

## A Novel Paradigm to Trigger Apoptosis in Chronic Lymphocytic Leukemia

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### Abstract

**Evasion of apoptosis is a hallmark of chronic lymphocytic leukemia (CLL), calling for new strategies to bypass resistance. Here, we provide first evidence that small-molecule X-linked inhibitor of apoptosis (XIAP) inhibitors in combination with the death receptor ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) present a novel approach to trigger apoptosis in CLL, including subgroups with resistant disease or unfavorable prognosis. XIAP, cellular IAP (cIAP) 1, and cIAP2 are expressed at high levels in primary CLL samples. Proof-of-concept studies in CLL cell lines show that subtoxic concentrations of XIAP inhibitors significantly enhance TRAIL-induced apoptosis and also sensitize for CD95-mediated apoptosis. Importantly also in primary CLL samples, XIAP inhibitor acts in concert with TRAIL to trigger apoptosis in 18 of 27 (67%) cases. This XIAP inhibitor-induced and TRAIL-induced apoptosis involves caspase-3 activation and is blocked by the caspase inhibitor zVAD.fmk. The cooperative interaction of XIAP inhibitor and TRAIL is even evident in distinct subgroups of patients with poor prognostic features (i.e., with 17p deletion, TP53 mutation, chemotherapy-refractory disease, or unmutated V<sub>H</sub> genes). Interestingly, cases with unmutated V<sub>H</sub> genes were significantly more sensitive to XIAP inhibitor-induced and TRAIL-induced apoptosis compared with V<sub>H</sub> gene-mutated samples, pointing to a role of B-cell receptor signaling in apoptosis regulation. By showing that XIAP inhibitors in combination with TRAIL present a new strategy to trigger apoptosis even in resistant forms and poor prognostic subgroups of CLL, our findings have important implications for the development of apoptosis-based therapies in CLL.** [Cancer Res 2009;69(23):8977–86]

### Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in adults in western societies (1). CLL is characterized by the abnormal accumulation of malignant monoclonal B cells, which has been largely attributed to defective apoptosis programs rather than aberrant proliferation (2–4). Indeed, the vast majority of clonal B cells of CLL are arrested at the G<sub>0</sub>-G<sub>1</sub> phase of the cell cycle, with only a very small population that actively proliferates (5). One of the key factors that contribute to malignant B-cell longevity is the misbalance between prosurvival

and prodeath molecules (2). Thus, new strategies to reactivate apoptosis may be crucial for the development of molecular targeted therapies in CLL.

Two principal pathways are involved in the initiation of apoptosis [i.e., the death receptor (extrinsic) pathway or the mitochondrial (intrinsic) pathway (6, 7)]. Ligation of death receptors of the tumor necrosis factor (TNF) receptor superfamily, such as CD95 (APO-1/Fas), by their cognate ligands or agonistic antibodies results in caspase-8 activation at the death-inducing signaling complex, which induces direct cleavage of downstream effector caspases, such as caspase-3 (8). In the mitochondrial pathway, the release of cytochrome *c*, second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis (IAP) binding protein with low isoelectric point (DIABLO), or Omi/high-temperature requirement protein A2 (HtrA2) from mitochondria into the cytosol results in caspase-3 activation via formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex and via Smac-mediated neutralization of caspase inhibition by IAP proteins (9).

The concept of inducing apoptosis in cancer cells by ligation of death receptors is of special interest for cancer therapy because death receptors are directly linked to the intrinsic death program of the cell (8). The death-inducing ligand TNF-related apoptosis-inducing ligand (TRAIL) is a prime candidate for clinical development because it has been reported to induce apoptosis in a wide spectrum of cancer cell lines with no or minimal toxicity on normal human cells (10). Recombinant TRAIL or fully human monoclonal antibodies that specifically target one of the two agonistic TRAIL receptors are currently evaluated in early clinical trials (10). However, many human tumors, including hematologic malignancies, are partially or completely resistant to monotherapy with TRAIL, limiting its therapeutic utility (8, 10–12). CLL cells have previously been reported to be inherently refractory to TRAIL-induced apoptosis despite the constitutive expression of the apoptosis-inducing TRAIL receptors (13–16). This highlights the demand to develop TRAIL-based combination therapies that counter resistance mechanisms of CLL cells toward TRAIL.

The molecular details that cause resistance of human cancers, including CLL, to TRAIL are still only partially understood (17). Increasing evidence suggests that high levels of IAP proteins may represent a key antiapoptotic mechanism in cancer cells (18, 19). Among the IAP family members, it is especially X-linked IAP (XIAP) that is known for its antiapoptotic function (20). Interestingly, high levels of XIAP were detected in CLL cells, possibly as part of a NF- $\kappa$ B-regulated survival program (20–22). Because XIAP blocks apoptosis at a central point via inhibition of caspases, there is currently much interest to exploit XIAP as a molecular target in human cancers (19). This has led to the development of small-molecule inhibitors or antisense strategies directed against XIAP (18). We previously showed proof-of-concept that Smac peptides that antagonize

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-09-2604

onize XIAP can be used to enhance TRAIL-induced killing *in vitro* and *in vivo* in a glioblastoma model (23). However, the question whether targeting XIAP is a suitable strategy to prime CLL cells for TRAIL-induced apoptosis has not yet been addressed. Therefore, we investigated the effect of small-molecule XIAP inhibitors on TRAIL-mediated antitumor activity in CLL in the present study.

## Materials and Methods

**Cell culture.** CLL cell lines (MEC1, JVM2, and JVM3), which were established from the peripheral blood of patients with CLL by EBV transformation (24, 25), were obtained from the German Resource Centre for Biological Material and cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies), 1 mmol/L glutamine (Life Technologies), 1% penicillin/streptavidin (Life Technologies), and 25 mmol/L HEPES (Biochrom). TRAIL was obtained from R&D Systems, Inc., and the broad-range caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem and used to determine the dependency of apoptosis on caspase activity. XIAP inhibitor 1, XIAP inhibitor 2, and control compound correspond to compounds 2, 11, and 15, respectively, described by Oost and colleagues (26), and XIAP inhibitors 3 and 4 were described by Chao and colleagues (27) and kindly provided by Idun Pharmaceuticals (now Pfizer, Inc.). The control compound, which is a close structural analogue that binds to the BIR3 domain of XIAP with a >100-fold lower affinity (26), was used to correlate the binding affinity of the XIAP inhibitors to the BIR3 domain of XIAP to their ability to induce apoptosis. All chemicals were purchased from Sigma unless indicated otherwise.

**Western blot analysis.** Western blot analysis was performed as described previously (28) using the following antibodies: mouse anti-caspase-8 (ApoTech Corp.), rabbit anti-caspase-3 (Cell Signaling), mouse anti-XIAP (BD), rabbit anti-Smac (MBL), rabbit anti-cellular IAP (cIAP) 2 (Epitomics), goat anti-cIAP1 and rabbit anti-survivin (R&D Systems), or mouse anti- $\beta$ -actin as loading control (Sigma) followed by goat anti-mouse IgG or goat anti-rabbit IgG or donkey anti-goat IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Enhanced chemiluminescence was used for detection (Amersham Biosciences).

**Determination of caspase activity.** Caspase activity was determined in living nonfixed, nonlysed cells as described (29) using the following caspase substrates conjugated to rhodamine R110: *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone-R110 (zDEVd-R110) as caspase-3 substrate and *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone-R110 (zIETD-R110) as caspase-8 substrate (Molecular Probes).

**Determination of apoptosis.** Apoptosis was determined by fluorescence-activated cell sorting (FACS) analysis (FACScan, BD Biosciences) of DNA fragmentation of propidium iodide-stained nuclei or by forward/side scatter analysis as described previously (28, 30) or by Annexin V staining (Roche) according to the manufacturer's instructions. Light scatter analysis is a FACS-based method to detect apoptotic cells (31).

**Determination of TRAIL receptors.** To determine surface receptor expression, cells were incubated with mouse anti-human TRAIL-R1, mouse anti-human TRAIL-R2, mouse anti-human TRAIL-R3, or mouse anti-human TRAIL-R4 (all from ApoTech) monoclonal antibodies for 30 min at 4°C, washed in PBS containing 1% FCS, incubated with rabbit anti-mouse F(ab')<sub>2</sub> IgG/biotin (BD Biosciences) for 20 min at 4°C in the dark, washed in PBS containing 1% FCS, incubated with streptavidin-phycoerythrin (BD Biosciences) for 20 min at 4°C in the dark, and analyzed by flow cytometry.

**Analysis of CLL patients and primary B cells.** A cohort of 27 CLL patients from our institution was analyzed after obtaining informed consent and approval of the Ethical Committee at Ulm University in accordance with the Declaration of Helsinki. Criteria for patient selection were a high lymphocyte count and a percentage of lymphocytes in excess of 80%. Clinical and genetic characterization was performed as previously described and is provided in Table 1 (32–34). Lymphocytes were isolated from blood samples

with Biocoll solution and aliquots were frozen viably at –196°C. Primary B cells were isolated from buffy coats of healthy donors over the age of 58 y by Ficoll separation (Biochrom) and magnetic bead isolation using human CD19 Micro Beads (Miltenyi Biotec) and cultured in RPMI 1640 (Biochrom) supplemented with 10% FCS, 1% penicillin/streptomycin, and 1% glutamine.

**Statistical analysis.** Statistical significance was assessed by Student's *t* test or Fisher's exact test, where appropriate, using Winstat (R. Fitch Software) or Statistical Package for the Social Sciences (SPSS GmbH Software) software. Interaction between XIAP inhibitors and TRAIL was analyzed by the combination index (CI) method using CalcuSyn software (Biosoft). A CI of 0.3 to 0.7 indicates synergism and a CI of 0.1 to 0.3 indicates strong synergism.

## Results

**XIAP inhibitors sensitize CLL cell lines for TRAIL-induced apoptosis.** In a first approach to investigate the role of XIAP in the regulation of apoptosis in CLL, we performed pilot experiments in CLL cell lines. Western blot analysis showed that the IAP proteins XIAP, cIAP1, cIAP2, and survivin as well as the endogenous IAP antagonists Smac/DIABLO and Omi/HtrA2 were all expressed in CLL cell lines (Fig. 1A, *left*). Next, we examined the effect of small-molecule XIAP inhibitors on apoptosis induction in CLL cell lines using two distinct small-molecule XIAP inhibitors that bind to the BIR3 domain of XIAP with high affinity at nanomolar concentrations (26). As control, we used a close structural analogue that weakly binds to the BIR3 domain of XIAP (26). Treatment with XIAP inhibitors as single agents caused no detectable increase in apoptosis in CLL cell lines (Supplementary Fig. S1), whereas we previously reported that they induce apoptosis in acute lymphoblastic leukemia cells at nanomolar to micromolar concentrations (29).

CLL cells have been described to be inherently resistant to the death receptor ligand TRAIL (13, 14), calling for strategies to enhance the efficacy of TRAIL in CLL. We therefore asked whether XIAP inhibitors could lower the threshold for TRAIL-triggered cell death in CLL cells. To address this question, we first examined expression of TRAIL receptors in CLL cell lines. Surface staining by flow cytometry showed high membrane expression of the agonistic TRAIL-R2 on all CLL cell lines, whereas TRAIL-R1 and the antagonistic TRAIL receptors TRAIL-R3 and TRAIL-R4 were expressed at much lower levels (Fig. 1A, *right*). Importantly, nontoxic concentrations of XIAP inhibitors significantly enhanced TRAIL-induced apoptosis in a dose-dependent manner (Fig. 1B). They also significantly increased CD95-mediated apoptosis, whereas they did not alter the susceptibility toward the chemotherapeutic agents fludarabine or chlorambucil (Supplementary Fig. S2). Analysis of DNA fragmentation, one of the biochemical hallmarks of apoptosis, confirmed that XIAP inhibitor 2 in combination with TRAIL enhanced apoptotic cell death (Fig. 1C). Similarly, several structurally distinct XIAP inhibitors cooperated with TRAIL in a dose-dependent manner to trigger apoptosis (Fig. 1D). Monitoring of caspase activation by Western blotting showed that XIAP inhibitor 2 substantially enhanced TRAIL-induced cleavage of caspase-3 into active p17/12 cleavage fragments, whereas it had no detectable effect on the processing of caspase-8 (Fig. 2A). Analysis of caspase activity by enzymatic caspase assay similarly showed that XIAP inhibitor 2 significantly increased TRAIL-induced caspase-3 activity, whereas it did not further augment caspase-8 activity compared with treatment with TRAIL alone (Fig. 2B). These findings are in line with a model that inhibition of XIAP primarily promotes activa-

**Table 1.** Characteristics of study patient samples

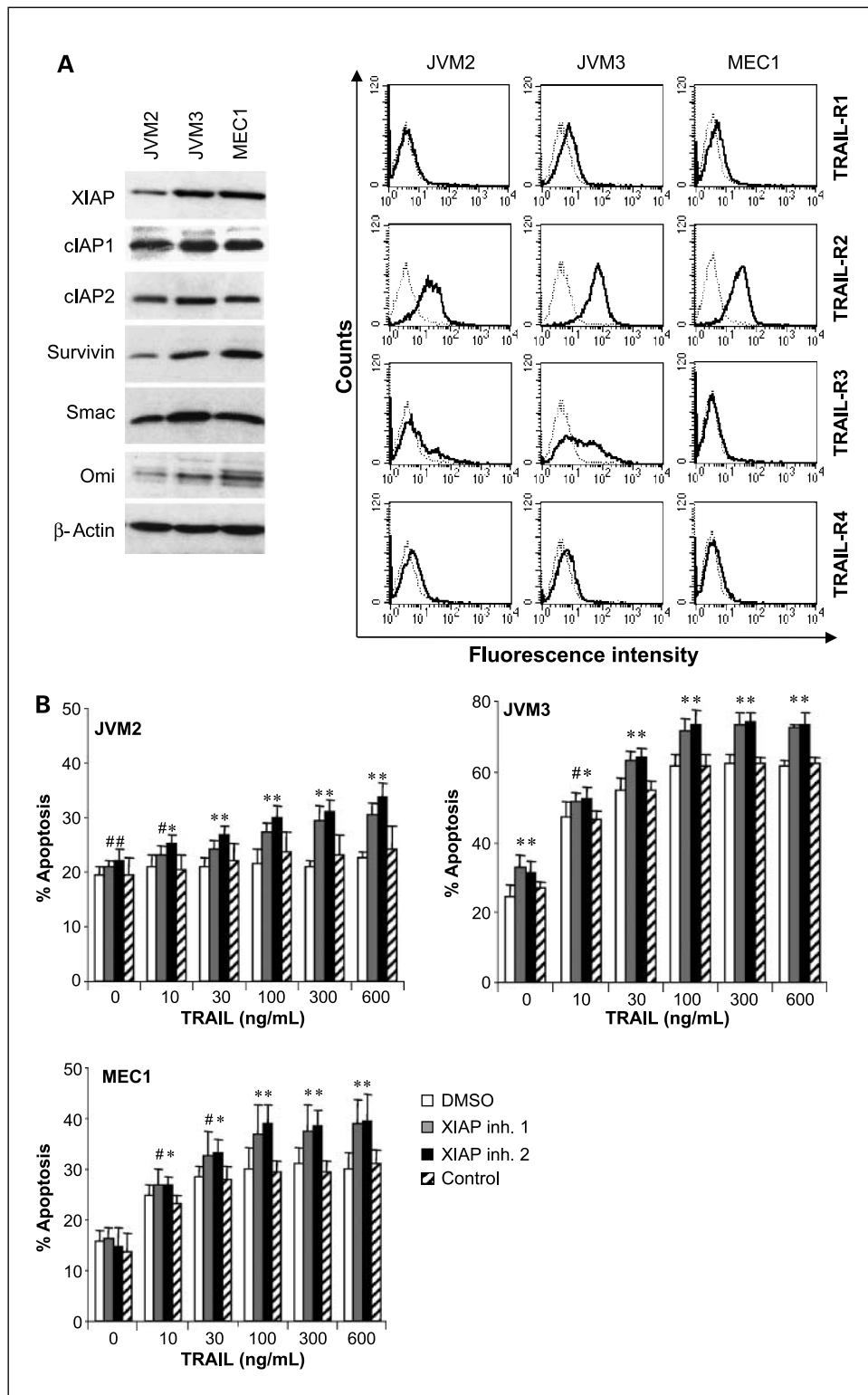
Patient no.	Age/sex	Karyotype (FISH)	V <sub>H</sub> status	% IgVH homology	V <sub>H</sub> usage	Zap70	TP53 mutation (y/n)	TP53 mutation type	Prior therapy	F-refractory	Effect of XIAP inhibitor
1	49/M	13q del	m	0.9796	V3-15	5.9 (-)	n.d.		n	n.a.	-
2a, 2b	71/F	13q del	m	0.918	V3-72	14.10 (-)	y	c.847C>T	n	n.a.	-
3	79/M	17p del, 12q+	u	1	V3-09	58.71 (+)	y	c.920-2A>G	y	y	+
4	34/F	Normal	u	1	V3-33	52.2 (+)	n		n	n.a.	+
5	64/M	Normal	u	1	V1-69	43.46 (+)	n.d.		n	n.a.	+
6	68/M	13q del	u	0.995	V3-30	35.75 (+)	n		n	n	+
7	63/F	Normal	m	0.88	V3-07	14 (-)	y	c.540_542delGCG	y	n	-
8	73/M	13q del	m	0.928	V3-43	1.4 (-)	n		n	n	+
9	79/M	Normal	m	0.94	V4-34	14 (-)	n		n	n.a.	+
10	72/M	11q del	u	1	V1-69	n.d.	n		n	n.a.	+
11	44/M	13q del	u	0.9817	V4-31	28.91 (+)	n.d.		n	n.a.	-
12	70/M	13q del	m	0.874	V3-23	1 (-)	n.d.		n	n.a.	-
13	57/M	11q del, 13q del	u	1	V3-11	1.4 (-)	n		y	n	+
14	49/F	17p del	u	0.995	V3-30	1.21 (-)	y	c.734G>A	n	n.a.	+
15	66/F	13q del	u	0.996	V5-a	9.5 (-)	n.d.		y	y	-
16	86/F	17p del, 12q+	u	1	V1-69	90.24 (+)	n		y	n.a.	+
17	63/F	13q del	m	0.899	V1-18	4 (-)	n.d.		y	y	+
18	52/M	Normal	u	1	V3-11	29.4 (+)	n.d.		n	n.a.	+
19	49/M	13q del	m	0.926	V3-53	6.47 (-)	n.d.		n	n.a.	-
20	52/M	13q del	u	1	V3-30	2.17 (-)	n.d.		y	n.a.	+
21	70/F	13q del	m	0.962	V3-21	16.53 (+)	n.d.		n	n.a.	+
22	59/M	11q del, 13q del	u	1	V2-05	29.13 (+)	n		y	y	+
23	63/F	13q del	m	0.971	V3-30	5.64 (-)	n		n	n.a.	-
24	75/M	13q del	m	0.872	V3-15	n.d.	y	c.716A>G	y	y	-
25	39/M	Normal	m	0.938	V4-34	0.09 (-)	n.d.		n	n.a.	+
26	72/F	17p del, 13q del	u	1	V1-69	30.06 (-)	n.d.		n	n.a.	+
27	74/F	Normal	m	0.872	V3-66	8.8 (-)	n		n	n.a.	+

NOTE: Difference (+, >10%)/no difference (-, <10%) in apoptosis between treatment with XIAP inhibitor and TRAIL compared with TRAIL and solvent. Abbreviations: FISH, fluorescence *in situ* hybridization; n.d., not determined; y, yes; n, no; M, male; F, female; n.a., not applicable; u, unmutated (<2% sequence divergence with the closest germ-line gene); m, mutated (>2% sequence divergence with the closest germ-line gene); F-refractory, fludarabine-refractory.

tion of effector caspases such as caspase-3 (20). Addition of the broad-range caspase inhibitor zVAD.fmk almost completely blocked apoptosis in response to the combined treatment with TRAIL and XIAP inhibitor 2 (Fig. 2C). Together, this set of experiments shows that XIAP inhibitors act in concert with TRAIL to induce caspase-3 activation and caspase-dependent apoptosis in CLL cell lines.

**XIAP inhibitor acts in concert with TRAIL to induce apoptosis in primary CLL cells.** In a second approach, we extended our studies to primary CLL cells to validate the results obtained in cell lines. Patients' characteristics are summarized in Table 1. Western blot analysis showed that XIAP, cIAP1, cIAP2, and Smac were all expressed in primary CLL cells (Supplementary Fig. S3). By comparison, survivin was expressed at low or undetectable levels in primary CLL cells compared with the CLL cell line MEC1 (Supplementary Fig. S3), consistent with a previous study of low survivin expression in primary CLL cells (35). Importantly, XIAP inhibitor 2 acted in concert with TRAIL to induce apoptosis in 18 of 27 (67%) primary CLL samples (Fig. 3A; Table 1). Analysis of apoptosis by Annexin V staining yielded similar results compared with forward/side scatter analysis, confirming that apoptotic cell death was induced (compare patient 3 in Fig. 3A and B). Of note, the cooperative interaction of XIAP inhibitor 2 and TRAIL was even evident in patients with an un-

favorable prognosis, for example, in all four patients with 17p deletion (patients 3, 14, 16, and 26; Fig. 3A; Table 1), the subgroup with the worst outcome (36). Furthermore, XIAP inhibitor 2 and TRAIL triggered apoptosis in patients with TP53 mutation (patients 3 and 14; Fig. 3A; Table 1), fludarabine-refractory disease (patients 3, 17, and 22; Fig. 3A; Table 1), and, strikingly, in almost all patients with unmutated V<sub>H</sub> genes (patients 3, 4, 5, 6, 10, 13, 14, 16, 18, 20, 22, and 26; Fig. 3A; Table 1). Interestingly, cases with unmutated V<sub>H</sub> genes were significantly more sensitive to XIAP inhibitor-triggered and TRAIL-triggered apoptosis compared with those with mutated V<sub>H</sub> genes ( $P = 0.04$ , Fisher's exact test), although these patients bear a worse prognosis (36). Moreover, XIAP inhibitor 2 induced apoptosis also as single agent in several primary CLL samples, whereas treatment with TRAIL alone caused no or minimal apoptosis in CLL samples, with the exception of patient 26 (Fig. 3A). Although the control compound showed some apoptosis-inducing activity alone or in combination with TRAIL, this effect was much weaker compared with XIAP inhibitor 2 (Fig. 3A), consistent with the strongly reduced ability of the control compound to bind to the BIR3 domain of XIAP (26). One patient was studied at an interval of 6 months and no difference in the response to treatment with XIAP inhibitor and TRAIL was observed (Fig. 3A, patients 2a and 2b).

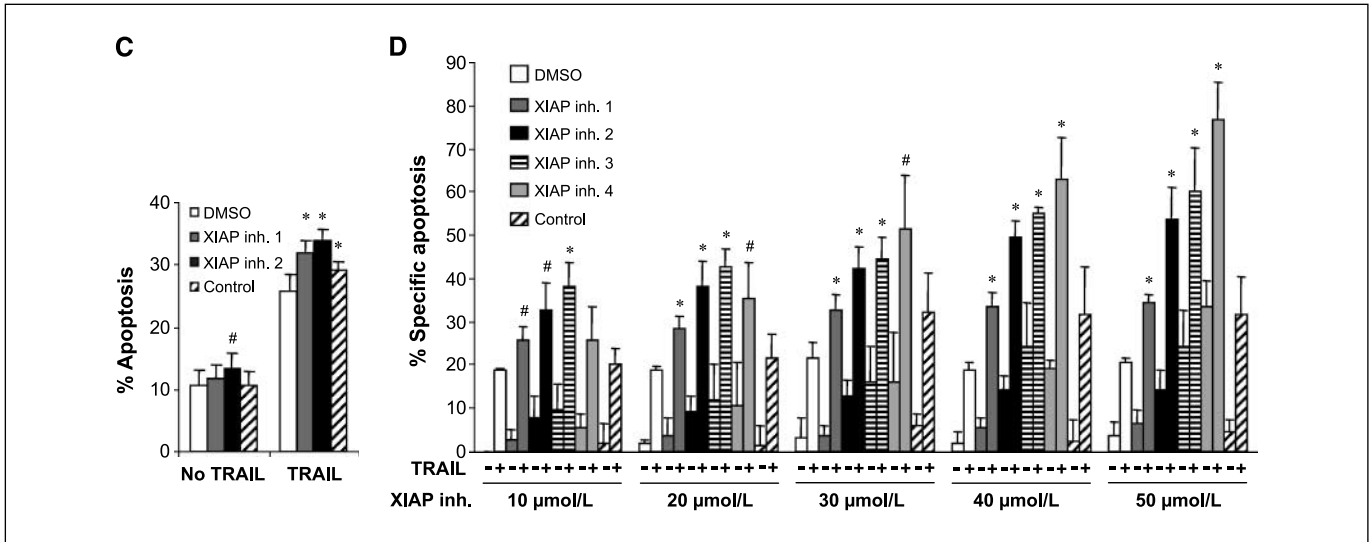


**Figure 1.** XIAP inhibitors sensitize CLL cell lines for TRAIL-induced apoptosis. *A, left*, protein expression of XIAP, cIAP1, cIAP2, survivin, Smac, Omi, and  $\beta$ -actin was assessed by Western blotting; *right*, surface expression of TRAIL receptors TRAIL-R1 to TRAIL-R4 was determined by fluorescence-conjugated antibodies and flow cytometry (*thin line*, cells stained with isotype control; *thick line*, cells stained with anti-TRAIL-R1 to anti-TRAIL-R4 antibodies). Fluorescence intensity (*X axis*) is binned against counts (*Y axis*). *B*, cells were treated for 72 h with indicated concentrations of TRAIL and/or 10  $\mu$ mol/L of XIAP inhibitors, control compound, or DMSO. Apoptosis was determined by forward/side scatter analysis and flow cytometry.

Moreover, we explored whether the interaction of XIAP inhibitor and TRAIL is additive or synergistic. Dose-response experiments and calculation of CI revealed a strong synergism of XIAP inhibitor 2 and TRAIL to induce apoptosis in primary CLL cells (Fig. 3C; Supplementary Fig. S4; CIs of <0.1 for 10  $\mu$ mol/L XIAP inhibitor and 10 ng/mL TRAIL). To test the broader relevance of our findings,

we extended our studies to an additional, structurally slightly different XIAP inhibitor, which also binds to the BIR3 domain of XIAP. XIAP inhibitor 4 similarly cooperated with TRAIL to induce apoptosis in primary CLL cells (Supplementary Fig. S5).

**Effect of XIAP inhibitors and TRAIL on normal B cells.** Furthermore, we assessed the effects of XIAP inhibitor and TRAIL on



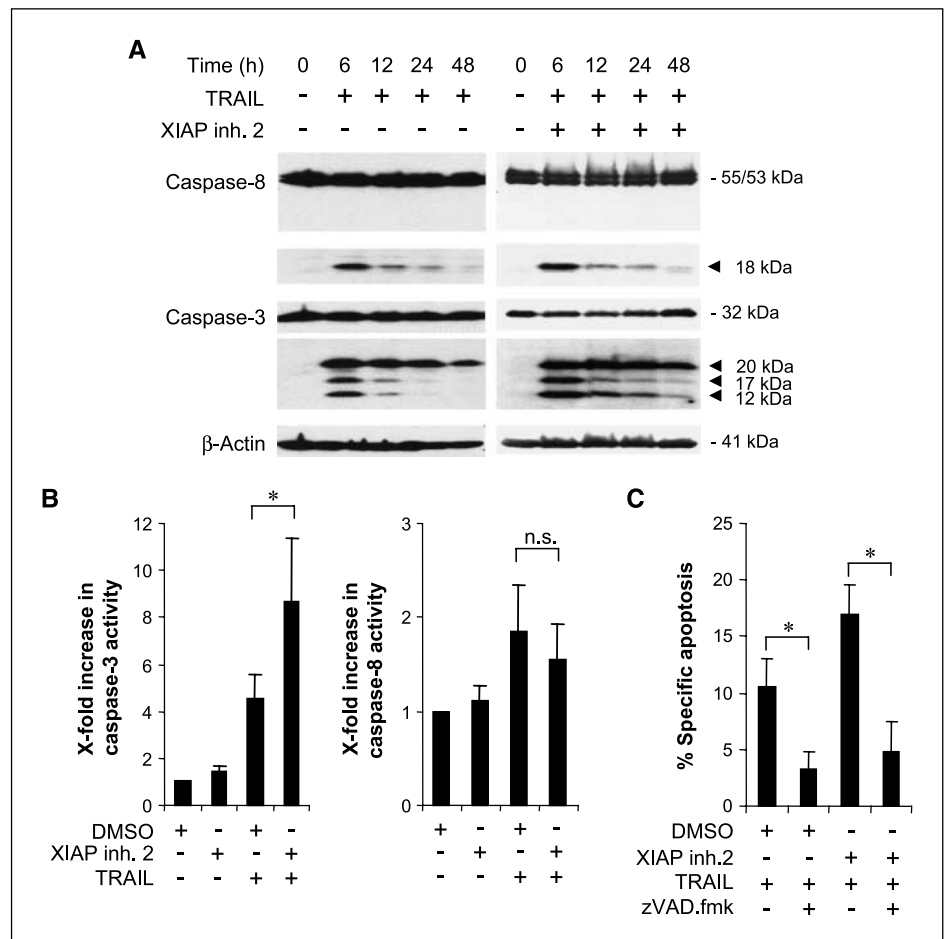
**Figure 1 Continued.** C and D, MEC1 cells were treated for 72 h with 100 ng/mL TRAIL and/or 10 μmol/L (C) or indicated concentrations (D) of XIAP inhibitors, control compound, or DMSO. Apoptosis was determined by FACS analysis of propidium iodide-stained nuclei (C) or by forward/side scatter analysis and flow cytometry (D). A, representative experiment of three independent experiments. B to D, columns, mean of three experiments each performed in triplicate; bars, SD. #,  $P < 0.05$ ; \*,  $P < 0.01$ , comparing XIAP inhibitors to solvent.

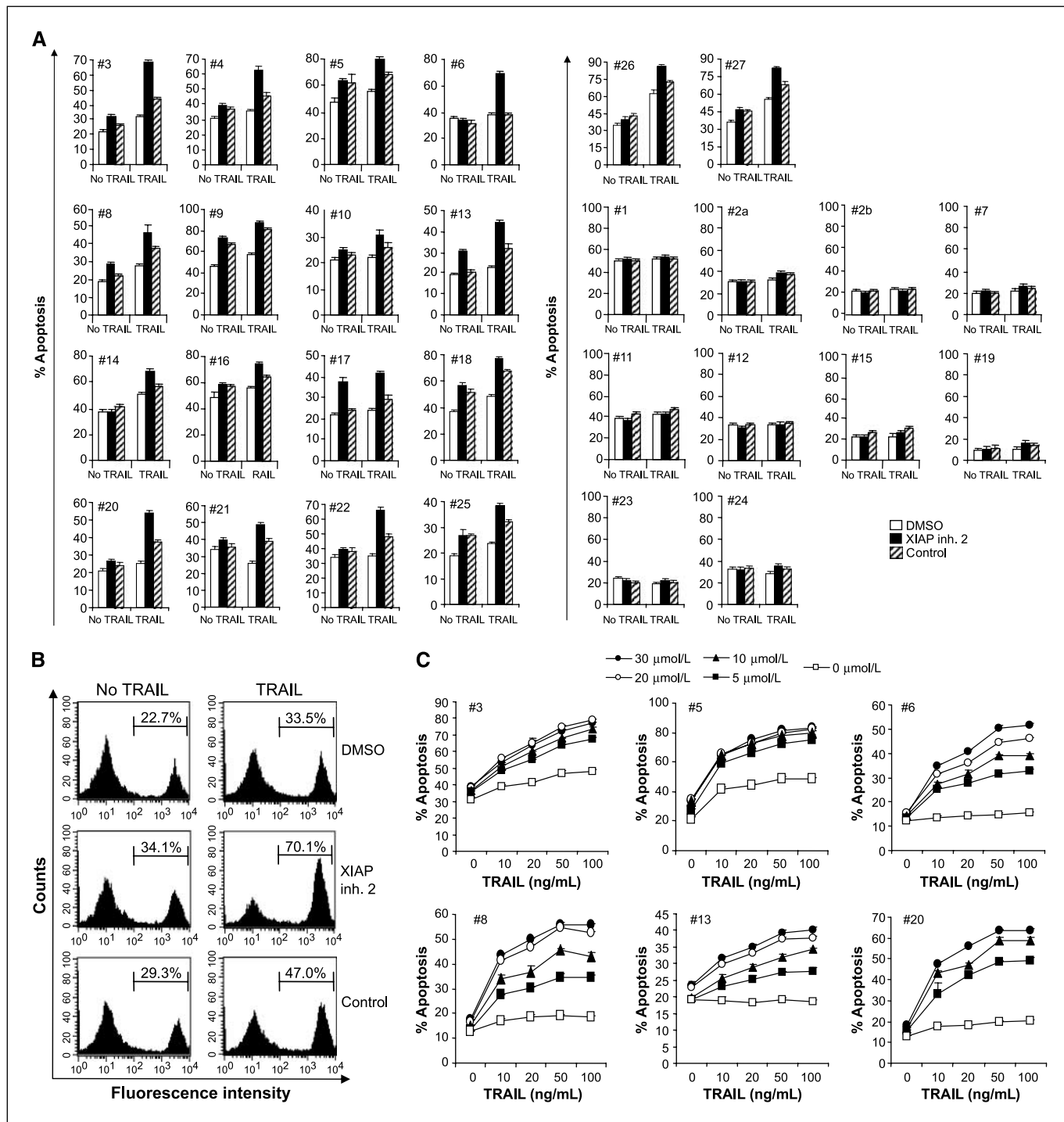
normal B cells that were isolated from healthy donors. Western blot analysis showed similar XIAP protein levels in nonmalignant B cells and CLL cells, whereas cIAP1 protein expression was substantially higher in CLL cells (Fig. 4A). XIAP inhibitors 2 and 4 increased

TRAIL-induced apoptosis to some extent in normal B cells, although less than in most CLL samples (Fig. 4B, compare Fig. 3A and C).

**XIAP inhibitor cooperates with TRAIL to induce caspase-3 activation and caspase-dependent apoptosis in primary CLL**

**Figure 2.** XIAP inhibitor enhances TRAIL-induced activation of caspases in CLL cell lines. A and B, MEC1 cells were treated for indicated times with 100 ng/mL TRAIL and/or 10 μmol/L (A) or 30 μmol/L (B) XIAP inhibitor 2. A, caspase activation was analyzed by Western blotting. Arrowheads, caspase cleavage fragments. A representative experiment of three independent experiments is shown. B, caspase activity was determined by FACS analysis using rhodamine-conjugated caspase substrates (zDEVD-R110 for caspase-3 and zIETD-R110 for caspase-8). Columns, mean fold increase in caspase activity of three independent experiments performed in triplicate; bars, SD. \*,  $P < 0.01$ . C, MEC1 cells were treated for 72 h with 100 ng/mL TRAIL and/or 10 μmol/L of XIAP inhibitor 2 or DMSO in the presence or absence of 20 μmol/L zVAD.fmk. Apoptosis was determined by forward/side scatter analysis and flow cytometry. Columns, mean of three experiments each performed in triplicate; bars, SD. \*,  $P < 0.01$ .





**Figure 3.** XIAP inhibitors sensitize primary CLL cells for TRAIL-induced apoptosis. **A**, primary CLL cells were treated *ex vivo* for 24 h (1, 2a, 2b, 5, 7, 10, 11, 12, 14, 15, 16, 18, 19, 20, 22, 23, 24, 25, 26, and 27) or for 48 h (3, 4, 6, 8, 9, 13, 17, and 21) with 100 ng/mL TRAIL and/or 30  $\mu$ mol/L of XIAP inhibitor 2, control compound, or DMSO. Apoptosis was determined by forward/side scatter analysis and flow cytometry. *Columns*, mean of one experiment performed in triplicate; *bars*, SD. Samples 2a and 2b were isolated from the same patient at an interval of 6 mo. **B**, primary CLL cells (patient 3) were treated *ex vivo* for 24 h with 100 ng/mL TRAIL and/or 30  $\mu$ mol/L of XIAP inhibitor 2, control compound, or DMSO. Apoptosis was determined by Annexin V staining. The percentage of Annexin V–positive cells is indicated. **C**, primary CLL cells were treated for 24 h with indicated concentrations of TRAIL and/or indicated concentrations of XIAP inhibitor 2. Apoptosis was determined by forward/side scatter analysis and flow cytometry. *Points*, mean of one experiment performed in triplicate; *bars*, SD.

cells. Mechanistic studies revealed that XIAP inhibitor 2 acted in concert with TRAIL to trigger cleavage of caspase-3 into active fragments p17 and p12 (Fig. 5A). Additionally, we assessed caspase-3 activity by an enzymatic assay. XIAP inhibitor 2 cooperated with TRAIL to trigger caspase-3 activity in CLL samples

that also underwent apoptosis on the combination treatment, whereas no increase in caspase activity was found in unresponsive cases (Fig. 5B; Supplementary Fig. S6), pointing to a link between caspase activation and apoptosis induction. Kinetic studies showed that caspase-3 activity was increased over several hours

on treatment with XIAP inhibitor 2 and TRAIL compared with either treatment alone (Fig. 5C). To directly test the requirement of caspases for apoptosis induction, we used the broad-range caspase inhibitor zVAD.fmk. Addition of zVAD.fmk almost completely inhibited apoptosis on treatment with XIAP inhibitor 2 and TRAIL, showing that apoptosis occurred in a caspase-dependent manner (Fig. 5D; Supplementary Fig. S7). Of note, the cooperative effect of XIAP inhibitor 2 and TRAIL to trigger proteolytic activation of caspase-3, enhanced caspase-3 activity, and caspase-dependent apoptosis was even observed in a patient with 17 deletion (patient 16 in Fig. 5A–C), the subgroup with the worst prognosis (36), and occurred irrespective of high Bcl-2 expression in primary CLL cells (Supplementary Fig. S8). Together, this set of experiments shows that the XIAP inhibitor acts in concert with TRAIL to induce caspase-3 activation and caspase-dependent apoptosis in primary human CLL cells even in subgroups with unfavorable prognosis or resistant disease.

## Discussion

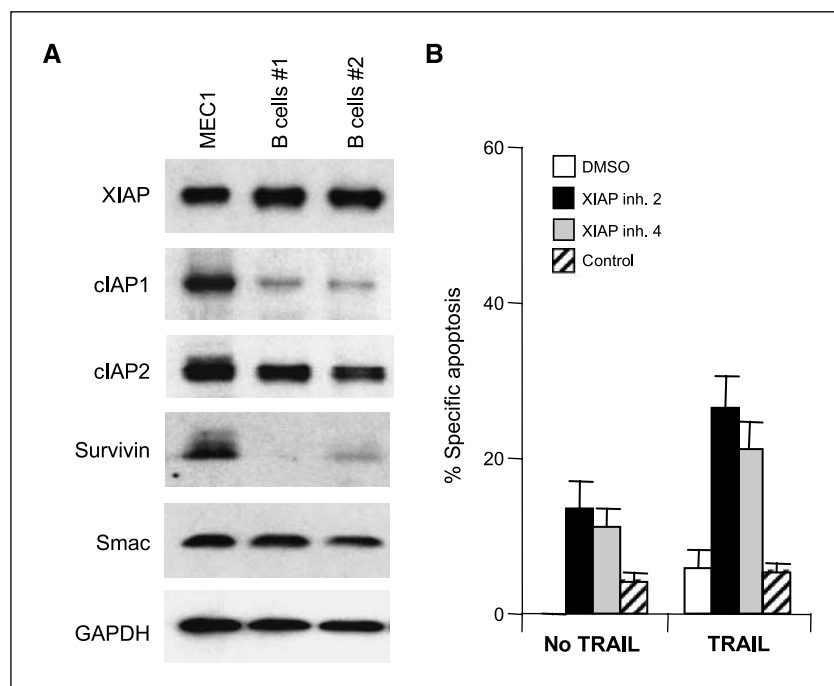
Defective apoptosis is an inherent feature of CLL and contributes to the accumulation of malignant monoclonal B cells as well as to treatment resistance (2, 3). Thus, novel approaches are required to restore apoptosis programs in CLL.

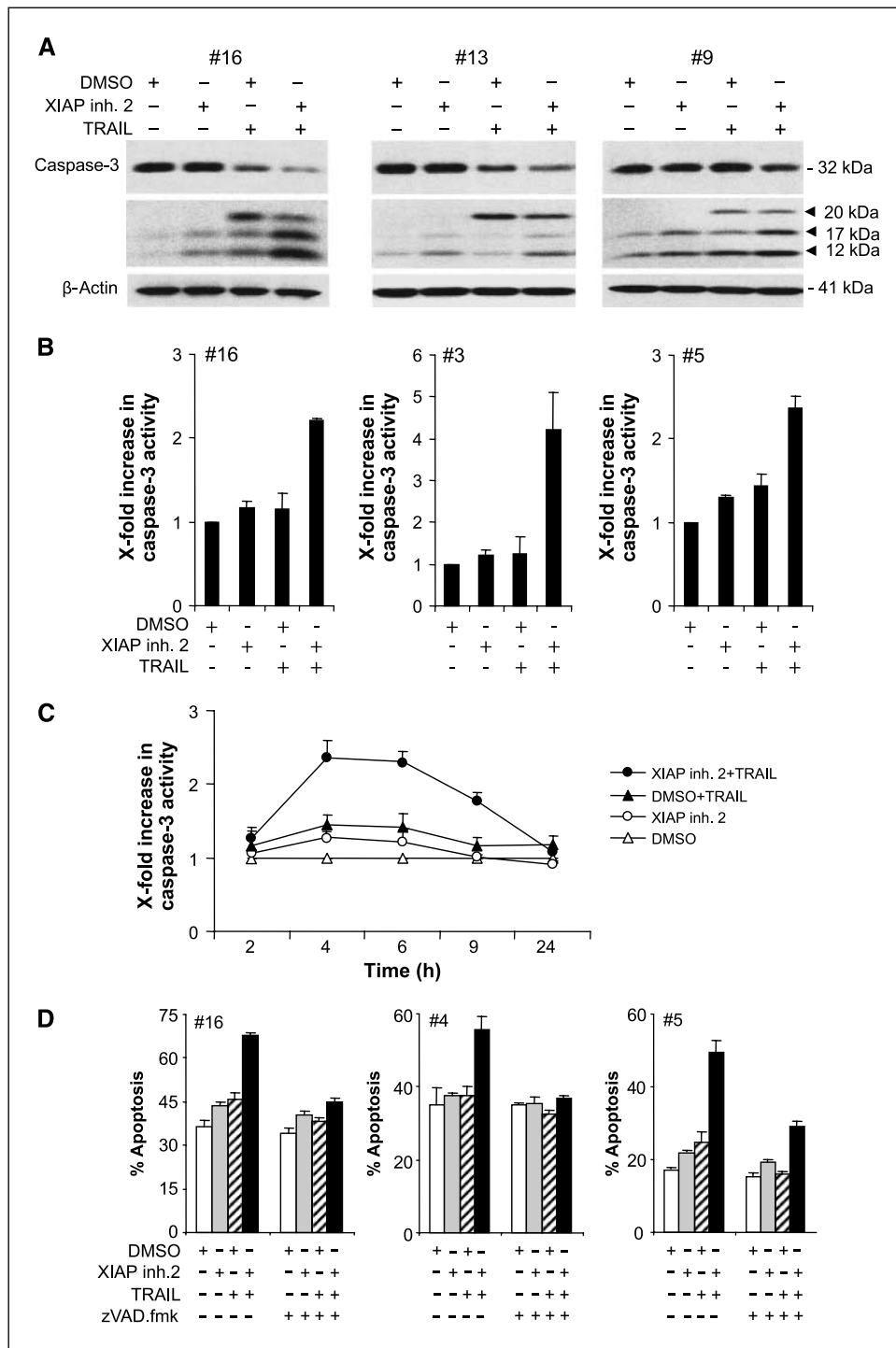
**XIAP inhibitors sensitize CLL cells to TRAIL-induced apoptosis.** Here, we provide for the first time evidence that small-molecule XIAP inhibitors in combination with the death receptor ligand TRAIL present a promising new strategy to trigger apoptosis in CLL, including poor prognostic and chemotherapy-resistant subgroups. This conclusion is supported by data obtained in a large panel of primary CLL samples showing that the XIAP inhibitor cooperates with TRAIL to induce apoptosis in 18 of 27 (67%) primary CLL cases. The mechanistic basis for this cooperative interaction was found to involve increased caspase-3 activation and caspase-dependent cell death. This is consistent with the concept that inhibition of XIAP relieves the

XIAP-imposed break on caspase-3 activation, which in turn promotes the effector phase of apoptosis and cell death execution (18, 19).

It is particularly worth to note that the cooperative interaction of XIAP inhibitor and TRAIL was observed in different subgroups of patients with unfavorable prognosis (36), including all four investigated patients with 17p deletion, the subgroup with the worst outcome (31), as well as cases with TP53 mutation, chemotherapy-resistant disease, or unmutated  $V_H$  genes. This suggests that the combination of XIAP inhibitor and TRAIL may be effective in at least some resistant forms of CLL. Because TRAIL has previously been described to trigger apoptosis independently of p53 (8), TRAIL in combination with XIAP inhibitors may present an alternative strategy to induce cell death in cases with TP53 mutation instead of DNA-damaging chemotherapeutics, which rely on intact p53 for cell death execution. Interestingly, our study reveals that CLL cases with unmutated  $V_H$  genes, a feature linked to poor prognosis (1), were significantly more sensitive to XIAP inhibitor-induced and TRAIL-induced apoptosis compared with those with mutated  $V_H$  genes. This finding is in line with the concept that CLL cells with unmutated  $V_H$  genes are more prone to respond not only to survival but also to apoptotic stimuli, and points to a role of B-cell receptor signaling in the regulation of apoptosis (1). We observed no differences in Bcl-2 expression or the phosphorylation status of Akt or extracellular signal-regulated kinase between cases with unmutated  $V_H$  genes, which responded to XIAP inhibitor and TRAIL, and nonresponsive cases with mutated  $V_H$  genes (data not shown). It therefore remains to be determined in future studies which signal transduction events are involved in the regulation of apoptosis sensitivity in CLL cells with unmutated  $V_H$  genes. Nevertheless, the IgVH mutation status may serve as a biomarker to predict the responsiveness of CLL patients toward the combination therapy with XIAP inhibitor and TRAIL, a hypothesis to be confirmed in a larger group of CLL patients.

**Figure 4.** Effect of XIAP inhibitors and TRAIL on normal B cells. Primary B cells were isolated from buffy coats of healthy donors. **A**, protein expression of XIAP, cIAP1, cIAP2, survivin, Smac, and  $\beta$ -actin was assessed in B cells from two different donors (*B cells #1* and *B cells #2*) by Western blotting, and MEC1 cells served as positive control. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. **B**, B cells were treated for 24 h with 100 ng/mL TRAIL and/or 30  $\mu$ mol/L of XIAP inhibitor 2 or 4 or DMSO. Apoptosis was determined by forward/side scatter analysis and flow cytometry. *Columns*, mean of two independent experiments performed in triplicate with B cells of two individual donors; *bars*, SD.





**Figure 5.** XIAP inhibitor acts in concert with TRAIL to induce caspase-3 activation and caspase-dependent apoptosis in primary CLL cells. Primary CLL cells were treated with 100 ng/mL TRAIL and/or 30 μmol/L of XIAP inhibitor 2 or DMSO. **A**, cleavage of caspase-3 after treatment for 6 h (#9), 9 h (#16), or 24 h (#13) was determined by Western blot analysis. *Arrowheads*, caspase cleavage fragments. **B**, caspase-3 activity was assessed after treatment for 3 h (#3) or 4 h (#5 and #16) by enzymatic caspase assay and x-fold increase in caspase-3 activity is shown. **C**, primary CLL cells from patient 5 were treated with 100 ng/mL TRAIL and/or 30 μmol/L of XIAP inhibitor 2 or DMSO and caspase-3 activity was assessed at indicated times. X-fold increase in caspase-3 activity is shown. **D**, apoptosis after treatment for 24 h in the presence or absence of 20 μmol/L zVAD.fmk was determined by forward/side scatter analysis and flow cytometry. **B** to **D**, columns, mean of one experiment performed in triplicate; bars, SD.

High expression of XIAP has previously been reported in CLL, possibly as part of a NF-κB-regulated survival program (20–22). In addition, XIAP has recently been implicated in apoptosis resistance that is imposed by constitutively activated Notch signaling in CLL cells (37), further indicating that XIAP may be a relevant target in CLL. However, the functional relevance of XIAP in the resistance of CLL toward TRAIL has not yet been explored. Thus, the present study is the first report that inhibition of XIAP by small molecules sensitizes CLL cells to TRAIL-induced apoptosis. Evasion of TRAIL-mediated apoptosis in CLL has previously been

linked to low TRAIL receptor expression in one study (13), whereas constitutive expression of both TRAIL-R1 and TRAIL-R2 and no correlation between agonistic TRAIL receptors and the sensitivity of CLL cells to TRAIL were observed in other studies (14, 15). Further, high levels of the antiapoptotic death effector domain-containing proteins c-FLIP and PED were reported to interfere with activation of caspase-8 at the receptor signaling complex in CLL (13, 15, 16). Moreover, impaired mitochondrial signaling because of high expression levels of Bcl-2 has been implicated in apoptosis resistance in CLL (38, 39). To this end, it is important to

note that the combination of XIAP inhibitor and TRAIL triggered apoptosis in primary CLL cells irrespective of high Bcl-2 levels, a characteristic feature of CLL (38, 39). These findings are in line with our recent results that XIAP inhibitors and TRAIL bypass Bcl-2-mediated resistance to TRAIL by directly triggering the effector phase of apoptosis (40).

Of note, our results also show that apoptosis sensitization by XIAP inhibitors is not restricted to TRAIL and extends to CD95 as another death receptor system. This suggests that the neutralization of XIAP more broadly enhances the susceptibility of CLL cells toward death receptor-mediated apoptosis. This notion is further supported by a report showing that phenylurea-based compounds that target the BIR2 domain of XIAP enhance the sensitivity of CD40-stimulated CLL cells to CD95-triggered apoptosis (41). Compared with death receptor stimulation via CD95 or TRAIL receptors, we observed no sensitization for apoptosis on treatment with commonly used chemotherapeutic agents such as fludarabine or chlorambucil. Along these lines, Silva and colleagues reported that the expression of XIAP, cIAP1, or cIAP2 in CLL cells did not correlate with sensitivity to fludarabine-induced apoptosis (42). Although the underlying mechanisms of this differential sensitization for death receptor-mediated versus chemotherapy-mediated cell death have not yet been identified, these findings indicate that inhibition of XIAP may be particularly promising to lower the threshold for death receptor-mediated apoptosis in CLL cells, which may also have implications for immune-mediated tumor control. Compared with CLL cells, nonmalignant B cells showed a lower susceptibility to the combined treatment with XIAP inhibitors and TRAIL. The molecular basis for this differential sensitivity is subject to future investigations.

Beyond CLL, XIAP inhibitors have recently been reported by our group and other investigators to act in concert with TRAIL to trigger apoptosis in childhood acute leukemia, pancreatic, colon, or breast carcinoma cells (29, 43, 44). In addition, low-molecular weight Smac mimetics displayed antitumor activities as single agents in multiple myeloma and several solid cancers (45–49). These results highlight the therapeutic potential of small-molecule XIAP inhibitors to promote apoptosis in cancer cells. Because IAP inhibitors and TRAIL receptor agonists are currently under evaluation in early clinical trials (10, 18), it is crucial to identify those cancers that may benefit from these new treatment options. As the present study indicates that the combination of XIAP inhibitors and TRAIL presents a new approach to bypass apoptosis resistance in CLL, our findings have important implications for the development of apoptosis-targeted therapies in CLL.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 7/13/09; revised 8/21/09; accepted 9/25/09; published OnlineFirst 11/17/09.

**Grant support:** Deutsche Forschungsgemeinschaft, Deutsche Krebshilfe, European Union (ApopTrain, APO-SYS), IAP6/18 (S. Fulda), and Else-Kröner-Fresenius Stiftung (S. Stilgenbauer, T. Zenz).

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We thank C. Hulford and M. Luzzato (Pfizer, Inc., Groton, CT) for providing XIAP inhibitors.

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