,[22-24,43] kinetic properties of CED-3–related caspases suggest these proteases to be the most involved in PARP cleavage.[21,23] Inhibition of PARP cleavage in $p210^{bcr\text{-}abl+}$ UT7/9 cells treated with etoposide suggests that $p210^{bcr\text{-}abl}$ prevents the activation of all CED-3–related caspases. Interestingly, the long isoform of caspase-2 is not cleaved and activated in any tested cell line when exposed to VP-16, indicating that all the caspases are probably not implicated in the VP-16–induced cell death pathway. Experiments performed in the cell-free system showed that neither $p210^{bcr\text{-}abl}$ nor other cellular components involved in its antiapoptotic effect could prevent apoptotic DNA fragmentation triggered by extracts from VP-16–treated U937 cells, further supporting the conclusion that $p210^{bcr\text{-}abl}$ acts at or upstream the procaspase-3 activation level.

Inhibition of the etoposide-mediated apoptotic pathway upstream of the activation of this CED-3–related caspases is in accordance with the recent observation that $p210^{bcr\text{-}abl}$-mediated resistance to apoptosis is overcome by cytotoxic T cells[44] and NK and LAK cells.[29] These lymphocytes use two systems to induce cell death in target cells, namely, the granzyme and the Fas systems. The substantial role played by granzyme B in the former has been confirmed by targeted gene disruption. Granzyme B is a serine protease with an unusual specificity for cleaving synthetic substrates after Asp residues. Granzyme B activates several CED-3–related procaspases including procaspase-3 and procaspase-7, whereas procaspase-1 is not a substrate for granzyme B.[45] Several recent studies established that Bcl-2 and Bcl-X_L function upstream of the CED-3–related caspases. The present study shows that $p210^{bcr\text{-}abl}$ similarly delays drug-induced apoptosis upstream of these proteases.

Several methods were proposed to overcome drug resistance of CML cells. Tyrosine kinase inhibitors inhibit the growth of the murine IL-3–dependent myeloid 32Dc13(G) cell line as well as a subclone transformed to IL-3–independent growth by retroviral transduction and expression of BCR-ABL. However, these compounds induced apoptosis in the parental cells and necrosis in *bcr-abl*–transformed cells, confirming that BCR-ABL could suppress apoptotic signal transduction.[11] Combination of antisense oligonucleotides that downregulate BCR-ABL protein with a conventional chemotherapeutic agent such as mafosfamide was shown to be an efficient strategy to eliminate CML cells in mice.[46] The present study indicates that targeting CED-3–related caspases could be another efficient way to overcome drug resistance of CML cells by acting downstream of the apoptotic pathway inhibition.

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