Targeting mutated protein tyrosine kinases and their signaling pathways in hematologic malignancies

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Over the last decade, major advances have been made in the elucidation of mechanisms involved in leukemogenesis, and this is particularly true with regard to deregulated protein tyrosine kinase (PTK) activation. This progress had led to the development of small molecules that specifically inhibit the abnormally activated kinase. The first example of such targeted therapy is imatinib-mesylate, an inhibitor of the BCR-ABL fusion gene that is found in more than 90% of patients with Philadelphia positive (Ph^+) chronic myeloid leukemia (CML) and in 20-30% of those with Ph^+ acute lymphoblastic leukemia (ALL). The excellent clinical results obtained with imatinib in CML have completely changed the therapeutic approach to this disease, and imatinib is now the gold standard for treatment of newly diagnosed CML. This has instigated a tremendous effort to develop targeted PTK therapy based on the presence of over 40 chromosomal translocations that lead to deregulation of 12 different PTK associated with various hematologic malignancies. That deregulated PTK are also involved in thepathogenesis of acute leukemia is underlined by the frequent occurrence of mutations leading to constitutive activation of the FLT3. Experimental as well as clinical evidence supports a model of acute leukemia based on the co-operation of constitutive active PTK with mutations of transcriptional regulators. Here we review the general impact of mutated PTK on the pathogenesis of various hematologic malignancies. We also discuss the development of new targeted therapies and strategies to circumvent the increasing problems related to the emergence of drug resistance by targeting downstream signaling mediators that are essential for transformation by deregulated PTK.

Key words: tuberculosis, cancer, risk factor, mortality.

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Intensive research efforts over the last decade have provided a better understanding of the molecular genetics underlying hematologic malignancies. Cloning and functional characterization of a large number of chromosomal aberrations associated with distinct hematologic malignancies has allowed the formulation of genetic models for the pathogenesis of acute leukemia.[1] Based on functional *in vitro* and *in vivo* studies, genetic alterations can be grouped in mutations that provide a proliferation and survival advantage, and mutations that regulate self renewal and impair cellular differentiation. In general, mutations targeting protein tyrosine kinases (PTK) are gain-of-function mutations (fusion genes, activating point mutations) leading to constitutive activation of the protein (Table 1). Overexpression of activated PTK has been shown to be sufficient to transform cells *in vitro* and to induce lethal myelo- and/or lymphoproliferative disorders in animals. In contrast, expression of mutations targeting transcriptional regulators that have a critical role in the differentiation of hematopoietic cells, although having some oncogenic potential, is generally not sufficient to induce a malignant phenotype *in vivo*.

Analysis of the transforming properties and structure-function studies have enabled the development of small molecule inhibitors specifically targeting constitutively activated PTK. The success of imatinib-mesylate (Gleevec® or Glivec®, Novartis Pharmaceuticals, Basel, Switzerland) for the treatment of patients with chronic myeloid leukemia (CML) instigated an intensive search for new small molecular compounds that may be able to target other PTK involved in the ransforming process of malignant hematologic and other cancer cells. However, relatively early on it became evident that overcoming the molecular resistance mechanisms against these compounds would be one of the challenges for future therapeutic approaches.

The goal of this article is to summarize the impact of mutated PTK on the pathogenesis of hematologic malignancies. We first list the most frequent genetic alterations in hematologic malignancies that lead to constitutive PTK activity. We then discuss the general impact of mutated PTK in the pathogenesis of hematologic malignancies. Next, recent progress made in therapy with small molecule inhibitors targeting these activated PTK is reviewed. Finally, we address new strategies for targeted therapy of hematologic malignancies, which include not only inactivation of the mutated PTK, but also targeting of their critical downstream signaling pathways.

Mutated PTK in hematologic malignancies

ABL

Almost half a century ago, Nowel and Hungerford described a distinct cytogenetic abnormality in cells from patients with chronic myeloid leukemia as the Philadelphia chromosome.[2] It then took more than two decades to characterize the Philadelphia chromosome as the consequence of a translocation between chromosome 9 and 22 [t(9;22)(q34;q11)] leading to a fusion of the BCR gene to the ABL PTK gene. This translocation is found in more than 90% of patients with CML and is believed to be the principal cause of CML.

It became evident that different chromosomal breakpoints lead to different forms of BCR/ABL (p185, p210, p230) of which the p185 variant is associated with 20-30% cases of adult pre B-cell acute lymphoblastic leukemia (ALL). During the following years intensive research efforts demonstrated the transforming activity of the BCR/ABL fusion in cell lines as well as in animal models. Structure-function studies have demonstrated that fusion of ABL (and PTK in general) to various oligomerization domains containing 5' partners leads to the cytoplasmic localization and constitutive PTK activation necessary for its oncogenic activity. In addition, a large number of downstream signaling pathways that are involved in malignant transformation by BCR/ABL were characterized. These studies, as summarized in several excellent review articles, are the basis for the development of successful targeted therapy by small molecule PTK inhibitors.[3,4] In contrast to CML and B-ALL, ABL PTK is rarely altered in malignancies of the T-cell lineage. However, a recent fluorescent *in situ* hybridization (FISH) based screening of a series of 90 T-cell ALL cases for the involvement of the ABL PTK resulted in the detection of a new mechanism for the generation of an oncogenic PTK fusion.[5] While no BCR/ABL fusion signals were detected, marked extrachromosomal episomal amplification of ABL was observed in >5% of the patients. Further mapping of the episomes showed a fusion of the ABL including the PTK domain to the nucleoporin 214 (NUP214; also known as CAN) gene. Screening of an additional 85 cases of T-cell ALL revealed a NUP214/ABL fusion in 5 individuals as well as in 3 of 22 T-cell lines. Whether the NUP214/ABL fusion also occurs in other hematologic malignancies, such as the Philadelphia-chromosome negative myeloproliferative disorders, is the subject of ongoing studies. In addition to the NUP214/ABL fusion, an EML/ABL fusion was detected in a case of T-cell ALL with a cryptic t(9;14)(q34;q32).[6] These studies demonstrate that constitutive PTK activation is a recurrent event not only in myeloid but also in T-cell leukemia, and may therefore open up a newavenue for the development of small molecule inhibitor based therapies.

PDGFR

Insights into the involvement of a *non-ABL* PTK in the pathogenesis of myeloid malignancies came from the molecular characterization of a balanced translocation t(5;12) (q33;p13) from a patient with chronic myelomonocytic leukemia (CMML). This translocation leads to a fusion of the TEL (also known as ETV6) gene to the PTK domain of the platelet-derived growth factor β receptor (PDGFβR).[7] The PDGFβR belongs to the type III receptor PTK family including PDGFR, KIT, CSFR1 and FLT3 that are characterized by a transmembrane domain, a juxtamembrane domain and a split kinase domain.[8] The impact of this study became evident from the fact that, over a decade later, the TEL gene has been shown to be a partner in >40 different chromosomal translocations, and that the TEL/PDGFβR is the first of >20 fusion genes involving non-ABL PTK currently known to be associated with hematologic malignancies (Table 1). Most of these fusion proteins are associated with chronic myelo-proliferative disorders such as atypical (Philadelphia chromosome-negative) CML or CMML. Therapeutic response to a small tyrosine kinase inhibitor (*see below*) and a t(1;4)(q44;q12) in a patient with hypereosinophilic syndrome (HES) suggested involvement of a PTK other than ABL. Cools and co-workers finally found that this translocation leads to the fusion of the kinase domain of the PDGFαR gene located on 4q12 to a new gene named FIP1-like1 (FIP1L1). However, in contrast to other PTK fusions, this fusion is not the result of a translocation but the consequence of a cryptic interstitial deletion.[9] Further analysis of HES patients showed the presence of a FIP1L1/PDGFαR fusion in almost 15% of the cases. Unless undergoing small molecule inhibitor treatment, FIP1L1/PDGFαR positive HES patients have been reported to have a poor prognosis with a tendency towards leukemic transformation.[10]

Table 1. Deregulated protein tyrosine kinases in hematologic malignancies.

PTK involved	*Fusion gene*	*Chromosomal aberration*	*Disease phenotype*	**Animal models*	
A. Fusion genes					
ABL (9q34)	BCR/ABL	t(9;22)(q34;q11)	CML, ALL	a, b	
(ABL1)	TEL/ABL	t(9;12)(q34;p13)	Atypical CML	a	
	NUP214/ABL	episomal amplification	T-ALL		
	EMS/ABL	t(9;14)(q34;q32)	T-ALL		
	SFQ/ABL	t(1;9)(p34;q34)	B-ALL		
ARG (1q24)	BCR/ARG	t(1;22)(q24;q11)	Atypical CML		
(ABL2)	TEL/ARG	t(1;12)(q24;p13)	Atypical CML		
PDGFβR (5q33)	TEL/PDGFβR	t(5;12)(q33;p13)	CMML, atypical CML	a, b	
	HIP1/PDGFβR	t(5;7)(q33;q11)	CMML, atypical CML		
	RAB5/PDGFβR	t(5;17)(q33;p13)	CMML, atypical CML		
	H4/PDGFβR	t(5;10)(q33;q21)	CMML, atypical CML	a	
	Myomegalin/PDGFβR	t(1;5)(q23;q33)	CMML, atypical CML		
	CEV14/PDGFβR	t(5;14)(q33;q32)	relapse AML		
	NIN1/PDGFβR	t(1;5)(q23;q33)	atypical CML		
	HCMOGT/PDGFβR	t(5;17)(q33;p11)	juvenile CMML		
	KIAA1509/PDGFβR	t(5;14)(q33;q32)	MPD		
	TP53BP1/PDGFβR	t(5;15)(q33;q22)	atypical CML		
PDGFαR (4q12)	FIIP1L1/PDGFαR	del(4)(q12q12)	HES		a
	BCR/PDGFαR	t(4;22)(q12;q11)	Atypical CML		
JAK2 (9p24)	BCR/JAK2	t(9;22)(p24;q11)	CML, atypical CML		
	TEL/JAK2	t(9;12)(p24;p13)	Atypical CML, ALL, AML	a, b	
	PCM1/JAK2	t(8;9)(p21-22;p23-24)	Atypical CML, AML, ALL		
SYK (9q22)	TEL/SYK	t(9;12)(q22;p13)	MDS		
TRKC (15q25)	TEL/TRKC	t(12;15)(p13;q25)	AML	a, b	
FGFR1 (8p13)	ZNF198/FGFR1	t(8;13)(p12;q12)	EMS	a	
	FOP/FGFR1	t(6;8)(q27;p12)	EMS	a	
	CEP110/FGFR1	t(8;9)(p12;q33)	EMS		
	HERVK/FGFR1	t(8;19)(p12;q13)	EMS		
	BCR/FGFR1	t(8;22)(p12;q11)	Atypical CML		
	FGFR1OP2/FGFR1	ins(12;8)(p11;p11p22)	EMS		
	TIF1/FGFR1	t(7;8)(q34;p11)	EMS		
FGFR3 (4p16)	TEL/FGFR3	t(4;12)(p16;p13)	PTL/AML		
FLT3 (13q12)	TEL/FLT3	t(12;13)(p13;q12)	MPD & eosinophilia		
FRK(6q21)	TEL/FRK	t(6;12)(q21;p13)	AML		
ALK (2p23)	NPM/ALK	t(2;5)(p23;q35)	ALCL	a, b,	
	TPM3/ALK	t(1;2)(p25;p23)	ALCL, IMT		
	TFG/ALK	t(2;3)(p23;q21)	ALCL		
	ATIC/ALK	inv(2)(p23;q35)	ALCL		
	CLTC/ALK	t(2;17)(p23;q23)	ALCL		
	MSN/ALK	t(2;X)(p23;q11)	ALCL		
	TPM4/ALK	t(2;X)(p23;q11)	ALCL, IMT		
	ALO17/ALK	t(2;17)(p23;q25)	ALCL		
	RANBP2/ALK	t(2;2)(p23;q13) or inv(2)(p23;q11)	IMT, ALCL?		
	MYH9/ALK	t(2;22)(p23;q11)	ALCL		
	CARS/ALK	t(2;11;2)(p23;p15;q31)	IMT, ALCL?		
B. Gain of function mutations					
FLT3 (13q12)	ITD (80%), activation loop kinase domain (15%)		AML	a	
KIT (4q12)	JM region, activation loop kinase domain		AML, mast cell leukaemia	a	
JAK2 (9p21)	JAK2 V617F mutation	9pLOH	PV, ET, myelofibrosis	a	
C. Deregulated expression					
FGFR3 (4p16)	Ectopic expression	t(4;14)(p16.3;q32)	Multiple myeloma		
FLT3 (13q12)	Overexpression	MLL alterations	ALL/AML		

*MPD: myeloproliferative disorder; CML: chronic myeloid leukemia; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; CMML: chronic myelomonocytic leukemia; EMS, 8p13 myeloproliferative syndrome; ALCL: anaplastic large cell lymphoma; IMF: inflammatory myofibroblastic tumor; ITD: internal tandem duplication; JM: juxtamembrane; PTL: peripheral T-cell lymphoma; PV: polycythemia vera; ET: essential thrombocythemia; LOH: loss of heterozygosity *Animal models: a: murine bone marrow transplant assay; b: transgenic animals.*

FLT3

The FMS-like PTK 3 (FLT3) gene (also known as FLK2, foetal liver kinase-2; or STK1, human stem cell kinase-1) encoding a transmembrane class III receptor PTK plays a crucial role in normal hematopoiesis. In normal bone marrow, FLT3 is expressed at high levels in early progenitor cells but is also expressed at abundant levels in a high proportion of cases of acute leukemia, suggesting that FLT3 may play a role in survival or proliferation of leukemic blasts.

Almost a decade ago, Nakao and co-workers first demonstrated FLT3 *length mutations* (internal tandem duplications (ITD) in the juxtamembrane domain), a finding that has been confirmed by several studies showing that more than 20% of adult and more than 10% of pediatric AML patients harbor an FLT3-ITD.[11] Subsequently *kinase domain* FLT3 mutations (most often targeting D835 in the activating loop) have been found to be present in the leukemic blasts of more than 5% of AML and in less than 5% of ALL patients. Both the FLT3 length and the kinase domain mutations lead to deregulated PTK activity.[12,13] *In vitro* studies have suggested that FLT3 mediates its proliferative and anti-apoptotic effects through several signaling pathways including the STAT5, RAS/MAPK and PI3K/AKT pathways.

In several clinical studies, activating FLT3 mutations were associated with a poor prognosis. In general, patients with mutant FLT3 show higher cell counts and decreased overall survival. It is noteworthy that absence of the wild-type allele in patients with FLT3-ITD predicted poor prognosis in adult *de novo* AML cases with otherwise normal cytogenetics.[14] Although elevated FLT3 mRNA expression levels have been found to be related to either length or kinase domain mutations, overexpression of FLT3 is an unfavorable prognostic factor, even in cases without activating mutations.[15] Interestingly, gene expression profile studies including a large series of AML samples showed that the presence of an FLT3-ITD (but not FLT3 kinase domain mutations) in patients with an otherwise normal karyotype was associated with a distinct expression signature.[16] Gene expression profiling of ALL patients demonstrated high expression levels of FLT3 in cases carrying a translocation involving the mixed-lineage leukemia (MLL) gene.[17] Further analysis revealed FLT3 activating kinase domain mutations (D835/I836) in infant ALL with MLL rearrangements as well as in paediatric ALL with hyperdiploidy.[18,19] Based on its clear prognostic impact, determination of the FLT3 status has become a standard analysis for the management of patients with acute leukemia.

JAK2

One of the downstream mediators of normal and oncogenic PTK is the JAK/STAT signaling pathway.[20] Molecular characterization of a T-ALL case with a t(9;12)(p24;p13) revealed a fusion of the JAK2 kinase domain to the N-terminus of TEL including its pointed or HLH oligomerization domain.[21] Subsequently, TEL/JAK2 isoforms, depending on the location of the breakpoints in the JAK2 gene, have been described in B-ALL and atypical CML.[22] In addition to TEL, other translocations have recently been cloned leading to fusions of JAK2 to BCR or PCM1.[23] In contrast to many other PTK fusions, the TEL/JAK2 fusion has been found in CML as well as in T- or B-cell ALL patients. Interestingly, expression of the TEL/JAK2 fusion gene in mouse bone marrow leads to induction of a lethal mixed lymphomyeloproliferative disorder, reflecting the multi-lineage transforming potential of this fusion gene.[24] To further explore the role of JAK2 in leukemogenesis, the genomic organization of the human JAK2 gene was determined. In fact, screening the sequence for the JAK2 domains regulating its tyrosine activity failed to detect any activating mutations in a series of leukemia samples.[25] However, loss of heterozygosity (LOH) studies have identified that a region on chromosome 9p harboring the JAK2 gene is genetically altered in patients with Philadelphia chromosome-negative chronic myeloproliferative syndromes.[26]

Recently, several groups independently described the presence of a distinct somatically acquired JAK2 mutation (V617F) in a substantial proportion of patients with Philadelphia chromosome-negative myeloproliferative disorders.[27-32] Kralovics and co-workers, were analyzing the 9p LOH region in samples from 244 patients with different myeloproliferative disorders by microsatellite polymerase chain reaction (PCR). Of the patients with 9pLOH, JAK2 showed a somatic homozygous G to T transversion in hematopoietic cells causing a phenylalanine to valine substitution. In patients without 9pLOH, one-third were heterozygous for V617F and two-thirds did not have the mutation. V617F was found in 65% of patients with polycythemia vera (PV), 57% of patients with idiopathic myelofibrosis (IMF), and 23% of patients with essential thrombocythemia (ET). Based on their findings, they proposed that a model of mitotic recombination was most probably the mechanism leading to both 9pLOH and the transition from heterozygosity to homozygosity for JAK2 V617F.[27]

In the studies published to date, the JAK2V617F mutation has been found in 472/944 (50%) of patients with Ph-negative chronic myeloproliferative disorders (including PV, IMF and ET) with predominance in PV (66%) followed by IMF (42%) and ET (26%). In contrast, JAK2V617F seems to be rarer in both atypical Ph-negative myeloproliferative disorders such as systemic mastocytosis or HES, and the myelodysplastic syndromes.[32,33] What are the implications of such an observation as concerns the signaling pathways regulating hematopoiesis, the pathogene-

sis of myeloproliferative disorders in general, or the diagnosis and management of patients with these disorders ?

The V617F transversion targets a highly conserved valine residue in the so-called JH2, or pseudokinase domain of JAK2. Previous work has proposed that this domain may serve as a negative regulatory domain for the C-terminal kinase domain (JH1), suggesting that a structural change in a highly conservative residue may lead to enhanced activity of the mutant variant.[34]

Indeed, in several of the above-cited studies, the JAK2 mutant has been found to be autophosphorylated and to enhance activation of its direct downstream target STAT5. In addition, expression of JAK2 617F provides growth and survival advantages as shown in several growth-factor dependent cell line models. These findings raise the question of whether deregulation of the JAK2/STAT5 signaling pathway may be a general mechanism in PV and perhaps in other forms of myeloproliferative disorders. It remains to be determined whether other factors in this pathway are altered in cases of myeloproliferative disorders without the JAK2 V617F mutation.

Myeloproliferative disorders, such as PV have previously been shown to be clonal disorders presumably induced by a genetic hit at the stem cell level.[35] It remains to be shown at which level of hematopoiesis the JAK2 V617F occurs. Identification of the JAK2 V617F mutation in CFU-GM and BFU-E colonies suggests a multipotent progenitor origin.[30] However, it cannot be excluded that the disease may be induced by a thus-far unidentified genetic alteration at the stem cell level, and the JAK2 V617F mutation may represent a second genetic hit in a more committed progenitor. In one study, patients with myeloproliferative disorders harboring the JAK2 V617F mutation had a significantly longer duration of disease and a higher rate of complications and treatment with cytoreductive therapy as compared to patients with wild-type JAK.[27] Expression of the JAK2 V617F in the mouse bone marrow seems to partly recapitulate the human disease as shown by induction of erythrocytosis with significant hematocrit values 4 weeks after transplant.[29] These observations indicate that JAK2 V617F plays an essential role in the development of the disease phenotype and suggest that inhibition of deregulated activity of JAK2 V617F by small molecule inhibitors would be of therapeutic benefit. Following the imatinib-mesylate paradigm of successful small molecule PTK inhibitors, new and more specific compounds blocking JAK2 are currently being evaluated.[36,37]

FGFR

Fibroblast growth factors (FGF) are a large family of pleiotropic heparin-binding growth factors that regulate cellular proliferation, migration and differentiation during embryogenesis, and are homeostatic factors that function in tissue repair.[38] Inappropriate expression of some FGF can contribute to cancer pathogenesis either through deregulated angiogenesis or direct stimulatory effects on tumor cells. FGF exert their functions through four related receptor PTK, fibroblast growth factor receptors 1-4 (FGFR1-4). Although several studies have proposed that the FGF/FGFR system may play a role in early hematopoietic development, evidence for a role in leukemogenesis came from molecular characterization of t(8;13)(p11;q12) associated with a distinct myeloproliferative syndrome.[39] Cases with a reciprocal translocation involving 8p13 are characterized by a Philadelphia chromosome-negative myeloproliferative yndrome associated with eosinophilia, generalized lymphadenopathy and an unusually high incidence of T-cell lymphoblastic leukemia/lymphoma. This disease was named *8p13 myeloproliferative syndrome* (EMS) or stem cell leukemia-lymphoma (SCLL). t(8;13)(p11;q12) results in a fusion that includes a gene called ZNF198 (also named RAMP or FIM) fused to the intracellular part of the FGFR1 known to map to the 8p13 region. Subsequently, there were reports of other translocations leading to variant translocations, including the FGFR1 fused to 5' partners such as FOP, CEP110, HERVK, BCR, TIF1 and FGF1OP2 (Table 1).[40–42] However, in contrast to ZNF/FGFR1, patients with a BCR/FGFR1 fusion present with a CML-like myeloproliferative disorder, but the development of T-cell leukemia/lymphoma has so far not been reported. To address the question of whether the 5' fusion partners (BCR vs. ZNF198) may direct the disease phenotype, Roumiantsev *et al*. used retroviral transfer of ZNF/FGFR1 or BCR/FGFR1 into mouse bone marrow cells, followed by transplantation into irradiated syngeneic recipients.[43] All mice expressing ZNF198/FGFR1 developed proliferation of both myeloid cells and T-cell lymphomas, closely mimicking the human EMS. In contrast, mice transplanted with bone marrow infected with equivalent titers of retrovirus expressing the BCR/FGFR1 fusion developed an aggressive myeloproliferative disease similar to BCR/ABL, suggesting that the FGFR1 partner gene may have an important effect on the disease phenotype. However, using a similar bone marrow transplantation approach, expression of the FOP/FGFR1 fusion (that has been found in patients with T-cell non-Hodgkin's lymphoma) in mice has been reported to result solely in a myeloproliferative disease.[44]

In addition to FGFR1, FGFR3 has recently been shown to be involved in the pathogenesis of hematologic malignancies. Molecular cloning of a translocation t(4;12)(p16;p13) from a patient with peripheral T-cell lymphoma revealed a TEL/FGFR3 fusion. Subsequent progression of the disease to AML retaining the same fusion suggests that, like EMS/SCLL induced by FGFR1

fusions, TEL/FGFR3-mediated disease also originates from a multi-potent stem or early progenitor cell.[45,46] The translocation t(4;14)(p16.3;q32) occurs unique in a subset of about 15% of patients with multiple myeloma.[47] This translocation seems to be mediated by isotype switch recombination errors and leads to deregulated expression of MMSET, encoding a SET-domain nuclear protein, and of the FGFR3 PTK gene. The tumor cells express very high levels of wild-type FGFR3 that induces a proliferative signal in these cells. In addition, some of these patients acquire subsequent activating mutations in FGFR3 that are associated with disease progression. Several experimental models have demonstrated that mutated FGFR3 has strong cellular oncogenic activity. The presence of a t(4;14)(p16;q32) in multiple myeloma is associated with marked reduction in overall survival. Interestingly there is strong experimental evidence that FGFR3 can be targeted by small molecule inhibitors (see below) that induce differentiation and apoptosis in t(4;14)(p16;q32) positive cells, showing that constitutively activated FGFR3 is a *bona fide* therapeutic PTK target that needs to be further evaluated.[48-50]

ALK

The ALK (anaplastic lymphoma kinase) receptor PTK was identified as a fusion partner to the nucleophosmin (NPM) gene upon molecular characterization of a t(2;5)(p23;q35) in anaplastic large-cell lymphoma (ALCL).[51] The ALK gene on 2p23 encodes for a receptor PTK of the insulin receptor type family that is highly conserved between species. ALK is physiologically highly expressed in the developing brain suggesting an important role in the nervous system. Its fusion partner, NPM, encodes for a ubiquitous highly expressed protein shuttling between the nucleus and cytoplasm involved in ribosome assembly. During the past decade, at least 9 different fusion genes containing the ALK PTK have been characterized (Table 1). Interestingly the subcellular localization of an x-ALK fusion protein in the tumor cells is mainly directed by the nature of the 5' ALK fusion partner. For instance, in contrast to ALK, which is mainly present in the cytoplasm, a significant amount of the NPM/ALK fusion protein is found in the nucleus, with the remaining cytoplasmic part presumably being due to the NPM-mediated cytoplasmic/nuclear shuttle mechanism. Chromosomal translocations involving the ALK PTK have so far only been reported in malignant lymphomas and in a rare proliferative lesion of the soft tissue called inflammatory myofibroblastic tumor. ALK-containing fusions are found in 60-80% of ALCL that are characterized by a cytotoxic T-cell phenotype or by a lack of both T- and B-cell markers (null phenotype). Detection of the ALK fusion proteins by immunohistochemistry or of alterations of 2p23 by FISH have become standard diagnostic procedures for undifferentiated lymphoproliferative disorders. In addition to ALCL and inflammatory myelofibroblastic tumor, ALK containing fusion genes have recently also been detected in rare cases of B-cell non-Hodgkin's lymphomas. Several studies have shown that the presence of an ALK fusion gene is a good prognostic marker with overall 5-year survival rate after chemotherapy of >70% in ALK-positive cases compared to <40% in ALK-negative cases. A large number of studies have investigated the molecular mechanisms that mediate the malignant potential of ALK-fusion proteins.[52,53] *In vitro* transforming activity of the NPM/ALK fusion protein was associated with constitutive activation of several downstream signaling pathways including PI3K/AKT, JAK/STAT and RAS/MAPK. In addition, the oncogenic potential of NPM/ALK was demonstrated in transgenic animals expressing the fusion protein in their T cells, as well as upon expression of NPM/ALK in transplanted bone marrow cells.[54,55]

KIT

c-KIT, the cellular homolog of the viral oncogene v-kit, encodes for a class III receptor tyrosine kinase (RTK) whose ligand is stem cell factor (SCF).[8] KIT plays an essential role in the development and proliferation of mast cells, melanocytes, interstitial Cajal cells and hematopoietic cells.[56] Activating mutations of KIT have been identified in systemic mastocytosis, gastrointestinal stromal tumors, seminoma/dysgerminoma, sinonasal NK/T cell lymphomas as well as in acute myeloid leukemia and rare cases of myeloproliferative disorders.[56,57] Similar to FLT3, KIT kinase domain mutations, of which D816V is the most frequent, affect the activation loop at the entrance of the enzymatic pocket, increasing the catalytic activity. In contrast, mutations outside the kinase domain located, for example, in the juxtamembrane domain (D550-580) are believed to disrupt a negative regulatory conformation thereby causing constitutive activation. Other regulatory mutations have been found in the extracellular domains of KIT (e.g. D52N, D 417-421) that lead to ligand-independent dimerization and subsequent activation.[57] In contrast to gastrointestinal stromal tumors, the role of KIT in the pathogenesis of leukemia is not completely understood. KIT protein is expressed in up to 80% of AML (predominantly in immature M0/M1 subtypes) and activation of KIT in the absence of added SCF is frequently seen in blasts from AML cases, suggesting an autocrine feedback mechanism and/or activating mutations. However, KIT mutations are relatively rare in AML (1-5%). Interestingly, up to 30% of AML cases with fusion targeting core-binding factor (CBF) [t(8;21), inv(16)] show activating KIT mutations in the extracellular domain targeting the asparagine residue 419 which suggests co-operation of the KIT gain of function

mutation with fusions such as AML1/ETO or SMMHC/CBFβ that interfere in a dominant-negative manner with normal CBF function.[58] In addition, molecular analysis of patients during disease progression indicated that in some cases the cooperating KIT mutation could be a subsequent event in CBF translocation (AML1/ETO) induced acute myeloid leukemia.[59]

Impact of mutated PTK in the pathogenesis of hematologic malignancies

Cloning of several hundred chromosomal translocations associated with various hematologic malignancies resulted in characterization of a large number of new putative oncogenic gene alterations. A recently proposed classification based on *in vitro* and *in vivo* functional analysis divides these alterations associated with human leukemia into at least two categories (Figure 1). Expression of *class I* mutations confers a proliferative and/or survival advantage to the cells. The current list of known leukemogenic class I mutations contains more than 10 different PTK that undergo constitutive activation either by being fused to different N-terminal partner proteins providing an oligomerization domain, or by activating mutations such as point mutations in their kinase domain or internal tandem repeats (length mutations) in the juxtamembrane domain (FLT3, KIT). Most of these alterations are associated with chronic myeloproliferative disorders such as CML/CMML or Philadelphia-negative myeloproliferative disorders, except activating mutations of FLT3 and KIT which are found almost exclusively in acute leukemia. Other class I alterations are gain of function mutations of the three main RAS isoforms (N-RAS, K-RAS, HRAS) which are frequently seen in different myeloid malignancies.[60] The protein tyrosine phosphatase SHP2 encoded by the PTPN11 gene plays an essential role in normal activation of RAS. PTPN11 activating mutations were found in over 30% of cases of juvenile myelomonocytic leukemia (JMML) and in some cases of childhood myelodysplastic syndrome and childhood acute leukemia.[61] Expression of mutant PTPN11 in murine bone marrow induces a fatal JMML-like disorder or, less commonly, lymphoproliferation. In addition, PTPN11 mutants also cause myeloproliferation in *Drosophila*, further demonstrating the *in vivo* transforming potential.[62] Interestingly, cases of JMML without any PTPN11 mutations show deregulation of the RAS pathway by either activating RAS mutations or by homozygous inactivation of the neurofibromatosis type-1 (NF1) gene, which has an endogenous RAS inhibitor function, further underlining the importance of deregulation of the RAS pathway in JMML.

When analyzed for their transforming potential, overexpression of *class I* mutations is generally sufficient to transform hematopoietic cells to growth-factor independence *in vitro* and to induce a lethal *leukemia-like* myeloproliferative disorder in mice[63] (Figure 2). In contrast to class I mutations, there is a large group of genetic alterations mostly associated with acute leukemia, referred to as *class II* mutations, which impair differentiation of hematopoietic cells but do not directly provide proliferative and/or survival advantage (Figure 1). Many of them are loss of function mutations (either through fusion formation or point mutations) of transcriptional regulators that are critical for normal hematopoietic development and differentiation. In contrast to class I alterations, expression of these mutations in the mouse bone marrow is usually not sufficient to mimic the human disease in mice by inducing a leukemia phenotype. However, after a long latency period, signs of myelodysplasia are often seen with a variable propensity to develop an immature and clonal hematologic disorder closely resembling human AML or ALL. These observations suggest that the development of an acute leukemia phenotype needs acquisition of *additional* genetic hits. The multi-genetic hit hypothesis of acute leukemia is further strongly supported by studies of twins developing leukemia during childhood showing a concordance rate of only 5% suggesting the need for additional post-natal events. In addition, analysis of unselected blood samples from neonates showed that about 1% have class II genetic alterations that are detectable by PCR. This rate is at least 100× higher than the cumulative risk of developing childhood leukemia suggesting that in the absence of additional genetic hits the pre-leukemic clones rarely convert into overt disease.[64]

There are several lines of clinical and experimental evidence that class II mutations co-operate with class I mutations to cause acute leukemia. The first evidence that activated PT may provide a proliferative signal in AML cases with class II mutations comes from rare but highly informative cases of CML in transformation. Several CML cases have been reported in which different class II mutations (e.g. CBFβ/MYH11, AML1/ETO, AML1/EVI1, NUP98/HOXA9) have been acquired upon transition into blast crisis.[65,66] Second, FLT3 activating mutations are frequently present in AML cases containing fusions targeting the retinoid acid receptor (RAR) or the CBF transcriptional regulator. In addition, activating mutations of KIT as well as RAS have been detected in a significant proportion of CBF leukemia patients.[58] Interestingly, RAS and activating PTK mutations seem to be mutually exclusive with only very few cases harboring two of these class I mutations. Third, co-operation of class I with class II mutations has been demonstrated in several animal models:

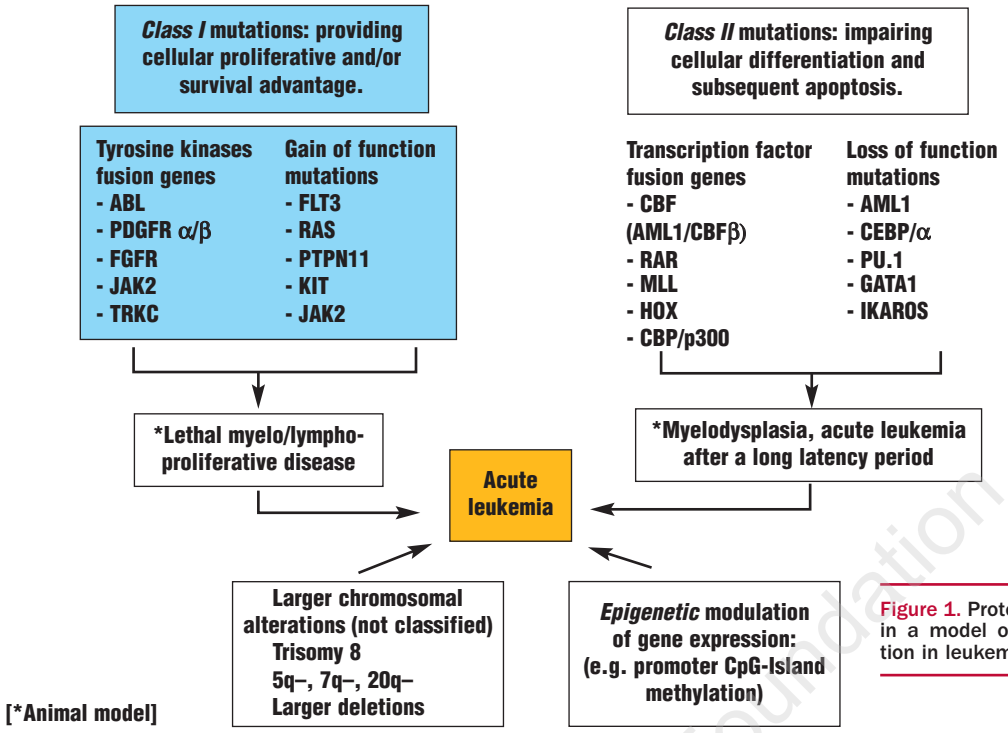

Figure 1. Protein tyrosine kinases in a model of genetic co-operation in leukemogenesis.

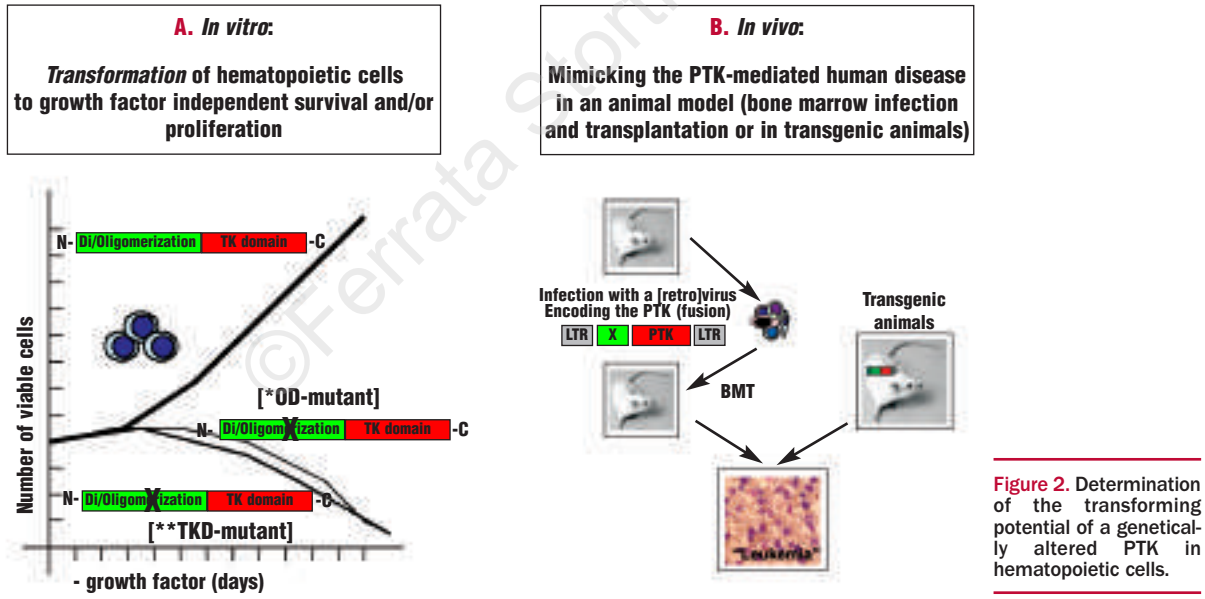

Figure 2. Determination of the transforming potential of a genetically altered PTK in hematopoietic cells.

[*OD: di-/oligomineralization domain; **TKD: tyrosine kinase domain]

expression of FLT3-ITD in cells harboring PML/RARα leads to the development of an acute promyelocytic leukemia (APL) phenotype; co-expression of TEL/PDGFβR and AML1/ETO or BCR/ABL with NUP98/HOXA9 or AML1/EVI1 induces AML.[67-71] The concept that alterations of transcriptional regulators are key events in leukemogenesis is not new. Over a decade ago, Rabbitts and colleagues proposed that translocations targeting master genes, mostly transcription factors of normal hematopoietic development, are key events in human leukemias.[72] Hereby, transcription factors either deregulated by juxtaposi-

tion to a heterologous promoter (e.g. as consequence of a translocation involving the TCR or IgH/L loci in T- or B-ALL respectively) or newly formed chimeric transcription factors resulting from fusion of part of a master regulator with another partner gene (e.g. AML1/ETO, E2A/HLF), lead to an altered transcriptional program preparing the ground for leukemogenesis.[73] This model of understanding leukemia as a consequence of altered gene programs of transcriptional master regulators of hematopoiesis does not stand against the above-mentioned hypothesis of two co-operating mutations. In fact there is increasing experimental evidence showing that chimeric transcription fusions do not only contribute to the leukemic phenotype as a loss of function of a critical regulator of normal differentiation, but also by activation of a gene expression program leading to a leukemic phenotype. An increasing number of gene expression profile studies, which use either cell lines or cells from patients with a distinct master gene translocation, are showing that expression of these fusion genes leads to deregulation of a large number of genes ranging from extracellular matrix proteins, regulators of hematopoietic differentiation, components of different signaling pathways and cell cycle regulators.[74–76]

Whether and how alterations of these master transcriptional regulators could directly prepare the ground for gene mutations targeting PTK and downstream signaling effectors leading to increased cell proliferation and/or survival is the subject of intensive research. Interestingly, expression of some of these fusion genes may directly lead to transcriptional deregulation of PTK as recently shown for the TRKA receptor PTK up-regulated by the AML1/ETO fusion protein.[77]

However, we are completely aware that this rather reductive view of co-operation of well characterized mutations being sufficient for the development of acute leukemia may be pure oversimplification of the task: non-random secondary chromosomal alterations, such as trisomy 8 or 22, or large deletions [(e.g. del(9q)] that are found in AML cases with class II mutations, must also be taken into account. In addition, an increasing number of genes have been shown to be epigenetically silenced in AML blasts through methylation of their CpG islands near the promoter and these cannot be directly grouped into the two major classes.[78,79] Despite these limitations, the *multi-hit* model of acute leukemia may have some clear implications for the therapeutic management of this disease. In the following section we will summarize success and limitations of small molecule PTK inhibitors that, after their success in chronic myeloproliferative disorders, may also find a place in therapy of acute leukemia.

Therapeutic targeting of oncogenic PTK activity

The development of small molecule PTK inhibitors represents a milestone in targeted cancer therapy. Only a decade after its first description, imatinib-mesylate (hereafter referred to as imatinib) is today seen as first-line therapy for newly diagnosed CML in chronic phase.[80] A complete cytogenetic response rate of 68% among patients with newly diagnosed CML treated with imatinib was achieved in a randomised trial including 1106 patients assigned to imatinib or interferon α plus cytarabine as initial therapy.[81] In addition, 83% of chronic phase CML patients treated with imatinib in combination with low-dose cytarabine had a complete cytogenetic response at 12 months.[82] A substantial, although much less impressive cytogenetic response (16%) was seen in patients with CML in myeloid blast crisis undergoing imatinib monotherapy.[83] Imatinib monotherapy for patients with BCR/ABL-positive ALL has been shown to be associated with a high proportion of early relapses under therapy. However, very promising data, although preliminary, have emerged recently from studies that combined intensive chemotherapy with imatinib.[84,85] Many other phase II studies are in progress, and phase III studies will start soon comparing imatinib + intensive chemotherapy with imatinib + less intensive chemotherapy. Based on its inhibitory activity against ABL, KIT, and PDGFR, imatinib is now also used for the treatment of chronic myeloproliferative disorders harboring PDGFβR fusions and for HES patients with the FIP1L1/PDGFαR.[10,86]

Modern methods of structure determination in combination with molecular imaging allowed the modeling of several new compounds that specifically interfere with PTK phosphorylation activity by blocking ATP binding (Table 2). A group of new small molecule inhibitors targeting the FLT3 PTK are currently being evaluated in clinical trials.[87] Although these drugs have been shown to be efficient in cell lines and animal models of leukemia mediated by FLT-length mutations, their efficiency is limited in cells with activating kinase domain mutations, indicating that a detailed molecular analysis of a patient's class I mutation is necessary for selection of the appropriate inhibitor.[88,89] The number of newly developed compounds with inhibitory activity against PTK is growing fast. Very recently, two new molecules were identified that inhibit FGFR3 (CHIR-258, Chiron) or ALK (Cmpd-1, Cephalon) at a nanomolar range, which opens up the possibility for targeted therapy for multiple myeloma and ALCL.[50,90] However, the therapeutic inactivation of an essential

signaling mediator exerts a selective pressure on the tumor cells to escape using various mechanisms of resistance.

Studies on acquired resistance to imatinib gave some important insights into molecular mechanisms of drug resistance. Mechanisms underlying resistance to imatinib may represent an inherent obstacle to any targeted, kinase-selective therapeutic approach. Imatinib resistance of BCR/ABL-positive leukemia has been attributed to at least five different molecular mechanisms: emergence of point mutations in the BCR/ABL kinase domain, overexpression of the BCR/ABL fusion gene, compensatory switch to another kinase pathway, extracellular sequestration of imatinib by α1-acid glycoprotein and p-glycoprotein-mediated active drug efflux from the target cells.[80,91-93] Moreover it has been suggested that some of these mutations might even be present before initiating imatinib therapy and may even confer a more aggressive phenotype.[94]

A large number of mutations associated with resistance to imatinib encode critical structural features of the tyrosine kinase. One of the most frequent is T315I, which directly interferes with the drug's binding by introducing a large isoleucine side chain. Other frequently found mutations are Y253Y and E255K, which seem to influence a distorted p-loop conformation essential for binding of imatinib. Mutations in the activation loop, such as H396R, lead to destabilization of a closed conformation that is necessary for optimal imatinib efficiency. Although frequently observed, the functional consequence of mutations, such as M351T, that are located outside the imatinib binding site is not fully understood.[91] Using a random mutagenesis approach in combination with a functional cellular analysis, a series of these mutations that are able to impair interaction with imatinib and contribute to resistance have been characterized.[95]

CML is a disease in which a rare, highly quiescent Philadelphia chromosome-positive stem cell population can be found.[96] BCR/ABL expressing quiescent hematopoietic stem cells have been demonstrated to be imatinib-insensitive *in vitro*.[97] Consequently, even when an apparently complete response is achieved by eliminating Philadelphia chromosome-positive progenitors, these cells remain and probably explain the persistence of molecular disease.

Whether clones expressing primary BCR/ABL kinase mutations co-exist among these cells remains a controversial issue. The elimination of quiescent leukemia stem cells may be crucial for complete disease eradication.[98] Not unexpectedly, mutations have been detected in leukemia cells containing PDGFR fusions or activated FLT3 leading to resistance against small molecule PTK inhibitors. Relapse of an HES patient with a FIP1L1/PDGFαR was associated with a T674I mutation that can be seen as analogous to T315I in BCR/ABL.[99] *In vitro* screening of FLT3-ITD detected a G697R kinase domain mutation that was resistant to all FLT3 inhibitors tested.[100] An AML patient with FLT3-ITD undergoing treatment with the PKC-412 inhibitor was recently reported to harbor an additional TK domain mutation at the time of relapse.[101] Interestingly, 1-2% of patients with combined activating FLT3 mutations, that show both the ITD length mutation and a TK domain mutation, are resistant to currently-used small drug inhibitors targeting FLT3.[102] The T674I mutation of FIP1L1/PDGFαR, though resistant to imatinib, was highly sensitive to PKC-412, a compound with the highest activity against FLT3 but which also targets PDGFR. This study clearly demonstrated the poten-

Table 2. Small molecule inhibitors for PTK in hematologic malignancies.

Target PTK	*Disease phenotype*	*Compound*	*Producer*
ABL	CML/ALL	Imatinib mesylate	Novartis
		ON012380	Onconova/Fels Research Institute
		AMN-107	Novartis
ABL/SRC	CML/ALL	BMS-354825	Bristol-Myers-Squibb
		AP-23464	Ariad
		SKI-606	Wyeth
		PD-166326	Pfizer
ALK	ALCL	CMPD-1	Cephalon
PDGFαR	HES	Imatinib mesylate	Novartis
PDGFβR	CMML, atypical	CML Imatinib mesylate	Novartis
KIT	AML/mast cell leukemia	Imatinib mesylate	Novartis
		MLN-518	Millenium
		BMS-354825	Bristol-Myers-Squibb
		AP-23464	Ariad
FLT3	AML/ALL	PKC-412	Novartis
		MLN-518	Millenium
		CEP-701	Cephalon
		SU-11248 (SU-5614)	Sugen
		KRN-383	Kirin
		GTP-14564	Chugai
		IMC-EB10, anti FLT3 ab	Imclone
FGFR1	EMS	PKC-412	Novartis
FGFR3	MM	CHIR-258	Chiron
		PKC-412	Novartis

AML: acute myeloid leukemia; CML: chronic myeloid leukemia; CMML: chronic myelomonocytic leukemia; ALL: acute lymphoblastic leukemia; HES: hypereosinophilic syndrome; EMS: 8p13 myeloproliferative syndrome; ALCL: anaplastic large cell lymphoma; MM: multiple myeloma.

tial of alternative PTK inhibitors to overcome resistance in target kinases.[99] BCR/ABL independence, but LYN dependence, was observed during *in vitro* selection of CML cells for imatinib resistance, suggesting that essential signals from BCR/ABL can be substituted through SRC family kinases.[103,104] In addition, several previous studies have proposed involvement of another SRC family kinase, HCK (hematopoietic cell kinase), in malignant transformation of myeloid cells by the BCR/ABL fusion.[105] Hu and co-workers have recently shown that the SRC-kinase family members LYN, FGR, and HCK are essential for induction of B-cell ALL by the BCR/ABL fusion.[106]

Interestingly, a group of small molecule inhibitors targeting SRC family kinases has also been shown to possess high sensitivity against ABL (Table 2).[107] Resolution of the crystal structure revealed that imatinib selectively binds to a distorted inactive conformation of the ABL kinase.[108,109] In contrast to imatinib, dual SRC/ABL inhibitors, such as BMS-354825 or AP-23464, seem to interact and block ABL in an active conformation.[110] These compounds have been shown to retain activity against the most frequent imatinib-resistance-associated BCR/ABL mutations including E255K, Y253F, Q252H, M351T, and H396P. In addition, a new direct derivative of imatinib (AMN-107) has recently been shown to be more potent than imatinib and to be active against a large number of imatinib-resistant BCR/ABL mutants *in vitro* and *in vivo*.[111]

However, cells harboring a T315I mutation in BCR/ABL did not respond significantly to any of these new compounds.[110-112] In contrast, a new small molecule compound (ON-012380) that, unlike the current tyrosine kinase inhibitors, is not ATP-competitive but substrate-competitive, was demonstrated to induce apoptotic cell death in a large number of imatinib-resistant BCR/ABL mutants, including T315I at a <10nM concentration.[113]

Clinical trials will be needed to evaluate the advantages and disadvantages of these 2nd generation, higher potency, tyrosine kinase inhibitors. It will be interesting to see whether the broader range of iological activity of combined SRC/ABL inhibitors will be linked to more severe side effects, which may limit their clinical use. In addition, the clinical efficacy of a combination of compounds which are ATP-, or substrate-competitive should be analyzed. Indeed, encouraging results have recently emerged from several ongoing clinical phase I/II studies testing the new SRC/ABL inhibitors (BMS-354825) for the treatment of imatinib-resistant advanced phase CML or relapsed/refractory Philadelphia chromosome-positive ALL.[114,115] It is foreseeable that we will soon have at hand a large number of potent small molecule inhibitors that target different oncogenic kinases involved in various hematologic malignancies. However, mutations of the targets as a consequence of adaptive resistance will always be a threat to this approach, and therefore the underlying molecular mechanisms need to be investigated. It is a fact that small molecule inhibitors, although proposed to be specific for a given PTK target, almost always have some activity against other known and unknown targets. Development of new *in vitro* strategies to determine PTK inhibitor specificity clearly demonstrates that specificity varies widely and is not strongly correlated with the chemical structure or the identity of the intended target.[116] By producing a BCR/ABL mutant (T315A) that can be inhibited uniquely by a distinct small molecule (NaPPI), Wong and collaborators showed that suppression of BCR/ABL activity alone was insufficient to eliminate BCR/ABL$^+$KIT$^+$ expressing immature murine myeloid leukemic cells, whereas imatinib, by targeting both ABL and KIT, effectively eliminated BCR/ABL$^+$KIT$^+$ expressing leukemic cells. These results suggest that the therapeutic effectiveness of small molecule drugs such as imatinib could be due to the ability of the inhibitor to suppress PTK in addition to the dominant target.[117]

Since some resistance mutations are difficult to target by small molecule inhibitors, other approaches to improve the treatment of PTK-mediated hematologic malignancies are needed (Figure 3). Oligomerization of a PTK fusion is crucial for its activation and therefore interference with oligomerization using synthetic peptides or antibodies against the protein/protein interface could offer an alternative means of reducing the biological activity of the kinase as demonstrated for BCR/ABL.[118] Increased imatinib sensitivity in cells expressing BCR-ABL mutants by selective down-regulation of the expression of an oncogenic PTK by RNA interference (siRNA) has been shown to be another possible therapeutic strategy.[119] In addition, down-regulation of the TEL/PDGFβR fusion by siRNA enhanced the efficiency of small drug inhibitors in a mouse model of leukemia.[120] Further improvements in siRNA delivery and bioavailability through chemical modification of siRNA may in the future allow clinical application to improve conventional therapy for hematologic malignancies.[121]

Improving anti-leukemia therapy by targeting critical downstream messengers

Intensive research focusing mainly on the BCR/ABL fusion provided evidence that transforming activity of oncogenic PTK is mediated by parallel activation of several downstream signaling pathways. In general, multiple phosphorylated tyrosine residues of a distinct PTK serves as a docking site for multiple adapter or signaling mediators to become themselves activated through phosphorylation by the constitutive active

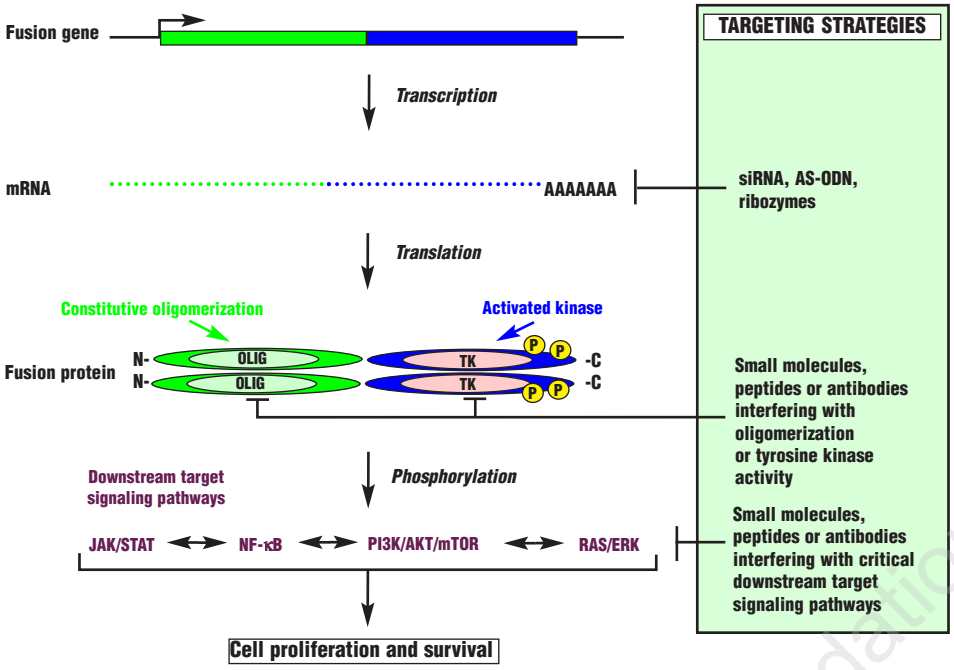

Figure 3. Therapeutic strategies targeting constitutive protein tyrosine kinases.

PTK. Final downstream mediators of this complex signalling network are phosphoproteins that translocate to the nucleus and act as transcriptional regulators activating a distinct group of target genes. As recently demonstrated by FACS-based single cell analysis, aberrant activation of this network of phosphoproteins correlated with chromosomal rearrangements and disease outcome in cells from leukemia patients.[122] Refined molecular techniques have allowed characterization of an increasing number of signaling pathways involved in transformation by deregulated PTK. As previously outlined in detail in several excellent review articles, the oncogenic activity of a given PTK is mediated by several signalling pathways including JAK/STAT, RAS/MAPK, PI3K/AKT, or NF-κB.[3,4,123,124] Interference with critical downstream signals may therefore offer a way of overcoming the limitations of resistance development against small molecule PTK inhibitor. However, how to determine whether activation of a distinct signalling mediator is critical for disease induction by a mutated PTK remains to be elucidated.

One method for genetically dissecting critical signaling pathways for *in vivo* leukemogenesis is to reconstitute lethally-irradiated mice with cells from syngeneic donors with a specific signalling defect expressing the oncogenic mutation of interest.[63] Using this approach as schematically outlined in Figure 4, it has been demonstrated that activation of STAT5 is essential for induction of a leukemia-like syndrome by TEL/JAK2 but not BCR/ABL.[125,126] In addition, a similar experimental strategy has demonstrated the importance of a signaling pathway involving the interaction of Grb2 and Gab2 in activating the RAS and PI3K/AKT axis: first, mutation of the Grb2 interaction site of BCR/ABL or TEL/ABL severely attenuated the induction of a CML-like disease by these PTK fusions; furthermore, bone marrow myeloid progenitors from Gab2 (-/-) were resistant to transformation by the BCR/ABL fusion protein.[127,128] There is increasing evidence that activation of the PI3K/AKT signaling pathway leading to downstream inactivation of FOXO transcription factors, activation of the mammalian target of rapamycin (mTOR), or induction of Skp2 (leading to degradation of the cell cycle inhibitor p27), plays a central role in transformation by several mutated PTK such as BCR/ABL, mutated FLT3 or KIT.[129,130] By using cells from mice lacking PI3K genes, Kharas *et al.* were able to show that products of the PIK3R1 gene directly contribute to transformation of B-cells by BCR/ABL.[131] In addition, constitutive activation of the PI3K pathway has been shown to be essential for survival of AML blasts.[132,133] Based on all these findings, it is not surprising that inhibition of the PI3K/AKT signaling cascade by various compounds, targeting PI3K itself or downstream mediators such as mTOR, has shown clear antiproliferative effects in PTK-transformed hematopoietic cells. Targeting of the PI3K/AKT pathway by the mTOR inhibitor of rapamycin synergizes with imatinib against BCR/ABL-mediated transformation of myeloid and lymphoid cells and increases survival in a murine CML model. In addition, a rapamycin/imatinib combination also inhibits imatinib-resistant mutants of BCR/ABL, and rapamycin plus PKC-412 synergistically inhibits cells expressing PKC412-sensitive or –resistant leukemogenic FLT3 mutants.[134] Likewise, the PDK1 inhibitor OSU-03012 in combination with imatinib showed a

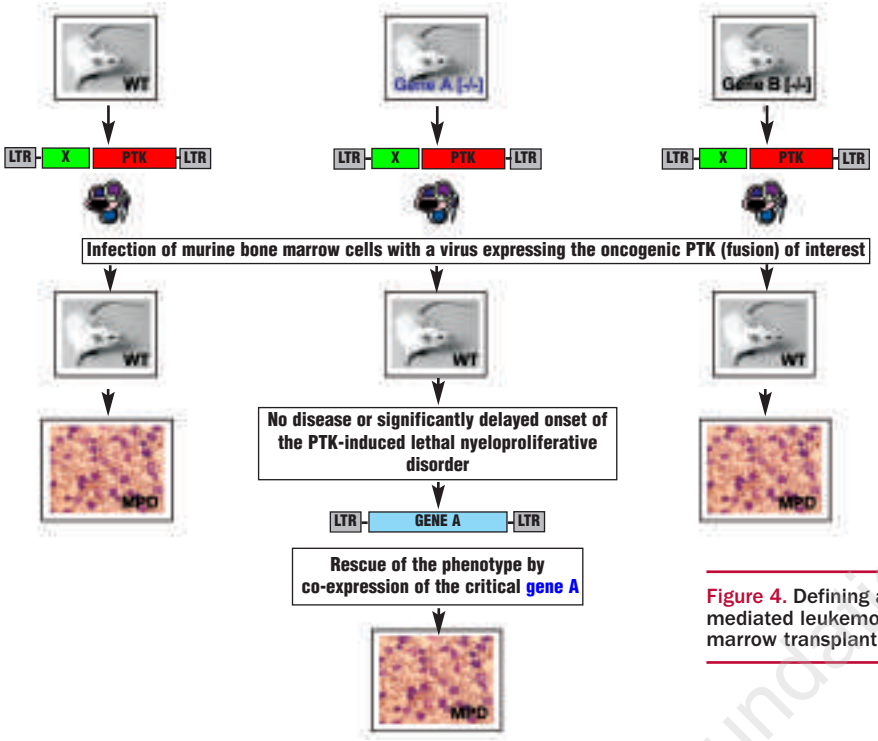

Figure 4. Defining a critical gene A involved in PTK-mediated leukemogenesis by using a murine bone marrow transplant assay.

synergistic effect on induction of apoptosis of cell lines expressing the E255K and T315I BCR/ABL mutants (Figure 5).[135]

Although the detailed molecular mechanisms are still not fully understood, it has been known for over a decade that the RAS/ERK signaling pathway is another major effector cascade mediating oncogenic activities of deregulated PTK.[136] To render RAS active, the molecule has to be farnesylated (transfer of an isoprenoid = farnesyl) to the C terminus allowing localization to the cell membrane, which is necessary to activate Raf1 and the MAP kinase pathway.[137] Therefore small molecules that interfere with farnesylation may be able to down-regulate these downstream pathways. Farnesyl-transferase inhibitors targeting the RAS/MAPK pathway (e.g. SCH-6636, R-115777) have been shown to inhibit proliferation of BCR/ABL-expressing cells.

Interestingly, farnesyl-transferase inhibitors not only enhanced imatinib-induced apoptosis but also co-operated with imatinib in resistant cells resulting from BCR/ABL mutation, suggesting that therapeutic regimens combining these compounds should be tested in clinical trials.[138] Exposure of hematopoietic progenitor cells from CML patients to imatinib produced enhanced MAP kinase activity. Treatment of the cells with a combination of imatinib plus a MAPK (MEK-1/2) inhibitor (U0126) resulted in significantly increased suppression of CML progenitor cell growth (Figure 5).[139]

Are there other ways of interfering with the RAS pathway? A substrate of the BCR/ABL PTK is the 62kD RAS GTPase-activating protein (RASGAP) p62/Dok-1 (downstream of tyrosine kinase).[140] Inactivation of the gene encoding for p62/Dok-1 resulted in increased proliferation of hematopoietic cells upon growth factor treatment, with sustained activation of RAS and MAPK after factor removal. In addition, p62/Dok-1 inactivation resulted in a significant shortening in the latency of the fatal myeloproliferative disease induced by retroviral-mediated transduction of BCR/ABL in bone marrow cells, suggesting that it acts as an endogenous negative regulator of RAS that can oppose BCR/ABL induced leukemogenesis.[141] Inactivation of two members of the dok family of proteins (p62/Dok1, p56/Dok-2) results in aberrant hematopoiesis and significantly increased activation of the RAS/MAPK pathway. In addition, these animals develop a CML-like myeloproliferative disease. Furthermore, in transgenic animals expressing the BCR/ABL PTK, loss of Dok-1/Dok-2 leads to disease acceleration and blast crisis.[142,143] Taken together, these observations suggest that imitating p62/Dok function by a small molecule could offer a future way of inhibiting deregulated RAS/MAPK activity in PTK-induced leukemia.

Several studies have proposed that constitutive activation of the NF-κB transcriptional regulator may represent another effector of the oncogenic effects of a mutated PTK.[144] Although it is not well understood how deregulated PTK activity leads to constitutive activation of NF-κB, there is emerging evidence that activation of NF-κB involves crosstalk between the PI3K and RAS/MAPK pathways.[145,146] Several NF-κB target genes, such as cIAP1/cIAP2, Bcl-XL, or Mcl1, are well-known

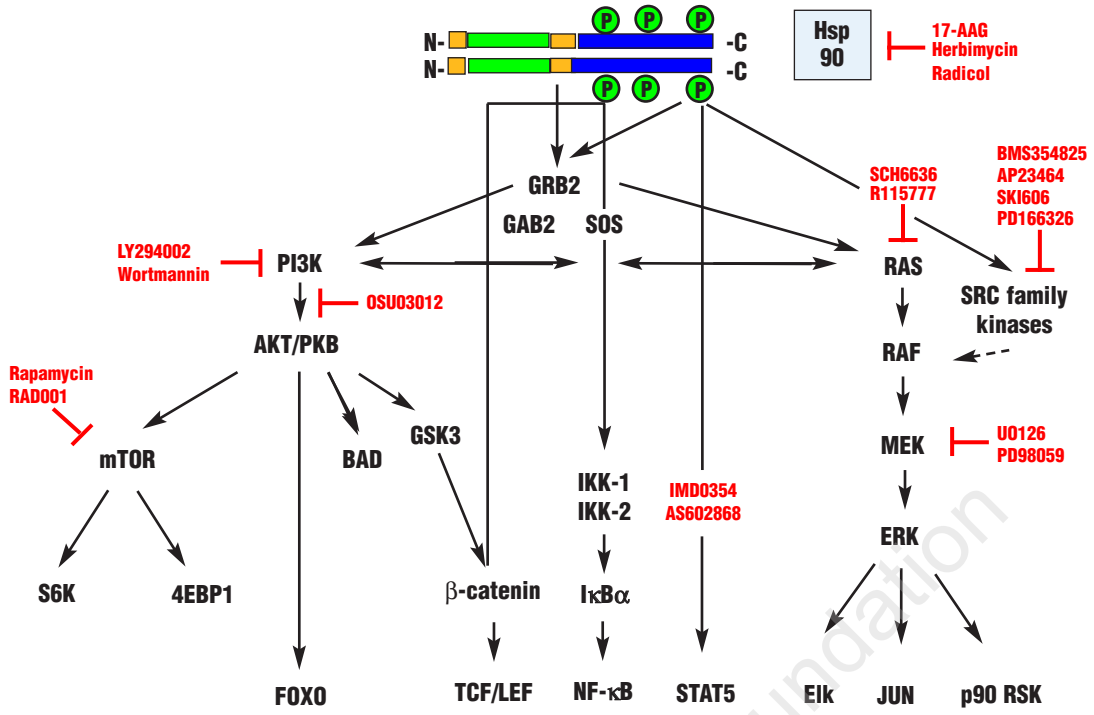

Figure 5. Targeting downstream signaling pathways of oncogenic PTK.

inhibitors of apoptosis that may co-mediate the anti-apoptotic effect of a constitutively activated PTK. Interestingly, Mcl-1 has been recently identified as a BCR/ABLdependent target in CML cells.[147] Since Mcl-1 plays an essential role in the survival of hematopoietic stem cells, it will be interesting to analyze its function in hematopoietic stem cells carrying an oncogenic PTK.[148] Several strategies have been proposed to target deregulated NF-κB activity in leukemic cells. The proteosome inhibitor PS-341 (bortezomid, Velcade™), a compound known to interfere with deregulated NF-κB activity, was able to suppress growth and to induce apoptotic cell death in BCR/ABL-positive cell lines that were sensitive or resistant to imatinib-mesylate.[149] In addition, two novel NF-κB inhibitors (IMD-0354; AS-602868) that selectively target IKK, (IKK2), a key element in the NF-κB pathway, were able to impair growth and survival of leukemia cells.[150,151] Moreover, parthenolide, a naturally-occurring small molecule found in herbal medicine, has very recently been shown to selectively induce apoptosis of leukemic stem cells, primary human AML cells and blast crisis CML cells through inhibition of NF-κB, proapoptotic activation of p53 and increased reactive oxygen species, while sparing normal hematopoietic cells.[152] Whether interference with the NF-κB pathway would be sufficient to overcome PTK-inhibitor resistance remains to be shown.

Are there other major signal transduction pathways involved in PTK-mediated leukemogenesis? New insights came from studies investigating signaling in cells from CML patients in blast crisis and from patients with imatinib-resistant CML. In these patients, increased levels of BCR/ABL and nuclear β-catenin were found in sorted granulocyte/macrophage progenitors. Unlike normal granulocyte/macrophage progenitors, CML-blast crisis progenitors formed self-renewing, replatable myeloid colonies. These findings suggest that activation of β-catenin provides progenitor cells with leukemic potential by enhancing self-renewal capacity.[153] β-catenin is a central mediator of the wingless-Int (WNT) pathway. Deregulated WNT activation through mutational alteration of several of its signaling mediators is frequently found in solid cancers.[154] However, there is increasing evidence suggesting that activation of the WNT pathway co-operates with PTK in leukaemic signal transduction. Expression of transcription factor fusions like AML1/ETO and PML/RARα in leukemic cells leads to induction of several genes associated with WNT signaling.[75] In addition, constitutive WNT signaling activation was found in a significant fraction of leukemic blasts from patients with AML-M0.[155] γ-catenin (plakoglobin), a functional (at least partially) homolog to β-catenin in the WNT pathway, has been shown to mediate self-renewal by several transcription factor fusions associated with

AML.[156] A direct link for co-operation of muted PTK with WNT signaling was observed in a murine hematopoietic cell line (32D) transformed by FLT3-ITD with significantly increased nuclear β-catenin levels. In addition, clonogenic growth of 32D-FLT3-ITD cells was strongly dependent on activation of T-cell factor 4 (TCF4, a central transcriptional regulator downstream of β-catenin) suggesting that FLT3-ITD and WNT signaling can synergize in myeloid transformation.[157] Although these observations suggest an important role of WNT in leukemogenesis, the detailed molecular links to define the critical mediators still need to be elucidated.

Are there mechanisms that would allow therapeutic interference with the oncogenic activity of a genetically altered PTK *other* than by targeting its kinase activity and/or blocking critical downstream signaling mediators? Growth factor and steroid receptors, as well as oncogenic PTK, seem to be protected against ubiquitinylation and proteosomal degradation by heat-shock proteins (Hsp) such as Hsp-90 acting as molecular chaperones.[158] Several compounds have been identified (e.g. geldanamycin, herbimycin A, or radicol) to be Hsp-active leading to destabilization of its client proteins.[159] Cells expressing various constitutively activated PTK [BCR/ABL (wild-type and imatinib-mesylate resistant mutants), FLT-ITD, NPM/ALK] have been shown to be sensitive to pharmacologic treatment with Hsp-90 inhibitors such as geldanamycin (17-AAG).[160-163] Since several Hsp-90 client proteins are known to exert chemoprotective anti-apoptotic function, Hsp-90 inhibitors are currently being tested for PTK-mediated leukemia in combination with other targeted compounds as well as with standard chemotherapeutic agents.[164-166]

Several of the new compounds targeting the PI3K/AKT, MAPK or NF-κB pathways have been tested for their effects on proliferation and survival of leukemia cells and have entered clinical trials. In addition, synergistic effects of small molecule PTK inhibitors are being reported with an increasing number of other compounds with anti-leukemia activity whose mechanisms of action are not well understood. Synergistic effects were observed for imatinib-mesylate and arsenic sulfide (As_4S_4) with enhanced *in vitro* cytotoxicity in BCR/ABL expressing cell lines.[167] In addition, antileukemic effects in BCR/ABL-transformed cell lines were observed when these cells were treated with perillyl alcohol, curcumin or mycophenolic acid. Perillyl alcohol and curcumin seem to exert their effects by interfering with RAS activation, whereas mycophenolic acid has been proposed to be a specific inosine monophosphate dehydrogenase inhibitor resulting in depletion of intracellular guanine nucleotides.[168-170] More natural and synthetic compounds will be characterized and these may help to overcome the resistance problem of targeting deregulated PTK activity by small molecules.

Most of the new compounds, that target signaling pathways *downstream* of an oncogenic PTK have been tested in PTK-transformed cells *in vitro*. Lack of specificity and toxic effects due to necessary high dosages may prevent the clinical use of many of these agents. However they can be used as a paradigm showing that targeting downstream pathways is a way to overcome resistance against small molecules directly blocking the constitutive activated PTK.

Most of these compounds have also been shown to act in synergy not only with small molecule PTK inhibitors but also with other reagents targeting a parallel signaling pathway. This observation is not surprising, since crosstalk between downstream signaling pathways has been demonstrated in numerous studies. In addition, one has to realize that downstream signaling by an oncogenic PTK may be a dynamic rather than a static process. Progressive changes, such as reactivation of new signaling pathways, have been observed in the leukemogenic signaling of BCR/ABL-transformed cells *in vitro*.[171] In addition, selection for imatinib-resistance of CML cells resulted in BCR/ABL-independent and LYN kinase overexpressing cells.[104] Moreover, inhibition of BCR/ABL kinase activity in CML progenitor cells ($CD34^+$) has been shown to result in a growth-factor dependent compensatory increase in MAPK activity that may contribute to incomplete elimination of CML progenitors by imatinib.[139]

Considering all the above mentioned studies, which are based on the current understanding of the molecular genetics of PTK-mediated hematological malignancies, one may propose a hypothetical ideal targeted therapeutic approach: after determination of the genotype showing the involved PTK, treatment would be started with a selective small molecule PTK inhibitor.

In case of signs of primary or secondary resistance against this compound, functional screening would be performed to enable an alternative compound to be selected.[172] To enhance the efficacy of the treatment, until compounds targeting critical downstream mediators become available, the PTK inhibitor could be combined with conventional chemotherapy. This approach has recently been demonstrated as effective even for patients with PTK-mediated chronic myeloid leukemia in progression.[84,85] We think that, in the near future, we will not only perform *antibiograms* to circumvent bacteria resistance, but we will also have to develop high throughput screening tools to select the optimal inhibitor cocktail targeting critical molecular lesions.

What is the role of compounds targeting PTKs and their critical downstream mediators in the treatment of acute leukemia? According to the proposed genetic model of human leukemia, blasts from patients with acute leukemia harbor at least two different alterations

targeting both classes. Is monotherapy targeting the class I mutation involved sufficient to achieve remission, or do we have to include compounds that target both classes of mutations? Expression of FLT3 containing an activating mutation (class I) in bone marrow from mice expressing the PML/RARα fusion (class II) clearly demonstrated cooperation of these mutations. Treatment of these mice with an FLT3 small molecule inhibitor (SU-11657) as a single agent only modestly impaired disease progression. However, application of this drug in combination with all-trans retinoid acid (ATRA) targeting PML/RARα led to rapid restoration of normal hematopoiesis.[173] Recent reports from two trials testing SU-11248 and PKC-412 demonstrated clinical activity in patients with AML and activating FLT3 mutations. However, partial remissions of short duration suggest that these compounds should be used in combination with other forms of treatment modalities such as chemotherapy.[174,175] Apart from the treatment of PML/RARα-induced acute promyelocytic leukemia by ATRA, the development of targeted therapeutic approaches against transcription factor fusions (class II targets)-induced leukemia is still in the early stages.

Several approaches are currently being investigated including chromatin modulation by histone deacetylase (HDAC) inhibitors in combination with conventional chemotherapy.[176] Based on our current knowledge, we think that an efficient targeted therapeutic protocol for acute leukemia should combine compounds that restore blocked differentiation induced by class II mutations with small molecule inhibitors against co-operating class I mutations reducing cell number.

Summary and Outlook

Deregulated PTK activation by either fusion gene formation or activating mutations is a key pathogenic event in human leukemogenesis (Figure 1). The development of efficient small molecule PTK inhibitors is a milestone in targeted anti-cancer therapy; a good decade after its first description, imatinib-mesylate is the gold standard for treatment of Philadelphia chromosome-positive CML. Following the imatinib principle, more and more small molecules targeting different PTK are currently entering clinical trials for hematologic malignancies induced by class I mutations (Tables 2, 3). Detailed molecular analysis of the patient's tumor cells before and during therapy will allow the selection of an optimal inhibitor for the mutation present. The vast number of inhibitor-containing compounds with a less restrictive target range may help prevent the development of resistance against the first inhibitor selected. In addition, new compounds that specifically block critical downstream signaling pathways could provide the means to overcome PTK inhibitor resistance (Figure 5). Since acute leukemia is characterized by class I and class II mutations, small molecule PTK inhibitors will also have an impact on therapeutic regimens for this disease. However, collaborating class I mutations are only known for a minority of cases of acute leukemia. Present efforts to analyze the mutational status of the whole set of human protein kinases (also referred to as the human *kinome*) in hematologic malignancies and other cancers will definitely determine which PTK have to be targeted in a given lesion. In addition, new molecular tools such as high-resolution DNA arrays will allow the identification of more cryptic translocations and small internal deletions leading to oncogenic PTK activation. Moreover, functional screens using siRNA technology in combination with large compound libraries will help to further define and target critical downstream signalling components of oncogenic PTK. Future therapeutic regimens for hematologic malignancies will most probably depend on a combination of specific agents acting at different levels to overcome resistances, as in infectious diseases. Based on all these elements, each patient will have an individually tailored combination treatment. Despite the success of small molecules targeting oncogenic PTK, we must be aware that the effect of these compounds is mostly based on reduction (debulking) of the leukemic clone at the committed progenitor cell level excluding the mostly quiescent leukemia stem cells. To develop curative targeted approaches, it will therefore be necessary to understand the molecular pathways that regulate leukemia stem cell self-renewal and survival.[177,178] New compounds selectively targeting leukemia stem cells, such as parthenolide, seem promising, but the activity of this drug needs to be confirmed in clinical trials and pharmacological properties limiting its use, such as water solubility, also need to be overcome.[152]

If the improvement in our understanding of functional cancer genetics continues at its present rate, anti-leukemia therapy, and perhaps anti-cancer therapy in general, will hopefully shift from an unselective chemotherapy to a regimen including a disease-driven selection of compounds which target critical signaling pathways, perhaps via a transitional step associating targeted therapy to chemotherapy.

YC: involved in design, writing and correction of the manuscript; JS: designed and wrote the text and performed all the figure artwork of the manuscript.

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