

# Deregulation of apoptosis in acute myeloid leukemia

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## ABSTRACT

Apoptosis, or programmed cell death, is central to the development and homeostasis of the hematopoietic system. Dysregulation of apoptosis plays an important role in the development of a variety of human pathologies, including cancer, autoimmune diseases and neurodegenerative disorders. Studies carried out in the last years have shown that leukemia cells invariably have abnormalities in one or more apoptotic pathways, determining a survival advantage of these cells over their normal counterpart. Furthermore, abnormalities in the apoptotic response also play a role in the development of drug resistance by leukemic cells. The identification of the different components of the apoptotic pathways has enabled the detection of various biochemical defects present in leukemic cells compared to their normal counterparts. These defects contribute to the survival advantage of the leukemic clone over the normal hematopoietic cells and are also frequently associated with a low rate of response to standard chemotherapy treatment and with poor survival. Furthermore, these findings have led to the identification of many potential apoptotic targets for the development of new drugs targeting anti-apoptotic molecules abnormally expressed or regulated in leukemic cells. Many of these drugs restore the sensitivity of leukemic cells to apoptotic stimuli and some of them are under investigation at a clinical level.

Key words: apoptosis, deregulation, acute myeloid leukemia.

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Acute myelogenous leukemia (AML) is a heterogeneous group of malignant disorders characterized by uncontrolled proliferation of clonal leukemic hematopoietic precursor cells blocked at various stages of normal myeloid development, associated with severely impaired normal hematopoiesis, thus leading to neutropenia, anemia and thrombocytopenia. The development of AML is associated with accumulation of acquired genetic alterations and epigenetic changes in hematopoietic progenitor cells which alter normal mechanisms of cell growth, proliferation, death and differentiation. Approximately 50-75% of adult patients with AML achieve complete remission when treated with cytarabine and daunorubicin or idarubicin, or with the topoisomerase II inhibitor, mitoxantrone.<sup>1,2</sup> Apoptotic processes play a key role in the control of tissue homeostasis, particularly of rapidly renewing tissues, such as hematopoietic tissue. Studies carried out during the last twenty years have dramatically improved our understanding of the role of apoptotic mechanisms in the control of differentiation, proliferation and survival of normal and leukemic cells.

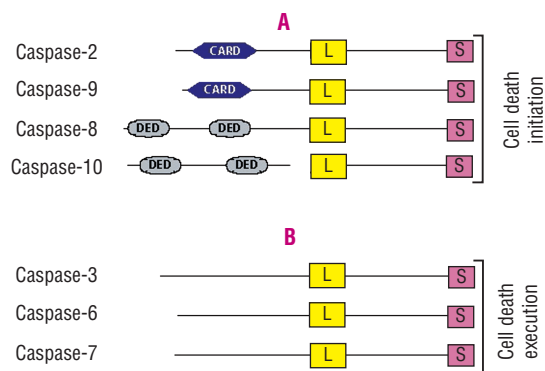
### Cell death pathways

The most common and well defined form of programmed cell death is apoptosis, a physiological process of cellular suicide required for the maintenance of cell homeostasis, embryonic development and for the differentiation and function of hematopoietic and lymphoid cells. A common feature of the apoptotic process is the constant involvement of caspases, a family of intracellular cysteine proteases (cysteine aspartyl-specific proteases) (Figure 1). These enzymes are present as inactive zymogens in all animal cells, but can be triggered to assume an active state, usually through proteolytic processing at conserved aspartic acid residues.

Based on their level of action caspases are distinguished in *initiator* caspases (which include caspase-8, -2, -9 and -10) and *effector* caspases (caspase-3, -6 and -7): the former act at the origin of the apoptotic process, while the latter act during the late steps of the apoptotic process (Figure 1). Once activated, the effector caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular proteins, which ultimately culminates with cell death. The *initiator* caspases

are structurally characterized by an extended N-terminal region, comprising one or two adaptor domains that are essential for their function; in contrast, the *effector* caspases contain 20-30 amino acid residues in their prodomain region. There are two pathways by which caspase activation is induced, the extrinsic and the intrinsic apoptotic pathways (Figures 2 and 3). Both the pathways converge on activation of effector caspases but require different initiation caspases to start the process. The *extrinsic* pathway is activated by engagement of death receptors on the cell membrane. Binding of ligands, such as FasL, tumor necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL) to their respective membrane receptors Fas, TNF-R and TRAIL-R induces the formation of the oligomeric death-induced signaling complex (DISC) (Figure 2). DISC in turn promotes caspase-8 recruitment and a cascade of activation of caspase enzymes that culminates with cell death. The activation of caspase-8 is antagonized by cellular FLICE inhibitory protein (c-FLIP), an enzymatically inactive relative of caspase-8 and -10 that binds to DISC. The knockdown of c-FLIP augments DISC recruitment, activation and processing of caspase-8, thereby enhancing effector-caspase stimulation and apoptosis. The *intrinsic* pathway is triggered by various extracellular and intracellular stresses, including growth factor deprivation, DNA damage, oncogene induction, hypoxia and cytotoxic drugs (Figure 3). Cellular signals originated by various mechanisms by these different stresses converge on a cellular target represented by mitochondria. Mitochondrial membrane permeability is controlled by pro-apoptotic (Bax, Bak, Bad, Bid, Bim, Bmf, NOXA, PUMA) and anti-apoptotic (Bcl-2, Bcl-X<sub>L</sub>, Mcl-1) members of the Bcl-2 family, inducing or preventing heterodimerization of pro-apoptotic members. A series of biochemical events is induced that lead to damage of the outer mitochondrial membrane, with the consequent release of cytochrome c and other pro-apoptotic molecules, such as Smac/DIABLO, from the inner membrane into the cytosol enabling the formation of the apoptosome, a large molecular complex formed by cytochrome c, apoptotic protease activating factor 1 (APAF-1) and caspase-9, and massive activation of caspases (Figure 3).

Members of the Bcl-2 family either promote or prevent apoptosis. The pro-survival Bcl-2 family members include Bcl-2, Bcl-X<sub>L</sub>, Bcl-w, A1 and Mcl-1, all characterized by the presence of four Bcl-2 homology domains (BH1-BH4), with the exception of Mcl-1 that contains only three BH domains. These proteins all play crucial roles for cell survival and the loss of any of these proteins causes major deregulation of survival of some cell types. The pro-apoptotic members of the Bcl-2 family can be subdivided into two groups: (i) Bax-like proteins (Bax, Bak, Bok, Bcl-G, and Bfk) contain three BH regions; (ii) *BH3-only* proteins (Bad, Bik, Bid, Bim, Noxa, PUMA, Bmf and Hrk) possess only the BH3 domain. BH3-only proteins are essential for cell death initiation through the intrinsic pathway, where-



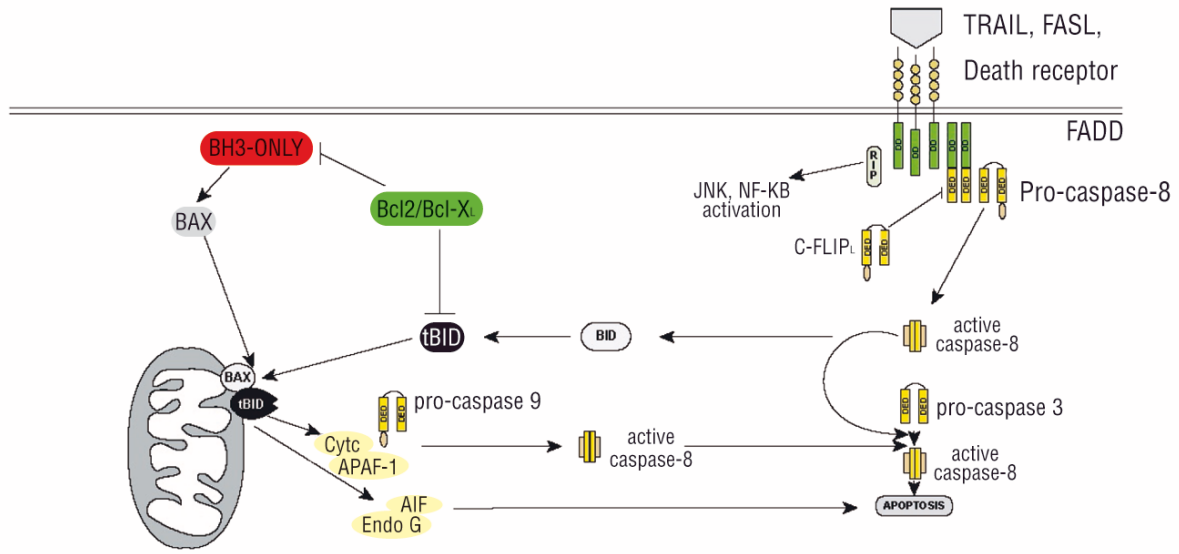
**Figure 1.** Basic structure of mammalian apoptotic caspases. According to their function and structure caspases may be subdivided into three groups: (A) caspases involved in cell death initiation; (B) caspases involved in cell death execution; (C) caspases involved in the inflammatory response (not shown). The adaptor-driven activation of the activator caspases depends on conserved motifs within their long prodomain. The caspase activation and recruitment domain (CARD) of caspase-9 and -2 and the death effector domain (DED) of caspase-8 and -10 mediate the homophilic interaction with CARD- and DEDS-containing adaptor protein (e.g., Apaf-1 or FADD).

as the Bax/Bak-like proteins play a key role in the progression of the apoptotic signaling stimuli at the level of permeabilization of the outer mitochondrial membrane. The molecular mode of action of BH3-only protein consists in binding to a surface groove, formed by the BH1, BH2 and BH3 domains, on the surface of Bcl-2-like molecules and, through this mechanism, antagonizing their pro-survival function.

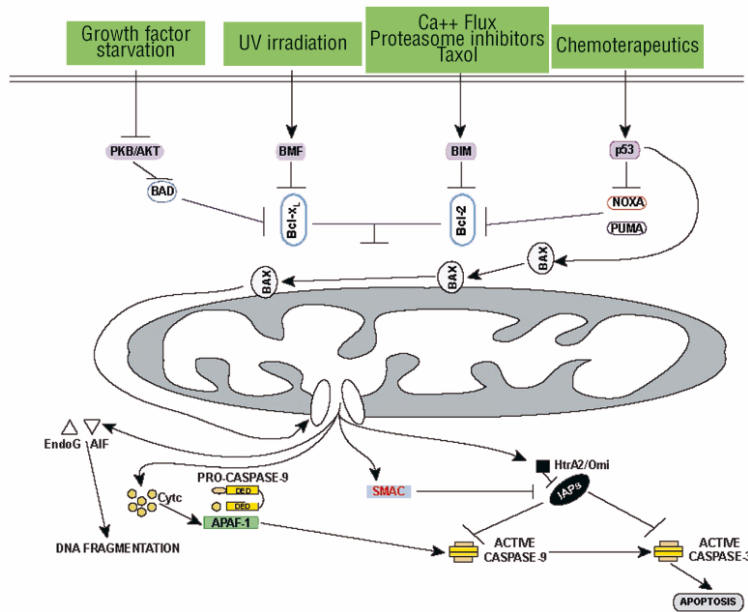
#### **Deregulation of apoptosis in acute myeloid leukemia: general mechanisms**

Deregulation of apoptosis disrupts the complex and delicate balance between cell proliferation, cell survival and cell death and plays a major role in the development of diseases such as cancer, and particularly leukemia. The evasion of programmed cell death has been regarded as one of the six essential alterations in cellular physiology that dictate the growth of cancer cells and is a hallmark of virtually all cancers.<sup>3</sup> Moreover, tumors that have alterations in proteins involved in cell death signaling are very frequently resistant to chemotherapy and are difficult to treat with chemotherapeutic agents that primarily act by inducing apoptosis.<sup>4</sup>

Leukemia development is regarded as a multistep process characterized by progressive genetic alterations that drive the transformation of normal human hematopoietic stem/progenitor cells into leukemic derivatives. Leukemia originates from a single cell that has undergone malignant transformation driven by frequent genetic mutations. These events are followed by a clonal selection of mutated cells that display increasingly aggressive behavior. There is clear evidence indicating that the cellular and molecular events leading to leukemia are orchestrated by leukemic stem cells.<sup>5</sup> Using *in vivo* models in which human AML cells were transplanted into immun-



**Figure 2.** FAS and TRAIL signaling pathway (extrinsic apoptotic pathway). The death-receptor apoptosis pathway is activated upon interaction of a death receptor (e.g. TRAIL-R1, TRAIL-R2 or FAS) with its cognate death inducing ligand, resulting in the recruitment of the adaptor protein FADD and procaspase-8 to the intracellular death domains of the receptor. Concomitantly, procaspase-8 is proteolytically cleaved and activates the effector caspases. In addition, caspase-8 can induce the cleavage of the BH3-only protein BID, thereby activating a mitochondrial amplification loop.



**Figure 3.** Intrinsic apoptotic pathway. The BH3-only proteins trigger apoptosis in response to insufficient trophic support (growth factor withdrawal), exposure to anti-tumor drugs (such as taxol and various chemotherapeutics) and UV-irradiation. Death signals activate some BH3-only members of the Bcl-2 protein family and other proteins which, in turn, induce oligomerization of the pro-apoptotic Bax and Bak proteins to insert into the outer mitochondrial membrane (OMM) and to cause the formation of pores in the OMM through which mitochondrial proteins are released in the cytoplasm.

odeficient mice, a leukemia initiating cell was identified that showed properties of an immature progenitor cell, expressing a CD34<sup>+</sup>/CD38<sup>-</sup> phenotype.<sup>67</sup> By contrast, despite the fact that they showed a leukemic blast phenotype, CD34<sup>+</sup>/CD38<sup>+</sup> leukemic cells were unable to initiate leukemia in immunodeficient mice.<sup>67</sup> Although the origin of acute leukemias from leukemic stem cells was clearly established, it should be noted that only about 50% of

AML are able to initiate leukemia in NOD/SCID mice.<sup>8</sup> The analysis of the membrane phenotype of leukemic stem cells provided evidence of a great similarity with normal hematopoietic stem cells, consistent with the hypothesis that AML arises from a hematopoietic stem cell.<sup>9</sup>

About 50-55% of AML are characterized by the presence of specific balanced chromosome abnormalities involved in the formation of specific fusion genes that

encode for fusion proteins, playing a key role in the development of leukemia through their effect on cell proliferation, survival and apoptosis.<sup>2</sup> Simultaneous alterations in the normal regulation of cell proliferation and apoptosis induce leukemic cells to accumulate, thus promoting further genetic alterations and leukemogenesis. The key role of apoptosis in the pathogenesis of AML has been elucidated over the last years. Some fusion proteins interact with mediators of apoptosis, sending anti-apoptotic signals that favor the preferential survival of leukemic cells: PML/RAR- $\alpha$  or CBF/SMMHC through the p53 pathway or AML1/ETO through the Bcl2-related pathway.<sup>10-12</sup> Furthermore, it has been suggested that MLL fusion proteins modify PPP1R15A function and inhibit apoptosis.<sup>13</sup> Finally, the PML/RAR $\alpha$  fusion protein was shown to exert an anti-apoptotic activity by downmodulating the expression of some death-inducing genes, such as TNF-R1<sup>14</sup> and TRAIL-R1/-R2.<sup>15</sup> Interestingly, a high frequency of mutations of nucleophosmin was observed in AML that do not display chromosomal translocations.<sup>16</sup> In fact, mutations affecting the carboxyterminal domain of nucleophosmin occur in a high percentage of adult patients with AML not associated with chromosomal translocations and these alterations create an additional nuclear export signal that relocalizes much of the protein from its normal nucleolar stores to the cytoplasm.<sup>16</sup> Furthermore, nucleophosmin is also frequently found in chromosomal translocations associated with myelodysplasia/AML.<sup>17</sup> Nucleophosmin acts as a cellular p53 negative regulator to protect hematopoietic cells from stress-induced apoptosis.<sup>18</sup> Furthermore, nucleophosmin has been implicated in the control of chromosomal ploidy and DNA repair.<sup>19</sup> The mutated nucleophosmin could perturb p53-dependent and independent activities of the ARF tumor suppressor protein, and through this mechanism could protect AML cells from apoptosis.<sup>20</sup>

These mutations are not sufficient to cause the development of AML and additional mutations, occurring at the level of signal transduction molecules (the receptor tyrosine kinases Flt3 or c-kit, NRAS and KRAS), are required for the generation of disease. Flt3 is overexpressed in the majority of AML and, importantly, is mutated in about 35% of AML [in about 25% of patients an internal tandem duplication (ITD) within the juxtamembrane domain of Flt3 is observed, while in about 10% of patients mutations, small deletions or insertion within the activation loop of the second kinase domain of Flt3 are observed].<sup>21</sup> These mutations determine a constitutive activation and signaling of Flt3, resulting in the triggering of anti-apoptotic pathways. Flt3 ITD mutants constitutively activate MAPK, AKT and Stat5, leading to Pim-1 activation and Bcl-XL hyperexpression.<sup>22,23</sup>

Another membrane receptor tyrosine kinase frequently mutated in AML is c-kit. In fact, c-kit mutations at the level of exon 8 (encoding for a part of the extracellular domain of the receptor) or mutations at the level of exon

17, codon 816 in the activation loop of the catalytic domain occur in about 20% and 30% of AML patients with t(8;21) or inv(16)t(16;26).<sup>24</sup> More recently, a third type of c-kit mutation was described in pediatric AML patients and was found to be associated with constitutive receptor signaling.<sup>25</sup> Extracellular c-kit mutations resulted in c-kit receptor hyperactivation in response to kit ligand, with subsequent strong activation of MAPK and PI3K, while codon 816 c-kit mutations induced constitutive Stat-3 activation and upregulation of Bcl-X<sub>L</sub> and c-myc. The KIT-D816 mutations occur in t(8;21)/AML-1/ETO-positive AML and represent a reliable molecular marker identifying patients with a poor prognosis in an otherwise prognostically favorable AML group.<sup>26</sup> Similarly, both types of c-kit mutations have an adverse impact on relapse risk and overall survival in AML with inv(16).<sup>27</sup>

KRAS and NRAS make part of a family of guanine nucleotide-binding proteins, activated by a large spectrum of cytokine receptors. KRAS and NRAS mutations are present in approximately 15% and 25% of AML patients: these mutations are usually point mutations and determine constitutive GTPase activity.<sup>28</sup> NRAS mutations lead to increased activity of the RAS pathway, resulting in increased proliferation and decreased apoptosis.<sup>29</sup> Constitutive RAS activation in AML without RAS mutations is also frequently observed, probably due to deregulation of signaling proteins acting upstream of RAS. Recent studies have shown that oncogenic NRAS mutants rapidly induce AML-like disease in mice, thus indicating that mutated NRAS can function as an initiating oncogene in the induction of myeloid malignancies.<sup>30</sup> Animal models indicate that abnormalities in the apoptosis pathway may lead to the development of AML. Thus, Fas-deficient mice constitutively expressing Bcl-2 in myeloid cells develop a disease closely resembling the M2 FAB subtype of AML.<sup>31</sup> These observations indicate that molecules involved in the control of apoptosis may act as tumor suppressors to control leukemogenic transformation in myeloid progenitor cells.

In addition to traditional cytogenetic analysis or the detection of mutations in individual genes, global gene expression profiling through microarray technology has provided an additional tool to probe and to try to classify in a rational way the marked biological heterogeneity of AML.<sup>32-35</sup> This approach has enabled the identification of several clusters of AML, exhibiting different molecular signatures, prognoses and responses to treatment. Cluster A is characterized by AML exhibiting a high frequency of NPM1 mutations, a so-called stem-cell molecular signature and overexpression of genes that promote apoptosis, such as LTBP1 and caspase-3. Cluster B, represented by a group of AML patients with a very poor response to induction therapy and a very bad prognosis, is characterized by overexpression of genes inducing chemoresistance, such as ABCG2, almost complete absence of NPM1 mutations and often a normal cytogenetic analysis. AML

**Table 1.** Features of the more frequent chromosome translocations observed in AML.

Chromosome abnormality	Frequency and distribution among AML subtypes	Altered genes	Associated molecular abnormalities	Phenotype	Response to treatment
t(15;17)(q22;q11?21)	7-10%, Acute Promyelocytic Leukemia (FAB M3)	PML/RAR $\alpha$ PML: promyelocytic leukemia RAR $\alpha$ : retinoic acid receptor $\alpha$	FLT3 ITD FLT3 ALD	Differentiation block at promyelocytic stage. Differentiation with ATRA, Apoptosis with ATO	Good response to treatment (chemoterapeutics+ATRA)
t(8;21)(q22;q22)	10-12% , AML with maturation (FAB M2)	AML1/ETO AML1: Acute Myeloid Leukemia 1 ETO: Eight Twenty-One	Kit 8 mutation Kit 816 mutation NRAS point mutations CEBPA downregulation	Early block of myeloid differentiation. Inhibition of normal AML1 allele and of C/EBP $\alpha$ function	Relatively favorable clinical outcome, in the absence of c-kit mutations
inv(16)(p13q22)10-12%, t(16;16)(p13;q22)	Acute myelomonocytic leukemia with eosinophils (FAB M4eo)	CBF $\beta$ -MYH11 CBF $\beta$ : $\beta$ subunit of core Binding factor MYH11: smooth muscle Myosin heavy chain	KIT8 mutation KIT 816 mutation NRAS mutation KRAS mutation CEBPA downregulation	Late myeloid differentiation block. Resistance to apoptosis	Relatively favorable clinical outcome, in the absence of c-kit mutations
t(9;11)(p22;q23) t(10;11)(p12;q23) t(6;11)(q27;q23) t(11;19)(q23;p13.3)	5% adult AML; 8-10% pediatric AML. Acute monocytic AML in the majority of cases (FAB M5)	MLL-AF9 MLL-AF10 MLL-AF6 MLL-ENL MLL: mixed lineage leukemia	FLT3 ITD (8%) FLT3 ALD (20%)	Monocytic differentiation block. Induction of the expression of some genes of the HoxA gene cluster. Immortalization of hemopoietic progenitors	Adult AMLs with MLL translocations are an intermediate risk prognosis. Pediatric MLL-AF10 AMLs have a worse prognosis.
t(6;9)(p23;q34)	1-2% AML, FAB M2 and M4 in the majority of cases; in a minority FAB M1	DEK-CAN CAN: Canio	FLT3 ITD (88%)	Early myeloid block. Hemopoietic dysplasia.	Poor prognosis
inv(3)(q21q26)	2% AML, mostly FAB M1	RPN1-EV1 RPN1: Ribophorin 1 EV1: Ecotropic Viral Integration Site 1	Transcriptional activation of the EV1 gene	"3q21q26" syndrome: normal or elevated platelet at diagnosis, hyperplasia with dysplasia of megakaryocytes	Poor response to chemotherapy. Poor prognosis
t(3;5)(q25;q35) t(3;3)(q21q26)	1% AML with multilineage dysplasia, mostly FAB M4 and M5	NPM/MLF1 NPM: nucleophosmin MLF1: myeloid leukemia Factor 1	Not known	AML with multilineage dysplasia	Early relapse and poor prognosis

patients in cluster C exhibit a high proliferative activity gene signature, a high incidence of karyotypic abnormalities, a low prevalence of NPM1 mutations and overexpression of IRF4 and IL10R. A high rate of response to induction therapy, but also a high rate of relapse have been observed in this cluster of patients. Cluster F is characterized by AML with monocytic features, with the highest white cell counts and a poor response to therapy. Finally, acute prolymphocytic leukemia (APL) form a separate cluster with a specific molecular signature and with a favorable prognosis related to the identification of specific first line and second line therapies.

Some signaling pathways, including phosphoinositide 3-kinase (PI3K)/AKT, JAK/STAT, RAS/Raf, /MEK/ERK and protein kinase C  $\alpha$ , are crucial to many aspects of cell growth, survival and apoptosis, and their constitutive activation has been implicated in both the pathogenesis and progression of AML. These pathways have been extensively studied in AML. AKT was found to be constitutively phosphorylated in the majority of AML patients with high blast cell counts and was an adverse prognostic factor.<sup>36</sup> Phosphorylated AKT, in turn, phosphorylates caspases, Bad, mTOR, Waf1, and NF- $\kappa$ B, resulting in protection from apoptosis and increased proliferation.<sup>37</sup> Activated AKT may promote leukemogenesis and inhibition of AKT activity induces apoptosis of AML blasts.<sup>38</sup> The RAS/Raf/MEK/ERK pathway is a key signaling trans-

duction pathway that induces increased cell proliferation and survival: constitutive activation of this pathway is observed in the majority of AML patients<sup>39</sup> and its blockade with specific pharmacologic agents induces blast cell death.<sup>40</sup> Finally, recent studies<sup>41</sup> have provided clear evidence that a significant proportion of AML exhibit the simultaneous activation of AKT, ERK and PKC $\alpha$  and exhibit a worse prognosis, associated with short survival.

### Death ligands and death receptors

There is growing interest in the development of therapeutic strategies that kill leukemic cells via activation of the extrinsic apoptotic pathway. Some members of the TNF-family directly trigger apoptosis and their activation could be used to induce the killing of tumor cells. Three ligands (TNF- $\alpha$ , FasL and TRAIL) of the TNF-family and their respective four receptors (TNF-R1, Fas, TRAIL-R1 and TRAIL-R2) have been considered for their potential use as anti-cancer therapeutics. The first of these molecules to be studied for its potential use as an anti-cancer drug was TNF- $\alpha$ . The demonstration that TNF- $\alpha$  selectively kills tumor cells but not normal cells, raised hopes about its potential use for cancer treatment.<sup>42</sup> Unfortunately, these hopes were not fulfilled since the marked pro-inflammatory effects of TNF- $\alpha$  precluded its systemic administration. Subsequent studies were focused on the two other molecules, FasL and TRAIL.

**Table 2.** Features of the most frequent gene abnormalities observed in AML.

Abnormality	Frequency and distribution among AML subtypes	Involved gene	Phenotype	Prognosis
NPM1 point mutations	46% in karyotypically normal (mostly FAB M4 and M5) AML 8% in karyotypically abnormal AML	Nucleophosmin gene	High bone marrow blasts High white blood cells	Improved response to treatment, in the absence of FIt3 mutations
NRAS point mutations	10% both in karyotypically normal and abnormal AMLs Particularly frequent in M4eo AMLs/inv(16);inv(3)t(8;3)	NRAS gene	Increased proliferation and decreased apoptosis	No significant prognostic impact
C/EBP $\alpha$ frameshift mutations	15%, preferentially observed in karyotypically normal AMLs	C/EBP $\alpha$ (CCAAT/enhancer binding protein $\alpha$ ) Gene	Block of cell differentiation	Improved response to treatment
c-kit point mutation D816	1.7%, mostly FAB M2 and also M4, frequently associated with either t(8;21) or trisomy 4	c-kit at the level of the PTK2 domain, codon D816	High white blood cell count	Negative impact on survival
c-kit point mutation	2.5%, mostly FAB M4eo, frequently associated with inv(16)	c-kit mutations at the level of exon 17	Spontaneous receptor dimerization Altered cell signalling Constitutive MAPK activation	High risk of relapse
c-kit ITD	7% pediatric AML, FAB M2 and M4, associated with either t(8;21) or t(9;11)	c-kit, ITD involves exons 11 and 12	Ligand-independent proliferation, apoptosis resistance and altered cell signaling	Negative prognostic impact
FLT3 ITD (internal tandem duplication)	20-35% in adult AML 15% in pediatric AML FAB M1 to M5 AML	FLT3 (fms-related tyrosine kinase 3), ITD occurring in exons 14 and 15	Constitutive receptor phosphorylation, proliferation advantage, Stat5 activation, apoptosis resistance and altered signaling	Reduced survival, event free-survival and complete remission duration in adult AML. Reduced survival in pediatric AML
FLT3 ALD (activation loop domain)	7% in both adult and pediatric AML FAB M1 to M5 AML	FLT3, point mutation at the level of D835 within the activation loop	Constitutive receptor phosphorylation	No significant prognostic impact in either adult or pediatric AML
MLL PTD	8%, associated with either normal karyotype or isolated trisomy of chromosome 11	MLL, in frame fusions of exons 11 and 12 upstream of exon 5 and duplication of the N-terminal region of MLL	MLL represses the transcription of the normal MLL allele that can be reactivated by methyltransferase and histone deacetylase inhibitors	Worse prognosis with shortened overall and event-free survival
JAK3 point mutations A572V V722I P132T	Acute megakaryoblastic leukemia, FAB M7	JAK3, point mutations	Constitutive JAK3 phosphorylation. The constitutive JAK3 activation induces Stat5, AKT and MAPK activation	Poor prognosis

Unlike TNF- $\alpha$ , these two cytokines do not have pro-inflammatory effects and are suitable as potential anti-cancer drugs. Unfortunately, agonistic antibodies triggering Fas activation are highly hepatotoxic causing death in mouse models.<sup>43</sup> In contrast, TRAIL and agonistic anti-TRAIL-R1/TRAIL-R2 antibodies appear to be well tolerated *in vivo*. In this context, initial studies have shown that TRAIL/Apo-2L exhibits potent anti-tumor activity and induces little cytotoxic effects in immunodeficient mice xenograft models implanted with several human tumor cell lines.<sup>44</sup> However, the *in vivo* half-life of the TRAIL-ligand is very short (<4 minutes), suggesting that agonistic anti-TRAIL-R1 or -TRAIL-R2 could have a better pharmacologic impact.<sup>45-46</sup> Another advantage of agonistic TRAIL-R1 and TRAIL-R2 antibodies is that they, at variance with the TRAIL ligand, do not bind to TRAIL decoy receptors, TRAIL-R3 and TRAIL-R4 are often present on the membrane of tumor cells.

Two anti-TRAIL-R have been developed for clinical use. One of these two antibodies is called HGS-ETR1 and is a fully human agonistic antibody with high affinity and

specificity for TRAIL-R1.<sup>47</sup> This antibody induces cell killing of tumor cell lines through activation of both extrinsic and intrinsic apoptotic pathways. Importantly, HGS-ETR1 was shown to have a long half-life *in vivo* (7-9 days in mouse) and suppressed the growth of several tumors in xenograft models in athymic mice.<sup>45</sup> Finally, EGS-ETR1 potentiated the anti-tumor efficacy of several chemotherapeutic drugs.<sup>47</sup> These observations clearly indicated that HGS-ETR1 has significant potential as a cancer therapeutic agent. The HGS-ETR1 antibody (Human Genome Sciences) was evaluated in phase I/II clinical trial in patients with advanced solid or hematologic tumors, revealing little toxicity.<sup>48</sup>

#### **Expression of death receptors and sensitivity to death ligands in AML**

Several studies have explored the sensitivity of AML to the pro-apoptotic effects of TRAIL showing that virtually all cases are resistant to this death ligand.<sup>49,50</sup> The analysis of the pattern of TRAIL-R expression showed that TRAIL-R3 and TRAIL-R4 are frequently expressed in AML,<sup>50,51</sup>

while TRAIL-R1 expression is usually associated with AML exhibiting monocytic features.<sup>50</sup> The presence of either TRAIL-R1/TRAIL-R2 expression is associated with TRAIL decoy receptor expression and does not confer sensitivity to TRAIL.<sup>51</sup> The analysis of HL60 TRAIL-resistant subclones provided evidence that TRAIL resistance is related either to TRAIL-R1/TRAIL-R2 down-modulation or to caspase-8 mutations.<sup>52</sup> Anti-leukemic drugs<sup>49</sup> or synthetic triterpenoids<sup>53</sup> are able to render AML blasts sensitive to TRAIL-mediated apoptosis. Finally, other studies have shown that histone deacetylase inhibitors induce TRAIL-mediated apoptosis of AML cells.<sup>53</sup>

Recently, natural dihydroflavonol compounds purified from plants were shown to increase TRAIL sensitivity of leukemic cells by enhancing TRAIL-R2 expression.<sup>54</sup> Similarly, arsenic trioxide (ATO), a compound used in the treatment of APL, sensitized AML cells to TRAIL via TRAIL-R2 upmodulation and c-FLIP downmodulation.<sup>55</sup> These effects elicited by ATO seem to be related to an inhibitory effect on PI3K: in fact, PI3K inhibitors enhanced TRAIL-R2 and down-modulated c-FLIP expression and sensitized AML cells to TRAIL.<sup>55</sup>

Particular emphasis was given to the study of APL. In this context, a first study provided evidence that retinoic acid induced TRAIL expression in APL cells and, through this mechanism, induced apoptosis of leukemic cells.<sup>56</sup> These findings, however, were not confirmed by a subsequent study since APL blasts, both before and after ATRA treatment, were resistant to TRAIL-mediated apoptosis; furthermore, undifferentiated APL blasts were shown to express TRAIL, whose levels greatly decreased after retinoic acid treatment.<sup>50</sup> Finally, studies performed on monocytic cell lines engineered to express the fusion protein PML/RAR- $\alpha$  clearly showed that its induction was associated with reduced TRAIL-R1 expression and reduced TRAIL sensitivity.<sup>50</sup> It is important to note that other studies have documented a stimulatory effect of this death ligand on cell proliferation<sup>57,58</sup> in a fraction of primary AML leukemic cells resistant to the pro-apoptotic effects of TRAIL.

#### ***Fas associated protein with death domain (FADD)***

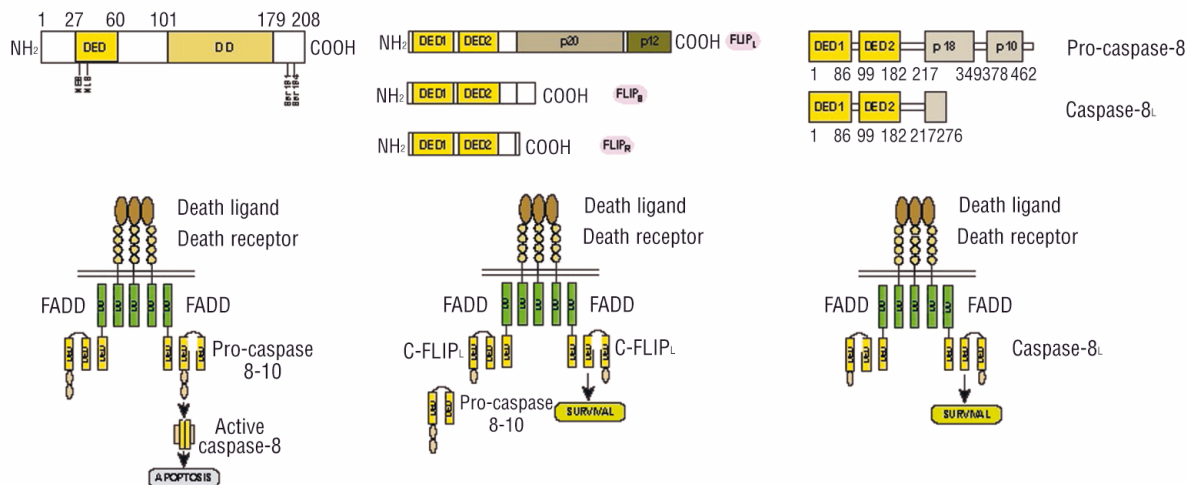
FADD is a key adaptor molecule transmitting the death signal mediated by death receptors.<sup>59</sup> FADD is the main signal-transducing intermediate adaptor molecule of several death receptors including Fas, TNF-R1, death receptor3, TRAIL-R1 and TRAIL-R2. The cytoplasmic tails of all these receptors possess a death domain able, once the receptors are activated, to recruit the death domains of FADD (Figure 4). The function of FADD in death receptor signaling is inhibited by c-FLIP, which, through their death domains, are able to bind to the death domains of FADD, thus preventing its recruitment by activated death receptors (Figure 4). However, FADD is also critical for cell cycle progression and proliferation, activities that are regulated by phosphorylation of its C-terminal Ser 194, which has

been implicated in the sensitivity of cancer cells to chemotherapeutic agents and in regulating intracellular localization.<sup>60</sup>

Studies in animal models of tumor development have shown that FADD may act as a tumor suppressor.<sup>59</sup> This interpretation is supported by recent studies showing that about two thirds of leukemic blasts of AML show low levels or no FADD protein. The absence of FADD protein in AML is associated with a poor prognosis.<sup>61</sup> Absence or low levels of FADD protein in AML may represent one of the factors responsible for the resistance of AML blasts to Fas and TRAIL-mediated apoptosis. In fact, the large majority of AML patients are resistant to Fas-mediated cell death despite expressing Fas<sup>62</sup> and Fas ligand.<sup>63</sup> The absence of FADD expression may confer AML blasts a resistance to immune-mediated attack and resistance to some chemotherapeutic drugs, such as anthracyclines and etoposide, which are known to enhance TRAIL-R2 expression and sensitivity to TRAIL and Fas-mediated apoptosis.<sup>64</sup>

#### ***FLIC-inhibitory protein (FLIP)***

The death effector domains (DED) present in caspase-8 and FADD are responsible for their reciprocal interaction with consequent formation of death receptor signaling complexes. However, other DED-containing proteins such as FLIP (also known as flame, casper, usurpin) exert a negative role in the control of death receptor signaling. FLIP exhibits extensive homology with pro-caspase-8 and -10,<sup>65</sup> containing two N-terminal DED, followed by a pseudo-caspase domain that lacks critical residues required for protease activity, including the catalytic cysteine (Figure 4).<sup>66,67</sup> FLIP associates with pro-caspase-8 and competes with pro-caspase-8 and -10 for binding to FADD, thus inhibiting the assembly of the machinery required for death receptor signaling. On the mRNA level c-FLIP exists in multiple splice variants, whereas on the protein level three forms, c-FLIP<sub>L</sub> (55kDa), c-FLIP<sub>S</sub> (26kDa) and c-FLIP<sub>R</sub> (24kDa) have been detected so far (Figure 4).<sup>68</sup> All three FLIP contain two NH<sub>2</sub>-terminus DED domains, essential for their biological activity. The C-terminus of c-FLIP<sub>L</sub> consists of two inactive caspase-like domains (p20 and p12) (Figure 4). c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> block caspase activation at the level of DISC. c-FLIP<sub>L</sub> promotes the activation of full length caspase-8, and its initial processing to p43 caspase-8, but inhibits further cleavage to fully active caspase-8. Selective knockdown of c-FLIP<sub>L</sub> provided definitive evidence that the long variant of c-FLIP functions primarily as an inhibitor of death receptor-mediated apoptosis.<sup>69</sup> c-FLIP is abundantly and constitutively expressed in a wide array of normal cell types, including CD34<sup>+</sup> hematopoietic progenitors. The transcription of the c-FLIP gene is controlled by NF- $\kappa$ B.<sup>70</sup> Overexpression of c-FLIP occurs frequently in a variety of B-cell malignancies, some AML and some chronic lymphocytic leukemias. The presence of elevated c-FLIP levels was strongly related to a



**Figure 4.** Schematic representation of the structure of FADD, FLIP and caspase-8 proteins. **Left, Top:** the structure of FADD protein, with two boxes indicating one DED domain and one DD domain. The numbers indicate the amino acid residue. Within the DED domain, a nuclear export sequence (NES) and nuclear localization sequence (NLS) have been identified: they determine the nuclear localization of FADD either in the nucleus or in the cytoplasm. In the COOH terminal site two serine residues (Ser 191 and Ser 194), essential for FADD function, are indicated. **Middle, Top:** the structure of the three c-FLIP isoforms, FLIPL, FLIPs and FLIPr with their structural domains is shown. All FLIP molecules contain two death-effector domains, similar to caspase-8. The long splice variant of cellular FLIP (cFLIPL) contains a C-terminal inactive caspase-like domain, containing the p20 and p12 subdomains, which confers the molecule structural homology with caspase-8 and caspase-10. The short splice variant of cFLIP (cFLIPs) and a variant cloned from the Raji B-cell line (cFLIPr) lack the caspase-like domain. **Right, Top:** the structure of pro-caspase-8 and caspase-8L with their structural domains is shown. Caspase-8 is synthesized as an inactive zymogen, pro-caspase-8. Pro-caspase-8 contains two death effector domains (DED1 and DED2) in tandem at its terminal NH2 and at its C-terminal an activable caspase domain, subdivided into p18 and p10 subdomains, with a conserved consensus active site sequence of QACQG. Pro-caspase-8 is proteolytically processed at specific cleavage sites: these cleavage events remove the prodomain and separate the enzyme proform into a large and a small subunit that constitute active caspase-8. In contrast, caspase-8L lacks the catalytically active caspase domain and acts as a competitor of caspase-8. **Left, Bottom:** signaling along the extrinsic pathway based on FADD and caspase-8 activation with consequent induction of the effector apoptotic machinery. **Middle, Bottom:** block of the apoptotic signaling along the extrinsic pathway when c-FLIP interacts with FADD, hampering the subsequent caspase-8 recruitment and activation. **Right, Bottom:** block of the apoptotic signaling along the extrinsic pathway as a consequence of the interaction of caspase-8L with FADD, thus hampering caspase-8 binding and activation.

poor prognosis in Burkitt's lymphoma.<sup>71</sup> In HTLV-1<sup>+</sup> T-acute lymphoblastic leukemia (T-ALL) Tax protein induces strong expression of c-FLIP in leukemic blasts, responsible for their resistance to TRAIL and FasL-mediated apoptosis.<sup>72</sup>

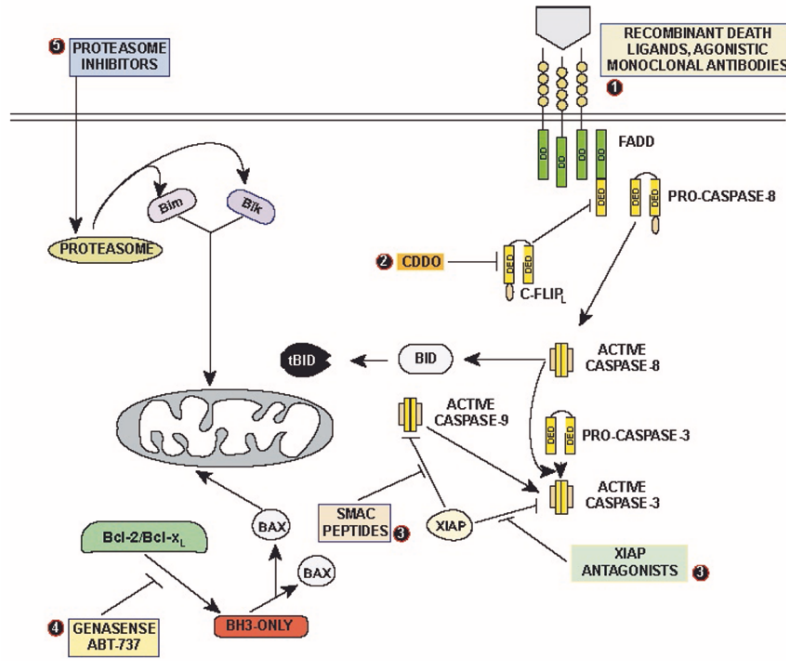
No compound directly able to inhibit c-FLIP has been described. However, several experimental agents have been reported to decrease c-FLIP expression and seemingly to predispose through this mechanism to apoptosis. One of these agents is the synthetic triterpenoid CDDO (2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid), another is its derivative CDDO-Im. Both CDDO and CDDO-Im have been shown to inhibit cellular proliferation and induce apoptosis of leukemic cells.<sup>50,73</sup> The mechanisms of the pro-apoptotic effects induced by CDDO and CDDO-Im are certainly complex and only in part defined. One of the main effects induced by CDDO is ubiquitination and proteasome-dependent destruction of FLIPs in cultured cancer cells<sup>72</sup> (Figure 5). CDDO and CDDO-Im down-regulate the c-FLIP and upregulate TRAIL-R1 and TRAIL-R2 in many tumor cell types, rendering them sensitive to TRAIL. CDDO and CDDO-Im are well tolerated in mice, administered alone or in combination with TRAIL.<sup>75</sup> CDDO is now under investigation in phase I clinical stud-

ies. Recently, the screening of a 50,000-compound library led to the identification of small molecules that sensitize various types of tumor cells to TNF death receptors through c-FLIP down-modulation.<sup>76</sup>

#### Initiator caspases in AML

The initiator caspases are able to activate effector caspases through two different molecular mechanisms: either by direct activation or by an indirect mechanism involving a secondary messenger.<sup>77</sup> Potential abnormalities of the various initiator caspases in AML have been explored. Pro-caspase-8 has two DED in its N-terminal region, required for interaction with FADD and two catalytic regions termed p18 and p10 (Figure 4). In addition to this standard pro-caspase-8, there are several caspase-8 isoforms: one of them, caspase-8L has several important biological properties. In fact, caspase-8L is shorter than standard caspase-8, lacks the catalytic site of caspase-8, but retains the two N-terminal repeats of the DED.<sup>78</sup> Caspase-8L acts as an inhibitor of caspase-8 by interfering with the binding of caspases-8 to FADD.<sup>78</sup> The expression of caspase-8L was examined both in hematopoietic progenitor cells and in leukemic blasts derived from AML.<sup>79</sup> Interestingly, CD34<sup>+</sup> cells selectively express the anti-





**Figure 5.** Therapeutic strategies for the targeted induction of apoptosis in AML. (1) Apoptosis is activated by triggering death receptors (e.g., TRAIL-R1 or -R2) either by recombinant forms of the cognate death-inducing ligand or by agonistic monoclonal antibodies. (2) Apoptosis is induced or sensitivity to apoptotic stimuli is greatly increased by lowering the levels of the caspase-8 inhibitor c-FLIP through the addition of various drugs, such as the synthetic triterpenoid CDDO. (3) Inhibition of the XIAP-mediated block on active caspase-9, using synthetic SMAC peptides, markedly increases the sensitivity of AML cells to apoptotic stimuli. Inhibition of XIAP-mediated block on active caspase-3, either using small-molecule inhibitors or a specific antisense oligonucleotide, results in activation of apoptosis of leukemic cells. (4) Inhibition of BCL-2, using the oligonucleotide ODN Genasense, and inhibition of BCL-2/BCL-XL using small-molecule inhibitor ABT-737, results in a block of the anti-apoptotic function of these molecules and induction of mitochondrial apoptosis. (5) Inhibition of the proteasome, using proteasome inhibitors such as bortezomib, deregulates protein homeostasis, leading to activation of mitochondrial apoptosis through stabilization and activation of the BH3-only proteins Bim and Bik.

apoptotic splice variant of caspase-8, caspase-8L; in line with this observation, AML with an immature phenotype (i.e., M0 and M1 AML) predominantly express caspase-8L.<sup>79</sup> The analysis of caspase-8 expression in AML showed a great heterogeneity in the levels of this caspase, with <10% of cases exhibiting no or low caspase-8 levels.<sup>80</sup> The levels of caspase-8 in AML do not seem to be a determinant of drug sensitivity of AML blasts.

There have also been studies exploring possible abnormalities in the expression of other effector caspases in AML. Caspase-2 was found to be involved in several apoptotic settings, including death receptor ligation, endoplasmic reticulum stress and genotoxic stress. Recent studies clearly support a major role of caspase-2 as a proximal mediator of heat shock-induced apoptosis<sup>81</sup> and in apoptosis in response to DNA damage.<sup>82,83</sup> As observed for caspase-8, caspase-2 levels varied greatly among AML patients and only a minority of them (ranging from 10-20% in the various studies) displayed low pro-caspase-2 levels.<sup>84,85</sup> The significance of caspase-2 levels in AML remains unclear since some studies have suggested that high caspase-2 levels, associated with high caspase-3 levels, represent a negative prognostic factor,<sup>84</sup> while other studies carried out on pediatric ALL and AML patients have shown that low pro-caspase-2 levels are associated with cellular drug resistance.<sup>85</sup> Finally, yet other studies have failed to find any correlation between caspase-2 levels and response to therapy in AML.<sup>86</sup> Interestingly, a recent report provided evidence that caspase-2 is localized at the level of the promyelocytic leukemia protein nuclear bodies, nuclear macromolecular complexes involved in the control of apoptosis and disrupted in APL.<sup>86</sup> In spite of

this localization, caspase-2 activation does not seem to be involved in the mechanism of cell death induced by arsenic trioxide in APL cells.<sup>87,88</sup>

### Effector caspases in AML

Levels of caspases-3 have been explored in AML, providing some interesting findings. Particularly, in a study carried out on newly diagnosed AML increased levels of procaspase-3 in leukemic cells were correlated with a poor prognosis.<sup>84</sup> However, patients exhibiting spontaneously active caspases-3 at the time of diagnosis showed improved survival.<sup>84</sup> Likewise, in ALL patients increased levels of active caspase-3 correlated with an improved rate of complete remission.<sup>89</sup> The poor prognosis among patients with high levels of procaspase-3 may be related to defects in caspase activation with subsequent accumulation of uncleaved caspase-3. The better prognosis observed among patients with increased levels of cleaved caspase-3 may be related to the activation of apoptotic pathways in these leukemic cells.

### Bcl-2 in AML

Overexpression of the anti-apoptotic protein Bcl-2, a key member of a family of proteins operative in the mitochondrial pathway of apoptosis, results in resistance to a wide variety of pro-apoptotic stimuli, including radiation, chemotherapeutic agents and corticosteroids.<sup>90,91</sup> Bcl-2 was found to be overexpressed in AML patients showing a low rate of complete remission<sup>92</sup> and downregulated in patients with the favorable t(8;21) karyotype.<sup>93</sup> Furthermore a high ratio of Bcl-2 to Bax, a pro-apoptotic member of the Bcl-2 family of proteins, was shown to be

predictive of worse outcome.<sup>94</sup> Bcl-2 is over-expressed preferentially among CD34<sup>+</sup> AML.<sup>95</sup> The combined expression of Bcl-2 and MDR-1 identifies M4/M5 AML associated with a very poor prognosis.<sup>91</sup> CD34<sup>+</sup> fractions are more resistant to apoptosis than are corresponding CD34<sup>-</sup> AML fractions, and this is paralleled by higher Bcl-2, Bcl-X<sub>i</sub>, Mcl-1 and lower Bax expression.<sup>96</sup> Recently a sub-analysis of Bcl-2 in AML patients stratified into low-risk, intermediate-risk and high-risk groups showed that in low-risk AML patients there is a perfect correlation between Bcl-2 levels and C/EBP $\alpha$  levels: according to these observations it was suggested that the transcription factor C/EBP $\alpha$ , which is mutated in about 10% of AML, could inhibit apoptosis via induction of Bcl-2 expression.<sup>97</sup> It seemed conceivable that Bcl-2 downregulation might lower the apoptotic threshold of leukemic cells and, through this mechanism, favor response to chemotherapy<sup>98</sup> (Figure 5). A phase I study using an antisense to Bcl-2 in elderly AML patients showed promising results:<sup>94</sup> in this study AML patients received induction with oblimersen, cytarabine and daunorubicin and, on achievement of complete remission, consolidation with oblimersen (antisense Bcl-2) and high-dose cytarabine. Of the 29 treated patients, 14 achieved a complete remission; during 12.6 months of follow-up, seven of these patients relapsed.<sup>98</sup> A phase II multicenter study of oblimersen plus gentuzamab, a cytotoxic antibody directed against CD33<sup>+</sup> cells, in relapsed AML patients has been reported: 12 out of 39 treated patients achieved a complete remission.<sup>99</sup> Ten of the 12 responders survived more than 6 months.<sup>100</sup> Finally, pharmacodynamic studies showed that therapy with oblimersen produced intracellular concentrations of the drug sufficient to down-regulate Bcl-2.<sup>100</sup> The findings from these studies have been extended into an ongoing phase III study of about 400 patients with untreated AML who are receiving daunorubicin plus cytosine arabinoside with or without oblimersen.

The *MCL-1* gene encodes a 36kDa protein with a carboxy terminal domain similar to Bcl-2. However, in contrast to Bcl-2, which is a long-lived protein, Mcl-1 has a short half-life. The analysis of Mcl-1 protein expression in AML showed great heterogeneity, but the levels of the protein do not seem to correlate with response to standard chemotherapy.<sup>101</sup> Interestingly, Mcl-1 levels increase at the time of a leukemic relapse.<sup>101</sup>

AML blasts frequently express elevated levels of the anti-apoptotic Bcl-2 family member, Bcl-X<sub>i</sub>. Elevated levels of Bcl-X<sub>i</sub> have been observed in CD34<sup>+</sup> AML cells and contribute to chemoresistance of these cells.<sup>102</sup> This finding is not surprising since Bcl-X<sub>i</sub> is a target gene of Stat-5, whose constitutive activation is frequently observed in AML.<sup>103</sup> Recent studies have shown that AML with Flt3 mutations are usually associated with elevated Bcl-X<sub>i</sub> levels, seemingly induced via constitutive Stat-5 activation.<sup>104,105</sup> Furthermore Flt3 internal-tandem mutants induce BAD phosphorylation.<sup>106</sup>

### **The X-linked inhibitor of apoptosis protein (XIAP) in AML**

XIAP was found to be expressed in the large majority of adult cases of AML.<sup>107,108</sup> In a significant proportion of patients high levels of XIAP were detected. An initial study carried out on 78 adult AML patients suggested a potential relationship between the level of XIAP expression and response to therapy, patients with high XIAP levels having a shorter survival than that observed in patients with low XIAP levels.<sup>108</sup> However, in a second study, carried out on 172 adult patients with primary AML, the same authors failed to observe a significant correlation between the level of XIAP expression in AML blasts and complete remission rate or overall survival or other clinical parameters.<sup>109</sup> In a subsequent study the same authors explored XIAP expression in a group of 92 adult patients with AML and observed that the level of XIAP expression correlated with the monocytic differentiation of leukemic blasts; in this group of patients, those with low levels of XIAP enjoyed a better overall survival in response to standard treatment.<sup>110</sup>

Studies on the prognostic value of XIAP and survivin levels have also been carried out in pediatric AML. In a group of 45 children with *de novo* AML an inverse correlation was observed between XIAP levels (high levels of XIAP being observed mostly in M/M1 and M4/M5 AML) and overall survival.<sup>111</sup> Survivin expression was detected in the majority of AML, but there was no evidence that its levels in leukemic blasts correlated with the response to treatment or with survival.<sup>108,112</sup>

Recently, an *in vitro* assay was used to explore the downstream part of the mitochondrial pathway of caspase activation in patients with *de novo* and with relapsing AML. Interestingly, impaired caspase activation was frequent in samples from patients with rapidly relapsing disease, but was rare in samples from patients with newly diagnosed AML.<sup>113</sup> Defects in caspase activation did not correlate with IAP or caspase-3 levels.<sup>113</sup> Similarly, the simultaneous detection of cytochrome c release, a key initial event in the activation of the intrinsic apoptotic pathway, and caspase-3 activation in leukemic cells seems to represent an important approach to assess the functional integrity of apoptosis signaling in leukemic cells.<sup>114</sup> Using this approach it was shown that patients with B-acute lymphoblastic leukemia (B-ALL) with deficient caspase-3 activation have a poorer prognosis than those with normal caspase-3 activation mediated by cytochrome-c release.<sup>115</sup>

Two approaches are currently being used to develop XIAP inhibitors: (i) antisense oligonucleotides that are in clinical trials and (ii) small molecule inhibitors that are in preclinical development.<sup>116,117</sup> Interestingly, polyphenylurea-based small-molecule XIAP antagonists induce apoptosis of AML cells at low micromolar concentrations through a mechanism involving activation of downstream caspase-3 and -7, independently of Bcl-2 levels.<sup>118</sup> Triptolide, a diterpenoid isolated from the Chinese herb

*Tripterygium Wilfordii*, decreases XIAP and potently induces caspase-dependent apoptosis of primary AML blasts mediated through the mitochondrial pathway, even at low concentrations.<sup>119</sup>

### Abnormalities in apoptosome formation in AML cells

Following cytochrome c release, the next step in the apoptotic process is apoptosome formation. Correct apoptosome formation requires APAF-1. The primary structure of APAF-1, a key member of the apoptosome, consists of an amino-terminal domain with 12 or 13 WD-40 repeats, a central ATPase domain with homology to CED-4, and a carboxy-terminal caspase recruitment domain (CARD). Four major isoforms of the protein have been identified: only isoforms containing an additional WD-40 repeat between repeats 5 and 6 are able to activate caspase-9 in response to cytochrome c and ATP. Activated APAF-1 interacts with procaspase-9 via a CARD-CARD interaction.

Defects in apoptosome proteins, such as APAF-1, have been involved in the development of some human malignancies, including AML. Studies carried out in leukemic cell lines, as well as in few cases of fresh primary leukemic cells, have shown that leukemic cells express low levels of APAF-1 in a significant proportion of cases and that their pattern of APAF-1 expression directly correlates with ultraviolet light-induced apoptosis.<sup>120</sup> Subsequent studies showed that 25% of human leukemic cell lines and 42% of primary AML cells express low levels of APAF-1 mRNA, due to methylation silencing of the APAF-1 gene.<sup>121</sup> Treatment of leukemic cells with 5-aza-2'-deoxycytidine, a specific inhibitor of DNA methylation, restored APAF-1 expression in leukemic cells.<sup>121</sup> The deregulation of APAF-1 expression in leukemic cells is very complex: in fact, other studies found that APAF-1 mRNA

levels did not correlate with APAF-1 protein levels in AML blasts,<sup>122</sup> thus suggesting the existence of post-transcriptional defects, in addition to the transcriptional defects.

### Conclusions

AML is an aggressive hematologic malignancy. The development and progression of the leukemic disease invariably involve deregulation in the apoptotic response. Despite major efforts during the past 40 years, limited improvements in disease survival have been made. The current primary treatment for AML is chemotherapy, which induces cell death by apoptosis mainly mediated through the intrinsic mitochondrial pathway but also through the extrinsic death receptor pathway, both of which lead to caspase activation and cell death.

The progress made in our understanding of the central role of apoptosis deregulation in the pathogenesis of leukemia and in the development of chemoresistance has launched the academic community, as well as the pharmaceutical and biotechnology industries into a search to identify new compounds for the treatment of leukemia. Targeted therapies that are designed to induce apoptosis in leukemic cells are currently the most promising anti-leukemia strategies. These strategies are aimed at targeting and killing the leukemia cells specifically with no or limited collateral damage to normal hematopoietic progenitor cells. The elucidation of the molecular apoptotic machinery and of its defects in AML lays the basis for developing new drugs able to trigger apoptosis of leukemia cells (Figure 5).

### Author Contributions

Both authors contributed equally to this work.

### Conflict of Interest

The authors reported no potential conflicts of interest.

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