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Caspase-Dependent Cleavage of 170-kDa P-Glycoprotein During Apoptosis of Human T-Lymphoblastoid CEM Cells

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Multidrug resistance (MDR) mediated by the drug efflux protein, 170-kDa P-glycoprotein (P-gp), is one mechanism that tumor cells use to escape cell death induced by chemotherapeutic drugs. Moreover, evidence suggests that cell lines expressing high levels of 170-kDa P-gp are less sensitive to caspase-mediated apoptosis induced by a wide range of death stimuli, including Fas ligand, tumor necrosis factor, and ultraviolet irradiation. However, the fate of 170-kDa P-gp during apoptosis is unknown. In this study, we demonstrate for the first time that 170-kDa P-gp is cleaved during apoptosis of VBL100 human T-lymphoblastoid CEM cells. Apoptotic cell death was induced by LY294002 (a pharmacological inhibitor of the phosphoinositide 3-kinase/Akt survival pathway), H_2O_2, and Z-LEHD-FMK (a caspase-9 inhibitor which has been recently reported to induce apoptosis in CEM cells). Using an antibody to a common epitope present in both the third and the sixth extracellular loop of P-gp, two cleavage products were detected, with an apparent molecular weight of 80 and 85 kDa. DEVD-FMK (a caspase-3 inhibitor), but not VEID-CHO (a caspase-6 inhibitor), blocked 170-kDa P-gp cleavage. Recombinant caspase-3 was able to cleave in vitro 170-kDa P-gp yielding two fragments of equal size to those generated in vivo. Considering the size of the cleaved fragments and their reactivity with antibodies, which recognize either the N-half or the C-half region of the protein, it is conceivable that the cleavage occurs intracytoplasmically. Since 170-kDa P-gp has been reported to counteract apoptosis, its cleavage may be a mechanism aimed at blocking an important cell survival component. J. Cell. Physiol. 207: 836–844, 2006. © 2006 Wiley-Liss, Inc.

The development of novel anti-tumor drugs has gradually improved the outcome of cancer chemotherapy, but the effectiveness of these treatments has often been limited by side effects on normal tissues and cells and by drug resistance of tumors. In fact, many tumors are intrinsically resistant to several of the more potent cytotoxic agents used in cancer therapy. Other tumors, initially sensitive, recur and are resistant not only to the initial therapeutic agents, but also to drugs not used in the treatment. The important clinical phenomenon in which a tumor cell exposed to a single antiproliferative agent becomes resistant to a large number of structurally and functionally unrelated cytotoxic compounds is called a multidrug resistance (MDR) phenotype (Schinkel et al., 1997; Nuti and Rao, 2002; Mahadevan and List, 2004). The MDR phenotype is associated with increased drug efflux from the cells that is mediated by an energy-dependent mechanism. Studies on the MDR phenotype have led to discovery of the ATP-binding cassette (ABC) transporter superfamily, such as MDR-associated proteins (MRPs) and P-glycoprotein (P-gp). The latter is a 170-kDa efflux pump molecule, encoded by human *MDR*1 gene, located on chromosome 7, band p21-21.1, and consists of 1280-residue polypeptide chain organized into two homologous halves (Nuti and Rao, 2002; Mahadevan and List, 2004). The N- and C-terminal halves of the molecule, which share a 43% homology, are considered to be linked by a cytoplasmically located region comprising amino acids 633–709, commonly called the "linker region" (Nuti and Rao, 2002). Like other ABC transporters, P-gp consists of four domains, two hydrophilic nucleotide-binding domains, which catalyze substrate export, and two transmembrane domains, each composed of six putative membrane-spanning helices that form a drug-binding pore (Van Helvoort et al., 1996; Mahadevan and List, 2004). Some lines of evidence suggest that ABC transporters can act not simply as transmembrane channels traversing the lipid bilayer, but rather as flipases that scan the lower leaflet of the bilayer for lipophilic substrates, accepting them from either the cytoplasmic interface or within the lipid bilayer to flip substrates to the outer membrane leaflet (Mahadevan and List, 2004; Leslie et al., 2005).

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P-gp is believed to be involved in protecting cells from xenobiotic accumulation and resulting toxicity (Leslie et al., 2005). In fact, it confers energy-dependent resistance to a number of naturally occurring, structurally unrelated, therapeutic agents and dozens of different commonly used drugs (Gottesman et al., 1995; Schinkel et al., 1997). The localization of 170-kDa P-gp in the intestinal mucosa, at blood–brain barrier sites, in biliary hepatocytes and in proximal tubules of the kidney indicates its significant role in drug pharmacokinetics (Gottesman et al., 1995; Schinkel et al., 1997). High P-gp expression is also found in hematopoietic pluripotent stem cells and specific lymphocyte lineages, including natural killer cells and mature thymocytes (Gupta and Gollapudi, 1993; MacDonald et al., 1995; Borst and Schinkel, 1996). MDR1 expression can also be upregulated as a consequence of tumor progression, such as mutation of tumor suppressor gene *p*53 and activation of *ras* oncogene (Tsuruo et al., 2003).

It has been observed that resistance to different drugs due to the expression and function of P-gp on the cell surface, leads to a parallel resistance to certain apoptotic stimuli (St Croix and Kerbel, 1997). In fact, apoptosis and anti-apoptosis pathways are deeply related to drug sensitivity and resistance. Despite this observation, very little is known about P-gp fate during apoptosis. In this paper, we show that 170-kDa P-gp transporter is cleaved in a caspase 3-dependent manner during apoptosis of human T-lymphoblastoid CEM cells. Our findings may suggest that cells cleave P-gp to downregulate its function, which could be detrimental for the apoptotic process.

MATERIALS AND METHODS

Cell cultures

The parental drug-sensitive human T-lymphoblastoid CEM cell line (wt CEM) and its MDR variant CEM-VBL100 (VBL100) (Matarrese et al., 2001; Meschini et al., 2003) were cultured at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 5 mM L-glutamine, penicillin (100 i.u./ml), and streptomycin (100 mg/ml) in 5% CO_2–95% air, at an optimal cell density of 3 to 8×10^5 cells/ml. MDR variant VBL100 cells were supplemented once a week with 100 ng/ml vinblastine (Sigma-Aldrich, St. Louis, MO). To induce apoptosis, VBL100 cells were treated with LY294002 at different concentrations (see Figure legends), H_2O_2 (100 μM), or Z-LEHD-FMK (50 μM). Z-DEVD-FMK (a caspase-3 inhibitor), and VEID-CHO (a caspase-6 inhibitor) (Calbiochem, La Jolla, CA) were used at 40 μM for 2 h prior to apoptotic induction.

Flow cytometric analysis of P-gp expression and apoptosis

For detection of P-gp surface expression, cells were washed with PBS and incubated with 4 μl/10^6 cells of a phycoerythrin (PE)-conjugated anti-P-gp monoclonal antibody (clone 17F9, BD Biosciences Pharmingen, Milan, Italy) for 1 h at 4°C. This antibody, according to the manufacturer's specifications, recognizes an extracellular epitope. Then, samples were washed with PBS and analyzed by flow cytometry. For sub-G_1 (apoptotic cells) peak analysis, cells were harvested by centrifugation at 200*g* for 10 min, washed once with phosphate buffered saline (PBS, pH 7.4) and fixed with 70% cold (4°C) ethanol for at least 4 h and subsequently stained with propidium iodide (PI, DNA-Prep kit, Beckman Coulter, Miami, FL). The subdiploid DNA content was analyzed and calculated using an Epics XL flow cytometer with the appropriate software (System II, Beckman Coulter). At least 10,000 events/sample were acquired.

Preparation of cell homogenates and western blot analysis

Cells were washed twice in PBS, containing the complete protease inhibitor cocktail (Roche Applied Science, Milan, Italy) and phosphatase inhibitor cocktail (1 mM Na_3VO_4, 2.5 mM Na pyrophosphate, 1 mM 2-glycerolphosphate, 25 mM NaF). Cells were then lysed at ~10^7/ml in boiling electrophoresis sample buffer containing the protease and phosphatase inhibitor cocktails. Lysates were briefly sonicated to shear DNA and reduce viscosity, and boiled for 5 min to solubilize proteins. Proteins separated on SDS–polyacrilamide gels (SDS–PAGE), were transferred to nitrocellulose sheets using a semi-dry blotting apparatus. Sheets were saturated in PBS containing 2% bovine serum albumin (BSA) and 3% non-fat dry milk for 60 min at 37°C (blocking buffer), then incubated overnight at 4°C with mouse monoclonal antibodies to P-gp (clone F4, Sigma-Aldrich; clone C494, Calbiochem), with rabbit polyclonal antibody to P-gp ($NH_2$11, a kind gift from Dr. U.S. Rao, Texas Tech University Health Sciences Center Amarillo, TX, see Nuti and Rao, 2002), with rabbit polyclonal antibodies to either phospho-Akt (Ser 473 p-Akt, Cell Signaling, Beverly, MA) or to poly(ADP-ribose) polymerase-1 (PARP-1, Apotech Corporation, Epalinges, Switzerland), or with goat polyclonal to lamin B (Santa Cruz Biotechnology, Santa Cruz, CA), all diluted 1:1,000 in PBS containing 0.1% Tween-20 and 2% BSA. Rabbit polyclonal antibodies to caspase-3 or caspase-6 were from Cell Signaling. Monoclonal antibody to β-tubulin was from Sigma-Aldrich. After four washes in PBS containing 0.1% Tween-20, blots were incubated for 90 min at room temperature with the appropriate peroxidase-conjugated secondary antibody, diluted 1:10,000 in PBS-Tween-20, and washed as above. Bands were visualized by the enhanced chemiluminescence (ECL) method (Lumi-Light^{Plus} ECL detection kit, Roche Applied Science).

Multidrug resistance direct dye efflux assay

This was performed essentially as described in the manufacturer's instructions (Chemicon International, Temecula, CA). Briefly, approximately 2.5×10^5 cells were centrifuged and the pellet was resuspended in cold Loading Buffer containing Rhodamine 123 and incubated on ice. Cells were washed twice and kept at different temperatures for 15 min. Then, test tubes were put on ice to stop the reaction, samples were washed twice again with PBS and cells resuspended in cold PBS. Samples were finally analyzed by flow cytometry.

In vitro P-gp cleavage

This was performed as reported previously (Martelli et al., 2004). Briefly, VLB100 cells (4×10^6) were lysed in 100 μl of 25 mM HEPES-NaOH, pH 7.4, 10% sucrose, 0.1% CHAPS, 2 mM EDTA, 5 mM dithiothreitol. Fifty nanograms of human recombinant activated caspase-3 or caspase-6 (from Calbiochem) was added to 50 μg of protein and incubation proceeded for 3 h at 37°C. Reaction was stopped by adding an equal volume of 2× electrophoresis sample buffer. Samples were then separated by SDS–PAGE, blotted to nitrocellulose paper, and probed with F4 monoclonal antibody.

Preparation of plasma membrane/particulate fraction

This was performed essentially following manufacturer's instructions (FractionPREP cell Fractionation System, BioVision Research Products, Mountain View, CA). After 16 h of incubation with 50 μM LY294002,VBL100 cells (10^7) were collected and washed twice in ice-cold PBS. Then, cells were resuspended by pipetting several times in 400 μl of Cytosol Extraction Buffer-Mix and incubated for 20 min on ice with gently tapping 3–4 times every 5 min. Samples were centrifuged at 700*g* for 10 min and the supernatant, which was the cytosolic fraction, was discarded. The remaining pellets were resuspended in 400 μl of ice-cold Membrane Extraction Buffer-A Mix and vortexed for 10–15 sec. Then, 22 μl of Membrane Extraction Buffer-B was added and samples were vortexed for 5 sec and incubated on ice for 1 min. Subsequently, samples were vortexed again for 5 sec and centrifuged for 5 min at 1,000*g*. The supernatants, which were the plasma membrane/particulate fraction, were transferred to a clean tube. Sample buffer was added to plasma membrane/particulate samples and they were separated on SDS–PAGE.

Flow cytometric P-gp detection in apoptotic cells

To detect expression of P-gp in apoptotic cells, we performed a dual parameter flow cytometric analysis with antibody to P-gp and TdT-mediated dUTP nick end labeling (TUNEL) reaction (In Situ Cell Death Detection kit, Roche Applied Science). After pretreatment for 16 h with 50 μM LY294002, cells were washed in PBS and put in a test tube at a concentration of 2×10^6 cells/ml. Then, samples were incubated with PE-conjugated anti-P-gp antibody for 1 h at 4°C and washed twice with PBS before fixation with 2% formaldehyde for 1 h at 4°C. After centrifugation and a washing in PBS, cells were permeabilized with a freshly prepared solution of 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice and washed again twice in PBS, before incubation in 50 μl/sample of TUNEL reaction mixture for 1 h at 37°C in a humidified atmosphere in the dark. Finally, samples were washed twice in PBS and directly analyzed by flow cytometry.

RESULTS

Expression of P-gp in wt CEM and VBL100 cells

We first evaluated P-gp expression in wt CEM and VBL100 cells by both flow cytometric analysis and western blot. For flow cytometric analysis, we employed an antibody which, according to the manufacturer, recognizes an extracellular epitope of the protein. In comparison with wt CEM cells, VBL100 cells expressed very high levels of P-gp (Fig. 1A). The flow cytometric results were corroborated by western blot analysis, which showed a very high P-gp amount in VBL100 cells, whereas wt CEM cells were completely negative (Fig. 1B). These findings were also confirmed by a flow cytometric functional efflux assay based on the extrusion of the fluorescent molecule, Rhodamine 123. The efflux activity of P-gp is highly temperature-sensitive: P-gp functions optimally near 37°C, but it is inactive at 4°C. When P-gp expressing cells are preloaded with fluorescent substrates, such as Rhodamine 123, and incubated at 4°C, they retain the dye and consequently have high fluorescence levels. Conversely, cells incubated at 37°C more readily efflux the dye and show reduced fluorescence. The drug vinblastine, which is also substrate for P-gp, competitively reduces efflux of Rhodamine 123. Inclusion of an excess of vinblastine in the efflux reaction at 37°C therefore results in higher fluorescence.

As expected, VBL100 cells displayed a reduction of the fluorescence due to Rhodamine 123 when incubated at 37°C in the presence of the carrier dimethylsulfoxide (DMSO) (Fig. 1C). The addition of vinblastine resulted in a double-peak histogram, in which the peak on right displayed a fluorescence higher than the one observed at 4°C, because these cells pumped out the dye at a slower rate due to the presence of the competitor. We performed this functional assay also on wt CEM cells (Fig. 1C) and, as expected for a cell line, which does not express P-gp, there were no differences in the fluorescence intensity between the three experimental conditions.

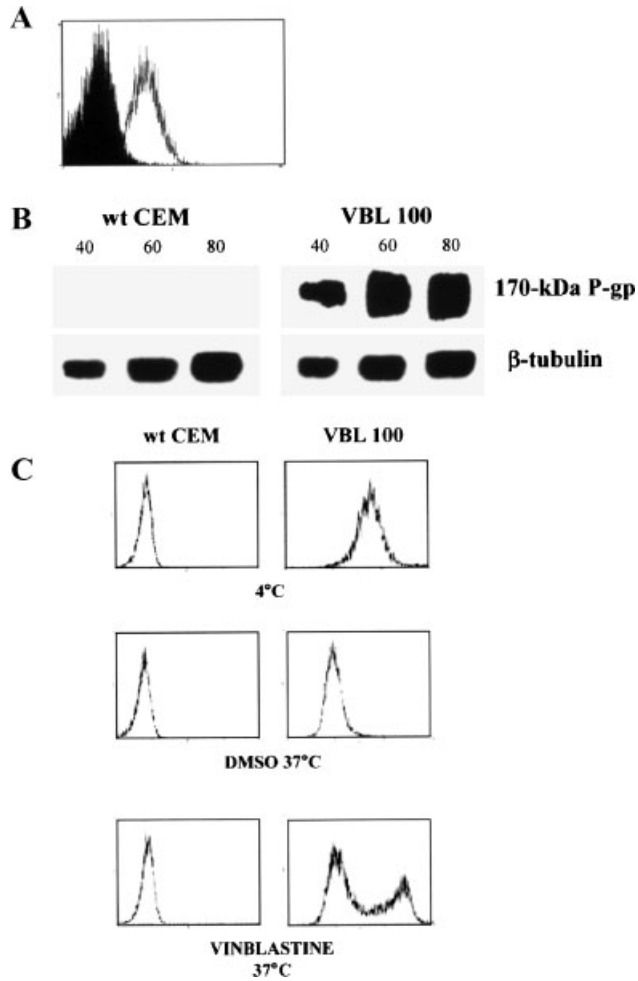

Fig. 1. Expression and functional activity of 170-kDa P-gp in wt CEM and VBL100 cells. **A**: Flow cytometric analysis for P-gp in wt CEM (black peak) and VBL100 cells (white peak). **B**: Western blot analysis for P-gp in wt CEM and VBL100 cells (40, 60, 80 μg of proteins were blotted). An antibody to β-tubulin was employed to verify equal loading of gels. **C**: Flow cytometric assay for P-gp functional activity. The efflux activity of MDR1 is highly temperature sensitive because P-gp functions optimally near 37°C, but is inactive at 4°C. wt CEM and VBL100 cells were preloaded with fluorescent substrate, Rhodamine 123 or the carrier dimethylsulfoxide (DMSO). The drug vinblastine behaves as a competitive inhibitor of Rhodamine 123 extrusion, thus increasing cell fluorescence at 37°C. One representative of three different experiments is shown.

P-gp is cleaved during apoptosis

To investigate the fate of P-gp during apoptosis in VBL100 cells, we treated them for 24 h with increasing concentrations of LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K)/Akt pathway, the most important signaling network for cell survival (Chang et al., 2003; Hanada et al., 2004; Martelli et al., 2005). Indeed, it is well established that CEM cells have elevated levels of p-Akt due to the lack of the lipid phosphatase PTEN and that inhibition of the PI3K/Akt pathway results in apoptosis (Uddin et al., 2004). Flow cytometric analysis of PI-stained samples demonstrated a marked increase in the percentage of apoptotic cells at 50 μM LY294002 (Fig. 2A), where 50% of cells were apoptotic.

As expected, Western blot analysis showed a progressive reduction in the levels of Ser 473 p-Akt, accompanied by the appearance of the cleaved form (85 kDa) of the apoptotic marker PARP-1. Cleavage of P-gp was evident only at 50 μM LY294002 (Fig. 2B). At this inhibitor concentration, Western blot analysis revealed that, simultaneously with the decrease of the 170-kDa native P-gp form, the antibody recognized a broad band in the range of 80–85 kDa (see also later, Fig. 2B).

A time course analysis of apoptosis induced in VBL100 cells by 50 μM LY294002, revealed that an increase in the percentage of apoptotic cells was apparent already after a 3-h treatment and progressively increased (Fig. 3A). Initial cleavage of P-gp was also apparent at 3 h of treatment, and interestingly, at this time point,

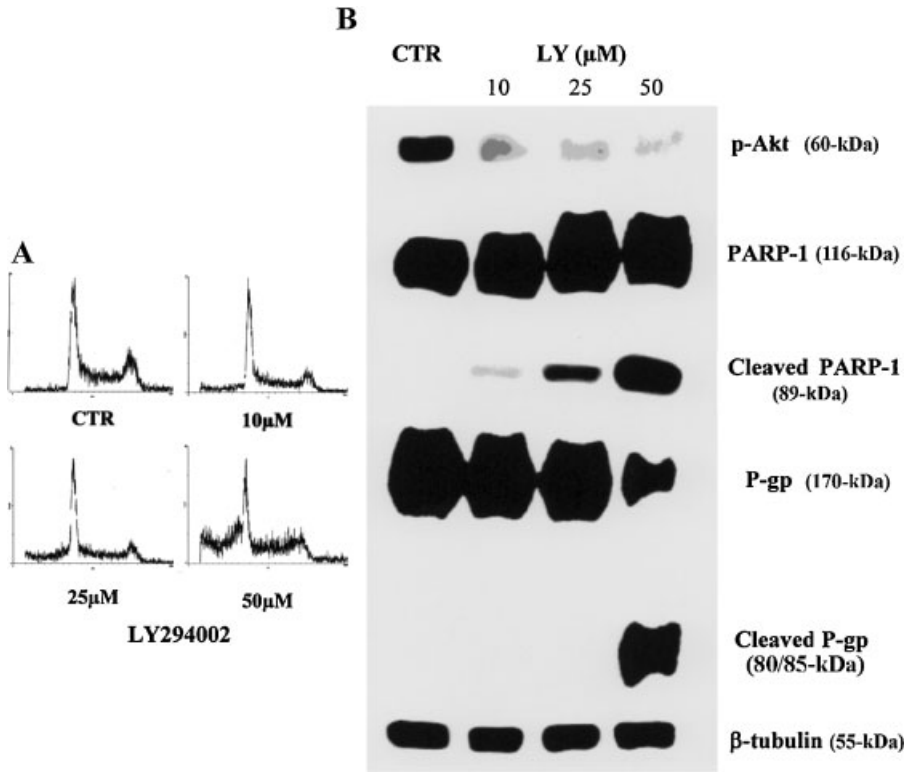

Fig. 2. P-gp (170 kDa) is cleaved during LY294002-induced apoptosis of VBL100 cells. **A**: Flow cytometric analysis of apoptosis in VBL100 cells treated for 24 h with increasing concentrations of LY294002. CRT, control (untreated) cells. **B**: Western blot analysis for Ser 473 p-Akt, PARP-1, and 170-kDa P-gp in VBL100 cells treated with 10, 25, and 50 μM of LY294002 (LY) for 24 h. Proteins (80 μg) were blotted to each lane, β-tubulin served as loading control.

two cleavage products were detected, one at 80 and the other at 85 kDa. At the following time points, due to the massive increase in the amount of cleaved P-gp, it was not always possible to clearly distinguish the two cleaved fragments (Fig. 3B). P-gp cleavage paralleled that of lamin B, a well-established substrate of caspases during apoptosis (Fig. 3B) (e.g., Slee et al., 2001). Since the antibody to lamin B, which we employed for this study does not recognizes the cleaved fragment, we detected a decrease in the amount of full-size (70 kDa) lamin B.

P-gp is cleaved following treatment with other apoptosis inducers

To determine whether or not P-gp cleavage is general feature of apoptotic death in VBL100 cells, we investigated it also using two other established apoptosis inducers, that is, 100 μM H_2O_2 (Clement et al., 1998;

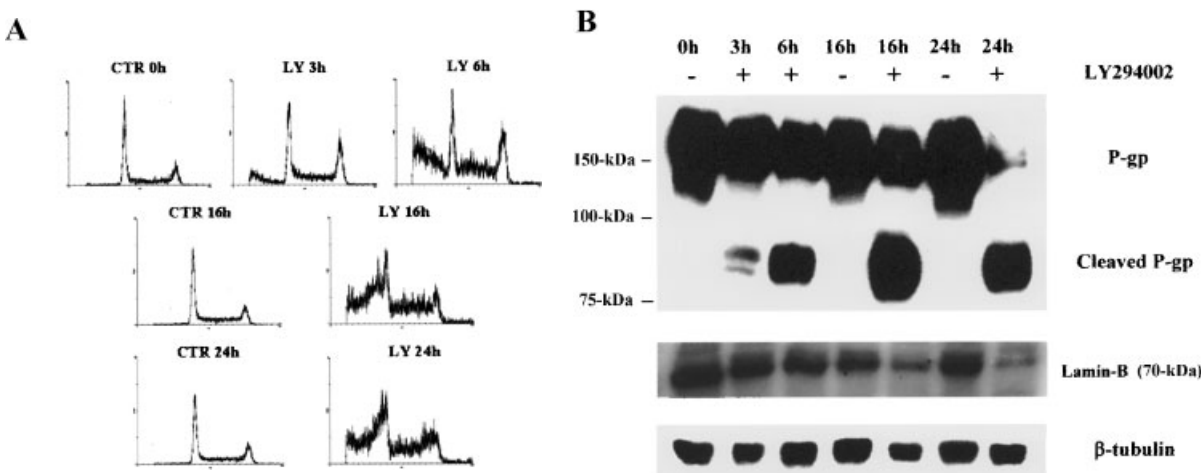

Fig. 3. Time course of 170-kDa P-gp cleavage in LY294002-treated VBL100 cells. **A**: Flow cytometric analysis of apoptosis in VBL100 cells treated with 50 μM of LY294002 (LY) for different times. CRT, control (untreated) cells. **B**: Western blot analysis for P-gp and lamin B in VBL100 cells treated with 50 μM LY294002 for 3, 6, 16, and 24 h. Proteins (80 μg) were blotted to each lane, β-tubulin served as loading control.

Pervaiz et al., 1999; Ahmad et al., 2004) and the caspase-9 inhibitor Z-LEHD-FMK (50 μM), a recently described paradoxical apoptosis inducer for CEM cells (Shah et al., 2004). Flow cytometric analysis demonstrated an increase in apoptotic VBL100 cells in response to either H_2O_2 or Z-LEHD-FMK (Fig. 4A, C). P-gp cleavage was detected in response to both apoptosis inducers (Fig. 4B,D).

P-gp cleavage is dependent on caspase-3 activity

We first assessed activation of effector caspases in response to apoptosis inducers. As presented in Figure 5A, caspase-3 was activated in response to LY294002 or H_2O_2 as documented by Western blot which showed the appearance of the distinctive cleavage products migrating at 17/20 kDa, as well as a decrease in the amount of full length procaspase-3. As to caspase-6, the antibody we employed revealed a decrease in the amount of full length procaspase-6, a clear indication of cleavage. Similar results were obtained when apoptosis was induced by Z-LEHD-FMK (data not shown).

We next employed selective irreversible pharmacological inhibitors of either caspase-3 (Z-DEVD-FMK) or caspase-6 (VEID-CHO) to determine whether or not P-gp cleavage was dependent on the activity of one (or both) of these effector caspases. As shown in Figure 5B, Western blot analysis demonstrated that Z-DEVD-FMK, but not VEID-CHO, was capable of completely blocking P-gp cleavage in LY294002-treated VBL100 cells. As a further proof that P-gp could be cleaved by caspase-3, we performed experiments on whole extracts of VBL100 cells. Although some P-gp cleavage was detected in cell extracts not treated with recombinant caspases, the addition of activated human recombinant caspase-3 (but not of caspase-6) resulted in a dramatic cleavage of P-gp, as documented by Western blot analysis which showed the appearance of the two 80/85 kDa cleaved fragments, co-migrating with those produced in vivo in cells treated with LY294002 (Fig. 5C).

Antibodies to either a C-half or N-half epitope of P-gp recognize only one cleaved band

To better characterize the cleaved fragments of P-gp, we employed two other antibodies, which had been raised to different epitopes. Antibody C494, recognizes an epitope located only in the C-half of the molecule, whereas antibody $NH_2$11 was generated against an epitope present in the N-half of P-gp (Nuti and Rao, 2002). Alignment of Western blots developed with F4, C494, and $NH_2$11 antibodies revealed that both C494 and $NH_2$11 recognized only one band. C494 antibody

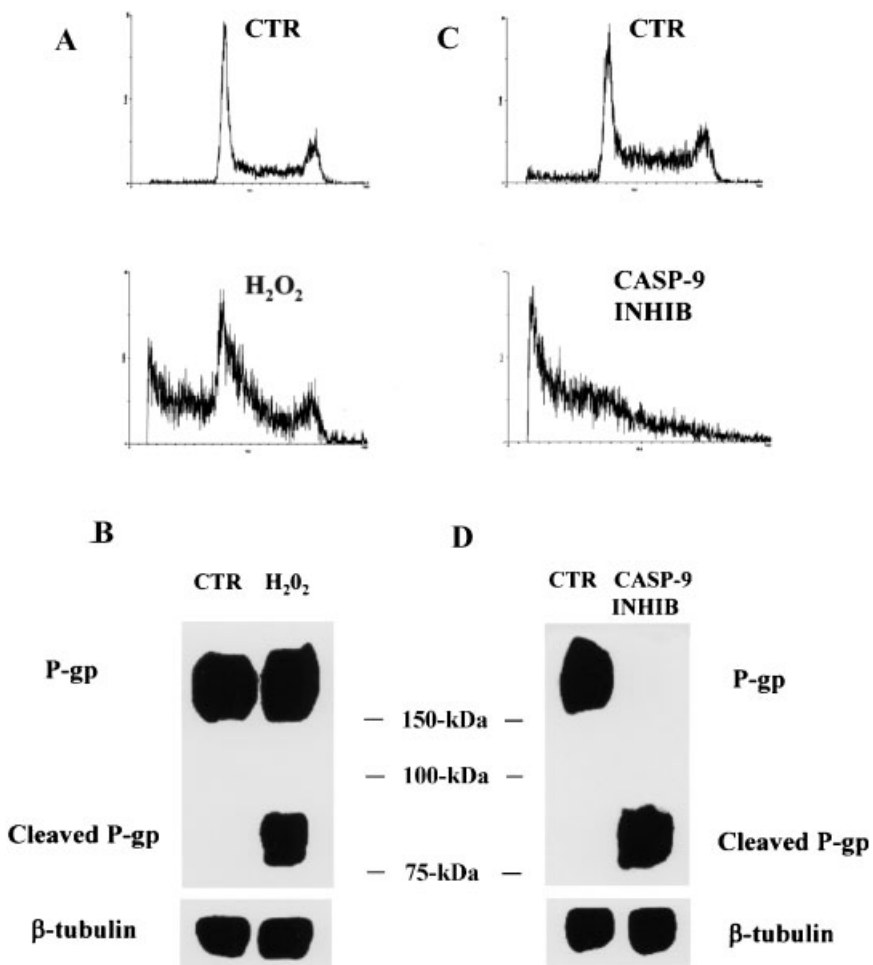

Fig. 4. P-gp (170 kDa) is cleaved in response to other apoptosis inducers. **A**: Flow cytometric detection of apoptosis in control VBL100 cells and in cells treated with 100 μM H_2O_2 for 8 h. **B**: Western blot analysis for 170-kDa P-gp in control VBL100 cells and in cells treated with H_2O_2 for 8 h. **C**: Flow cytometric detection of apoptosis in control VBL100 cells and in cells treated with 50 μM Z-LEHD-FMK caspase-9 inhibitor (CASP-9 INHIB) for 24 h. **D**: Western blot analysis for 170-kDa P-gp in control VBL100 cells and cells treated with caspase-9 inhibitor for 24 h. In (B) and (D), 80 μg of proteins were blotted to each lane, β-tubulin served as loading control. CTR, control (untreated) cells.

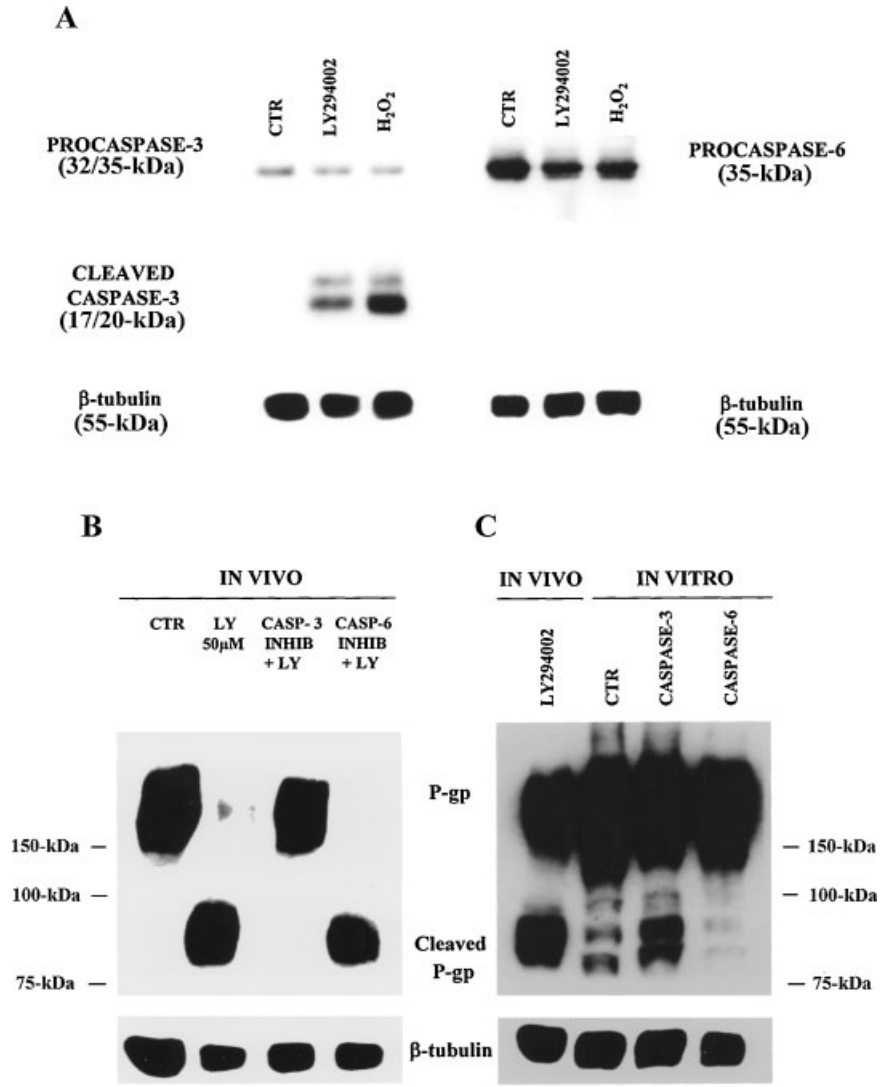

Fig. 5. P-gp (170 kDa) cleavage is dependent on caspase-3 activity both in vivo and in vitro. **A**: Western blot analysis for caspase-3 and caspase-6 cleavage. VBL100 cells were treated with 50 μM LY294002 for 16 h or with 100 μM H_2O_2 for 8 h. Proteins (80 μg) were blotted to each lane, β-tubulin served as loading control. CTR, control (untreated) cells. **B**: Western blot analysis for in vivo cleaved P-gp in VBL100 cells treated with LY294002 (50 μM) alone or pretreated with caspase-3 (casp-3 inhib) or caspase-6 inhibitor (casp-6 inhib) for 2 h and then with LY294002 (LY, 50 μM) for 24 h. Proteins (80 μg) were blotted to each lane, β-tubulin served as loading control. CTR, control (untreated) cells. **C**: In vitro cleavage of P-gp by active human recombinant caspase-3. VBL100 cell extracts (50 μg of protein) were incubated at 37°C for 3 h in the presence of 50 ng of either activated caspase-3 or activated caspase-6. The incubation was stopped by adding 2× electrophoresis sample buffer, then samples were run on SDS–polyacrylamide gels, transferred to nitrocellulose sheets and probed with an antibody to P-gp (clone F4). Immunostaining with a monoclonal antibody to β-tubulin confirmed equal loading. CTR = con-control (untreated) cells. For a comparison, whole extracts from cells treated in vivo for 6 h with 50 μM LY294002 were probed with F4 antibody.

stained the slower migrating band, which conceivably corresponds to the C-terminal half of P-gp, while $NH_2$11 recognized the faster migrating band which most likely originated from the N-terminal half of the protein (Fig. 6A). As a further proof that caspase-dependent cleavage of P-gp occurred in the linker region, we prepared the plasma membrane/particulate fraction from control and LY294002-treated VBL100. Western blot analysis with F4 antibody, demonstrated that the two cleaved fragments were still associated with the plasma membrane particulate fraction, as it would have been expected if the cleavage would have taken place intracytoplasmically (Fig. 6A).

We then employed TUNEL reaction, which is used to identify endonucleolysis, one of the key biochemical events of apoptosis, to detect surface expression of P-gp in apoptotic cells. We performed TUNEL reaction on VBL100 cells treated with 50 μM of LY294002 for 16 h and counterstained them with PE-conjugated anti-P-gp monoclonal antibody (Fig. 6B). The double stained apoptotic cell population (see quadrant A2) displayed almost the same mean PE fluorescence intensity as healthy cells (see quadrant A1). This indicates that the extracellular epitope of P-gp recognized by the antibody employed or flow cytometric detection of 170-kDa P-gp is still reactive and further supports the notion of a cleavage taking place in the intracytoplasmic region.

Sensitivity of VBL100 cells to apoptosis inducers

Since a previous report indicated that VBL100 cells were paradoxically more sensitive to apoptosis inducers acting through the mitochondrial pathway than wt CEM cells (Matarrese et al., 2001), we tested the

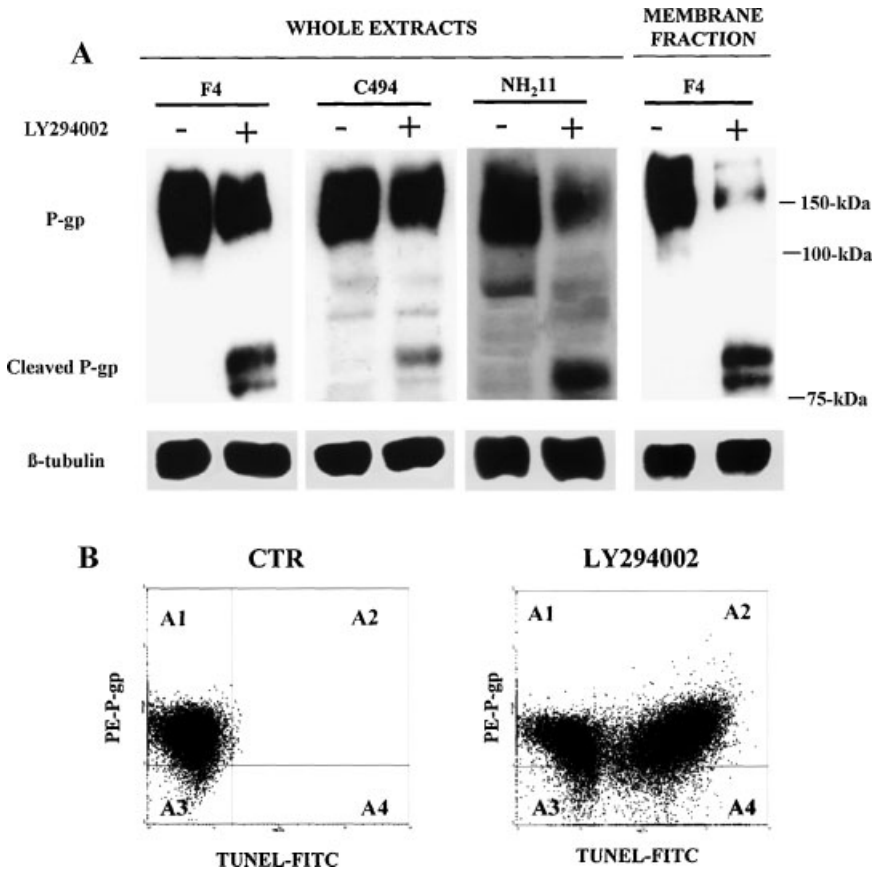

Fig. 6. Reactivity of different antibodies to P-gp in LY294002-treated VBL100 cells. **A**: Western blot analysis employing three different antibodies to P-gp (F4, C494, $NH_2$11). Cells, treated for 6 h with 50 μM LY294002, were lysed and whole cell extracts, separated by SDS–PAGE, were blotted to nitrocellulose membrane. Bands were visualized by ECL technique. Note how antibody C494 recognizes only the higher molecular weight fragment of P-gp, whereas $NH_2$11 stains the lower molecular weight fragment. The plasma membrane/particulate fraction, separated by SDS–PAGE and blotted to nitrocellulose paper, was probed with F4 antibody. Immunochemical staining for β-tubulin confirmed equal loading of gels. **B**: Double staining with PE-conjugated anti-P-gp antibody and TUNEL-FITC of VBL100 cells treated for 16 h with 50 μM LY294002. CTR, control (untreated) cells.

sensitivity of the two cell subtypes to the apoptotic inducers employed in this study, that is, LY294002, H_2O_2, and Z-LEHD-FMK. As shown in Figure 7, after a 6-h incubation, both LY294002 (either 25 or 50 μM) and H_2O_2 (100 μM) were able to induce a dramatic increase in the percentage of sub-G_1 (apoptotic) VBL100 cells, whereas wt CEM cells were much more resistant. Even a 200 μM LY294002 concentration killed less wt CEM

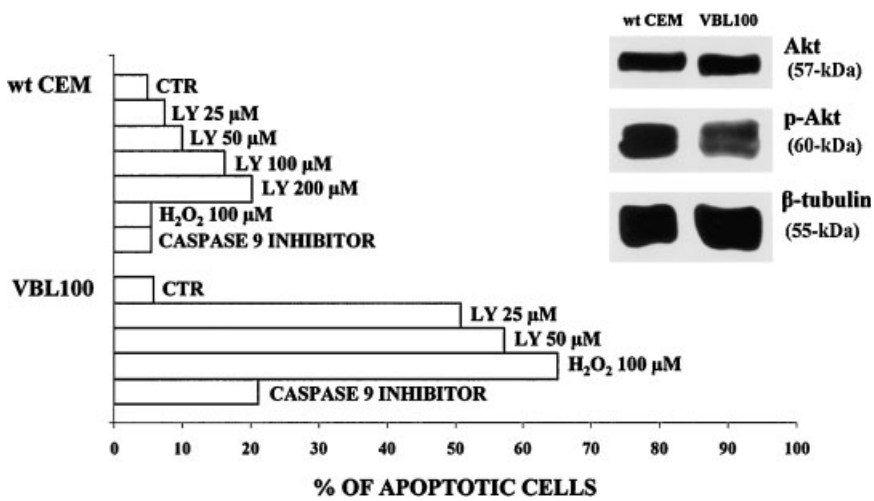

Fig. 7. VBL100 cells are more sensitive to apoptotic stimuli than wt CEM cells. Cells were incubated for 6 h in the presence of the indicated concentrations of apoptosis inducers. The percentage of apoptotic cells was assessed by flow cytometric analysis of PI-stained samples. CTR, control (untreated cells); LY, LY294002. One representative of three different experiments is shown. Western blot analysis shows the levels of total Akt, Ser 473 p-Akt, and β-tubulin in wt CEM and VBL100 cells.

cells than a 50 μM LY294002 concentration employed on VBL100 cells.

Therefore, we also investigated the status of Akt phosphorylation in the two cell subtypes. As presented in Figure 7, wt CEM cells had much higher levels of Ser 473 p-Akt when compared to VBL100 cells, as demonstrated by Western blot analysis.

DISCUSSION

In this article, we have reported, for the first time to our knowledge, the cleavage of 170-kDa P-gp during apoptosis of MDR VBL100 cells. Cleavage of P-gp was seen in response to various apoptotic stimuli, including the pharmacological inhibitor of PI3K LY294002, H_2O_2, and a caspase-9 inhibitor. Cleavage of P-gp was apparent early (3 h) during the apoptosis induction. The P-gp cleavage was caspase-3-dependent in that it could be completely inhibited by a caspase-3 inhibitor, but not by a caspase-6 inhibitor. Moreover, activated recombinant human caspase-3 was capable to cleave P-gp in vitro, yielding two fragments of the same apparent molecular weight as those generated in vivo.

As to the cleavage site, it should be recalled here that caspases are specific cysteine proteases recognizing four amino acids, named S4, S3, S2, S1. The preferred S3 position is an invariant glutamine for all mammalian caspases, while S1 is an aspartate residue. Thus, specificity for caspase cleavage can be described as X-Glu-X-Asp. Caspase-3 preferred cleavage motif is DEXD (Lavrik et al., 2005). Such a motif is not present in the human 170-kDa P-gp sequence. However, numerous exceptions to this rule have been reported (Fischer et al., 2003). Interestingly, a SEID motif is present at amino acids 646–649. This motif is included within the cytoplasmically located linker region. However, if the cleavage would indeed occur after aspartate 649, the fragment containing the N-half of P-gp (649 amino acids) would migrate slower than the fragment containing the C-half (631 amino acids), whereas in Western blot experiments it actually migrated faster. Considering that: (a) the antibody used for flow cytometry still recognizes its P-gp extracellular epitope also in apoptotic cells; (b) that the F4 antibody we mostly used for Western blots recognizes a common epitope located in both the third and sixth extracellular loops of P-gp (Chun et al., 1993); and (c) that the two cleaved fragments are recovered in the membrane fraction; we feel that apoptotic P-gp cleavage takes place intracytoplasmically, however the SEID motif of the linker region does not seem the most likely cleavage site. In any case, the sequence of P-gp contains several other aspartate residues, which might act as cleavage sites for caspase-3.

It should be reminded here that others (Nuti et al., 2000; Nuti and Rao, 2002) have demonstrated that the linker region is the site where proteases such as trypsin and chymotrypsin cleave 170-kDa P-gp, thus producing fragments of the size in the range of approximately 80 kDa (N-half) and 60 kDa (C-half), but in that case the cleavage site was mapped at amino acids 680 and 682.

The effect of P-gp upregulation on cell sensitivity to apoptosis is controversial. It has been suggested that P-gp could protect cells against apoptotic stimuli which function in a caspase-dependent manner by transporting a key caspase or adaptor molecules out of the cell or by inhibiting caspase activation by decreasing intracellular ATP or altering intracellular pH. P-gp overexpression was capable of blocking both the intrinsic and the extrinsic apoptotic pathways (Smyth et al., 1998; Johnstone et al., 1999; Ruefli et al., 2002). More recently, overexpression of an ATP-ase-mutant P-gp showed that cells could not efflux chemotherapeutic drugs but remained relatively resistant to apoptosis. Therefore, it seems that P-gp can inhibit apoptosis by a dual action, which involves both ATP-ase-dependent drug efflux and ATP-ase-independent inhibition of apoptosis (Tainton et al., 2004).

However, these findings have been questioned by others who showed that cells with high levels of P-gp are paradoxically more sensitive to apoptosis inducers acting through either the mitochondrial (intrinsic) or the death receptor-mediated (extrinsic) apoptotic pathways (Matarrese et al., 2001; Cenni et al., 2004).

Our results confirmed the increased sensitivity of VBL100 cells to apoptotic stimuli, when compared to wt CEM cells. However, a caveat is necessary here, because we have demonstrated that wt CEM cells, in comparison to VBL100 cells, have much higher levels of Ser 473 p-Akt, a clear indication of the activation of the PI3K/Akt pathway, which is the most important survival network in mammalian cells (Martelli et al., 2005). Indeed, our unpublished results indicate that if VBL100 cells were pretreated with LY294002 (which downregulates the PI3K/Akt-signaling network) they became much more sensitive to H_2O_2. These findings indicate that the PI3K/Akt axis is a very important determinant of VBL100 cell sensitivity to apoptosis inducers. Moreover, it is known that VBL100 cells have higher levels of pro-apoptotic proteins Bcl-XA, Bad, and Bax localized at the mitochondrial level when compared with wt CEM cells (Jia et al., 1999). It is important to emphasize that Bad, when phosphorylated by Akt, is not capable of localizing to mitochondria anymore (Hanada et al., 2004; Martelli et al., 2005). This observation is consistent with the lower sensitivity of wt CEM cells (which have an overactive PI3K/Akt network in comparison with VBL100 cells) to apoptotic inducers acting through the mitochondrial pathway.

Interestingly enough, a recent report has highlighted that also wt U2OS human osteosarcoma cells have much higher levels of Ser 473 p-Akt than their multidrug resistant subline (MDR-U2OS) which overexpresses 170-kDa P-gp (Cenni et al., 2004). MDR-U2OS are more sensitive than parental cells to the pro-apoptotic cytokine TRAIL which mainly acts through the extrinsinc apoptotic pathway, even if the apoptotic signal can also be amplified through the mitochondria (Thorburn, 2004). Indeed, overexpression of a dominant negative Akt form in parental U2OS cells increased their sensitivity to TRAIL, whereas a constitutive activated Akt form upregulated TRAIL-dependent apoptosis of MDR-U2OS (Cenni et al., 2004). Moreover, MDR-U2OS exhibited reduced activity of the Erk1/2 pathway (Cenni et al., 2004), which could also convey important survival signals (Thorburn, 2004).

At present the relationship (if any) between MDR-1 gene expression and activation of the PI3K/Akt pathway is unclear. It will be interesting to overexpress 170-kDa P-gp to determine whether or not this would result in activation or downregulation of PI3K/Akt signaling. Whatever the case, since both VBL100 and MDR-U2OS sublines have been selected by exposing parental cells to chemotherapeutic drugs, it could not be ruled out that, in addition to inducing 170-kDa P-gp expression, these treatments also had other effects on key cellular metabolic pathways, which can then influence sensitivity to apoptosis inducers. However, it should also be recalled here that others have shown that cells induced

to express P-gp either by drug selection or by retroviral gene transduction with MDR1 cDNA are intrinsically resistant to cell death (Johnstone et al., 1999).

In conclusion, we have demonstrated for the first time a caspase-dependent cleavage of 170-kDa P-gp during apoptosis. If 170-kDa P-gp counteracts apoptosis independently from its ATP-ase activity, the cleavage may be envisaged as a mechanism aimed at blocking an important cell survival component. Identification of the cleavage site followed by overexpression of a mutated, cleavage-resistant form of 170-kDa P-gp should help in better understanding its role during apoptosis.

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