

The Mitochondrial Effects of Small Organic Ligands of BCL-2 SENSITIZATION OF BCL-2-OVEREXPRESSING CELLS TO APOPTOSIS BY A PYRIMIDINE-2,4,6-TRIONE DERIVATIVE*

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We have investigated the mitochondrial effects of BH31-2', Chelerythrine, and HA14-1, small organic molecules that share the ability to bind the BH3 domain of BCL-2. All compounds displayed a biphasic effect on mitochondrial respiration with uncoupling at low concentrations and respiratory inhibition at higher concentrations, the relative uncoupling potency being BH31-2' (half-maximal uncoupling at about 80 nM) > Chelerythrine (half-maximal uncoupling at about 2 μM) > HA14-1 (half-maximal uncoupling at about 20 μM). At concentrations lower than required for uncoupling all compounds sensitized the permeability transition pore (PTP) to opening both in isolated mitochondria and intact cells. To assess whether the effects on BCL-2 binding, PTP induction and respiration could be due to different structural determinants we have tested a set of HA14-1 analogs from the Hoffmann-La Roche chemical library. We have identified 5-(6-chloro-2,4-dioxo-1,3,4,10-tetrahydro-2H-9-oxa-1,3-diaza-anthracen-10-yl)-pyrimidine-2,4,6-trione (EM20-25) as a molecule devoid of effects on respiration that is able to induce PTP opening, to disrupt the BCL-2/BAX interactions *in situ* and to activate caspase-9 in BCL-2-overexpressing cells. EM20-25 neutralized the antiapoptotic activity of overexpressed BCL-2 toward staurosporine and sensitized BCL-2-expressing cells from leukemic patients to the killing effects of staurosporine, chlorambucil, and fludarabine. These results provide a proof of principle that the potentially toxic effects of BCL-2 ligands on mitochondrial respiration are not essential for their antiapoptotic activity and represent an important step forward in the development of tumor-selective drugs acting on BCL-2.

Antiapoptotic proteins of the BCL-2 family contribute to neoplastic cell expansion by suppressing physiological cell death mechanisms and by increasing the resistance to anticancer drugs. High levels of the BCL-2 protein can be found in cells selected for their resistance to chemotherapeutic agents (1), and this makes BCL-2 an attractive target

for cancer therapy (2, 3). Despite intense research on this topic, the mechanisms through which BCL-2 prevents apoptosis are not fully understood (4). The protein dimerizes with other members of the family, which comprises both pro- and antiapoptotic members; and it is widely believed that the outcome for cell survival depends on the ratio of pro- to antiapoptotic BCL-2-like proteins (5).

A computer screening based on the structure of the close BCL-2 relative, BCL-XL, has identified HA14-1,⁶ a small organic ligand that is able to displace a peptide modeled on the BCL-2 binding region of BAK, a proapoptotic member of the family (6). Remarkably, HA14-1 was able to cause cell death that was preceded by activation of caspase-9 and -3 and caused mitochondrial depolarization *in situ* (6). Similar results have been reported for antimycin A, which displays a striking BCL-2 binding activity that is retained by a 2-methoxy derivative devoid of inhibitory effects on respiration (7), and for BH3Is (8) and Chelerythrine (9), which were identified by high throughput screening of chemical libraries. These findings point to mitochondria as the targets for the effects of BCL-2 ligands.

Mitochondria are important players in the pathways to cell death through at least three mechanisms: (i) changes of ATP production, (ii) alteration of Ca²⁺ homeostasis, (iii) release of apoptogenic proteins like cytochrome *c* and Smac-Diablo that activate procaspase-9 and then downstream caspases (10). A mechanistic link between BCL-2, mitochondria, and cell death is provided by the reported inhibition of the release of cytochrome *c* and of other mitochondrial proteins by BCL-2 overexpression (11–13). Although it appears reasonable that the proapoptotic effects of HA14-1, BH3Is, and Chelerythrine are mediated by interactions with BCL-2 in mitochondria, the mechanistic basis for the mitochondrial effects of these drugs has not been established. Specifically, the depolarizing and/or cytochrome *c*-releasing effects of BCL-2 ligands could be due to opening of the PTP, an inner membrane channel that is reportedly inhibited by BCL-2 overexpression (11), or to interference with energy coupling and/or respiration. Assessing whether BCL-2 ligands directly affect electron transfer appears particularly important in the light of the finding that antimycin A, the selective inhibitor of electron transfer at the *bc₁* complex, also binds to BCL-2 at the same site as HA14-1 (7).

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⁶ The abbreviations used are: HA14-1, ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate; B-CLL, B-chronic lymphocytic leukemia; BH31-2', 3-iodo-5-chloro-N-[2-chloro-5-((4-chlorophenyl)sulfonyl)phenyl]-2-hydroxybenzamide; Chelerythrine, 1,2-dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium; Cs, cyclosporin; EM20-25, 5-(6-chloro-2,4-dioxo-1,3,4,10-tetrahydro-2H-9-oxa-1,3-diaza-anthracen-10-yl)-pyrimidine-2,4,6-trione; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, human influenza hemagglutinin; Mops, 4-morpholinopropanesulfonic acid; PBS, phosphate-buffered saline; PT, permeability transition; PTP, permeability transition pore; siRNA, small interfering RNA; TMRM, tetramethylrhodamine methyl ester.

In this paper we have investigated the mitochondrial and cellular effects of BH3I-2', Chelerythrine, and HA14-1. All three compounds displayed a biphasic effect on mitochondrial respiration with uncoupling at low concentrations and respiratory inhibition at higher concentrations, the relative uncoupling potency being BH3I-2' > Chelerythrine > HA14-1, and they all sensitized the PTP to opening at concentrations lower than required for uncoupling both in isolated mitochondria and intact cells. BCL-2 overexpression did not sensitize but rather protected cells from the cytotoxic effects of the BCL-2 ligands. We show that the BCL-2 binding and PTP-inducing effects can be separated from the potentially toxic effects on respiration through the identification of EM20-25, a molecule devoid of effects on mitochondrial respiration that is able to induce PTP opening in isolated mitochondria and intact cells, to disrupt the BCL-2/BAX interactions *in situ* with activation of caspase-9 and to sensitize leukemic cells to the killing effects of staurosporine, chlorambucil, and fludarabine.

EXPERIMENTAL PROCEDURES

Measurements on Isolated Mitochondria—Liver mitochondria were isolated from albino Wistar rats weighing about 300 g by standard centrifugation techniques, as described previously (14). Oxygen consumption was measured polarographically with a Clark oxygen electrode in a closed 2-ml vessel equipped with magnetic stirring and thermostated at 25 °C. Mitochondrial swelling was followed as the change of light scattering of the mitochondrial suspension at 545 nm with a PerkinElmer Life Sciences 650-40 fluorescence spectrophotometer equipped with magnetic stirring and thermostatic control.

Cell Cultures—PC3 human prostate cancer cells were grown in RPMI 1640 medium supplemented with 2 mM glutamine. HeLa Neo and HeLa BCL-2 cells (a generous gift of Dr. Naoufal Zamzami, Institut Gustave Roussy, Villejuif, France) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine. The media were all supplemented with 10% fetal calf serum, 50 units \times ml⁻¹ penicillin, and 50 μ g \times ml⁻¹ streptomycin. Cells were kept in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C in a Forma tissue culture water jacketed incubator.

Analysis of BCL-2, BCL-XL, and GAPDH Expression in Different Cell Lines—One day before the experiment, 1 \times 10⁶ PC3, HeLa Neo, and HeLa BCL-2 cells were plated onto 100-mm diameter tissue culture dishes in the appropriate growth medium and incubated at 37 °C. Cells were then harvested, sedimented, washed once with ice-cold PBS, resuspended in 1 ml of ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 100 μ M phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin), incubated 30 min on ice, and finally Dounce-homogenized. The homogenates were sedimented at full speed in a microcentrifuge for 10 min at 4 °C to remove cell debris and nuclei. The supernatants, corresponding to the soluble cellular extracts, were transferred to clean tubes, and the protein concentration was determined by the Bradford assay. Equal protein amounts (100 μ g) were solubilized in Laemmli gel sample buffer containing 5% 2-mercaptoethanol, separated electrophoretically by SDS-PAGE, and subjected to Western blotting analysis using a mouse anti-human BCL-2 antibody (clone 7, BD Biosciences) or a rabbit anti-human BCL-XL antibody (clone 54HD, Cell Signaling Technology®), as described below. The same membranes were then washed, stripped as described below, and probed with a mouse monoclonal antibody against rabbit skeletal muscle GAPDH (clone 6C5, Chemicon International, Inc.).

Purification of B-CLL Cells—B-CLL was diagnosed according to standard clinical and laboratory criteria, and patients who had not yet

received treatment were studied. Mononuclear cells were recovered following centrifugation on Ficoll-Hypaque gradient (15). Cell samples were washed three times with PBS and resuspended in endotoxin free RPMI 1640 medium (Sigma) supplemented with 20 mM HEPES and L-glutamine, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum (ICN Flow, Costa Mesa, CA). T cells were removed from the entire cell suspension by rosetting with neuroaminidase (Sigma)-treated sheep red blood cells. Additional enrichment of B cells was obtained by removing residual CD3⁺, CD16⁺, and CD56⁺ lymphocytes using high gradient magnetic separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany), as described previously (16). Briefly, 10 \times 10⁶ cells obtained as above were incubated for 30 min at 4 °C in 80 μ l of PBS with purified azide-free CD3 (OKT3, Ortho Pharmaceuticals, Raritan, NJ), CD16 (Leu-11c, BD Biosciences) and CD56 (Leu-19) monoclonal antibodies. After two washes with PBS supplemented with 0.5% bovine serum albumin, 20 μ l of colloidal superparamagnetic microbeads conjugated with goat-anti-mouse-IgG antibodies were added. The CD3⁺, CD16⁺, and CD56⁺ cells rosetting with microbeads were then isolated and removed applying a magnetic system to the outer wall of the columns. Following this multistep negative selection procedure, more than 98% of the resulting cell population was CD19⁺ and BCL-2⁺ with high density expression of BCL-2 as defined by mean fluorescence intensities, which were comparable in all cells. The expression of BCL-2 was detected using flow cytometric analysis with fluorescein isothiocyanate-labeled mouse anti-hBcl-2 monoclonal antibody (Clone 124, Dako, Glostrup, Denmark). Cells were fixed and permeabilized using Fix and Perm kit (Caltag) for 15 min at room temperature and then stained with anti-BCL-2 antibody for 30 min. A fluorescein isothiocyanate-labeled mouse IgG1 monoclonal antibody was used as a negative control.

Fluorescence Microscopy—One-hundred thousand cells were seeded onto 24-mm diameter round glass coverslips in 6-well plates and grown for 1 day. The coverslips were then transferred onto the stage of a Zeiss Axiovert 100TV inverted microscope equipped with a HBO mercury lamp (100 watts), and epifluorescence was detected with a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments). Cells were incubated in Hanks' balanced salt solution without bicarbonate and phenol red and allowed to equilibrate with 20 nM TMRM in the presence of 1.6 μ M CsH or 1 μ M CsA for 30 min at 37 °C prior to further additions. Fluorescence images were acquired with a 560-nm dichroic mirror using a 40 \times /1.3 oil immersion objective (Zeiss), with excitation at 546 \pm 5 nm and emission at 580 \pm 15 nm. Exposure time was 80 ms, and data were acquired and analyzed with the MetaMorph Metafluor Imaging Software. Clusters of several mitochondria were identified as regions of interest, whereas background was taken from fields not containing cells. Sequential digital images were acquired every 2 min for 60 min, and the average fluorescence intensity of all the regions of interest and of the background was recorded and stored for subsequent analysis. Mitochondrial fluorescence intensities minus background were normalized to the initial fluorescence for comparative purposes.

Cell Viability—The number of viable cells was assessed based on the Resazurin assay as described (17, 18). Briefly, cells were grown in 96-well microtiter plates (20 \times 10³ cells/well) in their medium (0.2 ml/well) for 1 day. Cells grown on each well were then treated for 16 h as described in the legend to Fig. 5 in the Forma incubator. After treatment the medium was replaced with fresh medium supplemented with 10% (v/v) Resazurin for 3 h. The ratio of oxidized to reduced Resazurin (which reflects the metabolic activity of viable cells) was detected at 540/620 nm with a microplate reader (Spectracount™ Packard). We verified that

The Mitochondrial Effects of BCL-2 Ligands

the ratio increased linearly with the number of cells in the range used in the experiments.

Immunoprecipitation—The day before the experiment, 1×10^6 HeLa BCL-2 cells were plated onto 100-mm diameter tissue culture dishes in the appropriate growth medium and incubated at 37 °C until a confluence of about 80–90% was reached. Cells were then transiently transfected with 4 μ g of the eukaryotic expression vector pcDNA3 containing the BAX cDNA sequence fused to a HA tag sequence (a generous gift of Atan Gross, Weizmann Institute of Science, Rehovot, Israel) or with 4 μ g of pcDNA3 alone, using the LipofectamineTM reagent (Invitrogen) and following the protocol described in the product technical sheet. Exactly 46 h after the start of transfection, the medium was removed, and the cells were incubated for 6 h with fresh medium containing EM20-25 at the concentrations indicated in the legend to Fig. 8 or the same volume of Me₂SO. Cells were then harvested, sedimented, washed once with ice-cold PBS, resuspended in 1 ml of ice-cold lysis buffer, incubated 30 min on ice, and finally Dounce-homogenized. The homogenate was sedimented at full speed in a microcentrifuge for 10 min at 4 °C to remove cell debris and nuclei. The supernatant, corresponding to the soluble cellular extract, was transferred to a clean tube and the protein concentration determined by the Bradford assay (Bio-Rad). Equal protein amounts (450 μ g in a final volume of 1 ml) were incubated overnight at 4 °C on a rocker platform with 50 μ l of an anti-HA affinity matrix (Roche Applied Science) previously equilibrated with lysis buffer. The matrix was then sedimented at full speed in a microcentrifuge for 10 s and the supernatant (corresponding to the flow-through) carefully removed and stored at –20 °C. The matrix was washed with (i) 1 ml of ice-cold lysis buffer, (ii) 1 ml of ice-cold buffer containing 500 mM NaCl and 0.1% Nonidet P-40 and otherwise identical to the lysis buffer, and (iii) 1 ml of ice-cold buffer containing 0.1% Nonidet P-40 and otherwise identical to the lysis buffer except that it did not contain NaCl. The matrix was carefully pelleted and the supernatant removed at each wash step. The matrix was finally resuspended in 40 μ l of Laemmli gel sample buffer containing 5% 2-mercaptoethanol, boiled for 5 min, and pelleted again. The supernatants were transferred to clean tubes and subjected to Western blotting analysis using a mouse anti-human BCL-2 antibody, as described below. The same membrane was then washed, stripped as described below, and probed with a rat monoclonal anti-HA antibody (clone 3F10, Roche Applied Science).

Detection of Caspase-9 Cleavage—The flow-through fractions from the anti-HA affinity matrix incubation were precipitated with 4 volumes of ice-cold acetone for 10 min followed by centrifugation at full speed in a microcentrifuge for 15 min at 4 °C. The acetone was carefully removed and the pellets were air-dried at room temperature, resuspended in 40 μ l of Laemmli gel sample buffer containing 5% 2-mercaptoethanol, and boiled for 5 min. SDS-PAGE and Western blotting were performed as described below. Both full-length and cleaved caspase-9 were detected by using a rabbit polyclonal anti caspase-9 antibody (Santa Cruz Biotechnology, Inc.).

Small Interfering RNA (siRNA) Transfection—The human Bcl-2 siRNA sequence was designed as 5'-GCUGCACCUGACGCCCU-Uctg-3', and the corresponding annealed oligonucleotide was purchased from Ambion, Inc. (Austin, TX). A validated, non-targeting siRNA (negative control comprised of a 19-bp scrambled sequence with no significant homology to any known human gene sequences) was also purchased from Ambion, Inc. Transient transfection of siRNA was carried out using LipofectamineTM reagent (Invitrogen) according to the manufacturer's protocol. One day before transfection with siRNA, HeLa BCL-2 cells were plated on 12-well plates and grown in the appropriate medium supplemented with 10% serum and without antibiotics

until a confluence of about 30–50% was reached. Cells were transfected with 100 pmol of Bcl-2 siRNA or 25 pmol of negative control RNA per well in serum-free medium for 4 h and then cultured in the presence of serum (10%) and without antibiotics at 37 °C until they were ready to be assayed for cell viability or Western blotting analysis (40 h after RNA addition). The number of viable cells after RNA addition was assessed based on the Resazurin assay, as described previously. For Western blotting analysis, cells were harvested, sedimented, washed once with ice-cold PBS, resuspended in 1 ml of ice-cold lysis buffer, incubated 30 min on ice, and finally Dounce-homogenized. The homogenates were sedimented at full speed in a microcentrifuge for 10 min at 4 °C to remove cell debris and nuclei. The supernatants, corresponding to the soluble cellular extracts, were transferred to clean tubes and the protein concentration was determined by the Bradford assay. Equal protein amounts (100 μ g) were solubilized in Laemmli gel sample buffer containing 5% 2-mercaptoethanol, separated electrophoretically by SDS-PAGE, and subjected to Western blotting analysis, as described below, using a mouse anti-human BCL-2 antibody. The same membrane was then washed, stripped as described in the following paragraph, and probed with a mouse monoclonal antibody against rabbit skeletal muscle GAPDH (clone 6C5, Chemicon International, Inc.).

SDS-PAGE and Western Blotting—The proteins from each solubilized sample, obtained as described in the preceding paragraphs, were separated electrophoretically in SDS-polyacrylamide 1.5-mm-thick minigels (12% acrylamide-0.4% bisacrylamide) and electroblotted onto nitrocellulose membranes. For immunoblotting analysis, the membrane was blocked in PBS containing 0.05% Tween 20 and 5% nonfat milk (blocking buffer) and incubated with the proper antibody for 2 h (anti-BCL-2 and anti-HA antibodies) or overnight (anti-BCL-XL, anti-GAPDH, and anti-caspase-9 antibodies). The membrane was then washed with PBS containing 0.05% Tween 20 and incubated with blocking buffer containing horseradish peroxidase-conjugated goat anti-mouse, anti-rat, or anti-rabbit IgG (1:5,000 dilution) for 1 additional hour. After further washing in PBS containing 0.05% Tween 20, labeled proteins were visualized with an ECL Western blotting detection kit (Bio-Rad). Membrane stripping for sequential blotting was carried out using the *Re-Blot* Plus Western blot recycling kit (Chemicon International, Inc.) according to the manufacturer's protocol.

Reagents—TMRM was purchased from Molecular Probes (Eugene, OR); CsA was purchased from Fluka Riedel-de Haen. BH3I-2' and Chelerythrine were from Calbiochem and Sigma, respectively, while HA14-1 and EM20-25 were supplied from Hoffmann-La Roche (Basel, Switzerland). The antibody against caspase-9 was from Santa Cruz Biotechnology, Inc., and it recognized both the uncleaved and cleaved forms of caspase; the secondary peroxidase-conjugated antibodies were from Southern Biotechnology, and the peroxidase detection kit was from Pierce. All other chemicals and tissue culture reagents were purchased from Sigma and were of the highest available grade.

RESULTS

We tested the effects of BH3I-2', Chelerythrine, and HA14-1 on the respiration of isolated rat liver mitochondria. All compounds displayed a biphasic effect, with uncoupling at lower concentrations and respiratory inhibition as the concentration was raised further (Fig. 1). The most effective was BH3I-2' (half-maximal activity at about 80 nM, which is equivalent to the uncoupling activity of the most potent protonophore, FCCP). Chelerythrine had an intermediate potency (half-maximal uncoupling at about 2 μ M), followed by HA14-1 (half-maximal uncoupling at about 20 μ M).

We next investigated whether these compounds affect the PTP with

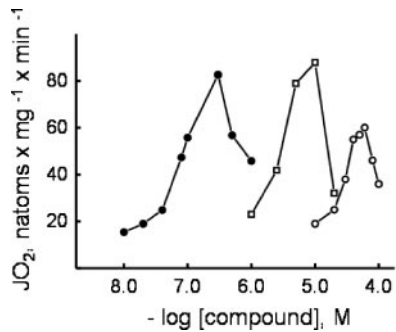


FIGURE 1. Effects of BH3I-2', Chelerythrine, and HA14-1 on respiration in isolated rat liver mitochondria. The incubation medium contained 250 mM sucrose, 10 mM Tris-Mops, 5 mM glutamate-Tris, 2.5 mM malate-Tris, 1 mM P_i -Tris, 20 μ M EGTA-Tris, 0.8 μ M CsA. The final volume was 2 ml at pH 7.4 and 25 °C. The experiments were started by the addition of 0.5 mg \times ml⁻¹ of rat liver mitochondria. After 1 min 30 μ M Ca^{2+} was added, followed after 1 further min by the indicated concentrations of each compound. Values on the ordinate refer to the rate of oxygen consumption observed in the presence of the indicated concentrations of BH3I-2' (closed circles), Chelerythrine (open squares), or HA14-1 (open circles).

a sensitive technique that we introduced in 1993 (19), which is illustrated for HA14-1 (Fig. 2A). In these protocols mitochondria are first loaded with a small Ca^{2+} pulse that is not sufficient to open the PTP *per se*. Under these conditions, the addition of a low concentration of FCCP (40 nM in this experiment) was not sufficient to trigger opening of the voltage-dependent PTP because the threshold potential for PTP opening was not reached (Fig. 2, trace a). Yet, increasing concentrations of HA14-1 in the range between 1 and 10 μ M caused a marked effect on PTP opening by 40 nM FCCP, which now occurred in increasing fractions of the mitochondria (traces b–f). The effect was due to PTP opening because it was fully inhibited by CsA (trace g). It should be stressed that 10 μ M HA14-1 did not depolarize mitochondria nor did it potentiate the depolarizing effects of concentrations of FCCP between 10 and 150 nM (results not shown). Of note, all BCL-2 ligands displayed a prominent PTP-sensitizing effect at concentrations that did not affect mitochondrial respiration (Fig. 2B).

To test whether these complex effects of BCL-2 ligands could also be observed in intact cells, we preliminarily studied the pattern of expression of BCL-XL and BCL-2 in three cell lines. Western blot analysis (Fig. 3) revealed that PC3 cells (lane 1) expressed high levels of BCL-XL but no detectable BCL-2, while HeLa Neo (lane 2) and HeLa BCL-2 cells (lane 3) expressed comparable levels of BCL-XL, BCL-2 being expectedly up-regulated in the latter cell type only.

We then incubated human prostate cancer PC3 cells with TMRM, which accumulates inside energized mitochondria. Mitochondrial depolarization causes probe release, which can be detected as the decrease of mitochondrial fluorescence with sensitive imaging techniques (20). The experiments of Fig. 4 document that the addition of 10 nM BH3I-2' (A), 1 μ M Chelerythrine (B), or 5 μ M HA14-1 (C) caused a fluorescence decrease (A–C, closed symbols) that is consistent with *in situ* mitochondrial depolarization, as demonstrated by the similar effect elicited by the protonophore FCCP (all panels). As expected of a PTP-dependent event, the TMRM fluorescence decrease elicited by the BCL-2 ligands was effectively prevented by CsA, which desensitizes the PTP (A–C, open symbols). As the concentration was increased to 30 nM BH3I-2' (A'), 10 μ M Chelerythrine (B'), and 30 μ M HA14-1 (C') mitochondrial depolarization was faster (A'–C', closed symbols) and could no longer be inhibited by CsA (A'–C', open symbols).

We then tested the effects of BCL-2 ligands on the survival of PC3, HeLa Neo, and HeLa BCL-2 cells (Fig. 5). BCL-2-overexpressing cells (open squares in all panels) were significantly protected from the killing effects of staurosporine (A) as compared with Neo-transfected cells

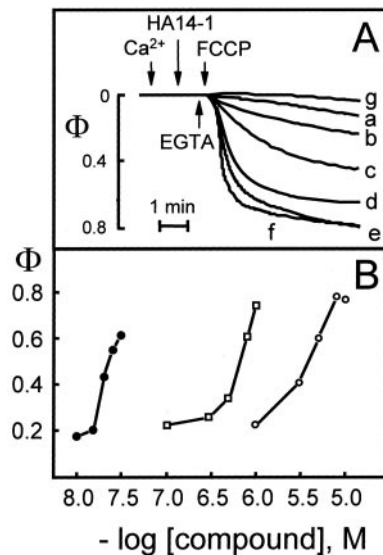


FIGURE 2. Sensitization of PTP opening by BH3I-2', Chelerythrine, and HA14-1. A, the incubation medium was the same as in Fig. 1, except that CsA was omitted. The final volume was 2 ml at pH 7.4 and 25 °C. The experiments were started by the addition of 0.5 mg \times ml⁻¹ of mitochondria. Where indicated 30 μ M Ca^{2+} , HA14-1 (traces b–g), 0.5 mM EGTA-Tris, and 40 nM FCCP were added. The concentration of HA14-1 was 1 μ M (trace b), 3 μ M (trace c), 5 μ M (trace d), 8 μ M (trace e), or 10 μ M (traces f and g). No HA14-1 was added in the experiment of trace a, and the incubation medium was supplemented with 0.8 μ M CsA in the experiment of trace g. B, fraction (Φ) of mitochondria that has undergone the permeability transition as a function of the concentration of BH3I-2' (closed circles), Chelerythrine (open squares), or HA14-1 (open circles) in protocols identical to those illustrated in A for HA14-1. Values on the ordinate were calculated from the changes of absorbance at 540 nm, which is a linear function of the number of permeabilized mitochondria (19).

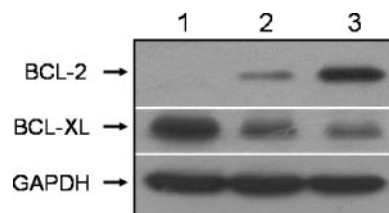


FIGURE 3. Pattern of BCL-2, BCL-XL, and GAPDH expression in PC3, HeLa Neo, and HeLa BCL-2 cells. PC3 (lane 1), HeLa Neo (lane 2), and HeLa BCL-2 (lane 3) cells were washed in serum-free medium, extracted with Laemmli gel sample buffer, separated by SDS-PAGE, and probed for expression of BCL-2, BCL-XL, and GAPDH by Western blotting as described under "Experimental Procedures." One-hundred micrograms of protein were loaded in each lane.

(closed squares in all panels). In striking contrast, HeLa BCL-2 cells, as well as PC3 cells, were as sensitive as HeLa Neo cells to the toxic effects of BH3I-2' (B), Chelerythrine (C), and HA14-1 (D). These results suggest that the cytotoxicity of BCL-2 ligands may be largely independent of expression of BCL-2 and BCL-XL and that it may rather be mediated by the effects of these compounds on respiration.

To assess whether the toxic mitochondrial effects were inevitably linked to the BCL-2 binding activity, we screened a series of HA14-1 analogs from the Hoffmann-La Roche chemical library. Fig. 6 reports the structure of one such compound, whose synthesis has been reported in the literature (21) and which we named EM20-25, together with that of HA14-1. EM20-25 did not cause uncoupling, and it only slightly inhibited uncoupled respiration at concentrations above 30 μ M (Fig. 7A), but it did cause sensitization of the PTP at concentrations higher than 2 μ M (Fig. 7B).

To address the key issue of whether EM20-25 interacts with BCL-2, HeLa BCL-2 cells were transiently transfected with HA-BAX or empty vector, and transfected cells were then treated with 1 mM EM20-25 or vehicle (Me₂SO) for 6 h. After detergent extraction, equal amounts of

The Mitochondrial Effects of BCL-2 Ligands

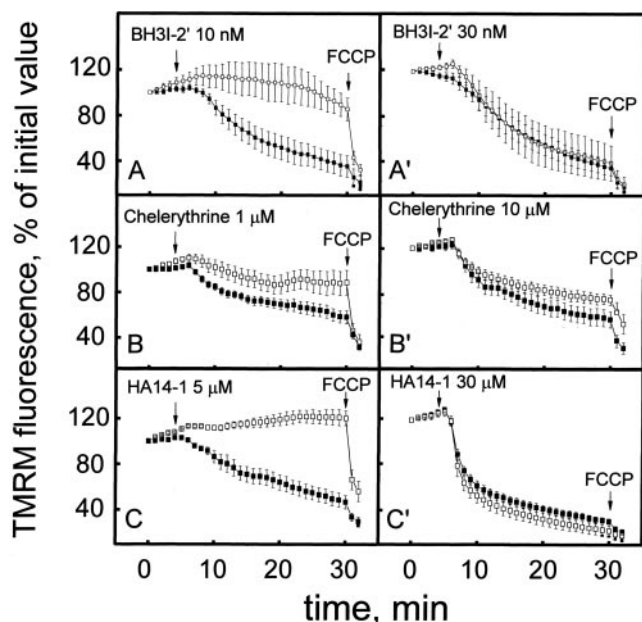


FIGURE 4. Effects of BH3I-2', Chelerythrine, and HA14-1 on mitochondrial membrane potential in intact cells. One-hundred thousand PC3 cells were loaded for 30 min at 37 °C with 20 nM TMRM and 1.6 μM CSH (closed squares) or 0.8 μM CsA (open squares). Where indicated, the indicated concentrations of BH3I-2' (A and A'), Chelerythrine (B and B'), or HA14-1 (C and C') were added, followed by 2 μM FCCP (all panels). For the analysis clusters of several mitochondria were identified as regions of interest, and background was taken from fields not containing cells. Sequential digital images were acquired every 2 min for 1 h, and the average fluorescence intensity of all the regions of interest and of the background was recorded and stored for subsequent analysis. Values on the ordinate report mitochondrial fluorescence intensities minus background after normalization to the initial fluorescence for comparative purposes.

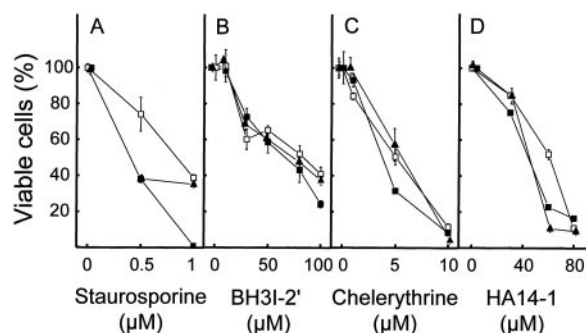


FIGURE 5. Effects of staurosporine, BH3I-2', Chelerythrine, and HA14-1 on the viability of PC3, HeLa Neo and HeLa BCL-2 cells. Twenty-thousand PC3 (closed triangles), HeLa Neo (closed squares), or HeLa BCL-2 (open squares) cells grown on 96-well plates were treated for 16 h with the indicated concentrations of staurosporine (A), BH3I-2' (B), Chelerythrine (C), or HA14-1 (D). Viable cells were estimated with the Resazurin assay.

protein were immunoprecipitated using an anti-HA affinity matrix, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antibody against BCL-2. One millimolar EM20-25 displaced BCL-2 (Fig. 8, compare lane 3 with lane 2 in the upper part of A; note that the signal arising from immunoglobulin light chains leaching from the affinity matrix, lane 1, overlaps with the BCL-2 signal), while the same amount of BAX could be detected in the immunoprecipitates (middle part of A). Consistent with these results, EM20-25 activated caspase-9 (lower part of A) and sensitized HeLa BCL-2 cells toward staurosporine (panel C, compare hatched with open bars) in the same range of concentrations that displaced BCL-2 from BAX (B).

It has been shown that BH3I-2' is a substrate of the multidrug resistance P-glycoprotein (22). To assess whether EM20-25 was transported by the same system, we tested the effects of CSH, which inhibits the pump but not the PTP (23). CSH did not modify the cytotoxic concen-

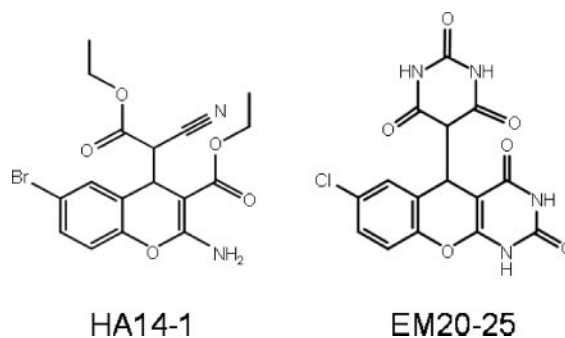


FIGURE 6. Structure of HA14-1 and EM20-25.

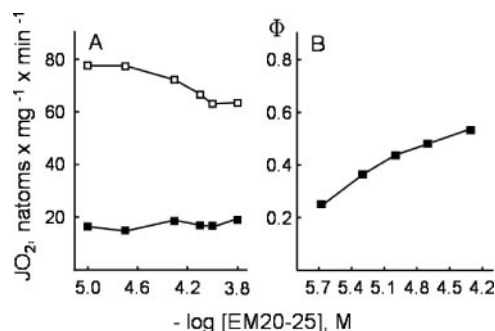


FIGURE 7. Effects of EM20-25 on respiration and volume in isolated rat liver mitochondria. A, the experimental conditions were exactly as described for Fig. 1. One min after the addition of mitochondria the indicated concentrations of EM20-25 were added, followed by 0.2 μM FCCP after a further 3 min. Values on the ordinate refer to the rate of respiration before (closed symbols) or after (open symbols) the addition of FCCP. B, the incubation medium was the same as in Fig. 2, except that CsA was omitted. The final volume was 2 ml at pH 7.4 and 25 °C. The experiments were started by the addition of 0.5 $\text{mg} \times \text{ml}^{-1}$ of mitochondria followed by 30 μM Ca^{2+} . One min later, the indicated concentrations of EM20-25 were added, followed by 0.5 mM EGTA-Tris and 40 nM FCCP after one further min. Values on the ordinate refer to the fraction (Φ) of mitochondria that had undergone the permeability transition as measured from the absorbance changes at 540 nm.

tration of EM20-25 in PC3 cells (Fig. 9A), while it considerably decreased the cytotoxic concentration of EM20-25 both in HeLa Neo and HeLa BCL-2 cells (Fig. 9, B and C, respectively).

We next assessed whether the cytotoxic effects of EM20-25 involved the PTP by studying the mitochondrial membrane potential *in situ* (Fig. 10). Cells were loaded with TMRM and analyzed with the same technique described in Fig. 4. Depending on the cell type, EM20-25 depolarized mitochondria at concentrations between 0.4 and 3 mM (open symbols), and this effect was sensitive to pretreatment with CsA (closed symbols). It should be mentioned that despite the clear PTP-inducing ability of EM20-25 cell death could not be prevented by CsA (results not shown), a finding that will be further addressed under "Discussion."

In an attempt to resolve the question of whether EM20-25 had cytotoxic effects independent of binding to BCL-2, we successfully suppressed BCL-2 expression in HeLa BCL-2 cells by siRNA interference. Treatment with BCL-2 siRNA was able to kill all cells surviving toxicity by the transfection technique itself (about 40% of the total), a finding that prevented further investigation of this problem (results not shown).

We finally tested whether EM20-25 affected the response of BCL-2-expressing leukemic B-CLL cells from untreated patients to staurosporine, chlorambucil, and fludarabine. A measurable cytotoxic effect of EM20-25 could be observed at concentrations between 20 and 40 μM (Fig. 11). Most remarkably, these concentrations of EM20-25 potentiated the killing effects of staurosporine (A), chlorambucil (B), and fludarabine (C).

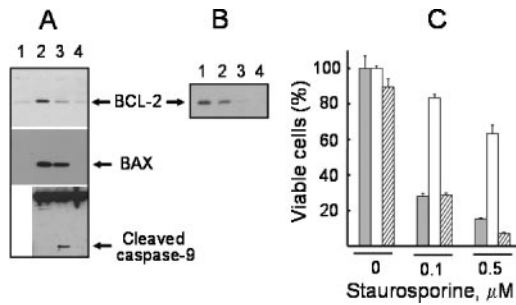


FIGURE 8. Effects of EM20-25 on the BCL-2/BAX interactions and on staurosporine-induced cell death in HeLa cells. *A*, HeLa cells overexpressing BCL-2 were transiently transfected with HA-BAX (lanes 2 and 3) or empty vector (lane 4). Transfected cells were then treated with 1 mM EM20-25 (lane 3) or vehicle (Me₂SO, lanes 2 and 4) for 6 h. Cells were extracted with detergent, and equal amounts of protein extracts were immunoprecipitated using an anti-HA affinity matrix, separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibody against BCL-2 (upper part of *A*). In lane 1 the supernatant obtained from an affinity matrix not incubated with protein extract was loaded. The membrane was then stripped and re-probed with antibody against HA (middle part of *A*). Note that the signal arising from immunoglobulin light chains leaching from the affinity matrix (lane 1) overlaps with the BCL-2 signal. In the lower part of *A* proteins not bound to the HA immunoaffinity matrix were separated by SDS-PAGE and immunoblotted with an anti-caspase-9 antibody, and the position of cleaved caspase-9 is indicated (arrow). *B*, HeLa cells stably overexpressing BCL-2 were transiently transfected with HA-BAX (lanes 1–3) or with empty vector (lane 4). Cells were then treated with vehicle (Me₂SO, lanes 1 and 4) or with 0.5 mM (lane 2) or 1 mM (lane 3) EM20-25 for 6 h. Cells were extracted with detergent, and equal amounts of protein extracts were immunoprecipitated with anti-HA affinity matrix, separated by SDS-PAGE, and immunoblotted with antibody against BCL-2. *C*, HeLa cells stably overexpressing BCL-2 (open and hatched bars) or HeLa cells containing the empty vector (gray bars) were seeded in 96-well plates (20×10^3 cells per well⁻¹) and treated for 16 h at 37 °C in a CO₂ incubator with the indicated concentrations of staurosporine in the absence (gray and open bars) or presence (hatched bars) of 0.5 mM EM20-25. Viable cells were then assessed with the Resazurin test.

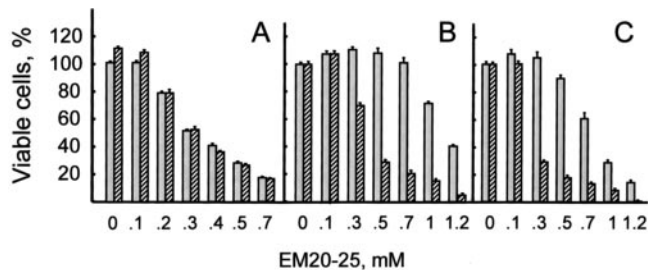


FIGURE 9. Effects of CsH on the cytotoxicity of EM20-25. PC3 cells (*A*), HeLa cells containing the empty vector (*B*), or HeLa cells stably overexpressing BCL-2 (*C*) were seeded in 96-well plates (20×10^3 cells per well⁻¹) and treated for 16 h at 37 °C in a CO₂ incubator with the indicated concentrations of EM20-25 in the absence (gray bars) or presence (hatched bars) of 1.6 μM CsH. Viable cells were then assessed with the Resazurin test.

DISCUSSION

In this manuscript we have characterized the complex mitochondrial effects of BH3I-2', Chelerythrine, and HA14-1, small organic molecules that share the ability to bind the BH3 domain of BCL-2. We have shown that these compounds also share a series of mitochondrial effects that can be summarized as follows: (i) sensitization of the PTP to opening at concentrations that do not interfere with energy coupling and (ii) uncoupling of mitochondrial respiration, which is then superseded by inhibition as the drug concentration is increased further. Analysis of structural analogs of HA14-1 from the Hoffmann-La Roche chemical library has then allowed to identify EM20-25 as a molecule devoid of toxic side effects on respiration but possessing the ability (i) to sensitize PTP opening in isolated mitochondria and intact cells, (ii) to displace BAX from BCL-2 in HeLa cells, with activation of caspase-9, (iii) to sensitize BCL-2 overexpressing cells to treatment with staurosporine, and of B-CLL cells from patients to treatment with staurosporine, fludarabine, and chlorambucil. Our results on BH3I-2', Chelerythrine, and

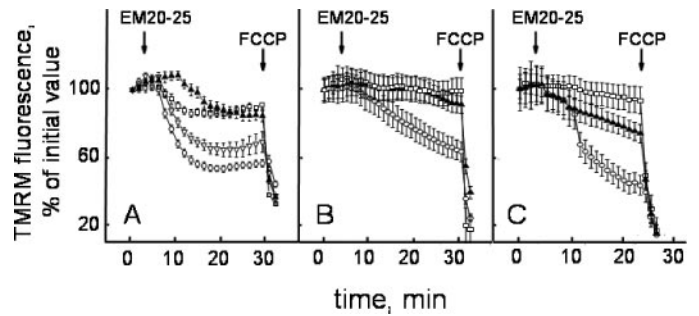


FIGURE 10. Effects of EM20-25 on mitochondrial membrane potential *in situ*. Approximately 100,000 PC3 cells (*A*), HeLa cells containing the empty vector (*B*), or HeLa cells stably overexpressing BCL-2 (*C*) were loaded for 30 min at 37 °C with 20 nM TMRM and 1.6 μM CsH (open symbols) or 0.8 μM CsA (closed triangles). Additions of EM20-25 were as follows: *A*, 0.4 mM (open squares), 0.6 mM (open triangles), or 1 mM (open circles and closed triangles); *B* and *C*, 2 mM (open squares) or 3 mM (open circles and closed triangles) followed in all panels by 2 μM FCCP. The addition of vehicle alone did not cause fluorescence changes (omitted for clarity). For the analysis clusters of several mitochondria were identified as regions of interest. Background was taken from fields not containing cells. Sequential digital images were acquired every 2 min for 1 h, and the average fluorescence intensity of all the regions of interest and of the background was recorded and stored for subsequent analysis. Values on the ordinate refer to mitochondrial fluorescence intensities minus background after normalization of the initial fluorescence for comparative purposes.

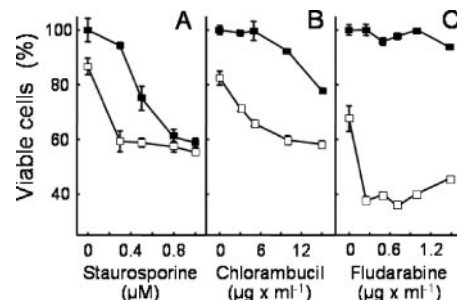


FIGURE 11. Effects of EM20-25 on lymphoma cell killing by staurosporine, chlorambucil, and fludarabine. B-CLL cells were treated with the indicated concentration of drugs in the absence (closed symbols) or presence (open symbols) of 30 μM (*A*), 40 μM (*B*), or 20 μM (*C*) EM20-25, and viability was assessed 20 h later by the Resazurin assay. Cells in each panel were from a different patient, and each condition was tested in quadruplicate samples.

HA14-1 provide a novel reading frame for previous studies where the cytotoxic effects of these drugs have been addressed.

Independent studies from two laboratories have shown that treatment with HA14-1 and/or BH3I-2' depolarized mitochondria *in situ* (22, 24, 25) and increased mitochondrial respiration, an effect that sensitized to cell death by tumor necrosis factor-related apoptosis-inducing ligand the human T lymphoblastic leukemia cell CEM (25). A similar effect was observed with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone, which by itself was devoid of apoptosis-inducing properties (25). The results of An *et al.* (24) are entirely consistent with our finding that at low concentrations HA14-1 sensitizes the mitochondrial proapoptotic pathway, while at higher concentrations the direct effects on mitochondria predominate. Indeed, low concentrations of HA14-1 caused activation of caspase-9 and -3 and DNA fragmentation, while higher concentrations of the drug caused caspase-independent cell death. In the study of Hao *et al.* (25) caspases were apparently not involved in sensitization to tumor necrosis factor-related apoptosis-inducing ligand by HA14-1 and BH3I-2', consistent with a direct effect of these drugs on mitochondrial energy coupling in that model system.

It must be stressed that the effective concentrations cannot be readily compared in different studies and in different cell lines because the drugs are substrates of the multidrug resistance P-glycoprotein, as shown for BH3I-2' (22) and EM20-25 in HeLa cells (Fig. 9). These obser-

The Mitochondrial Effects of BCL-2 Ligands

vations probably also account for our observation that B-CLL cells from untreated leukemic patients were much more sensitive to the proapoptotic effects of EM20-25 than HeLa cells. The study of Feng *et al.* (22) also demonstrated that the proapoptotic effects of BH3I-2' vary widely depending on the cell type, yet mitochondrial depolarization could always be observed, and this event could be counteracted by both BCL-2 and BCL-XL. These findings easily explain the depolarizing effects of EM20-25 in mitochondria of PC3 cells, which express large amounts of BCL-XL (Fig. 3).

A new entry among the ligands of BCL-2 family members is Chelerythrine, a natural product of plants that has been identified through high throughput screening of disruptors of the interaction of BCL-XL with a fluoresceinated peptide modeled on the BCL-2 homology 3 domain of BAK (9). Remarkably, a dual effect of Chelerythrine can be easily deduced from the results of Chan *et al.* (9). Indeed, overexpression of BCL-XL protected from the cytotoxic effects of low concentrations of Chelerythrine; and the protective effects could only be overcome by high drug concentrations (9) that according to our studies are more likely to affect mitochondrial energy conservation directly.

It is tempting to speculate that the effects of BH3I-2', Chelerythrine, HA14-1, and EM20-25 on the PTP are related to BCL-2 binding. BCL-2 overexpression does desensitize the PTP to a variety of stimuli when assessed in isolated mitochondria (11); and we observed onset of PTP desensitization with the same time course of mitochondrial BCL-2 up-regulation during hepatocarcinogenesis by 2-acetylaminofluorene, a condition that conferred resistance to hepatocyte apoptosis *in vivo* (26). On the other hand, the properties of the permeability transition are indistinguishable in mitochondria isolated from isogenic human colon cancer *bax*^{-/-} and *bax*^{+/+} HCT116 cell lines (27). On balance, we think that establishing whether the interactions of BH3I-2', Chelerythrine, HA14-1, and EM20-25 with BCL-2 play a mechanistic role in PTP regulation will require further work and will probably require characterization of the PTP at the molecular level.

Our results do not necessarily imply that apoptosis induction by EM20-25 can be exclusively explained by its PTP-inducing effects on mitochondria. Indeed, CsA did not prevent cell killing by EM20-25, suggesting that the latter compound may also influence BCL-2- and BCL-XL-dependent events at different cellular locations (*e.g.* the endoplasmic reticulum) or affect cell survival through additional mechanisms. However, it should also be noted that CsA is not a blocker of the PTP but rather a desensitizer whose inhibitory effects can be overcome by proper stimuli such as an increased Ca²⁺ concentration (28). Thus, the failure of CsA at inhibiting cell death is not in contrast with a role of PTP opening in triggering the process that will eventually lead to cell demise.

Irrespective of this issue, a clear point emerges from the present results, *i.e.* that the potentially toxic effects of BCL-2 ligands on respiration and energy coupling can be dissociated from the PTP-inducing effects. Indeed, EM20-25 possesses the ability to activate the mitochondrial proapoptotic pathway through the PTP without affecting energy coupling directly. It is also remarkable that EM20-25 displayed the unique property of sensitizing BCL-2-overexpressing cells to staurosporine and B-CLL cells from leukemic patients to staurosporine, chlorambucil, and fludarabine. These results represent a proof of principle that BCL-2 ligands with a selective effect on BCL-2-overexpressing

cells can be developed and used for the selective killing of apoptosis-resistant cells. Consistent with this prediction, after this manuscript was submitted a novel inhibitor of BCL-2 family proteins has been described, which induces regression of solid tumors in mice (29).

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REFERENCES

1. Sartorius, U. A., and Krammer, P. H. (2002) *Int. J. Cancer* **97**, 584–592
2. Reed, J. C. (2002) *Nat. Rev. Drug Discov.* **1**, 111–121
3. Letai, A., Sorcinelli, M. D., Beard, C., and Korsmeyer, S. J. (2004) *Cancer Cell* **6**, 241–249
4. Igney, F. H., and Krammer, P. H. (2002) *Nat. Rev. Cancer* **2**, 277–288
5. Adams, J. M., and Cory, S. (2001) *Trends Biochem. Sci.* **26**, 61–66
6. Wang, J.-L., Liu, D., Zhang, Z.-J., Shan, S., Han, X., Srinivasula, S. M., Croce, C. M., Alnemri, E. S., and Huang, Z. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 7124–7129
7. Tzung, S. P., Kim, K. M., Basanez, G., Giedt, C. D., Simon, J., Zimmerberg, J., Zhang, K. Y., and Hockenbery, D. M. (2001) *Nat. Cell Biol.* **3**, 183–191
8. Degterev, A., Lugovskoy, A., Cardone, M., Mulley, B., Wagner, G., Mitchison, T., and Yuan, J. (2001) *Nat. Cell Biol.* **3**, 173–182
9. Chan, S.-L., Lee, M. C., Tan, K. O., Yang, L. K., Lee, A. S. Y., Flotow, H., Fu, N. Y., Butler, M. S., Soejarto, D. D., Buss, A. D., and Yu, V. C. (2003) *J. Biol. Chem.* **278**, 20453–20456
10. Bernardi, P., Petronilli, V., Di Lisa, F., and Forte, M. (2001) *Trends Biochem. Sci.* **26**, 112–117
11. Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. (1996) *J. Exp. Med.* **184**, 1331–1341
12. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. L., Jones, D. P., and Wang, X. (1997) *Science* **275**, 1129–1132
13. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* **275**, 1132–1136
14. Costantini, P., Petronilli, V., Colonna, R., and Bernardi, P. (1995) *Toxicology* **99**, 77–88
15. Cerutti, A., Trentin, L., Zambello, R., Sancetta, R., Milani, A., Tassinari, C., Adami, F., Agostini, C., and Semenzato, G. (1996) *J. Immunol.* **157**, 1854–1862
16. Trentin, L., Zambello, R., Agostini, C., Enthammer, C., Cerutti, A., Adami, F., Zamboni, S., and Semenzato, G. (1994) *Blood* **84**, 4249–4256
17. O'Brien, J., Wilson, I., Orton, T., and Pognan, F. (2000) *Eur. J. Biochem.* **267**, 5421–5426
18. Gugliucci, A., Ranzato, L., Scorrano, L., Colonna, R., Petronilli, V., Cusan, C., Prato, M., Mancini, M., Pagano, F., and Bernardi, P. (2002) *J. Biol. Chem.* **277**, 31789–31795
19. Petronilli, V., Cola, C., Massari, S., Colonna, R., and Bernardi, P. (1993) *J. Biol. Chem.* **268**, 21939–21945
20. Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and Di Lisa, F. (1999) *Eur. J. Biochem.* **264**, 687–701
21. Figueroa-Villar, J. D., and Cruz, E. R. (1993) *Tetrahedron* **49**, 2855–2862
22. Feng, W. Y., Liu, F. T., Patwari, Y., Agrawal, S. G., Newland, A. C., and Jia, L. (2003) *Br. J. Haematol.* **121**, 332–340
23. Nicolli, A., Basso, E., Petronilli, V., Wenger, R. M., and Bernardi, P. (1996) *J. Biol. Chem.* **271**, 2185–2192
24. An, J., Chen, Y., and Huang, Z. (2004) *J. Biol. Chem.* **279**, 19133–19140
25. Hao, J. H., Yu, M., Liu, F. T., Newland, A. C., and Jia, L. (2004) *Cancer Res.* **64**, 3607–3616
26. Klöhn, P. C., Soriano, M. E., Irwin, W., Penzo, D., Scorrano, L., Bitsch, A., Neumann, H. G., and Bernardi, P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 10014–10019
27. De Marchi, U., Campello, S., Szabo, I., Tombola, F., Martinou, J. C., and Zoratti, M. (2004) *J. Biol. Chem.* **279**, 37415–37422
28. Soriano, M. E., Nicolosi, L., and Bernardi, P. (2004) *J. Biol. Chem.* **279**, 36803–36808
29. Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A. R., Letai, A., Li, C., Mitten, M. J., Nettesheim, D. G., Ng, S., Nimmer, P. M., O'Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B., Wendt, M. D., Zhang, H., Fesik, S. W., and Rosenberg, S. H. (2005) *Nature* **435**, 677–681