The evolving role of FLT3 inhibitors in acute myeloid leukemia: quizartinib and beyond

Seth A. Wander, Mark J. Levis and Amir T. Fathi

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Abstract: Acute myeloid leukemia remains associated with poor outcomes despite advances in our understanding of the complicated molecular events driving leukemogenesis and malignant progression. Those patients harboring mutations in the FLT3 receptor tyrosine kinase have a particularly poor prognosis; however, significant excitement has been generated by the emergence of a variety of targeted inhibitors capable of suppressing FLT3 signaling *in vivo*. Here we will review results from preclinical studies and early clinical trials evaluating both first- and second-generation FLT3 inhibitors. Early FLT3 inhibitors (including sunitinib, midostaurin, and lestaurtinib) demonstrated significant promise in preclinical models of FLT3 mutant AML. Unfortunately, many of these compounds failed to achieve robust and sustained FLT3 inhibition in early clinical trials, at best resulting in only transient decreases in peripheral blast counts. These results have prompted the development of second-generation FLT3 inhibitors, epitomized by the novel agent quizartinib. These second-generation inhibitors have demonstrated enhanced FLT3 specificity and have been generally well tolerated in early clinical trials. Several FLT3 inhibitors have reached phase III clinical trials, and a variety of phase I/II trials exploring a role for these novel compounds in conjunction with conventional chemotherapy or hematopoietic stem cell transplantation are ongoing. Finally, molecular insights provided by FLT3 inhibitors have shed light upon the variety of mechanisms underlying the acquisition of resistance and have provided a rationale supporting the use of combinatorial regimens with other emerging targeted therapies.

Keywords: acute myeloid leukemia, FLT3, quizartinib, targeted therapies

Introduction: acute myeloid leukemia and the impact of aberrant FLT3 signaling

Acute myeloid leukemia (AML) represents a malignant proliferation of hematopoietic progenitor cells of the myeloid lineage within the bone marrow. These poorly differentiated precursor cells cease to function normally and disrupt normal hematopoiesis causing infection, bleeding, and multi-organ dysfunction. AML has an overall incidence rate of 3.7 per 100,000 individuals; the median age at diagnosis is 67 years and more than 50% of all AML cases occur in individuals over the age of 65 [Howlader *et al.* 2013]. The prognosis for individual patients with AML varies greatly but is generally poor, particularly so in subgroups of AML patients including the elderly, individuals with a poor performance status, those with preceding myelodysplastic syndrome (MDS), those with treatment-associated AML, and patients who present with leukocytosis greater than 20,000

or elevated serum lactate dehydrogenase levels [Lowenberg *et al.* 1999]. Identification of an increasing array of cytogenetic and molecular abnormalities has become important for both risk stratification and selection of appropriate therapeutic approaches. As molecular characterization of the events leading to AML developed, a ‘two-hit’ model of leukemogenesis emerged: first, that AML blasts required disruption and hyperactivation of signal transduction pathways promoting survival or proliferation and, second, that lesions occurred to disrupt pathways leading to normal differentiation [Kelly and Gilliland, 2002]. However, recent genomic studies suggest that the process is far more complex and requires an interconnected network of aberrant signal transduction pathways [Ley *et al.* 2013]. The most common mutation in AML occurs in the FMS-like tyrosine kinase 3 (FLT3) gene and, like other class I mutations, promotes cell growth

Correspondence to:

Amir T. Fathi, MD
Massachusetts General
Hospital, Harvard Medical
School, Zero Emerson
Place, Suite 118, Boston,
MA 02114, USA
afathi@partners.org

Seth A. Wander, MD, PhD
Massachusetts General
Hospital Cancer Center,
Harvard Medical School,
Boston, MA, USA

Mark J. Levis, MD, PhD
Johns Hopkins Sidney
Kimmel Comprehensive
Cancer Center, Baltimore,
MD, USA

and proliferation. Mutations in *FLT3* occur in approximately one-third of all *de novo* AML patients and portend a particularly poor prognosis [Gilliland and Griffin, 2002].

The *FLT3* protein is encoded by a gene located on chromosome 13q12 and is a member of the class III receptor-tyrosine kinase (RTK) family, sharing a high degree of structural homology with the KIT, FMS and PDGFR receptors [Van Der Geer *et al.* 1994; Carow *et al.* 1995]. *FLT3* plays a well-established role in normal growth and differentiation of hematopoietic precursor cells. *FLT3* expression is typically limited to CD34+ cells while that of its ligand is virtually ubiquitous [Lyman *et al.* 1994; Small *et al.* 1994]. Like other RTKs, upon binding of ligand the *FLT3* receptor dimerizes at the plasma membrane, leading to autophosphorylation and activation of a host of downstream effector signaling cascades. These include the RAS/MEK, PI3K/AKT/mTOR, and STAT-5 pathways, all of which play a myriad of roles in the promotion of cell cycle progression, inhibition of apoptosis, and activation of differentiation [Hay and Sonenberg, 2004; Roux and Blenis, 2004; Manning and Cantley, 2007; Bar-Natan *et al.* 2012]. Mutant *FLT3* is often expressed at higher levels, has lost its association with CD34 positivity, and demonstrates ligand-independent, constitutive autophosphorylation and activation of downstream signaling [Carow *et al.* 1996; Rosnet *et al.* 1996; Kiyoi *et al.* 1998].

The most common group of mutations are internal tandem duplication (ITD) mutations, impacting the juxtamembrane portion of the receptor [Nakao *et al.* 1996]. ITD mutations may be of variable length and location and occur in approximately 23% of patients with *de novo* AML [Kayser *et al.* 2009; Marcucci *et al.* 2011; Schnittger *et al.* 2012]. While the potential to achieve complete remission is approximately equivalent to *FLT3* wild type (WT) patients, *FLT3*-ITD mutations are associated with a much higher relapse rate and a poor response to salvage therapy (reviewed by Levis and Small [Levis and Small, 2003]). The length of the ITD mutation also appears to carry prognostic import, with an association between increasing ITD size and decreased overall survival [Stirewalt *et al.* 2006]. In addition, patients with an elevated *FLT3*-ITD mutant:WT ratio have been noted to have worse outcomes [Thiede *et al.* 2002], but they may be more responsive to *FLT3*-directed therapies [Pratz *et al.* 2010b].

Point mutations within the activation loop of the receptor also occur (*FLT3*-TKD, tyrosine kinase domain), albeit at a lower frequency of approximately 7% in *de novo* AML [Abu-Duhier *et al.* 2001; Yamamoto *et al.* 2001]. TKD mutations most frequently occur at codon 835 where a tyrosine residue replaces aspartic acid, stabilizing the activation loop in the ATP-bound configuration and promoting constitutive activation. While *FLT3*-ITD patients clearly have a poor prognosis relative to *FLT3*-WT patients, this association is less clear with cases of *FLT3*-TKD disease [Moreno *et al.* 2003].

Finally, prognosis in *FLT3* mutant AML may be impacted by additional background mutations. For example, there is some debate regarding the effect of concurrent *NPM1* and *FLT3* mutations: evidence suggests that the negative impact of *FLT3* may be somewhat counter-balanced by coexisting *NPM1* mutations while, in other studies, this was not found to be the case [Gale *et al.* 2008; Schnittger *et al.* 2011].

Given the prevalence and propensity for poor outcome in AML patients harboring *FLT3* mutations, a sustained effort has been underway to develop targeted inhibitors of the *FLT3* RTK. A variety of compounds have entered clinical trials and some have met with success. The following will review the clinical experiences with early, first-generation *FLT3* inhibitors, many of which are limited by their suboptimal propensity for sustained *FLT3* attenuation *in vivo* as well as by their off-target effects. We will then address newer, more potent and specific second-generation *FLT3* inhibitors, principally quizartinib, which may hold significant promise in this high-risk subtype of AML.

First-generation *FLT3* inhibitors

Tyrosine kinase inhibitors (TKIs) have been developed to disrupt oncogenic RTK signaling in a variety of solid and liquid malignancies. Most of these inhibitors function via competitive inhibition of ATP in the active site of RTKs, thereby preventing autophosphorylation and activation of downstream substrates. Early *FLT3* inhibitors include sunitinib (SU11248), sorafenib (BAY 43-9006), midostaurin (PKC412), and lestaurtinib (CEP-701). These inhibitors, rather than being specifically designed to target *FLT3*, were developed against a variety of other RTKs in a host of malignancies (including KIT, PDGFR,

VEGFR, and JAK2, among others; reviewed by Kayser and Lewis [Kayser and Lewis, 2013]). While several trials remain ongoing, these agents generally failed to show robust and sustainable responses as single agents in phase I/II trials in various groups of relapsed or refractory AML patients (Table 1 lists completed and ongoing clinical trials assessing FLT3 inhibitors other than quizartinib). Molecular insights into the mechanisms underlying these poor responses have informed our understanding of FLT3 pathobiology and prompted development of improved, second-generation FLT3 inhibitors.

Sunitinib has been approved for use in solid malignancies such as metastatic renal cell carcinoma and gastrointestinal stromal tumors [Demetri *et al.* 2006; Motzer *et al.* 2007]. In early clinical trials evaluating safety and efficacy in AML, sunitinib demonstrated a transient antileukemic effect (typically a reduction in peripheral blast count), but this was limited by short duration and significant toxicities [O'Farrell *et al.* 2003; Fiedler *et al.* 2005]. Recent results from a combined phase I/II trial have been more encouraging, with sunitinib able to demonstrate a complete remission rate of 59% in elderly patients with mutant FLT3 [Fiedler *et al.* 2012]. Interestingly, a substantial portion of those complete responders harbored a FLT3-TKD mutation.

Sorafenib, another potent TKI, has also been approved for use in renal cell carcinoma and hepatocellular carcinoma [Escudier *et al.* 2007; Llovet *et al.* 2008]. In contrast to sunitinib, sorafenib has demonstrated less toxicity in early clinical trials. In several small phase I trials of AML, sorafenib demonstrated reliable reductions in peripheral blood and bone marrow blast counts, particularly in patients harboring FLT3-ITD mutations [Zhang *et al.* 2008; Pratz *et al.* 2010a; Metzelder *et al.* 2012]. Sorafenib has been extensively used as the FLT3 inhibitor of choice for patients who are not eligible for or unable to partake in clinical trials of FLT3 inhibitors. This has been based upon data from earlier-phase studies, as well as from case series suggesting that sorafenib-mediated disease control allowed for bridge to hematopoietic stem cell transplantation (HSCT) [Sharma *et al.* 2011]. Larger phase II trials demonstrated that sorafenib could be safely administered with conventional cytotoxic therapies, although a clinical benefit has not been established [Ravandi *et al.* 2010; Serve *et al.*

2010]. Two studies are currently recruiting patients to address the usefulness of sorafenib in the peritransplant setting [ClinicalTrials.gov identifiers: NCT01398501 and NCT01578109].

As with other early FLT3 inhibitors, midostaurin also induced transient decreases in peripheral and bone marrow myeloblasts and manifested selectively toward those harboring mutant FLT3 [Stone *et al.* 2005; Fischer *et al.* 2010]. In a phase Ib study of younger patients with newly diagnosed AML, midostaurin was safely administered in conjunction with conventional cytotoxic chemotherapy, resulting in high rates of complete remission in both WT (74%) and mutant FLT3 (92%) patients [Stone *et al.* 2012]. Several trials evaluating midostaurin in FLT3 mutant AML are recruiting or ongoing, including one large phase III randomized, double-blind study, following earlier studies of this combination, assessing the impact of midostaurin with daunorubicin/cytarabine induction and high-dose cytarabine consolidation in newly diagnosed patients younger than 60 years [ClinicalTrials.gov identifier: NCT00651261]. Additional studies exploring a role for midostaurin in the peritransplant setting and in combination with other targeted therapies are currently recruiting or ongoing. Lestaurtinib has been extensively studied in AML, even progressing to a completed phase III randomized trial in 2011 [Levis *et al.* 2011]. Early phase I/II trial results demonstrated transient reductions in peripheral and marrow myeloblasts and, furthermore, that these clinical outcomes were strongly correlated with robust suppression of FLT3 phosphorylation *in vivo* [Smith *et al.* 2004; Knapper *et al.* 2006]. These results prompted a large, multicenter phase III clinical trial evaluating lestaurtinib in combination with chemotherapy in relapsed/refractory patients. While lestaurtinib could be safely administered with conventional re-induction chemotherapies, no differences in complete remission rate or overall survival could be appreciated between the two arms of the trial [Levis *et al.* 2011]. Importantly, however, only a minority of patients achieved sustained FLT3 inhibition in this study. We do not as of yet have published data on additional phase III trials evaluating lestaurtinib in younger patients with mutant FLT3 AML (ISRCTN 17161961 and 55675535).

Our experiences with these early, nonspecific FLT3 inhibitors have informed our understanding of FLT3 biology in important ways. Several of

Table 1. FLT3 tyrosine kinase inhibitors under clinical investigation.

Agent	Identifier	Phase	Status	Findings/objectives	Reference
Sunitinib		I	Completed	Sunitinib monotherapy produced reductions in peripheral blast counts with significant toxicities; significant FLT3 inhibition was noted	O'Farrell <i>et al.</i> [2003]
		I	Completed	Sunitinib monotherapy in relapsed/refractory AML selectively induced transient responses in <i>FLT3</i> mutant patients	Fiedler <i>et al.</i> [2005]
		I/II	Completed	Sunitinib in combination with standard induction and consolidation induced a significant number of complete remissions in elderly patients with newly diagnosed AML (active against both FLT3-ITD/TKD)	Fiedler <i>et al.</i> [2012]
Sorafenib		I	Completed	Sorafenib was well tolerated as monotherapy in relapsed/refractory AML with transient reductions in peripheral and marrow myeloblasts and relative selectivity toward <i>FLT3</i> mutant patients	Zhang <i>et al.</i> [2008], Pratz <i>et al.</i> [2010a], Metzelder <i>et al.</i> [2012]
		I/II	Completed	Sorafenib was safe to administer with conventional cytotoxic chemotherapy (AraC, idarubicin) with >70% rate of complete remission in both FLT3-ITD and WT patients	Ravandi <i>et al.</i> [2010]
		II	Completed	Sorafenib was tolerated in combination with AraC/daunorubicin induction followed by consolidation and sorafenib maintenance, but no differences in survival between placebo and sorafenib groups	Serve <i>et al.</i> [2010]
	NCT00516828	I/II	Completed	The combination of sorafenib and low-dose cytarabine was assessed in older patients with AML or high-risk MDS	
	NCT01398501 NCT01578109	I	Recruiting	Two studies exploring the use of sorafenib to maintain remission before, during, and after bone marrow transplant (8109) and after allogeneic bone marrow transplant (8501)	
	NCT00943943	I	Recruiting	To establish maximum tolerated dose and safety profile of sorafenib in combination with G-CSF and the immunostimulant plerixafor/mozobil in FLT3-ITD relapsed/refractory AML	
	NCT01861314 NCT00908167 NCT00665990 NCT01534260	I	Recruiting	Multiple phase I studies to evaluate the use of sorafenib in combination with various regimens including bortezomib/decitabine	
		I/II	Recruiting	A study to assess the combination of sorafenib, vorinostat, and bortezomib in younger patients with high-risk AML, including those with FLT3-ITD	
	NCT00875745 NCT01254890	I/II	Active, not recruiting	Two studies, one assessing the combination of sorafenib + vorinostat and the other, sorafenib + 5-azacitidine in younger patients with AML/high-risk MDS	
	NCT01253070	II	Active, not recruiting	Cooperative group study assessing efficacy of sorafenib combined with conventional chemotherapy followed by sorafenib maintenance in older patients with newly diagnosed, <i>FLT3</i> mutant AML	
Midostaurin		III	Recruiting	A randomized trial assessing the combination of sorafenib and bortezomib in younger AML patients with and without <i>FLT3</i> mutations	
	NCT01371981	I	Completed	Midostaurin was safely administered with conventional cytotoxic chemotherapy (daunorubicin/cytarabine induction, Ara-C consolidation); >70% complete response rate in FLT3-WT and mutant patients	Stone <i>et al.</i> [2012]

(Continued)

Table 1. (Continued)

Agent	Identifier	Phase	Status	Findings/objectives	Reference
	NCT01174888	II	Completed	Midostaurin induced decreases in peripheral and bone marrow blast counts with some selectivity toward <i>FLT3</i> mutant patients	Stone <i>et al.</i> [2005], Fischer <i>et al.</i> [2010]
	NCT01477606 NCT01883362	I II	Recruiting Recruiting	Midostaurin in combination with bortezomib and conventional chemotherapy in relapsed/refractory AML Midostaurin as a component of induction/consolidation/maintenance therapy in <i>FLT3</i> -ITD patients with newly diagnosed AML, including those who have received allogeneic HSCT; Midostaurin maintenance therapy in <i>FLT3</i> -ITD patients following allo-HSCT	
	NCT01830361	II	Recruiting	Midostaurin with standard therapy in newly diagnosed AML patients with <i>c-KIT</i> or <i>FLT3</i> -ITD mutations	
	NCT01846624	II	Recruiting	Midostaurin with decitabine for older patients with newly diagnosed AML	
	NCT00819546	II	Active, not recruiting	Midostaurin with RAD001 for patients with relapsed, refractory or poor prognosis AML	
	NCT01202877 NCT01093573	I/II	Active, not recruiting; recruiting	Combination of midostaurin and 5-azacytidine for patients with refractory/relapsed AML or MDS	
	NCT00651261	III	Active, not recruiting	Midostaurin with standard cytotoxic chemotherapy (daunorubicin/cytarabine induction with cytarabine consolidation) in young patients with <i>FLT3</i> mutant AML	
Lestaurtinib		I/II	Completed	Lestaurtinib monotherapy resulted in transient reductions in peripheral and marrow blast counts with some selectivity toward <i>FLT3</i> mutant AML	Smith <i>et al.</i> [2004], Knapper <i>et al.</i> [2006]
		III	Completed	Lestaurtinib can be safely administered with standard cytotoxic chemotherapy in patients with <i>FLT3</i> mutant AML, but this did not result in overall benefit	Levis <i>et al.</i> [2011]
	ISRCTN17161961 ISRCTN55675535	III	Ongoing	British MRC trials assessing the effect of lestaurtinib in combination with standard induction and consolidation regimens in younger patients with newly diagnosed <i>FLT3</i> mutant AML	
Crenolanib	NCT01522469 NCT01657682	II	Recruiting	Two phase II trials exploring a role for crenolanib in relapsed/refractory <i>FLT3</i> mutant AML with or without prior <i>FLT3</i> TKI treatment	
PLX3397	NCT01349049	I/II	Recruiting	Safety/efficacy study of PLX3397 alone in adult patients with relapsed/refractory AML	
AML, acute myeloid leukemia; <i>FLT3</i> , FMS-like tyrosine kinase 3; HSCT, hematopoietic stem cell transplantation; ITD, internal tandem duplication; MDS, myelodysplastic syndrome; TKI, tyrosine kinase inhibitor; WT, wild type. Note: The status of these clinical trials are updated as of the time of manuscript preparation.					

the early TKI trials in AML revealed, first, improved efficacy in *FLT3* mutant patients and, second, a correlation between effective, sustained inhibition of *FLT3* *in vivo* and clinical response. Additional evidence indicates that drug plasma levels may correlate with sustained *FLT3* inhibition and thereby serve as a useful metric to maximize patient response rates [Ravandi *et al.* 2013]. The relative nonspecificity of these early TKIs has been implicated as a cause of the transient clinical responses noted in *FLT3*WT and mutant patients alike. While *FLT3* has been shown to be a key mediator of leukemic cell growth and survival, a great number of related and parallel signal transduction pathways contribute to leukemic progression, many of which are likely attenuated by targeted therapy with TKIs. This same nonspecificity may also contribute to the significant toxicities associated with these inhibitors and may limit their clinical utility. New, second-generation *FLT3* inhibitors have begun to emerge, including quizartinib (AC-220), crenolanib (CP-868596), and PLX3397. These inhibitors demonstrate a significantly greater specificity for *FLT3* and, as the mutant allelic burden increases in relapsed patients, hold tremendous promise for *FLT3* mutant patients.

Quizartinib and next-generation *FLT3* inhibitors

Quizartinib was originally identified in a library-based small molecule inhibitor screen and demonstrated enhanced potency and selectivity for *FLT3* *in vitro*. The IC_{50} of quizartinib for *FLT3* inhibition in plasma is 18 nM in contrast to the various first-generation TKIs outlined above, all of which are associated with IC_{50} measurements of greater than 400 nM in patient plasma [Zarrinkar *et al.* 2009; Pratz *et al.* 2010a]. Early preclinical xenograft assays revealed exquisite sensitivity to quizartinib treatment. In one model using immortalized MV4-11 cells, mice were transiently treated with quizartinib or sunitinib for 28 days and then followed for an additional 60-day post-treatment period: mice receiving quizartinib demonstrated rapid and complete tumor regression, sustained throughout the post-treatment period. Sunitinib treatment, in contrast, resulted in only partial reduction in tumor burden, and tumor growth resumed immediately in the post-treatment period [Zarrinkar *et al.* 2009]. In another xenograft model of leukemia engrafted into the bone marrow, quizartinib resulted in a dose-dependent improvement in

overall survival: at 10 mg/kg, 80% of the animals survived until the endpoint of the experiment (a 250% increase in life span over control) [Zarrinkar *et al.* 2009].

Of note, quizartinib is also associated with a significantly longer half-life *in vivo*, and a much greater capacity for sustained *FLT3* inhibition [Cortes *et al.* 2013]. These factors have translated into greater efficacy as a single agent in patients with relapsed/refractory AML. In phase I/II trials, quizartinib monotherapy has been found to be generally well-tolerated and has demonstrated an appreciable number of complete and partial responses in *FLT3* mutant patients [Cortes *et al.* 2012; 2013; Levis *et al.* 2012]. A phase I trial with 76 patients suffering from refractory or relapsed AML (age 23–86 years, *FLT3* status unselected at outset) revealed an acceptable toxicity profile and enhanced clinical activity in *FLT3*-ITD patients [Cortes *et al.* 2013]. Side effects included gastrointestinal (GI) upset, reversible QT prolongation, and myelosuppression. Despite quizartinib's enhanced specificity, these toxicities may in part be related to off-target effects on other RTKs, particularly KIT [Kampa-Schittenhelm *et al.* 2013]. Partial or complete responses were noted in 30% of study participants: 53% of *FLT3*-ITD patients responded *versus* 14% of *FLT3*-WT patients. These results were followed by a phase II study to assess quizartinib efficacy in two cohorts of patients. Cohort I ($n = 133$) included patients greater than 60 years of age with relapsed or refractory AML. As before, quizartinib was noted to be particularly effective in patients harboring *FLT3*-ITD mutations, who demonstrated a 54% composite complete remission rate compared with 31% in the *FLT3*-WT group [Cortes *et al.* 2012]. These results constitute the highest degree of single-agent efficacy for elderly patients with refractory/relapsed AML and, of note, a number of patients (8%) were able to successfully bridge to HSCT. Cohort II ($n = 137$) included younger patients, greater than 18 years of age who had relapsed or were refractory to second-line treatment or HSCT. *FLT3*-ITD patients again demonstrated a higher composite complete remission rate: 44% *versus* 34% in the *FLT3*-WT subset [Levis *et al.* 2012]. Of the *FLT3*-ITD patients who were refractory to prior therapy, 47% achieved composite complete remission. Of particular note, in this population of pretreated patients, more than one-third (37%) were successfully bridged to HSCT. It is important to acknowledge that a substantial proportion of

these remissions were characterized by incomplete peripheral count recovery, and therefore are not complete remissions per strict definitions. This may be related to the fact that the remissions which result from FLT3-targeted therapy may not be the kinetic remissions traditionally seen with cytotoxic therapy, where repopulation of the marrow with normal cells follows effective chemotherapy-induced ablation. Rather, effective and sustained FLT3 inhibition appears to promote differentiation of ITD myeloblasts, leading over time to replacement of the diseased marrow. Indeed, evidence for this process of differentiation has emerged [Sexauer *et al.* 2012; Fathi *et al.* 2013].

Several additional trials assessing quizartinib efficacy are currently recruiting or ongoing (see Table 2 for completed and ongoing quizartinib clinical trials). These include a phase II, randomized study of quizartinib monotherapy in FLT3-ITD positive patients (18 years or older) with relapsed/refractory AML [ClinicalTrials.gov identifier: NCT01565668] and two phase I trials addressing the potential usefulness of quizartinib therapy in conjunction with conventional chemotherapy or HSCT. The first of these ongoing phase I trials will explore the combination of quizartinib with standard 7+3 cytarabine and daunorubicin induction followed by high-dose cytarabine consolidation and quizartinib maintenance [ClinicalTrials.gov identifier: NCT01390337]. The second will assess the use of quizartinib as maintenance therapy following allogeneic HSCT performed in AML patients in first or second remission [ClinicalTrials.gov identifier: NCT01468467]. In addition, new combination phase I/II trials have been initiated to assess the tolerability, safety, and efficacy of quizartinib therapy in combination with other standard chemotherapies including etoposide and azacitidine for patients with AML or high-risk MDS [ClinicalTrials.gov identifiers: NCT01236144 and NCT01892371]. The molecular mechanisms underlying potential synergy between these compounds remain unclear. As discussed in detail below, FLT3 ligand levels appear to be dramatically altered following cytotoxic therapy [Sato *et al.* 2011]; this finding has important implications when considering the timing of combinatorial therapy as well as the mechanisms governing synergy. Results from these ongoing studies will provide insight into the role quizartinib might soon play in the context of these established therapies for AML.

Even as these clinical trials proceed, new evidence points toward mechanisms governing emerging resistance to quizartinib (and other FLT3 TKIs). Resistance to quizartinib in FLT3-ITD patients appears to be due, at least in part, to acquired TKD mutations at the D835 and F691 sites [Moore *et al.* 2012; Smith *et al.* 2012]. More recently, crenolanib has emerged as an alternative, highly potent inhibitor of FLT3. Originally designed to inhibit PDGFR and under study in several solid malignancies, crenolanib was found to inhibit FLT3 in both mutant and WT cells at similar plasma concentrations to quizartinib [Galanis *et al.* 2012]. Furthermore, crenolanib demonstrated no QTc prolongation in patients and less inhibition of cKIT *in vitro*, which will perhaps correlate with reduced myelosuppression *in vivo*. Of particular interest, crenolanib has demonstrated efficacy against tumor cell lines and primary blasts that have developed D835 activating mutations and resistance to quizartinib [Galanis *et al.* 2012; Zimmerman *et al.* 2013]. Based on these findings, a phase II study assessing the effect of crenolanib in relapsed/refractory AML patients with FLT3-TKD D835 mutations was recently initiated [ClinicalTrials.gov identifier: NCT01522469] (see Table 1).

The novel compound PLX3397 has also emerged as a potent, specific inhibitor of FLT3-ITD mutant AML. In various leukemic cell lines expressing either ITD or WT FLT3, the agent was shown to selectively inhibit FLT3-ITD variants at 10-fold lower concentrations (24–30 nM *versus* 240 nM) [Burton *et al.* 2011]. PLX3397 was also effective against primary AML samples harboring FLT3-ITD mutations in cell culture and inhibited cell growth in a dose-dependent fashion, while no significant effects were noted in FLT3 WT samples at equivalent doses [Burton *et al.* 2011]. A phase I/II study of PLX3397 in adults with FLT3-ITD relapsed/refractory AML is currently recruiting [ClinicalTrials.gov identifier: NCT01349049] (see Table 1).

Combinatorial therapy and overcoming acquired resistance

Despite promising results in preclinical models and early clinical trials, acquired resistance to TKIs continues to present a significant challenge. Delineating the molecular mechanisms driving resistance will simultaneously enhance our understanding of the complex signal transduction networks underlying leukemia progression while

Table 2. Quizartinib in clinical trials.

Identifier	Phase	Status	Findings/objectives	Reference
	I	Completed	Quizartinib monotherapy generally well tolerated; enhanced clinical activity in FLT3-ITD patients	Cortes <i>et al.</i> [2013]
	II (Cohort 1)	Accrual completed	Quizartinib monotherapy was well tolerated in older patients with relapsed/refractory AML, with enhanced efficacy in those with FLT3-ITD mutations, and facilitating bridge to HSCT in a subset	Cortes <i>et al.</i> [2012]
	II (Cohort 2)	Accrual completed	Quizartinib monotherapy was efficacious in younger patients with refractory/relapsed AML, particularly so in FLT3-ITD patients, with 37% successfully bridged to HSCT	Lewis <i>et al.</i> [2012]
NCT01565668	II	Active, not recruiting	Randomized trial of quizartinib monotherapy in FLT3-ITD mutant patients greater than 18 years old with relapsed/refractory AML	
NCT01390337	I	Active, not recruiting	Quizartinib in combination with standard 7+3 induction therapy, high-dose cytarabine consolidation, and quizartinib maintenance in newly diagnosed AML	
NCT01468467	I	Active, not recruiting	Quizartinib maintenance therapy in AML patients s/p allo-HSCT transplant in first or second remission	
NCT01236144	I/II	Recruiting	Quizartinib in combination with standard daunorubicin, ara-C, etoposide therapy in older patients with AML or high-risk MDS	
NCT01892371	I/II	Not yet recruiting	Quizartinib in conjunction with azacitidine or cytarabine for patients with AML or MDS	
AML, acute myeloid leukemia; FLT3, FMS-like tyrosine kinase 3; HSCT, hematopoietic stem cell transplantation; ITD, internal tandem duplication; MDS, myelodysplastic syndrome; WT, wildtype. Note: The status of these clinical trials are updated as of the time of manuscript preparation.				

providing rationale for new therapeutic approaches in relapsed or refractory patients. To that end, several mechanisms have been shown to contribute to TKI resistance in *FLT3* mutant AML. As mentioned above, secondary TKD mutations have been shown to occur during the course of *FLT3* TKI treatment in both murine models and in patients. This was first described in a single patient who developed resistance to midostaurin: subsequent genetic analysis revealed a new TKD mutation at position 676 (N676K) and reconstitution of this mutation *in vitro* conferred immediate resistance to midostaurin [Heidel *et al.* 2006]. A similar pattern has been identified following treatment with other *FLT3* TKIs, including quizartinib [Smith *et al.* 2012]. Interestingly, it is clear that different *FLT3* TKIs elicit distinct and largely reproducible patterns of acquired TKD resistance mutations [Von Bubnoff *et al.* 2009]. In a study of midostaurin, sorafenib, and SU5614-resistant *FLT3*-ITD cell lines with newly acquired TKD mutations, nonoverlapping mutations occurred in distinct regions of the TKD in all three cases. Importantly, when challenged with an alternate *FLT3* TKI, resistant cell lines retained their sensitivity to the new agent [Von Bubnoff *et al.* 2009]. Similar results have been shown in quizartinib-resistant cells that were subsequently challenged with crenolanib [Galanis *et al.* 2012; Zimmerman *et al.* 2013]. Taken together, these results suggest that empiric use of combinatorial *FLT3* TKI therapy may prevent the emergence of *FLT3*-TKD resistance mutations. Whether this can translate into clinical use requires further study, although individual pharmacokinetics, toxicities and drug interactions may be a significant challenge.

Upregulation of parallel and downstream signal transduction pathways has also been implicated in *FLT3* TKI resistance. Recent work has demonstrated the importance of PI3K/AKT signaling in *FLT3*-mutant, TKI-resistant cell lines: selective AKT inhibitors contributed synergistically to *FLT3* inhibition in resistant cell lines and in *FLT3* mutant patient samples [Weisberg *et al.* 2013]. Similar data has implicated STAT and MEK/ERK pathway upregulation in *FLT3* TKI-resistant cells [Piloto *et al.* 2007; Zhou *et al.* 2009]. These findings remind us that the complex interplay between various progrowth and pro-survival signal transduction pathways within the leukemic blast is a plastic, dynamic process. Selective pressure applied by a single targeted agent might prompt compensatory upregulation of alternative

signaling pathways, fundamentally changing the susceptibility profile of the evolving disease. A great variety of PI3K, AKT, mTOR, and MEK inhibitors are under development in a host of malignancies, and may in time come under study as a mechanism to prevent the emergence of FLT3 TKI resistance in AML [Gollob *et al.* 2006; Markman *et al.* 2009; Wander *et al.* 2011].

Other mechanisms extrinsic to the leukemic cells also appear to be contributing to resistance. As in many paradigms of solid tumor progression, accumulating evidence implicates the tumor microenvironment as an active and important contributor to drug resistance. In the case of AML, stromal components of the bone marrow microenvironment appear to provide important progrowth and anti-apoptotic signals to the developing blast population [Parmar *et al.* 2011]. This process is not distinct from the upregulation of parallel and downstream signal transduction pathways discussed above. In fact, stromal-dependent signaling, whether via cell–cell contact or cytokine-mediated paracrine effects, has the potential to upregulate the survival and proliferation pathways necessary for drug resistance and disease progression. Recent work has specifically implicated ERK and AKT signaling in this process. ERK upregulation following coculture of FLT3-ITD AML blasts with stromal cells correlated with FLT3 TKI resistance, and the addition of Janus kinase (JAK) inhibitors overcame stromal-mediated resistance in these models [Yang *et al.* 2011; Weisberg *et al.* 2013]. Stromal coculture was also shown to upregulate PI3K/AKT activation, which can be attenuated by application of selective AKT inhibitors [Weisberg *et al.* 2012]. These findings have been implicated as a possible explanation for the frequent discrepancy identified in early FLT3 TKI clinical trials: namely that FLT3 inhibitors selectively attenuated the peripheral blast count while the bone marrow blasts were relatively spared. Furthermore, these experiments have provided rationale for combinatorial therapies designed to target the stromal compartment within the bone marrow in an attempt to potentiate FLT3 TKIs.

Finally, in the setting of combined therapy with cytotoxic regimens, the timing of FLT3 TKI incorporation may be of critical importance in preventing therapeutic resistance. FLT3 ligand (FL) levels appear to be influenced by exposure to cytotoxic chemotherapy, and indeed studies have demonstrated a dramatic increase in FL

levels following chemotherapy treatment [Sato *et al.* 2011]. Plasma FL levels rose precipitously following intensive chemotherapy in newly diagnosed patients and, interestingly, rose to even greater levels in relapsed patients and after successive cycles. In cell culture experiments, recapitulation of these FL levels abrogated FLT3 attenuation by five FLT3 TKIs, including lestaurtinib, midostaurin, sorafenib, and quizartinib [Sato *et al.* 2011]. This too is likely a function of the bone marrow stroma: FL levels have been shown to be inversely proportional to bone marrow cellularity [Haidar *et al.* 2002]. Bone marrow aplasia following myeloablative chemotherapy may induce stromal upregulation of FL expression, thereby blunting the effect of concurrent or subsequent FLT3 TKI treatment. These findings should prompt careful consideration of the timing of FLT3 TKI administration in conjunction with conventional chemotherapy. FLT3 inhibition may be maximal once FL levels have stabilized or reached their nadir, either early in induction or as maintenance therapy following consolidation or transplantation.

Conclusions

Despite significant advances in our understanding of disease initiation and progression, prognosis for many patients with AML remains poor and successful long-term treatment options remain elusive. *FLT3* mutant AML presents both a unique challenge and an important opportunity for the application of targeted therapies. *FLT3* mutations were among the first mutations discovered in AML, a malignancy which in recent years has been determined to be a genetically heterogeneous disease. As we achieve an ever increasing understanding of the genetic processes underlying leukemogenesis, important scientific, clinical, and ethical questions regarding the impact of individual genes on the nature, diagnosis, and prognosis of the disease will arise.

Lessons gleaned from nonspecific, first-generation FLT3 inhibitors have informed our understanding of the molecular events driving disease initiation, therapy-related toxicities, and the acquisition of resistance. Now, a new generation of FLT3 inhibitors have demonstrated safety, increased potency, and a high degree of specificity. The precise role these inhibitors might play in juxtaposition to standard chemotherapy remains an important question and an active area of research. In the future, FLT3 inhibitors may be

used alongside conventional chemotherapy in induction regimens, as a maintenance therapy, or in relapsed/refractory patients as a bridge to transplantation; additional advanced-phase clinical trials will be required to explore these possibilities. Early results with quizartinib have been promising, and continued investigation at both the bench and bedside will continue to inform our understanding of *FLT3*-mutant AML, with the hope that effective *FLT3* inhibitors will become standard antileukemic approaches alongside conventional cytotoxic chemotherapies and HSCT.

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Conflict of interest statement

M.L. is on the clinical advisory board for Ambit Biosciences. A.F. is on the advisory boards for Seattle Genetics and Agios Pharmaceuticals.

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