

Cell death in hematological tumors

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Abstract Evasion of apoptosis is a hallmark of human cancers, for example in hematological malignancies. Apoptosis is an intrinsic cell death program that is crucial to maintain tissue homeostasis, for example in the hematopoietic system where there is a high turnover rate of cells. As a result, a decrease in the rate of apoptosis besides an increase in proliferation favors tumorigenesis as well as tumor progression. Further, the anti-leukemic 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 leukemia and lymphoma cells have set the ground for the development of novel diagnostic tools and molecular therapeutics targeting apoptosis pathways in hematological malignancies.

Keywords Apoptosis · Leukemia · Lymphoma · TRAIL · IAPs · Bcl-2

Introduction

Apoptosis or 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 and which is highly conserved throughout evolution [1]. One of the key

advances in cancer research is the recognition that evasion of apoptosis is one of the hallmarks of human cancers [2]. Proper regulation of programmed cell death might be especially relevant for hematologic malignancies, since tissue homeostasis in cellular compartments with a high intrinsic proliferative capacity and cell turnover such as the hematopoietic system critically depend on a tight balance between proliferation and cell death [3, 4]. Accordingly, too little apoptosis coupled with malignant transformation may result in the development of leukemia and lymphoma [3, 4]. Some oncogenic mutations that block apoptosis may promote tumor initiation and progression by creating a permissive environment for genetic instability and accumulation of gene mutations, by imposing resistance to immune-based destruction and by facilitating growth factor-independent survival as well as anchorage-independent growth during metastasis [2, 5]. Conversely, other oncogenic events such as aberrant expression of the myc oncogene can promote apoptosis, thereby producing selective pressure on cancer cells to evade apoptosis during the multistep process of carcinogenesis [6]. In addition, killing of tumor cells by cytotoxic therapies such as chemotherapy, γ -irradiation, suicide genes or immunotherapy, has been reported to depend on the induction of cell death in target cells [7, 8]. Since the same oncogenic alterations and defects in apoptosis programs that suppress cell death during tumor development can also confer resistance to cytotoxic therapies [5], apoptosis provides a conceptual framework to link cancer formation and cancer therapy.

The core apoptotic machinery

Most apoptosis signaling pathways finally result in the activation of caspases, a family of cysteine proteases that

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act as common death effector molecules in various forms of cell death (Fig. 1) [9, 10]. There are two major apoptosis signaling pathways, i.e., the death receptor (extrinsic) pathway and the mitochondria (intrinsic) pathway. Stimulation of death receptors of the tumor 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 to form the death inducing signaling complex (DISC) [11–13]. 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 IAP binding protein with low PI (DIABLO), or Omi/high temperature requirement protein A2 (HtrA2) [14]. 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 [15]. Smac/DIABLO promotes caspase activation through by neutralizing ‘Inhibitor of apoptosis proteins’ (IAPs) that inhibit caspase-3, -7 and -9 [16–18].

Apoptosis signaling 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_L and Mcl-1, as well as proapoptotic molecules [19]. The later comprise multidomain proteins such as Bax, Bak and Bad as well as Bcl-2 homology domain-3 (BH3)-domain only molecules, e.g., Bim, Bid, Bmf, Noxa or Puma [19]. 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 [19].

Although caspases are crucial for cell death execution in many systems, caspase-independent apoptosis as well as nonapoptotic 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 [20]. 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 hematopoietic system will be an area of future studies.

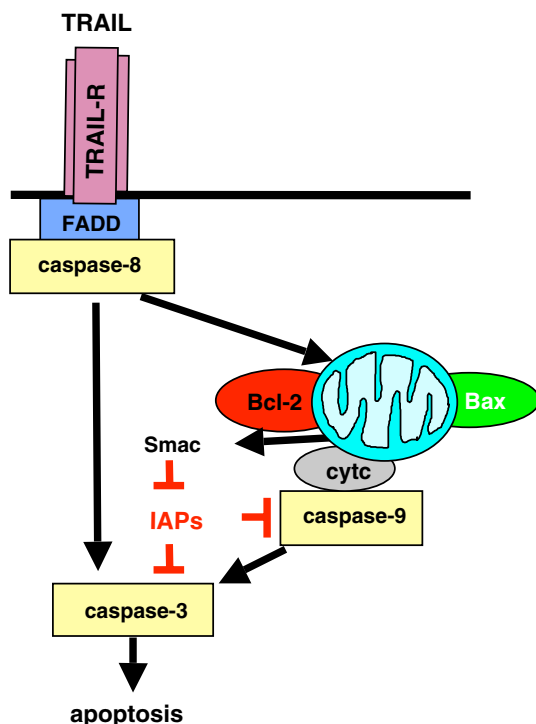


Fig. 1 Apoptosis pathways. Apoptosis pathways can be initiated by ligation of death receptors, e.g., TRAIL receptors (TRAIL-R) by TRAIL, which triggers receptor trimerization, recruitment of adaptor molecules such as FADD and activation of caspase-8 (receptor pathway). The mitochondrial pathway is initiated by the release of cytochrome *c* and Smac from mitochondria in the cytosol. Apoptosis can be inhibited by Bcl-2 or by ‘‘Inhibitor of Apoptosis Proteins’’ (IAPs). Smac promotes apoptosis by neutralizing IAP-mediated inhibition of caspase-3 and -9. See text for more details

Deregulated apoptosis and leukemogenesis

Cancer usually develops through a multistep series of events that is characterized by a deregulation of the physiological tissue homeostasis caused by enhanced proliferation and/or reduced cell death [5, 6]. It is well established that an abnormally high rate of proliferation can foster tumor growth [5, 6]. By comparison, it has been recognized much later that also a reduced rate of cell death can facilitate the carcinogenic process [5, 6]. One key clue to the concept that evasion of apoptosis is another characteristic trait of cancers that foster tumor development came from the identification of the Bcl-2 proto-oncogene at the chromosomal breakpoint of the t(14;18) translocation in follicular lymphoma [21]. Chromosomal translocation of the *bcl-2* oncogene into the immunoglobulin heavy chain locus results in aberrant expression of Bcl-2 [21]. Bcl-2 was found to favor cell survival by blocking apoptosis in contrast to many other oncogenes that were previously identified and that promote malignant transformation by driving proliferation. Experiments in Bcl-2 transgenic mice provided further evidence that ectopic expression of Bcl-2 can promote neoplastic

transformation of B and T lymphocytes as well as myeloid cells [22, 23]. The concept that defective apoptosis is a key factor that contribute to tumorigenesis was also supported by the discovery of the crucial role of p53 in the regulation of apoptosis and the high incidence of p53 mutations in the majority of human cancers often that is associated with poor prognosis [24].

Alterations in the expression of anti- or pro-apoptotic members of the Bcl-2 family proteins have been described in a variety of human cancers including hematologic malignancies [25]. Further, the Bcl-2 family protein Bax is one of the key proapoptotic target gene of the tumor suppressor gene p53 [26]. Accordingly, loss of p53 function also leads to loss or dysfunction of this proapoptotic effector molecule. Studies in *bax*^{-/-} mice established Bax as a bona fide tumor suppressor gene by demonstrating that Bax can suppress tumorigenesis in vivo [27]. Further, loss of functions mutations in Bax have been identified in human cancers including hematopoietic malignancies [28].

Besides mutational deregulation of some apoptosis regulators, i.e., p53 or Bax, impaired apoptosis in human cancers is often the consequence of increased expression of prosurvival molecules such as NF- κ B, IAPs, or because of reduced sensitivity for apoptosis induction [3–6, 18, 29–31]. Expression profiling of *Bcl-2* family and *IAP* genes in lymphoid malignancies revealed a specific expression pattern of IAP family proteins in CLL, B-ALL and follicular lymphoma samples, i.e., cIAP1, cIAP2, and survivin, that could be used to discriminate these entities from each other as well as from other diseases and controls [32]. Analysis of Smac/DIABLO expression in non-Hodgkin's lymphoma and Hodgkin's lymphoma tumors by immunohistochemistry demonstrated that Smac/DIABLO is expressed in most non-Hodgkin's lymphoma and all Hodgkin's lymphoma cell lines and in 47% primary Hodgkin's lymphoma, while its expression levels varied among non-Hodgkin's lymphoma with 29–68% of tumors being positive [33].

A characteristic feature of leukemias and lymphom are chromosomal translocations that can lead to deregulated expression of genes that promote proliferation such as *c-myc*, enhanced expression of genes that block apoptosis such as *bcl-2* or to the generation of fusion proteins such as BCR-Abl or PML-RAR α that cause cell death resistance [3, 4]. Constitutive expression of *c-myc* is a hallmark of a highly aggressive malignant lymphoma, Burkitt's lymphoma as a consequence of the t(8;14) translocation [34]. Interestingly, while increased constitutive expression of *c-myc* leads to rapid proliferation of malignant cells, these cells are also prone to apoptosis probably due to the concomitant activation of apoptosis pathways by deregulated *c-myc* expression [35]. As a recapitulation of the disease in humans, *myc*-transgenic mice rapidly develop aggressive

leukemia/lymphoma. The concomitant overexpression of Bcl-2, however, produces an even more aggressive phenotype demonstrating that inhibition of apoptosis in addition to deregulated proliferation control may be a secondary hit in malignant transformation [36].

Prognostic significance of apoptosis regulators in leukemia and lymphoma

A series of clinical studies have been conducted to explore the relevance of apoptosis regulators for treatment response and patient outcome in leukemia and lymphoma. Mutations in CD95 were detected in various hematological malignancies including acute leukemia, Hodgkin's disease and non-Hodgkin's lymphoma [37–40]. Further, most NHL cells were found to be constitutively CD95 resistant despite expression of the CD95 receptor [41]. In AML CD95 expression has been positively correlated to response to chemotherapy [42, 43]. Enhanced serum levels of soluble CD95 were found to be associated with reduced survival and outcome in adult T cell leukemia [44]. Moreover, elevated expression of c-FLIP that block apoptosis signaling via death receptors at the level of the DISC were detected in clinical samples from several tumors including Burkitt lymphoma [45].

Bcl-2 gene rearrangements or overexpression have been associated with poor prognosis in large-cell non-Hodgkin lymphomas in several studies [46–50]. In AML, high levels of Bcl-2 are correlated with a low level of response to chemotherapy in vivo [51]. However, some studies also demonstrated that enhanced expression levels of Bcl-2 bear no prognostic impact or paradoxically correlate with improved survival, for example in ALL [52–54]. Further, ectopic expression of Bcl-2 in a various malignant lymphoid cell lines conferred multidrug resistance to chemotherapeutic drugs of different classes, e.g., DNA damaging agents and nucleoside analogs [55, 56]. Similar to Bcl-2, no consistent correlation between expression levels of Bax and outcome can be drawn from clinical studies in leukemia. While a lack of correlation between Bax expression and response to induction chemotherapy and survival was found in one large cohort study, increased levels of Bax expression correlated with improved rates of overall survival in another study [57, 58].

Also, the influence of IAPs on clinical response and treatment outcome has been studied. IAP proteins are frequently deregulated in human cancers, e.g., due to increased mRNA or protein expression or loss of endogenous antagonists such as XAF1 [18, 59]. Survivin was identified as the fourth most common transcriptome of the human genome in human tumors in gene profiling studies [60]. By comparison, survivin was expressed at low or

undetectable levels in normal adult tissues suggesting that survivin may promote malignant progression of cancer cells [60]. Abnormal expression of cIAP2 frequently occurs in mucosa-associated lymphoid tissue (MALT) lymphoma, since the cIAP2 gene is affected by the t(11;18)(q21;q21) translocation that is present in 50% of MALT lymphoma [61–63].

In various retrospective trials, expression of IAPs in tumor samples has been correlated to clinical parameters. For example, high survivin expression predicted poor prognosis in AML, anaplastic or diffuse large-cell lymphoma [64–66]. Furthermore, AML patients with lower levels of XIAP protein were found to have significantly longer survival in one study [66]. However, a subsequent study demonstrated that expression levels of XIAP have no prognostic impact in AML patients [67]. In childhood de novo AML, high expression levels of XIAP and survivin correlated with poor overall survival [68]. Also, expression levels of XIAP were lower in patients with favorable rather than intermediate or poor cytogenetics [68]. 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 [68]. Together, these data suggest that the prognostic relevance of apoptosis regulators in leukemia and lymphoma is rather complex.

Apoptosis pathways and drug discovery in hematological malignancies

Based on the concept that resistance to apoptosis is a characteristic feature of human cancers that contributes to tumor formation and progression, strategies designed to restore defective apoptosis programs in tumor cells may overcome intrinsic or acquired resistance of cancers. Also, apoptosis targeted therapies may enhance the responsiveness of human cancers towards conventional treatments that are currently used in the clinic, e.g., chemo- or radiotherapy, since these therapies primarily exert their anti-tumor activity by triggering apoptosis in cancer cells [7]. In principle, apoptosis-based cancer therapies aim at disabling the antiapoptotic function of molecules involved in the leukemogenic process and/or in treatment resistance or alternatively, at directly activating the apoptotic machinery as discussed in more detail below.

Anti-leukemic therapy targeted at “Inhibitor of Apoptosis Proteins”

One promising therapeutic strategy directed at apoptosis regulators is the neutralization of IAPs. IAPs are a family of endogenous caspase inhibitors and comprise eight human

analogs, i.e., X-linked Inhibitor of Apoptosis Protein (XIAP), cIAP1, cIAP2, survivin, livin/melanoma-IAP (ML-IAP), apollon, NAIP and ILP-2 [18]. Among the IAP family members, XIAP is best known for its antiapoptotic function [69]. Structurally, XIAP contains three Baculovirus IAP Repeat Domains (BIR) and a RING finger. The BIR domains mediate the binding of XIAP to effector caspases, i.e., the BIR2 domain with its N-terminal linker binds and inhibits caspase-3 and -7, while a surface groove of the BIR3 domain interacts with caspase-9 [70]. The RING finger domain contains an E3 ligase activity that is involved in autoubiquitination as well as ubiquitination of a number of proteins, for example caspase-3, -9, Smac and AIF [71–74]. XIAP blocks apoptosis by binding to active caspase-3 and -7 and also by interfering with caspase-9 activation [69]. In addition, XIAP inhibits apoptosis via mechanisms unrelated to its ability to inhibit caspases. For example, XIAP can activate the NF- κ B pathway by forming a complex with the TAK1 kinase and its cofactor TAB1 [75–79]. Mechanistically, the XIAP-mediated NF- κ B activation can be dissociated from its caspase inhibitory effects and has been reported to require the E3 ubiquitin ligase activity of XIAP or the BIR1/TAB1 interaction and BIR1 dimerization [75–79]. The role of survivin in the regulation of apoptosis and proliferation is more complex compared to other IAP family proteins [80]. Besides its role as a regulator of apoptosis, survivin is also involved in the control of mitosis [80].

There is mounting evidence that cancer cells including leukemia or lymphoma have an intrinsic drive to apoptosis that is held in check by IAPs. In support of this notion, high basal levels of caspase activity in the absence of apoptosis were detected in tumor cell lines and cancer tissues, but not in normal cells [81]. Tumor cells, but not normal cells simultaneously expressed high levels of IAPs suggesting that upregulated IAP expression may counteract the high basal caspase activity selectively in tumor cells [81]. Thus, targeting aberrant expression of XIAP in leukemia and lymphoma may open new perspectives to trigger the apoptotic machinery selectively in cancer cell. Recently, several strategies to inhibit or downregulate XIAP have been developed for therapeutic purposes.

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 [70]. Ectopic expression of Smac or Smac peptides harboring 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 leukemia, lymphoma or multiple myeloma cells for apoptosis induced by death-receptor ligation, anticancer drugs or cytolytic T-cell attack [82–87]. In some studies, Smac peptides were linked to a carrier to facilitate their intracellular delivery, for example the protein transduction motif of the HIV Tat

protein, the *Drosophila* antennapedia penetrating sequence or a polyarginine stretch [88–90]. 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 [91–100]. 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 [101]. These XIAP antagonists were reported to bind to the BIR3 domain of XIAP with nanomolar affinity and promoted cell death in several human cancer cell lines including leukemia cells [101]. The low-molecular-weight Smac mimetic LBW242 was reported to enhance the killing of FLT3 mutant acute myeloid leukaemia cells that were resistant to the protein tyrosine kinase inhibitor PKC412, when combined with either PKC412 or standard chemotherapeutic drugs [87]. In multiple myeloma LBW242 was shown to trigger apoptosis in cells resistant to conventional therapies or bortezomib [86]. LBW242 even overcame multiple myeloma drug-resistance conferred by interleukin-6 or insulin-like growth factor-1 or by microenvironmental factors, e.g., adherence to bone marrow stromal cells [86].

In addition, the natural product embelin from the Japanese *Ardisia* herb was discovered as a cell-permeable, non-peptidic, small-molecular weight inhibitor of the BIR3 domain of XIAP through structure-based in-silico screening of a traditional herbal medicine three-dimensional structure database [102]. Embelin was shown to effectively overcome the protective effect of XIAP in Jurkat cells transfected with XIAP through binding to the XIAP BIR3 domain [102].

Besides the BIR3 domain of XIAP, its BIR2 motif has also served as 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 [103, 104]. These compounds caused apoptosis in leukemia cells including primary AML blasts without the requirement of an additional cytotoxic stimulus by derepressing downstream effector caspases [103, 105]. Of note, these XIAP antagonists also killed acute leukemia cells with high Bcl-2 expression levels suggesting that they may bypass some forms of resistance [103, 105]. Further, XIAP BIR2 antagonists were reported to restore caspase-9 mediated apoptosis in both chemotherapy-refractory and -responsive diffuse large B-cell lymphoma cells [106]. XIAP antagonists were also reported to enhance CD95-mediated apoptosis in CD40-activated chronic lymphocytic leukemia cells [107].

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 leukemia, either as single agents or in combination with chemotherapeutic drugs [67, 108, 109]. In animal models, AEG35156, a synthetic 19-mer, second generation, mixed backbone antisense oligonucleotide to human XIAP, reduced XIAP mRNA and protein levels in representative tissues at therapeutically feasible doses [110–112]. Importantly, the antitumor activity of XIAP antisense oligonucleotides correlated with downregulation of XIAP levels in targeted tissues isolated from pre-clinical models [110, 111]. Currently, XIAP antisense oligonucleotides are evaluated in phase I/II clinical trials in combination with chemotherapy, i.e., docetaxel, cytarabine and idarubicin, for example in AML [111, 112] (Table 1). In a phase I clinical trial of XIAP antisense AEG35156 administered as a continuous intravenous infusion, one patient with non-Hodgkin's lymphoma 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 [113]. 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 leukemia and lymphoma cells.

Anti-leukemic therapy targeted at Bcl-2 family proteins

Another approach to target apoptosis pathways for cancer therapy is to antagonize antiapoptotic Bcl-2 family members. There are currently two models how BH3-only proteins activate Bax and Bak during the course of apoptosis. According to the direct activation model [114], 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 [115, 116]. Imbalances in the ratio of anti- versus pro-apoptotic Bcl-2 proteins may tip the balance towards tumor cell survival and thus, may contribute to tumor formation and progression, for example in hematological malignancies [25].

Since high expression of anti-apoptotic Bcl-2 family proteins may confer resistance to chemo- or radiotherapy by blocking the mitochondrial pathway of apoptosis [117], there has been much interest to develop strategies that overcome the cytoprotective effect of Bcl-2 and related molecules. To this end, nuclease-resistant Bcl-2 antisense oligonucleotides downregulating Bcl-2 mRNA were tested in clinical trials for hematological malignancies including

Table 1 Examples of apoptosis targeted drugs in clinical trials for hematological tumors

Name	Cancer type	Single/combined	References
IAP targeting agents			
XIAP antisense	AML, NHL	Single, combination (chemotherapy)	[111–113]
Survivin antisense	AML	Single	[80]
Bcl-2/Bcl-X _L targeting agents			
Bcl-2 antisense	Leukemia/lymphoma	Combination (chemotherapy)	[118–120]
Bcl-2/Bcl-X _L inhibitor	Leukemia/lymphoma	Single	[143]
TRAIL receptor agonists			
TRAIL	NHL	Single, combination (rituximab)	[193, 199]
TRAIL-R1 mAb	NHL	Single	[196]
TRAIL-R2 mAb	Hodgkin disease	Single	[192]

non-Hodgkin lymphoma and CLL, as single agent or in combination with chemotherapy [118–121] (Table 1). In addition, BH3 peptides, which mimic BH3-only proteins in activating proapoptotic Bax and Bak proteins, are under preclinical evaluation [114]. 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_L and Bcl-w that normally interacts with the BH3 domain of Bax or Bak [122]. 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. ABT-737 as single agent has been reported to directly trigger apoptosis in a panel of hematological malignancies including CLL, AML, ALL, MCL, MM, DLBCL, especially in those that critically depend on antiapoptotic Bcl-2 proteins for survival [122–127].

To augment the antitumor 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 [122, 123, 128, 129]. In pediatric ALL, ABT-737 synergized with other chemotherapeutic agents, including drugs that are commonly used for remission induction in primary and relapsed childhood leukemia, such as vincristine, glucocorticoids, and L-asparaginase amidohydase, in ALL cell lines and also in vivo in mouse xenografts derived from patients with ALL [130].

Further, ABT-737 potentiates the anti-neoplastic activity of proteasome inhibitors in several lymphoid malignancies including CLL, MCL, and DLBCL [131]. In primary AML samples with activating FLT3 mutations that exhibit high expression of Bcl-2 protein, ABT-737 synergized efficiently with FLT3 inhibition [132]. Moreover, ABT-737 was shown to markedly enhance apoptosis induction by imatinib or by the second-generation Bcr-Abl inhibitor INNO-406, even in cells with Bcr-Abl point mutations, by

triggering a Bcl-2 family-regulated intrinsic apoptosis pathway [133, 134]. Mechanistic studies revealed that Bim and Bad account for most imatinib-induced killing of Bcr/Abl+ leukemic cells and that Bcl-2 overexpression causes resistance to imatinib [134]. These findings suggest that a dual targeting of both Bcr-Abl-driven oncogenic signaling and antiapoptotic Bcl-2 family proteins is a novel approach for Philadelphia-positive leukemias.

Since ABT-737 targets Bcl-2/Bcl-X_L 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 [123, 128, 135, 136]. Consequently, Mcl-1 downregulation by genetic approaches or pharmacologic 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 [123, 128, 135–139]. Further, the synthetic cytotoxic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR) has recently been reported to antagonize Mcl-1 through the generation of reactive oxygen species (ROS) and ROS-mediated activation of c-Jun kinase (JNK), which in turn phosphorylates and inhibits Mcl-1 [140]. This resulted in synergistic cytotoxicity of ABT-737 in combination with 4-HPR in ALL cell lines with minimal cytotoxicity for normal lymphocytes [140].

An orally bioavailable Bcl-2 family inhibitor, ABT-263, has recently been developed that exerts cytotoxic activity against a panel of lymphoid cancer cell lines and in xenograft mouse models of ALL, DLBCL, MCL and MM [141]. Further, ABT-263 has recently been evaluated by the pediatric preclinical testing program [142]. Interestingly, ABT-263 demonstrated highest in vitro activity against ALL cell lines among the panel of pediatric tumor cell lines tested and also showed significant activity against ALL xenografts [142]. In vivo, ABT-263 induced significant prolongation of the event-free survival distribution in

5 of 6 (83%) of the evaluable ALL xenografts and caused complete remissions in 3 of 6 evaluable xenografts in the ALL panel [142]. These results argue for further clinical evaluation of ABT-263 alone and in combination with chemotherapeutics in ALL. Moreover, ABT-263, is currently evaluated in early clinical trials [143]. In a phase I clinical trial, ABT-263 showed early evidence of activity in refractory or relapsed lymphoid malignancies, while ABT-263-associated toxicities are considered to be mechanism-based, for example a decrease in circulating platelet count that is mediated by inhibition of Bcl-X_L [143–145].

Obatoclox (GX15-070) presents another BH3 mimetic that has shown activity against several hematologic malignancies, including AML, CLL, mantle cell lymphoma and multiple lymphoma [146–148]. Obatoclox is predicted to occupy a hydrophobic pocket within the BH3 binding groove of antiapoptotic Bcl-2 family proteins. An important difference to ABT-737 resides in its ability to also antagonize Mcl-1 in addition to Bcl-2, Bcl-X_L and Bcl-w [146, 147]. Accordingly, obatoclox is capable to also overcome MCL-1-mediated resistance to the Bcl-2/Bcl-X_L/Bcl-w-selective antagonist ABT-737 [147]. Obatoclox was reported to inhibit cell growth and to trigger apoptosis in AML cell lines and primary AML samples and also synergized with chemotherapeutic drugs such as AraC [146]. Obatoclox induces activation of the mitochondrial apoptotic pathway by releasing Bak from Mcl-1 as well as Bim from both Bcl-2 and Mcl-1, followed by the formation of an active Bak/Bax complex [146–148]. Recently, phosphorylation of Bcl-2 at serine 70 residue has been shown to attenuate sensitivity to obatoclox suggesting that a combination with ERK inhibitors that interfere with Bcl-2 phosphorylation may enhance the cytotoxicity of obatoclox [148]. In mantle cell lymphoma (MCL), obatoclox was shown to trigger apoptosis in MCL cell lines *in vitro* and also in primary cells from patients with MCL, including those that bear defective DNA damage-sensor genes or cell-cycle regulators [148]. Notably, obatoclox also synergized with the proteasome inhibitor bortezomib by neutralizing the bortezomib-caused accumulation of Mcl-1 and by acting in concert with Noxa to initiate the displacement of Bak from Mcl-1 [148]. In contrast, obatoclox alone or in combination with bortezomib exhibited no significant cytotoxicity on normal peripheral blood mononuclear cells [148], while it was described to inhibit normal bone marrow-derived colony formation [149]. Also, in multiple myeloma, obatoclox demonstrated cooperative cytotoxic activity together with bortezomib as well as with melphalan and dexamethasone [149]. Collectively, these findings suggest that small molecule inhibitors of antiapoptotic Bcl-2 family proteins may open new perspectives to reactivate the mitochondrial pathway of apoptosis in cancer cells.

Anti-leukemic therapy targeting TRAIL receptors

Tumor necrosis factor related apoptosis-inducing ligand (TRAIL)/Apo-2L is another prime candidate for translation of apoptosis targeted therapeutics into the clinic. TRAIL was identified in 1995 based on its sequence homology to other members of the TNF superfamily [150–152]. TRAIL is a type II transmembrane protein and its extracellular domain can be proteolytically cleaved from the cell surface. TRAIL is constitutively expressed in a wide range of tissues [152]. Five different receptors have been described for the TRAIL receptor system. TRAIL-R1 and TRAIL-R2, the two agonistic TRAIL receptors, contain a conserved cytoplasmic death domain motif, which enables them to engage the cell's apoptotic machinery upon ligand binding [152]. TRAIL-R3 to R-5 are antagonistic decoy receptors, which bind TRAIL, but do not transmit a death signal [152]. TRAIL-R3 is a glycosyl-phosphatidylinositol-anchored cell surface protein, which lacks a cytoplasmic tail, while TRAIL-R4 harbors a substantially truncated cytoplasmic death domain [152]. In addition to these four membrane-associated receptors, osteoprotegerin is a soluble decoy receptor, which is involved in regulation of osteoclastogenesis [152].

The concept to selectively trigger death receptors of the TNF receptor gene superfamily to induce cell death in cancer cells is attractive for a potential clinical application, since death receptors are directly linked to the cell's intrinsic death machinery. Among the death receptors, the TRAIL system is considered to be most suitable for clinical development, since TRAIL predominantly kills cancer cells, while sparing normal cells [153]. Several strategies have been developed to target TRAIL receptors therapeutically [150, 151]. One approach is the use of trimeric TRAIL itself as a recombinant natural ligand [152]. Recombinant soluble TRAIL triggered apoptosis in a wide range of cancer cell lines including hematological malignancies and also *in vivo* in several xenograft models of human cancers [138, 154–161]. 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 antitumor activity in cancer cell lines and xenograft-bearing mice [162–164]. The existence of decoy receptors that can bind TRAIL, yet 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, it remains to be determined in future studies which of these agents triggering the TRAIL pathway will turn out to be superior for clinical application.

Another level of complexity relates to the fact that cancer cells may signal to apoptosis primarily or even

exclusively only via one of the agonistic TRAIL receptors, although they express both receptors on their surface. Since each of the agonistic receptors TRAIL-R1 and TRAIL-R2 can initiate apoptosis independently of the other and since many human cancer cell lines express both receptors, the relative contribution of TRAIL-R1 versus TRAIL-R2 to ligand-induced apoptosis has been explored using receptor-selective mutants of TRAIL or TRAIL receptor specific monoclonal antibodies. For example, TRAIL-R2-selective mutants were found to exert increased apoptosis-inducing activity compared to TRAIL-R1-selective mutants in several carcinoma cell lines [165]. In contrast, TRAIL-R1 targeting agents in combination with HDAC inhibitors have been shown to trigger apoptosis in primary CLL and mantle cell lymphoma cells, e.g., using TRAIL-R1 selective TRAIL mutants or TRAIL-R1 specific antibodies [166–168]. These studies indicate that expression analysis of TRAIL receptors in human tumors without any functional studies may not accurately predict which primary patient sample from a particular tumor type signals via TRAIL-R1 or TRAIL-R2. Such information, however, may be crucial to provide a rational approach for the optimal use of TRAIL receptor agonists in the clinic. Interestingly, TRAIL-R2 antibody-based therapy was reported as an efficient strategy not only to eliminate TRAIL-sensitive tumor cells, but also to induce tumor-specific T-cell memory that afforded long-term protection from tumor recurrence [169].

It is important to note that so far few studies have been carried out testing the efficacy of TRAIL receptor agonists on primary cells from human hematopoietic malignancies or solid tumors. While many cancer cell lines are susceptible to TRAIL-induced apoptosis, many primary tumor cells turned out to be refractory towards TRAIL despite the expression of the agonistic TRAIL receptors on their cell surface. For example, primary CLL cells have been reported to be resistant towards TRAIL [170]. In pediatric acute lymphocytic leukemia, 50% of primary samples obtained from children before the onset of chemotherapy were refractory to TRAIL-mediated apoptosis [171]. In some of these resistant samples, TRAIL even attenuated spontaneous apoptosis and stimulated proliferation [171], pointing to a pro-survival function of TRAIL under certain conditions. This resistance to TRAIL-induced cell death was not restricted to a specific TRAIL formulation, but extends from several form of soluble TRAIL to agonistic monoclonal antibodies for TRAIL-R1 or TRAIL-R2 [161, 166, 167, 171].

Genetic alterations in components of the TRAIL receptor pathway have been identified only in a small subset of tumors. For example, somatic mutations of TRAIL-R1 and TRAIL-R2 genes were found in a small proportion of non-Hodgkin's lymphoma [172].

Interestingly, TRAIL-R1 and TRAIL-R2 genes map to chromosome 8p21-22 [173], which is a frequent site of allelic deletions in many types of human tumors including non-Hodgkin's lymphoma [174, 175]. In a series of 117 human non-Hodgkin's lymphoma, eight tumors (6.8%) were found to have two TRAIL-R1 gene mutations or six TRAIL-R2 gene mutations [172]. Six of these mutations (2 TRAIL-R1 and 4 TRAIL-R2) were detected in the death domains and one nonsense mutation of TRAIL-R2 was detected just before the death domain [172]. This study suggests that somatic mutations of TRAIL-R1 and TRAIL-R2 genes may play a role in the pathogenesis of some non-Hodgkin's lymphoma [172].

Since primary resistance towards TRAIL will likely limit the success of TRAIL in the clinic, there have been many efforts over the last 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 γ -irradiation elicit markedly enhanced antitumor activity. For example, several chemotherapeutics or γ -irradiation were reported to synergistically interact with TRAIL in hematological malignancies [164, 176–178]. In addition, the concomitant use of TRAIL together with new compounds, e.g., proteasome inhibitors or kinase inhibitors enhanced the therapeutic effects of TRAIL in leukemia [179, 180]. Also, histone deacetylase (HDAC) inhibitors have been reported to enhance TRAIL-induced apoptosis in Jurkat T-cell acute lymphocytic leukemia and HL-60 acute myeloid leukemia cells as well as in primary CLL cells [83, 181–185]. Further, rituximab, an antibody directed at CD20, was shown to increase TRAIL-induced apoptosis in non-Hodgkin lymphoma cell lines [186]. Likewise, rituximab augmented the antitumor activity of TRAIL in a subcutaneous and disseminated xenograft mouse model *in vivo* suggesting that the combination presents a promising approach for further clinical evaluation [186]. Moreover, the multikinase inhibitor sorafenib was shown to enhance TRAIL lethality in leukemia and lymphoma cells, at least in part via mechanisms that involve downregulation of Mcl-1 [187, 188]. Furthermore, various natural products were reported to act in concert with TRAIL to induce apoptosis in hematological malignancies, e.g., 1-methoxy-canthin-6-one that is isolated from the medicinal plant *Ailanthus altissima* Swingle [189], Wogonin that is derived from the popular Chinese herb Huang-Qin [190], Fuligocandin B, an extract of myxomycete *Fuligo candida* [191] and Dihydroflavonol BB-1, an extract of natural plant *Blumea balsamifera* [191].

Soluble recombinant TRAIL and fully human monoclonal antibodies against TRAIL-R1 or -R2 are currently undergoing evaluation in early clinical trials (Table 1). Initial trials 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 [192–195]. Results of a Phase 2 clinical trial demonstrate that HGS-ETR1, a fully human monoclonal antibodies against TRAIL-R1 (mapatumumab; Humane Genome Sciences, Rockville, MD, USA), is well tolerated and capable of producing clinical responses in 3 out of 40 patients (8%) when administered as monotherapy in patients with advanced non-Hodgkin's lymphoma [196]. In a phase I clinical trial using a fully human monoclonal antibody to TRAIL-R2 (HGS-ETR2), one patient with chemotherapy-refractive Hodgkin's disease had a tumor regression of abdominal disease [192–194]. Since preclinical studies demonstrated cooperative interaction of TRAIL receptor agonists with chemotherapeutic drugs in several types of cancer, clinical trials using HGS-ETR1 in combination with anticancer agents were recently initiated in solid tumors, e.g., using carboplatin and paclitaxel or gemcitabine and cisplatin [197, 198]. Further, recombinant human TRAIL is currently under investigation in a phase I trial in combination with rituximab in patients with low-grade non-Hodgkin's lymphoma [199].

Conclusions

Based on the concept that cellular systems with a high intrinsic cell turnover in the body crucially depend on control mechanisms, defective regulation of the cell's intrinsic death program within the lympho-hematopoietic system may promote leukemogenesis as well as resistance to current treatment regimens. A variety of apoptosis regulatory molecules have been identified that play an important role in the regulation of cell death in leukemia and lymphoma cells. These molecules present promising targets for therapeutic intervention. 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 been translated into a clinical setting and are currently evaluated in early clinical trials in several malignancies, including leukemia and lymphoma. Thus, the application of basic knowledge on apoptosis pathways into medical practice is anticipated to yield new biomarkers and better cancer therapeutics for the treatment of hematologic malignancies.

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