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Novel X-linked inhibitor of apoptosis inhibiting compound as sensitizer for TRAIL-mediated apoptosis in chronic lymphocytic leukaemia with poor prognosis

Lukas P. Frenzel,[1–3] Michaela Patz,[1] Christian P. Pallasch,[1–4] Reinhild Brinker,[1] Julia Claasen,[1] Alexandra Schulz,[1] Michael Hallek,[1–3] Hamid Kashkar[3,5]* and Clemens-Martin Wendtner[1–3]*

[1]Department I of Internal Medicine, [2]Centre of Integrated Oncology, [3]Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases, University of Cologne, Cologne, Germany, [4]David H. Koch Institute for Integrative Cancer Research, MIT, Cambridge, MA, USA, and [5]Institute for Medical Microbiology and Immunology, and Centre for Molecular Medicine Cologne, University of Cologne, Cologne, Germany

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Correspondence: Lukas P. Frenzel, MD, Department I of Internal Medicine, Center for Integrated Oncology Cologne Bonn, University of Cologne, D-50937 Cologne, Germany.
E-mail: Lukas.frenzel@uk-koeln.de
*These authors contributed equally to this work.

Summary

Given that aggressive DNA damaging chemotherapy shows suboptimal efficacy in chronic lymphocytic leukaemia (CLL), alternative therapeutic approaches are needed. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is able to induce tumour-specific apoptosis. However, apoptosis might be inhibited by elevated levels of X-linked inhibitor of apoptosis (XIAP). Use of XIAP-inhibiting compounds might sensitize primary CLL cells towards TRAIL-mediated apoptosis. A novel small molecule, compound A (CA), an inhibitor of XIAP, was used in combination with TRAIL to induce apoptosis in primary CLL cells ($n = 48$). XIAP was significantly more highly expressed in primary CLL cells ($n = 28$) compared to healthy B cells ($n = 16$) ($P = 0{\cdot}02$). Our data obtained by specific knock-down of XIAP by siRNA identified XIAP as the key factor conferring resistance to TRAIL in CLL. Combined treatment with CA/TRAIL significantly increased apoptosis compared to untreated ($P = 8{\cdot}5 \times 10^{-10}$), solely CA ($P = 4{\cdot}1 \times 10^{-12}$) or TRAIL treated ($P = 4{\cdot}8 \times 10^{-10}$) CLL cells. CA rendered 40 of 48 (83·3%) primary CLL samples susceptible to TRAIL-mediated apoptosis. In particular, cells derived from patients with poor prognosis CLL (ZAP-70$^+$, *IGHV* unmutated, 17p-) were highly responsive to this drug combination. Our highly-effective XIAP inhibitor CA, in concert with TRAIL, shows potential for the treatment of CLL cases with poor prognosis and therefore warrants further clinical investigation.

Keywords: chronic lymphocytic leukaemia, second mitochondria-derived activator of caspase-mimetic, X-linked inhibitor of apoptosis, tumour necrosis factor-related apoptosis-inducing ligand, therapy.

The disease course of chronic lymphocytic leukaemia (CLL) is heterogeneous. While patients with a good prognosis can be managed effectively, although not cured, with conventional chemotherapy or antibody-based chemo(immuno)therapy, high-risk patients often suffer early disease progression and death. Patients with poor prognosis are defined as ZAP-70$^+$, *IGHV* unmutated, CD38$^+$ and/or carry deletions on chromosomes 11q and/or 17p, are grouped in Binet C stage or show an early relapse after treatment (Binet *et al*, 2006). For this reason, novel therapeutic strategies are needed to further refine the treatment options for patients and improve the efficacy by exploiting the specific biology of CLL.

The consensus based on previous experimental and clinical studies of CLL cells is that the abnormal accumulation of malignant monoclonal B cells in patients is largely attributed to defective apoptosis programmes rather than aberrant proliferation. With the discovery of the death receptors the opportunity has arisen to directly trigger apoptosis externally of tumour cells, thereby circumventing the intrinsic apoptotic machinery, which is mainly triggered by conventional chemotherapeutic treatments. In particular, tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) has recently received the most attention because of its unique property to selectively kill malignant tumour cells but not healthy cells. Recombinant

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TRAIL or monoclonal antibodies targeting TRAIL receptors are currently being evaluated in early clinical trials (Ashkenazi, 2008; Johnstone *et al*, 2008). Human TRAIL can bind to five different receptor molecules. TRAIL-R1 and -R2 contain a cytoplasmic so-called death domain that is capable of recruiting and promoting caspase-8 activation, which, in turn, activates downstream executioner caspases, such as caspase-3. The proteolytic activity of caspase-3 can be inhibited by X-linked inhibitor of apoptosis (XIAP) that directly binds caspase-3 (Eckelman *et al*, 2006). Therefore, for effective death receptor-mediated killing in cells with XIAP expression there is an essential need to disrupt the mitochondrial outer membrane and release the second mitochondria-derived activator of caspase (SMAC) which can directly bind and antagonize XIAP, thereby enabling the enrichment of the critical level of effector caspase activity (Du *et al*, 2000; Jost *et al*, 2009).

Although TRAIL receptors are constitutively expressed, CLL cells have been previously shown to be resistant to TRAIL treatment (Olsson *et al*, 2001; MacFarlane *et al*, 2002; Secchiero *et al*, 2005). However, the underlying molecular mechanisms giving rise to the resistance to TRAIL in CLL cells are still undetermined. Given its role in TRAIL-induced apoptosis and its elevated expression in CLL cells, this study aimed to inhibit XIAP in order to overcome the TRAIL-resistance in CLL. One strategy for targeting XIAP involves agents that mimic the amino terminus of SMAC and thus block critical XIAP/caspase interactions. Here we used a cell permeable small molecule XIAP antagonist, compound A (CA), which was developed based on the crystal structure of the four amino acids of SMAC which enable SMAC to efficiently bind the BIR3 domain of XIAP. In contrast to the active compound CA, which consists of an amino terminal methyl alanine, the inactive compound CB used in our studies as a negative control has an amino terminal methyl glycine. This specific substitution results in a significant reduction of IAP binding capability of CB as CA has binding affinity to XIAP in the picomolar range and CB is a weak binder with micromolar binding affinity to XIAP (Chai *et al*, 2000; Vince *et al*, 2007).

Materials and methods

Patients and cells

After informed consent was given, blood was obtained from patients fulfilling the diagnostic criteria for CLL. Only patients without prior therapy or patients who had a period of at least 6 months since their last chemotherapy were included in this study. Fresh CLL samples were enriched by applying B-RosetteSep (StemCell Technologies, Vancouver, BC, USA) and Ficoll- Hypaque (Seromed, Berlin, Germany) density gradient purification, resulting in purity of more than 98% of CD19/CD5 CLL cells (Pallasch *et al*, 2008a). Control cells were isolated from healthy blood donors using untouched depletion (naive B-cell isolation kit II; Miltenyi, Bergisch-Gladbach, Germany) resulting in at least 90% purity of B cells. Isolated cells were cultured as previously described (Pallasch *et al*, 2008a, 2009). This study was approved by the ethics committee of the University of Cologne and blood samples were given with informed consent according to the Helsinki protocol.

Transient XIAP knock-down

Transient gene knock-down of XIAP was achieved with 1 μg of the specific ON-TARGETplus SMARTpool siRNA (Dharmacon, Lafayette, CO, USA). The non-targeting ON-TARGETplus siCONTROL pool was used as negative control. Program U-013 was used with 100 μl Solution V for nucleofection of $1{\cdot}2 \times 10^7$ primary CLL cells with siRNA (Lonza, Cologne, Germany). Two days after transfection protein lysates were prepared and cytotoxicity assays were performed.

Treatment of cells

Small molecule active compound A (CA) and the negative control compound B (CB) were dissolved in dimethyl sulfoxide (DMSO) and were applied at a concentration of 100 nmol/l (Tetralogic Pharmaceuticals, Malvern, PA, USA) (Vince *et al*, 2007). Both alanine residues are carbamoylated in the control compound B in comparison to active compound A (Li *et al*, 2004). TRAIL was dissolved in aquaeous buffer and applied in 100 ng/ml concentration (Enzo Life Sciences GmbH, Lörrach, Germany).

Apoptosis

Apoptosis was assessed by flow-cytometry using Annexin-V-fluorescein isothiocyanate (FITC)/7-Aminoactinomycin D (7-AAD) staining (BD, Heidelberg, Germany). In the figures, apoptosis levels are given as Annexin-V positive cells, including also Annexin-V/7-AAD double-positive cells. Apoptosis statistics were normalized to untreated controls.

XIAP protein expression analysis

Cellular lystates were processed with radioimmunoprecipitation assay buffer, sonicated and further blotted onto nitrocellulose membranes. A XIAP-specific monocolonal antibody (clone 48/hILP/XIAP; BD) was used for detection of XIAP protein. Monoclonal antibody against actin (clone C4; Millipore, Schwalbach/Ts., Germany) served as housekeeping gene expression control and was used as an indication of protein loading. Western blot detection and density measurements were preformed on an Odyssey infrared imaging system (Licor, Lincoln, NE, USA).

Assessment of TRAIL receptor expression

Purified primary CLL cells were incubated for 30 min on ice with human IgGs to block unspecific binding and then incubated with phycoerythrin (PE)-conjugated anti-human

TRAIL receptor antibodies (DR4, clone: DJR1; DR5, DJR2-4) (NatuTec, Frankfurt, Germany) or an IgG1-PE isotype control antibody (Beckman Coulter, Krefeld, Germany) for 30 min at 4°C. The samples were washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in PBS and analysed by flow cytometry.

Statistics and data processing

Excel (Microsoft, Redmond, WA, USA) and Sigma-Plot (Stata-Corp, College Station, TX, USA) were applied for statistical analysis; in particular the Student *t*-test and Wilcoxon Ranged Sum Test were used.

Results

Elevated XIAP expression in primary CLL cells

As it is established that primary CLL cells are resistant to TRAIL treatment and in view of the fact that XIAP can efficiently inhibit TRAIL-induced apoptosis, we first examined the XIAP expression level in primary CLL cells compared to B cells from healthy donors. Of note, the first evidence indicating the elevated expression XIAP was derived from our microarray gene expression profiles, showing significantly higher expression of *XIAP* mRNA (2·36-fold; $P = 0{\cdot}0002$) in CLL cells ($n = 8$) in comparison to healthy B cells ($n = 5$) (Pallasch *et al*, 2008b). To confirm this result at the protein level, we performed Western blot analysis on 28 CLL samples and 16 healthy donor B cell samples. Calculation of the ratio between β-actin and XIAP expression revealed a significantly higher XIAP expression (2·22-fold; $P = 0{\cdot}02$) in CLL cells at the protein level (Fig 1). This indicated that XIAP protein was significantly over-expressed in purified primary CLL cells compared to healthy B cells, suggesting XIAP as a possible key target for improving death receptor-induced apoptosis in CLL.

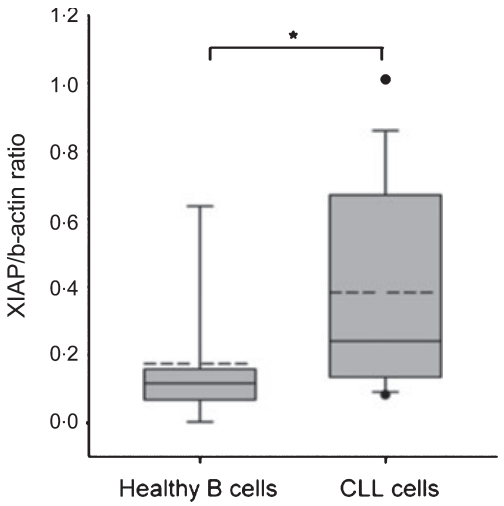

Fig 1. Elevated XIAP expression in primary CLL cells. To determine XIAP expression in 28 primary CLL cell samples and 16 B-cell samples from healthy donors, Western blot was performed with mouse-monoclonal antibody against human XIAP (clone 48/hILP/XIAP; BD). Monoclonal antibody against actin (clone C4; Millipore) served as housekeeping gene expression control and was used as an indication of protein loading. Calculation of the ratio between β-actin and XIAP expression revealed a significantly higher XIAP protein expression (2·22-fold; $P = 0{\cdot}02$) in CLL cells. Each lane was loaded with 30 μg of protein. *Statistics calculated by *t*-test; *$P < 0{\cdot}05$. Dashed lines indicate means; solid lines indicate medians.

XIAP confers resistance to TRAIL-induced cell death in primary CLL cells

The impact of XIAP on inefficient TRAIL-mediated killing was further analysed by specific siRNA-mediated knock-down of XIAP. Efficiency of specific XIAP knock-down was examined by Western blotting and showed efficient down-regulation of XIAP in primary CLL cells transiently transfected with XIAP-specific siRNA but not control siRNA after 48 h (Fig 2A). Additional TRAIL administration for 24 h resulted in significantly higher cell death of CLL cells ($P = 0{\cdot}02$) compared to control siRNA transfected cells (Fig 2B).

Active compound A sensitizes primary CLL cells towards TRAIL-mediated caspase-dependent apoptosis

Based on these analyses it was tempting to speculate that the combination of XIAP inhibition with TRAIL treatment might display an efficient and highly specific treatment for CLL. Therefore we next examined the susceptibility of freshly isolated primary CLL cells from 48 patients towards treatment with 100 nmol/l of XIAP inhibitor CA or its CB as a negative control either alone or in combination with TRAIL. Patients' characteristics are summarized in Table I. Of note, concentrations of up to 100 nmol/l CA or CB showed no direct cytotoxicity to healthy B cells and primary CLL cells (Fig 3 and data not shown). Compared to untreated samples, single TRAIL-treatment resulted in a mean level of apoptotic cells of 6·3% ($P = 0{\cdot}00083$) (Fig 3A). Meanwhile, simultaneous treatment with TRAIL and CA dramatically increased the rate of apoptotic CLL cells with a mean rate of apoptotic cells of 32·8% and an overall response (defined by more than 10% of apoptotic cells) rate of 83·3% (40 of 48 samples) (Table I, Fig 3A). Compared to untreated ($P = 8{\cdot}5 \times 10^{-10}$), solely CA ($P = 4{\cdot}1 \times 10^{-12}$) or TRAIL-treated ($P = 4{\cdot}8 \times 10^{-10}$) CLL cells the apoptosis rate was significantly increased by combined treatment with XIAP inhibitor and TRAIL.

To assess whether TRAIL treatment is CLL-cell specific, healthy B cells ($n = 4$) were exposed to TRAIL alone or CA(CB)/TRAIL and showed significantly lower susceptibility towards CA/TRAIL administration than CLL cells (Fig 3B).

As a potent cellular caspase inhibitor we next examined the involvement of caspases in CA/TRAIL-mediated apoptosis. Not surprisingly, co-application of the pan-caspase inhibitor zVAD.fmk inhibited cell death induced by CA/TRAIL, underscoring the apoptotic caspase-dependent cytotoxicity of CA/

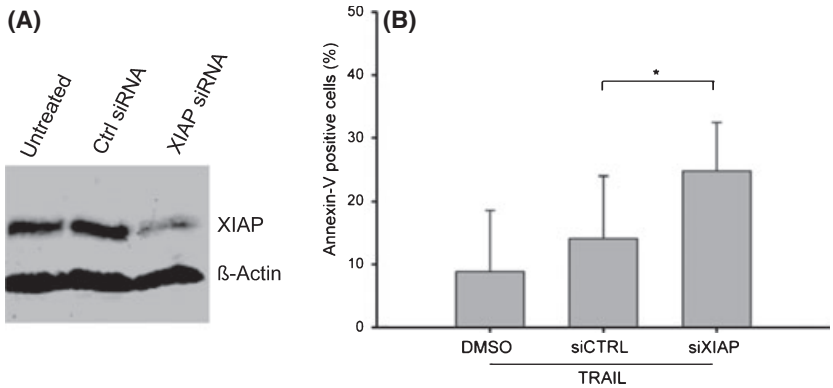

Fig 2. XIAP confers resistance to TRAIL-induced cell death in primary CLL cells. (A) Knock-down efficiency of XIAP was determined by Western blot for XIAP and Actin and shows down-regulation of XIAP in siXIAP samples compared to samples treated with control siRNA. Lysates were prepared 48 h after nucleofection of XIAP-specific and control (Ctrl) siRNAs. Each lane was loaded with 30 μg protein. (B) Knock-down of XIAP by nucleofection of siRNA sensitized primary CLL cells ($1{\cdot}2 \times 10^7$ cells in 100 μl solution V, Lonza, Cologne, Germany) ($n = 4$) to TRAIL. 48 h after nucleofection cells were exposed to TRAIL for additional 24 h. Knock-down of XIAP resulted in significantly higher apoptosis ($P = 0{\cdot}02$). Results are expressed as the mean ± SD of four independent experiments. Note: *Statistics calculated by *t*-test; *$P < 0{\cdot}05$

TRAIL treatment in CLL cells (Fig 3C). Previous studies have shown that, in addition to functional inhibition, IAP antagonists may potentiate caspase activation by directing IAP protein degradation. In order to address this issue we examined the XIAP expression level in primary CLL cells after treatment with CA. As shown in Fig 3D, concentrations up to 100 nmol/l CA or CB did not promote any XIAP protein level reduction, suggesting that the pro-apoptotic effect of CA is a result of the functional antagonization of XIAP. This is in line with a previous report that demonstrated that CA does not promote XIAP protein degradation (Vince *et al*, 2007).

ZAP-70$^+$, IGVH unmutated and CD38$^+$ poor-prognosis patients significantly benefit from CA/TRAIL treatment

CLL patients who are ZAP-70$^+$ and CD38$^+$ and harbour unmutated *IGHV* genes are known to suffer from early disease progression and shorter treatment-free intervals.

Strikingly, ZAP-70$^+$ (13%; $P = 0{\cdot}002$), *IGHV* unmutated (12·6%; $P = 0{\cdot}043$) and CD38$^+$ (11·2%; $P = 0{\cdot}032$) CLL samples were significantly more susceptible to TRAIL compared to ZAP-70$^-$ (2·0%), *IGHV* mutated (3·9%) and CD38$^-$ (4·5%) CLL cells (Fig 4A,B,C).

Interestingly, ZAP-70$^+$ samples ($n = 9$) expressed higher levels of TRAIL-R1 (not significant) and significantly higher levels of TRAIL-R2 ($P = 0{\cdot}0016$) on their surface in comparison to ZAP-70$^-$ ($n = 10$) CLL samples (Fig 4D).

Samples derived from CLL patients with poor-prognostic features were highly susceptible to combined treatment with TRAIL and CA. Co-treatment with CA and TRAIL dramatically increased the mean fraction of apoptotic cells in ZAP-70$^+$ (45·2 vs. 29·2%; $P = 0{\cdot}005$), *IGHV* unmutated (48·8 vs. 31·3%; $P = 0{\cdot}035$) and CD38$^+$ (42 vs. 28·9%; $P = 0{\cdot}09$) cohorts compared to their counterparts (no compound and CB; Fig 3A) (Fig 4A–C).

Poor-prognosis patients show same response to CA/TRAIL application compared to low-risk patients in terms of prior treatment, cytogenetics and Binet stage

In addition, previously treated patients and especially CLL cases with 17q-deletion often do not show durable responses to chemotherapeutic approaches. Samples derived from high-risk patients did not demonstrate significant differences concerning their mean of apoptosis response to the CA/TRAIL regimen in comparison to CLL samples derived from low-risk patients. In detail, 14 of 17 (82·4%) CLL cells from patients who had already received at least one chemotherapy, responded equally compared to leukaemic cells derived from therapy-naive patients (26/31; 83·8%) with respect to combined CA/TRAIL administration (Fig 5A). Also, no significant differences could be detected regarding the mean of apoptotic cells between clinical stage Binet A (31·9%), B (37·6%) and C (24·9%) patients, although the response rate in Binet C group was lowest (9/12; 75%) compared to the Binet A (18/22; 81·8%) and B (13/14; 92·9%) cohorts.

Most importantly, all patients (5/5) with 17q-deletion and four of five patients with 11q- deletion responded to CA/TRAIL treatment with a mean level of apoptosis of 31·7% for 17p-del and 41% for 11q-del patients, which was comparable to those from low-risk patients with normal cytogenetics (36·9%) or 13q deletion (26·4%).

Discussion

Here we describe a potent novel therapeutic option for CLL patients, especially those with poor prognostic features like ZAP-70$^+$, *IGHV* unmutated status, CD38$^+$ and/or 17p-, using highly efficient compound A to inhibit XIAP function and thereby to promote TRAIL-mediated apoptosis in CLL cells, but not in healthy B cells.

Table I. Characteristics of patient samples.

ID	Age (years)	Sex	Treatment	Binet	CD38	ZAP-70	*IGHV*	Cytogenetics	Response
1	59	M	y	C	−	−	m	13q del	23
2	70	M	n	B	−	+	u	Normal	29
3	68	M	y	C	+	+	n.d.	11q del, trisomy 12	16
4	61	F	n	B	n.d.	n.d.	n.d.	n.d.	18
5	69	M	y	C	+	+	u	6q del, 13q del	45
6	66	M	n	C	−	−	m	13q del	0
7	69	M	n	A	n.d.	n.d.	n.d.	n.d.	0
8	62	F	y	C	−	−	m	Normal	24
9	70	F	n	A	+	−	n.d.	Normal	61
10	54	F	y	B	n.d.	n.d.	n.d.	17p del	38
11	51	F	n	B	−	+	n.d.	Normal	47
12	76	M	n	B	−	+	u	Trisomy 12	67
13	45	M	n	A	−	−	n.d.	n.d.	31
14	54	M	y	A	+	−	u	11q del	75
15	63	M	y	C	−	−	n.d.	Normal	0
16	73	M	y	C	+	+	n.d.	n.d.	5
17	56	M	n	A	+	−	m	Normal	33
18	59	F	n	B	−	−	n.d.	n.d.	2
19	65	F	n	A	−	−	n.d.	n.d.	10
20	69	M	n	A	n.d.	−	n.d.	n.d.	0
21	53	M	y	C	+	+	n.d.	Normal	32
22	66	M	y	A	−	−	n.d.	n.d.	23
23	61	M	n	A	−	+	m	Normal	86
24	32	M	n	A	−	−	n.d.	17p del	13
25	69	M	y	B	−	−	m	13q del	51
26	73	M	n	A	−	−	m	Normal	8
27	79	M	n	B	−	+	n.d.	17p del	66
28	46	M	n	A	−	−	m	13q del	17
29	59	M	n	A	n.d.	n.d.	n.d.	13q del	25
30	65	M	y	C	+	+	m	n.d.	82
31	70	M	y	B	−	−	m	11q del, 13q del	17
32	53	M	n	A	+	+	u	13q del	25
33	69	M	y	C	−	+	n.d.	n.d.	33
34	64	M	n	B	+	+	u	11q del	89
35	63	M	y	C	n.d.	−	n.d.	11q del, 13q del	8
36	55	M	n	A	−	+	m	13q del	15
37	61	M	n	B	+	−	m	n.d.	31
38	69	M	n	A	+	−	m	17p del	25
39	56	F	n	B	−	+	u	13q del	19
40	61	F	n	B	+	+	u	13q del	39
41	88	M	n	A	−	−	m	13q del	38
42	84	M	n	A	−	−	u	Normal	60
43	77	F	n	A	+	−	u	13q del	19
44	81	M	n	A	+	+	u	13q del	56
45	68	M	n	A	+	+	u	n.d.	62
46	75	M	y	C	−	−	m	Normal	35
47	90	M	y	B	+	−	m	17p del	17
48	72	M	n	A	−	n.d.	n.d.	n.d.	19

M, male; F, female; y, yes; n, no; m, mutated; u, unmutated; n.d., not determined; del, deletion; response: percentage of apoptotic cells by Annexin-V/7-AAD staining after combined treatment with compound A and TRAIL; for ZAP-70 and CD38 respectively, a 20% (ZAP-70) and 30% (CD38) threshold of positive stained cells was applied for definition of positivity for these prognostic markers.

TRAIL-induced cell death remains a very attractive biologically targeted therapeutic option, because cancer cells are killed while normal cells are not affected. In fact, TRAIL administration in phase I and II trials with monoclonal antibodies against TRAIL-R1, -R2 or with recombinant TRAIL were shown to be safe and well tolerated (Plummer *et al*, 2007;

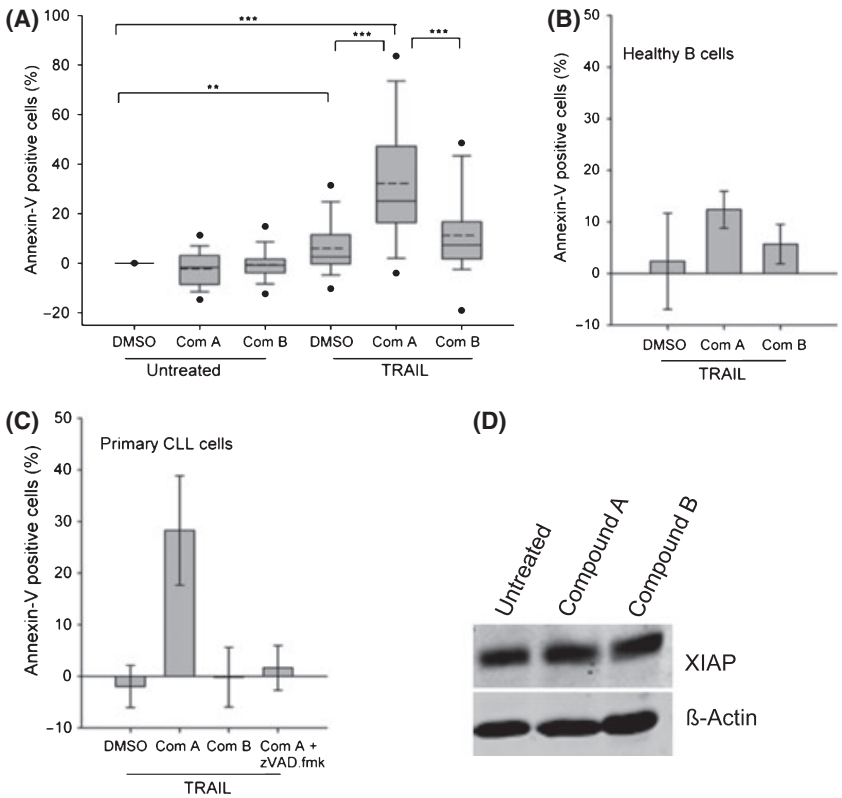

Fig 3. Compound A sensitizes primary CLL cells towards TRAIL-mediated caspase-dependent apoptosis. (A) Primary CLL cells from 48 different patients were treated for 24 h with 100 nmol/l compound A (CA) or negative control compound B (CB) and/or 100 ng/ml TRAIL. Apoptosis was significantly increased in CA/TRAIL treated compared to solely CA, TRAIL or untreated samples Dashed lines indicate means; solid lines indicate medians. (B) Healthy B cells ($n = 4$) are significantly weaker lysed by CA/TRAIL application than CLL cells. (C) Pan-caspase inhibitor zVAD.fmk restores survival in primary CLL cells ($n = 4$) after 24 h CA/TRAIL treatment. Results are expressed as mean ± SD of at least four independent experiments (for B+C). (D) Primary CLL cells were treated for 24 h with 100 nmol/l of compounds A and B, because this time-point was also chosen for measurement of apoptosis. Lysates were prepared after 24 h. Each lane was loaded with 30 μg protein. Note: *Statistics calculated by *t*-test; $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}001$; $^{***}P < 0{\cdot}0001$.

Greco *et al*, 2008; Soria *et al*, 2010; Trarbach *et al*, 2010). However, the benefit of these approaches has been critically discussed, because many primary cancers exhibit distinct resistance mechanisms against TRAIL-induced cell death in contrast to cell-lines, which are often more susceptible (Dyer *et al*, 2007). Primary CLL cells show low susceptibility towards TRAIL apoptosis (MacFarlane *et al*, 2002; Loeder *et al*, 2009). However, this paradigm does not seem to be the case for all CLL samples. Comparing ZAP-70$^+$ *versus* ZAP-70$^-$ (*IGHV* unmutated *versus IGHV* mutated; CD38$^+$ *versus* CD38$^-$) samples, we report here for the first time, that poor-prognosis samples (ZAP-70$^+$, *IGHV* unmutated, CD38$^+$) were significantly more susceptible to TRAIL-mediated apoptosis than their counterparts.

The reason for this observation might be the slightly higher expression of TRAIL-R1 and the significantly enhanced expression of TRAIL-R2 in ZAP-70 positive *versus* ZAP-70 negative CLL samples ($P = 0{\cdot}0016$). TRAIL mediates apoptosis in CLL cells via TRAIL-R1 and -R2 (Natoni *et al*, 2007). Initially it was thought that CLL cells exhibit apoptotic signalling via TRAIL-R1 after CLL cells were sensitized by HDAC1 inhibitors (MacFarlane *et al*, 2005). However, this result might be due to the efficacy of different TRAIL-R1 and -R2 agonistic antibodies and recombinant TRAIL, as supported by the observation that CLL cells are also efficiently killed by TRAIL-R2 agonistic antibodies after cross-linking (Natoni *et al*, 2007). Here we used recombinant TRAIL, which did not require cross-linking to induce apoptosis in CLL cells. However, further studies need to determine why ZAP-70$^+$ CLL samples express higher levels of TRAIL-R1 and -R2 and whether the higher expression and/or the ZAP-70$^+$ status is responsible for higher apoptosis by TRAIL.

Given that death-inducing signalling complex (DISC) formation in CLL cells – although at low level – is mediated by TRAIL (MacFarlane *et al*, 2002), further mechanisms might be involved in low susceptibility of CLL cells towards TRAIL-induced apoptosis. Because CLL is known to show general anti-apoptotic properties, we hypothesized that effector caspases-3/-9 might be inhibited by their major inhibitor XIAP. Indeed, analyses performed with peripheral blood mononuclear cells from CLL patients suggested an elevated XIAP expression in CLL cells from patients with progressive

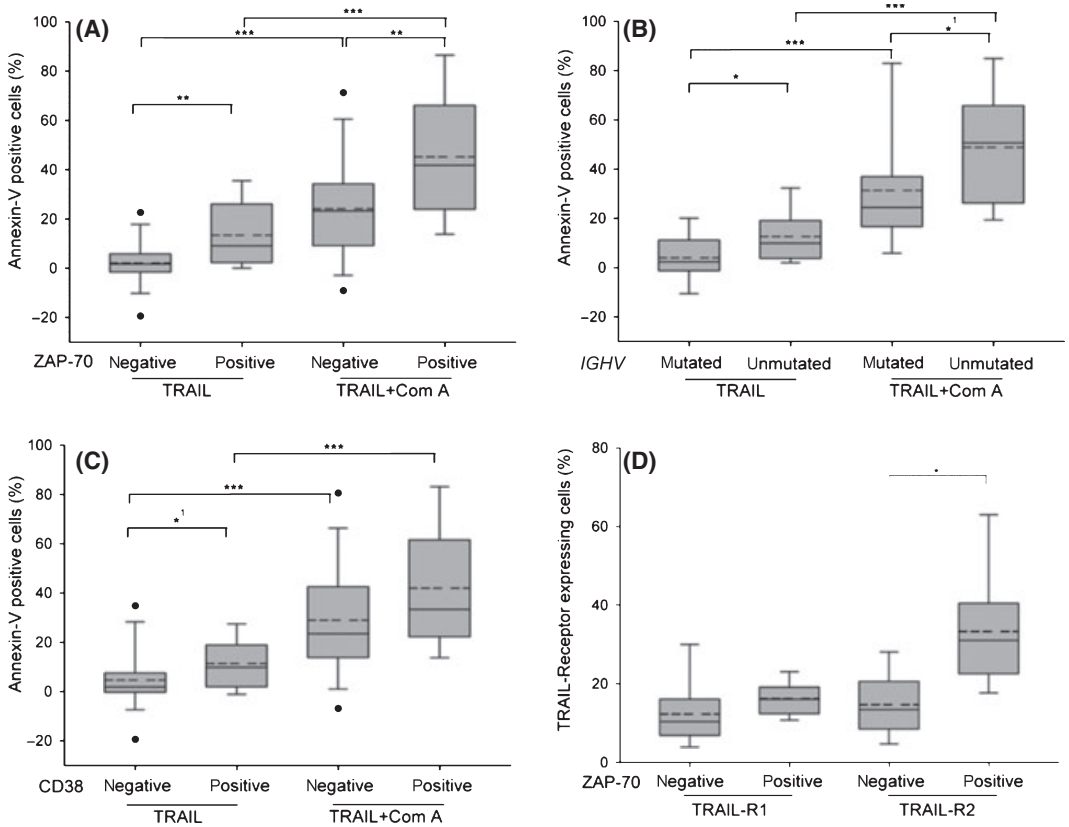

Fig 4. ZAP-70$^+$, *IGHV* unmutated and CD38$^+$ poor-prognosis patients significantly benefit from compound A/TRAIL treatment. (A) ZAP-70 positive samples showed significantly higher rates of apoptosis either by TRAIL alone or by compound A/TRAIL co-treatment compared to ZAP-70 negative CLL patients. (B) Samples with unmutated *IGHV* showed significantly higher apoptosis rates either by TRAIL alone or by compound A/TRAIL co-treatment compared to CLL patients with mutated *IGHV* genes. (C) TRAIL treatment alone resulted in CD38 positive individuals in significantly higher apoptosis than in CD38 negative samples. Addition of XIAP inhibitor to TRAIL induced significantly higher apoptosis in both CD38 negative and positive samples. (D) ZAP-70$^-$ (n = 10) and ZAP-70$^+$ (n = 9) CLL samples were stained with PE-conjugated TRAIL-R1 (clone: DJR1) and -R2 antibodies (clone: DJR2-4) and percentage of positive cells was assessed by flow-cytometry. Cells from ZAP-70$^+$ CLL donors express higher levels of TRAIL-R1 (not significant) and -R2 (P = 0·0016) compared to ZAP-70$^-$ CLL donors on their surface. Note: *Statistics calculated by t-test; *^{1}Statistics calculated by Wilcoxon Ranged Sum Test; */*$^1P < 0{\cdot}05$; **$P < 0{\cdot}001$; ***$P < 0{\cdot}0001$ Dashed lines indicate means; solid lines indicate medians.

disease, whereas those with stable disease had decreased XIAP protein level compared to lymphocytes from healthy donors (Grzybowska-Izydorczyk *et al*, 2010). Our analyses of XIAP expression however, revealed a significantly elevated expression of XIAP in purified, primary CLL cells versus non-malignant B-cells isolated from several healthy donors. Although not quantified, Loeder *et al* (2009) also showed different expression patterns of XIAP between CLL cells and normal B cells by Western Blot. Two prior studies that investigated XIAP expression at the transcript level did not detect significant differences between CLL cells and healthy donor B cells (Munzert *et al*, 2002; de Graaf *et al*, 2005). Moreover, Munzert *et al* (2002) revealed that *TRAF1* was the only nuclear factor-kappa B-regulated antiapoptotic gene that was significantly over-expressed in CLL, but did not correlate with markers of disease progression or overall survival. Furthermore, de Graaf *et al* (2005) compared apoptosis-regulating genes and IAP family members by quantitative reverse transcription polymerase chain reaction between different lymphoid malignancies. In CLL, XIAP expression was highest compared to the other malignant entities and to B cells from hyperplastic tonsils. Peripheral B cells showed similar levels of *XIAP* transcript in comparison to CLL cells (de Graaf *et al*, 2005). In our study, specific knock-down of XIAP by siRNA significantly enhanced TRAIL-induced apoptosis identifying XIAP as the key factor establishing resistance to TRAIL and further underscored the impact of XIAP as a therapeutic target for improving death receptor-induced apoptosis in CLL.

These results encouraged us to use CA in order to inhibit XIAP function. Single treatment with CA did not induce any toxicity in primary CLL cells, healthy B cells and PBMCs. However, low-dose CA was highly sufficient to render CLL cells, in 40 of 48 (83·3%) cases, susceptible towards caspase-dependent TRAIL-induced cell death. Our data are in line with several recent reports demonstrating the synergistic effect of XIAP antagonizing protocols with TRAIL in different tumour

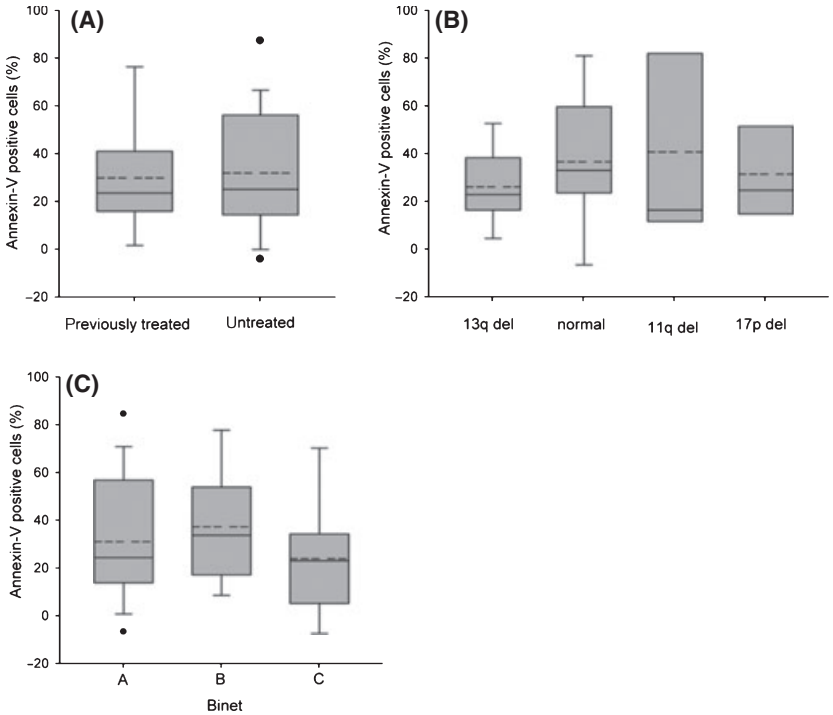

Fig 5. Poor-prognosis patients show same response to compound A/TRAIL application compared to low-risk patients in terms of prior treatment, cytogenetics and Binet stage. No significant differences are detectable in terms of apoptosis between (A) patients with prior chemotherapy and untreated patients, (B) patients with different cytogenetic feature and (C) patients with different clinical stages (Binet) of CLL. There is no statistically difference between the box-plots according to *t*-test. Dashed lines indicate means; solid lines indicate medians.

models including childhood acute leukaemia, pancreatic, colon and breast cancer (Karikari *et al*, 2007; Vogler *et al*, 2008, 2009; Fakler *et al*, 2009). Interestingly, a recent study using another XIAP inhibitor in 27 primary CLL samples showed that this XIAP inhibitor acted in concert with TRAIL to trigger apoptosis in 18 of 27 (67%) cases (Loeder *et al*, 2009). In contrast to the XIAP inhibitor used in the aforementioned study, the XIAP inhibitor used in our study was applied at 30-times lower concentrations, had almost no direct cytotoxic effect and did not induce any significant cytotoxic activity in non-malignant B cells at the concentrations used in combination with TRAIL.

Impressively, the administration of CA in our settings, i.e. especially using samples derived from CLL patients with poor prognostic features like ZAP-70$^+$, CD38$^+$ and/or *IGHV* unmutated disease, resulted in significantly greater susceptibility towards TRAIL-mediated cell death. These observations are supported by Lopez-Guerra *et al* (2009), who showed that ZAP-70$^+$ CLL cells were more susceptible towards selective IkB kinase inhibitor and TRAIL-mediated apoptosis.

Another major finding of our study is that samples derived from patients that had received prior chemotherapy showed similar response and apoptosis rates comparable to those samples derived from therapy-naïve patients. This observation can also be extended to p53-deficient patients, who generally had the worst clinical outcome (Binet *et al*, 2006), but showed maximum response under CA/TRAIL. It can be speculated that CA/TRAIL treatment might also be an attractive therapeutic option for all patients, who are not fit enough to receive standard chemo(immuno)therapy since CA/TRAIL did not show unspecific toxicity *in vitro*. Nevertheless, the definitive toxicity profile of CA is currently being defined in ongoing phase I clinical trials. Besides combination treatment of CA with TRAIL, another route of investigation will be to test these targeted drugs in combination with classic alkylating agents or purine analogues.

Since the discovery of XIAP in the second half of the 1990s, research on this unique inhibitor of apoptosis has been exponential giving us a detailed structural and mechanistic view of its activity in addition to abundant cell biology data. Through its ability to inhibit caspases, it has been suggested that XIAP renders cells resistant to multiagent chemotherapy. As a result, efforts have been undertaken to develop potential drugs targeting XIAP as a new way to counteract cancer and overcome drug resistance. In line with previous observations our results showed that XIAP is highly expressed and efficiently inhibits TRAIL-mediated cell death in CLL cells. The successful use of a novel potent pharmaceutic inhibitor of XIAP to restore TRAIL-induced cell death in CLL cells of 48 patients has a strong therapeutic implication, especially for treatment of CLL patients with poor prognosis, which needs to be further investigated in clinical trials to hopefully improve the outcome for patients with CLL.

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Authorship and disclosures

LPF, HK and C-MW conceived and designed the present work; LPF, MP, CPP, RB, JC and AS performed the research; LPF and MP conducted statistical analysis; LPF, MH, HK and C-MW analysed the data; LPF, HK and C-MW wrote the manuscript. HK and C-MW contributed equally to this work. The authors declare no competing financial interests.

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