CHAPTER

INTRODUCTION
1.1 Normal hematopoiesis

Hematopoiesis is organized as a hierarchy, with the most undifferentiated hematopoietic stem cell (HSC) at the basis of this process (Figure 1.1). These HSCs are able to give rise to new HSCs via self-renewal and to more mature multipotent progenitors via differentiation. In a multi-step process these progenitors further differentiate into all the mature blood cell lineages. Self-renewal of HSCs is required to sustain blood cell production during the lifespan of an organism [1, 2]. However, whether the life-long blood production is solely due to stem cells is currently under debate. This classical model has been challenged by the finding that hematopoietic progenitors rather than stem cell drive steady-state hematopoiesis in adult mice [3].

Figure 1.1: Schematic representation of the hematopoietic system

Hematopoiesis is a highly organized process and self-renewal, differentiation and proliferation are controlled in a spatial and temporal manner by cytokines and growth factors [4, 5]. These stimuli are generated by the blood cell themselves or by the bone marrow microenvironment in a so called autocrine or paracrine dependent manner. The interplay between the hematopoietic (stem) cells and the bone marrow microenvironment is essential for a proper maintenance of hematopoiesis [6, 7]. In malignant hematopoietic malignancies the intrinsic factors as well as the communication with the micro-environment are often deregulated.

1.2 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a hematological malignancy characterized by an increased number of immature myeloid cells in the bone marrow (> 20%) that has limited capacity to differentiate [8]. A rapid accumulation of these blasts
Table 1.1: French-American-British classification of AML

<table>
<thead>
<tr>
<th>Code</th>
<th>Description</th>
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<tbody>
<tr>
<td>M0</td>
<td>Myeloid leukemia with minimal differentiation</td>
</tr>
<tr>
<td>M1</td>
<td>Myeloid leukemia without maturation</td>
</tr>
<tr>
<td>M2</td>
<td>Myeloid leukemia with maturation</td>
</tr>
<tr>
<td>M3</td>
<td>Promyelocytic leukemia</td>
</tr>
<tr>
<td>M4</td>
<td>Myelomonocytic leukemia</td>
</tr>
<tr>
<td>M4eo</td>
<td>Myelomonocytic leukemia with abnormal eosinophils</td>
</tr>
<tr>
<td>M5</td>
<td>Monocytic leukemia</td>
</tr>
<tr>
<td>M6</td>
<td>Erythroid leukemia</td>
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<tr>
<td>M7</td>
<td>Megakaryocytic leukemia</td>
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results in the suppression of normal hematopoiesis leading to thrombocytopenia, granulocytopenia and/or anemia [9]. Therefore, patients often present with various symptoms including fatigue, increased bleeding, infections as well as fever and AML is fatal within weeks when the patients are not treated.

AML patients are treated with intensive chemotherapy, which is composed of an induction and consolidation therapy. In general a distinction in treatment intensity is made for patients between 18 – 65 years versus > 65 years due to pre-existing co-morbidities. For younger patients the treatment consists of 1 – 2 courses of cytarabine (Ara-C) combined with an anthracycline (e.g. daunorubicin). In approximately 80% of patients complete remission is obtained. The induction chemotherapy is followed by consolidation chemotherapy but the type depends on the risk classification of the leukemia. Patients with a favorable risk score are treated with autologous stem cell transplantation while patients with intermediate or poor risk score with an allogeneic stem cell transplantation. Elderly AML patients are treated with less intensive chemotherapy or with epigenetic drugs such as decitabine. In view of the high relapse rate in this elderly patient population, allogeneic stem cell transplantation should always be applied if feasible.

AML is a heterogeneous group of diseases and was for several decades classified according to the French-American-British (FAB) classification which was based on the differentiation stage of the leukemic blasts (Table 1.1) [10]. However, during the last decade a more clinically useful classification has been developed by the World Health Organization (WHO) (Table 1.2) [11]. Cytogenetic abnormalities are implemented in this classification. Moreover, these cytogenetic abnormalities are independent prognostic factors for treatment outcome (Table 1.3). However, the karyotype analysis shows only abnormalities in 50% of the AML patients. The implementation of next-generation sequencing has provided much more insight into the spectrum of mutations in AML. At the moment, mutations in more than 200 genes are identified, and a number of these mutations also have a strong prognostic impact [12] (Table 1.3). Interestingly, some of these mutations have also been found in peripheral blood mononuclear cells of normal individuals without hematological malignancies [13–16]. The frequency of these mutations increases upon ageing, consistent with the fact that AML is especially a disease of the elderly. These
### Table 1.2: WHO classification of AML

<table>
<thead>
<tr>
<th>Acute myeloid leukemia with recurrent genetic abnormalities</th>
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<tr>
<td>AML with t(8;21)(q22;q22); RUNX1-RUNX1T1</td>
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<tr>
<td>AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11</td>
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<td>Acute promyelocytic leukemia with t(15;17)(q22;q11); PML-RARA</td>
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<tr>
<td>AML with t(9;11)(p22;q23); MLLT3-MLL</td>
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<tr>
<td>AML with t(6;9)(p23;q34); DEK-NUP214</td>
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<tr>
<td>AML with inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1</td>
</tr>
<tr>
<td>AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1</td>
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<tr>
<td>AML with mutated NPM1 (provisional entity)</td>
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<tr>
<td>AML with mutated CEBPA (provisional entity)</td>
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<td>Acute myeloid leukemia with myelodysplasia-related changes</td>
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data suggest that these ‘driver’ mutations are required but not sufficient for the development of AML.

### 1.3 Leukemic stem cells

AML consists of a heterogeneous group of cells and it is proposed that these cells are organized in a hierarchical manner similar to the normal hematopoietic system. In the current model it is thought that outgrowth and maintenance of leukemia is driven by leukemic stem cells (LSCs). These LSCs are the most primitive cells at the top of the hierarchy and are capable of generating more differentiated leukemic cells [17, 18]. These LSCs likely survive the current treatments and are responsible for the leukemic growth at relapse [19, 20].

Leukemic stem cells originate from normal hematopoietic stem or progenitor cells in a process called leukemic transformation, which has been proposed to be a multi-step process, characterized by a chromosomal translocation and/or mutations resulting in increased proliferation and a block in differentiation [21]. Moreover, during the leukemic transformation these cells have often acquired resistance to apoptosis, a phenomenon which has been recognized to be one of the hallmarks of cancer [22]. Understanding the difference in dependency on apoptotic or pro-survival signaling of leukemic (stem) cells versus normal cells can be of importance to find new targets for the treatment of AML patients.

### 1.4 Apoptosis

Apoptosis or programmed cell death is a highly regulated and tightly controlled process during tissue development and homeostasis. The number of cells is controlled by the induction of apoptosis of cells that are no longer required or a danger to the tissue. Apoptosis is, in contrast to necrosis, beneficial to tissues. Whereas necrosis is characterized by membrane disruption and the excretion of cellular
Table 1.3: HOVON Risk classification

<table>
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<tr>
<th>Risk group</th>
<th>Criteria at diagnosis and early/late CR</th>
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<tr>
<td>Good risk</td>
<td>t(8;21) or RUNX1-ETO, WBC ≤ 20 Inv(16)/t(16;16) or CBF-MYH11 MK−, CEBPA-biallelic mutant* MK−, FLT3-ITD <em>/NPM1</em></td>
</tr>
<tr>
<td>Intermediate risk</td>
<td>Cyogenetically normal (CN) - X - Y, WBC ≤ 100, early CR t(8;21) or RUNX1-ETO, WBC &gt; 20, or mutant KIT NPM* /FLT3-ITD*</td>
</tr>
<tr>
<td>Poor risk</td>
<td>CN - X - Y, WBC ≤ 100; not early CR CN - X - Y, WBC &gt; 100 CA, but non-CBF, MK−, no 3q26, EVI1−</td>
</tr>
<tr>
<td>Very poor risk</td>
<td>CN - X - Y, WBC &gt; 100 Biallelic FLT3-ITD with FLT3-ITD /FLT3wt ratio &gt; 0.6 MK+ 3q26 Non-CBF, EVI1+ mutant p53, mutant RUNX1, mutant ASXL1</td>
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CR: complete remission
MK: monosom al karyotype (two or more autosomal monosomies or a single autosomal monosomy combined with a structural cytogenetic abnormality)
CN: Cytogenetically normal, CA: Cytogenetically abnormal
WBC: White blood cell count (10⁹/L)
CBF: core-binding factor leukemia including t(8;21)(q22;22), RUNX1-ETO, inv(16)(p13;q22), t(16;16)(p13;q22) and CBF-MYH11

Components leading to an inflammatory response and damage to other cells, apoptosis is characterized by membrane blebbing, fragmentation and degradation of cellular components followed by apoptotic body formation which are then engulfed by macrophages. Apoptosis can be activated by intrinsic and extrinsic pathways (Figure 1.2). The extrinsic pathway is activated by the association of TNF ligand to their receptors, such as Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) to the death receptors 4 and 5 (DR4 and DR5) [23]. Binding of TRAIL results in oligomerization of the death receptors and formation of the death-inducing signaling complex (DISC), which is composed of the death domain (DD) of the death receptor, Fas-associated protein with death domain (FADD) and the initiator caspases caspase-8 or 10 [24, 25]. The formation of this complex is repressed by cellular FLICE-like inhibitory protein (cFLIP), which competes with the caspases for the binding to the DISC. Upon activation of these caspases, the effector caspases 3, 6 and 7 are cleaved and activation results in execution of apoptosis by the degradation of cellular proteins and fragmentation of DNA. TRAIL-mediated apoptosis is counteracted by the surface decoy receptors 1 and 2 (DcR1 and DcR2), both lacking a functional death domain, and the soluble receptor osteoprotegerin
Figure 1.2: Schematic representation of the apoptotic process

(OGP). These receptors have an antagonistic function and are unable to induce apoptosis.

The intrinsic pathway is triggered by cellular stress such as DNA damage that activates the pro-apoptotic BCL-2 family members [26, 27]. The proteins of the BCL-2 family can be classified into three distinct groups: the anti-apoptotic proteins, the pro-apoptotic proteins and the pro-apoptotic BH3-only proteins. These proteins can bind to each other and thereby activate or repress their function. The anti-apoptotic proteins such as BCL-2, BCL-XL, MCL-1, BCL-W, A1 and BCL-B, prevent cell death by preserving the integrity of the mitochondrial outer membrane. The pro-apoptotic proteins BAK and BAX induce apoptosis by clustering into large oligomers which permeabilize the mitochondrial outer membrane, likely by the formation of pores. The third group is the BH3-only proteins, like BIK, BID, BIM, PUMA, NOXA and BAD, which can be activated by caspase 8 and 10. These proteins trigger the pro-apoptotic proteins in a direct and/or in an indirect manner [28]. Following the direct model it is assumed that BH3-only proteins bind directly to the inactive pro-apoptotic proteins and that association of both proteins is required for the activation of the pro-apoptotic proteins. In the indirect model it is proposed that BH3-only proteins will bind to the anti-apoptotic proteins, thereby releasing the pro-apoptotic proteins from the anti-apoptotic proteins which results in the destabilization of the mitochondrial outer membrane by the pro-apoptotic proteins.
Thereupon, cytochrome c and SMAC/DIABLO will be released from the mitochondria. Cytochrome c will bind to the APAF-1 complex leading to the formation of the apoptosome resulting in the activation of the executor caspases [29, 30]. At the same time, SMAC/DIABLO represses the inhibitor of apoptosis proteins (IAPs), which inhibit the activation of the executor caspases 3, 7 and 9 [31, 32]. Thus, the induction of cell death is controlled by the activity of apoptosis executors and inhibitors.

1.5 MCL-1 and BCL-2 in normal hematopoiesis

The most intensively studied apoptosis-related genes in hematopoiesis and leukemia are MCL-1 and BCL-2. The anti-apoptotic protein MCL-1 is highly expressed in normal HSCs and is crucial for the survival of normal hematopoietic stem cells [33]. Conditional deletion of MCL-1 using the Mx-Cre system resulted in ablation of bone marrow cells, including bone marrow progenitor cells and HSCs, whereas other tissues, such as the liver, were largely unaffected [34]. Furthermore, upon knockdown of MCL-1 in primitive HSCs, no engraftment of hematopoietic cells could be observed in a transplantation model, indicating the crucial role of MCL-1 in the survival of normal hematopoietic stem cells [35].

In the early nineties, studies have shown that knockout of BCL-2 resulted in a decreased output of the lymphoid lineage which coincided with an increased level of apoptosis, whereas the myeloid and erythroid compartments were largely unaffected [36–38]. The milder phenotype of BCL-2−/− mice compared to MCL-1−/− mice might be related to the low expression of BCL-2 compared to MCL-1 in isolated cord blood cells and reconstituted HSCs [35]. Upon overexpression of BCL-2 in HSCs, cell death induced by a variety of chemotherapeutic agents was impaired and the quality of HSCs was improved by means of long term reconstitution after 5-FU treatment [39]. Furthermore, HSCs overexpressing BCL-2 showed higher engraftment levels in primary and secondary recipients indicating that BCL-2 improved the survival of these HSCs under these stress conditions [40]. Increased engraftment levels in primary and secondary recipients of transplanted BCL-2 HSCs were also observed after irradiation of these HSCs, suggesting that BCL-2 directly enhances the survival of HSCs [40]. Moreover, mice transplanted with BCL-2 bone marrow progenitor cells were resistant to radiation-induced death [41].

1.6 MCL-1 and BCL-2 in leukemia

Both anti-apoptotic proteins are nowadays regarded as important factors for the survival of leukemic stem cells. MCL-1 is consistently highly expressed in primary AML patient cells [33]. It has been suggested that anti-apoptotic signaling via MCL-1 can directly cooperate in malignant transformation which was illustrated by the fact that overexpression of MCL-1 accelerated MYC-driven lymphomagenesis [42]. Over the past few years the requirement for MCL-1 in AML has been studied in various AML mouse models. Leukemia formation and maintenance was significantly delayed upon heterozygous deletion of MCL-1 in a MYC-induced AML.
model, upon conditional knockout of MCL-1 in a MLL-ENL-induced AML model and upon conditional deletion of MCL-1 in a BCR-ABL model [33, 43, 44]. In line with these data, the anti-apoptotic protein BCL-2 is also essential for the survival of leukemic stem cells. High BCL-2 expression levels have been observed in different patient AML samples which were correlated with a poor outcome and poor response to chemotherapy [45–47]. Interestingly, it has been shown that BCL-2 could interact with mutant NRAS and more importantly that overexpression of BCL-2 can directly act as a secondary hit in the progression to MDS/AML in a mutant NRAS background [48]. Recently, it has been shown that leukemic stem cells, which were defined by low levels of ROS, also express high levels of BCL-2. BCL-2 inhibition resulted in decreased oxidative phosphorylation and specifically induced cell death in these ROS-low LSCs [49]. Furthermore, it has been observed that the expression of BCL-2-family members increased during ongoing malignant transformation of CML cells. Combined inhibition of the BCL-2 family members and BCR-ABL by tyrosine kinase inhibitors eliminated blast-crisis LSCs while sparing normal cells [44, 50, 51].

Surprisingly, besides the increased expression and dependency of the anti-apoptotic proteins MCL-1 and BCL-2 in AML, also high levels of the pro-apoptotic BCL-2 family members in leukemic cells have been observed [52]. Moreover, high expression of both anti-apoptotic as well as pro-apoptotic genes was related to overall survival of AML patients. It has been suggested that the increased expression of pro-apoptotic BCL-2 family members is a response to the increased expression of anti-apoptotic BCL-2 family members pushing these cells into a 'primed to death' status [53]. These cells might therefore be more dependent on the presence of anti-apoptotic BCL-2 family members resulting in a so-called oncogenic addiction. This primed to death-model might explain why leukemic cells are selectively sensitive to BCL-2 family inhibitors and targeting of these components might therefore specifically induce cell death in leukemic cells compared to normal cells. Since 10 years, multiple BCL-2 family member inhibitors have been evaluated in various preclinical studies. It has been shown that targeting of MCL-1 and BCL-2 by chemical compounds such as ABT-737 [54–57], ABT-263 (navitoclax) [49], obatoclax [58, 59], sabutoclax [50], PIK-75 [60] and ABT-199 [61–63] induced cell death in primary AML samples or AML models in vitro and in vivo. ABT-737 and ABT-263 bind to BCL-2, BCL-XL and BCL-W and ABT-263 was the first agent to be tested in clinical trials. Obatoclax and sabutoclax showed improved effectiveness as these compounds also inhibited the anti-apoptotic protein MCL-1. However, whereas platelets are largely dependent on BCL-XL for their survival, these drugs induce a thrombocytopenia due to their binding to BCL-XL. Recently, ABT-199 has been developed, which does not bind to BCL-XL and the first results showed a reduction in the frequency of thrombocytopenia, which is now further evaluated in clinical studies.
1.7 Scope of this thesis

The NF-κB pathway is an important signaling module for the survival of leukemic cells and consequently, the transcription factor NF-κB drives the expression of variety of genes involved in anti-apoptosis and proliferation, such as BCL-2, BCL-XL, cFLIP, TNFα and IL-1 [64]. It has been observed that NF-κB is constitutively active in many primary AML cells [65, 66]. Various studies showed that AML cells were more dependent on NF-κB signaling compared to normal bone marrow cells as AML cells were highly sensitive to NF-κB inhibition whereas normal bone marrow cells were largely insensitive [66, 67]. Therefore, targeting of NF-κB has been proposed to be a promising strategy for the treatment of AML. Recently, various molecular mechanisms have been shown to contribute to the increased NF-κB activity in AML. It has been observed that chromosomal translocations or mutation resulting in myeloid transformation induced NF-κB activity. Moreover, also autocrine/paracrine cytokine signaling and increased expression and activation of NF-κB regulators could result in enhanced NF-κB activation. Besides these mechanisms, increased activation of the proteasome could contribute to the constitutive activation of NF-κB in AML as NF-κB activity is dependent on proteasome activity via the proteasomal degradation of IκBα, a negative regulator of NF-κB activity. The various mechanisms of NF-κB activation are extensively reviewed in Chapter 2. Also the various treatment strategies that target NF-κB and thereby result in cell death of AML cells are described in this chapter.

It has been shown that the proteasome inhibitor bortezomib induces apoptosis in AML cells in vitro. Although clinical studies have been performed in AML patients with bortezomib, it is not known whether AML stem cells are efficiently targeted in this context [68–70]. In addition, it has been suggested that NF-κB inhibition by bortezomib or IKK inhibition sensitizes AML cells to TRAIL-induced apoptosis [71–73]. In Chapter 3, we investigated the sensitivity of the primary stem cell-enriched (CD34+) AML subpopulation to the proteasome inhibitor bortezomib alone or in combination with TRAIL in short and long-term culture conditions. In particular the roles of NF-κB and MCL-1 were studied.

TRAIL is a promiscuous ligand and can bind to five receptors of which OPG is one of the decoy receptors. To improve the efficacy of TRAIL, receptor-specific variants have been developed which have an increased affinity to either the DR5 receptor (rhTRAIL D269H/E195R) or DR4 receptor (rhTRAIL 4C7 (G131R/R149I/N199R/K201H/S159R/S215D)) [73–75]. Interestingly, it has been shown that the DR4-specific variant has an improved efficacy compared to wild-type TRAIL to induce apoptosis in AML cell lines [73]. However, so far it has not been addressed whether these TRAIL-specific variants display a decreased affinity to OPG. In Chapter 4, we evaluated the change in affinity of these variants compared to wild-type TRAIL by using surface plasmon resonance (SPR)-techniques and (competitive) ELISA experiments. Since OPG is expressed and secreted by osteoblasts residing in the bone microenvironment, OPG could decrease the efficacy of TRAIL. Therefore, we studied in co-culture experiments with osteoblasts and multiple myeloma cells whether the DR5-specific variant could overcome the resistance mediated by OPG.
and therefore could still result in the induction of apoptosis.

In Chapter 5 we examined the efficacy of the recently developed second-generation proteasome inhibitors carfilzomib and oprozomib in comparison to bortezomib in inducing apoptosis in primary AML CD34+ cells. Different assays were used to study the efficacy on the primitive and more mature AML subpopulations in vitro. In addition, the efficacy was correlated to the in vitro proteasome activity.

To obtain a full spectrum of the deregulated expression of apoptosis related genes in AML, we investigated the expression pattern of 386 apoptosis associated genes in AML CD34+ cells compared to normal bone marrow CD34+ cells (Chapter 6). Amongst these apoptosis related genes, TGF-β-activated kinase 1 (TAK1) is frequently upregulated in AML cells. Therefore, we evaluated whether TAK1 could be a potential target for the treatment of AML patients by performing genetic downmodulation or pharmacologic inhibition of TAK1 in AML cells under in vitro and in vivo conditions. Furthermore, we investigated the molecular signaling pathways that resulted in the apoptotic phenotype upon TAK1 blockade.

A summary of the results and future perspectives are finally described in Chapter 7.
BIBLIOGRAPHY


