

## Inhibition of the Intrinsic Apoptosis Pathway Downstream of Caspase-9 Activation Causes Chemotherapy Resistance in Diffuse Large B-Cell Lymphoma

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**Abstract Purpose:** Inhibition of the apoptosis cascade is an important cause of therapy resistance in diffuse large B-cell lymphomas (DLBCL). In this study, we investigated possible mechanisms and expression levels of apoptosis-related genes in the apoptosis pathway that may be responsible for differences in chemotherapy sensitivity between DLBCL patients.

**Experimental Design:** Twenty-eight DLBCL patient samples were investigated for their expression levels of apoptosis-related genes using reverse transcription-multiplex ligation-dependent probe amplification analysis. Functional analysis of the intrinsic, caspase-9-mediated pathway was done using fluorescence-activated cell sorting analysis, Western blot analysis, and immunohistochemistry.

**Results:** Two DLBCL groups were identified: one with low expression levels of both proapoptotic and antiapoptotic genes and one group with high expression levels of these genes. DLBCL with high expression levels of proapoptotic and antiapoptotic genes frequently seemed to be refractory to clinical chemotherapy. Functional analysis in these latter DLBCL samples and DLBCL cell lines with comparable expression profiles revealed high levels of spontaneous caspase-9 activity without induction of apoptosis, indicating disruption of the apoptosis pathway downstream of caspase-9 activation. This disruption of the apoptosis pathway could be restored using a small-molecule XIAP antagonist.

**Conclusions:** We conclude that the intrinsic, caspase-9-mediated apoptosis pathway is constitutively activated in part of chemotherapy-refractory DLBCL with concomitant downstream inhibition of the convergence apoptosis pathway and that inhibition of XIAP might be an alternative therapy for chemotherapy-refractory DLBCL.

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma. Despite intensive chemotherapy, approximately 30% to 40% of the patients will die within 5 years after diagnosis. The addition of the anti-CD20 monoclonal antibody rituximab to standard chemotherapy has improved clinical outcome considerably in these patients and has recently become the standard form of treatment (1). Failure to achieve complete remission or the occurrence of an early relapse usually results in fatal outcome. The intrinsic sensitivity of lymphoma cells to chemotherapy is an important determinant of the patient's response to chemotherapy and clinical

outcome. Recently, *in vitro* studies have shown that the response to chemotherapy depends on activation of the apoptosis cascade (2–4). Two major apoptosis pathways have been identified: the intrinsic, caspase-9-mediated apoptosis pathway and the extrinsic, caspase-8-mediated apoptosis pathway. Most chemotherapeutic drugs used in the treatment of DLBCL primarily induce cell death via induction of the intrinsic apoptosis pathway (5–8). Previous studies have shown that inhibition of this apoptosis pathway is an important factor in the development of chemotherapy resistance in chemotherapy-refractory DLBCL (9–13).

The intrinsic and extrinsic apoptosis pathways converge on the downstream effector caspases: caspase-3, caspase-6, and caspase-7. Activation of these caspases results in the execution of apoptosis (14). Effector caspases can be suppressed by antiapoptotic proteins called inhibitor of apoptosis proteins (IAP). These proteins contain one or more copies of the baculoviral repeat domain and, in some cases, a zinc ring-finger domain (15). The human genome encodes eight IAP family members: XIAP, cIAP1, cIAP2, NIAP, survivin, MLIAP, ILP2, and apollon. Overexpression of these proteins has been shown to result in resistance to agents that trigger the intrinsic and extrinsic apoptosis pathways (16, 17).

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Using microarray expression analysis, we have recently shown that a subset of chemotherapy-refractory DLBCL is characterized by high expression levels of proapoptotic genes, particularly genes that are under transcriptional control of p53 and are involved in the intrinsic, caspase-9-mediated apoptosis pathway (18). These DLBCL samples were also characterized by high expression levels of apoptosis-inhibiting genes, providing an explanation of why expression of proapoptotic genes does not result in effective induction of apoptosis.

One of the limitations of microarray expression profiling of snap-frozen lymphoma tissues is that the tumor samples also contain tumor-infiltrating cells, including high numbers of nonneoplastic T lymphocytes. Infiltrating T cells express detectable levels of antiapoptotic genes, including *Bcl-2* (19) and *XIAP*,<sup>3</sup> and thus interfere with expression profiling of the lymphoma cells.

To obtain apoptosis expression profiles of only the neoplastic cell population, we determined expression levels of a previously validated panel of 36 apoptosis-related genes in isolated and purified neoplastic cells of DLBCL using a highly sensitive and quantitative reverse transcription-multiplex ligation-dependent probe amplification (RT-MLPA) analysis (20). Subsequently, we investigated whether the intrinsic apoptosis pathway is constitutively activated in DLBCL tumor cells that show high expression levels of proapoptotic genes.

Next, to understand the possible mechanisms that may underlie the differential chemotherapy sensitivity observed in the clinical samples, we investigated whether failure of chemotherapy to induce apoptosis is caused by downstream inhibition of the intrinsic apoptosis pathway in DLBCL cells and a panel of cell lines with apoptosis expression profiles that match with the lymphoma samples. Previously, we have shown that expression of *XIAP* is correlated with a poor clinical outcome in DLBCL (11). Schimmer et al. generated small antagonistic molecules that specifically interfere with the inhibitory function of *XIAP* (21–23). We used these antagonists to test whether neutralizing the effect of *XIAP* would result in restoring caspase-9-mediated apoptosis.

## Materials and Methods

**Lymphoma samples and cell lines.** DLBCL samples tested were obtained from patients diagnosed between 2000 and 2005 in the comprehensive cancer center in Amsterdam according to the WHO criteria (24). Twenty-eight DLBCL samples, including chemotherapy-refractory cases, were used for these studies. DLBCLs were considered responsive if the patient reached complete remission (according to standard clinical evaluation, including physical examination, chest X-ray, and computed tomography of chest, abdomen, and pelvis) without early relapse (within 2 years). All other cases were considered refractory (follow-up period, 9–43 months). As a control, tonsil germinal center B cells (GCB) and peripheral blood B cells from healthy donors were used. The institutional review board of the VU University Medical Center approved the study. Informed consent was provided according to the Declaration of Helsinki.

Lymphoma and tonsil cell suspensions were isolated from tissue biopsies by a combination of mechanical and enzymatic dissociation and frozen until further testing (25). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll density gradient centrifugation

and frozen until further testing. Cells were thawed 1 h before experimental testing and cultured in Iscove's modified Dulbecco's medium (BioWhittaker) supplemented with 10% FCS and 1% penicillin and streptomycin at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere. Nonneoplastic T cells were removed by using superparamagnetic dynabeads coated with anti-CD3 antibodies (Dyna). The purity of the resulting tumor cell fractions was determined by morphologic examination of the tumor cells in CD3-stained (clone CBC.37, DakoCytomation) and CD20-stained (clone L26, DakoCytomation) cytospin preparations. This procedure resulted in <5% contaminating, nonneoplastic CD20-negative cells in all cases tested. GCBs were fluorescence-activated cell sorting (FACS) sorted from tonsil B-cell suspensions using antibodies against the cell surface markers CD38 (clone HB7, BD Biosciences) and IgD (DakoCytomation; ref. 26).

The SUDHL4, SUDHL5, and HT DLBCL cell lines and the Nalm6 (originally obtained from the German Collection of Microorganisms and Cell Cultures) and CEM leukemia cell lines (originally obtained from the American Type Culture Collection) used in this study were cultured in RPMI 1640 (BioWhittaker) containing 10% FCS and 100 IU penicillin/100 µg/mL streptomycin.

**RT-MLPA analysis.** Total RNA of isolated lymphoma cells and cell lines was prepared using RNABee solution (Tel-Test, Inc.) according to the manufacturer's recommendations.<sup>4</sup> RT-MLPA was done on total RNA as described previously (20).

Data were analyzed using Genotype and GeneScan software (Applied Biosystems). The  $\beta$ -glucuronidase housekeeping gene was used as internal reference to minimize possible effects of unequal amounts of mRNA. The ratios were subsequently divided by the mean expression ratios for that particular gene for optimal comparison of the samples in all patients. To identify clusters of correlated genes, hierarchical cluster analysis using TIGR software<sup>5</sup> was used (27). Cluster analysis was done by average linkage analysis using Euclidian distance.

**Assessment of caspase-dependent cell death.** Induction of apoptosis in lymphoma cells and cell lines was determined as described previously and done in triplicate (25). Etoposide (Sigma) was used to assess the functional status of the intrinsic, caspase-9-mediated pathway. Briefly, cell lines were treated with 500 nmol/L etoposide for 4, 7, 16, and 24 h. Cell lines were preincubated with 25 µmol/L of the caspase-8 inhibitor LETD-fmk or the caspase-9 inhibitor LEHD-fmk (Alexis Biochemicals) 1 h before treatment. *XIAP* was inhibited using small-molecule *XIAP* antagonists. Lymphoma cells and cell lines were incubated with 10, 25, and 50 µmol/L of *XIAP* antagonist 1396-12 or the inactive control 1396-28 for 4 or 24 h (21–23).

Apoptosis was measured using a standard number of fluorescent beads (Fluorospheres, Becton Dickinson) in combination with 7-aminoactinomycin (ViaProbe, BD PharMingen) to determine the number of viable (7-aminoactinomycin negative) cells in each individual experiment. Fluorescence was detected by the FACSCalibur flow cytometer and analyzed using CellQuest software (both Becton Dickinson).

**Detection of caspase-9 and caspase-3/7 activity.** Caspase-9 activity was determined using a luminescent assay (Caspase-Glo 9 assay, Promega Benelux). Cells were lysed and incubated with the LEHD-containing substrate for 25 min at 37°C. Subsequently, luminescence was measured using a microplate luminometer (Victor2, Wallac, Perkin-Elmer). In addition, caspase-9 activity was measured in each individual cell using FACS analysis according to the manufacturer's instructions [CaspaTag, caspase-9 (LEHD) activity kit]. Briefly, cells (10<sup>6</sup> per mL) were incubated with 30× working dilution FAM-LEHD-fmk for 1 h at 37°C under 5% CO<sub>2</sub>. Cells were washed and fluorescence was measured on a FACSCalibur flow cytometer. The caspase-9 activity

<sup>4</sup> <http://www.mrc-holland.com>

<sup>5</sup> <http://www.tm4.org/mev.html>

<sup>3</sup> Cillessen et al., unpublished data.

was calculated as follows: (mean fluorescence intensity of FAM-LEHD-fmk-treated cells) / (mean fluorescence intensity of unstained cells).

Caspase-3/7 activity was determined using a fluorescent assay (Roche) according to the manufacturer's instructions. Cells were lysed and incubated with the DEVD-rhodamine 110 substrate for 1 h at 37°C. The amount of free rhodamine was measured with a 492-nm excitation filter and a 535-nm emission filter using a microplate fluorescence reader (TECAN Spectrafluor). Caspase activity was determined as caspase activity levels of treated samples minus caspase activity levels of untreated samples. For cell lines and lymphoma samples, determination of caspase activity levels was done in triplicate.

**Measurement of  $\Delta\psi_m$ .** Mitochondrial membrane depolarization was detected using the fluorescent probe tetramethylrhodamine ethyl ester perchlorate (Invitrogen) that accumulates in mitochondria. Briefly, cells ( $10^6$  per mL) were incubated with 25 nmol/L tetramethylrhodamine ethyl ester perchlorate at 37°C for 10 min in the dark and analyzed using FACS analysis. Mitochondrial membrane depolarization was observed as a shift to the left in the emission spectra.  $\Delta\psi$  was determined as the % unstained cells in etoposide-treated samples minus the % unstained cells in untreated samples.

To further investigate whether the mitochondrial potential is continuously decreased in active caspase-9-positive cells, we used the uncoupler SF6847 (tyrphostin A9, Sigma-Aldrich), which dissipates the mitochondrial membrane potential. Cells were incubated with 10  $\mu$ mol/L SF6847 for 15 min.

**Western blot analysis.** Total protein extracts were prepared from untreated cell lines using lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 0.5% NP40, 5 mmol/L EDTA] containing protease inhibitors. Antibodies against the following proteins were used: Bax (1:1,000; Dako), Bak (1:1,000; Cell Signaling Technology), AIF (1:1,000; Cell Signaling Technology), Puma (1:1,000; Cell Signaling Technology), MCL1 (1:1,000; Dako), and XIAP (clone 2F1, 1  $\mu$ g/mL; MBL). Proteins were visualized with the enhanced chemiluminescence technique (Amersham Pharmacia Biotech). Cellular  $\beta$ -actin (AB-1 kit, 6.25 ng/mL; Oncogene Research Products) was used as a loading control for each Western blot sample.

**Immunohistochemistry.** Expression of AIF, Bim, MCL1, and XIAP was investigated at the protein level in three high apoptosis expression profile cases and three low apoptosis expression profile cases as determined by RT-MLPA analysis. Sections (3  $\mu$ m thick) from paraffin-embedded biopsy specimens of DLBCL were stained with the following antibodies: Bim (NeoMarkers, Lab Vision Corp.), AIF, MCL1, and XIAP. The antibodies were incubated overnight. For detection of AIF, Bim, MCL1, and XIAP, a standard highly sensitive EnVision horseradish peroxidase system (DakoCytomation) was used. Antibodies were visualized with the chromogen 3,3'-diaminobenzidine.

**Statistical analysis.** The Mann-Whitney *U* test was used to compare means between groups. Qualitative variables were analyzed using the Pearson  $\chi^2$  test or the Fisher's exact test. Analyses were done using Statistical Package for the Social Sciences software (version 11.5; SPSS, Inc.). *P* values of <0.05 were considered significant.

## Results

**Chemotherapy-refractory DLBCLs frequently show high expression levels of proapoptotic and antiapoptotic genes.** We investigated the expression levels of 36 apoptosis-regulating genes (20) in isolated lymphoma cells of 28 DLBCL samples. Unsupervised cluster analysis revealed two groups of DLBCL: one with relatively low expression levels of both proapoptotic and antiapoptotic genes and one group of DLBCL with relatively high expression levels of proapoptotic genes in combination with high expression levels of antiapoptotic genes. High expression of apoptosis-regulating genes in this latter DLBCL group included proapoptotic members of the

Bcl-2 family (*Bax*, *Bak*, *Bad*, *Puma*, and *Bid*) and antiapoptotic genes of the downstream IAP family (*ciAP2*, *XIAP*, *NIAP*, and *apollon*) and *MCL1-long*, *c-Flip*, and *Pi-9*. When comparing the DLBCL groups for expression levels of each individual gene, it seemed that *survivin*, *Bcl-2*, *Bcl-x<sub>L</sub>*, *BMF*, and *Noxa* did not add significantly to the clustering of the lymphoma samples (Fig. 1A). Clinical data were available for 21 of the 28 patients; 6 of these 21 samples were relapsed DLBCL. In the remaining 15 patients with primary DLBCL, the clinical response to chemotherapy in cases with high expression of apoptosis-regulating genes was significantly less favorable than cases with low levels of apoptosis-regulating genes (*P* = 0.04). In addition, *XIAP* expression alone tended to correlate with an unfavorable outcome (*P* = 0.06). No significant correlation between the apoptosis profile and activated B cell (ABC)- or GCB-like phenotype was observed (Table 1). Using immunohistochemical analysis, we could confirm the mRNA expression levels with actual protein expression levels of Bim, XIAP, AIF, and MCL1 in the neoplastic cells of DLBCL samples (Fig. 1C).

Peripheral blood B cells from healthy donors also showed high expression levels of antiapoptotic genes. However, in contrast to DLBCL with the high apoptosis profile, blood B cells showed low expression levels of the proapoptotic genes, except for *Bim* and *Smac*. In DLBCL with a high apoptosis profile, higher expression levels of both proapoptotic and antiapoptotic genes were observed compared with the tonsil GCBs.

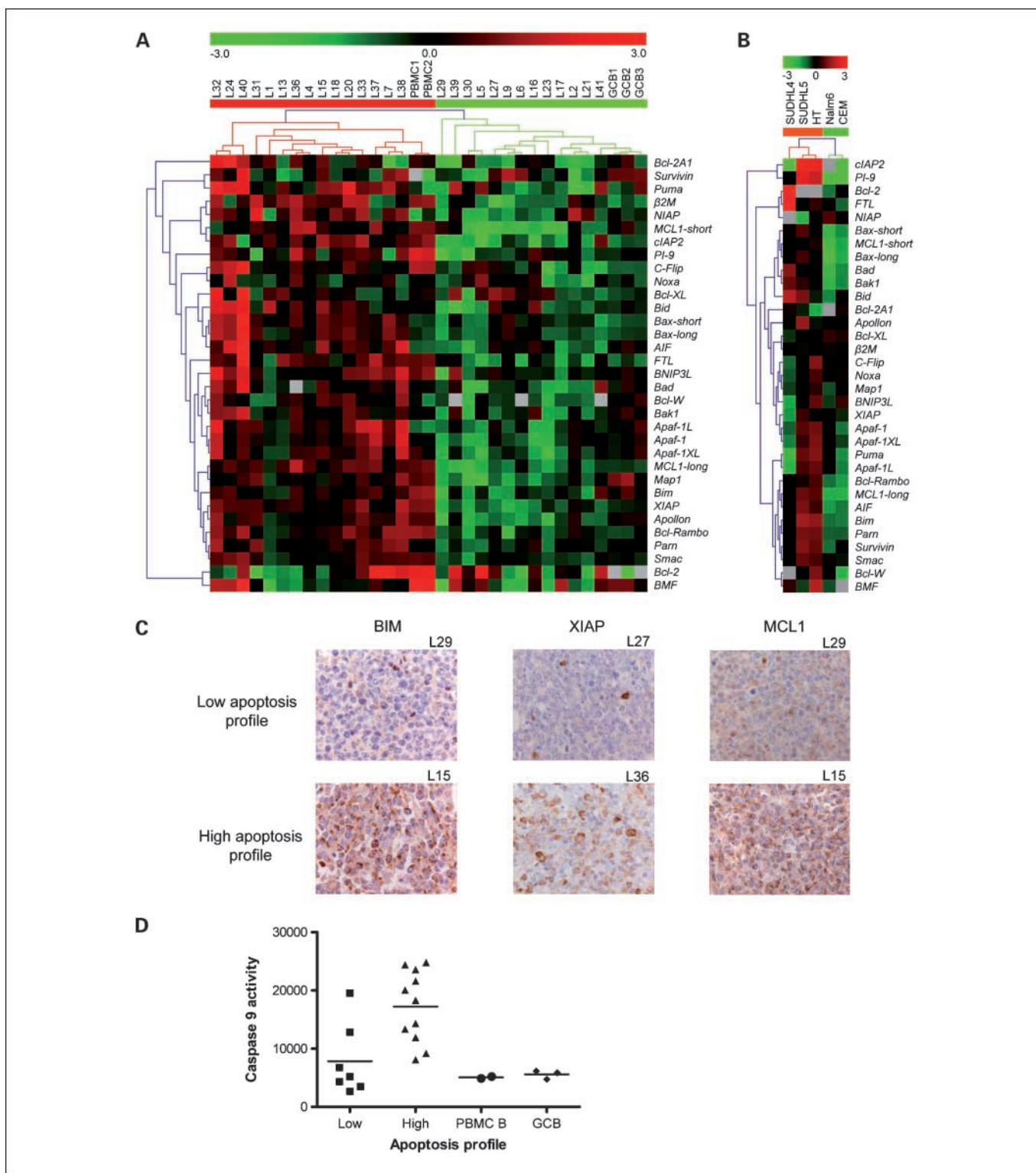
**High expression levels of proapoptotic genes correspond with high levels of spontaneous caspase-9 activity in DLBCL samples.**

To investigate whether high expression levels of proapoptotic genes reflect constitutive activation of the intrinsic apoptosis pathway, we compared levels of spontaneous caspase-9 activity in available isolated lymphoma cells of 11 cases characterized by high expression levels of proapoptotic and antiapoptotic genes with 7 cases with low expression levels of these genes. DLBCL samples with high expression levels of proapoptotic genes showed significantly higher levels of caspase-9 activity compared with the lymphoma cells with relative low levels of proapoptotic genes (*P* = 0.008; see Fig. 1D). The peripheral blood B cells and GCBs showed low levels of caspase-9 activity similar to the DLBCL cells with low expression levels of proapoptotic genes.

**Constitutive caspase-9 activation without caspase-3 activation and apoptosis in cell lines with high expression levels of proapoptotic genes.**

A more detailed functional analysis of the intrinsic apoptosis pathway was done in cell lines with apoptosis profiles comparable with both groups of DLBCL. Cluster analysis of RT-MLPA-detected gene expression values was done identical to that for the DLBCL samples. All the three available DLBCL cell lines (HT, SUDHL4, and SUDHL5) showed high expression levels of proapoptotic and most antiapoptotic genes, whereas the two leukemia cell lines (Nalm6 and CEM) showed low expression levels of proapoptotic and most antiapoptotic genes (Fig. 1B). Western blot analysis confirmed the relatively high expression of the proapoptotic genes *Bax*, *Bak*, and *AIF* at the protein level in HT, SUDHL4, and SUDHL5 cell lines (Fig. 2A).

Consistent with the results obtained in DLBCL lymphoma cells with a low apoptosis profile, Nalm6 and CEM cells showed relatively low levels of spontaneous caspase-9 activity (although higher than that in most DLBCL samples), whereas in HT, SUDHL4, and SUDHL5 cells very high levels of caspase-9



**Fig. 1.** High expression of proapoptosis- and antiapoptosis-regulating genes in a subset of DLBCL samples and cell lines. **A**, RT-MLPA analysis of apoptosis-regulating gene expression in isolated DLBCL cells. The results following unsupervised cluster analysis are shown. Two groups of DLBCL could be identified: one with relatively low levels of both proapoptotic and antiapoptotic gene expression and one with high levels of apoptosis-regulating gene expression. The expression levels of *BCL-G*, *Harakiri*, *ciAP*, and *BIRC7* were too low or not detectable. Cluster analysis was restricted to those genes that were expressed in at least 80% of the samples. Omitted genes were *BNIP3* and *Bik*. **B**, RT-MLPA analysis of apoptosis-regulating gene expression in cell lines. Unsupervised cluster analysis was done. Similarly to the DLBCL samples, two groups could be identified: one group with high levels of apoptosis-regulating gene expression, including *AIF*, *Bad*, *Bak*, *Bim*, *MCL1-long*, and *survivin*, and one group with low apoptosis-regulating gene expression. Expression levels of *BCL-G*, *Harakiri*, and *BIRC7* were too low or not detectable. **C**, immunohistochemical detection of Bim, XIAP, and MCL1 in DLBCL samples with low or high expression of proapoptotic and antiapoptotic genes. Original magnification,  $\times 400$ . **D**, caspase-9 activity was detected using a luminescent assay in the isolated lymphoma cells of 18 DLBCL samples. Experiments were done in triplicate. DLBCL samples with relatively high expression of proapoptotic genes show high expression of caspase-9 activity ( $P = 0.008$ ). Peripheral blood B cells and GCBs show lower levels of caspase-9 activity.

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**Table 1.** Characterization of DLBCLs

| Clinical response*              | Apoptosis profile |        | P    |
|---------------------------------|-------------------|--------|------|
|                                 | Low               | High   |      |
|                                 | n = 13            | n = 15 |      |
| Primary DLBCL                   |                   |        |      |
| Unfavorable †                   | 1                 | 7      | 0.04 |
| Favorable                       | 5                 | 2      |      |
| No follow-up                    | 3                 | 4      |      |
| Relapsed DLBCL                  | 1                 | 1      |      |
| Transformed follicular lymphoma | 3                 | 1      |      |
| GCB or ABC phenotype DLBCL ‡    |                   |        |      |
| GCB                             | 6                 | 5      | n.s. |
| ABC                             | 7                 | 6      |      |
| n.i.                            | 0                 | 4      |      |

Abbreviations: n.s., not significant; n.i., not interpretable.

\*Clinical data could be retrieved for 21 of 28 patients.

†Lymphoma samples were divided into samples with a favorable and an unfavorable clinical response. DLBCL showed a favorable response if therapy induced complete remission (according to standard clinical evaluation) and no relapse occurred during the follow-up time (at least 2 y).

‡According to Hans et al. (41).

activity were found. High levels of caspase-9 activity in HT, SUDHL4, and SUDHL5 cells were not accompanied by high levels of caspase-3/7 activity and apoptosis, indicating inhibition of the intrinsic apoptosis pathway downstream of caspase-9 (Fig. 2B). FACS analysis using a fluorescent active caspase-9 substrate showed that caspase-9 is constitutively activated in all cells of the three DLBCL cell lines (Fig. 2C and D).

As active caspase-8 is also capable of activating caspase-9 via truncation of Bid, we investigated whether active caspase-8 was responsible for the activation of caspase-9 in the cell lines with high levels of caspase-9 activity. High levels of caspase-9 activity in HT, SUDHL4, and SUDHL5 cells could not be blocked by simultaneous incubation with the caspase-8 inhibitor LETD-fmk (Fig. 2B).

**Etoposide induces caspase-9 activation and apoptosis only in cell lines without constitutive caspase-9 activation.** Cell lines were treated with etoposide to test the functionality of the intrinsic, caspase-9-mediated apoptosis pathway. After induction with etoposide, a strong increase in caspase-9 and caspase-3/7 activity and apoptosis in Nalm6 and CEM cells was found (Figs. 2C and 3A). Simultaneous incubation with the specific caspase-9 inhibitor LEHD-fmk showed that etoposide induced caspase-3/7 activation, and apoptosis in these cell lines was dependent on activation of caspase-9 (Fig. 3A, left). Furthermore, we observed mitochondrial depolarization of these cells after incubation with etoposide (Fig. 3B and C). In contrast, depolarization of the mitochondrial membrane already occurred in untreated DLBCL cell lines with continuous caspase-9 activity. In these cell lines, incubation with the uncoupler SF6847 hardly resulted in an additional loss of mitochondrial potential ( $\psi_m$ ). Etoposide failed to further increase caspase-9 activity and to induce caspase-3/7 activation (Figs. 2D and 3A, right). Moreover, no additional mitochondrial depolarization was observed in SUDHL4, SUDHL5, and HT (Fig. 3B and C). In SUDHL5 cells, a little increase in caspase-9 and caspase-3/7 activity was observed after induction with etoposide (Fig. 3A, right).

**Downstream inhibition of the inhibitory effect of XIAP results in induction of caspase-9-mediated apoptosis in cell lines and DLBCL samples with constitutive caspase-9 activation.** We next investigated whether neutralizing the effect of the downstream inhibitor XIAP results in caspase-9-mediated apoptosis in cell lines and DLBCL samples with constitutive caspase-9 activation (Fig. 4A). Three DLBCL samples of each apoptosis profile group were treated with increasing concentrations of the XIAP antagonist, 1396-12, or its inactive control, 1396-28, for 4 h. In DLBCL 13, 18, and 32, which showed high levels of caspase-9 activity and expression of XIAP, a strong increase in cell death was observed, whereas in the DLBCL samples with low caspase-9 activity and relatively low levels of XIAP expression, low or no sensitivity to the XIAP antagonist was found ( $P = 0.03$ ; Fig. 4B). The inactive control 1396-28 did not show any induction of cell death (data not shown).

A strong increase in cell death was observed after 24 h of treatment in the cell lines with high expression of caspase-9: HT, SUDHL4, and SUDHL5 (Fig. 5A, right). In these cell lines, the XIAP antagonist induced an increase in caspase-3/7 activity without further increasing caspase-9 activity (Fig. 5B and C, right). Simultaneous incubation with the caspase-9 inhibitor neutralized the apoptosis-inducing effect of the XIAP antagonist, indicating that the induction of cell death in these cell lines by the XIAP antagonist is caspase-9 dependent (Fig. 5D, right). Unexpectedly, the XIAP antagonist also induced apoptosis in Nalm6 and CEM cells (Fig. 5A, middle). In these cases, the XIAP antagonist induced a relatively low increase in caspase-3/7 activity, again without increasing caspase-9 activity (Fig. 5B and C, middle). In addition, in these cell lines, simultaneous incubation with the caspase-9 inhibitor neutralized the apoptosis-inducing effect of the XIAP antagonist, indicating that XIAP antagonist-induced cell death is dependent on caspase-9 activity (Fig. 5D, left). Apparently, the relatively low levels of caspase-9 activity in Nalm6 and CEM are sufficient to induce caspase-3/7 activity and apoptosis when cells are treated with the XIAP antagonist. This is explained by the fact that, although caspase-9 activity levels are relatively low, they are considerably higher than caspase-9 activity levels observed in nonneoplastic PBMC B cells (Fig. 5C, left) and in the DLBCL cells with low expression levels of proapoptotic genes (Fig. 1B). In these latter cases and in the PBMC B cells, caspase-9 activation levels are very low and the XIAP antagonist fails to induce apoptosis [Fig. 5A (left) and B].

## Discussion

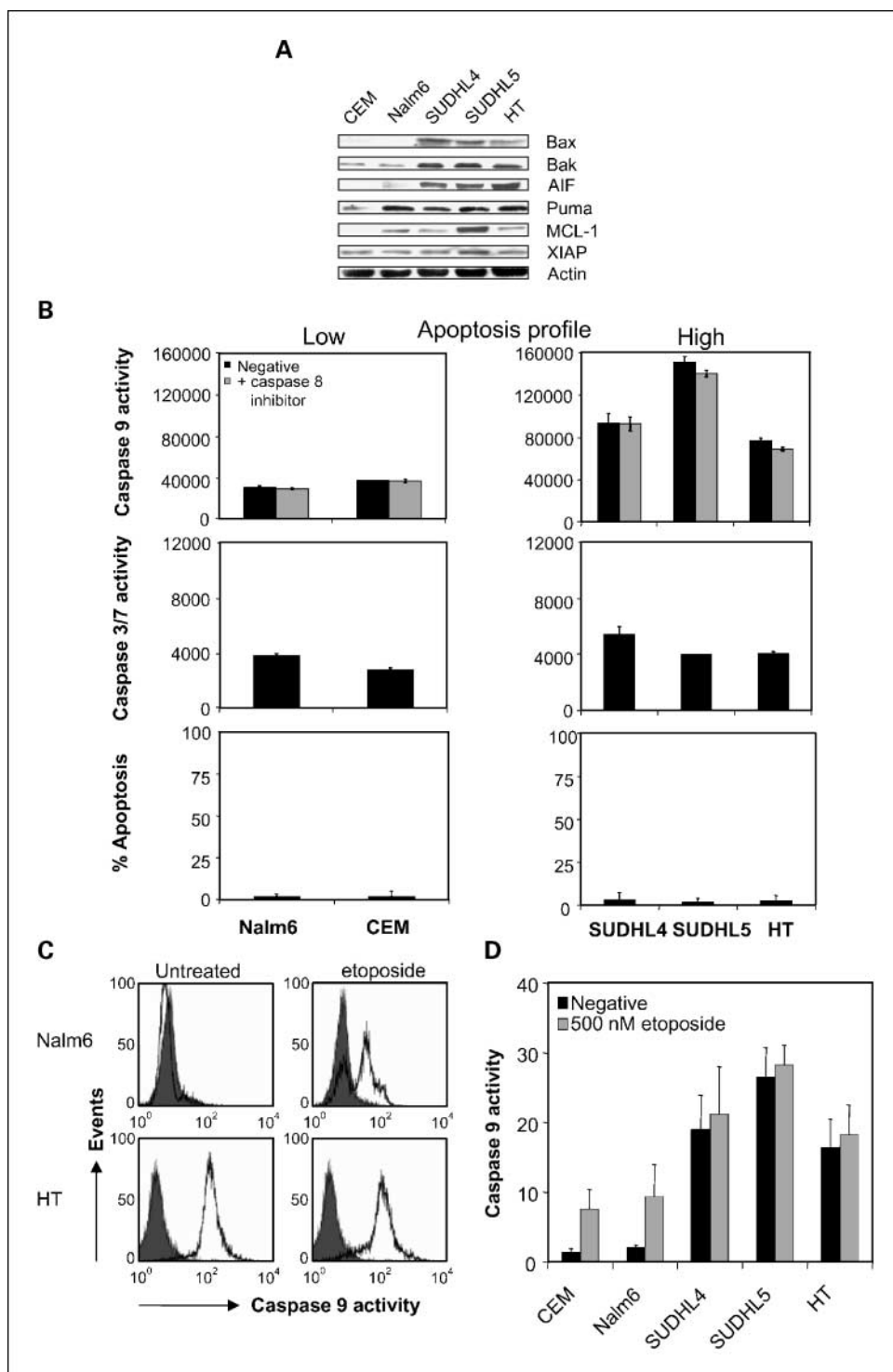
In this study, we have shown that a subset of nodal DLBCL cases displays high expression levels of both proapoptotic and antiapoptotic genes, primarily involved in the intrinsic, caspase-9-mediated apoptosis pathway. Consistent with our previous expression microarray results (18) and with a previous study by Davis et al. (28), we have found that cases with high levels of proapoptotic and antiapoptotic genes are frequently clinically refractory to chemotherapy. Although the association between high expression levels of antiapoptotic genes and a poor response to chemotherapy has been shown by many other studies (2-4, 10-12), the high expression levels of proapoptotic genes in chemotherapy-resistant cases were unexpected.

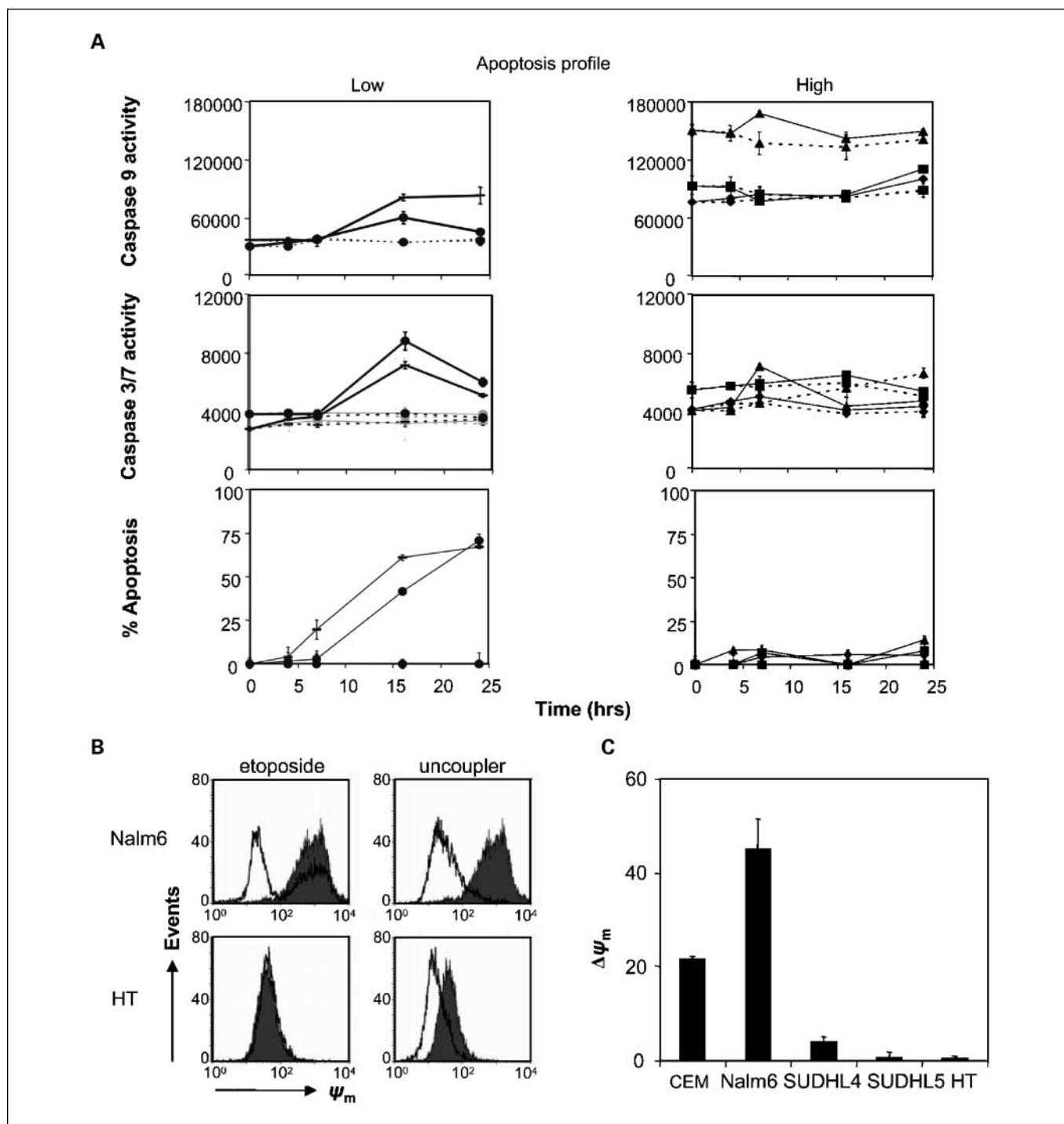
We further investigated this using functional analysis in isolated lymphoma cells of DLBCL samples and lymphoma cell lines (SUDHL4, SUDHL5, and HT). We could show that high

expression levels of proapoptotic genes are indeed associated with high levels of spontaneous caspase-9 activity and loss of mitochondrial membrane potential. Apparently, in DLBCL cells with a high apoptosis profile, the intrinsic apoptosis pathway is already triggered and part of the mitochondrial membrane potential is gone. These results might implicate that the normal oxidative phosphorylation is (partly) disrupted in cells with constitutive caspase-9 activation. However, the role of mitochondrial dysfunction in tumor cells is controversial. Recent articles

show that mitochondrial outer membrane permeabilization with cytochrome *c* release might be compatible with the preservation of mitochondrial integrity and function (29, 30). However, it has also been shown that tumor cells frequently depend on glycolysis even under aerobic conditions, suggesting a disruption of oxidative phosphorylation (31). Our studies do not allow drawing definitive conclusions on this issue. Thus, whether oxidative phosphorylation is indeed disrupted in cells with constitutive caspase-9 activity will be subject to further studies.

**Fig. 2.** Constitutive caspase-9 activation without caspase-3 activation and induction of apoptosis in cell lines with high expression of proapoptotic genes. **A**, protein expression of Bax, Bak, AIF, Puma, MCL1, and XIAP was detected in cell lines using Western blot analysis. **B**, levels of caspase-9 and caspase-3/7 activity and the percentage of apoptotic cells were determined in nontreated cell lines. Similar to a subset of DLBCL samples, cell lines with relatively high expression of proapoptotic genes show high expression of caspase-9 activity. Caspase-9 activity was also determined in the presence of 25  $\mu\text{mol/L}$  caspase-8 inhibitor. h $\text{TRAIL/Apo2L}$ -sensitive 8226 cells served as positive control and showed no sensitivity for h $\text{TRAIL/Apo2L}$  in the presence of the caspase-8 inhibitor (data not shown). **C**, caspase-9 activity was detected using FACS analysis in cells with a low apoptosis profile (Nalm6) and cells with a high apoptosis profile (HT) that were treated with or without 500 nmol/L etoposide for 24 h. The depicted graphs were obtained after staining with FAM-LEHD-fmk. Black peaks, isotype control staining; white peaks, staining with the caspase-9 substrate. **D**, levels of caspase-9 activity were determined in untreated and etoposide-treated cells using FACS analysis. Caspase-9 activity was calculated as follows: (mean fluorescence intensity of the FAM-LEHD-fmk – treated cells) / (mean fluorescence intensity of unstained cells).





**Fig. 3.** Cell lines with constitutive caspase-9 activation are resistant to etoposide-induced caspase-3/7 activation and induction of apoptosis. *A*, cell lines were incubated with 500 nmol/L etoposide for 4, 7, 16, and 24 h: Nalm6 (●), CEM (–), SUDHL4 (■), SUDHL5 (▲), and HT (◆). Levels of caspase-9 and caspase-3/7 activity and the percentage of apoptotic cells were determined in triplicate. Cell lines with a low apoptosis profile were also induced with 500 nmol/L etoposide in the presence of 25  $\mu$ mol/L caspase-9 inhibitor and caspase-3 activity was determined. Etoposide-induced apoptosis was entirely dependent on caspase-9 activation. —, negative; —, etoposide; —, caspase-9 inhibitor; —, etoposide + caspase-9 inhibitor. *B*, cells with a low apoptosis profile (Nalm6) and cells with a high apoptosis profile (HT) were treated with or without 500 nmol/L etoposide for 24 h or incubated with 10  $\mu$ mol/L uncoupler SF6847 for 15 min and stained with tetramethylrhodamine ethyl ester perchlorate. After treatment, Nalm6 cells showed mitochondrial membrane depolarization, which was observed as a shift to the left in the emission spectra. Black

Cases with high levels of spontaneous caspase-9 activity did not show concomitant caspase-3/7 activation and apoptosis. Consistent with our previous microarray data (18), DLBCL cases with a high apoptosis profile were clinically refractory to

chemotherapy, which was further substantiated by our observation that etoposide failed to induce additional caspase-9 activation, loss of mitochondrial membrane potential, and apoptosis in cell lines with high levels of proapoptotic genes.

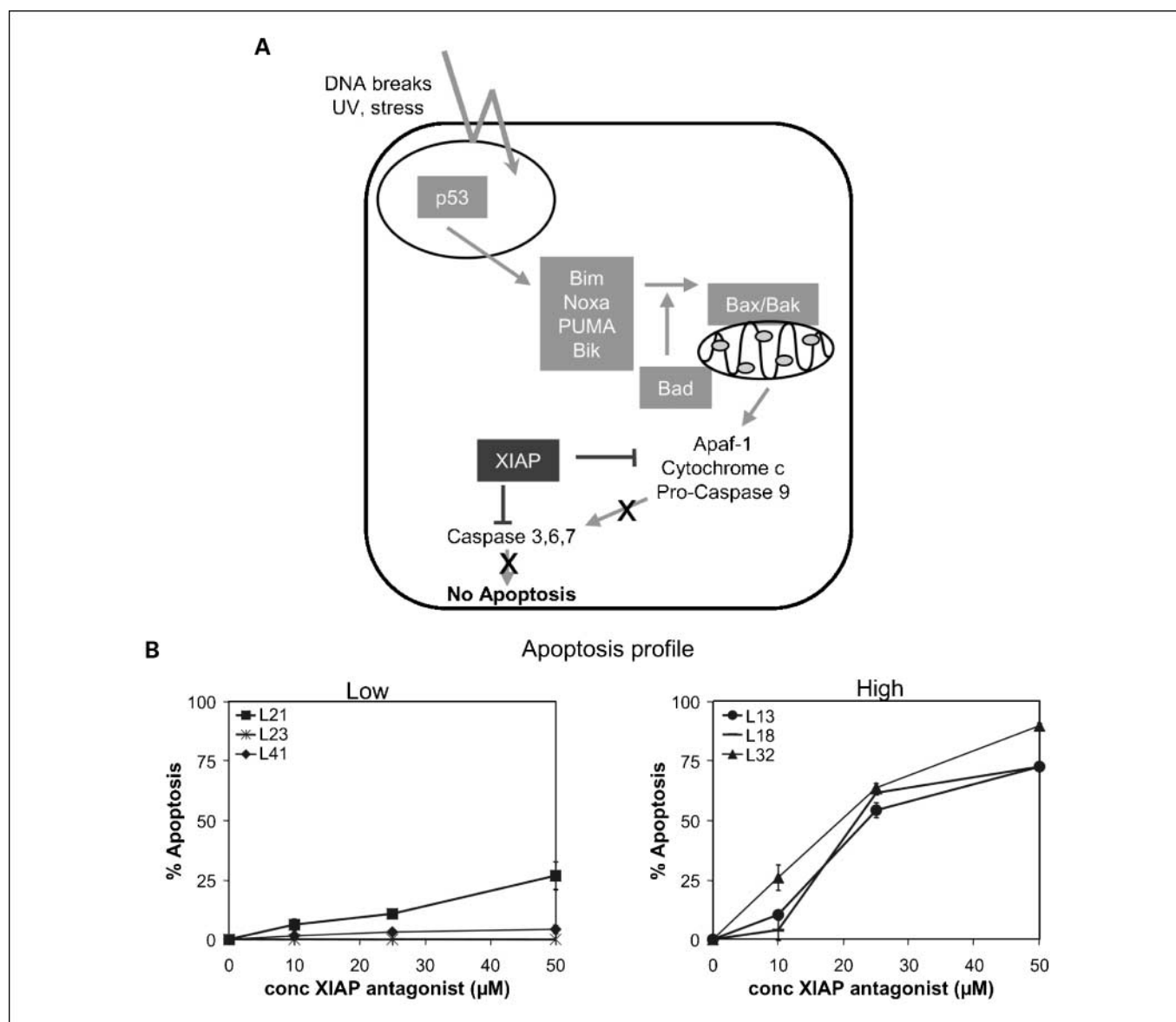
Apparently, failure of chemotherapy to induce apoptosis in chemotherapy-refractory DLBCL is caused by inhibition of the intrinsic pathway downstream of caspase-9 activation.

Downstream inhibition of the apoptosis pathways can be caused by expression of the members of the IAP family, including XIAP. DLBCL samples with high expression levels of proapoptotic genes also showed high levels of antiapoptotic genes, including most members of the IAP family, which explains the inhibition of the intrinsic apoptosis pathway in these cases. This notion was supported by our previous observation that expression of XIAP in neoplastic cells of primary nodal DLBCL is associated with a poor clinical outcome (11). Inhibition of the inhibitory effect of XIAP resulted in a rapid increase in caspase-3/7 activity and apoptosis without an increase in caspase-9 activity, indicating that the XIAP antagonist mediates its effect downstream from caspase-9 activation in

the apoptotic pathways, consistent with data by Carter et al. (21) and Wang et al. (23). However, the effect of the XIAP antagonist was completely blocked by the caspase-9 inhibitor, indicating that XIAP antagonist-induced apoptosis is caspase-9 dependent in these DLBCL cells and that caspase-9 activation itself is not caused by the XIAP inhibitor but results from an intrinsic cellular drive for constitutive caspase-9 activation.

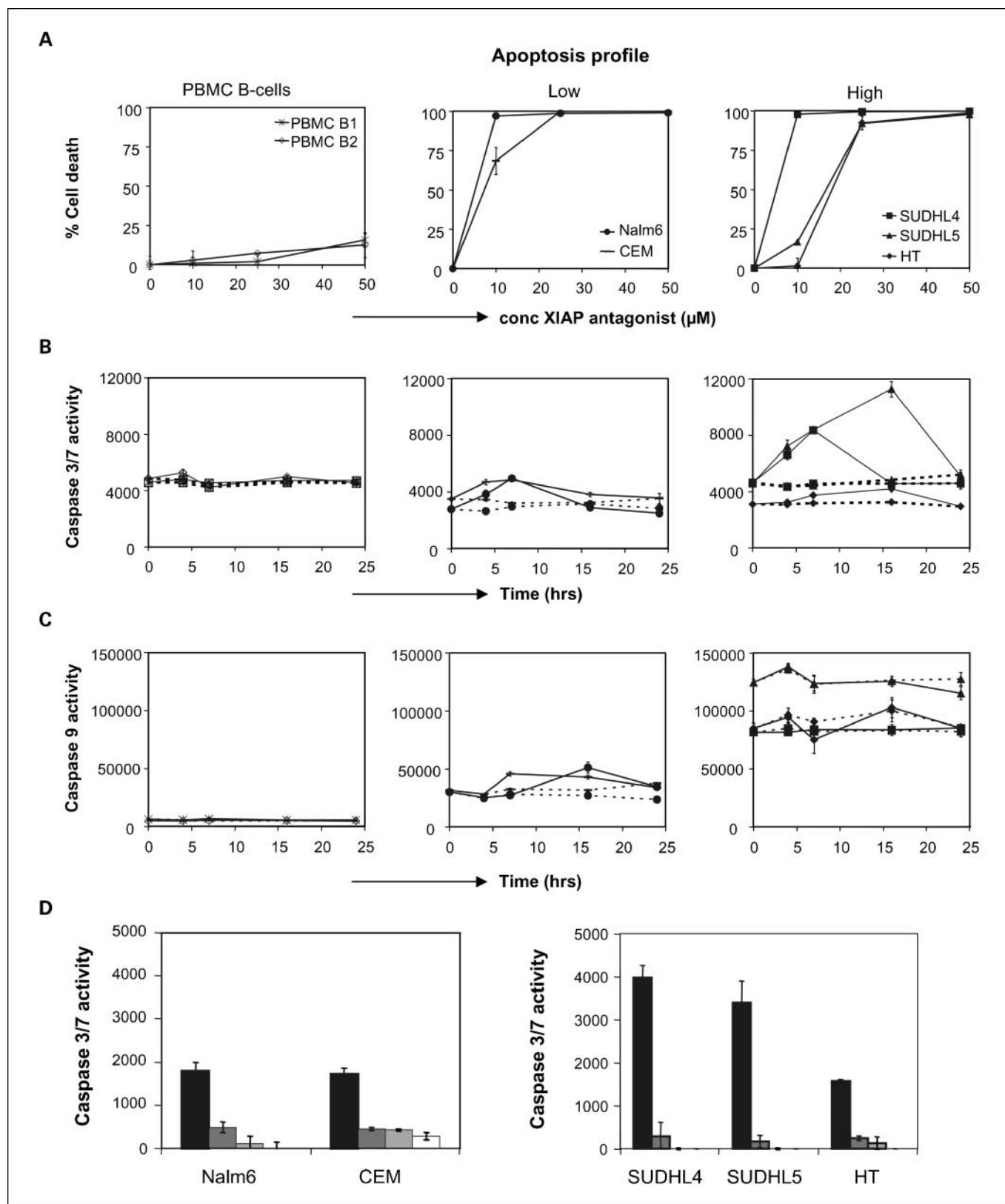
Taken together, these data indicate that resistance to chemotherapy-induced apoptosis in DLBCL may be caused by downstream inhibition of the intrinsic apoptosis pathway and not by failure of chemotherapy to activate the intrinsic apoptosis pathway. Furthermore, our study implies that neutralizing the apoptosis-inhibiting function of XIAP may be important in the treatment of chemotherapy-refractory DLBCL.

Previous genome-wide microarray expression profiling studies showed that DLBCL can be categorized in two major



**Fig. 4.** Inhibition of XIAP induces cell death in DLBCL cells with constitutive caspase-9 activation. **A**, schematic representation of the inhibition of the intrinsic, caspase-9-mediated apoptosis pathway by XIAP. **B**, percentage cell death in three DLBCL samples with a low apoptosis profile (*left*) and three DLBCL samples with a high apoptosis profile after treatment with increasing concentrations of the XIAP antagonist 1396-12 for 4 h ( $P = 0.03$ ).





**Fig. 5.** Inhibition of XIAP induces caspase-9–mediated caspase-3/7 activity and cell death in cell lines. *A*, percentage cell death in PBMC B cells from two healthy donors and cell lines with low and high levels of caspase-9 activity treated for 24 h with increasing concentrations of the XIAP antagonist 1396-12. *B*, caspase-3/7 activity in PBMC B cells and cell lines with low and high levels of caspase-9 activity after incubation with 25 μmol/L XIAP antagonist. *C*, caspase-9 activity in PBMC B cells and cell lines with low and high levels of constitutive caspase-9 activity before and after treatment with 25 μmol/L XIAP antagonist. *D*, induction of caspase-3/7 activity in cell lines after treatment with 25 μmol/L XIAP antagonist for 7 h in the presence and absence of increasing concentrations of caspase-9 inhibitor. ■, 25 μmol/L XIAP antagonist; ■, 25 μmol/L XIAP antagonist + 5 μmol/L caspase-9 inhibitor; ■, 25 μmol/L XIAP antagonist + 10 μmol/L caspase-9 inhibitor; □, 25 μmol/L XIAP antagonist + 25 μmol/L caspase-9 inhibitor.

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subtypes: one resembling GCBs and the other resembling *in vitro* ABCs (32–34). Nuclear factor- $\kappa$ B-mediated up-regulation of antiapoptotic genes probably explains the poor outcome in patients with ABC-like DLBCL (35, 36). However, in our study, DLBCLs with high levels of proapoptotic and antiapoptotic genes were of both the GCB- and ABC-like phenotypes, indicating that downstream disruption of the apoptosis pathway is not restricted to only ABC-like DLBCL.

What may cause constitutive activation of the intrinsic apoptosis pathway? In nonneoplastic lymphoid cells, expression of the proapoptotic BH3-only proteins (Puma, Noxa, Bad, Bim, Bik, Bmf, and Bid) can be induced by a variety of cellular signals, including cytokine withdrawal, antigen receptor cross-linking, glucocorticoid exposure, and DNA damage (37). Expression of these proapoptotic genes is mostly induced in a p53-dependent manner (38). In addition, several mitogenic oncogenes, including activated E2F1, also induce apoptosis in a p53-dependent manner as a “fail-safe” program, which, when intact, will prevent tumor development (38–40). Which of the above mechanisms causes the constitutive activation of the

intrinsic apoptosis pathway remains uncertain. In our previous microarray experiments, high expression levels of proapoptotic genes correlated with high expression levels of E2F1, suggesting that high expression levels of proapoptotic genes might be explained by constitutive expression of E2F1 (38, 39).

We conclude that constitutive activation of the intrinsic apoptosis pathway with concomitant inhibition downstream of caspase-9 activation plays a central role in chemotherapy resistance in DLBCL. Moreover, our results suggest that inhibition of XIAP function might be an alternative therapy for chemotherapy-refractory DLBCL and that profiling of apoptosis genes in DLBCL cells may allow for prospective identification of patients that are expected to benefit from therapy with the small-molecule XIAP antagonist.

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