

# Therapeutic opportunities for counteracting apoptosis resistance in childhood leukaemia

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## Summary

Evasion of apoptosis is a hallmark of human cancers, for example in haematological malignancies. Apoptosis is an intrinsic cell death program that is crucial in maintaining tissue homeostasis, for example in the haematopoietic system where there is a high turnover rate of cells. As a result, a decrease in the rate of apoptosis as well as an increase in proliferation favours tumorigenesis as well as tumour progression. Further, the anti-leukaemic action of current treatment approaches, including chemo-, radio- or immunotherapy, critically relies on intact cell death programs in cancer cells. Therefore, defects in apoptosis pathways are frequently associated with the resistance to anticancer therapies. In recent years, the identification and characterization of the molecules and pathways that are involved in the regulation and execution of cell death in leukaemia and lymphoma cells, for example tumour necrosis factor-related apoptosis inducing ligand (TRAIL), 'inhibitor of apoptosis' (IAP) proteins and Bcl-2, have set the ground for the development of novel diagnostic tools and molecular therapeutics targeting apoptosis pathways in haematological malignancies.

**Keywords:** apoptosis, childhood leukaemia, tumour necrosis factor-related apoptosis inducing ligand, inhibitor of apoptosis proteins, Bcl-2.

Apoptosis (programmed cell death) is the cell's intrinsic death program, which plays a crucial role in the regulation of many normal physiological processes during embryological development as well as in the adult organism (Hengartner, 2000). The fundamental role of apoptosis in normal physiology is also highlighted by the fact that it is highly conserved throughout evolution (Bergmann *et al*, 1998). A tight balance between proliferation on one side and cell death on the other side is crucial to maintain tissue homeostasis in cellular compartments with a high intrinsic cell turnover and proliferative capacity, such as the haematopoietic system (Reed & Pellec-

chia, 2005; Schimmer, 2008). Therefore, defective regulation of programmed cell death might be especially relevant for haematological malignancies, including paediatric leukaemia.

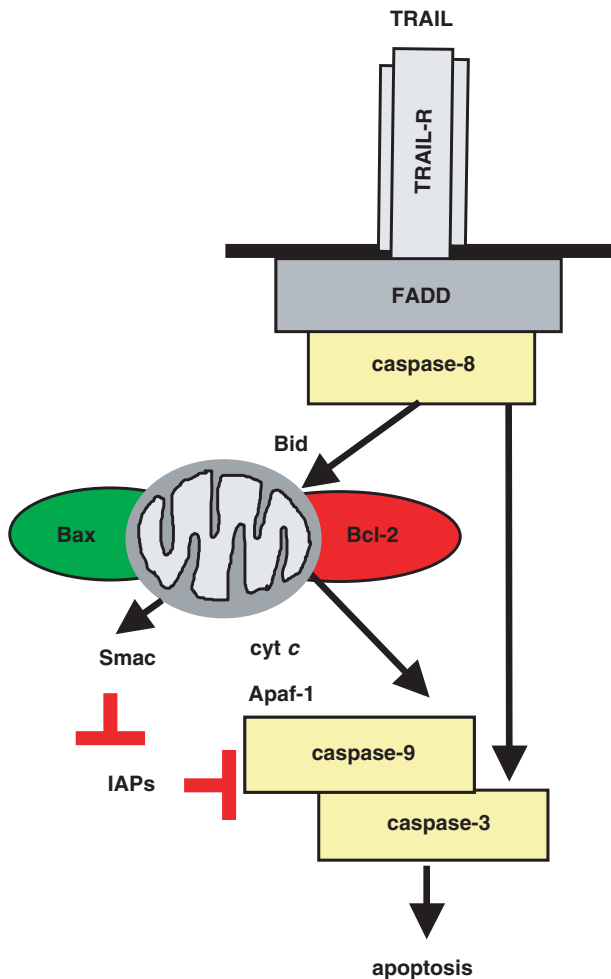
Evasion of apoptosis in leukaemia and lymphoma may be caused by oncogene activation as the consequence of chromosomal translocations (Rowley, 2008), which may result in abnormal expression or activation of oncogenes that promote proliferation, for example the *MYC* oncogene (O'Neil & Look, 2007). Alternatively, chromosomal translocations may lead to activation of oncogenes, such as *BCL2*, that promote tumour initiation and progression by blocking apoptosis (Schimmer *et al*, 2003). Further, the generation of fusion proteins such as BCR-ABL or PML-RAR $\alpha$  as the consequence of chromosomal translocations can also confer resistance to cell death in tumour cells (Bernardi & Pandolfi, 2003; Melo & Deininger, 2004). Further, oncogenic mutations that block apoptosis may favour genetic instability and accumulation of gene mutations as well as growth factor-independent survival and anchorage-independent growth during metastasis (Hanahan & Weinberg, 2000; Johnstone *et al*, 2002). Moreover, killing of tumour cells by cytotoxic therapies, such as chemotherapy,  $\gamma$ -irradiation, suicide genes or immunotherapy, has been reported to largely depend on the induction of cell death in target cells (Fulda & Debatin, 2006; Taylor *et al*, 2006).

This review provides an overview on the deregulation of apoptosis in haematological malignancies with the focus on childhood leukaemia and lymphoma.

## The core apoptotic machinery

Most apoptosis signalling pathways finally result in the activation of caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death (Fig 1) (Boatright & Salvesen, 2003; Degtrev *et al*, 2003). There are two major apoptosis signalling pathways, i.e. the death receptor (extrinsic) pathway and the mitochondria (intrinsic) pathway. Stimulation of death receptors of the tumour necrosis factor (TNF) receptor superfamily, such as CD95 or TNF-related apoptosis inducing ligand (TRAIL) receptors, by their respective ligands or agonistic antibodies results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8

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**Fig 1.** Apoptosis pathways. Apoptosis pathways can be initiated at the plasma membrane by death receptor ligation (death receptor or extrinsic pathway) or at the mitochondria (mitochondrial or intrinsic pathway). In the extrinsic pathway, stimulation of death receptors of the tumour necrosis factor (TNF) receptor superfamily such as TRAIL receptors (TRAIL-R) by their cognate ligand, e.g. TRAIL, results in receptor oligomerization and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8. Caspase-8 becomes activated upon recruitment to activated TRAIL receptors and initiates apoptosis either by direct cleavage of downstream effector caspases, such as caspase-3, or alternatively, by activating the mitochondrial pathway via cleavage of the BH3-only protein, Bid. In the intrinsic pathway, apoptogenic factors, such as cytochrome *c* (cyt *c*) and Smac, are released from the mitochondrial intermembrane space into the cytosol upon the induction of apoptosis. The release of cytochrome *c* into the cytosol triggers caspase-3 activation through the formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex. Smac promotes caspase activation through neutralizing the inhibitory effects of 'inhibitor of apoptosis' (IAP) proteins. Apoptosis is regulated at the mitochondria by anti-apoptotic Bcl-2 family proteins such as Bcl-2 as well as pro-apoptotic Bcl-2 family proteins such as Bax. See text for more details.

to form the death-inducing signalling complex (DISC) (Scaffidi *et al*, 1998; Falschlehner *et al*, 2007; Ashkenazi, 2008). Upon recruitment, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases.

The mitochondrial pathway is engaged by the release of apoptogenic factors from the mitochondrial intermembrane space into the cytosol including cytochrome *c*, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (Smac)/direct inhibitor of apoptosis (IAP) binding protein with low pI (DIABLO), or Omi/high temperature requirement protein A2 (HtrA2) (Kroemer *et al*, 2007). The release of cytochrome *c* into the cytosol triggers caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex (Riedl & Salvesen, 2007). Smac/DIABLO promotes caspase activation by neutralizing 'inhibitor of apoptosis proteins' (IAPs) that inhibit caspase-3, -7 and -9 (Du *et al*, 2000; Verhagen *et al*, 2000; Hunter *et al*, 2007).

Apoptosis signalling pathways are tightly regulated by pro- and anti-apoptotic mechanisms to allow some robustness of the system. For example, the Bcl-2 family of proteins consists of both antiapoptotic members, e.g. Bcl-2, Bcl-X<sub>L</sub> and Mcl-1, as well as proapoptotic molecules (Adams & Cory, 2007). The latter comprise multidomain proteins, such as Bax, Bak and Bad as well as Bcl-2 homology-3 (BH3)-domain only molecules, e.g. Bim, Bid, Bmf, Noxa or Puma (Adams & Cory, 2007). Bcl-2 family proteins play an important role in the regulation of the mitochondrial pathway of apoptosis, since they are involved in the control of mitochondrial outer membrane permeabilization (Adams & Cory, 2007).

Although caspases are crucial for cell death execution in many systems, caspase-independent apoptosis as well as non-apoptotic modes of cell death have also to be considered. For example, necrosis, autophagy, paraptosis or some forms of cell death that cannot be easily classified at present have been described (Okada & Mak, 2004). Although the signaling pathways and molecules involved in these alternative forms of cell death have not yet exactly been defined, non-caspase proteases, such as calpains or cathepsins, may be involved. The relative contribution of these diverse cell death mechanisms under various conditions both *in vitro* and *in vivo* in malignant cells of the haematopoietic system will be an area of future studies.

## Inhibitor of apoptosis (IAP) proteins

### *IAPs: structure and function*

IAP proteins are endogenous caspase inhibitors that are highly conserved throughout evolution from *Drosophila* to vertebrates (Salvesen & Duckett, 2002; Hunter *et al*, 2007). This protein family consists of eight human analogues, i.e. neuronal apoptosis inhibitory protein (NAIP/BIRC1/NLRB) cellular IAP1 (cIAP1)/human IAP2 (HIAP2)/BIRC2, cellular IAP2 (cIAP2)/human IAP1 (HIAP1)/BIRC3, X-linked inhibitor of apoptosis (XIAP)/BIRC4, survivin/BIRC5, BIR-containing ubiquitin conjugating enzyme (BRUCE)/apollon/BIRC6, livin/melanoma-IAP (ML-IAP)/BIRC7/KIAP and testis-specific IAP (Ts-IAP)/hILP-2/BIRC8 (Salvesen & Duckett,

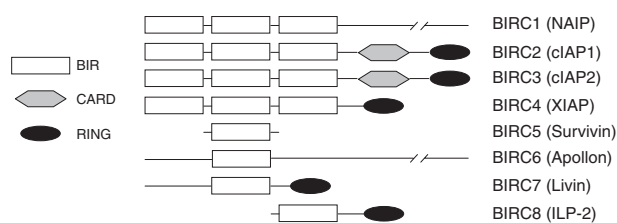


Fig 2. Structure of human IAP proteins. BIR, Baculovirus IAP repeats; CARD, caspase activating and recruitment domain; RING, really interesting new gene.

2002; Hunter *et al*, 2007). All IAPs contain at least one baculoviral IAP repeat (BIR) domain, a 70–80 amino acid long motif which is required for their classification as IAP protein (Fig 2). In addition to the BIR motif, all IAP proteins, except survivin, harbour one or more of the other functional domains, i.e. the really interesting new gene (RING) domain, which possesses E3 ubiquitin ligase activity and the caspase activating and recruitment domain (CARD) domain, a protein–protein interaction domain (Salvesen & Duckett, 2002).

Among the mammalian IAP proteins, XIAP possesses the most potent anti-apoptotic properties (Eckelman *et al*, 2006). The second BIR domain of XIAP mediates inhibition of the active site of caspase-3 or -7 (Chai *et al*, 2001; Huang *et al*, 2001; Riedl *et al*, 2001), while the third BIR domain of XIAP sequesters active caspase-9 in a monomeric state through two separate interaction sites (Srinivasula *et al*, 2001; Shiozaki *et al*, 2003). In addition to its function as caspase inhibitor, XIAP prevents apoptosis via the E3 ligase activity of its RING domain, which triggers proteasomal degradation of pro-apoptotic proteins, e.g. caspases (Vaux & Silke, 2005). In addition, XIAP can stimulate nuclear factor (NF)- $\kappa$ B activation via several mechanisms, i.e. by enhancing the translocation of NF- $\kappa$ B from the cytoplasm into the nucleus, by increasing the degradation of I $\kappa$ B protein and via its association with TAK1 kinase and its cofactor TAB1 (Hofer-Warbinek *et al*, 2000; Birkey Reffey *et al*, 2001; Levkau *et al*, 2001; Lewis *et al*, 2004; Lu *et al*, 2007).

Survivin is the smallest mammalian member of the IAP family, as it contains a single BIR domain only (Altieri, 2008). The gene that encodes survivin (*BIRC5*) is located on chromosome 17q25 in humans, and gives rise to five alternatively spliced survivin transcripts, i.e. wild-type survivin, survivin-2 $\alpha$ , survivin-2B, survivin-DeltaEx-3 and survivin-3B (Altieri, 2008). The anti-apoptotic function of survivin has been linked to its interaction with Smac/DIABLO, to the stabilization of XIAP protein via its binding to XIAP and to inhibition of mitochondrial and AIF-dependent apoptotic pathways (Blanc-Brude *et al*, 2003; Song *et al*, 2003; Dohi *et al*, 2004).

Livin/ML-IAP contains a single BIR domain at the N-terminus as well as a C-terminal RING domain (Vucic *et al*, 2000; Kasof & Gomes, 2001). Livin blocks apoptosis by binding and inhibiting caspases, by serving as a sink for Smac

and by stimulating TAK1-dependent JNK1 activation (Vucic *et al*, 2000, 2002, 2005; Sanna *et al*, 2002).

### IAP antagonists

Smac and its murine homologue DIABLO are nuclear encoded mitochondrial proteins, which are imported into the mitochondrial intermembrane space via a mitochondrial localization signal (Du *et al*, 2000; Verhagen *et al*, 2000). Removal of this mitochondrial localization signal generates the mature 23 kDa protein and exposes the IAP-binding motif at the N-terminus of Smac/DIABLO that is now able to interact with IAPs (Du *et al*, 2000). Intriguingly, the same surface groove on the BIR3 domain of XIAP, which binds to the IAP-binding motif of the N-terminus of Smac also binds to the IAP-binding motif exposed at the N-terminus of the small subunit of caspase-9 following the autocatalytic processing of caspase-9 (Srinivasula *et al*, 2001; Shiozaki *et al*, 2003). This structural homology allows Smac/DIABLO to displace caspase-9 from XIAP and to promote caspase activation and apoptosis (Srinivasula *et al*, 2001).

### Prognostic significance of IAPs in paediatric leukaemia and lymphoma

The relevance of IAPs for treatment response and outcome in childhood leukaemia and lymphoma has been explored in a number of clinical studies. In childhood *de novo* acute myeloid leukaemia (AML), high expression levels of XIAP and survivin correlated with poor overall survival (Tamm *et al*, 2004). Also, expression levels of XIAP were lower in patients with favourable rather than intermediate or poor cytogenetics (Tamm *et al*, 2004). In addition, XIAP showed maturation-dependent expression differences, with the highest expression levels detected within the immature M0/1 subtypes according to the French-American-British (FAB) morphology (Tamm *et al*, 2004).

In paediatric precursor B cell acute lymphoblastic leukaemia (ALL), overexpression of survivin was found to identify patients with a high risk of early relapse, as higher survivin expression was detected in relapse patients than those with a favourable outcome (Troeger *et al*, 2007). Analysis of survivin splice variants in childhood ALL revealed an association between lower expression of survivin-2B, an isoform of survivin with pro-apoptotic properties, and affiliation to the high risk group (Tröger *et al*, 2007). Expression analysis of survivin and its splice variants survivin-2B and survivin-DeltaEx3 in children with *de novo* AML revealed that survivin was the predominant transcript variant in AML cells, while significantly lower expression levels of survivin-2B and survivin-DeltaEx3 were detected (Wagner *et al*, 2006). There was no association of survivin or of any survivin splice variant expression with cytogenetic risk groups or maturation stage (FAB subtypes, immunophenotype) (Wagner *et al*, 2006). Notably, high survivin-DeltaEx3 expression correlated with a

significantly shorter overall survival, demonstrating that the expression of certain survivin splice variants has potential prognostic impact for long-term therapy outcome in childhood *de novo* AML (Wagner *et al*, 2006).

Surprisingly, the expression rate of the anti-apoptotic protein livin was recently identified as a favourable prognostic factor in childhood ALL (Choi *et al*, 2007). Notably, livin expression rate was high in *t*(12;21) and very low in *t*(9;22)/11q23 rearrangements (Choi *et al*, 2007), which have been reported to be associated with the best and worst clinical outcomes respectively (Chen *et al*, 1993; Pui *et al*, 1994; Arico *et al*, 2000). In this context, it is interesting to note that livin can be converted from an anti-apoptotic to a pro-apoptotic factor by caspase-mediated cleavage (Nachmias *et al*, 2003). In addition, the subcellular localization of livin may determine the balance between its anti- and pro-apoptotic activities (Nachmias *et al*, 2003). The prognostic relevance of livin in paediatric ALL, however, remains to be defined in future studies.

#### Anti-leukaemic therapy targeted at IAPs

Recently, several strategies to inhibit or downregulate XIAP have been developed for therapeutic purposes (Table I). For the design of small molecules to target XIAP, the binding groove of the BIR3 domain of XIAP, to which Smac binds to after its release from mitochondria, has attracted most attention (Shiozaki & Shi, 2004). Ectopic expression of Smac or Smac peptides harbouring the N-terminal part of Smac that is essential for binding of Smac to XIAP were reported to either directly trigger apoptosis or to sensitize leukaemia or lymphoma cells for apoptosis induced by death-receptor ligation, anticancer drugs or cytolytic T cell attack (Jia *et al*, 2003; Kashkar *et al*, 2003, 2006; Guo *et al*, 2004; Chauhan *et al*, 2007; Weisberg *et al*, 2007). In some studies, Smac peptides were linked to a carrier to facilitate their intracellular delivery, for example the protein transduction motif of the human immunodeficiency virus Tat protein, the *Drosophila* antennapedia penetrating sequence or a polyarginine stretch (Arnt

*et al*, 2002; Fulda *et al*, 2002; Yang *et al*, 2003). On the basis of the three-dimensional structure of Smac in complex with XIAP BIR3, Smac mimetics were designed, which bind to one or several of the BIR domains of IAP family proteins (Li *et al*, 2004; Sun *et al*, 2004a,b, 2006; Bockbrader *et al*, 2005; Zobel *et al*, 2006; Gaither *et al*, 2007; Petersen *et al*, 2007; Varfolomeev *et al*, 2007; Vince *et al*, 2007). Furthermore, capped tripeptides targeting the Smac binding site of XIAP BIR3 were developed by structure-based design of the interaction of Smac with the BIR3 domain of XIAP (Oost *et al*, 2004). These XIAP inhibitors bind to the BIR3 domain of XIAP with nanomolar affinity and promoted cell death in several human cancer cell lines including leukaemia cells (Oost *et al*, 2004). Recently, these XIAP inhibitors were reported to co-operate with TRAIL to induce apoptosis in ALL cell lines as well as in primary leukaemic blasts from children with ALL (Fakler *et al*, 2008). Importantly, XIAP inhibitors also significantly reduce leukaemic burden *in vivo* in a mouse model of paediatric ALL engrafted in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Fakler *et al*, 2008).

Besides the BIR3 domain of XIAP, its BIR2 motif has also served as a target for the development of small molecule compounds. To this end, non-peptidic XIAP antagonists were identified by screening of a polyphenylurea library using a caspase derepression assay (Schimmer *et al*, 2004; Wang *et al*, 2004). These compounds caused apoptosis in leukaemia cells, including primary AML blasts, without the requirement of an additional cytotoxic stimulus by derepressing downstream effector caspases (Schimmer *et al*, 2004; Carter *et al*, 2005). Of note, these XIAP antagonists also killed acute leukaemia cells with high Bcl-2 expression levels suggesting that they may bypass some forms of resistance (Schimmer *et al*, 2004; Carter *et al*, 2005).

Furthermore, antisense oligonucleotides were designed to downregulate aberrant XIAP expression in human cancers. Cellular studies have shown the anti-cancer effects of XIAP antisense oligonucleotides in leukaemia, either as single agents or in combination with chemotherapeutic drugs (Carter *et al*, 2003a,b; Lima *et al*, 2006). Importantly, the antitumour

**Table I.** Examples of apoptosis-based therapeutics in clinical trials for haematological malignancies.

Strategy	Name	Cancer type	References
<i>Targeting IAPs</i>			
XIAP antisense	AEG35156	AML, NHL	LaCasse <i>et al</i> (2006a) and Jolivet <i>et al</i> (2008)
Survivin antisense	LY2813008	AML	Altieri (2008)
<i>Targeting TRAIL receptors</i>			
Soluble TRAIL	Apo-2L/TRAIL	NHL	Herbst <i>et al</i> (2006)
TRAIL-R1 mAb	HGS-ETR1	NHL	Younes <i>et al</i> (2005)
TRAIL-R2 mAb	HGS-ETR2	Hodgkin disease	Patnaik <i>et al</i> (2006) and Saleh <i>et al</i> (2008)
<i>Targeting Bcl-2/Bcl-X<sub>L</sub></i>			
Bcl-2 antisense	Oblimersen	Leukaemia/lymphoma	Webb <i>et al</i> (1997) and Waters <i>et al</i> (2000)
Bcl-2/Bcl-X <sub>L</sub> inhibitor	ABT-263	Leukaemia/lymphoma	Wilson <i>et al</i> (2008)

AML, acute myeloid leukaemia; NHL, non-Hodgkin lymphoma.

activity of XIAP antisense oligonucleotides correlated with downregulation of XIAP levels in targeted tissues isolated from preclinical animal models (LaCasse *et al*, 2005, 2006a). Currently, XIAP antisense oligonucleotides are being evaluated in phase I/II clinical trials in adult oncology in combination with chemotherapy, for example in AML (LaCasse *et al*, 2006a,b) (Table I). In a phase I clinical trial of XIAP antisense AEG35156 administered as a continuous intravenous infusion, one patient with non-Hodgkin lymphoma (NHL) was reported to have marked, although short-lived, decreases in peripheral lymphoblasts during administration of XIAP antisense that was closely associated with knockdown of XIAP mRNA (Jolivet *et al*, 2008). Taken together, Smac mimetics, small molecule XIAP antagonists or XIAP antisense oligonucleotides are promising approaches to target XIAP in order to trigger apoptosis or to lower the threshold for apoptosis induction in leukaemia and lymphoma cells.

## The death receptor system

### *Biology of the death receptor system*

Death receptors belong to the TNF receptor superfamily, which comprises more than 20 proteins with various biological functions, e.g. regulation of cell death and survival, differentiation or immune regulation (Lavrik *et al*, 2005; Ashkenazi, 2008).

Members of the TNF receptor family share a characteristic cytoplasmic domain called the 'death domain', which is pivotal for transducing the death signal from the cell's surface to intracellular signalling pathways (Lavrik *et al*, 2005; Ashkenazi, 2008). CD95 (APO-1/Fas), TNF receptor 1 (TNFR1) and TRAIL receptors are the best-characterized death receptors and their corresponding ligands of the TNF superfamily are CD95 ligand, TNF $\alpha$  and TRAIL (Lavrik *et al*, 2005; Ashkenazi, 2008).

### *Prognostic relevance of death receptor molecules in paediatric leukaemia*

The death receptor pathway of apoptosis can be impaired in childhood leukaemia by mutational or epigenetic inactivation of key molecules of the pathway or by altered signalling events. For example, mutations in CD95 were identified in childhood T-ALL and Hodgkin lymphoma (Beltinger *et al*, 1998a; Gronbaek *et al*, 1998). By comparison, no mutations in the coding and proximal promoter region were detected by single-stranded conformation polymorphism analysis in a study that included 32 cases of primary B-lineage childhood ALL (Beltinger *et al*, 1998b). Expression levels of CD95 or sensitivity to CD95-induced apoptosis did not correlate with the response to induction chemotherapy or relapse rate in childhood ALL (Wuchter *et al*, 2000). Also, no consistent changes in CD95 expression were observed in children at both initial diagnosis and relapse (Wuchter *et al*, 2000). These results indicate that CD95 expression and CD95 function do

not present predictors of the response to induction chemotherapy or relapse rate in childhood acute lymphoblastic leukaemia (Wuchter *et al*, 2000). Recently, a single nucleotide polymorphism that causes guanine-to-adenine transition in the *FAS* promoter region (position-1377) and has been associated with decreased *FAS* expression, has been investigated in children treated for *de novo* AML (Mehta *et al*, 2008). While adult AML patients were reported to bear an increased risk of developing AML with a variant allele at this site (Sibley *et al*, 2003), children diagnosed with *de novo* AML showed no significant differences in overall survival, event-free survival, treatment-related mortality, or relapse rate with *FAS* 1377GG genotype *versus* 1377GA/1377AA genotypes (Mehta *et al*, 2008), pointing to a differential role of this polymorphism during tumorigenesis compared to tumour progression and treatment resistance.

### *Targeting the death receptor pathway by TRAIL*

Among the death receptor ligands, TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2L is a prime candidate for translation of apoptosis targeted therapeutics into the clinic, because TRAIL predominantly kills cancer cells, while sparing normal cells, although the molecular basis of this tumour selectivity has not been exactly elucidated (LeBlanc & Ashkenazi, 2003). Several strategies have been developed to target TRAIL receptors therapeutically (Ashkenazi & Herbst, 2008; Humphreys & Halpern, 2008). One approach is the use of trimeric TRAIL itself as a recombinant natural ligand (Ashkenazi *et al*, 2008). Recombinant soluble TRAIL triggered apoptosis in a wide range of cancer cell lines including haematological malignancies (Snell *et al*, 1997; Ashkenazi *et al*, 1999; Gazitt, 1999; Walczak *et al*, 1999; Clodi *et al*, 2000; Chen *et al*, 2001; Liu *et al*, 2001; Mitsiades *et al*, 2001; Plasilova *et al*, 2002). An alternative therapeutic strategy is based on agonistic monoclonal antibodies that specifically target one of the agonistic TRAIL receptors, TRAIL-R1 and -R2, which demonstrated antitumour activity in cancer cell lines and xenograft-bearing mice (Humphreys & Halpern, 2008). The existence of decoy receptors that can bind TRAIL, yet do not deliver a death signal, suggests a potential advantage to the use of antibodies that specifically target one of the two agonistic TRAIL receptors. Another potential advantage of these antibodies is their longer half-life compared to that of recombinant TRAIL. However, future studies will need to determine which of these agents triggering the TRAIL pathway will be superior for clinical application.

It is important to note that, to date, few studies have been carried out testing the efficacy of TRAIL receptor agonists on primary cells from human haematopoietic malignancies. While many cancer cell lines are susceptible to TRAIL-induced apoptosis, primary tumour cells were found to be refractory towards TRAIL despite the expression of the agonistic TRAIL receptors on their cell surface. For example, in paediatric ALL, 50% of primary samples obtained from children before the

onset of chemotherapy were refractory to TRAIL-mediated apoptosis (Ehrhardt *et al*, 2003). In some of these resistant samples, TRAIL even attenuated spontaneous apoptosis and stimulated proliferation (Ehrhardt *et al*, 2003), pointing to a pro-survival function of TRAIL under certain conditions.

Since primary resistance towards TRAIL will probably limit the success of TRAIL in the clinic, there have been many efforts in recent years to develop rational combination regimens to overcome resistance mechanisms. To this end, numerous studies have shown that combinations of soluble TRAIL or TRAIL receptor antibodies with conventional anticancer therapeutics, such as chemotherapy or  $\gamma$ -irradiation, elicit markedly enhanced antitumour activity. For example, several chemotherapeutics or  $\gamma$ -irradiation were reported to synergistically interact with TRAIL in haematological malignancies (Wen *et al*, 2000; Belka *et al*, 2001; Olsson *et al*, 2001; Georgakis *et al*, 2005). In addition, the concomitant use of TRAIL together with new compounds, e.g. histone deacetylase (HDAC) inhibitors has been reported to enhance TRAIL-induced apoptosis in Jurkat T-ALL (Guo *et al*, 2004; Inoue *et al*, 2004, 2006, 2008; Nakata *et al*, 2004; Tsapis *et al*, 2007). Moreover, the multikinase inhibitor sorafenib was shown to enhance TRAIL lethality in leukaemia and lymphoma cells, at least in part via mechanisms that involve downregulation of Mcl-1 (Meng *et al*, 2007; Rosato *et al*, 2007).

Soluble recombinant TRAIL and fully human monoclonal antibodies against TRAIL-R1 or -R2 are currently under evaluation in early clinical trials (Table I). Initial trials in adults revealed no major dose-limiting toxicities for recombinant TRAIL or human monoclonal antibodies to TRAIL-R1 and defined the maximal tolerated dose for monoclonal antibodies to TRAIL-R2 (Herbst *et al*, 2006; Patnaik *et al*, 2006; Tolcher *et al*, 2007; Saleh *et al*, 2008). Also in paediatric patients, a clinical trial with TRAIL-R2 monoclonal antibodies has recently been launched including children and young adolescents with lymphoma (<http://clinicaltrials.gov/ct2/show/NCT00428272>, <http://www.cancer.gov/clinicaltrials/NCI-07-C-0040>). TRAIL-R1 monoclonal antibodies are currently under evaluation in a phase II clinical trial in NHL (Younes *et al*, 2005).

## Bcl-2 family proteins

### *Biology of Bcl-2 family proteins*

The Bcl-2 family of proteins consists of both anti-apoptotic proteins, for example Bcl-2, Bcl-X<sub>L</sub> and Mcl-1, as well as pro-apoptotic molecules, such as Bax, Bak and BH3 domain only molecules, such as Bid, Bim, Bik, Noxa, Puma (Adams & Cory, 2007). There are currently two models as to how BH3-only proteins activate Bax and Bak during the course of apoptosis. According to the direct activation model (Letai *et al*, 2002), putative activators, such as Bim and cleaved Bid (tBid), bind directly to Bax and Bak to trigger their activation, while BH3-only proteins that act as sensitizers, e.g. Bad, bind to the pro-survival Bcl-2 proteins. By comparison, the indirect

activation model holds that BH3-only proteins activate Bax and Bak by binding and thus inactivating the various antiapoptotic Bcl-2 proteins that in turn inhibit Bax and Bak (Chen *et al*, 2005; Willis *et al*, 2007). Imbalances in the ratio of anti- versus pro-apoptotic Bcl-2 proteins may tip the balance towards tumour cell survival and thus, may contribute to tumour formation and progression, for example in haematological malignancies (Reed, 2008).

### *Prognostic relevance of Bcl-2 family proteins in childhood leukaemia*

Analysis of expression levels of Bcl-2 family proteins including Bcl-2, Bcl-xL, Mcl-1, Bax, Bad and Bak in childhood leukaemia showed no associations with features at presentation, *in vitro* or *in vivo* drug response and long-term clinical outcome (Salomons *et al*, 1999). As far as Bcl-2 is concerned, expression levels of Bcl-2 did not correlate with the response to induction chemotherapy, the relapse rate or disease aggressiveness in paediatric ALL (Campos *et al*, 1996; Coustan-Smith *et al*, 1996; Charpin *et al*, 1998; Wuchter *et al*, 2000). Also, no difference in Bcl-2 expression was found at initial diagnosis and at relapse (Wuchter *et al*, 2000). By comparison, good responders to initial therapy with prednisone exhibited significantly higher expression levels of Bcl-2 compared to ALL patients that poorly responded to prednisone therapy (Wuchter *et al*, 2000). In one study, high Bcl-2/Bax ratio correlated with good prognostic features (Narayan *et al*, 2007). Analysis of Bcl-X<sub>L</sub> and Bax expression in a series of 62 children with ALL showed that both proteins were frequently expressed at high level, while only Bcl-X<sub>L</sub> was found to be an independent predictor of event-free survival (Addeo *et al*, 2005). Bax expression levels and the Bax/Bcl-2 ratio were reported to be significantly lower in children with ALL at relapse as compared with samples at initial diagnosis (Prokop *et al*, 2000). However in another study, increased Bax expression was associated with an increased risk of relapse in childhood ALL (Hogarth & Hall, 1999). Overall, the heterogeneous correlation of Bcl-2 family members, especially of Bcl-2 and Bax, and clinical parameters point to a more complex regulation of these molecules in childhood leukaemia *in vivo*.

A more consistent picture is emerging for the prognostic relevance of the anti-apoptotic Bcl-2 protein, Mcl-1, and the pro-apoptotic Bcl-2 family protein, Bim. To this end, *MCL1* was recently identified in gene expression profiling studies to be overexpressed in childhood ALL samples as part of a gene signature of glucocorticoid resistance (Holleman *et al*, 2006; Wei *et al*, 2006). Vice versa, upregulation of the pro-apoptotic Bcl-2 family protein Bim was observed upon exposure to glucocorticoids in sensitive ALL cells (Planey *et al*, 2003; Wang *et al*, 2003; Webb *et al*, 2003; Schmidt *et al*, 2006). Of note, this glucocorticoid-induced increase of Bim was substantially attenuated in response to prednisone in glucocorticoid-refractory primary ALL cells (Bachmann *et al*, 2005). Also, studies in Bim or Puma knockout mice revealed that deletion

of Bim or Puma conferred resistance of normal lymphoid tissues to glucocorticoids (Erlacher *et al*, 2005).

#### *Anti-leukaemic therapy targeted at Bcl-2 family proteins*

Given that high expression of anti-apoptotic Bcl-2 family proteins may confer resistance to chemo- or radiotherapy by blocking the mitochondrial pathway of apoptosis (Galluzzi *et al*, 2006), there has been much interest in developing strategies that overcome the cytoprotective effect of Bcl-2 and its related molecules. To this end, nuclease-resistant Bcl-2 antisense oligonucleotides downregulating *BCL2* mRNA were tested in clinical trials for haematological malignancies including childhood leukaemia, as single agent or in combination with chemotherapy (Tolcher, 2005; Szegedi *et al*, 2008) (Table I). Bcl-2 antisense Oligonucleotide was recently reported to induced apoptosis in childhood leukaemia cell lines and primary patient samples (Szegedi *et al*, 2008).

Moreover, the attempt to target the protein–protein interaction site between antiapoptotic Bcl-2 proteins and Bax or Bak has resulted in the generation of the small molecule antagonist ABT-737, which binds to the surface groove of Bcl-2, Bcl-X<sub>L</sub> and Bcl-w that normally interacts with the BH3 domain of Bax or Bak (Oltersdorf *et al*, 2005). By preventing the binding of antiapoptotic Bcl-2 proteins to Bax or Bak, ABT-737 frees Bax and Bak to engage the mitochondrial pathway of apoptosis. The use of ABT-737 as single agent has been reported to directly trigger apoptosis in a panel of haematological malignancies, especially in those that critically depend on anti-apoptotic Bcl-2 proteins for survival (Oltersdorf *et al*, 2005; Konopleva *et al*, 2006; Del Gaizo Moore *et al*, 2007, 2008; Kline *et al*, 2007; Vogler *et al*, 2008).

To augment the antitumour activity of ABT-737 and to overcome potential mechanisms of resistance, ABT-737 has also been evaluated in a series of combination studies with conventional chemotherapeutics (Oltersdorf *et al*, 2005; Konopleva *et al*, 2006; Van Delft *et al*, 2006; Kuroda *et al*, 2008). In paediatric ALL, ABT-737 synergized with other chemotherapeutic agents, including drugs that are commonly used for remission induction in primary and relapsed childhood leukaemia, such as vincristine, glucocorticoids and L-asparaginase, in ALL cell lines and also *in vivo* in mouse xenografts derived from patients with ALL (Kang *et al*, 2007). Mechanistic studies showed that ABT-737 plus L-asparaginase caused increased activation of Bax and Bid, mitochondrial damage, release of cytochrome *c* from mitochondria into the cytosol, caspase activation and finally apoptosis compared to either drug alone (Kang *et al*, 2007), indicating that the combination synergistically triggered the mitochondrial pathway of apoptosis.

As ABT-737 targets Bcl-2/Bcl-X<sub>L</sub> but not Mcl-1, high expression of Mcl-1 may confer resistance to this agent. Indeed, several reports have provided evidence that Mcl-1 represents a key determinant of ABT-737 sensitivity and

resistance in cancer cells (Konopleva *et al*, 2006; Van Delft *et al*, 2006; Chen *et al*, 2007; Lin *et al*, 2007). Consequently, Mcl-1 downregulation by genetic approaches or pharmacological compounds, including CDK inhibitors (e.g. roscovitine, flavopiridol, seliciclib) or Raf/Mek inhibitors (e.g. sorafenib), has been demonstrated to dramatically increase ABT-737 cytotoxicity in malignant cell types (Chen *et al*, 2001, 2007; Konopleva *et al*, 2006; Van Delft *et al*, 2006; Dai & Grant, 2007; Lin *et al*, 2007; Tahir *et al*, 2007). To this end, CDK inhibitors have been shown to reduce Mcl-1 expression by blocking Mcl-1 transcription (Chen *et al*, 2007), while sorafenib was reported to downregulate Mcl-1 at the translational level (Lin *et al*, 2007). In paediatric ALL cell lines, the synthetic cytotoxic retinoid N-(4-hydroxyphenyl) retinamide (4-HPR) has recently been shown to synergize with ABT-737 to trigger cell death via inactivation of Mcl-1 (Kang *et al*, 2008). Mechanistic studies revealed that 4-HPR causes generation of reactive oxygen species (ROS) and ROS-mediated activation of c-Jun kinase (JNK), which in turn phosphorylates and inhibits Mcl-1 (Kang *et al*, 2008). This resulted in synergistic cytotoxicity of ABT-737 together with 4-HPR in ALL cell lines with minimal cytotoxicity for normal lymphocytes (Kang *et al*, 2008). In this context, it is also interesting to note that rapamycin was reported to sensitize ALL cells to glucocorticoid-induced apoptosis via modulation of the anti-apoptotic protein Mcl-1 (Wei *et al*, 2006).

Furthermore, obatoclox (GX15-070) presents a BH3 mimetic that also antagonizes Mcl-1 in addition to Bcl-2, Bcl-X<sub>L</sub> and Bcl-w (Nguyen *et al*, 2007; Konopleva *et al*, 2008). Accordingly, obatoclox has been reported to overcome Mcl-1-mediated resistance to the Bcl-2/Bcl-X<sub>L</sub>/Bcl-w-selective antagonist ABT-737 (Nguyen *et al*, 2007). Obatoclox has demonstrated anti-leukaemic activity against several adult haematological malignancies, including AML, chronic lymphocytic leukaemia, mantle cell lymphoma and multiple lymphoma (Nguyen *et al*, 2007; Konopleva *et al*, 2008; Perez-Galan *et al*, 2008) and is currently being evaluated in early clinical trials (O'Brien *et al*, 2007).

An orally bioavailable Bcl-2 family inhibitor, ABT-263, has recently been developed (Tse *et al*, 2008). Interestingly, the evaluation of ABT-263 by the paediatric preclinical testing program revealed that ABT-263 exhibits highest *in vitro* activity against ALL cell lines across the panel of paediatric tumour cell lines tested (Lock *et al*, 2008). In addition, ABT-263 showed significant anti-leukaemic activity against ALL xenografts *in vivo* (Lock *et al*, 2008). To this end, ABT-263 induced significant prolongation of the event-free survival distribution in five of six (83%) of ALL xenografts and caused complete remissions in three of six ALL xenografts (Lock *et al*, 2008). Together, these data suggest that ABT-263 is a promising candidate for further clinical evaluation alone and in combination with chemotherapeutics in paediatric ALL. In adult haematology/oncology, ABT-263 has currently entered clinical trials (Table I) (Wilson *et al*, 2008). In a phase I clinical trial in refractory or relapsed lymphoid malignancies,

ABT-263 demonstrated early evidence of activity (Wilson *et al*, 2008). The toxicities that were associated with ABT-263 treatment are considered to be mechanism-based, since the reduction in circulating platelet counts has been shown to be due to the critical role of Bcl-X<sub>L</sub> in platelet survival (Mason *et al*, 2007; Zhang *et al*, 2007; Wilson *et al*, 2008).

Collectively, these findings suggest that small molecule inhibitors of antiapoptotic Bcl-2 family proteins may open new perspectives for reactivating the mitochondrial pathway of apoptosis in cancer cells.

## Conclusions

Given that cellular systems with a high intrinsic cell turnover critically rely on control mechanisms that maintain the subtle balance between proliferation and cell death, defective regulation of programmed cell death within the lympho-haematopoietic system may promote leukaemogenesis and also treatment resistance. Various apoptosis molecules have been identified that play an important role in the regulation of cell death in paediatric leukaemia. These molecules also present promising therapeutic targets for the design of anticancer drugs. Indeed, several apoptosis-based cancer therapeutics, for example TRAIL receptor agonists, antisense oligonucleotides against Bcl-2, XIAP or survivin and small molecule inhibitors of IAPs or anti-apoptotic Bcl-2 proteins, have already recently entered clinical trials in several malignancies, including leukaemia and lymphoma. Thus, the application of basic knowledge on apoptosis pathways into medical practice is anticipated to eventually yield new biomarkers and better cancer therapeutics for the treatment of childhood leukaemia.

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