

The Future of Targeting FLT3 Activation in AML

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Abstract Internal tandem duplications (ITD) and tyrosine-kinase domain (TKD) mutations of the FMS-like tyrosine-kinase 3 (*FLT3*) can be found in up to one third of patients with acute myeloid leukemia (AML) and confer a poor prognosis. First discovered 20 years ago, these mutations were identified as viable therapeutic targets, and FLT3 tyrosine-kinase inhibitors (TKIs) have been in development for the last decade with steadily increasing potency. However, FLT3-mutated AML often acquires resistance to the growing armamentarium of *FLT3* inhibitors through a variety of mechanisms. In this review, we discuss the distinct clinical phenotype of *FLT3*-mutated AML, historically and currently available therapeutics, mechanisms of resistance, ongoing trials, and future outlook at treatment strategies.

Keywords Acute myeloid leukemia (AML) · FLT3 · Tyrosine-kinase inhibitor (TKI)

Introduction

Essentially incurable half a century ago, durable remissions now occur in up to 40% of adult AML patients via optimization of cytotoxic chemotherapy regimens and supportive care [1•]. However, further permutations to cytotoxic chemotherapy regimens have not resulted in significantly improved outcomes [2]. Now, rather than being viewed (and treated) as a single entity, AML has been recognized as being composed of a diverse group of genetically disparate subtypes that require unique management strategies [3], with progress being made among specific subtypes (e.g., acute promyelocytic leukemia (APL)), but still with an unmet need in others.

In a model of mutational cooperativity in AML developed in 2002 [4], it was hypothesized that the development of AML requires two distinct classes of mutations working together to promote leukemogenesis. Class I mutations, in genes such as *FLT3*, *N-RAS*, and *K-RAS*, confer a proliferative and survival advantage to hematopoietic progenitors, but do not affect differentiation. Class II mutations, which include *AML1/ETO*, *CBFβ/SMMHC*, *PML/RARα*, and *MLL*-related fusion genes, impair differentiation but are also not sufficient alone to cause leukemia. Both of these mutations working in concert result in leukemogenesis. Recently, this model has been further refined using whole genome sequencing data of AML samples and healthy hematopoietic stem/progenitor cells, demonstrating that most mutations in AML are random events (passengers) prior to leukemogenesis, and that single driver mutations (*PML-RARA* or *NPM1*) confer a clonal survival advantage [5]. Mutations in *NPM1*, *DNMT3A*, and *IDH1* or *IDH2* are thought to be a part of a central AML pathway (drivers), while *FLT3*-ITD is the most frequently mutated gene that is thought to cooperate with these central events as a second driver and leads to developing of the founding leukemic clone detected at presentation [5]. While aberrant activation of a tyrosine kinase

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and/or growth factor signaling pathway may be a central feature in many AML cases, as postulated in the original model of cooperativity, whole genome sequencing studies seem to suggest that more than a third of AML cases lack such mutations [6•].

FLT3-Mutated AML

FLT3 is a member of the class III “split kinase domain” family of receptor tyrosine kinases (RTKs) which also includes PDGFR, KIT, and FMS [7]. FLT3 is composed of five extracellular domains, a transmembrane domain, a juxtamembrane domain (JM), and two tyrosine-kinase domains and is expressed on leukemic blasts in most cases of AML [8]. FLT3 binds to FLT3 ligand (FL) and then dimerizes, changing to an open conformation which allows ATP access to the binding pocket. The receptor then undergoes autophosphorylation and transduces cell growth and apoptotic inhibitory signals via Ras-GTPase activating protein, phospholipase C β , STAT5, and ERK1/2 [9].

The two most common types of FLT3 activating mutations are the so-called internal tandem duplication mutations (FLT3-ITD mutations) and point mutations within the tyrosine-kinase domain (FLT3-TKD mutations). The FLT3-ITD mutations were first discovered by Nakao et al. in 1996 [10]. FLT3 mutations are carried by up to 30% of patients with AML, most (23%) of which are FLT3-ITD, with the remainder (7%) composed of FLT3-TKD mutations primarily at residue D835 (along with some less common point mutations) [11–13]. Most FLT3-ITD mutations occur in or near the sequence for the JM domain of the receptor, while point mutations occur largely within the sequence for the activation loop of TKD1.

FLT3-ITD AML is a distinct clinical entity conferring a poor prognosis [14–17]. Patients with FLT3-ITD AML tend to present at a younger age on average (though overall it is less common in childhood AML) [18, 19], with higher white blood cell (WBC) counts and with normal karyotype. It is more likely to present de novo rather than as a consequence of some antecedent disorder such as MDS and to confer a significantly worse prognosis than what is seen for FLT3 wild-type patients [14–17, 20]. FLT3-TKD mutations appear to occur more frequently with high WBC, cytogenetically favorable risk, and primarily in de novo AML. FLT3-TKD mutations have been shown to be constitutively activating, though clinical outcomes are significantly better than in FLT3-ITD AML and actually trend towards superiority to FLT3-WT disease in inv.(16) AML [11, 12, 21]. In one study at 10 years, interestingly, overall survival among higher-level mutants (>25% mutant TKD) was 59%, which was superior to lower-level mutants (<25%) and FLT3-TKD-wt patients (37 and 33%, respectively) [21].

FLT3-ITD and FLT3-TKD mutations constitutively activate the receptor in different fashions. The former lesions result in in-frame duplications involving the JM which plays a role in regulating the kinase activity [22]. This is potentially mediated by two key tyrosine residues that serve as STAT5 binding sites, Y589 and Y591, which in the context of ITDs, are activating [23]. ITDs commonly vary from 2 to 42 AA (though may be more than 400 bp) and are always in frame. Specifically, arginine R595 in the JM domain is duplicated in 77% of FLT3-ITD patients and has been shown to confer factor-independent growth in cell lines and is activating of STAT5 [24]. Up to 30% of FLT3-ITD-positive patients may have activating insertions in other locations, most commonly the nucleotide binding loop (612–623) [25]. ITD location may be important with some data supporting that location of ITDs within the β 1-sheet of TKD1 is an independent unfavorable prognostic factor possibly outweighing allelic ratio, and may predict nonresponsiveness to TKI therapy [26]. ITD length may be prognostically relevant as well with some support for the notion that longer ITDs confer a worse outcome [27].

TKD mutations are common at aspartate 835, and also occur at aspartate 842 and isoleucine 836. However, based on receptor data from the structurally related receptor c-KIT, these mutations may have very different mechanisms of activation resulting in different clinical and biologic effects [28].

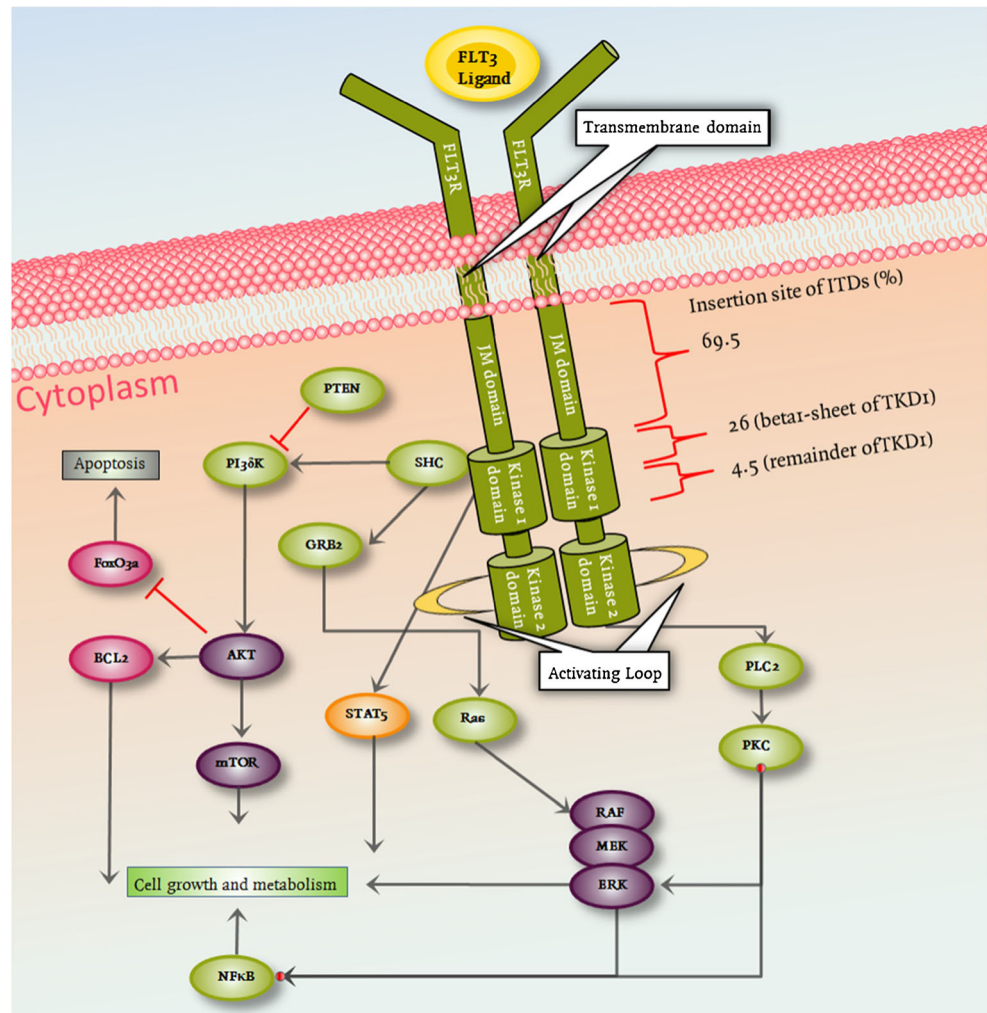
FLT3-ITD mutations were found to occur in to 20–40% of patients APL with 6–10% having TKD mutations in two studies with nearly 500 combined APL patients [29, 30]. In this subtype, FLT3-ITD mutations are associated with higher WBC at presentation, without a clear impact on outcomes. Otherwise, FLT3-ITD mutations are uncommon in cytogenetically abnormal AML except in t(6;9) [16].

The eventual signal cascade of FLT3-ITD activation culminates in multiple pathways including phosphatidylinositol3-kinase (PI3K)/AKT, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and signal transducer and activator of transcription 5 (STAT5) (Fig. 1) [31, 32]. FLT3-ITD expression induces independent growth in cell lines and causes a fatal myeloproliferative disorder in transgenic mouse models [33], while mice harboring the TKD mutation develop an oligoclonal lymphoid disorder with longer latency with better survival outcome [34].

Assays for FLT3 Mutations

The assay for FLT3 activating mutations is polymerase chain reaction (PCR)-based and interrogates genomic DNA from a given patient’s malignant cells from either whole blood or bone marrow. The assay is subject to dilution from nonmalignant DNA which may decrease amount of mutant alleles measured. In the most commonly used platform, PCR primers

Fig. 1 Key signaling pathways of the FLT3-ITD receptor



flanking the JM coding sequence amplify *FLT3*-ITD mutations and, simultaneously in the same assay, a second set of primers amplify the TKD domain [35]. Amplified fragments are separated by capillary gel electrophoresis. EcoRV digestion of PCR products cleaves the kinase domain unless a TKD mutation is present (D835 or I836). The assay is able to determine if a TKD mutation is present at 835 and the length of any potential ITD mutation.

Using these primers flanking the juxtamembrane domain, the wild-type *FLT3* PCR fragment is 330 bases while an ITD mutation may increase this length by dozens of bases. During the PCR reaction, the shorter wild-type fragment gains a significant competitive advantage as synthesis of the shorter allele will occur more rapidly. The resultant “PCR bias” can be demonstrated even with a 1:1 mixture of mutant and wild-type gene templates. The result will be a more wild-type product than mutant (ratio less than one of mutant-to-wild type). Longer insertions lead to a greater PCR bias. This distortion can be limited by using fewer PCR cycles; however, this limits sensitivity, particularly if a low number of leukemic blasts are present in the sample being assayed [16]. Regardless, because

the *FLT3* mutant-to-wild-type allelic ratio derived from the PCR assay is predictive of clinical outcome (see below), the lack of a standardized assay in this field is a genuine impediment to interpreting the results of clinical studies which include this parameter. A high allelic ratio in one study may be an intermediate ratio in another.

The allelic ratio describes the amount of ITD-mutated alleles relative to the amount of wild-type alleles. It is affected by the number of leukemic cells in the sample, and amount of cells with one, both, or no allelic mutations. The dominant cell in most cases is heterozygous for the mutation. Not uncommonly, however, AML subclones may be homozygous via uniparental disomy [36], or lack the mutation altogether. Cells may also be hemizygous for the mutation and completely lack a wild-type allele (associated with a worse prognosis) [37]. In general, higher allelic ratio has been associated with worse prognosis. However, in concert with other favorable cooperating mutations (e.g., *NPM1*) combined with low allelic ratio (<0.5), patients may have prognosis no different than wild-type *FLT3*-ITD patients [38, 39]. These conclusions are all complicated by the lack of uniformity in assay method,

which, as described above, influences the estimated ratio. Reference labs often use a cutoff of, for example, 10% mutant allelic burden to label a sample as “positive.” In some cases, the lab might receive a bone marrow sample from a FLT3-ITD patient in early relapse with relatively few blasts present. Based on the lab’s pre-determined cutoff, such a sample is likely labeled as “negative” for a FLT3-ITD mutation, even if this is clearly not the case. Although there are readily found examples of patients who were positive for a FLT3-ITD mutation at diagnosis and negative at relapse, this artifact in the assay has resulted in the belief that the FLT3-ITD mutation is unstable [40] and therefore of little value as a marker of minimal residual disease (MRD). More often, the *FLT3*-ITD burden actually increases from diagnosis to relapse [41].

Treatment

Most *FLT3*-ITD patients are able to achieve complete responses (CR) at similar rates to wild-type patients, with exceptions for patients with very high allelic ratio and those who are hemizygous who may often be primary refractory [37]. However, patients with *FLT3*-ITD AML on average relapse much sooner compared with wild type with a median time to relapse of 6–7 vs. 9–11 months. For example, in a prospective study of FLT3-mutated AML patients, the median time to relapse was 6 months [42]. The presence of a FLT3-ITD mutation is an especially poor prognosticator in first relapse resulting in very short survival [43]. The chance of achieving a second CR after relapse in less than 6 months with conventional chemotherapy was as low as 11% [42].

FLT3 Tyrosine-Kinase Inhibitors

Inhibition of *FLT3* signaling via blockade of its kinase activity with small molecules has been a longstanding goal in drug development. An arsenal of small molecule TKIs has been moving through the developmental pathways for some years now. These agents compete with ATP for binding to the active pocket of the kinase, resulting in inhibition of autophosphorylation and phosphorylation of substrates [44].

The initial wave of potential *FLT3* inhibitors were coopted from drugs used to treat solid tumors and were nonselective kinase inhibitors. The use of these agents often resulted in substantial toxicities. Complete, prolonged in vivo FLT3 inhibition has been difficult to achieve [44], with in vitro studies pointing towards the need for sustained blockade for efficacy [45]. Given the relatively poor potency of the older inhibitors, they have largely been used as part of combination therapy [46–48]. First-generation TKIs include midostaurin (PKC412), lestaurtinib (CEP-701), sunitinib (SU11248), KW2449, and sorafenib (BAY43–9006). Second-generation

inhibitors include gilteritinib (ASP2215), PLX3397, quizartinib (AC220), crenolanib (CP-868596), and ponatinib (AP24534). Clinical trial data from these agents is summarized in Table 1.

Plasma Inhibitory Activity Assay

Conventional pharmacokinetic studies of TKI drug levels in patients can be often misleading due to the highly protein-bound nature of the drugs, which can even vary between patients. In general, it is often difficult to confirm that the kinase being targeted has been inhibited in vivo as target tissue (leukemia cells) may be absent from the peripheral blood. A plasma inhibitory activity assay, in which a FLT3-ITD cell line is exposed to plasma from a TKI-treated patient and then assayed for FLT3 inhibition, has been shown to be a useful surrogate for in vivo activity and outcomes [51, 71]. An immunoblot is performed which identifies the phosphorylated-FLT3 which is the active form of the receptor. This can be quantified via densitometry and compared with baseline levels. Inhibition to <15% of baseline has been used to define adequacy for inhibitors.

First-Generation FLT3 TKIs

Sunitinib

Sunitinib is an indolinone derivate, multikinase inhibitor with activity against FLT3, CSF, VEGFR, c-KIT, and PDGFR approved for use in metastatic renal cell carcinoma and gastrointestinal stromal tumors. Some in vitro evidence shows that sunitinib may be effective against TKD mutations [72]. It has been tested in the phase I setting and was shown to be an effective in vivo FLT3 inhibitor [70]. Another phase I trial with 15 patients (4 *FLT3* mutations) found that all mutated patients (4/4) had a morphologic or partial response compared with 2/10 wild-type patients [69].

Midostaurin

Midostaurin is an indolocarbazole multikinase inhibitor with activity against c-KIT, PDGFR, FLT3, and VEGFR (among many others). In the phase II setting in relapsed refractory AML patients harboring *FLT3* mutations, treatment with midostaurin resulted in a reduction by more than 50% in peripheral blood blasts in most patients [53]. A subsequent larger phase IIB study in relapsed and refractory patients (or those unfit for intensive chemotherapy) with 95 patients with either *FLT3*-ITD mutations or wild type showed greater blast reduction in the *FLT3*-ITD group [54]. In a phase IB combination study of midostaurin at 50 mg twice daily (BID) or 100 mg BID with daunorubicin and cytarabine for induction of 69

Table 1 Overview of reported data from clinical trials investigating FLT3 TKIs

Drug	Phase	Population	Median age	FLT3 status	Treatment schedule and dose	Response	Reference
Crenolanib	II	$n = 20$ R/R AML + D835 mutation; many with prior TKI Tx	47	TKD D835 $n = 20$	Oral monotherapy, 100 mg tid, then individualized to 200 mg m ⁻² day ⁻¹ in 3 divided doses	1/20 CR, 2 CRi, 4 PR with 4 patients going to transplant	[49]
Gilteritinib	I–II	$n = 120$, R/R AML	NR	Mutated, $n = 82$; wt, $n = 38$	Oral monotherapy 20 mg escalated to max 450 mg Qday	ORR was 57%, (65% with >80 mg dose) in FLT3+ patients; wt ORR, 11%	[50]
KW2449	I	$n = 36$ R/R AML, CML, ALL	NR	Not reported	Oral monotherapy from 25 mg to max 500 mg Qday	Trial discontinued due to poor in vivo activity	[45]
Lestaurtinib	I–II	$n = 17$ R/R AML	61	ITD, $n = 16$; TKD, $n = 1$	Oral, monotherapy, 40 mg BID, 28-day cycle, dose escalation to 80 mg BID	5/14 treated at a dose of 60 mg with PB decrease and decreased transfusion requirement. 1 patient with CR.	[51]
Lestaurtinib	II	$n = 29$ newly diagnosed AML, age > 70 or 60–70 with comorbidity	73	ITD, $n = 2$, TKD $n = 3$, wt, $n = 24$	Oral monotherapy, 60 mg BID escalating to 80 mg BID	PB clearance or PR in 3/5 of mutFLT3, 5/22 of wt	[52]
Lestaurtinib	III	$n = 224$, first relapse AML	59	ITD, $n = 198$; TKD, $n = 17$; both, $n = 8$; not confirmed $n = 1$	Randomized with ($n = 112$) or without ($n = 112$) lestaurtinib; oral 80 mg BID with chemo: either mitoxantrone 8 mg m ⁻² , etoposide 100 mg m ⁻² , AraC 1 g m ⁻² on days 1–5, if 1st CR 6 months or AraC 1.5 g m ⁻² on days 1–5, if 1st CR lasted from 6 to 24 months	CR for chemo + lestaurtinib, 26% vs. chemo 21% ($p = 0.35$), no difference in OS.	[47]
Midostaurin	II	$n = 20$ R/R AML ($n = 19$)/high grade MDS ($n = 1$), secondary AML ($n = 5$)	62	ITD $n = 18$; TKD $n = 2$	Oral, single agent, 75 mg, TID	PB blasts <50%: in 70% of patients; BM blasts <50%: in 30% of patients	[53]
Midostaurin	IIB	$n = 95$ R/R AML or newly dx unfit for intensive chemo ($n = 85/95$), high-grade MDS ($n = 10/95$)	NR; 61/95 with age >60	ITD $n = 26$; TKD $n = 9$; wt $n = 60$	Oral, single agent, randomized, 50 or 100 mg, BID	PB or BM blasts <50%: in 71% of FLT3+ patients and 42% of wt patients; 1 PR at 100 mg dose in FLT3+	[54]
Midostaurin	IB	$n = 69$ newly diagnosed AML aged 18–60	49	ITD $n = 9$; TKD = 4	Oral, 50 mg ($n = 40/69$), or 100 mg ($n = 29/69$) BID either with (days 1–7, 15–21) or sequentially(8–21) with chemo: induction daunorubicin 60 mg m ⁻² IV on days 1–3 and cytarabine 200 mg m ⁻² continuously IV on days 1–7; consolidation: 3 cycles AraC 3 g m ⁻² IV q12h (days 1, 3, 5)	wtFLT3/mutFLT3 CR 74%/92%, 1-year OS 78%/85%, 2-year OS 52%/62%	[48]
Midostaurin	III	$n = 717$ newly diagnosed mutFLT3 AML patients	48	FLT3/ITD allelic ratio (0.05–0.7), $n = 341$ (>0.7), $n = 214$; TKD, $n = 162$	Oral, randomized 50 mg ($n = 360$) or placebo ($n = 357$) days 8–22, daunorubicin 60 mg m ⁻² IV on days 1–3 and cytarabine 200 mg m ⁻² on days 1–7. If CR 4 cycles of cytarabine 3 g m ⁻² over 3 h q12hr days 1, 3, 5 plus placebo or	Midostaurin/placebo CR 59%/54% ($p = 0.18$), median OS 74 months/26 months, HR for OS 0.77 ($p = 0.007$)	[55•]

Table 1 (continued)

Drug	Phase	Population	Median age	FLT3 status	Treatment schedule and dose	Response	Reference
Midostaurin	I–II	<i>n</i> = 54 R/R AML or newly dx unfit for intensive chemo (<i>n</i> = 34), secondary AML (<i>n</i> = 14), MDS (<i>n</i> = 3)	65	ITD, <i>n</i> = 40; TKD, <i>n</i> = 3; wt, <i>n</i> = 14	midostaurin 50 mg on days 8–22, followed by 1 year of maintenance with either placebo or midostaurin AZA 75 mg m ⁻² on days 1–7, midostaurin 25 mg BID (cohort 1 of phase I, <i>n</i> = 14) or 50 mg BID (cohort 2 of phase I and in phase II, <i>n</i> = 40) orally on days 8–21 during first cycle, then continuously	ORR 26%, median remission duration 20 weeks, significantly longer if patient had not received prior TKI or transplant	[56]
Midostaurin	II	<i>n</i> = 149 newly diagnosed AML patients, FLT3-ITD positive	54	ITD, <i>n</i> = 149; TKD, not reported	Induction: daunorubicin (60 mg m ⁻² , d1–3), cytarabine (200 mg m ⁻² , continuously, d1–7); midostaurin 50 mg BID, day 8 onwards. Second cycle is optional. Consolidation: alloHSCT as first priority (<i>n</i> = 94); if alloHSCT not possible, 3 cycles of HIDAC with midostaurin from day 6 onwards (<i>n</i> = 12). 1 year maintenance intended in all patients	Overall response to induction CR 75%, 94 patients received alloHSCT (<i>n</i> = 70 MUD, <i>n</i> = 24 MRD). Relapse risk for allelic burden <0.5 or ≥0.5, 12%/5% after alloHSCT, and 28/29% after HIDAC.	[57]
Quizartinib	I	<i>n</i> = 76, R/R AML	60	ITD, <i>n</i> = 17; wt, <i>n</i> = 45; unknown, <i>n</i> = 13	Oral monotherapy escalation from 12–450 mg	CR + PR in ITD 9/17, OS 14 weeks (18 weeks in ITD, 11 weeks in wt)	[58]
Quizartinib	II	<i>n</i> = 138, AML, relapsed after or refractory to 2nd line treatment or alloHSCT	ITD 50, wt 55	ITD, <i>n</i> = 100; wt <i>n</i> = 38	Oral monotherapy 135 mg in men, 90 mg daily for women	Cumulative CR ITD vs. wt (44/99) vs. (13/38); OS 23.1 vs. 25.6 weeks, 37% bridged to alloHSCT	[59]
Quizartinib	II	<i>n</i> = 133, AML aged >60, 1st relapse within 1 year or refractory to 1st line	70	ITD, <i>n</i> = 92; wt, <i>n</i> = 41	Oral monotherapy 135 mg in men, 90 mg daily for women	OS (weeks) ITD = 25.3/wt = 19; Cumulative CR 50/92 ITD and 13/41 in wt	[60]
Sorafenib	I	<i>n</i> = 16, R/R AML	62	ITD, <i>n</i> = 6; TKD, <i>n</i> = 3; both, <i>n</i> = 1; wt, <i>n</i> = 7	Oral monotherapy, 200–400 mg BID escalating doses	PB and BM blast decrease in 6/6 ITD patients, 43% of wt.	[61]
Sorafenib	I	<i>n</i> = 65, R/R AML; two cohorts 1) <i>n</i> = 29, after alloHSCT, 2) <i>n</i> = 36, after chemo	58	ITD, <i>n</i> = 65	Oral monotherapy, 400 mg BID	HR, <i>n</i> = 54; PR, <i>n</i> = 10; CMR 7/29 alloHSCT; 3/36 chemo group; median time to resistance in alloHSCT 197 days vs. 136 days in chemo group. Resistance developed in 11/29 alloHSCT and 17/36 chemo group	[62]
Sorafenib	I	<i>n</i> = 15, R/R AML, <i>n</i> = 12; ALL, <i>n</i> = 2; biphenotypic, <i>n</i> = 1	63	ITD, <i>n</i> = 2	Oral monotherapy, dose escalation from 400 to 600 BID	11/15 with stable disease, 1/5 on 2-week (vs. 3 weeks) schedule responded (this was the only ITD patient in the study)	[63]
Sorafenib		<i>n</i> = 50, 2 cohorts; relapsed or refractory FLT3-ITD AML	61	ITD, <i>n</i> = 50	Cohort 1: oral sorafenib BID 400 mg Monday–Friday; cohort 2: sorafenib	Bone marrow blast decrease in 34%, 10% CR rate.	[64]

Table 1 (continued)

Drug	Phase	Population	Median age	FLT3 status	Treatment schedule and dose	Response	Reference
Sorafenib	I	<i>n</i> = 22, AML FLT3/ITD after alloHSCT	54	ITD, <i>n</i> = 22	400 mg day BID 14 days out of 21. Oral monotherapy started between days 45 and 120, continuous dosing either 200 mg BID, 400/200 mg daily, 400 mg BID	1 year PFS 85%, OS 95%	[65•]
Sorafenib	II	<i>n</i> = 267 AML patients with newly diagnosed disease (placebo, <i>n</i> = 133; sorafenib, <i>n</i> = 134)	50	ITD, <i>n</i> = 46	Randomized, double-blind, placebo-controlled (1:1) 2 cycles daunorubicin (60 mg m ⁻² on days 3–5), cytarabine (100 mg m ⁻² on days 1–7), followed by 3 cycles HIDAC consolidation (3 g m ⁻² twice daily on days 1, 3, and 5) with either sorafenib (400 mg twice daily) or placebo on days 10–19 of induction cycles 1 and 2, from day 8 of each consolidation, and as maintenance for 12 months	3 year event-free survival 22% in placebo vs. 40% in sorafenib, HR 0.64 (<i>p</i> = 0.013). Among ITD relapse-free survival 18 vs. 6 months (though not statistically significant)	[66•]
Sorafenib	I–II	<i>n</i> = 61, phase I: R/R (<i>n</i> = 10); phase II: newly diagnosed (<i>n</i> = 51)	Phase I 34, phase II, 53	ITD, <i>n</i> = 20; TKD, <i>n</i> = 2; wt, <i>n</i> = 39	Phase I: oral escalating from 400 mg qod to 400 mg BID; phase II: oral, 400 mg BID, days 1–7 with chemo: AraC 1.5 g ⁻¹ m ⁻² on days 1–4, idarubicin 12 mg m ⁻² on days 1–3	Phase II: CR in 38/51; 12/13 ITD, 2/2 TKD, 24/36 wt, increased probability of CR in ITD than wt (<i>p</i> = 0.033), 1 year OS 74%	[46]
Sorafenib	II	<i>n</i> = 197, newly diagnosed AML age > 60, (<i>n</i> = 102 sorafenib, <i>n</i> = 95 placebo)	NR	ITD, <i>n</i> = 28	Randomized, placebo-controlled 400 mg BID with AraC/daunorubicin (“7 + 3”), consolidation: 2 cycles AraC 1 g m ⁻² ; 1 year sorafenib maintenance	Placebo vs. Sorafenib: median OS 15 vs 13 months (HR 1.025, <i>p</i> = 0.89), no improved outcomes in ITD subgroup	[67]
Sorafenib	II	<i>n</i> = 37, R/R AML patients	64	ITD, <i>n</i> = 37	5-azacytidine (AZA) 75 mg m ⁻² intravenously daily for 7 days and sorafenib 400 mg orally twice daily continuously	RR 46% with 10 Cri, 6 CR, and 1 PR	[68]
Sunitinib	I	<i>n</i> = 15, R/R AML or newly dx unfit for intensive chemo	72	ITD, <i>n</i> = 2; TKD, <i>n</i> = 2; wt, <i>n</i> = 10; unknown, <i>n</i> = 1	Oral monotherapy 50–75 mg	PR in 4/4 mutFLT3, 2/10 in wt	[69]
Sunitinib	I	<i>n</i> = 29, R/R AML or newly dx unfit for intensive chemo	67	ITD, <i>n</i> = 3; TKD, <i>n</i> = 2; wt, <i>n</i> = 24	Oral, 50–350 mg dose escalation	Inhibition of FLT3 phosphorylation in >50% of patients with 200 mg dose	[70]

ALL acute lymphatic leukemia, AML acute myeloid leukemia, BM bone marrow, CR complete remission, FLT3 FMS-like tyrosine kinase 3, ITD internal tandem duplication, MDS myelodysplastic syndrome, MRC Medical Research Council, OS overall survival, PB peripheral blood, TKD tyrosine kinase domain, TKI tyrosine-kinase inhibitor, wt wild type, NR not reported

newly diagnosed AML patients found similar rates of overall survival at 2 years (0.62 vs. 0.52) for *FLT3*-ITD and wild type, respectively, with 12 of 13 *FLT3*-ITD patients achieving CR with induction at the 50-mg BID dose. A significant number

of patients at the 100-mg BID dosing discontinued therapy due to primarily gastrointestinal side effects [48]. The subsequent phase III trial (“RATIFY”) was recently presented at the 2015 American Society of Hematology Meeting Plenary

Session. Seven hundred seventeen previously untreated patients with *FLT3* mutations aged 18–60 were enrolled in 17 countries in a randomized, placebo-controlled fashion. Midostaurin was combined with daunorubicin and cytarabine induction, and patients were stratified by *FLT3*-TKD, ITD high allelic ratio (>0.7), or low allelic ratio (0.05–0.7). The hazard ratio (HR) of midostaurin compared with placebo was 0.77 for OS and showed a benefit in TKD, as well as high and low allelic ratio ITD mutations [55•]; 402/717 patients received an allogeneic transplant (58% of those receiving midostaurin, and 54% receiving placebo) with similar median time to transplant. Interestingly, patients randomized to the midostaurin arm who then proceeded to allogeneic transplant achieved significantly longer survival than those patients who were randomized to placebo who were then transplanted. Given the importance of depth of remission in achieving success with allogeneic transplant [73], it is possible that treatment with the combination of chemotherapy and midostaurin resulted in a better quality of remission. Based on RATIFY, midostaurin was granted breakthrough status by the FDA in 2016 for newly diagnosed *FLT3* mutation-positive AML. Midostaurin has also been used in combination with azacitidine in phase I–II with 54 patients that were relapsed, refractory, or unfit for intensive chemotherapy finding a response rate of 26% with longer remissions in those who had not previously undergone transplants and in those who had not previously used *FLT3* inhibitors [56]. Midostaurin was studied with induction chemotherapy in 149 newly diagnosed *FLT3*-ITD patients with a median age of 54, followed by consolidative therapy with either allogeneic transplant or HIDAC. Overall 75% of patients achieved a completed response to one or more cycles of induction. Of 52 patients who started maintenance therapy, 40 had undergone transplant and 12 received HIDAC. Relapse at 1 year was 12% for allelic ratio <0.05 and 5% for ratio ≥ 0.5 for those undergoing allogeneic transplant, and 28 and 29%, respectively, after HIDAC, with only four suffering adverse events grades 3–4 attributable to midostaurin, though 55% of patients required dose-reduction or interruption of midostaurin during induction [57].

Lestaurtinib

Lestaurtinib is an indolocarbazole multikinase inhibitor and is active against *FLT3*, *JAK2*, *VEGFR*, and tropomyosin-related kinase A (TrKA; and even more so than midostaurin and many other kinases) [74–76]. In a phase I–II study of lestaurtinib in 17 relapsed or refractory AML patients with *FLT3* mutations, one patient had a CR and 5/14 patients had reduction in blast counts with correlative data showing sustained inhibition of *FLT3*; however, all responses were of short duration ranging from 2 weeks to 3 months [51]. Another phase II trial of lestaurtinib looked at untreated elderly patients not suitable for chemotherapy, irrespective of *FLT3*

mutation status, and found that 3/5 *FLT3*-mutated patients achieved a hematologic response vs. 5/22 wild type [52]. A randomized phase III trial of *FLT3* patients in first relapse compared chemotherapy alone to combination with lestaurtinib, no difference was found between the two groups and only limited *in vivo* inhibition was demonstrated on correlative studies [47].

Sorafenib

Sorafenib is approved for renal cell and hepatocellular carcinoma and has activity against *FLT3*, *VEGFR*, *c-KIT*, and *RAF* kinase [77]. As a single agent, and like the other multi-targeted TKIs, sorafenib's efficacy is somewhat limited by side effects at doses necessary to inhibit *FLT3* *in vivo*. One phase I trial of 15 relapsed or refractory patients with single agent sorafenib failed to show any complete or partial responses; however, Plasma Inhibitory Activity (PIA) data showed inhibition of *FLT3* *in vivo* at doses below the maximum tolerated dose [63]. In a phase I trial of 16 relapsed or refractory patients, sorafenib was shown to reduce leukemic blasts in the peripheral blood and the bone marrow only in those patients harboring a *FLT3*-ITD mutation [61]. In a somewhat larger phase I study, sorafenib induced complete remissions with incomplete count recovery in 10% of AML patients with a *FLT3*/ITD mutation [64]. Finally, a study of 13 relapsed or refractory *FLT3*-ITD patients with sorafenib found that 12 patients had clearance or near clearance of bone marrow myeloblasts; however, most patients relapsed after 72 days. Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice transplanted with leukemia cells from patients prior to and after relapse recapitulated the clinical findings. Leukemia cells harboring the D835 mutation expanded at relapse providing the likely culprit for resistance [78].

Sorafenib has been combined with conventional induction chemotherapy, but with as yet uncertain results. A small single institution phase II study demonstrated suggested that the combination was feasible and potentially effective [46]. However, a large phase II randomized, placebo-controlled trial of sorafenib plus standard 7 + 3 induction in those age >60 revealed worse outcomes for the sorafenib, arm and no benefit was seen even in analysis limited to the *FLT3*-ITD subgroup [67]. The SORAML trial was a randomized, double-blind, placebo-controlled phase II study involving 267 patients aged 18–60, in which sorafenib was given during two cycles of 7 + 3 induction and three cycles of HIDAC consolidation. Outcomes revealed a 3-year event-free survival of 22% in placebo vs. 40% in the sorafenib arm (HR = 0.64, $p = 0.013$) but no improvement in the overall survival [66]. Forty-six patients harbored the *FLT3*-ITD mutation had a trend towards increased overall survival (not reached vs.

19 months) and relapse-free survival (18 vs. 6 months), though these were not statically significant.

There have been a number of reports of the clinical activity of sorafenib in the post-transplant setting. A retrospective review of 65 FLT3-ITD patients with relapsed or refractory disease who were treated with sorafenib monotherapy found that patients who had undergone allogeneic hematopoietic stem cell transplant (HSCT) developed resistance less frequently (38%) and significantly later (197 days) vs. those had not undergone alloHSCT (47% and 136 days) [62]. Sustained remissions were seen exclusively in the alloHSCT group. In light of this, a phase I trial of sorafenib maintenance in patients with *FLT3*-ITD after allogeneic HSCT found a very encouraging 1 year OS of 95% [65•].

Second-Generation TKIs

Quizartinib

Quizartinib, originally identified in a library-based small molecule inhibitor screen, has an IC_{50} of less than 1 nM in culture medium and an IC_{50} of 18 nM in plasma. This is an order of magnitude more potent than any previously studied FLT3 TKI [79]. The drug appears to completely inhibit FLT3 in vivo at doses with a minimum of off-target effects, although prolongation of the QT interval and myelosuppression from c-KIT inhibition is a problem at higher doses [58]. A phase I trial of quizartinib monotherapy in 76 relapsed or refractory patients found that 9/17 FLT3-ITD patients responded (CR + PR) compared with 5/37 wild-type patients [58].

A large phase II trial of quizartinib in 333 relapsed/refractory patients was divided into two cohorts: (1) with age >60 and (2) patients refractory to two lines of chemotherapy or who had relapsed after transplant [59, 60]. Cohort 2 revealed a composite complete remission rate of 44% compared with 34% for those without *FLT3*-ITD ($n = 99$ and 38, respectively) allowing over a third of the relapsed cohort to undergo HSCT. Cohort 1 (median age, 70) had a composite complete remission rate of 54% in those with *FLT3*-ITD and 32% in those who were negative ($n = 92$ and 41, respectively). Correlative studies revealed that quizartinib induced terminal differentiation of bone marrow blasts without changing the *FLT3*-ITD allelic ratio resulting in the generation of peripheral blood neutrophils that harbored the same mutations as the leukemic blasts [80]. Occasionally, there was an associated clinical differentiation syndrome, similar to that seen in acute promyelocytic leukemia [81]. Similar effects on differentiation of in vitro blasts have been shown with sorafenib and lestaurtinib [78, 82, 83]. Among those patients who responded to quizartinib, a number of them developed resistance-conferring point mutations at residues D835 and F691 [84]. While obviously troublesome from a clinical perspective, the emergence of these resistance mutations provided a clear

evidence of the importance of FLT3-ITD mutations as a driver in this disease.

Crenolanib

Crenolanib was initially developed to inhibit PDGFR in solid malignancies [85]; however, it was found to have activity against FLT3-ITD and FLT3-TKD mutations [86]. In vitro studies have shown a number of possible advantages including lack of significant activity against c-KIT (avoiding myelosuppression), no QTc prolongation, low plasma protein binding, and a high degree of activity against D835 resistance mutations. The compound is in a number of single agent and combination trials for the treatment of FLT3-mutated AML [49].

Ponatinib

Ponatinib is a third-generation TKI developed to target the T315I mutation in BCR-ABL [87]. Ponatinib also inhibits the SRC, VEGFR, FGFR, and PDGFR families of receptor tyrosine kinases, including FLT3. Importantly, it has been shown in vitro to induce apoptosis in the multiresistant FLT3-F691I mutation but not against FLT3-D835 [88].

Gilteritinib

Gilteritinib is a selective inhibitor of FLT3 and AXL and has demonstrated activity against ITD and D835 mutations. The first in human study of gilteritinib, a phase I–II trial in 215 relapsed or refractory patients of which 137 had FLT3-ITD found an overall response rate of 68/114 (60%) among the ITD carriers and 4/14 (29%) in those with either D835 or D835 and ITD. Median overall survival for all FLT3+ patients was 29 weeks and was independent of prior treatment with TKIs [50]. PIA data confirmed in vivo FLT3 inhibition at doses of 80 mg day⁻¹ and higher.

Combinations with Hypomethylating Agents

Like the proposed mechanism for TKIs in FLT3-ITD AML, DNMT inhibition also can induce terminal differentiation of myeloid blasts [89]. Ongoing work with these well-tolerated agents (azacitidine and deoxy-5-azacitidine) and FLT3 inhibitors in cell lines and patients has shown a promising synergy and may be especially useful in the elderly who have limited reserve for cytotoxic chemotherapy [68, 90]. A phase II trial in the relapsed setting, median age 64, of *FLT3*-ITD-mutated patients treated with 5-azacitidine and sorafenib found a response rate of 46% including 27% complete responses [68].

Resistance

Resistance to FLT3 inhibition has been classified into three primary etiologies and is addressed extensively elsewhere [44]: (1) extrinsic mechanisms (drug metabolism CYP3A4 and drug interactions, plasma protein binding, FLT3 ligand), (2) receptor intrinsic mechanisms (resistance point mutations, overexpression, ITD length/site), and (3) cell intrinsic mechanisms (parallel pathways, apoptosis evasion, nonaddicted subclones).

One of the first receptor intrinsic mechanisms identified was the point mutation N676K which induced resistance in a patient treated with midostaurin [91]. Similar work identified resistance conferring TKD mutations to quizartinib [84]. Cell-based screening approaches have identified unique, non-overlapping resistance profiles to sunitinib, midostaurin, and sorafenib, suggesting that combinatorial therapy may overcome a development of resistance [92]. Newer TKIs, such as gilteritinib, have shown an efficacy in the phase II setting regardless of prior TKI exposure [50] and crenolanib has shown in vitro efficacy against quizartinib-resistance mutations including D835 [86], as well as in the phase II setting in patients with the D835 mutation [49]. Saturation mutagenesis of *FLT3*-ITD followed by selection of transfected cells has been identified (F621L, A627P, F691L, and Y842C) as mutations that confer varying levels of resistance to TKIs and may be encountered in patients [93].

Alterations in key signaling pathways in FLT3 TKI-resistant cell lines and primary samples reveal other forms of resistance. These cell lines have activation of PI3k/AKT and/or Ras/MEK/MAPK pathways as well as continued expression of genes involved in FLT3-mediated cellular transformation. Inhibition of these pathways via other agents may at least partially restore sensitivity to FLT3-TKIs [94].

FL may play a key role in resistance to FLT3 inhibition as well. Gene profiling of MV4–11 cells after long-term exposure to linifanib revealed up-regulation of FL and BIRC5 (survivin). Short-hairpin RNA targeting of survivin induced apoptosis and enhanced TKI cytotoxicity [95]. Treatment of *FLT3*-mutated AML patients with chemotherapy leads to high levels of FL throughout recovery and consolidation, which then acts directly on the FLT3-ITD receptor maximizing its activity and promoting survival of blasts. Furthermore, this effect seems to shift the IC₅₀ of FLT3 inhibitors upwards by 2–4-fold and can influence their inhibitory effect in vitro [96]. Given this understanding, it may be more ideal to begin FLT3 inhibition early after induction and transplant to avoid the surge in ligand levels that might blunt the effect of the inhibitors [97]. In vitro studies with a variety of FLT3 inhibitors in primary patient blast samples showed substantially more efficacy in those with high allelic burden and those in relapsed patients, possibly indicating greater addiction to FLT3 signaling [98].

Alteration of the *FLT3*-ITD insertion site (essentially, longer insertions that begin in the first tyrosine-kinase domain,

rather than the juxtamembrane domain) may be important for resistance [26]. The N676K mutation has been shown to result in up-regulation of the anti-apoptotic protein MCL-1, which conferred resistance to midostaurin. RNAi knockdown of MCL-1 was able to rescue TKI sensitivity [99].

Leukemia cells within the bone marrow do not reliably undergo apoptosis, even with maximal FLT3 inhibition, and the bone marrow microenvironment clearly plays an important role in this phenomenon. Co-culture with bone marrow stromal cells revealed that FLT3 inhibition (via sorafenib and quizartinib) in leukemic cells results in cell cycle arrest rather than apoptosis. Persistent activation of extracellular regulated kinase (ERK) through parallel signaling pathways contributes to the protective effect. Inhibition of the upstream kinase, MEK, blocked stromal-mediated resistance [100]. In another study, stromal niche cells were shown to protect leukemic stem cells from the effects of sorafenib and sunitinib—allowing the maintenance of leukemic progenitors, even in the presence of these agents [101].

Allogeneic Bone Marrow Transplant

The role of allogeneic stem cell transplant in *FLT3*-ITD AML had been controversial until perhaps more recently [102]. One of the first large retrospective analyses on the topic performed in 2005 out of the UK did not find a benefit for transplant [103]. However, only 35 of 68 patients with a donor actually underwent transplant and some have argued that in an as-treated analysis, there was a benefit [104]. Furthermore, the risk of relapse after allogeneic transplant was similar between *FLT3*-ITD-mutated and wild-type patients suggesting amelioration of the negative influence of the lesion. However, a large study of 872 adults less than 60 years of age by the German-Austrian Acute Myeloid Leukemia Study Group from 2008 revealed that among cytogenetically normal AML, only *FLT3*-ITD and wild-type CEBPA/NPM1 without *FLT3*-ITD benefitted from transplant [105]. Transplant-related mortality was only 21% (compared with 30% in the UK study), and transplant rate was 82% compared with 63%. Further studies have corroborated the finding of a benefit of allogeneic HSCT in *FLT3*-ITD patients [106–108]. Of more recent interest is the impact of allelic ratio and co-expression of mutations in *NPM1* [38, 39, 109]. Due to the short duration of remissions in *FLT3*-ITD AML prior to relapse after an initial remission, many have argued to begin planning a transplant immediately upon detection of the mutation [97].

Conclusions

Over the past 20 years, retrospective clinical studies, in vitro and clinical studies of FLT3 inhibitors, and whole genome and whole exome studies of AML

patient samples, along with a large body of basic hematopoietic laboratory research has generated a wealth of information about the disease entity we know as FLT3-ITD AML. We are now in a position to speculate about how the disease evolves during treatment, and how we might best approach it with currently available therapeutics. FLT3-ITD AML presents in polyclonal fashion, with only a relatively small subset of leukemia cells at diagnosis being dependent or addicted to FLT3-mutant signaling [98, 110]. Following induction chemotherapy and subsequent rounds of consolidation chemotherapy, FL levels rise, conferring a protective effect on, and possibly selecting for, a subset of cells that are both chemo-resistant and FLT3 addicted [97]. These are the cells that emerge at relapse and lead to the death of the patient. To counter this, a reasonable approach might be to induce the patient with chemotherapy combined with a polyclonal TKI such as midostaurin (as in the RATIFY trial) which has broad anti-leukemic effects against FLT3, PDGFR, c-KIT, PKC, and VEGFR [55], followed by rapid transition to an allogeneic transplant (rather than multiple rounds of chemotherapy). If necessary, rapid transplant with an alternative donor (e.g., a haplo-identical relative or cord blood) may be preferable to administering successive rounds of chemotherapy while waiting to find the “right” donor. The net beneficial effect of midostaurin in RATIFY may be due to the use of induction chemotherapy and nonselective kinase inhibition to debulk the polyclonal tumor followed by rapid consolidation with allogeneic transplant and maintenance FLT3 inhibition to prevent relapse of any residual FLT3 clones. After allogeneic transplant, a more selective FLT3 inhibitor may be an even more effective maintenance therapy [65], since it is often the FLT3-addicted clones that are identified as the culprit at relapse, although this concept should be first studied in the context of a randomized trial. The field is moving rapidly. Several FLT3 inhibitors are now in phase III pivotal trials (see Table 1), and it seems likely that one or more will be approved over the next 5 years. How we take advantage of these new tools remains to be seen, but the prospects for our patients are hopeful.

Compliance with Ethical Standards

Conflict of interest Mark B. Leick declares no potential conflicts of interest. Mark J. Levis reports grants from Novartis and Astellas and consultancies for Novartis and Daiichi-Sankyo.

Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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