Molecular-Targeted Therapies for Hematologic Malignancies

Guest Editors: Kevin D. Bunting, Cheng-Kui Qu, and Michael H. Tomasson
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Major advances in the disciplines of hematology, genetics, biochemistry, and chemistry over the past decades have empowered investigators with the background and methods required for development of customized molecular-targeted therapies. The ability to identify signaling pathways that are dysregulated, to determine the associated mutations, and to develop chemical drugs toward a desired correction is now a realistic work flow. The landmark demonstration that BCR-ABL could be molecularly targeted and could have a major impact upon disease progression really ignited the field of targeted therapies for hematologic malignancies. Through much hard work, we now know the key drivers of some hematologic malignancies, and depending on the particular disease, we have an arsenal of agents available to act at multiple nodal points. Some enabling technologies that have been a key for these advances include small molecule screening, high throughput whole genome sequencing, mouse models for cancer, and gene and microRNA expression array analyses. In this special issue, we present a collection of seven articles that contribute to our understanding of molecular targets and the development of approaches for their inhibition or rationale use of existing agents or their derivatives. These targets range from very early initiators of malignancy to molecules that are already advanced into clinical studies.

The first paper in this issue by A. Fathi and T. Abdel-Wahab “Mutations in epigenetic modifiers in myeloid malignancies and the prospect of novel epigenetic-targeted therapy” addresses the series of mutations identified that alter DNA and/or histone lysine methylation. These early epigenetic changes predispose to leukemogenesis and are relevant in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) patients. The second paper by J. Okabe-Kado et al. “Extracellular NM23 protein as a therapeutic target for hematologic malignancies” describes extracellular NM23-H1 protein and its relationship with altered signaling pathways and growth/survival in AML. The third paper by S. Verma et al. “Gab adapter proteins as therapeutic targets for hematologic disease” describes the Grb2-associated adapter proteins (Gabs) as potential therapeutic targets playing major roles in regulation of multiple signaling pathways. These first three papers describe potential new targets that require further validation and greater specificity, but could have significant impact on the initiation and progression of hematologic malignancy.

The fourth paper by X. Liu et al. “Molecular targets for the treatment of juvenile myelomonocytic leukemia” describes the outstanding progress made in identifying the mutations associated with activation of the Ras pathway in juvenile myelomonocytic leukemia (JMML), several of which are already targets of drugs that are being tested such as Ras and SHP-2. This paper additionally points toward protein:protein interactions as potential therapeutic targets such as SHP-2/Gab2 in hematopoiesis. The fifth paper by P. Argyriou et al. “The role of mTOR inhibitors for the treatment of B-cell lymphomas” focuses on the downstream activation of the mTOR pathway and the development and testing of new rapalogues and ATP-competitive inhibitors for clinical use. The mTOR pathway is central to cell survival and metabolism and represents a common target for many types of cancers. The sixth paper by F. Tzifi et al. “The role of Bcl2 family of apoptosis regulator proteins in acute and
chronic leukemias” describes the exciting new advances in understanding and targeting the Bcl-2 family of proteins and gives a comprehensive update on new agents that are in clinical studies targeting survival in acute and chronic leukemias. All of these three papers describe bona fide targets that are already subject to significant validation and commercial drug development.

The seventh paper by P. Koehler et al. “Engineered T cells for the adoptive therapy of B cell-chronic lymphocytic leukemia (B-CLL)” in the issue addresses immunotherapy using T-cell therapies against CD19, which has recently been very successful and received widespread attention for treatment of chronic lymphocytic leukemia. This approach promises to provide sustained targeted therapy based on cell surface phenotype and although it has to deal with issues such as B-cell deficiency and intravenous immunoglobulin infusions, such approaches when combined with chemotherapy are very promising as a form of targeted gene-based therapy.

In summary, the articles in this special issue address the spectrum of new targeted therapy development, from basic understandings of structure-function to mature rationale drugs already being tested in patients. We sought to cover the full spectrum of therapeutic development and are pleased to present a series of papers that do just that. As editors of this issue, we appreciate the important contributions of the authors of these review articles and hope that this issue will encourage expanded translational research toward developing novel therapies for hematologic malignancies.

Kevin D. Bunting
Cheng-Kui Qu
Michael H. Tomasson
In the recent years, the discovery of a series of mutations in patients with myeloid malignancies has provided insight into the pathogenesis of myelodysplastic syndromes (MDSs), myeloproliferative neoplasms (MPNs), and acute myeloid leukemia (AML). Among these alterations have been mutations in genes, such as \( \text{IDH1/2}, \text{TET2}, \text{DNMT3A}, \text{and} \ \text{EZH2} \), which appear to affect DNA and/or histone lysine methylation. Large clinical correlative studies are beginning to decipher the clinical importance, prevalence, and potential prognostic significance of these mutations. Additionally, burgeoning insight into the role of epigenetics in the pathogenesis of myeloid malignancies has prompted increased interest in development of novel therapies which target DNA and histone posttranslational modifications. DNA demethylating agents have been demonstrated to be clinically active in a subset of patients with MDS and AML and are used extensively. However, newer, more specific agents which alter DNA and histone modification are under preclinical study and development and are likely to expand our therapeutic options for these diseases in the near future. Here, we review the current understanding of the clinical importance of these newly discovered mutations in AML and MDS patients. We also discuss exciting developments in DNA methyltransferase inhibitor strategies and the prospect of novel histone lysine methyltransferase inhibitors.

1. Introduction

The increasing use of systematic genome-wide discovery efforts in patients with a variety of myeloid malignancies has led to the rapid discovery of a series of recurrent genetic abnormalities underlying these disorders. Remarkably, a large number of these alterations appear to be in genes whose function is known, or suspected, to be involved in epigenetic regulation of gene transcription. In the last 3 years, alone mutations in the genes \( \text{TET2}, \text{IDH1}, \text{IDH2}, \text{DNMT3a}, \text{and} \ \text{EZH2} \) have all been found in patients with myeloproliferative neoplasms (MPNs), myelodysplastic syndromes (MDSs), and/or acute myeloid leukemia (AML). Although the functional implications of these mutations and how precisely they contribute to abnormal hematopoiesis and leukemogenesis is being heavily investigated and not yet clarified, a number of potentially clinically important implications of these mutations may already be apparent. First, mutations in several of these genes likely hold prognostic importance for patients, and these genetic alterations, thereby, may serve as prognostic markers for risk stratification and aid in therapeutic decision making. Secondly, mutations in several of these genes may specifically impact DNA methylation and/or histone posttranslational modifications in such a manner that is therapeutically targetable. Mutations in several of these genes, such as \( \text{IDH1} \) and \( \text{IDH2} \), have been proven to result in a gain-of-enzymatic function which holds the prospect for development of novel targeted therapeutics. This review focuses on the clinical relevance of recently discovered epigenetic alterations in...
patients with myeloid malignancies and the prospect for novel targeted therapeutics against aberrant epigenomic characteristics in patients with MDS and AML.

2. Recently Identified Mutations in EpigeneticModifiers in Myeloid Malignancies

2.1. IDH1 and IDH2 Mutations. Genome sequencing of AML has recently led to the discovery of mutations in the genes encoding isocitrate dehydrogenase (IDH1 and IDH2). IDH1 is a key cytosolic enzyme in the Krebs cycle. It catalyzes the decarboxylation of isocitrate to a keto-glutarate (α-KG), leading to the production of nicotinamide adenine dinucleotide phosphate (NAD-P). The isocitrate dehydrogenase 2 (IDH2) gene encodes a homologous protein which catalyzes the same reaction in mitochondria. IDH2 mutations have been extensively studied and are frequently found alterations in low-grade gliomas. They have also been discovered in a small subset of the highly aggressive glioblastomas, where they confer a more favorable prognosis [1–6].

IDH mutations were subsequently identified in AML and other myeloid malignancies, including MDS and MPNs [7, 8]. All discovered IDH mutations reside in the active site of the enzyme and participate in isocitrate binding [9]. They are missense alterations affecting arginine-132 (R132) in IDH1, and either the analogous arginine residue (R172), or the arginine-140 (R140) residue in the IDH2 protein [7, 10–15]. The common recurrence of IDH mutations in AML suggests an importance in leukemogenesis. All mutations are missense and heterozygous, suggesting that the alterations lead to a “gain of function” [9]. It has been shown that the mutant forms of IDH cannot catalyze the conversion of isocitrate to α-KG [16]. Dang et al. reported that the mutated R132H IDH1, in place of the normal process of isocitrate decarboxylation, catalyzes an NADPH-dependent reduction of α-KG to 2-hydroxylutarate (2-HG). 2-HG is a metabolite which is normally present at very low levels in cells [17], 2-HG levels have been found to be elevated in IDH-mutant glioma samples, and Gross et al. reported that IDH1 R132 mutations also lead to production and accumulation of 2-HG in AML blasts, greater than 50-fold higher than their nonmutant counterparts. Elevated 2-HG levels in IDH-WT samples led to the first discovery of IDH2 mutations, which accounted for elevated 2-HG levels in these AML cells. The elevation in 2-HG levels has also been noted in sera of patients with IDH-mutant AML [9].

Studies of IDH mutations in gliomas have suggested that they are an early event in the pathogenic process [5]. Their exact mechanism in leukemogenesis of AML is uncertain. Normal IDH function appears essential for normal cell growth and proliferation. IDH1 is one of only three cytosolic proteins which contribute to NADPH production which is essential for nucleotide and lipid synthesis. Ward et al. demonstrated that siRNA silencing of the IDH1 and IDH2 proteins led to a significantly reduced proliferative capacity [15]. Some investigators have suggested that accumulation of 2-HG plays an important role in this process, the “gain of function” neomorphic enzyme activity that promotes cancer [9, 18, 19]. Patients with a rare inherited condition called 2-hydroxyglutaric aciduria have elevated levels of 2-HG with an increased propensity for brain tumors. 2-HG has indeed been shown to increase reactive oxygen species in these patients [20, 21]. Additionally, 2-HG is homologous to α-KG in structure and thus may bind and interfere with essential enzymes that are activated by α-KG. Among these are prolyl hydroxylases which control the stability of and downregulate HIF-1α transcription factors, implicated in the pathogenesis of multiple malignancies [9, 19, 22].

The association of IDH mutations with aberrant hypermethylation has only recently been discovered. By studying samples from 398 AML patients in an Easter Cooperative Group (ECOG) E1900 trial, we found that IDH-mutant AML is associated with consistent and aberrant hypermethylation of various promoter sites involved in myeloid differentiation and leukemogenesis [23] (Figure 1). Promoter CpG sites are extremely important in the regulation of gene expression, specifically those of genes which mediate tumor suppression and differentiation, and DNA methylation can lead to transcriptional inactivation or chromosomal instability [24, 25]. Aberrant hypermethylation has been extensively described as a pathogenic process in forms of MDS and AML [26–29]. The discovery of aberrant hypermethylation and transcriptional inactivation of loci in relation to IDH-mutant AML is intriguing and significant. The potential mechanism for the hypermethylation and leukemogenesis in IDH-mutant disease may be related to downregulation of normal α-KG levels. Multiple enzymes are dependent on α-KG for their function, including the TET enzymes, which appear to play an important role in the differentiation of myeloid cells and promote demethylation by hydroxylating methylcytosine groups [30, 31].

Another recent study reported that altered 2-HG/α-KG levels present in IDH1/2 mutant cells additionally results in the inhibition of a different set of α-KG-dependent enzymes, the Jumonji family of histone lysine demethylases (JHDM) [32]. There are 3 classes of enzymes which are known to antagonize histone methylation: (1) peptidylarginine deiminase, which removes methylarginine modifications to produce citrulline [33], (2) lysine specific demethylase 1 which removes H3K4me1/H3K9me1 marks in a reaction requiring flavin is a cofactor [34], and the Jumonji C domain family of histone demethylases which require iron Fe(II) and α-KG as cofactors. Unlike LSD1, which can only remove mono- and dimethyl lysine modifications, the JHDMs can remove methyl groups from all three histone methylation states. So far, JHDM family members have been shown to reverse the following lysine methyl marks: H3K36me1/2 (JHD1) [35], H3K9me1/2 (JHD2) [36], H3K9me2/3 (JHD3) [35], and H3K36me3 (JHD3) [35], H3K4me2/3 ([JARID1] [37], and H3K27me2/3 (UTX/JMJD3) [38]. In addition, JMJD6 has been shown to encode an arginine-specific histone demethylase which demethylates H3R2me1/2 and H4R3me1/2 [39]. All of these marks may, therefore, be affected by the presence of IDH1/2 mutations, and Xu et al. indeed demonstrated hypermethylation of many of these marks following introduction of IDH1/2 mutations into cells [32] (Figure 1). These findings lend significant...
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Figure 1: Specific histone and DNA posttranslational modifications shown to be associated with mutations in epigenetic modifiers in hematologic malignancies. Only genetic alterations which have some evidence for resulting in a gain or loss of function are displayed here. Mutations which result in the acquisition of hyperactivation or new enzymatic activity are displayed on the left of nucleosome while mutations which have evidence as resulting in a loss of enzymatic function are displayed on the right (translocations known to directly affect histone posttranslational modifications are listed in Table 1). The majority of mutations in epigenetic modifiers in myeloid malignancies recently identified are known to affect posttranslational modifications on the N-terminal tail of histone H3 or at cytosines of DNA as displayed here. Currently, the function of DNMT3a mutations in AML has yet to be extensively clarified, particularly the recurrent R882 heterozygous mutations.

credence to the theory that hypermethylation of DNA and histone lysine/arginine modifications play a key role in the pathogenesis of AML in this subgroup of patients. Further work to delineate the complex epigenetic alterations to the transcriptional changes which promote leukemogenesis will be very enlightening.

Currently, our clinical use of genetics in AML for prognostication relies on (1) the use of cytogenetics to delineate patients into favorable, intermediate, or adverse cytogenetic categories and (2) molecular genotype of the genes FLT3, NPM1 (nucleophosmin 1), and CEBPA (CCAAT/enhancer-binding protein alpha) for those patients with a normal karyotype. Multiple studies have demonstrated that patients with a normal karyotype but with an internal tandem duplication of FLT3 (FLT3-ITD) have an inferior outcome compared to those without a FLT3-ITD mutation [40, 41]. In several additional studies, the presence of an NPM1 mutation with or without the presence of a FLT3-ITD mutation was associated with higher complete response and event-free survival [42, 43]. Normal karyotype AML with mutations in CEBPA also appear to represent a subset of AML with more favorable outcome [44, 45]. Given the identification of multiple new molecular genetic abnormalities in patients with AML, an increasing number of studies have been performed to delineate the mutational frequency and prognostic implications of IDH1/2, TET2, and DNMT3A mutations in AML patients. The initial report of IDH1 mutations in AML found that 8.5% of AML samples contained a mutation at amino acid R132. All mutations were associated with samples that displayed intermediate risk cytogenetics, with the large majority having normal cytogenetics. With this relatively small group of 187 samples, the investigators noted no independent prognostic value on overall survival (OS), but subgroup analysis suggested adverse effects in those patients with no NPM1 mutations [7]. Ward et al. also assessed for mutations in a series of 87 AML samples and found that 7.7% had IDH1 mutations. Additionally, they found that even a larger subset of samples (15.4%) displayed mutations in the IDH2 gene. All samples with IDH mutations were found to have normal karyotypes, without an effect on overall survival [15].

Larger studies, based in North America and Europe, have attempted to further investigate the prevalence and prognostic implications of these mutations. A CALGB study assessed bone marrow and peripheral blood marrow samples from 358 patients with cytogenetically normal AML. The investigators reported IDH mutations in the third of their patients, with 14% having IDH1 mutations and 19% displaying IDH2 mutations. AML patients with IDH mutations in this study tended to be younger with lower-risk disease as defined by a higher frequency of NPM1 mutations without the presence of FLT3-ITD mutations.
Specifically, *IDH1* mutations adversely affected disease-free survival (DFS) in this particular subgroup of patients with a favorable molecular profile. Interestingly, those patients with *IDH2* mutations achieved a lower rate of complete remission with induction therapy [12].

A study from The Netherlands also found a significant prevalence of *IDH* mutations in 893 samples from patients with newly diagnosed AML, with 6% and 11% having *IDH1* and *IDH2* mutations, respectively. They also reported an association with normal karyotype AML and the *NPM1* mutation. As in previous studies, no effect on OS was noted for the entire cohort of AML samples, but interestingly, in those samples without an NPM1 mutation, *IDH* mutations were associated with an inferior event-free survival (EFS) [10]. A subsequent study from the UK found *IDH1* mutations in 8% of patient samples, using data on 1333 adult patients from the UK MRC AML10 and AML12 trials. Similar to the Dutch study, they reported a strong association with intermediate cytogenetics and the *NPM1* mutation. However, in contrast to the previous study but similar to results of the CALGB trial by Marcucci et al. [12], the investigators found that an *IDH1* mutation preferentially reduced survival in the cohort of patients with a coexistent *NPM1* mutation [46]. These findings were supported by large studies from France and Germany, which again found that 12–16% of patients had *IDH* mutations, with the mutations adversely affecting rates of CR and survival in the subgroup of patients with CN-AML and with *NPM1* mutations [11, 13].

However, the prognostic significance of the copresence of *IDH* and *NPM1* mutations in CN-AML continues to be uncertain. A recent large German study reported shorter EFS and rates of CR in all AML patients with an *IDH1* mutation, and specifically within the population of patients without *NPM1* mutations [14]. The same authors have recently also presented their data at the annual American Society of Hematology (ASH) 2010 meeting on 526 patients with cytogenetically normal AML and found that 28.7% of patients had *IDH* mutations. 12.9% of patients were found to have *IDH1* mutations and 15.8% had *IDH2* mutations. In their survival analysis, no effect was found on OS or EFS. However, in this presentation, a trend for shorter EFS was reported in all IDH mutant patients with *NPM1*+/FLT3-ITD genotype, and, interestingly, a significant adverse effect on EFS in those with specifically IDH2 mutations in the *NPM1*+/FLT3-ITD-group [47]. The leukemia group from M.D. Anderson in Houston, Tex, USA, also presented their data at the ASH 2010 meeting on 358 AML patients treated with induction chemotherapy. They found that *IDH1* and *IDH2* mutations were associated with normal karyotype and *NPM1* mutations and were present in 12 (7%) and 24 (14%) patients and also found an *IDH1G165* single-nucleotide polymorphism (SNP) alteration in 24 (14%) patients. Overall, they found that 30% of patients had *IDH* alterations, but there was no association with achievement of CR, remission duration, OS or EFS, and *IDH* aberrations [48]. Caramazza et al. from the Mayo Clinic examined *IDH* mutations in 157 patients with hematologic malignancies and suggested an association with isolated trisomy 8. Eighteen *IDH* mutations were identified, with the majority (15) being *IDH2* mutations. Seventeen of the 18 *IDH* mutations occurred in myeloid malignancies, among which the large majority were MDS or AML. *IDH* mutations were fewer among 64 additional patients with AML or MDS without isolated trisomy 8. In MDS patients with trisomy 8 alteration, prognosis was similar between those with *IDH* mutations and those without [49].

2.2. TET2 Mutations. TET2, the ten-eleven-translocation gene 2, is located in chromosome 4 at band 4q24. Delhommeau and colleagues first described the presence of TET2 mutations in myeloid malignancies, by evaluating bone marrow samples from 320 patients with MDS, MPNs, and AML. TET2 defects, either mutations or deletions, were discovered in 17 of 90 patients with MDS (19%), in 24 of 198 patients with MPN (12%), and in 5 of 21 patients with secondary AML (24%) [50]. Other groups have since reported a similar prevalence of TET2 mutations in myelodysplastic syndromes and other myeloid diseases [51–53]. TET2 mutations were also found in the large majority (median of 96%) of the precursor cells in the bone marrow of patients with mutations, including CD34+ progenitor cells [54]. In addition, there has also been much interest in the role of TET2 as a transformative mutation in myelodysplasia and MPNs. By analyzing the DNA of paired samples from patients with transformed, secondary AML, it was found that TET2 mutations were detected most frequently after the transformation of MPNs to AML [55].

The pathogenic mechanism of TET2 mutations in myeloid diseases has been an area of intense investigation. Strong evidence supports the integral role of TET2 in demethylation of DNA and epigenetic regulation, specifically in the conversion of 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) [30, 31]. A recent intriguing study convincingly demonstrated that 5-hmC is an intermediary in the process of DNA demethylation, and that TET hydroxylases are essential mediators of this process [56]. Ko et al. further demonstrated that mutations of TET2 lead to alteration and suppression of catalytic activity of the enzyme. They reported low DNA levels of 5-hmC in bone marrow samples taken from patients with TET2 mutations. In addition, the depletion of TET2 in murine models led to a suppression of differentiation of hematopoietic precursors in culture [57]. This interruption in myelopoiesis may be a necessary “hit” or alteration in the process of leukemogenesis in patients with TET-2 mutant AML.

Most recently, a variety of groups have studied the prognostic impact of TET2 mutations in MDS and AML. They appear to have a particularly high prevalence in chronic myelomonocytic leukemias, in which they have been found to be associated with significant monocytosis and poor outcomes [58]. Data on prognosis has also been presented in patients with MDS and AML, where there has been some controversy. Nibourel et al. sequenced sample DNA from 111 patients who had achieved CR after induction chemotherapy for de novo AML. They reported an overall incidence of 17% of TET2 mutations, which were associated with *NPM1* mutations but did not appear to have an impact on survival. This lack of effect on outcomes was also reported by other
investigators [59, 60]. Another group from France reported that TET2 mutations were actually independently associated with significantly improved overall and progression-free survival in patients with MDS. Only a small percentage of patients (7%) in this study were reported to have secondary survival in patients with MDS. Only a small percentage of that TET2 mutations were actually independently associated with a lower rate of CR and shorter disease-free and overall survival, with outcomes particularly worse for patients with favorable risk (CEPBA and NPM1 mutant) disease [62].

Perhaps, more intriguing has been the consistent finding in recent studies that TET2 mutations rarely cooccur with mutations affecting isocitrate dehydrogenase (IDH1 and IDH2) [60, 62]. As mentioned, the possible mechanism for aberrant methylation and leukemogenesis in IDH-mutant AML may be related to the downregulation of α-KG levels, on which TET2 enzymes depend for their activity [26]. Others have reported that the metabolite 2-HG, markedly elevated in samples of patients with IDH mutant AML, can also directly inhibit TET2 function [32]. Therefore, TET2 and IDH mutations may be leukemogenic through a common mechanism, that of suppression of TET2 function. They, thus, likely act as a distinct mutational class in AML with overlapping effects on DNA methylation and leukemogenesis (Figure 1).

2.3. DNMT3A Mutations. DNA methyltransferases catalyze the methylation of cytosine residues of CpG dinucleotides in DNA and are encoded by the human genes DNMT1, DNMT3A, and DNMT3B. DNMT3A mutations in AML have only been recently described [63–65] and found to be present in approximately 20–22% of patients with de novo AML. Interestingly, these mutations seem to be associated with intermediate-risk AML, a finding also noted with TET2- and IDH-mutant disease. In contrast, no cases of favorable risk AML contained these mutations [63]. Unlike the exclusivity of TET2 and IDH mutations in recent studies of AML, DNMT3A mutations often cooccurred with IDH mutations, suggesting that these latter two mutations may not have overlapping functions in leukemogenesis.

Somatic mutations in DNMT3A have been reported as nonsense, frameshift, and missense mutations throughout the open-reading frame. However, a notable recurrent mutation in DNMT3A has been repeatedly reported in AML [63] and MDS [66] patients as a somatic missense mutation at amino acid R882. Although one study reported a decrease in DNA methylation activity of >50% with the DNMT3A R882 mutant [64] in an in vitro methyltransferase assay, AML patient samples with DNMT3A mutations were not found to have altered total 5-methylcytosine content or altered patterns of methylation [63] (Figure 1). Equally important is the fact that the DNMT3A R882A mutation appears to occur exclusively as a heterozygous mutation suggesting a potential gain of function which may or may not require a wildtype copy of DNMT3a for altered function. Future studies examining the function of the DNMT3A R882 mutation in vitro and in vivo in the presence of wildtype DNMT3A will hopefully shed further light on a pathogenic mechanism of DNMT3a mutations in AML.

DNMT3A mutations were subsequently noted in patients with MDS and secondary AML. Walter et al., sequencing samples from 150 patients, found that 12 harbored DNMT3A mutations. Similar to the trend noted in AML, the majority of mutations were at amino acid R882. These mutations were associated with worse overall survival and rapid progression to AML, although sample size was small and transplantation status was not considered [66]. The adverse impact on survival has also been reported in patients with AML. Ley and colleagues found, in their cohort, that those with DNMT3A mutations experienced a significantly worse median overall survival of 12.3 months as compared to 41.1 months for those without mutations. Interestingly, it appeared that DNMT3A mutations accounted for the majority of the adverse effect on survival seen even in patients with FLT3-ITD alterations [63]. A more recent study from China, studying patients with acute monocytic leukemias, also reported decreased overall survival and worse outcomes in those with DNMT3A mutations [65]. Lastly, DNMT3A mutations have also recently been reported in patients with primary myelofibrosis [67]. Larger studies of DNMT3A mutations in patients with additional MPNs will be needed to further understand the clinical and/or prognostic importance of DNMT3a mutations in MPNs.

2.4. EZH2 Mutations. EZH2 is a highly conserved enzyme which serves as a histone H3 lysine 27 (H3K27) methyltransferase. Although EZH2 has been known to be overexpressed in several epithelial malignancies for some time, only in 2010 was it discovered that EZH2 may be mutated in hematopoietic malignancies. Curiously, a recurrent monoallelic activating mutation has been identified in EZH2 at tyrosine 641 in patients with lymphomas [68] while a series of apparent loss-of-function mutations have been found in patients with MDS and primary myelofibrosis [69–71].

The fact that EZH2 is altered by overexpression/increased activity in epithelial cancers and lymphomas, yet inactivated in myeloid malignancy, argues that the biologic consequences of alterations in H3K27me3 may be tissue specific. At the same time, rigorous assessment of whether EZH2 mutations affect the abundance and/or the distribution of H3K27me3 in the chromatin of malignant versus paired normal nonmutated cells has not yet been published (Figure 1). In addition, investigation of the effects of EZH2 mutations on alterations in DNA methylation may be particularly important given that EZH2 physically interacts with DNA methyltransferases 1–3 [72], and data suggests that H3K27 methylation is a necessary prerequisite for DNA promoter methylation [72].

From a clinical standpoint, inactivation of EZH2 by loss or mutation in MDS is enlightening as cytogenetic abnormalities of chromosome 7 which have been long recognized in MDS and AML and linked to adverse outcome [73, 74]. In fact, several studies suggest that MDS patients with EZH2 mutations have worsened overall survival compared to those without EZH2 mutations, regardless of gross
cytogenetic findings [70, 71]. Larger studies incorporating EZH2 mutations in light of additional genetic abnormalities will be needed to further clarify the prognostic importance of EZH2 mutations in MDS and MPNs and are ongoing.

3. The Prospect of Novel Therapeutic Agents Targeting Epigenetic Modifiers in Myeloid Malignancies

3.1. DNA Methyltransferase Inhibitors (DNMTIs). The first three epigenetic targeted therapeutics which have been FDA approved for use in the United States include 2 drugs targeting DNMTIs (azacitidine (AZA) and decitabine (5-aza-2′-deoxycytidine)) as well as one histone deacetylase inhibitor (vorinostat). Although the use of DNMTIs has proven useful in the therapy of high-risk MDS as well as in AML, there are several questions which have lingered regarding the use of these therapies: (1) what is the ideal dose and schedule of DNMTIs? (2) what is the true mechanism of action of the nucleoside DNMTIs? (3) are there biomarkers which can be used for predicting response and/or resistance to DNMTIs? and (4) can we develop nonnucleoside direct inhibitors of DNMTs?

Despite questions regarding use and schedule of DNMTIs, there have been several interesting new developments in this class of therapeutics. Within the original category of nucleoside analog DNMTIs, an oral formulation of AZA has recently been developed [75]. Parenteral azacitidine is approved for administration at 75 mg/m² for 7 days every 28 days, and this dose is believed to result in DNA hypomethylation as well as cytotoxicity. Using several assays for DNA methylation, this dose of 5-aza has been shown to result in maximal DNA hypomethylation at approximately day 15 with gradual return of DNA methylation back to baseline around the time of next cycle [76]. Given this, use of orally administered AZA on a more frequent schedule may result in altered effects on DNA methylation and cellular cytotoxicity and holds the potential for greater therapeutic efficacy. The initial phase I trial of oral AZA on a 7 days schedule revealed that the drug is bioavailable, safe, and clinically active in patients with MDS and AML. At the 2010 ASH meeting, results of the multicenter phase I study of extended oral AZA schedules revealed that oral azacitidine on a 14- or 21-day schedule is well tolerated, with no AZA accumulation, and promising clinical responses were observed [75].

The direct cytotoxic effects of the clinically utilizing DNMTIs as well as their chemical instability have prompted continuous rationale for developing additional DNMTIs. A third nucleoside DNMTI which has been under development for some time is the cytidine analogue zebularine. Although zebularine has a similar mechanism of action as decitabine and azacitidine, resulting in the depletion of DNMTs through covalent bonding with DNMTs, zebularine has a much longer half-life making oral administration of the drug possible [77, 78]. Additionally, zebularine appears to be selectively incorporated into malignant and not normal cells admixed with tumor in at least one setting, a property not seen with decitabine or AZA [79]. Despite these properties, one limitation to the development of zebularine for clinical use has been the fact that higher concentrations of zebularine are needed to obtain similar levels of demethylation in cells in comparison with azacitidine and decitabine [78]. Further preclinical works addressing the practicality of the drug as a clinical therapeutic agent are ongoing.

In addition to the nucleoside DNMTIs, there has been considerable efforts at developing nonnucleoside targeted molecules to directly inhibit individual DNMTs. One approach has been the development of antisense oligonucleotides targeting DNMT1 for in vivo use. One such molecule, the phosphorothioate antisense oligonucleotide MG98, was developed based on its ability to knockdown DNMT1 expression in various model systems [80, 81]. However, phase I clinical trials of MG98 in solid and hematopoietic tumors was disappointing with very little consistent knock down of DNMT1 mRNA in patients [82, 83]. This likely resulted from inefficient intracellular uptake of MG98.

Rational design of small molecules targeting DNMTs through noncovalent interactions with the catalytic sites of these enzymes has resulted in the development and characterization of several test compounds. The first rationally designed DNMT1 inhibitor is RG108 which was designed utilizing a three-dimensional model of the human DNMT1 catalytic pocket [84]. RG108 has comparable demethylating activity to zebularine but appears to be less active than azacitidine and decitabine. Several additional small-molecular inhibitors of DNMTs have more recently been found within the NCI open database of compounds through a similar screening approach which led to the discovery of RG108 [85]. Further preclinical characterization of all of these compounds is underway.

The use of DNMTIs in patients with high-risk MDS and AML has proven that while durable complete remissions are possible with these drugs, responses can be quite variable with no current routinely used clinical parameter known to predict likelihood of response to therapy. With the discovery of mutations in TET2 in these patients and the postulated pathogenic mechanism of TET2 mutations in MDS/AML, it has been hypothesized that TET2-mutated patients may have higher rates of response to DNMTIs. This has recently been suggested by a small French study of 86 patients with MDS and secondary AML. The investigators reported that those with TET2 mutations experienced a response rate (RR) of 82% to AZA in comparison to the TET2-wt group, which had a significantly lower RR of 45%. However, there was no effect on survival parameters, and the study group was quite heterogeneous with few additional genetic parameters studied [86]. In contrast, other groups have found that TET2 alterations in a similar cohort of patients may actually predict for decreased responsiveness to demethylating therapies [87]. The small number of patients included in these studies and the limited genetic characterization of the patients must be considered. Larger studies with more comprehensive genetic evaluation will be critical in determining if mutations in genetic factors suspected to be important in regulating DNA methylation
(TET2, IDH1/2, and DNMT3a mutations, amongst others) affect response to DNMTIs.

4. The Prospect for Novel Histone Methyltransferase Inhibitors in Myeloid Leukemias: DOT1L Inhibition and Rational Design of Protein Methyltransferase Inhibitors

Recent discovery of the potential importance of aberrant hypermethylation of histone lysines in the pathogenesis of myeloid leukemias driven by MLL-translocations [88], NUP98-NSD1 translocations [89], and possibly IDH1/2-mutant disorders [32] suggests the possibly of targeting histone lysine methyltransferases in myeloid leukemias.

One novel and exciting prospect utilizing this therapeutic rationale is the study of DOT1L-targeted therapy for the selective treatment of MLL-translocated leukemias. In addition to the recently identified mutations in epigenetic modifiers in MDS and AML, a number of translocations disrupting the normal activity of epigenetic modifiers in myeloid malignancies have been recognized for a longer period of time (Table 1). Key amongst the frequent translocations altering the activity of an epigenetic modifiers in AML includes translocations involving mixed lineage leukemia 1 (MLL1) which occur in at least 10% of adult AML patients and >70% of infant acute leukemias. MLL1 normally serves as an histone H3 lysine 4 (H3K4) methyltransferase. MLL1 translocations result in fusion of the N terminus of MLL1 to one of >60 different translocation partners [90]. In a recent landmark survey of MLL rearrangements, 760 MLL-rearranged biopsy samples were reviewed and 104 different MLL rearrangements were found in adult and pediatric acute leukemia patients [90]. However, amongst AML patients with MLL rearrangements, 77% were accounted for by one of 7 translocations: MLL-AF9 (30.4%), MLL-AF10 (14.5%), MLL-ELL (10.9%), MLL-AF6 (10.1%), MLL-ENL (5.4%), MLL-AF17 (2.9%), and MLL-SEPT6 (2.5%). Many of these same MLL rearrangements are also common in acute lymphoblastic leukemia (ALL) along with MLL-AF4 which is the most common MLL rearrangement in ALL (accounting for 66% of MLL-rearranged ALL cases) [90].

Importantly, the four most frequent MLL translocations (MLL-AF4, MLL-AF9, MLL-AF10, and MLL-ENL) result in recruitment of DOT1L (disruptor of telomeric silencing 1-like) to the fusion protein and acquisition of histone 3 lysine 79 (H3K79) methyltransferase activity (Figure 1). A number of studies using both shRNA for DOT1L and conditional deletion of DOT1L have recently shown that the H3K79 methyltransferase activity is critical for leukemogenesis induced by MLL-fusion proteins [91, 92]. This has led to the concept of developing targeted therapy for DOT1L inhibition in the therapy of MLL-translocated leukemias. At the 2010 ASH meeting, investigators at Epizyme Inc. presented the initial results from in vitro studies of the first DOT1L inhibitor, EPZ01 [93]. EPZ01 acts as a competitive inhibitor of the cofactor S-adenosylmethionine (SAM), the universal methyl donor for all enzymatic methyltransferase reactions. Despite the ubiquity of SAM in protein methyltransferase reactions, EPZ01 is reported to have a 500-fold selectivity for DOT1L over other lysine histone and arginine methyltransferases. Consistent with this, the investigators revealed selective killing of EPZ01 for leukemia cell lines bearing MLL1 translocations over non-MLL rearranged cell lines. DOT1L-inhibition appeared to downregulate H3K79me3 abundance globally and at critical loci, serving both as proof of concept of the mechanism of activity and potentially as a biomarker of response [93].

As mentioned earlier, MLL rearrangements are also frequent in ALL with MLL-AF4 translocation being the most frequent MLL rearrangement in ALL [90]. Although two initial transgenic mouse models of MLL-AF4 fusion gene overexpression did not result in the development of acute leukemia, a conditional knockin model of MLL-AF4 [94] as well as a retroviral transplantation model of MLL-AF4 [95] did result in the development of ALL. Moreover, mice with overexpression of MLL-AF4 in the conditional knockin model by Krivstov et al. were clearly distinguished by increases in H3K79me3 indicating a clear link between the presence of MLL-AF4 fusion oncoprotein and acquisition of increased H3K79 methyltransferase activity. In addition, recent purification of the MLL-AF4 complex has clearly indicated binding of DOT1L to this complex [96]. Equally important, use of an shRNA against DOT1L inhibited the expression of several genes critical for MLL-AF4-mediated oncogenesis in the MLL-AF4-conditional knockin model underscoring the potential importance of DOT1L inhibition in the therapy of ALL with MLL-AF4 rearrangement [94].

One key question which must be further addressed in the preclinical development of DOT1L-targeted therapy is the question of potential adverse ramifications of DOT1L inhibition. Jo et al. recently reported that mice bearing conditional disruption of DOT1L-developed pancytopenia and failure of hematopoietic homeostasis revealing a critical role of DOT1L in normal hematopoiesis [91]. At the same meeting, Bernt et al. also developed a conditional deletion model of DOT1L in vivo using Vav-Cre technology for DOT1L deletion in the adult hematopoietic and endothelial cells but also in the germline [92]. DOT1L−/− mice in this system were born at Mendelian rations and with blood counts at lower border of normal range. In addition, cardiac-specific deletion of DOT1L resulted in increased mortality in mice due to cardiac dysfunction which closely resembled human dilated cardiomyopathy [97]. Interestingly, Nguyen et al. further discovered that DOT1L is downregulated in patients with idiopathic dilated cardiomyopathy, and the cardiac phenotype in mice could be rescued by expression of dystrophin [97]. Further characterization of these mouse models and use of DOT1L inhibitors in preclinical in vivo testing will hopefully clarify the potential utility and safety of this very promising new therapeutic strategy.

Development of other specific inhibitors of histone methyltransferases holds promise in myeloid leukemias. The challenges for this prospect have been twofold: (1) knowledge of the genome-wide and locus-specific effects of histone modifications due to direct genetic abnormalities found in myeloid leukemia patients has been less clear for the
malignancies. Although empiric use of these medications tant in the pathogenesis of prostate and breast carcinomas CARM1, a protein overexpressed and thought to be impor-
molecule serves to inhibit the arginine methyltransferase inhibitor was recently reported using this strategy [99]. This fact, the first selective and potent protein methyltransferase targeting of the ATP-binding pockets of protein kinases. In
of protein methyltransferase somewhat analogous to the inhibitors of the SAM-binding pocket, a universal feature mentioned earlier is the development of small-molecule methyltransferases have only recently begun. One strategy understand [98]. (2) In addition, from a drug development for targeted changes a occur in some of the same disorders making the rationale H3K27 demethylase UTX has also been suggested to primary myelofibrosis [69–71]. At the same time, loss of EZH2 loss-of-function mutations in the H3K27 methyltransferase majority of non-MLL translocated patients. For instance, loss-of-function mutations in the H3K27 methyltransferase EZH2 has been recently found in patients with MDS and primary myelofibrosis [69–71]. At the same time, loss of the H3K27 demethylase UTX has also been suggested to occur in some of the same disorders making the rationale for targeted changes affecting H3K27 methylation hard to understand [98]. (2) In addition, from a drug development standpoint, potent and selective inhibitors of histone protein methyltransferases have only recently begun. One strategy mentioned earlier is the development of small-molecule inhibitors of the SAM-binding pocket, a universal feature of protein methyltransferase somewhat analogous to the targeting of the ATP-binding pockets of protein kinases. In fact, the first selective and potent protein methyltransferase inhibitor was recently reported using this strategy [99]. This molecule serves to inhibit the arginine methyltransferase CARM1, a protein overexpressed and thought to be impor-
tant in the pathogenesis of prostate and breast carcinomas [100, 101]. Although empiric use of these medications in early-phase clinical trials has been utilized previously with some success, in-depth characterization of histone posttranslational modifications in patients with MDS/AML may shed light on rational strategy for specific histone methyltransferase inhibition as a therapeutic strategy in these disorders.

5. Conclusion
The exciting discovery of new genetic abnormalities in patients with myeloid malignancies holds the promise for furthering our understanding of the pathogenesis of these disorders but also in refining our risk stratification and therapeutic management of patients. As highlighted here, a series of studies have rapidly suggested that mutations in TET2, IDH1/2, and DNMT3a will likely refine our current prognostication of patients with AML if borne out repeatedly in large prospective trials of AML patients. Moreover, given the suggested effects of these genetic abnormalities on DNA methylation, the potential importance of these mutations on affecting response to DNMTIs will need to be more thoroughly investigated. The effects of the recurrent DNMT3a R882 mutation will particularly need to be scrutinized given its frequency and the fact that it is always present as a heterozygous mutation. Furthering our understanding of the specific altered epigenetic marks placed by genetic abnormalities in MDS/AML patients is critical as it may result in the development of novel epigenetic
targeted therapeutics. The clearest example of this currently is the exciting development of DOT1L inhibitors for MLL-translocated leukemias described here. Further characterization of DOT1L deficiency/inhibition in a variety of in vivo models is greatly anticipated. Moreover, development of additional protein methyltransferase inhibitors is likely forthcoming and prompts for greater understanding of the epigenetic alterations present in patients with MDS and AML.

References


Review Article

Extracellular NM23 Protein as a Therapeutic Target for Hematologic Malignancies

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An elevated serum level of NM23-H1 protein is a poor prognostic factor in patients with various hematologic malignancies. The extracellular NM23-H1 protein promotes the in vitro growth and survival of acute myelogenous leukemia (AML) cells and inversely inhibits the in vitro survival of normal peripheral blood monocytes in primary culture at concentrations equivalent to the levels found in the serum of AML patients. The growth and survival promoting activity to AML cells is associated with cytokine production and activation of mitogen-activated protein kinases (MAPKs) and signal transducers and activators of transcription (STAT) signaling pathways. Inhibitors specific for MAPK signaling pathways inhibit the growth/survival-promoting activity of NM23-H1. These findings indicate a novel biological action of extracellular NM23-H1 and its association with poor prognosis. These results suggest an important role of extracellular NM23-H1 in the malignant progression of leukemia and a potential therapeutic target for these malignancies.

1. NM23 Expression and Hematologic Malignancies

The NM23 gene was identified by differential hybridization of a cDNA library with total RNA extracted from slightly and highly metastatic melanoma cell lines [1]. The NM23 gene has been identified as a family of genes encoding different isoforms of nucleoside diphosphate kinase (NDPK) [2]. NM23 genes play critical roles in cellular proliferation, differentiation, oncogenesis, and tumor metastasis, and the mechanisms of these pleiotropic effects are not well understood [3, 4]. Ten isotypes of the human NM23 gene have been identified [5]. Among these, only NM23-H1 and NM23-H2 have been studied extensively in human cancers. The level of NM23-H1 expression is inversely correlated with the tumor's metastatic potential in experimental rodent cells and in human tumors, such as breast, ovarian, cervical, and gastric cancer, hepatocellular carcinoma, and melanomas [4]. Exogenous overexpression of NM23-H1 reduces the metastatic potential of multiple types of cancer cells and suppresses in vitro tumor cell motility and invasion [6]; therefore, NM23-H1 is implicated in the regulation of metastasis in a variety of human cancers, and its overexpression predicts a favorable patient prognosis. In contrast, elevated NM23-H1 expression is related to a more aggressive disease in neuroblastoma and many hematologic malignancies [7–11]. The significance of NM23-H1 overexpression as a prognostic factor is dependent on tumor cell types although the mechanism of this discrepancy is unknown.

We previously reported that a nondifferentiating myeloid leukemia cell line produced differentiation-inhibiting factors [12, 13]. We purified one of these factors as a homologue of mouse NM23-M2 [14]. The NM23-H1 gene was overexpressed in various hematologic neoplasms, including AML, acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia in blast crisis (CML-BC), and myelodysplastic syndrome (MDS), more than in normal blood cells (Figure 1(a)) [10]. The progression of CML was accompanied by the overexpression of NM23-H1 mRNA [15]. This increase in NM23-H1 was observed not only in leukemia, but also in malignant lymphoma. It has been reported that high-grade non-Hodgkin's lymphoma and Hodgkin's lymphoma
Figure 1: Clinical significance of NM23-H1 expression in hematologic malignancies. (a) Overexpression of NM23-H1 gene in hematologic malignancies [10, 20, 31]. Quantitative RT-PCR analysis on NM23-H1 mRNA in human bone marrow mononuclear cells (BM-MNC) in normal (n = 5), AML (n = 110), acute lymphoblastic leukemia (ALL, n = 9), and chronic myelogenous leukemia in the chronic phase (CML-CP, n = 9), CML in blast crisis (CML-BC, n = 7), myelodysplastic syndrome (MDS, n = 9), MDS overt leukemia (n = 5). The mRNA levels were normalized for gapdh mRNA. The positive control (the index = 100) is represented by RNA extracted from the human leukemia cell line (HEL). Mann-Whitney’s U test (versus normal), *P<0.05, **P<0.001. (b) Overall survival curves of patients with AML, according to NM23-H1 expression level [31]. High NM23-H1 (>100 index) patients (n = 32, solid line) had a worse prognosis than low NM23-H1 (≤100) patients (n = 71, broken line).

exhibited significantly higher levels of NM23-H1 expression than low-grade non-Hodgkin’s lymphoma [16–19]. NM23-H1 gene was overexpressed in AML cells, and a higher level of NM23-H1 expression was correlated with a poor prognosis in AML (Figure 1(b)) [10, 20, 21]. Multivariate analysis of putative prognostic factors revealed that elevated NM23-H1 mRNA levels significantly contributed to the prognosis of patients with AML [10]. NM23-H1 and NM23-H2 are highly expressed in normal CD34+ hematopoietic progenitors but are downregulated during normal hemopoietic maturation [22]. These genes are also downregulated during in vitro differentiation of AML cell lines [23, 24]. These findings suggest an important role for NM23-H1 and NM23-H2 in controlling hemopoietic differentiation and leukemic progression [22–24]. Taken together, these results indicate that the expression of NM23 is downregulated during hematoipoietic maturation, and its overexpression is found in many hematologic malignancies and predicts poor treatment outcome of patients with AML (Figure 1).

2. Clinical Significance of Extracellular NM23-H1 Protein in Hematologic Malignancies

NM23 has no secretion signal peptide but is nonetheless detected in conditioned medium of some tumor cell lines and in body fluids [14, 25–28]. The mechanisms by which NM23-H1 protein is secreted into the extracellular environment are unclear. Recently, Keller et al. [29] showed a new pathway for the secretion of many inflammatory response proteins without signal sequences. This unconventional secretion required the catalytic activity of caspase-1 and could rapidly release a wide variety of proteins involved in...
trigger detoxification, tissue repair, and cell survival. Furthermore, an exosome-associated export pathway of a number of proteins without signal sequences from the cells is reported [30]. It would be interesting to examine whether these new secretion pathways secrete NM23 protein; however, unlike secretion, it might be the release of NM23 protein by dying tumor cells overexpressing NM23.

We determined the serum level of NM23-H1 protein by enzyme-linked immunosorbent assay (ELISA) and assessed the association between this level and the clinical outcome of patients with hematologic malignancies [17, 25, 31, 32]. As shown in Figure 2(a), serum NM23-H1 levels were significantly higher in all of these hematologic malignancies used than in a normal control [31, 32]. The 102 patients with AML were divided into two groups with different serum NM23-H1 levels, to compare the overall survival of the two groups. All the patients with levels higher than 80 ng/mL died within 2 years. The 2-year survival rates for the high-NM23-H1 (≥80, n = 29) and low-NM23-H1 (<80, n = 73) groups were 0% and 33.3%, respectively (Figure 2(b)). The univariate analysis showed that unfavorable prognostic factors for overall survival were WBC count over 50,000/μL, LDH level over 5× normal, and NM23-H1 protein level over 80 ng/mL. Multivariate analysis using Cox's proportional hazard model showed that serum NM23-H1 level (≥80 ng/mL) was the strongest unfavorable factor, followed by WBC count and LDH. Thus, the elevated serum NM23-H1 levels significantly contributed to the prognosis of AML patients [32]. The 149 patients with aggressive (intermediate- and high-grade) non-Hodgkin's lymphoma were divided into groups with different NM23-H1 levels at a cutoff value of 80 ng/mL. The 3-year survival rates for the high (≥80, n = 28) and low NM23-H1 groups (<80, n = 121) were 6.7 and 76.4%, respectively (Figure 2(c)). These results suggest that an elevated serum NM23-H1 concentration predicts a poor outcome of aggressive non-Hodgkin's lymphoma [25]. The prognostic ability of serum NM23-H1 protein was confirmed by examining a number of patients with various types of malignant lymphoma in our study involving a different banks of patients are unclear. Very little information is available concerning extracellular expression and function although many studies have examined the expression of intracellular NM23 proteins; therefore, we focused on extracellular NM23-H1 protein derived from tumor cells, because its clinical significance is higher than that of intracellular overexpression [31], and the elevated extracellular expression of NM23-H1 has not been found in normal healthy plasma [25]. To demonstrate the clinical importance of extracellular NM23-H1 protein as a therapeutic target of patients with hematologic malignancies, we surveyed the biological functions of extracellular NM23-H1 protein. First, we investigated the extracellular functions of recombinant NM23 (rNH23) proteins on the survival and growth of normal and leukemic PBMNC and their association with the poor prognosis of AML patients.

3. Biological Functions of Extracellular NM23-H1 Protein

The mechanisms by which NM23-H1 protein is secreted into the extracellular environment and affects the outcome of patients are unclear. Very little information is available concerning extracellular expression and function although many studies have examined the expression of intracellular NM23 proteins; therefore, we focused on extracellular NM23-H1 protein derived from tumor cells, because its clinical significance is higher than that of intracellular overexpression [31], and the elevated extracellular expression of NM23-H1 has not been found in normal healthy plasma [25]. To demonstrate the clinical importance of extracellular NM23-H1 protein as a therapeutic target of patients with hematologic malignancies, we surveyed the biological functions of extracellular NM23-H1 protein. First, we investigated the extracellular functions of recombinant NM23 (rNH23) proteins on the survival and growth of normal and leukemic PBMNC and their association with the poor prognosis of AML patients.

3.1. Effect of Extracellular NM23 Protein on the In Vitro Growth/Survival of Primary Cultured AML Cells.

To investigate the potential pathological link between the elevated serum level of this protein and poor prognosis, we examined the extracellular functions of rNM23 protein on the in vitro growth/survival of primary cultured AML cells. rNM23-H1 protein promoted the in vitro growth/survival of AML cells at concentrations equivalent to the levels found in AML patients. This finding indicates a novel extracellular function of NM23-H1 and its potential link with poor prognosis (Figure 3). Both rNM23-H1 and rNM23-H2 promoted the growth/survival of AML cells; therefore, this activity of rNM23 is independent of H1/H2 isotypes. The mutant NM23-H1H1n protein, which does not have any NDPK activity, also promoted the growth/survival of AML cells. These results indicate that the activity of NM23 is independent of its NDPK activity [33]. How extracellular NM23-H1 protein promotes the growth/survival of AML cells remains unclear. We examined cytokine levels in the conditioned medium (CM) of AML cells treated with or without rNM23-H1, using cytokine antibody array and ELISA. Various cytokines and chemokines were detected in 48 h CM of AML cells growth/survival promoted by rNM23-H1 [33]. These cytokines included TNFα, IL-1β, IL-6, IL-8, IL-10, GM-CSF, and RANTES. The cytokine-inducing activity of rNM23-H1 was associated with its growth/survival-promoting activity for AML cells. Although the patterns of cytokine induction are different among cases, cytokines known as a growth factor for AML cells, such as GM-CSF and IL-1β, were induced in CM of NM23-sensitive cases but not NM23-unresponsive cases. Moreover, the induced-cytokine concentrations reached physiologically effective levels. To investigate the correlation between cytokine-inducing activity and the growth/survival-promoting activity of rNM23-H1, we tried to inhibit the cytokines using specific antibodies (anti-TNFα, anti-IL-1β, and anti-IL-6 antibodies). All these antibodies alone or some combinations tested partially
Figure 2: Clinical significance of extracellular NM23-H1 protein in hematologic malignancies. (a) Serum NM23-H1 protein levels in normal healthy control and hematologic malignancies [31]. Normal control ($n = 21$), AML ($n = 102$), ALL ($n = 6$), CML-CP ($n = 13$), CML-BC ($n = 9$), and MDS ($n = 6$). Mann-Whitney’s $U$ test versus control, $**P < 0.001$. (b) Overall survival curves of patients with AML [32]. High-NM23-H1 ($\geq 80$ ng/mL) patients ($n = 29$) had a worse prognosis than low-NM23-H1 (<80 ng/mL) patients ($n = 73$). (c) Overall survival curves of patients with intermediate and high-grade non-Hodgkin’s lymphoma [11, 25]. High-NM23-H1 (>80 ng/mL) patients ($n = 28$) had a worse prognosis than low-NM23-H1 (<80 ng/mL) patients ($n = 121$).

Figure 3: Extracellular function of NM23-H1 protein derived from tumor (AML) cells on primary cultured normal PBMC and AML cells. Extracellular NM23-H1 protein promotes the growth/survival of primary AML cells mediated by MAPK activation, STAT activation, and cytokine release [33]. On the other hand, extracellular NM23-H1 protein affects normal PBMC survival, activates monocytes, and induces cytokine production [34]. Some of these cytokines, especially GM-CSF and IL-1β, directly promote the survival/growth of primary cultured AML cells. Moreover, NM23-H1 induces immunosuppressive cytokine, such as IL-10. Therefore, the cytokine-inducing effect of extracellular NM23-H1 protein on normal PBMC may be associated with a poor prognosis in AML. Taken together, these observations suggest that extracellular NM23-H1 may play an important role in the malignant progression of leukemia and that inhibitors of extracellular NM23-H1 protein or inhibitors of extracellular functions of this protein should be evaluated for their treatment.
inhibited the rNM23-H1-induced growth/survival of AML cells. Anti-GM-CSF antibody also inhibited both GM-CSF-induced and rNM23-induced growth/survival of AML cells. These results suggest that the growth/survival-promoting activity of this protein may be partly mediated by the induction of these cytokines (Figure 3). We next investigated the NM23-induced-signal transduction pathways relating with cell proliferation, survival, and cytokine production, namely, MAPK (p38, Erk1/2, JNK), STAT, AKT/Pi3K, and NFkB in AML cells. Of these pathways, extracellular rNM23-H1 activated MAPK (p38, Erk1/2) and STAT, but not others [33]. STATs are known as key proteins playing roles as signal messenger and transcription factors that participate in normal cellular responses to cytokines. The constitutive activation of STAT3 has been reported to be associated with a wide variety of human malignancies containing AML. rNM23-H1 increased the total amount of STAT1 protein and the phosphorylation of Tyr and Ser. STAT3 was phosphorylated on Tyr705 in the absence of rNM23-H1, but Ser727 of STAT3 was also phosphorylated in the presence of rNM23-H1. Ser phosphorylation of STAT3 has been reported to be required for maximal transcriptional activity [37]. STAT5 (Tyr694) was also activated by rNM23-H1 [33]. The individual signaling pathways induced by rNM23-H1 protein were blocked in AML cells using specific pharmacological inhibitors, SB202190 and SKF86002 for p38 MAPK, PD98059 for extracellular signaling kinase (ERK, also known as MEK), and Curcumin for STAT3 [38]. These inhibitors suppressed the rNM23-induced growth/survival promotion of AML cells [33]. These findings indicate that the activity of rNM23-H1 is associated with the activation of the signaling pathways in AML cells [33]. MAPK/STAT activation by conventional growth factors takes only a few minutes; however, the activation of these signaling pathways by rNM23-H1 required a longer time than various mitogens [33, 39]; therefore, it might be an indirect activation rather than a direct activation by NM23 molecules. Taken together, these observations suggest that extracellular NM23-H1 may play an important role in the malignant progression of leukemia, and the inhibitors of extracellular NM23-H1 protein or inhibitors for the signaling pathways activated by extracellular NM23-H1 should be evaluated for their potential treatment as an approach to these malignancies (Figure 3).

Lilly and his colleagues reported heterogeneity in the ability of AML samples to bind and respond to extracellular NM23-H1 [40]. The authors offered evidence that binding was essential for support survival. Although rNM23-H1 promoted the survival of the most primitive blasts within responding AMLs, it was not these cells that actually bound the protein. Instead, rNM23-H1 bound to more mature CD34low/CD34+ and CD11b+ cells, showing an indirect survival benefit of rNM23-H1 on primitive blasts. Collectively, these results show that NM23-H1 preferentially binds to the more mature cells of the AML clone that are CD34low/CD11b+. However, the survival of both the more mature (CD34low/wCD11b+) and immature (CD34+/CD11b−) cells is enhanced. In support of this finding, the survival of purified blast cells was enhanced by conditioned medium (CM) of more mature cells from the clone that had been stimulated by rNm23-H1. Levels of IL-1β and IL-6 in rNM23-H1 CM mirrored the potency of the CM to promote blast cell survival. These data indicate that NM23-H1 indirect survival affects the CD34+/CD11b− cells, by inducing supportive cytokine release from the more mature CD34low/CD11b+ cells. The authors offered the first evidence of novel crosstalk between cell populations within the tumor (AML) clone. Moreover, these findings have implications for the role of NM23-H1 in AML and its use as a prognostic marker, well coinciding with our results (Figure 3).

3.2. Effect of Extracellular NM23 Protein on the In Vitro Growth/Survival of Primary Cultured Normal Peripheral Blood Mononuclear Cells (PBMNCs).

We examined the extracellular effects of rNM23-H1 protein on the in vitro survival of primary cultured normal PBMNC. rNM23-H1 inhibited the survival of PBMNC at concentrations equivalent to the levels found in the serum of AML patients [34]. The rNM23 responsible adherent cells were CD68-positive and nonspecific-esterase- (NSE-) positive monocytes. On the other hand, rNM23 did not inhibit, rather slightly stimulated, the survival of nonadherent PBMNC (mainly CD3-positive lymphocytes). These results indicate that extracellular rNM23-H1 protein affects the in vitro survival of normal immune cells, such as monocytes. The inhibitory effect of this protein on normal monocytes may be associated with the poor prognosis of hematologic malignancies, since monocytes/macrophages also play a crucial role in the immune system.

Moreover, rNM23-H1 protein promoted the production of various cytokines and chemokines, including proinflammatory cytokines in normal PBMNC, especially in monocytes [34]. By using a human cytokine array system, we analyzed NM23-regulated cytokines in CM of PBMNC treated with rNM23-H1. Cytokine arrays showed that the expressions of inflammatory cytokines (IL-1β, IL-6, IL-8, GM-CSF, and MCP-1) were significantly induced in CM of rNM23-H1-treated cells. It has been known that inflammation is an important component of the tumor microenvironment although the mechanisms through which immune cells might promote tumorigenesis are unclear [41]. Some cytokines induced by rNM23-H1 such as GM-CSF and IL-1β, practically and directly promoted the growth/survival of primary cultured AML cells (Figure 3). The cytokine arrays also showed that rNM23-H1 enhances the production of MCP-1 and IL-10 by normal PBMNC [34]. It has been described that MCP-1 increases recruitment of tumor-associated macrophages (TAMs), leading to a higher degree of angiogenesis [42]. TAMs generally express an M2-like phenotype [43], which is characterized by high IL-10 expression and low tumoricidal activity and promotes tissue remodeling and angiogenesis [41]. Buxton et al. reported the angiogenesis-promoting activity of extracellular NM23 protein in breast cancer [44]. In most human tumors, TAM infiltration is associated with poor prognosis, as seen in Hodgkin’s disease [45]. Collectively, these results indicate that NM23 protein in extracellular environment activates...
monocytes and induces the tumor-promoting inflammatory cytokines and the immunosuppressive cytokines in normal PBMCN. These results also show that extracellular NM23-H1 could offer tumor cells an environmental condition convenient for their growth/survival through the cytokine production of normal PBMCN, which in turn might contribute to the poor outcome of patients with elevated serum levels of NM23-H1 protein (Figure 3).

Although rNM23-H1 induced various cytokines in both normal and leukemic PBMCNs, it promoted only the growth/survival of AML cells but not normal PBMCNs and rather stimulated its apoptosis. Moreover, rNM23-H1 protein did not have any effects on the growth/survival of normal endothelial cells (HUVECs) and various tumor cell lines (leukemia, lymphoma, neuroblastoma, and lung) [34]. NM23 proteins promoted the induction of various cytokines in the normal monocytes but not in monocytic leukemia cell lines (THP-1 and U937). Therefore, the survival-inhibiting activity of rNM23 might be specific for normal monocytes. These results indicated that rNM23 induced TAM-like functions but did not increase the growth and survival of normal monocytes, in contrast to AML cells. Taken together, these observations suggest that extracellular NM23-H1 may play an important role in the malignant progression of leukemia through normal monocytes (Figure 3).

3.3. Effects of Extracellular NM23 Protein on the In Vitro Growth/Survival and Differentiation of Other Hematopoietic Cells. Willems et al. [22] previously reported that NDPK/NM23 was present in normal human plasma and that NDPK activity correlated with hemoglobin levels, indicating the presence of NM23 in plasma as a consequence of red blood cell lysis. Moreover, they reported a modulating effect of extracellular NM23 proteins on the terminal stages (CD34+CD38+ progenitor cells) of normal hematopoietic differentiation [46]. More erythroid burst-forming units (BFU-E) and fewer macrophage colonies (CFU-M) were observed in cultures containing any of the NM23 isoforms examined, even the enzymatically inactive H118N mutant of NM23-H1. They suggest that fairly high concentrations of NM23 constitutively present in plasma/serum could have a physiologic role in supporting erythropoiesis and inhibiting excessive macrophage formation. It is interesting that extracellular NM23 serves as an alarm molecule for informing on red blood cell lysis and as a supporting molecule for normal erythropoiesis. We also reported that extracellular NM23 could inhibit the erythroid differentiation of human leukemia cell lines (K562, HEL, and KU812) without any effect on proliferation [47] and that serum NM23-H1 levels in AML-M6 (acute erythroleukemia classified by FAB) were especially high and significantly higher than that in other FAB subtypes of AML [32]. The elevated levels of extracellular NM23 in AML-M6 might function as proliferation-supporting molecules of erythroblast leukemia cells as in normal erythropoiesis. In contrast to erythropoiesis, extracellular NM23-H1 seems to be inhibitory to the growth/survival of normal monocyte lineage cells [46]; however, it could promote the growth/survival of primary cultured AML-M5 (acute monocytic leukemia classified by FAB classification) cells [33]. Serum NM23-H1 levels in AML-M5 were higher than that in the other FAB subtypes of AML except AML-M6 [32]. Taken together, these results suggest that an elevated serum level of NM23-H1 protein in AML affects the biological properties of normal hematopoietic cells and leukemia cells of specific lineages and specific differentiation stages and causes poor prognosis.

4. Extracellular NM23-H1 Protein as a Potential Prognostic and Therapeutic Target for AML

Recent advances in genome technologies and the ensuing outpouring genomic information-related cancer have accelerated the conversion from a genome discovery into a tangible clinical endpoint. Successful examples of translating cancer genomics into therapeutics and diagnostics show the importance of establishing the biological relevance of a cancer genomic discovery in realizing its clinical potential [48]. NM23-H1 plays complex roles in the development of diverse cancers including carcinoma, high-grade lymphomas, and AML. As has been mentioned, in the case of AML and lymphomas, serum NM23-H1 protein is elevated with highest levels correlating with poorest prognosis. Moreover, the data of Lilly and colleagues [40] and our recent studies [33, 34] strongly indicate that extracellular NM23-H1 can act as a tumor-derived growth/survival factor in AML (Figure 3). These findings suggest an important biological role of extracellular NM23-H1 in the malignant progression/poor prognosis of leukemia and a potential therapeutic target for these malignancies.

As shown in Figure 3, extracellular NM23-H1 derived from tumor (AML) cells generates a supportive microenvironment convenient for their growth/survival of primary AML cell through cytokine production of AML cells and normal PBMCN; therefore, the reduction of extracellular NM23-H1 protein concentration or inhibitors of its action should be evaluated for therapeutic potential to combat these malignancies. Although it might be a useful technique to reduce the serum level of this protein using therapeutic filtration devices such as Hemopurifier that selectively target the removal of immunosuppressive proteins from the entire circulatory system [49], it will be very important to reveal the signal transduction pathways induced by extracellular NM23-H1 protein to increase growth/survival of AML cells.

4.1. NM23 Receptors. Lilly et al. [40] recently reported the heterogeneity in the ability of AML samples to bind and respond to extracellular NM23-H1 and offered evidence that binding was essential for improving survival. These data imply that some AMLs express an NM23-H1 receptor whereas others do not. It is interesting to examine the receptor molecules for extracellular NM23-H1 on AML cells. Recent evidence suggests that NM23-H1 can bind to a cleaved form of Mucin1 called MUC1*, which is present on the surface of many cancer cell lines and on pluripotent stem cells [50, 51]. The binding of NM23-H1 to MUC1* was reported to result in dimerization of MUC1*, and subsequent activation of the MAPK pathway.
to increase proliferation of the breast cancer cell line T47D [50]. Although these studies indicate that MUC1* can act as a receptor for NM23-H1, Lilly et al. [40] showed that cell surface binding to AML cells is independent of MUC1*, and therefore an alternative receptor must be present on these cells. Further studies should investigate the relationship between NM23-H1 binding and responses to AML therapies and aim to determine the nature of the NM23-H1 receptor in AML, which may provide a novel target for adjunctive therapies.

Apetoh et al. [52] identified Toll-like receptor 4 (TLR4) ligand, high-mobility group box 1 (HMGB1) alarmin protein from dying tumor cells. This indicated that the molecule from tumor cells elicits an immune response involving the induction of inflammatory cytokines in a TLR4-dependent fashion. Reportedly, a number of endogenous proteins bind and stimulate TLR4: heat-shock protein (HSP) 60, HSP70, oxidized LDL, surfactant protein A, hyaluronan breakdown product, fibronectin, and β-defensin-2 [53]. The mechanism by which extracellular NM23-H1 protein induces various inflammatory cytokines in normal monocytes and AML cells (Figure 3) is unknown. It will be interesting to determine whether extracellular NM23 binds and stimulates TLR4-like HMGB1.

4.2. Small Molecules Regulating NM23 Functions. The downstream signaling pathway induced by the extracellular NM23-H1 also would be a potential therapeutic target for AML. We previously reported that inhibitors of MAPK/STAT3 activity suppressed the NM23-induced growth/survival of AML cells [33]. Various inhibitors are now under development, since MAPK and STAT3 signaling activations and tumor-induced inflammatory conditions are widely observed in malignant progression [54–57]. These agents might have potential to improve treatment for AML patients with a poor treatment outcome predicted by measuring serum levels of NM23-H1.

NM23 has a large number of functions, including NDPK activity [1, 58, 59], a transcription factor PuF for the c-Myc promoter [60], and protein kinase activity [61, 62]. Coincident with these biological characterizations, the NM23 proteins are postulated to participate in multiple biochemical activities and associations. However, it is unknown how one small molecule like NM23 exerts these many functions. Nm23-H1 and H2 form homo- and heterohexamers. It may allow the formation of a variety of isoenzymes with subtly different functions. Moreover, NM23 proteins have been reported to be associated with other proteins; transcription factors such as the retinoic acid receptor-related orphan receptor α and the retinoic Z receptor β [63], the heat shock protein Hsc70 [64], telomeres [65], Epstein-Barr virus (EBV) nuclear protein EBNA-3C [66], prune [67], and a low-molecular-weight GTPase Rad [68, 69]. These protein:protein interactions involving NM23 protein might light on studies of the multifunction observed in NM23 protein. Although we have not yet had any evidence showing NM23-interacting proteins in extracellular conditions, development of small molecules, which can modify the protein:protein interactions, may provide a novel therapeutic tool to target malignant AML.

EBV is an oncogenic virus associated with a number of human malignancies including Burkitt lymphoma and lymphoproliferative disease. A subset of latent EBV antigens is required for mediating immortalization of primary B-lymphocytes. EBNA-3C is one of the six latent proteins essential for EBV transformation of B-lymphocytes and interacts specifically with the NM23-H1 protein. Moreover, EBNA-3C reverses the ability of NM23-H1 to inhibit migration of Burkitt lymphoma and breast carcinoma cells. Therefore, the NM23-H1 is identified as a cellular target for EBNA-3C. NM23-H1 is predominantly localized in the cytoplasm in B-lymphocytes, while NM23-H1 is predominantly nuclear and colocalized with EBNA-3C in EBV-transformed B-lymphoblastoid cell lines and in B cells transfected with EBNA-3C [66]. These results indicate that EBV may influence the subcellular localization and function of NM23-H1 in infected cells. These results also suggest that it would be important to examine the subcellular localization of NM23-H1 protein overexpressed in leukemia and lymphoma. Zhu et al. have reported an interesting small molecule, named stauprimide, that increases the efficiency of the directed differentiation of mouse and human embryonic stem cells (ESCs) in synergy with defined extracellular signaling cues, such as activin A [70]. Using an affinity-based approach, NM23-H2 was identified as the biological target of stauprimide. By binding to NM23-H2, stauprimide inhibits NM23-H2 nuclear localization, which, in turn, represses c-Myc expression, because NM23-H2 in nucleus functions as a transcription factor for c-Myc [71]. This study points to an important role for stauprimide in modulating the subcellular localization and function of NM23-H2. The amount of intracellular NM23-H1 and NM23-H2 is inversely correlated with differentiation, and NM23 overexpression is considered to function as a differentiation suppressor in AML cells. Therefore, the suppression of the NM23’s function by stauprimide should be evaluated for differentiation inducing therapy for AML.

Expression of the human isoforms, NM23-H1 and NM23-H2, is thought to be inversely associated with metastatic potential of a variety of cancers [72–74]. While NM23-H1 is strongly associated with metastasis mechanisms in many tumors, NM23-H2 is not [75–77]. The products of these two genes, NDPK-A and NDPK-B, were named for their function as nucleoside diphosphatase (NDP) kinases. NDPK-B is elaborated into the extracellular environment by the breast carcinoma cell line MDA-MB-435s as well as other cells derived from solid tumors such as colon, lung, and prostate [27]. The presence of NDP kinase activity on the surface and external environment of cancer cells that exist in the milieu of apoptosis and necrosis provides effective mechanism for regenerating extracellular purines. Buxton et al. have shown an attractive evidence to support secreted extracellular NM23-H2’s (sNDPK-B) putative role in promoting metastasis [44]. The authors have provided evidence for a nucleotide-dependent regulation of angiogenesis by breast cancer of secreted extracellular NM23-H2 [27, 44, 78–80]. This can be mechanistically
related to extracellular nucleotide elevation and subsequent activation of nucleotide receptors to regulate cancer growth and tumor angiogenesis [44]. These findings show that pathologically secreted NM23-H2 and its regulation of extracellular nucleotides utilize P2Y receptors to stimulate angiogenesis [44, 78]. These results also represent new therapeutic targets for antiangiogenic therapies to benefit patients. Furthermore, the authors have shown a number of inhibitors for extracellular NDPK-B activity [80]. Catechin gallates (EGCG, ECG), theaflavins, and ellagic acid (EA) are shown to inhibit NDPK-B completely with the rank order of potency: EA > theaflavins > EGCG > ECG. These compounds, are known to suppress cancer cell proliferation, inhibit invasion into Matrigel, and inhibit angiogenesis [78, 80]. EA is a potent NDPK-B inhibitor that may potentiate the suppression of metastasis and thus may be useful agents to use in conjunction with traditional chemotherapy or angiogenesis inhibitors such as bevacizumab (Avastin). Using EA, we tried to inhibit the function of NM23/NDPK as a differentiation suppressor in AML cells for increasing the efficiency of the directed differentiation of AML cells in synergy with all-trans-retinoic acid (ATRA). EA enhanced the ATRA-induced differentiation and the apoptosis of human acute promyelocytic leukemia cell lines [81]. These results might have implications for the incorporation of anti-NM23 agents such as EA into therapeutic intervention against leukemia and possibly other hematologic malignancies overexpressing NM23.

In conclusion, NM23 has a large number of biological functions including growth/survival-promoting activity for AML cells. Inhibitors of NM23 expression and its actions might hold promise for the treatment of AML. Especially extracellular NM23-H1 represents an important role in the malignant progression of leukemia. Therefore, its functional inhibitors and its downstream inhibitors for signaling pathways activated by extracellular NM23-H1 should be evaluated for their potential treatment as an approach to these malignancies.

References


Review Article

Gab Adapter Proteins as Therapeutic Targets for Hematologic Disease

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The Grb-2 associated binder (Gab) family of scaffolding/adaptor/docking proteins is a group of three molecules with significant roles in cytokine receptor signaling. Gabs possess structural motifs for phosphorylation-dependent receptor recruitment, Grb2 binding, and activation of downstream signaling pathways through p85 and SHP-2. In addition, Gabs participate in hematopoiesis and regulation of immune response which can be aberrantly activated in cancer and inflammation. The multifunctionality of Gab adapters might suggest that they would be too difficult to consider as candidates for “targeted” therapy. However, the one drug/one target approach is giving way to the concept of one drug/multiple target approach since few cancers are addicted to a single signaling molecule for survival and combination drug therapies can be problematic. In this paper, we cover recent findings on Gab multi-functionality, binding partners, and their role in hematological malignancy and examine the concept of Gab-targeted therapy.

1. Discovery and Similarities of Gab Family Members

The Gab proteins, Gab1, Gab2, and Gab3, comprise a family of scaffolding/docking molecules involved in multiple signaling pathways mediated by receptor tyrosine kinases (RTKs) and non-RTK receptors. Gab proteins integrate and amplify signals from a wide variety of sources including growth factor, cytokine, and antigen receptors, as well as cell adhesion molecules. They are subject to complex regulation by feedforward and feedback phosphorylation events as well as protein-protein interactions. Gab proteins range from 50 to 100 kDa in size [1] and were originally identified as the mammalian homologs of the daughter of sevenless (DOS) Drosophila adapter proteins [2, 3]. They also display sequence similarity to Suppressor of Clear 1 (Soc1), which was identified by genetic screen in C. elegans [3, 4].

Gab1 was originally identified as a binding protein for Grb-2 [5], and Gab2 was isolated by the purification of a binding partner for SHP [6]. The discovery of Gab3 was aided by a large sequencing project, and its isolation was based on sequence similarities to Gab1 and Gab2 [7]. Very recent entries at both the genomic DNA and transcript level have been recorded for Gab4 gene in both humans and chimpanzees, but this gene is not present in mice. The human Gab4 gene is located on chromosome 22q11.1 and its nucleotide sequence is most related to Gab2 [8].

The overall sequence homology between Gab family members is about 40–50%. All Gab proteins share a similar modular structure, including a Pleckstrin homology (PH) domain at their N-terminus, proline-rich regions in the central part, and multiple phosphorylated tyrosine residues (Figure 1). The PH domain is an approximately 100 amino acid domain that binds phosphoinositides. Gab2 binds preferentially to the PI-3K product phosphatidylinositol-3,4,5-trisphosphate (PIP3), which is only found within the plasma membrane [9]. The PH domain mediates recruitment of Gab2 to phagocytic cups induced by FcyRI and is required for fibroblast growth factor-induced tyrosine phosphorylation of this docking protein. The PH domain might play an important role to localize or to concentrate Gab proteins to membrane areas where receptors are activated [2].
The phosphotyrosine domains and the proline-rich sequences (PXXP) are potential binding sites for Src homology 2 (SH2) and SH3 domains, respectively. The positions of the SH2-binding tyrosine-based motifs and the SH3-binding proline-rich sequence are conserved among the Gab proteins. These are one of the most prominent motifs in signaling molecules due to their relevance in binding and “docking” phosphorylated tyrosine residues or directing protein-protein interaction [10, 11].

These adapter proteins serve important roles in cytokine receptor signaling by acting as a scaffold and coordinating interactions between signaling intermediates. Multiple protein binding motifs are present in many of the adapter molecules leading to multimeric complexes that may also include proteins such as CrkL [12], PLC-γ [13], SHIP [14], SHP-2 [15], STAT3 [16], and STAT5 [17].

2. Involvement in Normal and Leukemic Signaling Pathways

As mentioned above, one of the fundamental mechanisms for regulation of Gab-mediated signal transduction is site-specific tyrosine phosphorylation of these proteins. These molecules are involved in the phosphatidylinositol-3 kinase (PI3-K) and mitogen-associated protein kinase (MAPK) pathways and include multiple protein binding sites [18]. To further elaborate, Figure 2 illustrates how Gab2 is involved in PI3-K and MAPK pathways. These proteins are tyrosine phosphorylated following cytokine stimulation which enables interaction with a large number of partners. Table 1 summarizes a few key receptors which are associated with hematopoiesis, some of which are also found mutated in association with hematologic malignancy, Serine phosphorylation of Gabbs by downstream effectors also has been described [19, 20], which will be discussed later.

The significance of the phosphorylation sites present on Gabbs not only lies in aiding its interaction with crucial binding partners, but also on how it influences activation of downstream cytokine receptors. Interaction between the protein tyrosine phosphatase Shp2 and Gab2 regulates MAPK pathway activation. Notably, mast cells and macrophages from Gab2−/− mice have decreased Erk activation in response to SCF [27]. Gab2-Shp2 complex also appears to have an additional, distinct signaling role in response to other stimuli. For example, overexpression of Gab2Y604F/Y633F mutant fails to bind to Shp2 and blocks IL-3-evoked gene activation [6]. Further, the Gab/Shp2 complex also positively regulates other downstream pathways. These include c-Kit-induced Rac activation [28], where Gab2 via Shp-2 transmitted signals from Kit receptor (Tyr-567) to activate the Rac/JNK pathway. This in turn is significant for mast cell development [28]. Another example of downstream regulation is the Gab2-mediated PI3K activation wherein, this activation is necessary for epidermal growth factor (EGF)-induced DNA synthesis in rat hepatocytes [58, 59]. It is noteworthy that persistent activation of c-Kit and c-Mpl induces hematological malignancies. Activation of PI-3K by c-Kit is dependent both on the direct PI3K-binding site in c-Kit and on the phosphorylation of Gab2 [54]. The fact that c-Kit has been found mutated in numerous human malignancies, including acute myeloid leukemia, and that Gab2 is often overexpressed in acute myeloid leukemia suggests a potential role of Gab2-mediated PI3K activation in transformation [60]. TPO-induced stimulation of c-Mpl has been implicated in maintaining HSC quiescence and also in myeloproliferative disorders (MPDs) and Gabbs play a role in regulating PI-3K and MAPK pathways, in c-Mpl/TPO signaling [55, 61].

3. Functional Role in Hematopoiesis Defined by Knockout Mice

Given their integral role in cytokine signaling, it was proposed that Gabbs may play important roles in hematopoiesis. However, to date, very little is known about how multiple Gabbs regulate hematopoietic cytokine signaling.

Gab1 deficiency results in embryonic lethality due to developmental defects in heart, placenta, skin, and a reduced ratio of liver to body weight at E14.5 [62, 63]. Also associated with these defects was reduced Erk activation in embryonic fibroblasts in response to stimulation with platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF). These defects were initially observed to be similar to mice lacking expression of MET receptor, HGF, PDGF, and EGF growth factors with phenotypes such as open eye lids [64], abnormal hair follicles [65], hemorrhage and cardiac hypoplasia [66], and abnormal placenta [67–69]. Later generation of SHP-2 mutant mice revealed yet again similar defects [70] indicating an essential central role for Gab1/SHP-2 interactions in mediating growth factor activation of the Erk MAP kinase pathway. More recent conditional deletion of Gab1 led to deficient Erk signaling which allowed increased insulin receptor substrate
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Figure 2: Gab2 interactions with binding partners. Diagram shows generic Gab adapter docked at the plasma membrane by the PH domain. Interactions with p85 and SHP-2 are involved in Akt and Erk activation respectively. Binding of receptor tyrosine kinases to their receptive ligands triggers the kinase activity of the cytoplasmic domain of the receptor. The receptor becomes phosphorylated on tyrosine residues. Docking proteins such as Grb2 contain SH2 domains that bind to the phosphotyrosine residues of the activated receptor. Grb2-Gab interacts through the SH3 domains and activates downstream signaling pathways, PI-3K/Akt and SHP2/Erk. Grb-2 can bind to SOS via N-terminal SH3 domain while the C-terminal SH3 domains are used for its interaction with Gab proteins. SOS-1 has been known to associate with Grb2, leading to its autophosphorylation. This complex gets translocated to the activated receptor where it then associates with Ras. As Ras gets activated it induces the downstream ERK/MAPK pathway. Alternatively, stimuli from growth factors like EGF, VEGF, and so forth, causes binding of Gab2 to Grb2. This then leads to recruitment of SHP2, that binds to phosphorylated tyrosine residues on Gab, and in turn activates ERK/MAPK signaling. Negative feedback by serine phosphorylation of Gab at S160 by Akt and S623 by Erk (block arrows) plays an important role in control of function and signaling of Gabs.

Gab2 is tyrosine phosphorylated at any of 19 sites by several early-acting cytokine receptors such as Flt3, c-Kit, IL-3R, and c-Mpl. Gab2 contains SH2 domain binding sites and 5 PxxP sites (polyProline sites) that bind SH3 domain [6, 18, 29]. Consistent with binding of p85 and SHP-2, Gab2 activates the PI3-K and the MAPK pathways, respectively, which may participate in regulating hematopoietic cell migration functions [48]. Gab2−/− mice are viable but lack allergic response [72] since Gab2 deficiency has also been shown to alter mast cell development [27] in a manner similar to STAT5-deficient mice [73]. Use of point-mutants [36, 48, 74], that deter Gab2 binding to signaling partners, could help to dissect the structure-function relationship of Gab2 in vivo. A recent study has established Gab2 ΔSHP2 (Y603F/Y632F) and Gab2 ΔPI3K (Y441F/Y465F/Y573F) mutant knockin mice [75], with Gab2 mutated at SHP-2 and PI3K binding sites, respectively. Further assessment of these mice has shown that the PI3K or SHP2 binding sites in Gab2 are important for mast cell degranulation and the anaphylaxis response.

Despite the normal appearance, normal BM cellularity, and normal blood counts of mice lacking Gab2 expression, Gab2−/− mice show reduced colony forming ability in methylcellulose and impaired KLS (c-Kit+ Lin− Sca-1+ cell surface markers denoting mouse hematopoietic stem cell) cell growth in liquid culture [76]. The defects of these cells in response to early-acting cytokines like SCF, TPO, and IL-3 suggest that Gab2 may act as an intermediate relay protein that organizes signaling complexes and amplifies receptor activation. Owing to these findings, Gab2 can be a potential target molecule for better understanding steady-state and aberrant hematology.
Table 1: Gab proteins are associated with multiple common hematopoietic receptors, RTK: receptor tyrosine kinase.

<table>
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<td>GM-CSF receptor</td>
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<td>FDFms, BaF3, UT-7</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>gp130</td>
<td>IL-6, LIF</td>
<td>HepG2, BAF-B03, T47D, MM.1S, cardiomyocytes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-15 receptor</td>
<td>IL-15</td>
<td>T cells, MyLa2059</td>
<td>NR</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-2 receptor</td>
<td>IL-2</td>
<td>Kit225, KT-3, T-cell blasts, T cells, NK3.3, MyLa2059</td>
<td>NR</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-3 receptor</td>
<td>IL-3</td>
<td>BaF3, BAF-B03, primary hematopoietic cells, NIH 3T3</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Mpl</td>
<td>TPO</td>
<td>TF-1, UT-7, BaF3, primary megakaryocyte progenitors</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

NR: not reported.

In contrast to Gab1 and Gab2 which have ubiquitous expression in brain, kidney, lung, heart, and ovary [5, 6, 9], Gab3 is localized to hematopoietic tissues [7, 77]. An additional difference between Gab3 and Gab1/2 is that it may not be able to interact with Csk or Crkl but has unique potential binding sites that have yet to be characterized [7]. Gab3 SH3 domains have been shown to associate with a number of Src family kinases including Src, Fyn, and Lyn [7]. Increased Gab3 expression is observed following M-CSF stimulation of myeloid and macrophage cell lines [7], and differentiation is facilitated by overexpression. However, Gab3 knockout mice do not have obvious hematopoietic phenotypes [78] and have normal macrophage numbers.

4. Supporting Role for Gabs in Cancer Progression

It is well established that Gab proteins promote tumorgenesis by functioning as “accomplices” of certain oncoproteins or by amplifying signaling upon their overexpression. This type of “nononcogene addiction” has been described for molecules that become essential in the setting of cancer, but they are not mutated or capable of transformation on their own. In addition to normal cytokine activation, Gab1 and Gab2 can also be activated by oncogenic tyrosine kinases, oncoproteins, and Src family kinases (summarized in Table 2). Gab3 has not yet been described to play a role in cancer signaling.

The study of Gab1 in Met signaling and cancer has been researched in recent years. Overexpression of Gab1 promotes cell cycle progression when Met is expressed at suboptimal levels. For this response, it is required for Gab1 to possess an intact Met-binding motif, the PH domain, and the binding sites for PI3-K and SHP-2. In this model, Gab1 sufficiently promoted transformation and proliferation of fibroblasts [93]. A role for Gab1/Shp-2 interaction in growth and transformation of NIH 3T3 fibroblasts has also been reported [94], although this has not yet been validated in vivo for disease induction. It was shown in recent studies that Gab1 expression increased cell motility and adhesion of myeloid 32D cells in a hepatocyte growth factor (HGF) stimulated setting. In this setting, Gab1 was also seen to up

Table 2: Gab1 and Gab2 are activated by kinases, oncoproteins, and other adaptors in cancer cells.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Gab activator</th>
<th>Gab1</th>
<th>Gab2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast [79–83]</td>
<td>Amplification, ErbB2, Src</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Glioblastoma [84]</td>
<td>Met</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Thyroid [85]</td>
<td>Ret</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Gastric [86]</td>
<td>Amplification</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Myeloma [40]</td>
<td>Hck</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Chronic myelogenous leukemia</td>
<td>Bcr/Abl</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Friend virus erythroblastosis</td>
<td>Sf-Stk</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Anaplastic large cell lymphoma [89]</td>
<td>NPM-Alk</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Acute myeloid leukemia [90, 91]</td>
<td>Flt3-ITD</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Myeloproliferative disease [92]</td>
<td>JAK2V617F</td>
<td>?</td>
<td>Yes</td>
</tr>
</tbody>
</table>

In vivo is marked as [94].
regulate GATA-2, which has been implicated in CML and could be a key player in malignant transformation [95].

Involvement of Gab2 in leukemogenesis was highlighted when myeloid progenitors from Gab2-deficient mice were found to be resistant to transformation by Bcr-Abl [87]. Phosphorylation of Y177 within the Bcr moiety leads to recruitment of the Grb2/Gab2 complex and triggers downstream signaling via SHP2 and PI-3K, which is crucial for enhanced proliferation and survival. Similarly, the oncogenic Bcr-FGFR1 fusion protein, which is also the product of a chromosomal translocation, drives the tyrosine phosphorylation of Gab2 in murine bone marrow cells and their malignant transformation through phospho-Y177 mediated Grb2 association [96]. Another kinase implicated as a key component of the Bcr-Abl signaling network is Jak2 that in turn activates Lyn leading to Gab2 phosphorylation. These findings highlight the role of Gab2 phosphorylation in driving chronic myeloid leukemia (CML) [97]. After the pivotal role of Gab2 in Bcr-Abl-mediated transformation had been established, its involvement in the pathogenesis of several other leukemias was discovered. The oncogenic fusion kinases Tel-Abl and Tel-Jak2 engage Gab2 in a similar manner to Bcr-Abl [98, 99]. Likewise, it was seen that introduction of a germline gain-of-function SHP2 mutation, D61G/+ , induced MPD by aberrant activation of STAT5 that introduction of a germline gain-of-function SHP2 mutation, D61G/+ , induced MPD by aberrant activation of STAT5 is highly complex and difficult to discern [21, 90, 91, 100]. Both Erk and STAT5 can drive myeloproliferation, and the degree to which they cooperate in normal and leukemic hematopoiesis is not well defined.

It should be pointed out that although the focus of this review is on Gabbs as interaction partners of oncogenes involved in the transformation of hematopoietic cells, the most thoroughly described roles have been in solid tumors. Gab2 amplification has been seen in nonhematopoietic cancers such as breast cancer cell lines [79]. Gab2-mediated activation of the Shp2/Erk signaling pathway is important for the proliferation of mammary cells. Amplification of Gab2-containing region has been reported in 10–15% of human breast tumors [80]. Further supporting this is the in vitro study whereby overexpression of Gab2 in human MCF-10A cells using a retroviral vector approach [81] gave similar results. Furthermore, deletion of Gab2 delays migration of mouse mammary tumors generated using breast cancer cell lines and this defect can be fully restored by reintroduction of a plasmid expressing Gab2 [82]. A particular role for Gab2 and Akt activity has been shown following E2F1 hyperactivation in p27-deficient cells leading to enhanced cell migration and invasion [101]. A recent study in MCF-10A epithelial cells show Gab2 overexpression enhances cell migration and reduces formation of epithelial colonies. Further, modulation of focal adhesions by Gab2 was dependent on Shp2 binding sites. Shp2 binding defect mutant restored normal cell spreading. In contrast, the Shp2 affinity mutant promoted Vav2 phosphorylation and recruitment of some important RhoA family regulators leading to increased cell motility [80, 102, 103].

5. Regulation by Posttranslational Modifications

The key to figuring out the role of Gabs in hematological disease is to understand their role in signaling cascades. It is crucial to visualize these as intertwined loops. Firstly, phosphorylation of a particular residue might affect the phosphorylation of a nearby residue in either a positive or antagonistic fashion, due to phosphorylation-induced changes in protein conformation. Secondly, phosphorylation-induced conformational changes may alter the accessibility of key regions, such as the PH domain.

Negative feedback regulation of Gab2 can be achieved by serine phosphorylation at sites near the PH domain (Akt) or near the SHP-2 binding domain (Erk). Akt can constitutively associate with Gab2, phosphorylate it on a consensus phosphorylation site (Ser159) and inhibit Gab2 tyrosine phosphorylation [20]. Mutation of S159A (corresponding mutation in mouse is S160) resulted in increased tyrosine phosphorylation of Gab2 and the Gab2S159A mutant displayed transforming properties in fibroblasts and prolonged signaling through the PI-3K/Akt pathway. This might impact downstream STAT5 activity, as it has been shown that constitutively active STAT5 forms a complex with the p85 subunit of the PI3-K and Gab2 in leukemic bone marrow cells, resulting in the activation of Akt [17]. Gab2 is also regulated by Erk-mediated negative feedback phosphorylation, wherein the identified S623 is the site of phosphorylation of Gab2 by Erk. The Gab2/Shp2 interaction is enhanced by S623A mutation. This is in turn expected to strongly inhibit downstream STAT5 [19]. Thus, it will be of relevance to study the balance between positive and negative signaling through both in vitro models and further complement it by in vivo analysis. Negative feedback is deemed critical in shifting Gab2 signaling from Erk and Akt to STAT5. This mechanism may be particularly relevant during conditions of hematopoietic stress such as recovery from myelosuppression or leukemic hematopoiesis. These conditions are more likely to increase Gab2-mediated Erk and Akt signaling, while inhibiting STAT5, whereas blockage or complete deficiency of Gab2 might be expected to have a reverse effect (Figure 3).

6. Targeted Therapy of Gabs?

Since Gabbs bind many common receptors and are much less studied in hematopoiesis, it is pivotal to understand the role of binding to their partners and how it impacts oncogenic tyrosine kinase signaling. Due to the fact that all three family members are potential players in various signaling pathways, it will be interesting to see the shift in approach for targeting the Gab family. As discussed earlier, total knockdown of these proteins has varied physiological and phenotypic impacts. But, it cannot be emphasized enough that the Gab
family members are also key regulators/enhancers of other oncogenic proteins and could compensate for the deficit of a family member.

Synthetic peptides that specifically bind to directed targets offer an approach for the modulation of receptor signaling and subsequent gene expression. Stabilized α-helix, SAH, is a stapled peptide to p53 that has been shown to prevent p53-MDM4 binding, enabling activation of p53 response and tumor growth suppression both in vitro and in vivo models [104]. Recently, engineered photoreactive stapled BH3 peptide helices covalently trap both static and dynamic protein interactors, and enable rapid identification of interaction sites, providing a new scope for targeted drug design for the BCL-2 family targets [105]. Visualizing potential sites on Gabs for similar stapled peptides can provide fascinating insights into protein-binding disruption. These disruptions could also uncover the role of binding partners in disease regulation (Figure 3). In some ways these directed peptides could be more practical as targeted therapy, since they would not silence the entire molecule, like siRNA targeting, but rather, could disturb binding and interactions of the target with regulators. However, these interactions are complex and as mentioned earlier both Erk and STAT5 are important for leukemogenesis, so it remains unclear whether promoting Gab2/SHP2 interaction to reduce STAT5 activation would be advantageous. The disadvantage of such a targeted therapy approach would be increased Erk activation. The therapeutic potential could depend on the particular upstream activating mutation and the stage of disease development. The use of new knockin animal models expressing mutant Gabs would be needed to address these important issues.

An alternative approach to targeting the specific effectors of Gab functional interactions would be to target the entire molecule as a whole. The risk of this approach is that it would be predicted to have widespread effects on Erk and AKT activation, as well as loss of potential negative regulatory functions. The total Gab targeting approach is also complicated by the potential redundancy of Gab1, Gab2, and Gab3 for specific Gab signaling functions. Only through rational dissection of Gab structure-function as related to disease progression will targeted therapy for Gabs become more rationally guided. It is appealing to consider adapter proteins as drug targets from the respect that they have the ability to impact upon multiple key oncogenic signals which may be cooperative. This “killing a flock of birds with one stone” may be optimal over even “killing two birds with one stone,” and only adapter proteins permit widespread impact on receptor-signaling molecule interactions. Therefore, Gabs are of interest in the field of targeted therapeutics and their complete deletion shows therapeutic potential in mouse models; however, drug development for these targets will require moving forward with caution and greater understanding of structure-function relationships.

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Review Article

Molecular Targets for the Treatment of Juvenile Myelomonocytic Leukemia

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Significant advances in our understanding of the genetic defects and the pathogenesis of juvenile myelomonocytic leukemia (JMML) have been achieved in the last several years. The information gathered tremendously helps us in designing molecular targeted therapies for this otherwise fatal disease. Various approaches are being investigated to target defective pathways/molecules in this disease. However, effective therapy is still lacking. Development of specific target-based drugs for JMML remains a big challenge and represents a promising direction in this field.

1. Juvenile Myelomonocytic Leukemia (JMML) and Current Clinical Standard of Care

Juvenile myelomonocytic leukemia (JMML) is a rare hematologic malignancy of early childhood with high mortality. It represents 2% to 3% of all pediatric leukemias [1, 2], and its incidence is approximately 0.6 per million children per year [3]. Clinically, patients often present with pallor, failure to thrive, decreased appetite, irritability, dry cough, tachypnea, skin rashes, and diarrhea and are found to have lymphadenopathy and hepatosplenomegaly on examination [4–8]. JMML is characterized by leukocytosis with prominent monocytes, thrombocytopenia, elevation of fetal hemoglobin (HbF), and hypersensitivity of hematopoietic progenitors to granulocyte-macrophage colony-stimulating factor (GM-CSF) [4–8].

Prior to the revision in 2008, JMML was diagnosed based on the following criteria: presence of peripheral blood mononcytosis (>1000/μL); less than 20% blasts in the bone marrow; absence of Philadelphia (Ph) chromosome or BCR-ABL fusion gene AND at least two of the following criteria: increased HbF levels; presence of immature myeloid precursors in the peripheral blood; white blood cell count >10,000/μL; GM-CSF hypersensitivity of myeloid progenitors in vitro [5, 9]. In 2008, the JMML diagnostic criteria were revised to account for the molecular genetic abnormalities that were identified in this disease [5].

The natural course of JMML is rapidly fatal with 80% of patients surviving less than three years [10]. Low platelet count, age at diagnosis older than 2 years, and high HbF percentage have been shown to correlate with poor outcome [11]. Allogeneic hematopoietic stem cell transplantation (HSCT) is currently the only curative treatment for JMML, but controversy exists in identifying the patients that need to proceed to transplant immediately versus those that can be observed for a longer time. Patients with Noonan syndrome often develop a JMML-like myeloproliferative disorder that may resolve spontaneously within one year of presentation [12]. While awaiting transplant, most patients receive chemotherapy, and most clinicians will use cytarabine-based acute myeloid leukemia-like therapy [10, 13]. Identification of gene mutations in the RAS-MAPK pathway has increased interest in development of drugs that can specifically affect molecular targets. For more detailed review of genetic mutations in JMML and approaches to therapy, please refer to Dr. Loh’s recent article [14]. Here, we shall proceed to
briefly discuss molecular defects in JMML with focus on potential drug targets.

2. Identification of Genetic Mutations in JMML

The molecular defects in JMML result in deregulated signaling through the RAS pathway [15–17]. These mutations are mutually exclusive which highlights the major functional role of the RAS pathway activation in JMML pathophysiology and disease progression. The specific genes implicated in JMML are summarized in Table 1.

### 2.1. PTPN11

Somatic mutations within PTPN11, which encodes protein tyrosine phosphatase SHP-2, have been found in 35% of JMML patients [18, 19]. PTPN11 mutations were also associated with poor prognosis for survival. Mutation in PTPN11 was the only unfavorable factor for relapse after hematopoietic stem cell transplantation [20]. SHP-2 contains 2 Src homology domains (N-SH2 and C-SH2) at the amino terminus and a phosphatase domain at the carboxy terminus [21–25]. It is involved in a variety of signaling pathways, especially the RAS/MAPK/ERK pathway [26–28]. SHP-2 is normally self-inhibited by hydrogen bonding of the backside of the N-SH2 domain loop to the deep pocket of the PTP domain [29, 30]. The self-inhibition leads to occlusion of the phosphatase catalytic site and a distortion of the pY-binding site of N-SH2. PTPN11 mutations found in JMML are mainly localized in the N-SH2 domain. These mutations result in amino acid changes at the interface formed between N-SH2 and PTP domains, disrupting the inhibitory intramolecular interaction, leading to hyperactivation of SHP-2 catalytic activity [18, 31]. In addition, disease mutations enhance the binding of mutant SHP-2 to signaling partners [32–34]. Recent studies have shown that PTPN11 gain-of-function mutations induce cytokine hypersensitivity in myeloid progenitors [16, 34, 35] and myeloproliferative disease with some similarity to JMML in mice [32, 36–38], establishing the causal role of PTPN11 mutations in the pathogenesis of JMML. It is evident that increased signal transduction along SHP-2’s pathways leads to aberrant hematopoietic cell proliferation and differentiation. SHP-2 may, thus, be an ideal target of mechanism-based therapeutics for this disease.

### 2.2. RAS

The RAS subfamily includes three members: HRAS, KRAS, and NRAS. Twenty-five percent of JMML patients were found to have a somatic NRAS or KRAS point mutation [20, 39]. Flotho et al. analyzed 36 children with JMML. RAS mutations were detected in 6 cases. Two children had a mutation in codon 12 of NRAS, 3 children in codon 13 of NRAS, and 1 child in codon 13 of KRAS. No mutation in HRAS codons 12, 13, or 61 was found [40]. De Filippi et al. reported a 38G > A (G13D) mutation in the NRAS gene in all types of cells checked in a male infant who was diagnosed with JMML [41]. This case suggests that constitutively active mutations of NRAS may be responsible for the development of JMML in children [41].

### 2.3. NF1

In 1994, Shannon et al. demonstrated loss of the wild-type NF1 allele in the diseased bone marrow of children with JMML affected by neurofibromatosis type 1 (NF1) [42]. The protein product of NF1, neurofibromin (NF1), contains a GTPase-activating protein- (GAP-) related domain. It inhibits RAS signaling by increasing the intrinsic GTPase activity of RAS-GTP and, thus, the generation of inactive RAS-GDP [43]. Eleven percent of JMML patients have constitutive NF1 [44] and 15% of the JMML patients without clinical signs of NF1 [39, 45]. A mitotic recombination event in JMML-initiating cells led to 17q uniparental disomy with homozygous loss of normal NF1, providing confirmatory evidence that the NF1 gene is crucial for the increased incidence of JMML in NF1 patients [44]. In addition, children (but not adults) with NF1 show a 200- to 500-fold increase in the incidence of de novo malignant myeloid disorders, particularly JMML [46].

### 2.4. CBL

The Casitas B-cell lymphoma (CBL, c-CBL) protein is a member of the CBL family of E3 ubiquitin ligases. Loh et al. first reported that c-CBL mutations were detected in 27 of 159 JMML samples, and 13 of these mutations alter codon Y371 [47]. The same c-CBL mutation was also found in another study with a smaller cohort of JMML patients [48]. A recent study screened CBL mutations in

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### Table 1: Summary of genetic mutations in JMML.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Site of mutation</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPN11</td>
<td>E76K, D61Y, D61V, E69K, A72T, A72V, E76V/G/A,</td>
<td>35%</td>
</tr>
<tr>
<td>RAS</td>
<td>Codons 12 and 13</td>
<td>25%</td>
</tr>
<tr>
<td>NRAS</td>
<td>Codon 13</td>
<td></td>
</tr>
<tr>
<td>KRAS</td>
<td>No mutation in codons 12, 13, and 61 was found</td>
<td></td>
</tr>
<tr>
<td>HRAS</td>
<td>Loss of wild-type NF1 allele</td>
<td>11–15%</td>
</tr>
<tr>
<td>NF1</td>
<td>Codons 371, 380, 381, 384, 396, 398, 404, and 408.</td>
<td>17%</td>
</tr>
<tr>
<td>CBL</td>
<td>Splice sites 1227, 1228, and 1096</td>
<td></td>
</tr>
</tbody>
</table>
Advances in Hematology

GM-CSF antagonist

Cell membrane

JAK2 antagonist

GM-CSFR

SHC

Grb2

CRB1

Grb2

SHP2

Ras-GTP

Farnesyltransferase inhibitor

NF1

Farnesyl group

RAF1 enzyme

RAF1 inhibitor

Nucleus

STAT5

RAF1

MEK

ERK

AKT

TOR

SOS

PI3K

SHP-2 phosphatase inhibitor

3. Chromosomal Aberrations

Some chromosome abnormalities were found in JMML. The most common chromosome abnormalities in JMML patients are monosomy 7 or deletion 7q (-7/del(7q)) [56]. In addition, there are some case reports for other chromosomal aberrations. For example, a 11-month-old boy with JMML had deletion 5q as the sole clonal chromosome abnormality [57]. Another JMML patient had a chromosomal translocation at t(1;5) [58]. Also, leukemic cells in a JMML patient harbored a 46,XX,der(12)t(3;12)(q21–22;p13.33) karyotype and subsequently developed partial trisomy of 3q [59]. However, at this time specific genes associated with these breakpoints are not yet identified, and; thus, the relevance of these chromosomal aberrations remains to be determined.

4. Recent Experimental Therapy for JMML

The recent focus in JMML has concentrated on using the information gained from knowledge of these molecular defects in order to design targeted drug therapy. Animal models, especially mouse models of the disease, are commonly used to test molecularly targeted agents. Since RAS hyperactivation is very important in the pathophysiology of JMML, agents designed to decrease RAS activity are being evaluated. There are numerous approaches that have been tested to target this pathway (Figure 1).

4.1. RAF1 Enzyme. RAF1 is a MAP kinase (MAP3K) that functions downstream of the RAS subfamily of membrane-associated GTPases to which it binds directly and plays an important role in the MAPK/ERK signal transduction pathway as part of a protein kinase cascade. A DNA enzyme designed to specifically cleave mRNA for RAF1, named RAF1 enzyme, was tested on JMML cells cultured both in vitro and in a xenograft model of JMML. When immunodeficient mice engrafted with JMML cells were treated continuously with this enzyme for 4 weeks, JMML cell numbers in the recipient murine bone marrows were profoundly reduced. No effect of the enzyme on the proliferation of normal bone marrow cells was found in vitro, indicating its specificity and potential safety [60].
4.2. RAF1 Inhibitor: BAY 43-9006. BAY 43-9006 is a low-molecular-weight agent that inhibits both the wild-type BRAF and the activated V599E mutant BRAF by binding at the active site of the kinase [61]. BAY 43-9006 can significantly inhibit tumor growth in a dose-dependent manner and has demonstrated oral in vivo activity in three human tumor xenograft models with mutant KRAS genes (HCT116, MiaPaca-2, H460) and one human tumor xenograft with a wild-type KRAS but exhibiting overexpression of growth factor receptors for epidermal growth factor (EGF) and HER2 (SKOVC-3) [62]. Based on these findings, a phase II window clinical trial is under development to evaluate response rate and acute toxicity to JMML patients [5].

4.3. Farnesyltransferase Inhibitor (FTI). RAS is first activated at the cell membrane via the addition of a farnesyl group to the newly translated protein. Farnesyltransferase inhibitors (FTIs) can prevent RAS translocation to the plasma membrane, thus, leading to downregulation of RAS-activated cellular pathways, so its competitive inhibitors have been developed as a novel class of anticancer therapeutics. L-744,832 is one such farnesyltransferase inhibitor. It can inhibit HRAS prenylation in cell lines and in primary hematopoietic cells, abolish the in vitro growth of myeloid progenitor colonies in response to GM-CSF, and increase the amount of unprocessed HRAS in bone marrow cells. However, FTIs had no detectable effect on NRAS, and the mouse model with JMML features created by transplantation of Nfi−/− fetal liver cells did not respond to L-744,832 treatment [63]. L-739,749, another kind of FTI, also has significant growth inhibitory effects in vitro, indicating a potential treatment for JMML [64]. Unfortunately, FTIs have modest to little activity in clinical trials when used as a single agent to treat cancers, yet recent studies show that when combined with other inhibitors, such as AKT inhibitors, FTIs do show a therapeutic potential in some cancer models [65, 66].

4.4. SHP-2 Phosphatase Inhibitor. The direct connection between activating mutations in PTPN11 and JMML indicates that SHP-2 may be a useful target for mechanism-based therapeutics for this disease. It is very important to develop selective SHP-2 inhibitors. The availability of SHP-2-specific inhibitors could lead to the development of new drugs that would ultimately serve as treatments for JMML. However, development of selective SHP-2 inhibitors has been challenging as the catalytic site of SHP-2 shares a high homology with those of other tyrosine phosphatases, especially SHP-1 that plays a negative role in cytokine signaling in contrast to SHP-2 phosphatase [26–28]. Several groups have attempted to identify low molecular weight inhibitors for SHP-2 phosphatase using various approaches [67–70]. However, the inhibitors identified to date either show low or no selectivity between SHP-2 and highly related SHP-1 phosphatase. Furthermore, therapeutic effects of these inhibitors in mouse models or human JMML samples have yet to be determined. More efforts are still needed to advance this line of research.

4.5. GM-CSF Antagonist: E21R. GM-CSF markedly promotes proliferation and survival of JMML cells and, thus, contributes to the aggressive nature of this malignancy [71]. Iversen et al. developed a GM-CSF analogue (E21R) that carries a single point mutation at position 21 in which glutamic acid is substituted for arginine [72]. It can effectively antagonize GM-CSF in binding experiments and in functional assays. They administered E21R or isotonic saline to SCID/NOD mice transplanted by JMML cells or normal bone marrow cells and found that E21R reduced growth of JMML cell load in the mouse bone marrow [8]. As TNFα may increase the production of GM-CSF [71], E21R also synergizes with anti-TNFα monoclonal antibody (MoAb) cA2 in suppressing JMML cell growth. Remarkably, E21R preferentially eliminated leukemic cells [8]. These data suggest that E21R may have a therapeutic potential in JMML.

4.6. Inhibition of Angiogenesis. Angiogenesis is essential for growth and metastasis of solid tumors and probably also for hematological malignancies. Endostatin and PI-88, two kinds of angiogenic inhibitors, were used to treat JMML xenograft mice and resulted in a reduction of about 95% of the malignant cell load. Furthermore, it was evident that neither endostatin nor PI-88 interfered with the engraftment of normal cells [73].

4.7. STAT5 Activation by the RAS/RAF/MEK/ERK Pathway in JMML-Biomarker and Potential Therapeutic Target. In addition to activation of the RAS pathway in JMML, there are several studies that have also shown enhanced signal transducer and activator of transcription 5 (STAT5) activation downstream of activated RAS. In a KRAS G12D mouse model, STAT5 activation was also associated with ERK and S6K phosphorylation [74]. KRAS also led to hyper-active STAT5, AKT, and ERK pathways [75]. Furthermore, NRAS caused an adult CMML-like phenotype characterized by ERK shows promise for clinical application. Additionally, STAT5 activation downstream of activated RAS in KRAS G12D mouse model, STAT5 activation was also associated with ERK and S6K phosphorylation [74]. KRAS also led to hyper-active STAT5, AKT, and ERK pathways [75]. Furthermore, NRAS caused an adult CMML-like phenotype characterized by ERK and STAT5 activation via a GM-CSF-dependent induction mechanism [76]. In patient samples, Kotecha et al. [77] elegantly showed that both ERK and STAT5 activation are associated with human JMML, but, interestingly, it was the phosphorylated STAT5 that was prognostic in these patients, suggesting that effective suppression of STAT5 will also be an important biomarker in JMML-oriented targeted therapies. Therefore, monitoring pSTAT5 by phospho-flow cytometry shows promise for clinical application. Additionally, STAT5 can partner with the adapter protein GAB2 to provoke activation of the PI3-kinase pathway [78]. Interestingly, GAB2 is also a major partner of oncogenic SHP-2 with gain-of-function mutation D61G and is responsible for a significant contribution to the myeloproliferative disease phenotype in mice [38].

5. Discussions and Perspectives

The clinical therapy of JMML has significantly improved over the last 20 years. However, the low incidence of the disease has limited the capacity to perform large-scale pathophysiological studies and testing newer therapeutic
strategies. JMML is a disease that only occurs in children, and drug dosage modifications are needed in children as compared to adults. All these factors limit the development of JMML treatment to some extent. Specific inhibitors for the molecular targets identified in this disease are still lacking.

Molecular mechanisms of JMML have been elucidated in almost 85% of patients, but it is also true that a few JMML patients with Noonan syndrome can spontaneously recover without intervention [79, 80]. This means that we do not completely understand this disease, and there is much more to learn. It is indeed promising that there have been some novel agents evaluated in investigational phase II trials of JMML patients [81, 82], and there is legitimate hope that the knowledge we have gained about JMML will soon translate into more efficacious treatment modalities. Scientists and clinicians should continue to study molecular defects in JMML in a concerted effort to define novel therapeutic targets and to develop effective, less toxic, therapeutic interventions.

Acknowledgments

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[54] B. Pérez, O. Kosmider, B. Cassinat et al., “Genetic typing of CBL, ASXL1, RUNX1, TET2 and JAK2 in juvenile myelomonocytic leukaemia reveals a genetic profile distinct
Advances in Hematology


Review Article
The Role of mTOR Inhibitors for the Treatment of B-Cell Lymphomas

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Despite the fact that the majority of lymphomas initially respond to treatment, many patients relapse and die from disease that is refractory to current regimens. The need for new treatment strategies in lymphomas has led to the investigation and evaluation of novel agents that target cellular pathways. The mammalian target of rapamycin (mTOR) is a representative pathway that may be implicated in lymphomagenesis. Rapamycin and especially its derivatives (temsirolimus, everolimus, and deforolimus) represent the first described mTOR inhibitors. These agents have shown promising results in the treatment of lymphoid malignancies. On the other hand, new ATP-competitive mTOR inhibitors that provoke a broader inhibition of mTOR activity are in early stages of clinical development. The purpose of this paper is to summarize the existing knowledge about mTOR inhibitors and their use in the treatment of B-cell lymphomas. Relevant issues regarding mTOR biology in general as well as in B-cell lymphoid neoplasms are also discussed in short.

1. Introduction

Current approaches in treating lymphoid malignancies have focused on the development of therapeutic regimens that selectively target dysregulated signal transduction pathways in neoplastic cells. Among aberrantly activated signaling cascades that are implicated in the pathogenesis of lymphomas is the mammalian target of rapamycin (mTOR) pathway, which is involved in many vital cellular processes [1]. Rapamycin and its analogs (rapalogs) comprise the classical mTOR inhibitors. A number of completed as well as other ongoing preclinical and clinical trials have tested these drugs in lymphomas, either as monotherapy or in combination with established chemotherapy [1]. Moreover, other anti-mTOR molecules, such as specific active-site TOR inhibitors (aSTORi), with better pharmacological profiles are candidate drugs to be tested in clinical trials against lymphoid malignancies [2].

Herein we aim to review the results of trials with mTOR inhibitors in B-cell lymphomas. Firstly, the mTOR signaling network as well as possible aetiologic factors of aberrant activation of the mTORC1 signaling cascade in B-cell lymphoid malignancies are discussed in short.

2. mTOR Signaling Network

Rapamycin (also known as sirolimus or Rapamune, Wyeth) is the first described mTOR inhibitor [3]. This drug, originally developed as an antifungal agent, was soon found to have immunosuppressive and antineoplastic actions [4]. Systemic efforts to decipher the molecular mechanisms of these actions led to the isolation of the mTOR protein and the identification of two multimolecular complexes that are formed by mTOR, namely, the mTOR complex 1 (mTORC1) and 2 (mTORC2) [4, 5]. mTOR is the mammalian ortholog of a yeast serine-threonine kinase called target of rapamycin (TOR) [6]. Except for mTOR itself and the proteins mLST8/G\βL [mammalian LST8 (lethal with SEC13 protein 8), also known as G protein beta subunit-like] and DEPTOR, which are common in both mTORC1 and mTORC2, several different constitutional proteins associate to form the two mTOR complexes [7, 8]. These multipeptidic structures are
situated inside a signaling network, the mTOR network, characterized by many feedback loops and crosslinks among its various components [9].

Activity of mTORC1 is regulated by multiple molecular pathways that conduct input generated by growth factors, hormones, cytokines, amino acids, energy, stress- and oxygen-related signals [10–13]. Among these cascades are the PI3K/Akt (Phosphoinositide 3-kinase/Akt) and Raf/MEK/ERK (Raf/MEK/extracellular signal-regulated kinase) pathways, which are commonly activated in cancer and may cooperate in malignant transformation [9, 12]. Both pathways trigger the activity of mTORC1 through downregulation of the inhibitory effect of the TSC1–TSC2 (tuberous sclerosis complex 1–tuberous sclerosis complex 2) complex on RHEB (Ras homolog enriched in brain) protein [10, 12]. Akt kinase affects mTOR by way of two mechanisms. First, it lies upstream of mTORC1 and controls its activation. Second, Akt lies downstream of mTORC2 and depends on the latter as well as on PDK1 (pyruvate dehydrogenase kinase, isozyme 1) for full activation [7, 14].

Regulation of the two mTOR complexes bears some resemblance. For example, similarly to mTORC2, mTORC1 may also be activated by growth factors, hormones and amino acids, and this upregulation may be PI3K mediated [7, 15, 16]. In contrast, the TSC1–TSC2 complex, which suppresses mTORC1 activity, may promote mTORC2 signaling [10]. mTORC2 regulates Akt, SGK1 (serum- and glucocorticoid-induced protein kinase 1), and PKCa (protein kinase C, alpha) phosphorylation and controls organization of actin cytoskeleton as well as cell size, cell cycle progression, proliferation, and survival [7, 15, 16]. The best characterized targets of mTORC1 are the S6 kinases [S6K1 (also known as p70S6) and S6K2] and the eukaryotic initiating factor-4e (eIF4e) binding proteins 1 and 2 (4E-BP1 and 4E-BP2) [9–11]. Upon activation, mTORC1 triggers vital anabolic processes such as ribosome biogenesis, cap-dependent translation, uptake of nutrients including glucose and amino acids, biosynthesis of amino acids, proteins, and lipids as well as (adenosine triphosphate) ATP sensing. Moreover, gene transcription, cell growth, cell cycle progression, proliferation, and survival are induced [4–7, 9, 17]. In addition, active mTORC1 downregulates macroautophagy and other catabolic processes such as fatty acid oxidation and protein degradation, while, in contrast, it stimulates aerobic glycolysis [4, 5, 17, 18].

Dysregulated activation of the mTORC1 pathway has been associated with tumor biology. Aberrant mTORC1 signaling disrupts homeostatic cell balance and contributes to uncontrolled proliferation and cell growth, survival, as well as angiogenesis and metastasis [9]. The same malignancy-inducing processes may be also promoted by abnormally elevated mTORC2 signaling [16, 19–21].

3. Aberrant mTORC1 Pathway Activation in B-Cell Lymphomas

Several lines of evidence indicate that aberrant activation of the mTORC1 pathway is common in both Hodgkin (HLs) and many types of B-cell non-Hodgkin lymphomas (NHLs) (Table 1) [22–25, 27, 28, 30–33, 40–42, 46–49]. However, the cause of this upregulation is currently poorly defined. Molecular events that affect signaling pathways related to mTORC1 complex modulation may presumably have an impact on the mTORC1 pathway itself [9]. Notably, the PI3K/Akt pathway, which is abnormally activated in many types of B-cell lymphomas, seems to participate in mTORC1 upregulation at least in a subset of these entities [22, 25, 27–29, 31–39, 41, 43–45, 47–49] (Table 1).

Several possible mechanisms of PI3K/Akt pathway activation in mature malignant lymphoid B cells have been described. These include (a) overexpression of membrane receptors which may be mutation related, and/or stimulation by their ligands by autocrine/paracrine secretion [23, 36–39, 41, 50], (b) aberrant tyrosine kinase (TK) activity [31], (c) constitution of oncogenic singalosomes [52], (d) expression of Epstein Barr virus (EBV) latent membrane protein 2A (LMP2A), and high levels of activated Ras protein [53, 54], (e) expression of the K1 protein of Kaposi’s sarcoma-associated herpesvirus [55], (f) overexpression of the phosphodiesterase PDE4B gene and protein [56, 57], (g) overexpression of the T-cell leukemia/lymphoma 1 (TCL1) oncoprotein [58], (h) point mutations or amplification of the PI3K catalytic subunit alpha (PIK3CA) gene [29], and (i) genetic or epigenetic downregulation of phosphatase and tensin homolog (PTEN) suppressor gene [34, 35, 59, 60]. As for the latter, a very recent study in animal models, showed that PTEN cooperates with another negative modulator of PI3K-mediated signaling, the Src homology 2 domain-containing inositol phosphatase (SHIP), in order to suppress lymphoma pathogenesis [61].

Molecular alterations that activate the PI3K/Akt pathway could explain in part the upregulation of mTORC1 signaling in B-cell lymphomas (Figure 1(a)). The Raf/MEK/ERK pathway is another candidate inducer of mTORC1 activity in HLs and NHLs (Figure 1(b)). The fact that, on one hand, this pathway is another major upstream effector of mTORC1 and, on the other hand, it is activated in a subset of B-cell lymphomas justifies this hypothesis [24–26, 31, 38, 39, 42, 45] (Table 1). Indeed, there is evidence that upregulated Raf/MEK/ERK pathway may contribute to elevated mTORC1 signaling in the setting of follicular lymphoma (FL) and HL [25, 40, 42]. Apart from Erk, p38 is another mitogen-activated protein (MAP) kinase which was recently suggested to induce mTORC1 activity [13] and that may also become dysregulated in lymphomas [25, 62–64] (Figure 1(c)). Moreover, there is evidence for a role of the activated TK Syk in the upregulation of mTORC1 activity in FL, diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), and Burkitt lymphoma (BL) cells. Notably, Syk-in-duced mTORC1 activation in FL cells appears not to be PI3K/Akt dependent [40]. Furthermore, Syk gene amplification and elevated protein expression was found in Jeko-1 MCL cell line and a few MCL tissue samples. These alterations could potentially be related to the activation of Syk protein and mTORC1 [65]. In addition, phospholipase D (PLD) seems to mediate mTORC1 stimulation in two FL cell lines, while its possible implication
Table 1: Evidence of aberrant activation of mTORC1, PI3K/Akt, and Raf/MEK/ERK pathways in B-cell lymphomas.

<table>
<thead>
<tr>
<th>Lymphoma Type</th>
<th>mTORC1 activation</th>
<th>PI3K/Akt activation</th>
<th>MEK/ERK dysregulation</th>
<th>Ref/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hodgkin Lymphoma (HL)</td>
<td>Cell lines, tissue samples</td>
<td>Cell lines, tissue samples</td>
<td></td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>Tissue samples</td>
<td>Tissue samples</td>
<td></td>
<td>[23, 24]</td>
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<tr>
<td></td>
<td>Tissue samples</td>
<td></td>
<td></td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[26]</td>
</tr>
<tr>
<td>Mantle Cell Lymphoma (MCL)</td>
<td>Cell lines, tissue samples</td>
<td>Cell lines, tissue samples</td>
<td></td>
<td>[27, 28]</td>
</tr>
<tr>
<td></td>
<td>Cell lines</td>
<td>Cell lines, tissue samples</td>
<td></td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>Tissue samples</td>
<td>Cell lines, tissue samples</td>
<td></td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td>Tissue samples</td>
<td></td>
<td></td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Cell lines, Lymphoma cells from a MCL patient</td>
<td>Cell lines, Lymphoma cells from a MCL patient</td>
<td></td>
<td>[31]</td>
</tr>
<tr>
<td>Diffuse Large B-Cell Lymphoma (DLBCL)</td>
<td>Tissue samples</td>
<td>Cell lines, tissue samples</td>
<td></td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>Cell lines, tissue samples</td>
<td>Cell lines, tissue samples</td>
<td></td>
<td>[33]</td>
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<tr>
<td></td>
<td>Cell lines</td>
<td>Cell lines</td>
<td></td>
<td>[34, 35]</td>
</tr>
<tr>
<td></td>
<td>Tissue samples</td>
<td></td>
<td></td>
<td>[36, 37]</td>
</tr>
<tr>
<td></td>
<td>Cell lines, tissue samples</td>
<td></td>
<td></td>
<td>[38, 39]</td>
</tr>
<tr>
<td>Follicular Lymphoma (FL)</td>
<td>Cell lines, tissue samples</td>
<td>Cell lines</td>
<td></td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>Cell lines</td>
<td></td>
<td></td>
<td>[41]</td>
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<tr>
<td></td>
<td>Cell lines</td>
<td></td>
<td></td>
<td>[42]</td>
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<tr>
<td></td>
<td>Cell lines</td>
<td></td>
<td></td>
<td>[43, 44]</td>
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<tr>
<td></td>
<td>Cell lines</td>
<td></td>
<td></td>
<td>[45]</td>
</tr>
<tr>
<td>Burkitt Lymphoma</td>
<td>Cell lines</td>
<td></td>
<td></td>
<td>[24]</td>
</tr>
<tr>
<td>Primary Effusion Lymphoma (PEL)</td>
<td>Cell lines, animal model (mice)</td>
<td>Cell lines, animal model (mice)</td>
<td></td>
<td>[47, 48]</td>
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<tr>
<td></td>
<td>Cell lines</td>
<td></td>
<td></td>
<td>[24]</td>
</tr>
</tbody>
</table>

in mTORC1 activation in other lymphomas deserves further investigation [40] (Figure 1(d)). There are also data suggesting the contribution of serine/threonine kinase 11 (LKB1), a tumor suppressor kinase which negatively regulates mTORC1 activity, in lymphoma pathogenesis in animal models. However, whether LKB1 participates in human lymphoid malignancy induction remains uncertain at present [66] (Figure 1(e)). On the other hand, in a study in B-cell lymphoma cell lines mTORC1 upregulation was shown to be dependent on nutrients but not on other known upstream effectors [46]. As regards more proximal effectors of mTORC1, elevated levels of Rheb mRNA were found in some aggressive NHLs through an unknown mechanism and in individual cases of high increase were related to mTORC1 activation [67]. Finally, amplification of the RPS6KB1 gene, which encodes for p70S6/p85S6 protein, has been described in one third of a series of DLBCLs with unknown functional significance in mTORC1 signaling [33].

4. mTOR Inhibitors

4.1. Rapamycin and Rapalogs. The prototype of classical mTOR inhibitors is sirolimus [14]. The mechanism of action of sirolimus is rather complicated since it may inhibit mTORC1 or both the two mTOR complexes and either increase or in reduce Akt phosphorylation. These pharmaceutical effects are dependent on dose concentration, time after administration, and cell type [68–70]. Treatment with sirolimus may also activate ERK. Furthermore, sirolimus differentially affects the major substrates of mTORC1, S6K1 and 4E-BP1. It seems to downregulate S6K1, in most cell types, while in contrast inhibition of 4E-BP1 does not last long after treatment and is also cell specific. Consequently, a recovery in cap-dependent translation may be induced [17]. In malignant B-cells, sirolimus may cause cell cycle arrest, reduce proliferation, and inhibit growth in culture or delay tumor progression in animal models [47, 49, 70–72]. In addition, it may act similarly to amino acid deficiency as a positive modulator of genes which regulate nutrient catabolism and energy production and as a negative modulator of genes involved in the anabolic procedures of proteins, lipids, and nucleotides [73]. Moreover, it may potentiate the in vitro cytotoxicity of the chemotherapeutic agent doxorubicin, and the histone deacetylase inhibitor LBH [22, 70]. Of interest, sirolimus exhibits immunosuppressant properties and has been widely administered in patients with organ transplantation [3]. In addition, it may induce autophagy, both when given as monotherapy or in combination to radiation or dexamethasone [74–76].

Second generation rapamycin derivatives (rapalogs) with more favorable pharmacokinetic properties than the parent
molecule, facilitating their clinical use, have been developed [77]. Currently, three of these chemical agents are available for clinical trials: temsirolimus (CCI-779, Torisel, Wyeth Pharmaceuticals), everolimus (RAD001, Afinitor, Novartis Pharmaceuticals), and ridaforolimus (AP23573, ARIAD Pharmaceuticals, formerly deforolimus) [78]. Similarly to rapamycin, rapalogs inhibit mTORC1, may downregulate mTORC2, and exert either excitatory or inhibitory effects on Akt protein, both in vitro and in vivo. Moreover, these effects depend on tumor-specific characteristics, dose, and schedule of treatment [70, 79, 80]. In B-cell lymphomas rapalogs exhibit antiproliferative, cytostatic, and antiangiogenic properties and may also trigger autophagy. In contrast, they appear to have minimal or no effect on survival of malignant cells [70, 81, 82]. Despite many theoretical gaps concerning rapalogs’ mechanism of action, clinical trials with these agents show promising results in lymphoid neoplasms, apparently at the appropriate molecular background.

The pharmacokinetic and pharmacodynamic profiles of rapalogs differ. Temsirolimus is available in oral and
converted to rapamycin, its primary active metabolite [83]. Upon administration, it is rapidly intravenous formulations. Upon administration, it is rapidly converted to rapamycin, its primary active metabolite [83]. Phase I dose-finding studies for temsirolimus aimed to establish a maximum-tolerated dose through dose escalation, by testing either a schedule of a daily administration of 0.75 mg/m² IV every other week with a 20% dose escalation or a weekly schedule of doses ranging from 7.5 mg–220 mg/m² IV [83, 84]. Maximum tolerated dose was not established but the maximum acceptable dose was 19 mg/m²/day due to grade 3 stomatitis. Although temsirolimus was found to be well tolerated, the most common toxicities included neutropenia, thrombocytopenia, asthenia, diarrhea, and stomatitis. The severity of its adverse effects was dose related. Because drug activity ceased to increase after several dose levels, phase I studies supported the use of a flat dose for temsirolimus and the suggested dose for phase II studies was 25, 75, or 250 mg weekly.

Everolimus is orally available and typically administrated on a daily schedule. It has been also been tested on a weekly basis, mostly in combination regimens. Phase I studies showed that the efficacy of the drug was dose dependent and that mTOR inhibition was more profound with daily dosing. In addition, everolimus was found to have acceptable tolerability at the highest doses studied. The suggested dose for phase II studies was 10 mg daily or 50–70 mg weekly [85, 86].

Ridaforolimus, unlike temsirolimus, is not a prodrug [87], and it is given typically intravenously, although oral administration is currently under clinical testing [88]. Phase I studies tested a daily regimen of 3–28 mg and found that mTOR inhibition increased in a less than proportional manner. Maximum tolerated dose was 18.75 mg/day due to grade 3 mouth sores and a dose of 12 mg IV daily was proposed as suitable for phase II studies [87].

At present, numerous clinical trials are under way in order to evaluate the above drugs as single agents or in combination in aggressive and/or refractory lymphomas (Table 2) (http://www.cancer.gov/search/ResultsClinicalTrials).

### 5. Clinical Trials with Rapalogs in Lymphomas

#### 5.1. Mantle Cell Lymphoma

Mantle Cell Lymphoma (MCL) is an aggressive type of mature B-cell lymphoid neoplasm with a relative frequency of 7% among NHLs. The genetic hallmark of MCL is the translocation t(11;14)(q13;q32), which results in overexpression of cyclin D1 [89]. It is characterized by an aggressive clinical course and poor prognosis with median survival of 3 to 5 years [90]. Although frontline treatment induces a high rate of complete remission (CR), relapse is common. Therefore, new therapies are needed [91]. Aberrant activation of mTORC1 as well as PI3K/Akt signaling is frequent in the MCL [27, 28, 30, 49].

Additionally, in preclinical MCL models both temsirolimus and everolimus showed anti-proliferative effects, especially in combination with other therapeutic agents, such as vorinostat, doxorubicin, and vorozemib [81, 82, 92, 93].

Among rapalogs, temsirolimus has been thoroughly studied in clinical trials in MCL. In two phase II studies and in one large randomized phase III study performed by North Central Cancer Treatment Group (NCCTG), temsirolimus was found to display significant antitumor activity and clinical benefit as a single therapeutic agent in relapsed or refractory MCL. The first of phase II studies conducted by Witzig et al., assessed the efficacy of a 250 mg/week IV course of temsirolimus monotherapy in 35 patients with relapsed or refractory MCL that had received previous treatment. The overall response rate (ORR) was 38%, with one complete remission (CR) and one partial remission (PR) [94]. In the second phase II study, Ansell et al. administered a 10-fold lower dose of temsirolimus (25 mg weekly IV) in 29 patients with relapsed or refractory MCL and achieved a similar ORR (41%) with one CR and ten PRs. However, the lower dose was associated with lower rates of toxicity (50% versus 71% grade 3 and 4% versus 11% grade 4) [95]. In both studies, thrombocytopenia was the most common adverse effect and the most frequent cause of dose reduction. In addition, both studies included adults (median age 70 years old) that had

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### Table 2: Ongoing clinical trials with rapalogs in patients with NHL.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Locations</th>
<th>Clinical trial gov. number</th>
<th>Additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/II</td>
<td>USA</td>
<td>NCT01076543</td>
<td>Lenalidomide and Temsirolimus in patients with relapsed or refractory HL or NHL</td>
</tr>
<tr>
<td>I/II</td>
<td>USA</td>
<td>NCT00787969</td>
<td>Rituximab, Cladribine, and Temsirolimus in patients with newly diagnosed MCL</td>
</tr>
<tr>
<td>I/II</td>
<td>GERMANY</td>
<td>NCT01078142</td>
<td>Temsirolimus, Bendamustine, and Rituximab for relapsed FL or MCL (BERT)</td>
</tr>
<tr>
<td>I/II</td>
<td>USA</td>
<td>NCT00474929</td>
<td>Everolimus and sorafenib for relapsed or refractory NHL, HL, or MM</td>
</tr>
<tr>
<td>I</td>
<td>Cleveland OH</td>
<td>NCT00671112</td>
<td>Everolimus plus bortezomib for relapsed/refractory MCL and other NHL</td>
</tr>
<tr>
<td>I/II</td>
<td>USA</td>
<td>NCT00967044</td>
<td>Panobinostat (LBH589) plus Everolimus (RAD001) in patients with relapsed and refractory Lymphoma</td>
</tr>
<tr>
<td>I/II</td>
<td>USA</td>
<td>NCT 101075321</td>
<td>Everolimus and Lenalidomide in treating patients with relapsed or refractory NHL or HL</td>
</tr>
<tr>
<td>I/II</td>
<td>USA</td>
<td>NCT00918333</td>
<td>Panobinostat and Everolimus in treating patients with recurrent MM, NHL, or HL</td>
</tr>
<tr>
<td>II</td>
<td>USA</td>
<td>NCT00869999</td>
<td>Everolimus plus Rituximab for relapsed/refractory DLBCL</td>
</tr>
<tr>
<td>I/II</td>
<td>USA</td>
<td>NCT00704054</td>
<td>Deferolimus for relapsed/refractory NHL/HL</td>
</tr>
</tbody>
</table>
failed previous therapies with rituximab (monoclonal anti-CD20 antibody), cyclophosphamide, or doxorubicin [94, 95].

Based on these results, a randomized, large phase III study was conducted to evaluate the effect of temsirolimus in comparison to investigator’s choice therapy in 162 patients with relapsed/refractory MCL, previously treated with rituximab, alkylating agents, and anthracycline. The patients were randomized to receive treatment with temsirolimus applied in one of two therapeutic schemes (175 mg/week for three weeks followed by either 25 mg or 75 mg weekly IV) or treatment with a single agent of the investigator’s choice from approved protocols. It was shown that ORR was significantly higher in patients who received the 75 mg dose of temsirolimus compared to treatment with the investigator’s choice agent (22% versus 2%, \( P = .0019 \)). Median progression free survival (PFS) was also longer (4.8 months versus 1.9 months). Regarding patients who received 25 mg temsirolimus, ORR was 6% and PFS 3.4 months. Similar to the previous trials, hematological toxicity was the most frequent adverse effect. This study demonstrated that administration of 175/75 mg temsirolimus improved ORR and PFS significantly and showed a trend toward longer overall survival (OS) [96]. The results of this trial led to the European approval of temsirolimus as single agent therapy for the treatment of relapsed/refractory MCL [97].

More recently, Ansell et al. reported the results of the first phase II study that examined the efficacy of temsirolimus in combination with rituximab in patients with relapsed or refractory MCL [98]. In this study 69 patients were treated with temsirolimus (25 mg/week) and rituximab (375 mg/m² per week) for 4 weeks during the first cycle followed by a single dose of rituximab every other 28-day cycle for a total of 12 cycles. The ORR was 59% consisting of 19% CRs and 41% PRs. The ORR was 63% for rituximab-sensitive patients and 52% for rituximab-refractory patients. The median time to progression (TTP) for all patients was 9.7 months (10.9 months in the rituximab-sensitive patients and 5.4 months in the rituximab-refractory patients). The most common side-effect was hematological toxicity which did not differ from that in the previous studies of temsirolimus alone. Additionally, the other more frequent grades 3 and 4 toxicities included increased serum concentrations of cholesterol and triglycerides, hyperglycaemia, fatigue, and dyspnoea. The frequencies of these toxicities were also similar to that of temsirolimus as monotherapy suggesting that rituximab can be safely combined with temsirolimus without much increase in toxicity. The above results are promising with much higher ORR and CR rate than in the phase III study of temsirolimus alone without increasing toxicity [96]. However, more randomized trials are needed in order to establish the effectiveness of the combination of temsirolimus plus rituximab in the treatment of relapsed or refractory MCL patients.

The efficacy of everolimus in MCL was investigated in phase II clinical trials. Witzig et al. demonstrated the antitumor activity of the drug when applied as monotherapy in relapsed/refractory NHLs. In this trial, 19 patients with MCL, 47 patients with DLBCL and 3 patients with FL were included. Daily dose was 10 mg PO. All patients had been heavily pretreated with a median of three previous therapies and 32% of them had undergone autologous stem-cell transplantation. In this study, ORR in patients with MCL was 32%, lying in the middle of the 40% and 22% that were found in the two phase II and the one phase III trials of temsirolimus, respectively. In addition, ORR in DLBCL patients was 30% and in FL patients 38%. Hematological toxicity, mainly thrombocytopenia (38%), was again the most frequent adverse effect. Grade 3 or 4 toxicity was observed in 68% of the patients, which was managed easily with dose interruption or reduction [99]. On the other hand, in another phase I/II study designed to evaluate everolimus effect in 26 patients with hematological malignancies including MCL, none of the 4 patients with MCL responded to everolimus [93].

Furthermore, in the setting of MCL the activity of ridaforolimus has been evaluated as well. In a phase II study, 55 heavily pretreated patients with various hematological malignancies, including 9 patients with MCL, received ridaforolimus as single agent (12.5 mg IV once a week every 2 weeks). The most favorable response was observed in MCL patients, with 33% ORR and three PRs. Although the number of MCL patients involved in this study was small, it cannot be ignored that ORR achieved with ridaforolimus is similar to that demonstrated in the two phase II studies of single agent temsirolimus in MCL. Additionally, the fact that 44% of MCL patients had stable disease and only two experienced progressive disease probably reflects a promising antitumor activity of ridaforolimus that has to be further investigated. It is also noteworthy that ridaforolimus was well tolerated. Mouth sores was the most frequent adverse effect, while thrombocytopenia was less commonly encountered than with other rapamycin derivatives [100].

In summary, temsirolimus is the most extensively studied mTOR inhibitor in the setting of MCL, which has been shown to significantly improve objective response and progression-free survival compared to investigator’s choice therapy in patients with relapsed/refractory MCL in a phase III clinical trial. The effectiveness of temsirolimus in combination to immunotherapy or chemotherapy has already been under investigation. Everolimus and ridaforolimus have demonstrated promising antitumor activity against MCL but further investigation is needed in order to evaluate their potential efficacy.

5.2. Diffuse Large B-Cell Lymphoma (DLBCL). Diffuse large B-cell lymphoma (DLBCL) represents almost one third of all NHL subtypes [90]. Although standard chemotherapy regimens (R-CHOP, rituximab-cyclophosphamide, doxorubicin, vincristine, and prednisone) have shown effectiveness in the treatment of DLBCL, there is still a group of patients that die from the disease [101].

In preclinical studies, rapamycin analogue everolimus has been found to induce G1 cell-cycle arrest but not apoptosis in DLBCL cell lines of the germinal centre (GC) type and to increase the cytotoxicity of rituximab [71, 102].
Temsolim is has shown promising results as a single agent in DLBCL in a phase II study performed by the University of Chicago. The study included 89 pretreated patients with either DLBCL, follicular lymphoma (FL), chronic lymphocytic leukemia (CLL), or other indolent lymphomas that were stratified in three groups. Patients received a weekly course of temsirolimus of 25 mg. It was found that DLBCL patients had an ORR of 28.1% with four CRs and 5 PRs (9 patients out of 32). This result is promising, taking into account that all patients were heavily pretreated; however, the durability of response was short (2.4 months). Based on the fact that nearly half of DLBCL responders had transformed from a prior FL, authors suggest that temsirolimus might be more active in follicle derived lymphomas. The most common adverse effect of temsirolimus was myelosuppression, which was reversible, while other toxicities included stomatitis and metabolic dysregulation mainly grade one or two [103].

Everolimus has also been tested in clinical trials in DLBCL, demonstrating response rates similar to temsirolimus. In a phase II study by Witzig et al. that included 77 patients with DLBCL, FL, or MCL, the ORR in DLBCL patients was 30% (14 out of 47) [99]. However, the duration of response was longer than with temsirolimus (5.7 months). On the other hand, compared to the previous study with temsirolimus where 4 CRs were observed, no patient among DLBCL group who were treated with everolimus achieved a CR. The most important adverse effect in this study was grade 3 or 4 hematologic toxicity, which appeared in 68% of patients [103].

Deferolimus has not been investigated yet as a single agent in the treatment of DLBCL or other NHL subtypes. However, preliminary results show antitumor activity in many tumor types and numerous ongoing clinical trials are under way (Table 2) [87].

5.3. Follicular Lymphoma (FL). Follicular lymphoma (FL) is the second most common type of B-NHL in the West, accounting for 25–35% of all NHLs [90]. Patients with FL usually have an indolent clinical course, but they might eventually evolve to a refractory phase that can lead to death. Activation of mTOR pathway has been demonstrated in FL cell lines and tissue samples [40–42]. It has also been shown that mTOR activation in FL cell lines is enhanced by Syk independently of Akt and also by PLD [40]. However, mTOR inhibitors have not been tested in FL cell lines or animal models.

Both temsirolimus and everolimus have shown effectiveness in relapsed FL in phase II studies. In the above mentioned study from University of Chicago, patients with FL demonstrated an ORR of 53.8% with CR rates reaching 25.6%. Furthermore, median duration of response was 13 months, much longer than in DLBCL [103]. Similarly to temsirolimus, everolimus has shown antitumor activity in FL. In the previously mentioned phase II study by Witzig et al. the reported ORR in FL was 38%, with a median duration of response of 5.7 months. Of note, this study included only patients with FL grade 3 [99]. These results are very promising, but they need to be validated in further studies that will include larger groups of patients.

5.4. Hodgkin's Lymphoma (HL). Hodgkin's Lymphoma (HL) represents 30% of all lymphoma cases. It is characterized by the presence of neoplastic Hodgkin and Reed-Steinberg cells (HRS) in a background of inflammatory and accessory cells [90]. Although it has proven a highly curable disease, there is a subset of patients that either relapse after salvage chemotherapy or do not respond due to old age. Consequently, there is still need for new therapeutic approaches.

Various studies have demonstrated activation of the mTOR pathway in HL cell lines and primary tumors [104–107]. The mechanism of mTOR activation in HL has not been clarified yet, but Akt is considered to play a role once phosphorylated Akt has been reported to be activated in HL tumors [22, 108]. In HRS cells, rapamycin induces G1-S cell cycle arrest but not apoptosis and enhances the cytotoxic activity of doxorubicin [109]. Among rapamycin analogues, temsirolimus has been reported to induce cell cycle arrest followed by autophagy in HL cell lines [110]. In addition, everolimus has been demonstrated to have antiproliferative results in HRS cells. Furthermore, it has been shown to be effective in HL murine xenograft models [109].

Based on preclinical studies, a phase II study assessed the effectiveness of everolimus in patients with refractory/relapsed HL. This study evaluated a total of 19 patients with relapsed/refractory HL as part of a larger study evaluating everolimus in more rare forms of lymphoma. Patients had a median age of 37 years and had received a median of six prior therapies. They were treated with a daily oral dose of 10 mg and response was evaluated after two and six cycles of therapy. The ORR was 47%, with 8 PRs and one CR. Among those patients, 4 remained progression free at 12 years and 1 remains on therapy for more than 3 years. Of note, 74% patients experienced grade 3 or 4 toxicity. Although hematological toxicity was the most common adverse effect, a subset of patients (11%) developed pulmonary symptoms, such as dyspnea and cough that required dose reductions. This high rate of toxicity should be a matter of concern in older patients. Overall, the results of this study are encouraging, if we take into account that responders had stable disease on everolimus for a long period. The authors suggest that combination of everolimus with other agents might be even more effective [110].

6. Specific and NonSpecific ATP-Competitive mTOR Inhibitors

Recently a new category of mTOR inhibitors has come to prominence due to their ability to show a more profound impact on PI3K/Akt/mTOR pathway in relation to rapamycin and rapalogs. These drugs are small molecules that bind to the ATP-binding site of mTOR kinase and inhibit the catalytic activity of both mTOR complexes [2, 111–122]. Two subclasses of agents are included here. The first subclass comprises of nonspecific ATP-competitive mTOR inhibitors, which apart from mTORC1 and mTORC2 also
inhibit PI3Ks (Dual PI3K/mTOR inhibitors) [112–116]. The second subclass consists of drugs which selectively inhibit mTORC1 and mTORC2 without affecting other kinases [111, 117–122]. These molecules are known with different names such as specific active-site TOR inhibitors (asTORi) and TOR kinase domain inhibitors (TORKinis) [2, 115, 123].

7. Dual PI3K/mTOR Inhibitors

This subclass of ATP-competitive mTOR inhibitors includes several molecules which have been applied in preclinical models in hematologic malignancies [2, 27, 28, 49, 102, 116, 124, 125]. Two of these agents, LY294002 and wortmannin, were initially described as PI3K inhibitors and later found to target mTOR as well [124]. With regard to B-cell lymphomas, both of them have been tried in MCL and FL cell lines and were shown to downregulate Akt and/or mTOR activity [27, 28, 40, 49]. In addition, LY294002 was shown to decrease cyclin D1 protein levels in MCL cells, suggesting induction of cell cycle arrest [27]. LY294002 was also applied on DLBCL cell lines and found to trigger apoptosis in 3 out of 5 cell lines in one study. In the same study two DLBCL cell lines exhibited dephosphorylation of Akt upon LY294002 treatment [102]. Similar were the results regarding LY294002 effect on Akt inactivation in another study from China which also included DLBCL cell lines. Moreover, in this study, LY294002 decreased the ratio of S phase and of interest exhibited synergistic effect with doxorubicin on triggering apoptosis [126]. Another dual PI3K/mTOR inhibitor, NVP-BEZ235 was recently tried in FL cell lines and was found to inhibit cell growth and proliferation due to increased apoptosis. Furthermore, it showed a synergistic activity with bortezomib against FL cell proliferation [116]. Furthermore, Bhatt et al. reported that NVP-BEZ235 suppressed proliferation in primary effusion lymphoma (PEL) cell lines and xenograft models, more efficiently than selective inhibitors of PI3K/Akt mTOR pathway [48]. Although results from preclinical trials with dual PI3K/mTOR inhibitors are preliminary and further clinical trials are needed to confirm them, these agents seem that may be potentially effective in NHL treatment. Dual PI3K/mTOR inhibitors are currently being tested in phase I trials [112].

8. Active-Site TOR Inhibitors (asTORi)

All preclinical compounds in this category have been reported to have similar molecular behaviour, as they have been shown to reduce phosphorylation of both endogenous S6 kinase and Akt [115, 117–119, 122]. Interestingly, they are more effective mTORC1 inhibitors than rapamycin and rapalogs, since they completely inhibit S6 kinase and 4EBP1. Also, they trigger a more intense suppression on cap-dependent translation, protein synthesis, cell growth and proliferation [2, 115, 117–119, 122]. In addition, asTORi may induce apoptosis and autophagy and in relation to rapalogs cause a more profound decrease of lactate as well as the angiogenic hypoxia inducible factors (HIFs) and vascular endothelial growth factor (VEGF) [121, 122]. Among asTORi agents used in clinical models, AZD8055 shows similar in vitro effects with PP42, Torin-1, and Ku-0063794. However, in contrast to these inhibitors AZD8055 has also been found to inhibit tumor cell proliferation in vivo [121]. More specifically, it induces a dose-dependent inhibition and/or regression in human tumor xenograft models which is associated with a dose-dependent pharmacodynamic effect on both phosphorylated S6 and phosphorylated Akt. AZD8055 is currently being evaluated in phase I studies [122]. PP42 and Ku-0063794 are two other asTORi drugs which have shown important preclinical activity against hematological malignancies [2]. PP42 has been found to cause death to mouse and human Ph+ B-ALL cells, with great selectivity to leukemia cells compared to normal bone marrow and peripheral blood lymphocytes [120]. Moreover, PP42 has shown marked therapeutic response in transgenic mice that develop thymic lymphomas [127]. Regarding another asTORi, OSI-027, it has been reported to generate antileukemic effects in BCR/ABL transformed cells. Based on this finding, it is currently being evaluated in phase I studies in solid as well as lymphoid neoplasms. Finally, INK128 has demonstrated broad preclinical antitumor activity against a range of solid tumor types and multiple myeloma [2]. Currently, it is also being evaluated in phase I studies. None of these inhibitors have been tested in clinical trials in lymphomas yet. Further studies are currently conducted aiming to elucidate the potential therapeutic effect of asTORi-s in neoplasms, including lymphomas.

9. Conclusions

Through the last years it has become clear that mTOR pathway contributes to the pathogenesis of hematological malignancies by playing a key role in the regulation of many cell functions, such as cell proliferation, cell growth, and angiogenesis. The development of mTOR inhibitors has opened a new field in the clinical arena of lymphomas. Temsirolimus has been recently approved for the treatment of relapsed/refractory mantle cell lymphoma. In addition, everolimus and deferolimus have been evaluated in phase II clinical trials that reveal their potential for the treatment of aggressive lymphomas, justifying further evaluation with randomized phase III trials. In addition to rapalogs, other types of mTOR inhibitors have been currently developed, with promising results in preclinical studies. In our opinion, future research on the use of mTOR inhibitors in lymphoid malignancies should aim in three basic fields: (a) identification of the whole spectrum of molecular alterations that are related to mTOR signaling dysregulation in each lymphoma subtype, (b) definition of the subset of patients who are likely to respond best to anti-mTOR treatment, and (c) design of new clinical trials aiming to determine the effectiveness of mTOR-inhibitors in the context of established or other; targeted or nontargeted; treatment. Undoubtedly, there is evidence-based hope that lymphoma treatment will be substantially improved in the next decade.
Acknowledgments

P. Argyriou and P. Economopoulou contributed equally to this paper.

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Advances in Hematology


Review Article

The Role of BCL2 Family of Apoptosis Regulator Proteins in Acute and Chronic Leukemias

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The disturbance of apoptosis molecular signaling pathways is involved in carcinogenesis. BCL2 family of proteins is the hallmark of apoptosis regulation. In the last decade, new members of BCL2 gene family were discovered and cloned and were found to be differentially expressed in many types of cancer. BCL2 protein family, through its role in regulation of apoptotic pathways, is possibly related to cancer pathophysiology and resistance to conventional chemotherapy. It is well known that leukemias are haematopoietic malignancies characterized by biological diversity, varied cytogenetics, different immunophenotype profiles, and diverse outcome. Current research focuses on the prognostic impact and specific role of these proteins in the pathogenesis of leukemias. The understanding of the molecular pathways that participate in the biology of leukemias may lead to the design of new therapies which may improve patients’ survival. In the present paper, we describe current knowledge on the role of BCL2 apoptosis regulator proteins in acute and chronic leukemias.

1. Introduction

Apoptosis, or programmed cell death, is a cell-suicide program, distinct from necrosis, which is activated in physiological processes such as tissue development and differentiation as well as in pathophysiological conditions. The term is used to describe the situation in which a cell actively pursues a course toward death upon receiving certain stimuli. The morphological changes of apoptosis found in most cell types include nuclear pyknosis, DNA fragmentation and chromatin condensation, cytoskeleton destruction, membrane blebbing, and eventually the formation of membrane apoptotic bodies, which are phagocytosed by macrophages and other cells without promoting inflammatory response [1]. The mechanism of apoptosis is evolutionarily conserved and is executed by a family of proteins, called caspases. Caspases are cysteine proteases that are cleaved after an Asp residue in their substrates. They are synthesized as latent zymogens and activated by proteolytic cleavage; their activation is mainly regulated by the BCL2 family proteins [2–4].

BCL2 gene (otherwise B-cell lymphoma 2 gene, bcl-2) was first discovered in follicular B-cell lymphoma as a gene which is linked to the immunoglobulin heavy chain locus at the breakpoints of t(14;18) translocation [5]; the result of this translocation is the enhanced BCL2 protein transcription. In normal cells this gene is located on chromosome segment 18q21.3. BCL2 protein was found to inhibit cell death. This discovery was a revolution in the way of approaching cancer pathology, since it gave birth to the notion that tumor genesis could be due to not only unlimited proliferation, but also to impaired apoptosis. It should be noticed that BCL2
oncoprotein overexpression is found not only in follicular non-Hodgkin’s lymphoma but also in other haematopoietic malignancies and solid tumors, independent of t(14;18) chromosomal translocation.

There are two known distinct pathways which lead to apoptosis (Figure 1). The first, which is called the intrinsic cell death pathway, is evoked by intracellular stresses like radiation, growth factor withdrawal, cytokine deprivation, cytotoxic drugs and is regulated by BCL2 family proteins [6, 7]. Progression through this pathway leads to the release of cytochrome c from the damaged mitochondrion, which then binds to the adaptor molecule APAF-1 and an inactive “initiator” caspase, procaspase 9, within a multiprotein complex called the apoptosome. This leads to the activation of caspase 9, which then triggers a cascade of caspases activation (caspases 3 and 7) resulting in the morphological and biochemical changes associated with apoptosis. The second cell death pathway is the extrinsic pathway, which functions independently of mitochondria. This pathway is activated by the cell-surface death receptors CD95 (Apo-1 or Fas)/TRAIL/tumor necrosis factor (TNF) receptor 1 family proteins, which are located on the plasma membrane, and directly activates the caspase cascade via the recruitment of the “initiator” caspase-8 within a death-inducing signaling complex (DISC) [8].

Impaired apoptosis is a hallmark of the pathogenesis of many forms of cancer [9–16]. This paper focuses on the role of BCL2 family members in the biology, progression, prognosis, and therapy of acute and chronic leukemias.

2. BCL2 Family of Apoptosis Regulator Proteins

Mammalian BCL2 protein family consists of at least 30 related proteins, characterized by the presence of up to four relatively short sequence motifs (less than 20 amino acid residues in length) termed BCL2 homology (BH) domains [17–21]. BCL2 family is divided into three different subfamilies based on structural and functional features.

2.1. Prosurvival or Antiapoptotic Family Members. The pro-survival or antiapoptotic subfamily includes BCL2, BCL-XL, BCL-W, and MCL-1 proteins, which possess all four conserved BH domains, designated BH1-4, and a hydrophobic C terminal part. BH1-BH3 domains form a hydrophobic groove and the N-terminal BH4 domain stabilizes this structure. BH4 domain is usually absent in apoptotic proteins and therefore is a key factor for the antiapoptotic activity. The BH4 domain of BCL2 consists of 26 amino acids and its structure shows an amphipathic character upon interaction with the membranes, akin to antimicrobial peptides. The β-sheet conformation of BH4 in water is converted into an a-helical structure, appropriate to interact favorably with the negatively charged membranes [22]. It appears that the proteins are mainly located outside the membrane, however their exact insertion and complex formation is not well understood. BCL2 is permanently found in membranes, whereas BCL-XL and BCL-W are linked to the membrane after a cytotoxic signal [6].

BCL2 (and its antiapoptotic orthologues) seems to inhibit apoptosis by the preservation of mitochondrial membrane integrity as its hydrophobic carboxyl-terminal domain is linked to the outer membrane. BCL2 prevents BAX/BAK oligomerization, which would otherwise lead to the release of several apoptogenic molecules from the mitochondrion. It is also known that BCL2 binds to and inactivates BAX and other pro-apoptotic proteins, thereby inhibiting apoptosis. BCL2 might also regulate the activation of several initiator caspases like caspase-2 that act upstream or independently of cytochrome c release from mitochondria. Moreover, BCL2 directly blocks cytochrome c release and therefore prevents APAF-1 and caspase-9 activation.

BCL2 has not only been localized to the outer mitochondrial membrane but also to the nuclear envelope and the endoplasmic reticulum membrane (ER). In the ER, it regulates calcium storage, whose intracellular levels have been shown to affect apoptosis. ER-associated BCL2 is able to protect from apoptosis induced by various triggers. Beyond BCL2, BCL-XL also interacts with pro-apoptotic members like BAX and BAK thought their BH3 domains [22, 23]. It is possible that the antiapoptotic action of BCL2 and BCL-XL is converted to a pro-apoptotic one when these proteins are cleaved by caspases after initiation of apoptosis [24].

MCL-1 protein has a short half-life (estimated at less than 1 h) which is unique among antiapoptotic BCL-2 family members. Under basal conditions, human MCL-1 undergoes rapid protein turnover, but the control of this constitutive degradation pathway is incompletely understood. MCL-1 can be cleaved by caspases and granzyme B, which proteolytically degrade MCL-1 during cell death. In addition, human MCL-1 can be ubiquitylated and degraded by the proteasome. Several levels of degradation control have been postulated. The tight regulation of MCL-1 protein expression makes it an ideal regulator of cell survival. In response to cellular signaling, MCL-1 protein levels can be rapidly induced by inducing new MCL-1 transcription and by preventing MCL-1 protein turnover. When cells need to be eliminated, MCL-1 levels can be rapidly diminished by blocking new protein synthesis and degrading the existing MCL-1. Dysregulation of this balance, by inappropriately promoting its synthesis or by blocking its elimination, can lead to inappropriate stabilization of MCL-1 and promote cellular survival. Furthermore, dysregulated MCL-1 levels can lead to inappropriate cell survival or death; therefore, understanding regulation of MCL-1 levels is of great importance [25–27].

2.2. Proapoptotic Family Members. The pro-apoptotic members such as BAX, BAK, and BOK usually share sequence similarity in BH1, BH2, BH3 but not in BH4 domain [17, 28–30]. BAX protein is a monomeric protein in the cytosol, which integrates into the mitochondria during apoptosis and subsequently oligomerizes, resulting to the release of apoptogenic factors like cytochrome c and the activation of the caspase cascade. On the other hand, BAK is an integral mitochondrial membrane protein, which also undergoes conformational changes to form larger aggregates during apoptosis [29]. BAX and BAK are also present in the ER,
Figure 1: In this figure we show the two main pathways to apoptosis, and their interaction through the molecule BID, since death receptors activate the intrinsic pathway by activating BID. Either BAX or BAK are required for apoptosis, where they oligomerize in the mitochondrial outer membrane and induce the release of cytochrome c. DISC: death-inducing signaling complex, FADD: FAS-associated protein with death domain, TRAIL: TNF-related apoptosis-inducing ligand, APAF-1: apoptotic protease-activating factor, IAP: inhibitor of apoptosis proteins.

where they control apoptosis through the regulation of calcium levels [31]. The localization of BCL2 to the ER membrane supports the concept that it regulates ER-located BAX and BAK function. P53 can activate BAX, which lacks a clearly identifiable BH3 domain [32]. During drug-induced apoptosis BAX is cleaved by caspases and activated calpains [33].

2.3. BH3-Only Family Members. BH3-only proteins include a number of the proapoptotic proteins such as BID, BIM, BIK, BAD, BMF, HRK, DIVA, NOXA, and PUMA. These share similarity only in the BH3 domain. They are activated by different mechanisms, among which are transcriptional upregulation, proteolytic truncation, and phosphorylation. BIM and BMF are released upon phosphorylation, whereas BAD is released upon dephosphorylation [34]. BAD molecule is also cleaved by caspases when apoptosis is induced by transforming growth factor β1 [35]. BID is unique among the BH3-only BCL2 family members in interconnecting death receptors to the mitochondrial amplification loop of the intrinsic pathway. The potent proapoptotic activity and broad expression patterns of BID require that cells carefully regulate its apoptotic activation. Subcellular localization appears to play a role in directing the proapoptotic activity of BID. Following death receptor stimulation, BID is activated by caspase-8 cleavage and N-myristoylation to target mitochondria, where it activates BAX and BAK or is alternatively sequestered by antiapoptotic BCL2 members, preventing death. Full-length BID is also capable of translocation to the mitochondria in at least one case facilitated by other proteins such as PACS2. In the mitochondria, full-length BID has been shown to potentiate cell death following certain apoptotic signals, suggesting that caspase cleavage is not an absolute requirement for activating the proapoptotic function of BID. Recent studies indicate that activation of prodeath activity of BID may be negatively regulated by phosphorylation. Casein kinases have been implicated in BID phosphorylation, and ATM has been shown to phosphorylate BID following DNA damage [36–39]. Moreover, BID is proteolytically cleaved by caspase-8 and granzyme B. In acute T lymphoblastic leukemia cells, BID was found to be proteolytically activated by an aspartate-specific protease and to play a crucial role in mitochondrial activation in the p53-independent DNA damage response to etoposide and γ-radiation [40]. BH3-only proteins have dual function: both positive and negative regulation of BCL2 family members. Several BH3 peptides relieved the inhibition of BAX caused by the antiapoptotic BCL-XL and/or MCL-1 proteins and some of them display specificity for either BCL-XL or MCL-1. BIM and BID were the only BH3 peptides found to induce cytochrome c release from mitochondria in vitro. They are thought to trigger apoptosis by binding and therefore inactivating the antiapoptotic BCL2 relatives, whereas BID seems to promote apoptosis by activating BAX and BAK [41]. Recent studies show that there are two different subgroups in the BH3-only proteins: the first consists of BIM and BID which induce mitochondria permeabilization via BAX and BAK and are called death agonists. The second includes BAD and BIK. These proteins induce mitochondria permeabilization by opposing antiapoptotic proteins like BCL2, and they are called survival agonists. Peptides that resemble BIM and BID can directly activate BAD, whereas the rest BH3-domain-like peptides act indirectly and require the presence of BIM and/or BID that can directly activate BAD [41].

2.4. Newly Identified Proteins of the BCL2 Family Include BCL2L10 (BOO/DIVA), BCL2L12, BCL2L13 (BCL-RAMBO), BCL2L14 (BCL-G), and MAP-1 [9]. BCL2L10 is an antiapoptotic gene mapped on human chromosome 15q21.2. It encodes for the widely expressed protein BCL2L10
(BOO/DIVA) in adult human tissues, with its highest levels typically found in liver, pancreas, kidneys, brain, and lungs.

BCL2L12 gene maps on chromosome 19q13.3. It encodes for BCL2L12 protein which has a predominant molecular mass of 36.8 kDa. BCL2L12 protein contains the conserved BH2 domain of BCL2 family and a putative BH3 domain [9, 10]. There is evidence that BCL2L12 interacts with BCL-X<sub>i</sub> protein. Additionally, it bears repeated PXXP motifs and a proline rich region that is essential for the interaction with the src homology region (SH3) of tyrosine kinases, such as the protooncogenes c-Scr and c-Abl. It is worth mentioning that it is the first gene identified encoding for a protein which contains both a proline rich and a BH2 domain. The recent identification of the BAX-binding protein BIF-1 suggests a probable connecting role of BCL2L12 among the apoptotic proteins and the SH3-bearing oncoproteins [9, 10]. Recently, it was found that BCL2L12 neutralizes p53 signaling in glioblastoma [8]. One splicing variant missing exon 3 and expressing a 176 amino acid truncated protein with no BH2 homology domain has also been identified. The classic form of the BCL2L12 protein is highly expressed in the thymus, prostate, fetal liver, mammary, colon, placenta, small intestine, kidney, and bone marrow, with lower levels being expressed in all other tissues. The splice variant is highly expressed in fetal liver, spinal cord, and skeletal muscle, where it is present at higher levels than the classical form of the gene, compared to the other tissues. BCL2L12 is also overexpressed in many types of malignancies [9–13].

BCL2L13 widely expressed protein displays a significant similarity to the BCL2 family of proteins, containing all four conserved BH domains (BH1/BH2/BH3/BH4), separated by a 250 amino acid insertion with two tandem repeats rich in serine residues from the characteristic hydrophobic c-terminal membrane anchor (MA). It is characterized by proapoptotic activity and is localized to mitochondria in mammalian cells, although it appears that it induces apoptosis independently of the classical mitochondrial signaling pathways without involving BH4 or other BH domains.

BCL2L14 is another novel human proapoptotic member of the BCL2 protein family. The BCL2L14 gene maps on chromosome 12p12. It consists of six exons and undergoes alternative splicing producing three different proteins (BCL-G<sub>1</sub>, BCL-G<sub>M</sub>, BCL-G<sub>i</sub>), whose overexpression in various cell lines, such as COS-7 and HEK293T, induces apoptosis. The largest product, BCL-G<sub>i</sub> (327 amino acids), is diffusely distributed in the cytosol and displays a wide tissue distribution including bone marrow, prostate, pancreas, colon, testis, and spleen. It possesses both BH2 and BH3 domains and it can interact with BCL-X<sub>i</sub>, which blocks its proapoptotic function.

MAP-1 (modulator of apoptosis-1) is another proapoptotic BH3 domain-only protein. It interacts with BAX, BCL2, and BCL-X<sub>i</sub> and itself to form dimers in vivo and in vitro in mammalian cells. Its association with BAX through its BH3 motif seems to be responsible for its caspase-dependent proapoptotic function, which is evident upon overexpression [9]. The better studied members of BCL2 gene family are further presented in Table 2.

3. BCL2 Gene Family and ALL

A number of studies have linked impaired apoptosis and deregulation of BCL2 gene family with the pathogenesis and treatment failure in ALL. A recent study [42] indicated a high frequency of BCL2 mRNA overexpression and a relatively low frequency of BAX mRNA overexpression in ALL and AML, suggesting that altered transcription of these genes may be involved in leukemogenesis. Moreover, BCL2 expression in neoplastic cells from patients with precursor B-ALL, typical ALL and atypical ALL was found to be aberrant in 84%, 77%, and 75% of the cases, respectively, consistent with a diverse expression of BCL2 in the different types of ALL according to the stage of B-cell maturation [43]. In other words, abnormal BCL2 gene expression seems to influence the survival capacity of B-cell progenitors and contribute to leukemogenesis [44]. Additionally, Aref et al. [45] showed that the expression of BCL2 was higher in patients with ALL as compared to controls. Although there is a higher expression of BCL2 in ALL patients, clinical studies failed to correlate this with survival. Sahu and Das [46] found that there was no correlation between BCL2 expression and overall survival. Another study by Campos et al. led to similar results: high levels of BCL2 were not associated with clinical or biological characteristics in adult patients with ALL (survival of leukemic cells, outcome after intensive chemotherapy) [47]. Although ALL patients that responded to induction chemotherapy had lower BCL2 expression compared to the nonresponders, no correlation between BCL2 expression and the outcome was found.

Findings regarding lineage-dependent BCL2 expression in ALL are controversial. According to one study, T-ALL but not B-ALL blasts showed higher BCL2 expression in comparison to normal subjects. This finding could explain the poor outcome of the adult patients with T-ALL. On the other hand, another study showed that blasts from pediatric patients with T-ALL expressed lower BCL2 protein when compared to patients with B-ALL [48]. Recent data from more sophisticated techniques, such as DNA microarrays, are also informative of the role of apoptosis genes in ALL. The expression of apoptosis genes is different in the subtypes of ALL, according to lineage origin of the disease and the cytogenetic features. As far as other clinical features are concerned, CD10 positive B-ALL blasts produce higher levels of BCL2 and there is no correlation between BAX expression or BCL2/BAX ratio and other prognostic features of ALL like age, gender, karyotype, or WBC count at the time of diagnosis. Finally, lower expression of BCL2 protein among patients with ALL is observed in patients older than 45 years old and patients with an abnormal karyotype, that is, chromosome of Philadelphia or other translocations [49, 50].

The molecular events underlying the progression of T-lymphoblastic lymphoma (T-LBL) to acute T-lymphoblastic leukemia (T-ALL) remain elusive. A recent study revealed autophagy and increased levels of BCL2, SIP1, and ICAM1 in human T-LBL compared with T-ALL. Inhibition of SIP1 signaling in T-LBL cells led to decreased homotypic adhesion in vitro and increased tumor cell intravasation in vivo [51].
Table 1: This table summarizes the general findings regarding BCL2 family members in each type of leukemia.

<table>
<thead>
<tr>
<th>Type of Leukemia</th>
<th>BCL2 family members involved in disease</th>
<th>Correlation with overall survival and outcome</th>
</tr>
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<tbody>
<tr>
<td>ALL</td>
<td>High levels: BCL2, BAX, MCL-1</td>
<td>No correlation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCL-1  →  resistance to chemotherapy</td>
</tr>
<tr>
<td>AML</td>
<td>High levels: BCL2, BCL-XL, BAD, BCL2/BAX ratio especially in M4,M5,M6 subtypes and in CD34+ blasts</td>
<td>BCL2 and FAS  →  no correlation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAD and BAX, BCL2/BAX ratio  →  worse outcome</td>
</tr>
<tr>
<td>CLL</td>
<td>High levels: BCL2, BCL-W, BAD, BAX, BCL2/BAX ratio</td>
<td>Conflicting results</td>
</tr>
<tr>
<td></td>
<td>No participation of BIK and BCL-XL</td>
<td>MCL-1, BAX, BAG-1  →  resistance to chemotherapy</td>
</tr>
<tr>
<td>CML</td>
<td>High levels: MCL-1, BCL2 Low levels of BIM</td>
<td>BCL2: key protein in disease progression</td>
</tr>
</tbody>
</table>

Table 2: This table summarizes the properties of the most well-studied members of BCL2 family.

<table>
<thead>
<tr>
<th>Action</th>
<th>Mechanism of action</th>
<th>Subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL2</td>
<td>Antiapoptotic</td>
<td>Inhibits apoptosis by preservation of mitochondrial membrane integrity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(i) Outer mitochondrial membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Nuclear envelope</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) Membrane of the endoplasmic reticulum (ER)</td>
</tr>
<tr>
<td>BCL-XL</td>
<td>Antiapoptotic</td>
<td>Inhibits cytochrome c release through the mitochondrial pore that inhibits activation of the cytoplasmic caspase cascade by cytochrome c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transmembrane molecule in the mitochondria</td>
</tr>
<tr>
<td>BCL-W</td>
<td>Antiapoptotic</td>
<td>Reduced cell apoptosis under cytotoxic conditions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exclusively on the mitochondrion</td>
</tr>
<tr>
<td>MCL-1</td>
<td>Antiapoptotic</td>
<td>Short half-life, interaction with BAK1, Noxa, BCL2L11, Bcl-2-associated death promoter, PCNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mitochondria, nucleus</td>
</tr>
<tr>
<td>BAX</td>
<td>Proapoptotic</td>
<td>Release of apoptogenic factors like cytochrome c, activation of caspase cascade</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cytosol</td>
</tr>
<tr>
<td>BAK</td>
<td>Proapoptotic</td>
<td>Undergoes conformational changes to form larger aggregates during apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Integral mitochondrial membrane protein</td>
</tr>
<tr>
<td>BID</td>
<td>Proapoptotic</td>
<td>Direct activator of Bax</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cytosol and membrane</td>
</tr>
<tr>
<td>BIM</td>
<td>Proapoptotic</td>
<td>Free Bim binds to Bcl-2 or Bcl-XL and inactivates their antiapoptotic functions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free BIM in mitochondria</td>
</tr>
<tr>
<td>BAD</td>
<td>Proapoptotic</td>
<td>Dephosphorylated BAD forms a heterodimer with Bcl-2 and Bcl-xl, inactivating them and thus allowing Bax/Bak-triggered apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Free BAD in mitochondria</td>
</tr>
</tbody>
</table>

As far as other members of the BCL2 family are concerned, there are data concerning BAX and BCLXL that are worth mentioning. Studies investigating the expression of BAX protein and the probability of relapse in children with ALL are contractive. High levels of BAX protein have been associated with an increased probability of relapse [48]. However, both BAX expression levels and the BAX/BCL2 ratio were, according to another study, significantly lower in samples at relapse compared to samples at initial diagnosis. Moreover, at initial diagnosis ALL patients displayed spontaneous in vivo processing of caspase 3, whereas this was completely absent at relapse [52]. BCLXL has been shown in animal studies to demonstrate an oncogenic synergy with the c-myc oncogene towards the development of ALL [53], and in ALL pediatric patients it could represent an independent prognostic factor of overall survival [54].

Furthermore, BCL2 levels influence the sensitivity of leukemic cells to therapy [44]. According to a recent study, there is an association between lower expression levels of CASP3, CASP8, and FAS gene and a poor response to induction therapy at day 7 and prognosis in childhood ALL. The same study indicated an association between higher levels of BCL2 and white blood cell (WBC) count <50,000/mm³ at diagnosis and low risk group classification [55]. The differential regulation of pro- and antiapoptotic BCL2 family members appears to be a key event in the execution of dexamethasone-induced apoptosis in ALL cell lines and also indicates a role of these proteins in primary ALL cells [56]. Using primary lymphoblasts from ALL children during systemic glucocorticoid monotherapy and related cell lines, it was shown that a subsequent induction of the proapoptotic BH3 molecules BMF and BIM and also an unexpected
significant repression of the proapoptotic BCL2 protein Noxa take place [57]. In addition, a study of the expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood ALL indicated that MCL1 was significantly associated with prednisolone sensitivity, whereas BCL2L13 was correlated with L-asparaginase resistance and with unfavorable clinical outcome [58].

To summarize the most important findings regarding BCL2 gene family in ALL one should note the higher frequency of BCL2 mRNA overexpression and the lower frequency of BAX mRNA overexpression in ALL cases, and the diverse expression of BCL2 in the different types of ALL according to the stage of B-cell maturation. However, different studies have failed to correlate the altered expression of these genes with survival. Findings regarding lineage-dependent BCL2 expression in ALL are controversial. Furthermore, BCL2 levels influence the sensitivity of leukemic cells to therapy and it has been shown that differential regulation of pro- and antiapoptotic BCL2 family members appears to be a key event in the execution of dexamethasone-induced apoptosis in ALL cell lines.

4. BCL2 Gene Family and AML

BCL2 gene family is overexpressed in AML and seems to play an important role not only in disease pathogenesis but also in resistance to chemotherapy. The importance of BCL2 family members in AML is indicated by the expression of BCL2, BCL2L12, BCL-XL, and BAD in leukemic CD34+ cells, whereas normal promyelocytes (in non-APL AML cases) (CD34+CD33+) lack BCL2 and BCL-XL expression. A low BCL2/BAX ratio is found in >20% of CD34+ cells, in M0/M1 FAB subtypes, and in those patients with poor prognosis karyotype. Leukemic promyelocytes with the phenotype CD34+CD33+CD13—express only BCL-XL protein and not BCL2 [9, 59]. Moreover, the enhanced expression of BCL2 in CD34+ cells offers them a survival advantage and resistance to chemotherapy [59]. Finally, BCL2 expression plays an important role in maintaining a favorable antiapoptotic microenvironment for the survival of AML blasts. In vitro studies show that stable BCL2 protein levels reduce T-cell apoptosis and favour the survival of peripheral blood cells and malignant cells [60]. This microenvironment also prevents T-cell activation and proliferation by inhibition of several molecules like NF-κB, c-MYC, and pRB, that enables malignant cells escape from immune surveillance [61].

BCL2 expression levels have been associated with FAB classification, age, and cytogenetics of AML in several studies. BCL2 is not expressed in M2 FAB subtype, in contrast to M4, M5, M6 subtypes. Positive expression of BCL2 is also found in the more immature AML subtypes M0 and M1 [62]. Not only a higher BCL2 expression but also a lower CD95 (or FAS molecule) expression is found in immature FAB M0/M1 AML cells compared to the more mature M2/M5 subtypes. However, no maturation-dependent difference in BAX expression is observed [63]. On the other hand, Kornblau et al. [64] found no association between BCL2 expression and FAB classification, the percentage of blasts or cytogenetic abnormalities.

Cytogenetics is the most important predictive factor in AML and the association of apoptosis and several gene mutations or chromosomal abnormalities is interesting. The prognostically favorable chromosomal translocation t(8;21), which is commonly found in middle-aged adult AML patients, creates the AML1/ETO fusion protein and induces antiapoptotic BCL2 expression in vitro [65], but this is not confirmed in vivo [66]. Additionally, high BCL2 protein levels were detected by Western blotting in 198 patients with AML and were considered to be an adverse prognostic factor for patients with favorable or intermediate prognosis cytogenetics, for example, inversion (16), t(8;21), t(15;17). On the contrary, high BCL2 levels represent paradoxically a favorable prognostic factor for the group of patients with poor risk karyotype (e.g., 11q23, Ph+, deletion 5 and 7, or complex changes) [64]. Several studies indicate that BCL2 is a prognostic factor for AML [67]. Patients with higher BCL2 mRNA levels show lower complete remission (CR) rates and worse outcome. There is no association between remission rate or survival and BCL2 expression in patients >60 years and in patients with AML following myelodysplastic syndromes [67]. As far as other members of the BCL2 family proteins are concerned, high levels of BAD and BAX mRNA are associated with patient failure to enter CR and increased BAD or BAD and BAX expression predicted an adverse outcome regardless of the response to induction chemotherapy. Following induction chemotherapy, the presence of increased levels of BAX and BCL2/BAX ratio are independent predictors of unfavorable outcome [68]. In contrast, Ong et al. found that high BAX expression at diagnosis is correlated with significantly longer disease-free survival, event-free survival, and overall survival [69]. Abnormal expression profile of BCL-X gene is associated with recurrence in AML, but no mutation in BCL-X gene has been detected. There are two products of this gene, BCL-XL and BCL-XS. BCL-XL transcript is found in most patients at diagnosis and during relapse, but BCL-XS transcript is detected in fewer cases. There is an indication that the loss of BCL-XS expression is a prognostic factor in AML, but this requires further investigation [70].

Correlation of BCL2 family members with other proteins that appear to influence their levels of expression is also interesting. Protein kinase C (PKC) phosphorylates BCL2 protein and BAX modulates BCL2 dimerization. It was found that, in AML patients, BAX and PKCa levels are heterogeneous, do not correlate with the percentage of blasts in the sample, and their expression is similar among FAB groups with a greater range for M4. Patients with inversion 16 had lower BAX levels. No correlation with prognosis was found. Nevertheless, low BCL2/BAX and PKCaB2/BAX ratios correlate with longer survival. Patients with unfavorable cytogenetics are an exception to this finding and have the worst outcome [71].

A novel receptor tyrosine kinase, named AXL, was found to be expressed in AML specifically of monocytic origin. Thirty-five percent of AML patients express this kinase. CD34+ cells show high expression levels of both BCL2 and
AXL, suggesting a possible correlation between the two proteins. No difference in prognosis between patients positive or negative for AXL expression is found, but patients with very high levels of this protein have a dismal outcome [72].

Additionally, BCL2 expression is subjective to cytokines. Blasts produce high interleukin-1 (IL-1) levels, in the absence of exogenous growth factors. IL-1 enhances the autonomous growth of these cells [73]. Activation of IL-1 receptor leads to leukemic cell survival and poor outcome through three signaling pathways. The first is PIK3 pathway and interferes to leukemic cell survival and poor outcome through three growth of these cells [73]. On the other hand, there are some cytokines, such as interferon-γ [76], epidermal growth factor (EGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) that, under certain circumstances, have dual function by inducing not only antiapoptotic but also pro-apoptotic signals. For example, GM-CSF act by phosphorylating STAT-5, upregulating cyclin D and stimulating cell proliferation. It can also upregulate procaspase 3 levels and activate caspase 3, cleave PARP, upregulate Jak-STAT-dependent pro-apoptotic proteins like BAX, BCL2, BCL-XL, and XIAP and therefore induce cell death [77].

Another growth promoting pathway with prognostic value in AML is MEK/MAPK pathway, which is associated with an apoptosis-resistance phenotype due to its anti-apoptotic function. It is found that in primary AML cells, MAPK is constitutively active and promotes leukemic growth and survival. Therefore, patients with low antiapoptotic BAX/BCL2 ratio and constitutively active MAPK pathway have a poor prognosis, because these two factors synergistically act and enhance leukemic cell survival (Figure 2) [78].

Summarizing the most important findings concerning BCL2 family of genes in AML, BCL2 gene family is overexpressed in AML and seems to play an important role not only in disease pathogenesis, but also in resistance to chemotherapy. BCL2 expression levels have been associated in different studies with FAB classification, age, and cytogenetics of AML. Additionally, high BCL2 protein levels were considered to be an adverse prognostic factor for patients with favorable or intermediate prognosis cytogenetics and paradoxically a favorable prognostic factor for the group of patients with poor risk karyotype. As far as other members of the BCL2 family are concerned, high levels of BAD and BAX mRNA are associated with patient failure to enter CR while increased BAD or BAD and BAX expression predicted an adverse outcome regardless of the response to induction chemotherapy. The correlation of BCL2 family members with other proteins that influence their levels of expression, such as (PKC), AXL, cytokines, or MEK/MAPK pathway, is also interesting.

5. BCL2 Family of Genes and CLL

B-cell Chronic Lymphocytic Leukemia (CLL) is characterized by the accumulation of malignant clonal CD5+ CD23+ B cells.
proteins, since marked elevation of BCL2 was linked to worst outcome. Another assay [89] showed elevated expression of BCL2, MCL-1, BAG-1, BAX, BAK, and caspase 3 in contrast to absence of BCL-XL and BAD expression in cells from 58 patients with CLL. This study also indicated that higher levels of MCL-1 were associated with resistance to chemotherapy; higher levels of BAG-1 correlated with marginal failure to achieve complete remission; and high levels of BCL2 expression and a high BCL2/BAX ratio were associated with elevated WBC. Moreover, a study by Faria et al. that evaluated BCL2 protein levels before and after treatment with fludarabine indicated increased MCL1 and BAG-1 expression in fludarabine-resistant cells. Therefore, it could be assumed that BAG-1 expression might identify CLL patients who will need treatment earlier [94].

BCL2 family members have also been linked to resistance to chemotherapy in CLL. More specifically, BFL1 mRNA levels were inversely correlated with the apoptotic response to in vitro fludarabine treatment of B-CLL cells [95, 96]. In addition, an in vitro study of Pepper et al. [84] regarding resistance of CLL cells to chlorambucil treatment linked high levels of BCL2 and low levels of BAX protein expression to chemoresistance. It was observed that cells undergoing apoptosis demonstrated a remarkable elevation of BAX protein. Therefore, BAX was suggested as a critical protein in determining apoptosis in leukemic cells [91, 97]. Beyond BAX protein, MCL-1 and BAG-1 seem to be very important in apoptosis resistance. MCL-1 is associated with dismal prognosis [97], whereas BAG-1 protein is a very important protein involved in apoptosis resistance, since its expression is very high in viable cells after fludarabine incubation [94].

The relationship between BCL2 and other proteins in CLL is also interesting. BCL2 and p53 expression seem to be inversely related. There are indications that P53 protein overexpression downregulates BCL2 expression in a subgroup of B-CLL patients, but this assumption needs further investigation [98]. Another interesting point is that BCL2 is often overexpressed early in the course of the disease, whereas P53 is found in advanced stages [99]. However, P53 mRNA expression is similar between B-CLL cells and normal cells, while P53 overexpression is considered the result of posttranscriptional modification [91].

As far as the relationship of BCL2 and cytokines is concerned, interleukin-4 and interferon-γ are found to protect CLL cells from apoptosis, since high levels of IL-1 are associated with low cellular expression of BCL2 protein [100]. NF-κB is also found to inhibit apoptosis. NF-κB is a dimeric nuclear transcription factor. CLL cells exhibit high levels of NF-κB compared to normal cells [101], and in vitro death of B-CLL cells is accompanied with the loss of NF-κB and PI3K/AKT activities [102]. Moreover, specific inhibition of Akt induced extensive apoptosis of CLL cells, which was associated with both a rapid loss of MCL1 through
proteasomal degradation and increased expression of p53. CLL clones consistently contain activated Akt which plays a pivotal role in maintaining cell survival. Inhibition of the Akt pathway may be of potential value as a novel therapeutic strategy in the disease [103]. These examples show that the interaction of apoptosis proteins with factors like cytokines or transcription factors involved in pathogenesis of leukemias is important for the understanding of the complex biology of these malignancies.

To summarize the most important findings concerning the role of BCL2 family in CLL, we should highlight that malignant CLL B cells overexpress BCL2, possibly through downregulation of miR-15a and miR-16-1. These are located in a genomic region which is frequently deleted in CLL. miR-15a and miR-16-1 expression is inversely related to BCL2 expression in CLL and both miRNAs negatively regulate BCL2 at a posttranscriptional level. The role of epigenetic alterations is also under investigation in CLL. In the majority of patients, the promoter region for BCL2 is hypomethylated, which may contribute to increased transcription and BCL2 protein expression. Important findings of other studies include a link of marked elevation of BCL2 to worst outcome, association of higher levels of MCL-1 with resistance to chemotherapy and association of higher levels of BAG-1 with failure to achieve complete remission. Moreover, BCL2 family members have been linked to resistance to chemotherapy of CLL patients (Table 1).

6. BCL2 Gene Family and CML

BCR/ABL affects a number of molecular pathways, including apoptosis. Dysregulation of the expression of BCL2 protein seems to play a role in disease progression as shown in mouse models. It is overexpressed in CML cells and acts synergistically with BCR/ABL in inducing blast crisis. BCL2 is more important than c-MYC or RAS oncoproteins in the transformation of chronic to blastic phase [104]. In vivo studies showed that BCL2 expression is restricted to the lymphocyte and blast subpopulation cells at the chronic phase, whereas it is higher in the accelerated and the blastic phase. However, other in vivo studies show that c-MYC is more important than BCL2 protein in disease progression since (a) it is expressed in more immature cells, (b) its unregulated expression can inhibit myeloid differentiation, and (c) its levels are increased in the peripheral blood blast cell subpopulation in the accelerated and blastic phase [105, 106]. These studies also suggest that the expression of apoptosis oncoproteins such as BCL2, BAX, FAS and caspase-3 is not associated with the three different phases of CML, since no phase-related predominance of their levels was found.

BCR/ABL activates several signaling pathways, such as PIK3, STAT, Ras, and NF-kB which influence the expression of members of the BCL2 protein family. There are several examples of this: AKT is a serine/threonine kinase which regulates survival signals in response to several internal and external signals. It becomes activated through the PIK-3 signaling pathway and its role focuses on inhibiting cell death by two ways: the first is the inactivation of pro-apoptotic proteins like BAX or caspase-9, and the second is the inactivation of NF-kB and eventually BCL-XL. Another example is STAT proteins, which are cytoplasmic proteins activated by phosphorylation after recruitment to an activated receptor complex. When active STAT proteins translocate to the nucleus, they bind to specific DNA response elements and induce the expression of STAT-regulated genes. The latter play an important role in haemopoiesis and in haemopoietic cell function, such as in Th1 or Th2 response in lymphocyte function. STAT 1, 3, and 5 control cell cycle and apoptosis and STAT 5 is found constitutively active in CML patients [107]. The fusion BCR/ABL tyrosine kinase activates the PIK-3/AKT pathway and the result is either phosphorylation of BAD protein at its serine residues, or enhanced expression of BCL-XL protein. Overexpression of BCL-XL can be accomplished through the activation of the STAT-5 protein. BAD and BCL-XL are considered to be the most important regulators of apoptosis in CML [108, 109].

It has also been shown that high levels of BCR/ABL expression are responsible for the prevention of the early translocation of the pro-apoptotic proteins BAD and BAX from the cytosol to the mitochondrion following a cytotoxic signal, explaining the resistance of cells that express high BCR/ABL levels to cytotoxic drugs [110].

Another member of the BCL2 superfamily, the BH3-only pro-apoptotic protein BIM is considered to be an important target in CML cells, as its downregulation is associated to the survival of leukemic cells. It is shown in mouse models that BIM is an essential cytokine-dependent regulator of normal haemopoiesis [111]. BCR/ABL tyrosine kinase reverses the induction of BIM mRNA caused by cytokine deprivation in hematopoietic progenitor cells, and it also down-regulates BIM expression in human CML cell lines. Therefore BIM is considered to be an important downstream target in CML cells that express BCR/ABL. The most likely pathway involved in BIM mRNA down-regulation by BCR/ABL is considered to be PIK-3 pathway [112].

Antia apoptotic protein MCL-1 is reported to be another interesting target in CML, since BCR/ABL expressing cells show higher expression of MCL-1 mRNA and MCL-1 protein, and the use of BCR/ABL inhibitor imatinib results in the decrease of MCL-1 expression in CML cell lines [113].

In summary, in CML BCR/ABL is the key regulating mechanism in disease pathogenesis and affects many signaling pathways including apoptosis. Nevertheless, BCL2 family proteins do not seem to play a determining role in CML.

7. BCL2 Family Proteins and Leukemia Treatment

Since BCL2 family proteins are pivotal regulators of apoptotic cell death and given their deregulation in acute and chronic leukemias, the concept of manipulating their function towards enhancing their antitumor effects seems a reasonable strategy in the design of antileukemic therapeutic agents.
Impaired apoptosis related to overexpression of BCL2 protein, which is observed in approximately 76% of patients with CLL, is implicated in the resistance to chemotherapy. BCL2 antisense oligonucleotides like oblimersen are used in order to downregulate BCL2 oncprotein in several hematopoietic malignancies, including CLL, multiple myeloma and non-Hodgkin lymphoma [114–116]. In order to achieve better clinical results, these agents are used in combination with traditional drugs like fludarabine or cyclophosphamide and monoclonal antibodies like rituximab in several clinical trials. While the combination of chemotherapeutic agents (fludarabine, cyclophosphamide and rituximab) has good results in patients with CLL [117], the addition of BCL2 antisense oligonucleotides appears promising [118].

Oblimersen has also been administered during induction and consolidation treatment in untreated elderly AML patients. After 72-hour oblimersen infusion, Bcl-2/ABL mRNA copies were decreased compared with baseline in patients that achieved CR, whereas it was increased in nonresponders. Changes in Bcl-2 protein showed a similar trend. The degree of Bcl-2 downregulation may correlate with the response to therapy [119]. Another study demonstrated that oblimersen can also be administered safely with FLAG chemotherapy, downregulating its target, Bcl-2, in previously untreated high-risk AML patients (i.e., age at least 60 years) [120, 121]. Furthermore, expression levels of BCL2 and BCL2L12 were found to be significantly altered during apoptosis induced by widely used chemotherapeutic drugs in human leukemia cells, supporting their usefulness as biomarkers to predict response to therapy [9, 14, 122–128].

Studies investigating BCL2 family inhibitors, such as ABT-737 and ABT-263, in ALL are really interesting. ABT-737 is a pan-BCL2 inhibitor that has a wide range of single agent activity against ALL cell lines. Furthermore, ABT-737 has been shown to enhance the activity of vincristine, L-asparaginase and dexamethasone against ALL cells. On the other hand, ABT-263 is a potent, orally bioavailable BAD-like BH3 mimic that induces complete tumor regressions in xenograft models of ALL. A relationship between MCL1 expression and resistance to ABT-737 has been reported, which is abolished with the use of a synthetic cytotoxic retinoid, N-(4-hydroxyphenyl) retinamide, that phosphorylates and inhibits MCL1 [129–132].

Interestingly, CLL cells were previously reported to be highly sensitive to BCL2 inhibition and treatment with ABT-737. Moreover, recent data support that, although structurally similar and exhibiting similar binding affinities to antiapoptotic BCL2 family proteins, ABT-263 is less potent than ABT-737 in inducing apoptosis in CLL cells. Furthermore, binding of ABT-263 to albumin seems to markedly increase the concentration of drug required to induce apoptosis and clear CLL cells from the blood in vivo [133–138].

The use of BCL2 inhibitors in the treatment of leukemias is promising, either alone or in combination with classical treatments. Given the fact that therapeutic interventions in leukemias are often not adequate nor successful, new therapeutic plans are more than welcome.

8. Conclusions

BCL2 protein family plays an important role in regulating the cellular program of apoptosis. Normal cellular homeostasis appears to be dependent on the balance between pro- and antiapoptotic members of BCL2 family. BCL2 is overexpressed in almost all types and subtypes of leukemia, indicating the importance of this molecule in disease pathogenesis and evolution. BCL2 is the most well studied member of the family, but evidence shows that other BCL2 related family proteins like BAX and MCL-1 are important as well. More specifically, MCL-1 is related to almost all leukemias that show resistance to chemotherapy and bad prognosis. Expression levels of BCL2 and BCL2L12 were altered during apoptosis induced by widely used chemotherapeutic drugs in human leukemia cells. These molecules may not be used as disease markers in most cases, but their importance lies in (a) explaining drug chemoresistance and (b) in the effort to design new agents with a greater specificity. Further research should focus on the role of BCL2 family members in leukemogenesis. It is clear that clinical studies are only beginning to assess the expression of BCL2 family members. Further research is more than valuable in the understanding of the importance of this gene family in leukemias.

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Review Article

Engineered T Cells for the Adoptive Therapy of B-Cell Chronic Lymphocytic Leukaemia

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B-cell chronic lymphocytic leukaemia (B-CLL) remains an incurable disease due to the high risk of relapse, even after complete remission, raising the need to control and eliminate residual tumor cells in long term. Adoptive T cell therapy with genetically engineered specificity is thought to fulfill expectations, and clinical trials for the treatment of CLL are initiated. Cytolytic T cells from patients are redirected towards CLL cells by ex vivo engineering with a chimeric antigen receptor (CAR) which binds to CD19 on CLL cells through an antibody-derived domain and triggers T cell activation through CD3ζ upon tumor cell engagement. Redirected T cells thereby target CLL cells in an MHC-unrestricted fashion, secret proinflammatory cytokines, and eliminate CD19+ leukaemia cells with high efficiency. Cytolysis of autologous CLL cells by patient’s engineered T cells is effective, however, accompanied by lasting elimination of healthy CD19+ B-cells. In this paper we discuss the potential of the strategy in the treatment of CLL, the currently ongoing trials, and the future challenges in the adoptive therapy with CAR-engineered T cells.

1. Introduction

B-cell chronic lymphocytic leukaemia (B-CLL) is the most common leukaemia in the western hemisphere with escalating incidence. Although treatment of B-CLL has achieved significant progress during the last years based on the use of nucleoside analogs, monoclonal antibodies, and bone marrow transplantation [1–5], the disease is rarely cured, even in those patients with complete molecular remission [6–8]. Interest is therefore growing in activating the immune system, by single agents or in combination with chemotherapy, to control the disease. The application of monoclonal antibodies, including anti-CD20 and anti-CD52 antibodies, substantially improved response rates and progression-free survival [9]. Allogeneic haematopoietic stem cell transplantation induced a significant T cell-mediated graft-versus-leukemia response and durable remissions in a subset of patients with chemotherapy-refractory B-CLL [4, 5]. Genetically modified malignant cells enhanced the anti-tumor response [10, 11]. The isolation of B-CLL-reactive T cells from patients with long-lasting tumor regression [12] sustained the concept that adoptive cell therapy with CLL specific T cells may be successful in controlling the disease. Advances in genetic engineering of a recombinant T cell receptor (TCR) and of a chimeric antigen receptor (CAR) provide the technology to modify T cells ex vivo with predefined specificity for use in specific cell therapy. This paper summarizes recent experiences with CAR-engineered T cells for the use in adoptive therapy of B-CLL.

2. Redirecting T Cells towards B-CLL

Tumor-specific T cells can be genetically engineered in large quantities by engrafting with a recombinant TCR or alternatively with a CAR of predefined tumor specificity. In contrast to the TCR, the CAR consists of one trans-membrane polypeptide chain; the extracellular domain is composed of a single chain fragment of variable region (scFv) antibody for binding; the intracellular domain provides T cell activation
through the CD3ζ endodomain upon antigen engagement [13–15]. The "T-body" concept thereby combines the power of the targeting antibody with the effector mechanisms of cytolytic T cells [14, 16]. The CD3ζ molecule contains three immunoreceptor tyrosine-based activation motifs (ITAMs) which are phosphorylated to initiate T cell activation; the first and third ITAMs additionally cause apoptosis. Inactivation of these ITAMs by mutation consequently decreased apoptosis and enhanced survival of redirected T cells upon CAR signalling [17–19]. By using an antibody for target recognition, CAR-redirected T cells bypass the MHC haplotypes of the individual patients and undergo T cell activation in an MHC-unrestricted fashion. CAR-modified T cells can thereby be redirected towards antigens of various structure and composition. Alternatively, T cells can be redirected in an MHC-restricted fashion by using antibody-derived binding domains with TCR-like specificity [20–22]. Genetically engineered with a CAR, modified T cells are amplified ex vivo to numbers suitable for adoptive cell therapy and administered to the patient upon preconditioning. Preclinical and clinical data, which are discussed below, provide strong evidence that peripheral blood T cells from B-CLL patients can successfully be redirected to initiate an effective antitumor response even in advanced stages of the disease.

Success of adoptive therapy with modified T cells, however, depends upon efficient and durable expression of the transgenic CAR. Mostly murine y-retroviral vectors are used to modify T cells taking advantage of its small size, stable transgene integration, and the ability to generate vector batches of high titres. Up to 10^{10} gene-modified T cells can be obtained by retroviral transduction in a Good Manufacturing Procedure-conform manner. However, the strong mitogenic stimulus required for retrovirus transduction may result in T cells which have undergone excessive replication and are suboptimal for an efficient anticancer response. Lentiviral vectors, in contrast, require cytokine prestimulation of recipient cells which generates T cells with a less differentiated phenotype. Recent developments such as the incorporation of a measles virus envelope protein into viral particles allow transductions with less or without T cell stimulation. Alternatively, naked plasmid DNA or RNA by electroporation is used to obtain CAR-modified T cells; the DNA transfection efficiency is low requiring extensive T cell amplification prior-clinical application. Recent developments in transposon technology suggest that these technologies may also be amenable to clinically modifying T cells in the near future.

3. CD19 Is a Good Target for a Redirected T Cell Attack of B-CLL Cells

The target for CAR-mediated tumor cell recognition is crucial for the therapeutic success, and several issues have to be considered. The target must be expressed on the cell surface of the tumor cell to be recognized by CAR-modified T cells. Most "tumor-associated antigens", however, are self-antigens and not exclusively expressed on tumor cells but on cells of healthy tissues as well. Malignant cells moreover show extreme flexibility, loose target antigen expression, and the tumor may recover despite an ongoing immune response. Ideal would therefore be a target molecule which is causally associated with the malignant phenotype since antigen-loss tumor cell variants which are not furthermore recognized by CAR-redirected T cells would lose their malignancy and enter senescence. To selectively target B-CLL cells, CD19 seems to be a good target since it fulfills some although not all of the above-cited criteria. CD19 is physiologically expressed on B-lineage cells of almost all stages, from the pro–B–cell to mature B-cell, and is in particular absent from plasma cells, hematopoietic stem cells and other tissues. CD19 decreases the threshold for B-cell activation by assembling with the antigen receptor which enables B-cells to respond to different antigens in a specific and sensitive manner. B-lineage leukemia cells including B-CLL express CD19 at high levels, even during progression of the disease. Targeting CD19 is therefore ideal for redirected therapy of B-CLL, and no myelosuppression, apart from B-cell depletion, or other organ toxicities is expected due to the restricted CD19 expression. CD20 is expressed by nearly the same cells as CD19; targeting CD20 may be an alternative, however, with the same expected side effects.

The receptor tyrosine kinase-like orphan receptor 1 (ROR1) may be an alternative target for eliminating B-CLL cells [23]. Compared to CD19, ROR1 has the advantage that it is not expressed on normal B-cells. ROR1 is an oncofetal antigen and expressed by undifferentiated embryonic stem cells but not by major adult tissues apart from low levels in adipose tissue and at an early stage of B-cell development. CAR-modified T cells with specificity for ROR1 eliminate B-CLL cells but not mature normal B-cells. The expression on some normal tissues, however, suggests potential toxicity of ROR1-specific T cells.

4. The Car Redirected T Cell Antitumor Response Benefits from Costimulation

According to the “two-signal paradigm,” T cells require in addition to the TCR/CD3 signal (“signal 1”) a second signal called costimulation or “signal 2” to sustain pro-longed activation, to improve proliferation, to increase cytokine secretion, and to avoid anergy. CD28 costimulation increases bcl-2 and bcl-xL expression [24] and thereby improves resistance towards activation-induced cell death by preventing apoptosis. To provide CD28 costimulation along with CAR signalling, the CD3ζ endodomain was combined with the CD28 costimulatory domain in a so-called “second generation” CAR with combined CD28-CD3ζ signalling moiety [25, 26]. There is increasing support for the use of alternative costimulation, for example, via 4-1BB (CD137) or OX40 (CD134), both members of the CD28 family. Each of these costimulatory domains modulates the redirected effector functions in a different fashion including cytokine secretion, proliferation, and prevention from activation-induced cell death [27, 28]. CD28 costimulation mediates IL-2 secretion [26, 29, 30]; without simultaneous costimulation through the native B7-CD28, 4-1BB, and OX40 costimulation do not induce IL-2 although both increasing IFN-γ secretion. CD28-CD3ζ CAR stimulated T cells thereby indirectly increase antitumor
efficacy by sustaining survival, proliferation, and recruiting other activated bystander T cells in the tumor environment. OKT40 and 4-1BB costimulation, however, is superior in preventing activation-induced cell death and in sustaining T cell survival. These observations lead to CARs with two costimulatory domains to further improve T cell potency and persistence by augmenting the levels of anti-apoptotic proteins [31]. Combining CD28, OKT40 and CD3ζ as well as CD28 with 4-1BB and CD3ζ induced superior T cell expansion and cytokine secretion. 4-1BB-CD3ζ alone, however, is superior in antitumour activity in vivo compared to CD28-CD3ζ or CD28-OKT40-CD3ζ CARs [32]. CAR-mediated T cell cytotoxicity as revealed by in vitro short-term assays, however, is independent of costimulation. Taken together combining costimulatory domains with CD3ζ allows for specifically modulating T cell effector functions in order to sustain a long-lasting antitumour response.

Costimulation, moreover, provides benefit when T cells enter the immune-suppressive environment of tumors. Immune repression, mostly more pronounced in solid tumors, is mediated infiltrating suppressive cells and by tumor cells itself through repressive cytokines or the altered metabolism which results in the depletion from essential nutrients or the accumulation of immunosuppressive metabolites in the microenvironment. Metabolites with suppressive activity include indolamine-2,3-dioxygenase (IDO), arginase, inducible nitric oxide synthetase (iNOS), and lactate dehydrogenase (LDH)-A, all repressing the adaptive immune response. One of the repressive cytokines is TGF-β expressed by a variety of tumor cells on the cell surface and secreted into the tumor environment and expressed by repressive immune cells. CD28 costimulation counteracts repression of T cell proliferation by TGF-β thereby improving the antitumor response of redirected T cells [33]. Treg cells infiltrating the tumor mass repress a CAR-redirected T cell antitumor response [34]. Since Treg cells require IL-2 for survival and repression effector, T cells equipped with a CAR which is deficient in inducing CD28-mediated IL-2 secretion exhibit a superior antitumor response in presence of Treg cells [35]. Taken together, appropriate costimulation can, at least partially, counteract tumor-mediated immune repression.

5. In Vitro Evidence for the Efficacy of CAR-Redirected T Cells toward B-CLL Cells

T cells engineered with a CD19-specific CAR with CD3ζ or combined CD28-CD3ζ signalling domain are currently explored for targeting B-CLL cells. Both CARs can efficiently be expressed on peripheral blood T cells and activate T cells in a CD19-dependent fashion indicated by increase in proinflammatory cytokines including IFN-γ (Figures 1(a) and 1(b)). CAR-driven T cell activation is antigen-specific since unmodified T cells or T cells with a CAR of irrelevant specificity are not activated upon binding to CD19+ cells. In contrast to CD3ζ CAR signalling, T cells triggered by the CD28-CD3ζ CAR, furthermore, secrete IL-2 (Figure 1(c)). Anti-CD19 CAR T cells from healthy donors exhibit cytolytic activity towards B-CLL cells in vitro (Figure 1(d)). The redirected cytolytic activity in a short term in vitro cytotoxicity assay is not substantially higher by CD28-CD3ζ compared to CD3ζ CAR T cells which is in accordance to other reports using CARs of different specificities [29]. The efficacy in both the CD3ζ and CD28-CD3ζ CAR-redirected cytolysis does not furthermore increase with the level of CD19 expression on B-CLL cells (Figure 1(e)) implying that the CD19 levels on B-CLL cells are high enough to cross-link the anti-CD19 CAR for synapse formation and signalling. Anti-CD19 CAR-modified T cells, however, do not distinguish between normal B-cells and B-CLL cells leading to the elimination of normal B-cells as well (Figure 1(f)).

Peripheral blood T cells from B-CLL patients can be redirected towards autologous B-CLL cells. CAR-engineered T cells increase IFN-γ secretion when engaging autologous B-CLL and additionally secrete IL-2 when stimulated by the CD28-CD3ζ CAR (Figure 1(g)). Patient’s T cells efficiently lyse autologous B-CLL cells in a short term in vitro assay.

B-CLL cells are resistant to Fas-mediated cell death [36] rising the question how CAR-engineered T cells execute lysis of B-CLL cells. Basically, cytolytic T cells can lyse target cells by a granzyme/perforin-dependent mechanism, which requires Ca2+ release, via Fas/FasL interaction or via TNF-α. The cytolytic activity of CD19-specific CAR T cells is blocked by EGTA while nearly unaltered upon blocking Fas and TNFα (Figure 2) indicating that cytolysis is predominantly executed by a granule-dependent pathway to overcome Fas resistance of B-CLL cells.

High-serum thymidine kinase-1 levels identify a subgroup of patients with CLL at high risk for disease progression [37]. Thymidine kinase-1 is involved in the salvage pathway for DNA synthesis, found in the cytoplasm of dividing cells and is absent in resting cells [38]. Cycling tumor cells are more susceptible to a redirected T cell attack compared to resting cells. Accordingly, B-CLL cells with high proliferative capacities from patients with high-serum thymidine kinase-1 levels, that is, >10 U/L, are more efficiently eliminated by redirected T cells in vitro than B-CLL cells from patients with low-thymidine kinase-1 levels (Figure 3). Susceptibility to a CAR-redirected T cell attack is not correlated with other clinical prognostic factors like mutation of the immunoglobulin heavy chain variable region (IgVH) locus. B-CLL cells in the population of blood mononuclear cells from patients of younger age are more efficiently eliminated than cells from >70 year patients. This is likely due to the fact that regulatory T (Treg) cells increase in numbers in the blood with progression of the disease and thus with increasing patient’s age [39]. Consequently, depletion from Treg cells accordingly increased T cell-mediated elimination of B-CLL cells.

6. Murine Models Demonstrate Successful Targeting of CD19+ Leukaemia Cells In Vivo

The CAR-redirected T cell response towards CD19+ target cells was extensively studied in murine models. In most studies, immunodeficient mice were engrafted with primary human CD19+ leukaemia cells or cell lines before adoptive transfer of engineered T cells [36, 40, 41]. Other models use murine tumor cells which were equipped with the human target antigen and attacked by engineered murine T cells.
**Figure 1:** Continued.
Figure 1: Anti-CD19 CAR redirects engineered T-cells towards CD19+ B-CLL cells. (a) Schematic diagram depicting the modular composition of the recombinant CD19-specific chimeric antigen receptor (CAR). scFv: single chain fragment of variable region antibody; IgG1: hinge-CH2CH3 domain of IgG1; TM: transmembrane domain; CD3ζ: intracellular domain of CD3ζ; CD28: intracellular domain of CD28. (b) Peripheral blood T cells were transduced by retroviral gene transfer to express the respective anti-CD19 CAR. CAR expression was monitored by flow cytometry upon staining with a FITC-conjugated anti-CD3 antibody and a PE-coupled antihuman IgG1 Fc antibody directed against the extracellular IgG1 CAR domain. (c) CAR-mediated T-cell activation was monitored by recording IFN-γ and IL-2 secretion upon coincubation of anti-CD19 CAR-engineered T cells (5 × 10^5 cells/well) with primary CD19+ B-CLL cells (1 × 10^5 cells/well). After 24 hrs, IFN-γ and IL2 in the coculture supernatant were determined by ELISA. (d) Anti-CD19 CAR-engineered T-cells (10^5 cells/well) from healthy donors were coincubated with B-CLL cells (10^5 cells/well), and the viability of B-CLL cells was monitored by a flow cytometry-based assay after 24 hrs. B-CLL cells were identified by staining for CD5 and CD19, T cells by staining for CD3, dead cells by staining with 7-AAD. The number of viable B-CLL cells was determined using “Rainbow beads” (Becton Dickinson) as standard. Spontaneous cytolysis is recorded by incubation of B-CLL cells without T cells (−). CAR-redirected cytolysis was calculated in comparison to cytolysis by T cells without CAR (w/o). (e) The efficacy in specific cytolysis by anti-CD19 CAR-engineered T cells (data from D) is independent of the CD19 expression level on B-CLL cells as determined by mean fluorescence intensity of CD19 staining. (f) Anti-CD19 CAR-engineered T cells engineered with anti-CD19 CAR with CD3ζ and CD28-CD3ζ signalling domain, respectively, were incubated with allogeneic peripheral blood B-cells (purity > 95%) (1 × 10^5 cells/well each). B-cells alone (−) and B-cells mixed with un-modified T cells without CAR (w/o) were incubated as control. Specific cytotoxicity towards B-cells was recorded after 24 h by a flow cytometry-based assay. T-cells were identified by CD3 staining, B-cells by CD5 and CD19 staining, apoptotic cells by 7-AAD staining. (g) CAR-engineered T cells from B-CLL patients lyse autologous B-CLL cells. T cells from B-CLL patients (n = 3) were engineered with the CD3ζ and CD28-CD3ζ CAR, respectively, both with specificity for CD19, and coincubated with autologous CD19+ B-CLL cells (each 1 × 10^5 cells/well) for 24 hrs. Cytokine release into the culture supernatant was determined by ELISA. CAR-engineered patient’s T cells showed improved cytotoxicity towards autologous B-CLL cells, indicated by decrease in B-CLL cell viability, compared to nonmodified T cells. Data represent the mean ± standard error of mean. Statistic calculations are based on Student’s t-test; ** represents P < 0.001.
Although these studies demonstrated the elimination of malignant cells from immunodeficient mice, they do not reflect the clinical situation of the immunocompetent patient who is tolerant to CD19 self-antigen and experienced a long adaptation to the growing tumor cell mass.

A most recently reported model takes these issues into account [18]. Anti-CD19 scFv-CD28-CD3ζ CAR engineered syngeneic T cells which target murine CD19 were adoptively transferred to immune competent mice which expressed CD19 on healthy B-cells and on a transplanted, syngeneic lymphoma. Along with the antilymphoma activity anti-CD19 CAR-engineered T cells exhibited profound and long-lasting activity against healthy CD19+ B-cells without recovery up to 200 days after adoptive T cell transfer. This is in accordance with clinical experience where lymphoma patients treated with anti-CD19 CAR T cells showed lasting and complete depletion of B-cells [42]. In the clinical context, B-cell depletion is manageable and can, at least partially, be alleviated by immunoglobulin replacement.

An alternative model was described by Cheadle et al. [43]. In contrast to the above-described model, T cells were engineered with a CD3ζ CAR without CD28 costimulatory domain. CAR-modified T cells showed a profound antilymphoma effect in the syngeneic mouse accompanied by temporary depletion of healthy B-cells. Whether the difference depends on the presence or absence of CD28 costimulation in the context of CAR-mediated T cell activation or on the different CD19-binding domains remains to be explored.

### 7. Lymphodepletion Improves Antitumor Efficacy of Redirected T-Cells

The immunocompetent mouse model [18], moreover, indicated the crucial role of preconditioning for antilymphoma efficacy of adoptively transferred T cells. When CAR-modified T cells were transferred into mice without prior total body irradiation, only marginal antilymphoma activity was observed with minimal improvement in survival compared to untreated mice. In contrast, all mice survived when irradiated prior to adoptive T cell transfer. These and other data confirm that lymphodepletion before adoptive T cell therapy is crucial for antitumor efficacy [44, 45]. Increasing preconditioning improves antitumor efficacy of adoptive T cell therapy [46]. The lymphodepleting regimen is by itself not sufficient to elicit antitumor responses, their benefit obviously results from the produced environment which favours persistence and expansion of the adoptively transferred T cells.

Several mechanisms may contribute to the observation [47]. T cell homeostasis in number and function under normopenic conditions in a normal host is tightly controlled by multiple redundant mechanisms to protect the host from uncontrolled immune responses against pathogens and from harmful autoimmunity. Inducing lymphopenia by treatment with cyclophosphamide and fludarabine or by total body irradiation is assumed to provide a selective advantage to adoptively transferred T cells. Nonmyeloablative treatment eliminates regulatory T cells and other repressive cell populations and eliminates immature dendritic cells which anergize T cells. Cell populations competing for the same survival and stimulatory cytokines, like IL-2, IL-7, IL-15, and IL-21, are eliminated as well (“cytokine sinks”) which enhances the availability of those factors to adoptively transferred T cells. Under these conditions of an induced proinflammatory environment adoptively transferred T cells have selective advantage to undergo homeostatic expansion and to improve effector functions. In this context, it is worthwhile to note that the day of adoptive T cell transfer in relation to preconditioning seems to be crucial since T cells given at day 2 after stem cell transplantation show superior amplification and persistence than cells given at later days [48]. Safe nonmyeloablative lymphodepleting preconditioning protocols are developed and are currently used in adoptive T cell trials as summarized in Table 1.

Experimental data indicate that increased intensity lymphoablation by high-dose total body irradiation given together with haematopoietic stem cell transplantation further improves efficacy of adoptive T cell therapy [50]. With intensified ablation, the levels of pro-inflammatory cytokines increased and tumor treatment efficacy improved. Increased intensity of preconditioning, however, goes against the current trend in hematopoietic stem cell transplantation to reduce treatment-related adverse events by nonmyeloablative
Figure 3: ZAP70-positive B-CLL cells are more efficiently eliminated by CAR-redirected T cells in vitro. The efficacy in specific cytolysis by CAR-redirected T cells (data from Figure 1(d)) was plotted against (a) serum thymidine kinase-1 levels (<10 U/L versus >10 U/L), (b) mutated versus unmutated status of the immunoglobulin heavy chain variable region of B-CLL cells, and (c) patient’s age (<70 yrs versus >70 yrs). Closed circles represent CD3ζ CAR, open circles CD28-CD3ζ CAR-mediated B-CLL killing. Depletion from CD25^{high} Treg cells improves redirected cytolysis of B-CLL cells as exemplarily shown for two patients. Statistic calculations were performed using Student’s t-test, \( * P < 0.05; ** P < 0.001 \).
Table 1: Phase 1 clinical trials using anti-CD19 CAR modified T cells for the treatment of B-cell malignancies (updated and adapted from [49]).

<table>
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<th>Disease</th>
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<th>Preconditioning</th>
<th>Status of trial</th>
<th>Clinical trials.gov identifier</th>
<th>Clinical trial centre</th>
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<td>none versus cyclophosphamide</td>
<td>recruiting</td>
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<td>Baylor College of Medicine</td>
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<tr>
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<tr>
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</tr>
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</table>

8. Clinical Trials with CAR-Engineered T Cells in the Adoptive Therapy of B-CLL

A panel of anti-CD19 CARs were characterized by means of laboratory methods during the last decade. Good Manufacturing Processes (GMP) conform procedures are established to modify T cells from the peripheral blood ex vivo with a CD19-specific CAR and to subsequently expand engineered T cells to numbers sufficient for adoptive cell therapy [51]. The processes allow generating clinically relevant doses of CAR-engineered T cells in about 2-3 weeks in a semiclosed culture system. After expansion, the diversity of the TCR repertoire is preserved, and the CD4:CD8 T cell ratio did not change or rather increased. Modification of T cells by DNA transfection turned out to be less efficient compared to viral transduction [52].

CAR-modified T cells are currently explored in a number of phase I trials for the therapy of B-CLL and other B-cell malignancies (Table 1). Patients are receiving CAR-engineered T cells in advanced stages of the disease, and the optimal approach is currently being explored, in particular the optimal dose and the intensity of lymphodepletion [49]. Lessons learnt from pre-clinical animal studies moreover suggest superior antitumor performance of CD28 and 4-1BB costimulatory CARs.

Most severe side effects reported for trials with adoptively transferred CAR T cells were not treatment related; some, although manageable, required temporary discontinuation of therapy and protocol modification. In a phase I trial, however, a treatment-related death of an extensively pretreated CLL patient occurred shortly after lymphodepletion and infusion of CD28-CD3ζ CAR T cells at a total dose of $3 \times 10^7$ T cells per kg [53]. The patient was in the second dose escalation cohort. In contrast to patients in the first cohort who received the same number of T cells without developing significant adverse events, this patient was the first to receive cyclophosphamide pretreatment for lymphodepletion. The syndromes patient developed immediately after T cell transfer are consistent with an inflammatory cytokine cascade after lymphodepletion which gave the clinical picture of renal failure and adult respiratory distress syndrome. Although toxicity did not appear to be directly caused by the modified T cells, investigators modified the protocol by reducing T cell dose and administering T cells in split doses to improve safety. Two patients treated on this trial under the modified protocol tolerated treatment without notable toxicities.

There is a clear correlation between persistence of modified T cells and clinical outcome [54]. To improve T cell persistence, Epstein Barr virus- (EBV-) specific T cells are used assumed that those T cells receive optimal and continuous costimulation through their native TCR resulting in longer survival and redirected cytotoxicity-mediated through their CAR. Triggered by chronic EBV infection, CAR-modified EBV-specific T cells, like other virus-specific T cells, may be superior providing a long-lasting antitumor response [54]. A recently initiated trial at Baylor College of Medicine (NCT00608270) aims to address this issue for the treatment of B-cell malignancies. Alternative strategies avoiding the need to isolate EBV-specific T cell clones from strategies. Myeloablation-associated toxicity needs therefore be titrated against the benefit of improved antitumor efficacy.
The impact of the individual CAR domains on redirected T cell activation was recently discussed in detail [58]; we here focus on particular issues related to anti-CD19 CARs. The CAR-targeting domain has significant impact on redirected T cell activation. The anti-CD19 scFvs currently used are derived from monoclonal antibodies of different affinities targeting different epitopes of CD19. Since both affinity and epitope impact CAR-mediated T cell activation, the optimal combination needs to be identified.

Most binding domains were derived from murine antibodies. The generation of human antiidiotype antibody responses was reported in some trials including the generation of anti-idiotypic antibodies which blocked CAR-mediated antigen recognition [59,60]. An antibody immune response against modified T cells limits the persistence of modified T cells; CARs with humanized domains will therefore be beneficial.

A “spacer” domain between the scFv and the transmembrane domain improves binding to antigen by overcoming steric hindrance in attaining sufficient proximity to the target antigen. Most CARs therefore harbour the human IgG1 hinge-CH2CH3 region between the scFv and transmembrane domain. The same spacer, however, may lead to nonspecific activation of effector cells through interaction with Fc receptors which can be, at least partly, prevented by a modified Fc region [61]. Other spacer regions like CD8 can alternatively be used.

While CAR redirecting T cells clearly exhibit antigen-specific and dose-dependent recognition of target cells, engineered T cells sometimes produce small but potentially crucial amounts of pro-inflammatory cytokines, such as IFN-γ, even when the targeted antigen is absent [62]. A recent study demonstrates that inactivation of the first and third CD3ζ ITAM decreased non-specific IFN-γ production by anti-CD19 CAR-modified T cells without impairment of the antileukaemia activity [18]. The FceRI γ chain which harbours one ITAM in contrast to the three ITAMs in the CD3ζ chain may alternatively be used as implied by earlier studies [63].

“First generation” CARs transmit the signal through the CD3ζ intracellular chain, “second generation” CARs added a costimulatory domain like CD28, 4-1BB or OX40 to improve T cell persistence and activation. While each of these domains differentially modulates individual effector functions [28], the benefit of each costimulation in mounting the antitumor response needs to be determined. This is moreover required for the most recent “third generation” CARs with combined costimulatory domains.

9.2. The Effector T-Cell Population. Different T cell populations are currently explored for adoptive cell therapy; it is still unresolved which T cell subset shows best therapeutic performance. There is increasing evidence that cytotoxic effector T cells are not a homogenous population but consist of different subsets with individual phenotypes and functional capacities. Resting CD8+ T cells in the peripheral blood exist as naïve, central memory, and effector memory T cells. Effector and central memory T cells can be subdivided on the basis of their expression of homing receptors to lymphoid organs. Effector memory T cells develop effector functions more rapidly than central memory T cells, however, secrete lower amounts of IL-2. In mouse models, central memory T cells engraft, survive better, and exhibit superior antitumor activity than effector memory T cells [64]. Data were confirmed by a study of nonhuman primates [65]. Central memory T cells can efficiently be produced ex vivo by CD3 and CD28 stimulation which can be further augmented by IL-7 and IL-15; CD3 stimulation in presence of IL-2 showed less effective [66].

Naïve T cells, however, represent the most common CD8+ T cell phenotype and thereby the major source of effector cells. Using T cells transgenically or retrovirally, equipped with a tumor-specific TCR, [67] revealed that naïve T cell-derived effector cells are superior for proliferation and cytokine production than effector cells derived from central memory T cells. Longer persistence of those cells may result in superior antitumor efficacy compared to central memory T cells. The procedures for isolating and modifying naïve T cells from cancer patients in a GMP-compliant manner, however, still need to be developed.

CD4+ and CD8+ lymphocytes are most frequently transferred since a mixture of those T cell subsets performs better than either T cell subset alone in preclinical models [68]. Bulk T cells, however, contain regulatory T (Treg) cells which repress the antitumor response [34,35]. Since CD4+ T cell depletion eliminates helper CD4+ T cells along with Treg cells and depleting CD25 high T cells also eliminates proliferating T cells, a more specific strategy in eliminating Treg cells is needed.

Issues additionally to be addressed in the near future include the particular immune status and the decreasing T cell number in the peripheral blood of patients in advanced stages of the disease. The efficiency in collecting T cells with sufficient functional capacities will be dependent on the clinical situation in which T cells are collected, that is, a patient in remission with minimal residual disease versus a patient with bulk disease. This situation challenges collecting adequate numbers of T cells to be expanded. Apart from that, patients in advanced stages of the disease accumulate a large number of antigen experienced T cells with diminished
activation potential due to decreased CD3ζ expression and downstream signalling capacities. Engineering with a CD3ζ or CD28-CD3ζ signalling CAR may overcome some, but not all defects in "burn-out" T cells of progressed tumor patients. We assume that T cells in advanced stages of terminal differentiation may require additional stimuli to execute their effector functions. On the other hand, low T cell counts in patients with advanced disease may limit the overall efficiency in generating engineered T cells with the consequence that multiple rounds of ex vivo amplifications are required to provide clinically effective T cell numbers. While longer ex vivo stimulation provides higher numbers of CAR-modified effector cells, it remains questionable whether their antitumor potency and proliferative capacity conserves with expansion.

Protective immune response seems to be associated with the ability of adoptively transferred T cells to form memory [64]. Conditions which promote protective memory after adoptive T cell transfer need to be established.

Although cytotoxic T cells are extremely effective eliminating larger haematopoietic tumor mass and of residual tumor cells in preclinical model systems, other effector cells may be envisaged. Beside T cells, NK cells can be effectively redirected by engineering with CARs [69]. Anti-CD19 CAR NK cells, modified by RNA transfer, showed redirected lysis of CLL cells in vitro [70] providing hope for an alternative effector cell population in adoptive therapy.

T cells from each individual patient need to be modified, amplified, and tested prior to reinfusion. Local production at each clinical institution requires individually approved cell processing facilities and trained personnel to ensure guideline conform production and the uniformity of the cell product. From the regulatory point of view, one or few central facilities may be advantageous which receive cells from the individual patients and produce the cell product. Once tested for safety parameters the cell product can be shipped in a cryopreserved fashion to the clinical site and locally stored until adoptive transfer to the patient.

9.3. Toxicity. T cells are mostly transduced by retro- or lentivirus infection to obtain CAR modified cells with high efficiencies. As far as safety concerns, mutagenesis by insertion of the CAR encoding transgene needs to be addressed. There is so far no reported experimental evidence that retroviral modified, mature polyclonal T cells produce clonal amplification upon adoptive transfer [71]. Clinically, malignant transformation was not observed in any case of more than 100 patients who were treated so far with gene-modified T cells which is in contrast to the treatment with genetically modified haematopoietic stem cells. Apart from that, the search for a safer vector system using nonintegrating vectors [72], RNA transfer [73], or targeted recombination into safe sites [74] is still ongoing.

Since CD19 targeting is not tumor specific, CD19+ healthy B-cells are eliminated as well. While this situation is expected to be clinically manageable, selectivity for B-CLL cells may be improved by targeting alternative, more unique surface markers or by simultaneous targeting of two different markers. To improve tumor cell selectivity, CAR-redirected T cell killing can, moreover, be combined with the administration of therapeutic antibodies as shown for the anti-CD20 antibody rituximab which sustains the antitumor activity of anti-CD19 CAR T cells in the treatment of non-Hodgkin’s lymphoma [75].

Once adoptively transferred, controlling engineered T cell in vivo represents an important option. High-dose steroids showed effective in eliminating engineered T cells in a recent trial [76]; alternative strategies using tagged receptor molecules which can be targeted by depleting antibodies [77] and an inducible caspase-based suicide system [78] showed efficacy in experimental models.

10. The Way Ahead

We think it is quite possible that improvements in all of these and potentially of additional aspects are required for success in the T cell therapy of B-CLL in particular and of malignant diseases in general. Cell dose for minimal toxicity and maximal antitumor efficacy may be different for each CAR, for each effector cell population, for each preconditioning regimen, and others. The complexity of adoptive cell therapy challenges standard clinical trial strategies lastingly established in testing drug-based therapies. At least two aspects have to be taken into account.

First, a standard in assay systems to monitor cell therapy-induced immune responses needs to be defined to allow comparison of data sets from different clinical trials.

Second, trials differ in such a large number of parameters that it will be problematic to identify those aspects which are critical for the effectiveness or ineffectiveness of a protocol. Table 1 partly illustrates the issue for trials using CD19 CAR-modified T cells. To unambiguously identify the effects of defined changes in clinical trial protocols it will require systematic “one-parameter trials” on the basis of a standard format, in particular with respect to conditions for cell modification, a CAR format, the target, and for preconditioning. The currently recruiting trials using anti-CD19 CAR modified T cells give chance for standardizing and rapidly optimizing the strategy with respect to the discussed parameters [49]. Although toxicities occurred in early-phase trials and caution is still warranted, the potential benefits of adoptive cell therapy with redirected T cells for the therapy of B-cell malignancies should not be abandoned.

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