Title

FLT3 as a therapeutic target in AML: still challenging after all these years

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Abstract
Mutations within the *FMS-like tyrosine kinase 3* (*FLT3*) gene on chromosome 13q12 have been detected in up to 35% of acute myeloid leukemia (AML) patients and represent one of the most frequently identified genetic alterations in AML. Over the last years, FLT3 has emerged as a promising molecular target in therapy of AML. Here, we review results of clinical trials and of correlative laboratory studies using small molecule FLT3 tyrosine kinase inhibitors (TKI) in AML patients. We also review mechanisms of primary and secondary drug resistance to FLT3-TKI and from the data currently available we summarize lessons learned from FLT3-TKI monotherapy. Finally, for using FLT3 as a molecular target we discuss novel strategies to overcome treatment failure and to improve FLT3 inhibitor therapy.

Introduction
AML is a heterogeneous disorder of the hematopoietic progenitor cell, characterized by a block in differentiation and uncontrolled proliferation. Long-term survival rates are 25-70% in patients younger than 60 years, and only 5-15% in older patients. Currently, cytogenetic analysis at the time of diagnosis provides the most important prognostic information, predicting outcome after induction chemotherapy, relapse rate and overall survival.\(^1\) Approximately 45% of adult AML patients have no detectable chromosomal aberrations and, until recently, were considered to have an intermediate risk profile. However, outcome of patients with a normal karyotype is highly heterogeneous and suggests the necessity for further classification of this large patient group. Indeed, several specific acquired mutations have been described and shown to be significant in molecular pathogenesis of AML. Mutations within the *FLT3* gene represent one of the most frequently identified genetic alterations. FLT3 belongs to the class III receptor tyrosine kinase (RTK) family, including FMS, c-KIT, PDGFRα and PDGFRβ.\(^2\) In normal human hematopoiesis, FLT3 expression is restricted to immature hematopoietic progenitors including CD34\(^+\) hematopoietic stem cells (HSC).\(^3\) Binding of its ligand, FLT3-ligand (FL), is followed by a conformational change, homodimerization and subsequent activation of multiple downstream signaling pathways.\(^2\) FL stimulation of hematopoietic progenitors without other growth factors prompted monocytic differentiation, whereas combinations of SCF, IL-3 and FL induced proliferation and maintenance of human CD34\(^+\)/CD38\(^-\) progenitor cells.\(^4\) Of note, most human CD34\(^+\) HSCs capable to reconstitute non-
obese diabetic/severe combined immunodeficiency (NOD/SCID) mice express FLT3, suggesting an essential role of FLT3 in human hematopoiesis.7

In hematologic malignancies, high levels of FLT3 expression have been detected in AML blasts (70 – 100%) and acute lymphoblastic leukemia.8-10 Two major classes of activating FLT3 mutations have been identified in AML patients: internal-tandem-duplications (ITD) and tyrosine kinase domain (TKD) point mutations. ITDs in the juxtamembrane (JM) domain of FLT3 were first described by Nakao et al. and are detected in 20 – 25% of AML patients.11-16 ITDs are in-frame duplications of 3 – 400 base pairs. Recently, FLT3-ITD insertion sites were systematically reviewed in 753 unselected patients with AML positive for FLT3-ITD and it was demonstrated that 28.7% of ITDs integrate in the TKD1 and not as previously assumed in the JM domain of FLT3.17 ITD-mutations cause constitutive activation of FLT3, leading to aberrant activation of multiple downstream pathways such as PI3K/AKT, MAPK/ERK and STAT5.18, 19 FLT3-ITD expression confers factor independent growth in murine IL-3-dependent cell lines and causes a fatal myeloproliferative disorder in murine bone marrow transplantation models and in FLT3-ITD knock-in mice.20-23

In addition, about 5 – 10% of AML patients harbor point mutations within the second TKD. In most cases, these mutations result in a substitution of tyrosine for aspartic acid at codon 835 (D835Y). Similar to FLT3-ITDs, TKD-mutations cause constitutive activation of the FLT3 receptor, aberrant activation of downstream signaling pathways and factor-independent growth. In addition to FLT3-TKD-D835Y, several other mutations within the TKD have been reported.24-26 Finally, rare activating point mutations within the JM domain (less than 1%) have been described.27

Impact of FLT3 mutations on cell counts at presentation, CR rate and prognosis

The presence of FLT3-ITD mutations is highly associated with increased white blood cell counts, high percentages of peripheral blood (PB) and bone marrow (BM) blasts and cytogenetic normal (CN) AML (65-70%).12-14, 16, 28, 29 Whereas it appears that there is no uniformly detectable impact on complete remission (CR) rate, the presence of a FLT3-ITD mutation significantly correlates with an increased risk of relapse (RR) and dismal overall survival (OS) and therefore has become a widely accepted prognosis factor in CN-AML.12, 13, 28, 29 A smaller study failed to demonstrate
an adverse effect on treatment outcome based on the mere presence of a FLT3-ITD but suggested the absence of the wild-type (wt) allele as a predictor of poor prognosis in AML.\textsuperscript{15} In line with these data, two studies demonstrated that a high mutant/wt allelic ratio is associated with a particular high risk of early relapse within the first year and decreased OS, and is an independent prognostic factor in multivariate analysis.\textsuperscript{29, 30} However, others did not find an association of FLT3-ITD allelic-burden with poor prognosis.\textsuperscript{29, 31} The impact of the ITD size on prognosis is also discussed controversially.\textsuperscript{32-34}

Recently, Kayser et al. reported detailed molecular analysis of FLT3-ITDs in 241 FLT3-ITD positive AML patients. The authors found a strong correlation of the ITD insertion site with ITD length: the more C-terminal the insertion site is located, the longer is the size of the inserted fragment.\textsuperscript{31} In multivariable analysis, logarithm of WBC counts and presence of FLT3-ITD in the beta1-sheet of the TKD1 were associated with lower CR rates. FLT3-ITD length and mutant/wt allelic ratio were predictors for reduced CR rates in univariable analysis only.\textsuperscript{31} Multivariable analysis revealed a significantly dismal relapse free survival and OS for patients with FLT3-ITD insertions within the beta1-sheet of the TKD1 as compared to all other insertion sites. In this analysis, neither ITD size nor higher mutant/wt allelic ratio showed a significant impact on RFS or OS.

In 2005, Falini and colleagues described a novel mutation within the \textit{NPM1} gene detected in 35\% of AML patients.\textsuperscript{35} As \textit{FLT3-ITDs}, mutated \textit{NPM1} is significantly associated with CN-AML and a significant proportion of patients carry both, \textit{FLT3-ITD} and \textit{NPM1}\textsuperscript{mut}\textsuperscript{35-40}. Mutated \textit{NPM1} is associated with a high rate of CR, an increase in event-free survival and favorable OS. However, these positive effects are lost in the presence of a coexisting \textit{FLT3-ITD}.\textsuperscript{36-40} Whether the genotype \textit{NPM1mut/FLT3-ITD} is associated with intermediate or poor outcome is discussed controversially and warrants further analysis.\textsuperscript{36-39, 41}

As shown for \textit{FLT3-ITD} mutations, the presence of \textit{FLT3-TKD} point mutations in AML is associated with higher PB and BM blast counts and CN-AML.\textsuperscript{12, 29, 42} However, with respect to prognosis, the relevance of \textit{FLT3-TKD} point mutations is less clear. Whereas some studies reported dismal outcome, others described an association with good prognosis or no significant differences.\textsuperscript{12, 29, 43-46} These conflicting data are likely due to small patient numbers, different treatment regimens and selection within patient cohorts. Interestingly, Bacher et al. demonstrated that the
prognostic effect of \textit{FLT3-TKD point mutations} is dependent on concomitant mutations in other genes.\textsuperscript{42} For example, \textit{FLT3-TKD} point mutations had an additional positive prognostic impact in patients harboring \textit{NPM1} or \textit{CEBPA} mutations and negative effects in combination with already unfavorable alterations such as \textit{FLT3-ITD} or \textit{MLL-PTD}. However, another group failed to demonstrate improved survival in patients harboring \textit{FLT3-TKD} point mutations within the context of \textit{NPM1}\textsuperscript{mut}.\textsuperscript{45} In conclusion, the prognostic significance of \textit{FLT3-TKD} point mutations is currently unclear.\textsuperscript{12, 42, 47, 48}

\textbf{FLT3 as a therapeutic target}

Aberrantly activated FLT3-kinase is considered to represent an attractive therapeutic target in AML. Several small molecule FLT3-TKIs have been developed and examined in AML patients as single agents or in combination with chemotherapy. In addition, FLT3-directed antibody therapy (IMC-EB10) is currently being investigated in a phase 1 clinical trial. Preclinical characteristics of FLT3-TKI are summarized in Table 1. Clinical experience using FLT3-TKIs is reviewed as outlined below and summarized in Table 2.

\textit{Clinical experience using FLT3-TKIs as single agents}

\textbf{Midostaurin}

In a phase 2 trial, 20 patients with either relapsed/refractory FLT3-mutated AML, advanced myelodysplastic syndrome (MDS) or considered unfit for intensive chemotherapy were treated with midostaurin.\textsuperscript{49} In 18 patients a \textit{FLT3-ITD} mutation and in 2 patients a \textit{FLT3-D835Y} mutation was detected. Fourteen patients experienced a greater than 50% reduction in PB blast count with some patients achieving complete clearance of PB blasts. In addition, 6 patients achieved a more than 50% decrease in BM blast count. Median time to progression was 2 – 3 months. In correlative laboratory studies, the plasma inhibitor activity was determined in 10 patients.\textsuperscript{50} FLT3-ITD expressing cell lines were incubated with trough plasma samples obtained from patients during midostaurin therapy. FLT3 autophosphorylation was determined and compared to baseline levels. In 8 patient samples substantial inhibition of FLT3 tyrosine-phosphorylation was observed, 6 of these 8 patients showed clinical responses, whereas 2 patients appeared to be
intrinsically resistant to midostaurin. No responses were observed in patients with FLT3 tyrosine-phosphorylation levels >15% of baseline upon midostaurin therapy.

Lestaurtinib
Single agent lestaurtinib was tested in a phase 1/2 clinical trial in patients with refractory/relapsed AML expressing FLT3-activating mutations.51 Five patients treated at a dose of 60 mg orally twice daily experienced clinical responses as shown by a decrease in PB and BM blasts, recovery of absolute neutrophil counts and decreased transfusion requirements. In general, responses were of short duration, lasting from 2 weeks to 3 months. All patients, in whom clinical responses were observed, achieved strong and, sustained inhibition of FLT3 tyrosine phosphorylation to a level of less than 15% of baseline.51 Interestingly, 2 of 8 patients displayed no cytotoxic response to lestaurtinib in an in vitro bioassay, despite potent inhibition of FLT3 phosphorylation. As predicted by the in vitro data, both patients showed no obvious clinical response, suggesting the activation of unknown alternative pathways. In a follow-up phase 2 clinical trial, the effects of lestaurtinib monotherapy were examined in patients with untreated AML irrespective of their FLT3 mutation status.52 No complete or partial remissions were observed in this older AML patient group. Eight out of 29 patients treated at a starting dose of 60 mg BID demonstrated hematologic improvements, with some patients experiencing prolonged transfusion independence. The median time to progression was 25 days. Again, informative correlative in vitro and ex vivo studies demonstrated a clear relationship between the level of FLT3 tyrosine kinase inhibition, in vitro cytotoxicity and clinical response.53 Interestingly, in 5 out of 24 FLT3-WT patients (23%) clinical responses were seen, suggesting dependence on FLT3 signaling due to autocrine/paracrine mechanisms or due to so far undetected activating mutations in AML blasts of these patients.

Sorafenib
The therapeutic efficacy of sorafenib was evaluated in a phase 1 trial in 16 patients with refractory/relapsed AML. Dose levels ranged from 200 mg to 400 mg BID po and no dose limiting toxicity (DLT) has been observed. A significant decrease in the percentage of PB blasts and BM blasts was observed in all FLT3-ITD+ patients (6/6), whereas only 3 of FLT3-WT patients (3/7) and none of patients harboring FLT3-D835Y (0/3) responded.54 Recently, results from a compassionate use program of
sorafenib in 6 FLT3-ITD^{+}-patients, either refractory or in relapse, have been reported.\textsuperscript{55} All 6 patients showed evidence of clinical response with 3 patients achieving a complete remission (CR). Two patients could undergo allogeneic stem cell transplantation. In contrast to previously reported FLT3-TKI studies, treatment duration was prolonged with a median of 158 days. The long lasting responses and high response rates in this poor risk population are in line with recently published data demonstrating that blasts with high mutant to wild-type allelic ratios or from relapsed/refractory disease develop oncogene addiction and are more likely to respond to FLT3-TKIs such as sorafenib.\textsuperscript{56}

Semaxanib
Semaxanib was tested in a phase 2 trial in patients with refractory AML or MDS irrespective of their FLT3-mutation status.\textsuperscript{57} Single agent semaxanib had modest clinical activity with documented PR and hematologic improvement in 4 patients (7%). In AML patients, median survival was 12 weeks with median treatment duration of 9 weeks. Correlative studies revealed FLT3-phosphorylation in 17 out of 22 patients. Seven patients exhibited inhibition of FLT3-phosphorylation following semaxanib infusion. However, no correlation with clinical response could be demonstrated.\textsuperscript{58} In another multicenter phase 2 trial, enrollment was restricted to refractory AML patients or patients unfit for conventional chemotherapy and expression of c-KIT on leukemic blasts.\textsuperscript{59} Out of 42 patients, one patient achieved a morphological response with no evidence of blasts in PB and BM, 7 patients had a documented PR (19% overall response rate) and 17 patients were not evaluable due to rapid disease progression or early death. Responses lasted from 1 to 5 months with a mean duration of 1.6 months. Of 7 patients harboring FLT3-ITD mutations none responded to therapy.

Sunitinib
Pharmacodynamic (PD) and pharmacokinetic (PK) effects of sunitinib were assessed in a single-dose phase I study in AML patients.\textsuperscript{60} As expected, magnitude and duration of inhibition of FLT3 tyrosine-phosphorylation was dependent on dose and plasma drug levels. Significant and sustained (>24 hours) inhibition of phosphorylation of FLT3-ITD and FLT3-WT was observed in patients achieving plasma drug levels of >50 ng/ml and >100 ng/ml, respectively. In line with reported in
vitro data, FLT3-ITD blasts were more susceptible to inhibition by sunitinib compared to FLT3-WT blasts. In order to assess safety and tolerability as well as biological and molecular activity, a phase 1 study of sunitinib in relapsed or refractory AML patients was initiated. All 4 patients with FLT3 mutations achieved a morphological or partial response, whereas only 2 of 7 FLT3-WT patients showed evidence of clinical activity. Evaluable patients achieved drug plasma levels of 50 - 100 ng/ml and displayed modulation of FLT3 tyrosine-phosphorylation. All responses were of short duration (4 - 16 weeks).

Tandutinib
In order to evaluate safety, PK and PD, a phase 1 clinical trial with tandutinib in 40 patients with AML was initiated. Tandutinib was given twice daily with a starting dose of 50 mg followed by dose escalation up to 700 mg BID. The DLT proved to be reversible generalized muscular weakness, fatigue or both, probably due to inhibition of muscle-type nicotinic receptors. In a follow up phase 2 trial, 20 patients with FLT3-ITD-positive AML, either refractory, in relapse or not eligible for induction chemotherapy, were included. All patients achieved tandutinib trough plasma-concentrations of >150 ng/mL, the suggested IC₅₀ necessary to inhibit FLT3-autophosphorylation, and ex vivo assessment of FLT3 tyrosine-phosphorylation revealed partial or complete inhibition in 4 evaluable patients. Response was evaluable in 15 of 18 patients: seven patients experienced progressive disease, two patients had stable disease and 6 patients demonstrated transient (1-3 months) evidence of anti-leukemic effects with a decrease in PB blasts (mean decrease 92%) and in BM blasts (mean decrease 62%).

KW-2449
To assess PK, PD and safety, a phase 1 dose escalation study of KW-2449 in relapsed/refractory AML patients was initiated. KW-2449 was safe and well tolerated. Eight patients (26%) exhibited a >50% transient reduction of PB blasts, no CR or PR were observed. Out of the 8 responders, 5 harbored FLT3-ITD mutations. Interestingly, although the maximum tolerated dose was not defined, the trial was prematurely terminated because correlative laboratory studies suggested that effective and sustained inhibition of FLT3 was not achieved using a twice daily dosing schedule. Drug-plasma levels of >500 nM, the threshold necessary to achieve
inhibition of FLT3 tyrosine-phosphorylation to <20% of baseline level, were only maintained for 4 – 6 hours. Consequently, FLT3 tyrosine phosphorylation was completely downregulated at 2 and 4 hours post dose, but fully recovered at 8 and 12 hours time points as revealed by an \textit{ex vivo} analysis of primary AML blasts.\textsuperscript{65} These data underscore the importance of correlative laboratory studies necessary to understand the correlation of \textit{in vitro} cytotoxic effects, PK/PD and clinical activity.

**AC220**

A novel approach to identify promising TKIs was utilized by Zarrinkar et al., who screened a scaffold-focused library of compounds against several kinases.\textsuperscript{66} Based on binding affinity, they identified a novel bis-aryl urea derivate with high selectivity for FLT3. Optimization of this compound in terms of potency, selectivity and PK properties resulted in the second generation FLT3-TKI AC220. Recently, AC220 was investigated in a phase 1 dose escalation study in relapsed/refractory AML patients irrespective of their FLT3 mutation status.\textsuperscript{67} AC220 was administered once daily as an oral solution. At doses of 300 mg/day grade 3 QTc prolongations were observed in 2 patients, and therefore, 200 mg was declared as the maximum tolerated dose. In this trial, a total of 76 patients were treated with AC220. Of these, 23 (30\%) experienced clinical responses: 9 (12\%) patients had a CR and 14 (18\%) had a PR. Of note, responses were already observed in cohorts treated with doses as low as 18 mg and 40 mg/d. The median duration of response was 14 weeks. Interestingly, 10 of 18 FLT3-ITD\textsuperscript{+} patients (56\%) compared to 9 of 47 FLT3-WT patients (19\%) responded suggesting an increased susceptibility of FLT3 mutant AML. Currently, phase 2 follow up studies in FLT3-ITD and FLT3-WT patients are in progress.

**Clinical trials – combination therapy using TKI and chemotherapy**

Based upon \textit{in vitro} data showing synergistic effects for combinations of FLT3-TKIs with conventional chemotherapeutic agents, this approach is being tested in a number of clinical trials.\textsuperscript{68-71} Up to date, there is only little published data on results of these trials.\textsuperscript{72-75} Table 3 summarizes currently available data on clinical efficacy and toxicity. It appears that FLT3-TKI can be safely combined with conventional chemotherapy, produce high CR rates in FLT3-mutated patients, and inhibit FLT3 signaling. However, whether this translates in longer progression-free survival and
better OS rates is still unclear. Interestingly, results from a randomized trial of salvage chemotherapy followed by lestaurtinib for FLT3 mutant AML in first relapse were reported to be negative as to increase in response rates or prolongation of survival. However, these results need to be viewed carefully since pharmacokinetic factors and possible physiologic factors limited lestaurtinib’s ability to effectively inhibit FLT3. Currently, large international multicenter randomized studies are ongoing in newly diagnosed patients to test the efficacy of FLT3-TKI in combination with standard chemotherapy. Table 4 lists these yet unpublished phase II and phase III clinical trials. These trials are in progress and are expected to recruit a few hundred patients. Results are eagerly awaited and will have a major impact in this field. The optimal schedule (concomitantly or sequentially, during induction only or during all chemotherapy cycles) and duration (only during primary therapy or maintenance therapy) of TKI treatment needs to be carefully determined in future clinical trials. Last but not least, recently two phase I/II clinical trials testing the combination of a FLT3-TKI (Midostaurin) with hypomethylating agents (decitabine or azacitidine) have been initiated in the United States. The latter trials are mainly designed for AML patients ≥60 years of age and results may be of great importance for the growing population of older AML patients.

Preclinical experience using a FLT3-directed antibody-approach
The use of neutralizing antibodies directed against FLT3 may also prove as a successful therapeutic strategy. Recently, the neutralizing antibody IMC-EB10 was isolated from a human Fab phage display library and was shown to selectively bind cell-surface-FLT3 with high affinity and to block binding of FL. IMC-EB10 treatment of FLT-WT and FLT3-mutated cells inhibits FLT3 tyrosine-phosphorylation, activation of downstream pathways and cell growth. Furthermore, IMC-EB10 induced antibody-dependent cell-mediated cytotoxicity on FLT3-expressing cells. In a NOD/SCID BMT-model, treatment of mice decreased engraftment of primary human AML blasts without affecting engraftment of normal human CD34+ cells. In addition, IMC-EB10 significantly prolonged survival of NOD/SCID mice transplanted with FLT3-ITD+ MOLM14 cells. Interestingly, this neutralizing antibody therapy was also effective in NOD/SCID mice transplanted with FLT3-TKI resistant MOLM14 cells, highlighting the impact of antibody-dependent cell-mediated cytotoxicity. Safety of IMC-EB10 is currently tested in AML patients in a phase 1 clinical trial (NCT00887926).
Mechanisms of resistance to FLT3-TKIs

Generally, treatment failure is caused by inherent (primary) resistance of the malignant clone or development of secondary (acquired) resistance emerging after an initial response. In the following section we will discuss possible mechanisms of inherent and acquired resistance involved in failure of FLT3-TKI therapy. Figure 1A summarizes intrinsic mechanisms of primary and secondary FLT3-TKI resistance.

Primary resistance

Data derived from phase I and II trials using FLT3-TKI monotherapy suggest the existence of primary resistance in about 30% of FLT3-mutated AML patients. Interestingly, some FLT3-mutated leukemic blasts show inherent resistance despite almost complete inhibition of FLT3 tyrosine phosphorylation. In addition, some patients displayed persistent activation of STAT5 and MAPK downstream pathways. These data suggest activation of compensatory survival pathways rendering leukemic cells independent of FLT3-ITD (Fig. 1B). Alternatively, leukemic cells are independent of FLT3 autophosphorylation but still require FLT3-ITD expression. Support for this hypothesis comes from recently published data describing a novel mechanism of primary resistance. An ITD that atypically integrated in TKD-1 (FLT3-ITD627E) induced sustained binding to the adaptor protein GRB2 and enhanced MCL1 expression. Of note, these effects were independent of TKI-induced suppression of FLT3 tyrosine-phosphorylation and mediated primary resistance to FLT3-TKI. In contrast, siRNA-induced knockdown of FLT3-ITD expression abolished MCL1 up-regulation and caused apoptotic cell death. Sensitivity towards FLT3-TKIs may also depend on the type of FLT3 receptor mutations. Gilliland and colleagues tested the sensitivity of 8 activation loop mutations to the compound tandutinib. All mutants conferred cytokine-independent growth in Ba/F3 cells; however, there was broad variability in inhibition of FLT3 autophosphorylation and cytotoxicity among different mutants. Similar results have been reported for the compound SU5614. Moreover, it has been shown that different FLT3-TKIs exhibit distinct inhibitory activity against various FLT3-TKD point mutations. In the future, information on differential sensitivity of FLT3-TKD
mutations and crossreactivity of distinct FLT3-TKIs could have implications for selection of an appropriate FLT3-TKI.

**Secondary resistance**

The majority of patients treated with single agent FLT3-TKIs experienced a partial and transient response lasting for only a few weeks. For several compounds pharmacokinetic and –dynamic studies revealed poor bioactivity due to insufficient plasma drug levels, short plasma half-lives and/or hepatic metabolization. These findings likely correlate with incomplete/transient inhibition of FLT3 autophosphorylation followed by impaired cytotoxic effects as observed in correlative laboratory studies. Insufficient cytotoxicity resulting in incomplete elimination of the malignant clone is likely a prerequisite for the development of secondary drug resistance.

A major mechanism of TKI resistance is caused by acquisition of specific genetic alterations within the target kinase. These mutations may interfere with TKI binding to the FLT3 receptor similar as described for resistance to imatinib-mesylate in CML (Figure 1 C). Indeed, an in vitro screen designed to detect mutations in the ATP-binding pocket of FLT3 identified 4 mutations conferring resistance to midostaurin, SU5614 and K-252a (similar to CEP-701). Interestingly, one of these mutations was detected in a patient at the time of clinical relapse while on midostaurin monotherapy and was identified as the sole cause of resistance to midostaurin. Recently, the profile of resistance mutations upon treatment with sorafenib, midostaurin and SU5614 was investigated using a cell-based screening approach. In contrast to the situation using different BCR-ABL inhibitors, various FLT3-TKIs generated a distinct, non-overlapping molecular profile of resistance. These data provide a rationale for sequential and/or combinatorial treatment strategies of FLT3-TKIs in first-line therapy.

Autocrine FL-stimulation has also been identified as a potential resistance mechanism (Figure 1 D). Long-term treatment with the FLT3-TKI ABT-869 rendered FLT3-ITD⁺-MV4-11 cells resistant to several FLT3-TKIs. No mutations within the FLT3-TKD or up-regulation of FLT3-expression/phosphorylation were detected. However, gene expression analysis revealed an increase in FL expression accompanied by constitutive activation of STAT3 and subsequent up-regulation of
the anti-apoptotic protein survivin. shRNA-mediated knock-down of survivin or treatment with an FL-neutralizing antibody abrogated the resistance phenotype. 

*In vitro* data suggested amplification of the FLT3-locus on chromosome 13 and FLT3-ITD protein overexpression as additional potential mechanisms for secondary FLT3-TKI resistance (Figure 1E). In clinical trials, it has been shown that FLT3-TKI treatment and myelosuppressive chemotherapy may induce an increase in FL expression and/or FLT3 cell surface expression.

An alternative mechanism of resistance is activation of compensatory pathways rendering FLT3-mutated cells independent of FLT3-signaling. In an effort to recapitulate prolonged exposure to FLT3-TKIs *in vivo*, Piloto et al. treated MOLM14 cells with increasing doses of lestaurtinib for several months. Analysis of downstream-pathways showed constitutive activation of AKT and ERK in resistant cell lines although complete inhibition of FLT3 autophosphorylation was observed. Mutational screening of 100 tyrosine- and threonine-/serine-kinases revealed acquisition of novel mutations within NRAS in 2 resistant cell lines, likely responsible for the observed activation of AKT and ERK. Of note, inhibition of AKT and MAPK pathways partially restored sensitivity to FLT3-TKIs in cell lines exhibiting FLT3-independent activation. A common feature of FLT3-TKI resistance is the dysregulation and/or overexpression of anti-apoptotic proteins. As discussed earlier, the anti-apoptotic proteins MCL1 and survivin were found to be up-regulated in resistant AML cells. In addition, MCL1 is up-regulated in FLT3-ITD⁺-LSCs. Finally, the protein BCL2 has been shown to be up-regulated in FLT3-expressing cell lines and primary AML blasts resistant to TKIs. Treatment with the BH3-mimetic ABT-737 restored sensitivity to FLT3-TKI therapy. In conclusion, targeting apoptosis-related signaling proteins in combination with FLT3-TKIs may provide an interesting option for resistant leukemia.

**Lessons learned from FLT3-TKI clinical trials**

Up to now, 6 oral FLT3-TKIs, including midostaurin, lestaurtinib, sorafenib, sunitinib, tandutinib and KW-2449, the i.v. compound SU5416 and the second generation FLT3-TKI AC220 have been investigated as monotherapy in clinical trials. Analysis of clinical single agent FLT3-TKI studies allows to draw several important conclusions with respect to FLT3 mutation status, PK/PD and prediction of response:
1) Blasts from different patients display a high degree of heterogeneity in drug response irrespective of the FLT3 mutation status as demonstrated by in vitro cytotoxicity assays. High FLT3 expression and paracrine/autocrine stimulation may render FLT3-WT blasts dependent on FLT3 signaling and susceptible to FLT3-TKI therapy. In addition, it is possible that off-target effects may account for the observed effects. However, clinical activity in FLT3-WT patients is clearly seen less frequently as compared to patients harboring FLT3 mutations. Of note, some FLT3-ITD-patients showed no obvious response to FLT3-TKI therapy, although near complete inhibition of FLT3 autophosphorylation was observed in vitro and in vivo. Thus, activation of compensatory autophosphorylation pathways may render cells independent of FLT3 signaling. Therefore, some patients will not benefit from FLT3-TKI therapy due to inherent primary resistance.

2) Cytotoxic dose responses in AML patients closely mirrored the inhibition of FLT3 tyrosine-phosphorylation. In correlative laboratory studies, downregulation of FLT3 autophosphorylation to less than 20% of baseline levels was necessary for the achievement of a cytotoxic response in vivo. Furthermore, cytotoxicity is dependent on sustained inhibition of FLT3 tyrosine-phosphorylation as non-durable inhibition results in survival of leukemic blasts.

3) A prerequisite to achieve inhibition of FLT3 autophosphorylation and induction of cell death are sufficient plasma drug levels. In vitro studies of plasma inhibitor activity demonstrated that plasma concentration needs to reach levels of 1–2 orders of magnitude higher than values obtained from cell culture experiments to confer inhibition of FLT3 autophosphorylation and cell growth. This effect is most probably due to the high protein binding capacity of TKIs with a potentially wide variability in free active drug levels. The accumulation of active and inactive metabolites further underscores the complexity of this issue. For example, midostaurin is metabolized in the liver by cytochrome P450 to 2 major metabolites, CGP62221 and CGP52421. Pharmacokinetic studies demonstrated an increase of trough concentrations of midostaurin and its active metabolite CGP62221 with peak concentrations on days 3 and 8, respectively. During further follow up, a 2–4 fold decrease was observed reaching steady state levels on day 28. Of note, although CGP52421 is 22 times less potent than the parent compound, it is less protein bound and reaches steady state levels ranging from 20–30 µM, 3 fold higher than the required IC50 for suppression of FLT3 autophosphorylation. Consequently, in vitro cytotoxicity assays alone appear to
be unreliable as a surrogate marker of in vivo FLT3-TKI effects/activity and the use of plasma inhibitory assays may serve as a more appropriate alternative.

4) The quality of clinical response in general was minor. The majority of responding patients experienced hematologic improvement only with a decrease in PB blasts and a less pronounced decrease in BM blasts. Furthermore, responses were transient lasting only few weeks to months. Possible explanations for the relatively poor efficacy include the aforementioned PK/PD properties of the compounds or inherent resistance in some patients. However, in a few patients CR and CRi were observed. For example, 2 FLT3-ITD+ AML patients treated with sorafenib in a compassionate use program experienced a long lasting CR. Both patients had already received several cycles of chemotherapy and finally relapsed upon allogeneic stem cell transplantation. This experience suggests that clonal evolution of highly FLT3-dependent leukemic blasts may ultimately result in response. Three of 6 FLT3-ITD+-patients treated with AC220 at the MTD of 200 mg/d also experienced a CR. Both, sorafenib and AC220 have been shown to be highly selective FLT3-TKIs equipped with beneficial PK/PD features, thus likely inducing sustained and complete inhibition of FLT3 tyrosine-phosphorylation in leukemic blasts addicted to FLT3 signaling. In line with these observations, Pratz et al. recently demonstrated in in vitro studies that relapsed/refractory AML patients and patients with high mutant allelic burden are more likely to respond to selective FLT3-TKI therapy.

5) The therapeutic potential of using FLT3 as a molecular target is still not clearly defined. It will likely depend on further refinement of the intrinsic properties of FLT3 inhibitors and on defining useful combination partners and therapeutic algorithms.

**Future directions**

**FLT3-TKI in combination with other small molecules**

A hallmark of oncogenic signal transduction is the simultaneous activation of several survival pathways. In most primary AML blasts and cell lines redundant activation of the PI3K/AKT, MAPK and JAK/STAT pathway has been observed. These pathways are activated by mutated upstream receptor kinases, cross-activation between these pathways or autocrine/paracrine mechanisms. As mentioned earlier, FLT3-TKI treatment of primary AML blasts causes substantial inhibition of FLT3 but in some
samples only incomplete suppression of downstream pathway. Therefore, targeting leukemic blasts at multiple levels may further suppress protein phosphorylation below a threshold necessary to induce apoptotic cell death. Indeed, several in vitro studies demonstrated synergistic effects using midostaurin in combination with the mTOR inhibitor rapamycin or the dual PDK-1/PI3K inhibitor BAG956 or sunitinib in combination with the mTOR-inhibitor RAD001 or the MEK1/2 kinase inhibitor AZD6244. Currently, a phase I clinical trial testing the combination of the mTOR-inhibitor RAD001 with the tyrosine kinase inhibitor midostaurin (PKC412) is under way (NCT00819546). Furthermore, synergistic effects have been demonstrated in combination with the HSP-90 inhibitor 17-AAG and the HDAC inhibitor MS-275. However, the sequence of administration, toxicity profiles and optimal target combinations need to be defined.

**FLT3 and leukemia initiating cells (LIC)**

The concept of leukemic stem cells (LSCs) or LIC has been developed by John Dick several decades ago. In elegant xenotransplantation studies his group demonstrated that only a minor subset of leukemic blasts displayed self renewal capacity and was able to propagate leukemia in irradiated recipients. The transplanted cells had the capability to differentiate and copied the initial phenotype of the disease. These data indicate that hematopoietic stem cells (HSCs) and LICs share many characteristics, including phenotype, self renewal activity, and enhanced drug resistance. It is reasonable to assume that for the cure of AML patients, eradication of leukemia-initiating and -maintaining cells while sparing their normal counterpart is a prerequisite. However, the impact of WT- and mutated FLT3 on LIC survival and maintenance as well as the question whether FLT3-TKIs target the LIC-compartment and contribute to the eradication of leukemia-initiating cells remain elusive.

There is some evidence that FLT3-ITD mutations play an essential role in LIC function. Enforced expression of FLT3-ITD in human CD34⁺ cord blood cells conferred persistent, FLT3-dependent self renewal properties in vitro. Further, FLT3-ITD transduced human CD34⁺ HSCs demonstrated enhanced survival potential, increased proliferation and expansion of the CD34⁺/CD38dim population. Importantly, in these studies FLT3-ITD is expressed under the control of exogenous promoters causing non-physiological expression levels and thus may alter “normal”
FLT3-ITD induced function. Indeed, data derived from primary patient samples provide more heterogeneous results. For example, in several clinical studies analysis of paired patient samples at diagnosis and relapse have reported that (i) the originally identified ITD or TKD mutation was lost in some cases;\textsuperscript{106-109} (ii) a novel FLT3-ITD was detected at relapse;\textsuperscript{106} and (iii) FLT3-ITD and TKD mutations emerged in patients previously considered as FLT3-WT at diagnosis.\textsuperscript{107, 108} These data indicate that FLT3 mutations are unstable and late events in leukemogenesis and targeting these cells may eliminate a subclone, but not the LIC. However, most patients (88\%) retained the originally detected FLT3 mutation at relapse as revealed by a combined evaluation of 6 studies.\textsuperscript{110} Of note, the mutant-to-wild type ratio increased at relapse in most cases with some patients proceeding to a hemizygous state, suggesting evolving oncogene addiction.\textsuperscript{14, 108} Levis et al. analyzed the FLT3 mutant-wild type ratio in stem cell enriched CD34\(^+\)/CD38\(^-\) cells in comparison to unsorted AML blasts. No difference in FLT3-ITD expression levels was detected, suggesting that the mutation is already present in the stem/progenitor population. Further, the CD34\(^+\)/CD38\(^-\) population was able to confer leukemia in a NOD/SCID mouse model, whereas treatment with lestaurtinib significantly inhibited leukemic engraftment but not that of normal HSCs.\textsuperscript{111} In summary, in some cases FLT3 mutations appear to represent an early hit during malignant transformation and significantly contribute to survival and proliferation of leukemic blasts.

**FLT3 and microenvironment**

A consistent observation in all clinical trials testing FLT3-TKIs as single agents was rapid clearance of blasts in PB, but less pronounced effects on BM blasts in responding patients. Similar to conventional chemotherapeutics, small molecule inhibitors preferentially seem to ablate actively cycling leukemic blasts, but do not target blasts embedded in their BM niche and protected against drug induced apoptotic cell death. This is in line with early reports demonstrating that AML – stromal cell interactions are able to confer resistance to chemotherapeutics.\textsuperscript{112} These protective effects are mediated by direct cell-cell-interactions, soluble factors or extracellular matrix (ECM)-proteins. In _in vitro_ studies, addition of exogenous FL significantly decreased cytotoxic effects of several FLT3-TKIs.\textsuperscript{91} Therefore, expression of FL on surrounding stromal cells may enhance signaling through WT or mutated FLT3 and counteract the inhibitory effects of small molecule inhibitors.
Alternatively, other cytokines and growth factors, such as SCF, IL-3 or TPO, abundantly present in the BM, may compensate loss of constitutive FLT3 activation and render FLT3-mutated blasts independent of FLT3 signaling. Recently, niche-like conditions have been shown to completely abrogate FLT3-TKI induced cell death whereas inhibition of commonly shared downstream pathways like PI3K and MAPK markedly decreased cell survival in this setting. In addition to cytokines and growth factors, components of the ECM and cell adhesion molecules have been shown to confer cell adhesion-mediated drug resistance (CAM-DR). For example, expression of very late antigen 4 (VLA-4) on leukemic cells mediated attachment to fibronectin produced by stromal cells and conferred resistance to chemotherapy through the PI3K/AKT/BCL-2 signaling pathway. Blocking the interaction of fibronectin to VLA-4 using FNIII14, a peptide derived from fibronectin, restored sensitivity to cytarabine in leukemic cell lines. Therefore, disrupting the interaction of leukemic blasts with their niche may provide a therapeutic strategy to overcome CAM-DR (Figure 2).

The chemokine stromal-derived factor 1α (SDF-1α) and its cognate receptor CXCR4 have been shown to act as critical mediators in stromal – leukemic cell interactions. CXCR4 is involved in migration, homing and engraftment of AML cells to the BM of NOD/SCID mice. Interestingly, CXCR4 expression was demonstrated to be significantly higher in FLT3-ITD + AML than in FLT3-WT AML samples. Recently, the serine-/threonine-kinase PIM1 was found to be essential for CXCR4 surface expression and intracellular receptor processing. PIM1-deficient, FLT3-ITD expressing BM cells failed to reconstitute lethally irradiated recipients due to deficient homing and migration. As PIM1 is highly expressed in FLT3-ITD + AML cells, PIM1 seems to act as a central regulator of FLT3-ITD induced CXCR4 expression. Targeting CXCR4 may disrupt AML-niche interactions, sensitize leukemic blasts to chemotherapy and overcome CAM-DR. Indeed, blockade of CXCR4 using small molecule inhibitors caused mobilization of BM-resident leukemic blasts and synergized with conventional chemotherapeutics. AMD3465, a second generation CXCR4 inhibitor, inhibits CXCR4 phosphorylation and suppresses stroma-mediated activation of pro-survival signaling pathways. Of note, using stroma co-culture conditions, CXCR4 inhibition rendered FLT3-ITD expressing leukemic cells sensitive to the FLT3-TKI sorafenib. In vivo, combined treatment with AMD3465 and G-CSF mobilized FLT3-ITD expressing cells from the BM, rendered AML blasts susceptible to the FLT3-inhibitor sorafenib and significantly prolonged survival as
compared to single agent treatment. Based on these encouraging in vitro and in vivo data, a phase I clinical trial testing the combination of the CXCR4-inhibitor plerixafor plus G-CSF in addition to the tyrosine kinase inhibitor sorafenib recently started recruitment (NCT00943943). As most FLT3-TKIs have been shown to be less potent in inhibiting FLT3-WT as expressed on normal HSCs, targeting CXCR4 in combination with FLT3-TKI may selectively eradicate malignant FLT3-mutated blasts while sparing their normal counterparts (Figure 2).

Conclusions

FLT3-TKI monotherapy has been proven to efficiently target FLT3-mutated AML blasts. However, complete and sustained remissions will require the combination with standard chemotherapy or alternatively with other therapeutic agents. Novel strategies such as targeting oncogenic signaling at multiple levels or disruption of AML – stromal cell interactions may serve as clinically valuable partners in combinatorial approaches of FLT3-targeted therapy. Results from ongoing randomized clinical trials examining a combination of standard chemotherapy with and without FLT3-TKI in newly diagnosed patients will be available within the next few years and are expected to have a major impact in this area.

Acknowledgements

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Authorship

Contributions: T.K., D.L. and T.F. wrote and edited the manuscript.
Conflict-of-interest disclosure: The authors have no conflict of interest to declare.
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<table>
<thead>
<tr>
<th>TKI</th>
<th>Structural class</th>
<th>Targets</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; cell growth</th>
<th>Prolongation of survival in murine models of FLT3-ITD induced disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midostaurin (PKC412)</td>
<td>indolocarbazole alkaloid</td>
<td>c-FMS, c-KIT, PDGFRα/β</td>
<td>10 nM</td>
<td>&lt; 30 nM</td>
<td>+</td>
<td>89</td>
</tr>
<tr>
<td>Lestaurtinib (CEP-701)</td>
<td>indolocarbozole alkaloid</td>
<td>TrkA, VEGFR</td>
<td>2.5 nM</td>
<td>2.3 nM</td>
<td>+</td>
<td>50, 123</td>
</tr>
<tr>
<td>Sorafenib (BAY 43-9006)</td>
<td>biaryl urea derivate</td>
<td>c-RAF, VEGFR, PDGFR, c-KIT</td>
<td>2.8 nM</td>
<td>0.88 nM</td>
<td>+</td>
<td>54, 124, 125</td>
</tr>
<tr>
<td>Semaxanib (SUS416)</td>
<td>indolinone derivate</td>
<td>VEGFR, c-KIT</td>
<td>100 nM</td>
<td>250 nM</td>
<td>Not reported</td>
<td>126, 127</td>
</tr>
<tr>
<td>Sunitinib (SU11248)</td>
<td>indolinone derivate</td>
<td>VEGFR, PDGFR, c-KIT</td>
<td>50 nM</td>
<td>8 nM</td>
<td>+</td>
<td>128, 129</td>
</tr>
<tr>
<td>Tandutinib (MLN-518)</td>
<td>piperazinyl quinazoline</td>
<td>c-KIT, PDGFR</td>
<td>30-100 nM</td>
<td>10-30 nM</td>
<td>+</td>
<td>130-132</td>
</tr>
<tr>
<td>KW-2449</td>
<td>not disclosed yet</td>
<td>ABL, FGFR1, Aurora kinase</td>
<td>13.1 nM</td>
<td>11-24 nM</td>
<td>+</td>
<td>133</td>
</tr>
<tr>
<td>AC220</td>
<td>bis-aryl urea derivate</td>
<td>c-KIT, RET, PDGFR, CSF1R</td>
<td>1.1 nM</td>
<td>0.56 nM</td>
<td>+</td>
<td>66</td>
</tr>
</tbody>
</table>

PDGFR: platelet derived growth factor receptor; VEGFR: vascular endothelial growth factor receptor; FGFR: fibroblast growth factor receptor;
In addition, several other FLT3-TKI including ABT-869, dovitinib (CHIR-258) and AP24534 have been demonstrated to exhibit pronounced in vitro and in vivo inhibitory activity, but have not entered clinical trials yet or results have not been reported so far (reviewed in Weisberg et al.).

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Table 2: Summary of clinical trials using FLT3-TKI as single agent

<table>
<thead>
<tr>
<th>TKI</th>
<th>Trial (FLT3 status)</th>
<th>Dosage (MTD)</th>
<th>Best response</th>
<th>Duration of response</th>
<th>Side effects/ DLT</th>
<th>Comment</th>
</tr>
</thead>
</table>
| Midostaurin (PKC412) | Phase 2\(^{29}\)  
(FLT3 mut only) | p.o., 75mg, 3x/d                   | blasts BM <50%: 6/20  
blasts PB < 50%: 14/20               | 72- 330 d                   | Nausea, pulmonary events          | sustained responses in some patients                                  |
| Lestaurtinib (CEP-701) | Phase 1/2\(^{31}\)  
(FLT3 mut only) | p.o., 60mg BID                    | blasts PB<50%: 5/14                                                                 | 2 weeks- 3 month       | Nausea, emesis, diarrhea          | sustained responses in some patients                                  |
|                      | Phase 2\(^{28}\)  
(FLT3 mut + wt, age > 70 y) | p.o., 60 – 80mg BID               | blasts PB<50%:  
FLT3-mut: 3/5  
FLT3-WT: 5/22     | 2 weeks- 9 month                   |                                    |                                                                          |
| Sorafenib (BAY 43-9006)  | Phase 1\(^{34}\)  
(FLT3 mut + wt) | p.o., 400mg BID (range: 200-400mg, 2x/d) | blast response in PB:  
FLT3-ITD: 6/6  
FLT3-WT: 3/7  
FLT3-TKD: 0/3 | n.d.                               | Pleural effusion, nausea, vomiting, rash                      | 1000 fold more selective for FLT3-ITD                                 |
| Semaxanib (SU5416)   | Phase 2\(^{37}\)  
(FLT3 mut + wt) | i.v., 145 mg/m² 2x/week           | PR: 2/33  
HI: 1/33        | 3- 3.5 month                     | Fatigue, headache, bone pain    | AE likely caused by hyperosmolaric drug formulation                    |
|                      | Phase 2\(^{39}\)  
(AML, FLT3 n.d.) | i.v., 145 mg/m² 2x/week           | blasts PB and BM <50%:  
7/25 with 1 MR     | 1.6 month (1- 5 month)       |                                    |                                                                          |
| Sunitinib (SU11248)  | Phase 1\(^{41}\)  
(FLT3 mut + wt) | p.o., 50mg 1x/d                   | blasts PB and BM <50%:  
FLT3-ITD: 4/4 (1 HI)  
FLT3-WT: 2/7 | 4-16 weeks               | Hypertension (DLT), fatigue, edema |                                                                          |
| Tandutinib (MLN-518) | Phase 1\(^{42}\)  
(FLT3 mut + wt) | p.o., 50 – 700mg BID             | n.a.                                                           | n.a                   | Muscular weakness, fatigue, nausea, vomiting                         | Muscular weakness caused by inhibition of a muscle-type nicotinic receptor at high concentrations |
|                      | Phase 2\(^{43}\)  
(FLT3-ITD only) | p.o., 525mg BID                   | 6/18 responder:  
blast decrease in PB and BM   | 1-3 month        |                                    |                                                                          |
| KW-2449              | Phase 1\(^{44}\)  
(FLT3 mut + wt) | p.o., 500mg BID                   | blasts PB and BM <50% in 26%                        | n.d.                  | Vomiting, nausea, fatigue       | trial closed on basis of PD studies (MTD not reached)                    |
| AC220          | Phase 1[7] (FLT3 mut + wt) | p.o., 200mg 1x/d (range: 12 – 300 mg/d) | CR: 12%  
PR: 18%  
FLT3-ITD: 56%  
FLT3-WT: 19% | 14 weeks | QTc prolongation (DLT), peripheral edema, GI events |

MTD: maximum tolerated dose; DLT: dose-limiting toxicity; n.d.: not determined; n.a. not applicable; PB: peripheral blood; BM: bone marrow;  
PR: partial response; CR: complete response (PR and CR as defined by IWG criteria)  
MR: morphological response (clearance of PB blasts and less than 5% BM blasts)  
HI: hematologic improvement (CR except for platelet count 100 > 10^9/L)
Table 3: Summary of published phase I/II trials investigating safety and efficacy of FLT3-TKI in combination with chemotherapy

<table>
<thead>
<tr>
<th>TKI</th>
<th>Clinical phase</th>
<th>Chemotherapy</th>
<th>TKI-Therapy</th>
<th>Patient number</th>
<th>Age</th>
<th>Prior therapy</th>
<th>Cytogenetics</th>
<th>FLT3 mutation status</th>
<th>CR rate [%]</th>
<th>OS</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tandutinib (MLN518)</td>
<td>Phase I/II</td>
<td>Induction:</td>
<td>Cohort 1: 200 mg BID continuously until 6 months after completion of therapy (7 pts); Toxicity; GI intolerance</td>
<td>29 pts.</td>
<td>26-83 years (median: 60 years)</td>
<td>Newly diagnosed AML</td>
<td>9 pts. unfavorable</td>
<td>FLT3-ITD</td>
<td>Cohort 1: 5/7 pts</td>
<td>not reported</td>
<td>AEs: mainly diarrhea, nausea &amp; vomiting</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AraC 200 mg/m²/day, d 1-7; Daunorubicin 60 mg/m²/day, d 1-3</td>
<td>Consoliation:</td>
<td>HD-AraC (3000 mg/m² q12h, d 1,3,5)</td>
<td>(8 pts); during induction &amp; consolidation</td>
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<td></td>
<td>Cohort 2: 200 mg BID d 1-14</td>
<td>Cohort 2: 6/8 pts</td>
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<td></td>
<td>Cohort 3: 500 mg BID d 1-14</td>
<td>Cohort 3: not reported</td>
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<tr>
<td>Midostaurin (PKC412)</td>
<td>Phase 1b</td>
<td>Induction:</td>
<td>Phase I: Sorafenib 400 mg p.o. d 1-7 a) every other day b) 400 mg daily c) 400 mg BID</td>
<td>100 pts</td>
<td>20-65 years (median: FLT3-mut 46 years; FLT3-WT 50 years)</td>
<td>Newly diagnosed AML (de novo)</td>
<td>FLT3-mut: 85% intermediate and 15% unfavorable</td>
<td>FLT3-mut: 13 pts (9 pts. with FLT3-ITD)</td>
<td>FLT3-mut: 12/13 (92%)</td>
<td>FLT3-WT: 27 pts. (74%)</td>
<td>100 mg BID poorly tolerated (nausea &amp; vomiting); 5 pts. received maintenance therapy (3 FLT3-mut, 2 FLT3-WT)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AraC 100 mg/m²/day, d 1-7; Daunorubicin 60 mg/m²/day, d 1-3</td>
<td>Consoliation:</td>
<td>HD-AraC (3000 mg/m² q12h, d 1,3,5) elderly patients: 2000 mg/m²/day, d 1-5</td>
<td>(sequentially)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Consoliation: HD-AraC (3000 mg/m² q12h, d 1,3,5)</td>
<td>3 Cycles</td>
<td></td>
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</tr>
<tr>
<td>Sorafenib</td>
<td>Phase I/II</td>
<td>Induction:</td>
<td>Phase II: Newly diagnosed AML 1/10 pts unfavorable</td>
<td>7/10 pts. overall 40%</td>
<td>FLT3-mut: 3/7</td>
<td>FLT3-WT: 1/3</td>
<td>Overall: 12 months 74%</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>AraC 1.5 g/m²/day, d 1-4; Idarubicin 12 mg/m²/day, d 1-3</td>
<td>Consoliation: AraC 0.75 g/m²/day, d 1-3; Idarubicin 8 mg/m², d 1-2</td>
<td>(up to 3 cycles) Sorafenib 400 mg BID up to 28 days during consolidation</td>
<td>(up to 5 cycles)</td>
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<td></td>
<td></td>
<td></td>
<td>Sorafenib 400 mg p.o. BID d 1-7</td>
<td>Phase II:</td>
<td>18-65 years (median: FLT3-mut 53 years)</td>
<td>Newly diagnosed AML</td>
<td>27 pts.</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lestaurtinib (CEP701)</td>
<td>Randomized phase II</td>
<td>Induction: Mitoxantrone + etoposide + cytarabine or HD-AraC</td>
<td>Sorafenib 400 mg BID p.o. post chemotherapy (randomized) for 112 days, extension possible; crossover to TKI therapy possible if refractory to chemotherapy alone</td>
<td>80 mg BID p.o. post chemotherapy (randomized) for 112 days, extension possible; crossover to TKI therapy possible if refractory to chemotherapy alone</td>
<td>224 (220 receiving therapy)</td>
<td>median age: 55 years</td>
<td>FLT3-mut AML in 1. relapse</td>
<td>not reported</td>
<td>FLT3-ITD: 88%</td>
<td>Ctx only: 21%</td>
<td>Duration of CR 1-6 months in 47%; 7 pts. crossed over from Ctx only to the TKI arm; 31 pts received TKI on the extension protocol; pts. achieving &gt;85% target inhibition on day 15 had a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sorafenib 400 mg BID maintenance for up to 1 year</td>
<td>Phase II:</td>
<td>1/10 pts unfavorable</td>
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superior CR/CRp rate as compared to those not achieving this target (39% vs. 9%, respectively)

TKI: tyrosine kinase inhibitor; CR: complete response; CRp: complete response with incomplete platelet recovery; OS: overall survival; GI: gastrointestinal; pts: patients; AraC: cytarabine; HD-AraC: high-dose cytarabine; AML: acute myeloid leukemia; FLT3-mut: mutated FLT3-receptor (internal tandem duplication and/or tyrosine-kinase domain mutation); AE: adverse events; Ctx: chemotherapy
<table>
<thead>
<tr>
<th>TKI</th>
<th>Clinical phase</th>
<th>Title</th>
<th>Chemotherapy</th>
<th>Patient number</th>
<th>Age</th>
<th>Principal investigator</th>
<th>Remarks</th>
</tr>
</thead>
</table>
| Midostaurin (PKC412)    | Phase III      | A phase III randomized, double-blind study of induction (Daunorubicin/Cytarabine) and consolidation (high-dose Cytarabine) chemotherapy + Midostaurin or placebo in newly diagnosed patients >60 years of age with FLT3 mutated acute myeloid leukemia | **Induction:** ArA 200 mg/m²/day, d 1-7; Daunorubicin 60 mg/m²/day, d 1-3  
**Consolidation:** ArA 3 g/m²/day, d 1, 3, 5  
either plus Midostaurin or Placebo followed by 12 cycles maintenance with Midostaurin / Placebo | 514 FLT3 mutated patients planned | 18-60 years | Dr. Richard Stone, Dana Farber Cancer Institute, Boston, USA | Placebo controlled  
International  
Recruiting  
Reference: NCT00651261 |
| Lestaurtinib (CEP-701)  | Phase III      | MRC AML 15: A trial of directed therapy in younger patients with acute myeloid leukaemia: MRC AML 15 | **Induction:** ArA + Daunorubicin +/- Etoposide  
**Consolidation:** 2-3x HD-AraC or MACE + MidAC  
+/- Lestaurtinib for FLT3-mut pts (randomized) | Target number of patients: 2500 | 18-60 years | Dr. Alan Burnett, Cardiff University, Cardiff, UK | Open label, international.  
Recruitment completed.  
Results have not been published yet.  
Reference: ISRCTN 17161961  
| Lestaurtinib (CEP-701)  | Phase III      | MRC AML 17: A programme of treatment development in younger patients with acute myeloid leukaemia and high risk myelodysplastic syndrome | **Induction:** 2x ArA + Daunorubicin +/- Etoposide +/- Mylotarg  
**Consolidation:** 1x MACE +/- MidAC  
+/- Lestaurtinib for FLT3-mut pts after 1. induction (randomized) | Target number of patients: 2800 | 18–60 years | Dr. Alan Burnett, Cardiff University, Cardiff, UK | Open label, international.  
Recruiting  
Reference: ISRCTN 55675535  
http://aml17.cardiff.ac.uk/files/aml17_protocolv2.pdf |
| Sorafenib               | Phase II       | A double blind, placebo-controlled, randomized, multi-center phase-II trial to assess the efficacy of Sorafenib added to standard primary therapy in patients with newly diagnosed AML > 60 years of age. | **Induction:** 2x ArA + Daunorubicin  
**Consolidation:** 3x HD-AraC  
either plus Sorafenib or Placebo followed by maintenance therapy with Sorafenib / Placebo | 276 FLT3-mutated patients planned | 18–60 years | Dr. Gerhard Ehninger, Technical University Dresden, Germany | Placebo controlled  
Recruiting  
| Sorafenib               | Phase II       | Efficacy of Sorafenib added to standard primary therapy in elderly patients with newly diagnosed AML | 1-2x induction chemotherapy  
2x consolidation chemotherapy  
either Sorafenib or Placebo between chemo cycles, followed by maintenance therapy with Sorafenib / Placebo | 200 FLT3 mutated patients accrued | 18–60 years | Dr. Hubert Serve, Wolfgang-Goethe-University, Frankfurt, Germany | Double-blind, placebo controlled  
Completed  
Results have not been published yet.  
Reference: NCT00373373 |
| Sunitinib               | Phase III      | Clinical study of SU11248 (Sutent) combined with standard chemotherapy in patients with FLT3 mutated AML over 60 years | ArA + Daunorubicin plus | 30 FLT3 mutated patients planned | > 60 years | Dr. Walter Fiedler, University Medical Center, Hamburg-Eppendorf, Germany | Open-label, single group  
Recruiting  
Reference: NCT00373373 |
| Sunitinib (different dose levels and schedules) | Reference: NCT00783653 |
Legends

**Figure 1: Molecular mechanisms of intrinsic resistance to FLT3-TKI.**
(A) Overview: FLT3-TKI resistance of FLT3-mutated AML can be classified in primary resistance which is due to specific biologic characteristics of the disease, and in secondary resistance which occurs secondarily upon exposure to TKIs. Known mechanisms of resistance to FLT3-TKIs are: (B) In AML blasts expressing a mutated FLT3-receptor survival and proliferation signals are continuously mediated by the mutant receptor. FLT3-TKIs abrogate constitutive activation of the FLT3-receptor and its downstream signals followed by apoptotic cell death. Alternatively, activation of compensatory survival pathways, e.g. activating NRAS mutations, renders leukemic cells independent of FLT3. (C) Mutations in the ATP-binding pocket of the tyrosine-kinase domain impair binding of the TKI to the receptor; (D) Autocrine and/or paracrine FLT3-receptor stimulation via FLT3-Ligand (FL); (E) Overexpression of the mutated FLT3-receptor.

**Figure 2: Targeting LIC – stroma interaction in combination with FLT3-TKI.**
a) treatment of leukemic blasts with chemotherapy (CT) or FLT3-TKIs kills cycling cells in PB and BM whereas LIC and HSCs are embed in their niche and protected against apoptotic cell death; b) Targeting LIC-stroma cell interaction using neutralizing antibodies or small peptides disrupts stroma-mediated survival signals and releases LIC, but also HSCs from their environment; c) as a consequence FLT3-ITD expressing LIC enter the cell cycle and become sensitive to FLT3-TKIs while normal HSCs are spared; d) finally HSCs adhere to stromal cells again and initiate hematopoietic reconstitution.
Primary resistance

**FLT3-dependent:**
FLT3-mutations that are insensitive to specific TKIs

**FLT3-independent:**
Activation of alternative survival pathways (cells are not addicted to FLT3)

Secondary resistance

- Resistance mutations in the ATP-binding pocket of FLT3
- Autocrine FL-stimulation
- FLT3 overexpression
- Activation of alternative pathways (e.g., secondary mutations of NRAS)
Figure 1B - E

B

FLT3-ITD

Survival

Apoptosis

Survival

STAT5 activation

IRK activation

AKT activation

Binding of TKI to the FLT3-ITD receptor

Abrogation of downstream signaling

TKI resistance mutation inhibits TKI binding

C

FLT3-ITD

Binding of TKI may impair efficacy of TKIs

mechanism is unknown

D

FLT3-ITD

Autocrine/paracrine expression of FL

FL overexpression and binding to FLT3-ITD may impair efficacy of TKIs

mechanism is unknown

E

FLT3-ITD exposed to TKIs leads to FLT3 overexpression

FLT3 overexpression confers incomplete target inhibition

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Figure 2

a) 

b) 

peptides mAb

peptides mAb

HSC LIC

blast

stroma

CT

FLT3-TKI

FLT3-TKI

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FLT3 as a therapeutic target in AML: still challenging after all these years

Thomas Kindler, Daniel B. Lipka and Thomas Fischer