REVIEW

FLT3 inhibitors for acute myeloid leukemia: successes, defeats, and emerging paradigms

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FLT3 mutations are one of the most common genetic aberrations found in nearly 30% of acute myeloid leukemias (AML). The mutations are associated with poor prognosis despite advances in the understanding of the biological mechanisms of AML. Numerous small molecule FLT3 inhibitors have been developed in an effort to combat AML. Even with the development of these inhibitors, the five-year overall survival for newly diagnosed AML is less than 30%. In 2017, midostaurin received FDA approval to treat AML, which was the first approved FLT3 inhibitor in the U.S. and Europe. Following, gilteritinib received FDA approval in 2018 and in 2019 quizartinib received approval in Japan. This review parallels these clinical success stories along with other pre-clinical and clinical investigations of FLT3 inhibitors.

Introduction

The Fms-like tyrosine kinase 3 (FLT3) was discovered in 1991 and belongs to the type III receptor tyrosine kinase class expressed by hematopoietic stem cells.[1,2] The receptor is encoded by the *FLT3* gene located on chromosome 13q12.[1,3,4] The FLT3 receptor is comprised of four domains: (1) an extracellular domain that consists of five immunoglobulin-like (Ig-like) domains, (2) a transmembrane (TM) domain, (3) a juxtamembrane (JM) domain, and (4) a cytoplasmic domain with two split tyrosine kinases (TK) (Fig. 1).[1,3,4] Binding of the FLT3 ligand to the extracellular domain of the FLT3 receptor induces dimerization with a second FLT3 receptor (Fig. 1). This dimerization event activates the intracellular kinase domains followed by phosphorylation of downstream proteins and subsequent activation of signaling cascades, which ultimately promotes transcription of genes that regulate cell survival, proliferation, and differentiation.[5–10] The FLT3 ligand (FLT3G or FL) is a cytokine that belongs to a family of growth factors responsible for proliferation and differentiation of hematopoietic cells. FLs are known to act in a synergistic manner in the presence of other related cytokines (SCF, CSF-1, *etc.*).[11–15]

FLT3 is expressed in both myeloid and B-lymphoid progenitor cells.[1,16,17] FLT3 is overexpressed in hematological malignancies such as acute myeloid leukemia (AML), B precursor cell acute lymphocytic leukemia (ALL), and chronic myelogenous leukemia (CML).[1,8,18] Activation of FLT3 enhances proliferation and reduces apoptosis in leukemic cells.[19,20] Mutations within FLT3 are observed in nearly 30% of AMLs[5,21] with two main types of activating mutations (Fig. 2): (1) internal tandem duplications (FLT3-ITD) in the JM domain[22] and (2) point mutations in the TK domain (FLT3-TKD).[23] FLT3-ITD mutations have been reported in approximately 25% of AMLs while FLT3-TKD mutations have been reported in just 5%.[1] These mutations promote ligand-independent activation of FLT3, which stimulates continuous expression of cell survival and proliferation genes.[5,10] Both overexpression of and mutations within the *FLT3* gene have been associated with poor prognosis in hematological malignancies.[24–26]

FLT3 gene mutations

The FLT3-ITD mutation is formed by duplication of a fragment of the juxtamembrane domain coding sequence within the intracellular region of the *FLT3* gene.[22,27] The size and exact location of the duplicated region varies but the event produces a functional kinase domain with an elongated JM region.[28] FLT3-ITD promotes ligand-independent dimerization, autophosphorylation, and subsequent signal transduction.[22,26] This ligand-independent activation may be attributed to the elimination of naturally occurring regulatory regions of FLT3, which are believed to prevent dimerization without ligand stimulation. It has been shown that FLT3 with an ITD mutation can dimerize and activate wild-type receptors in a ligand-independent manner.[27]

FLT3-TKD mutations mainly result from a missense point mutation within the activation loop of the TK domain at residue D835.[23,26] Point mutations, deletions, and insertions

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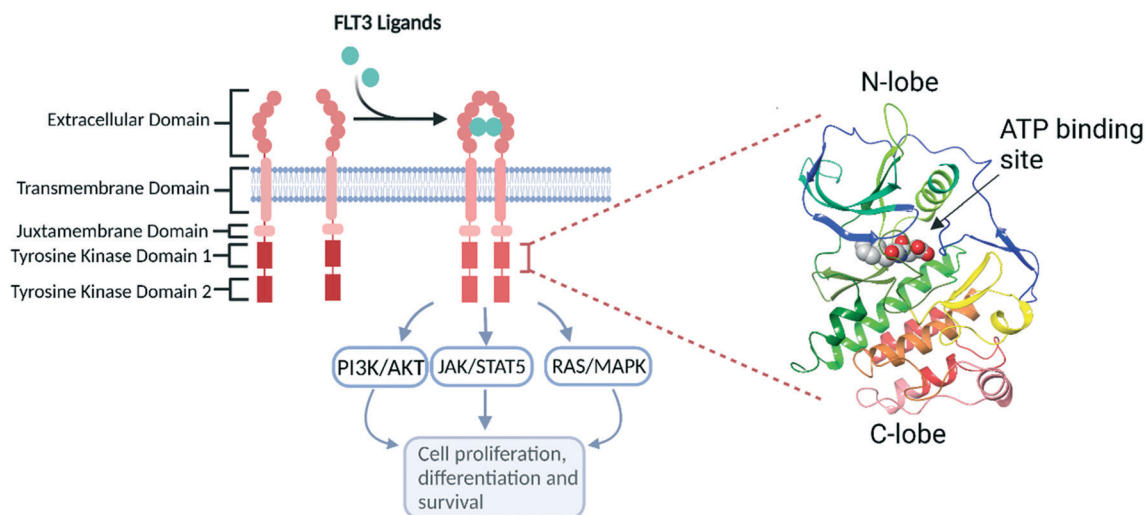
Fig. 1 Domains of the FLT3 receptor tyrosine kinase (PDB: 1RJB). Binding of the FLT3 ligand to the receptor initiates receptor dimerization and autophosphorylation of the intracellular tyrosine kinase domain, which further activates downstream signaling pathways.[1]

in codons surrounding D835 of the *FLT3* gene have also been reported.[23,26] FLT3-TKD mutations result in autophosphorylation and ligand-independent cell growth.[29] Additionally, mutations at the gatekeeper residue F691 have also been reported.[30] Mutations at F691 have been shown to confer resistance to kinase inhibitors.

FLT3 mutations are common in myeloid neoplasms such as AML and myelodysplastic syndromes (MDS)—a form of pre-leukemia.[24] In MDS, FLT3 mutations are infrequent and are only observed in 3% of patients, yet nearly 15% of patients that progress to AML have FLT3 mutations, suggesting FLT3 activation is a key event in progression of the disease. FLT3-ITD mutations occur in 25–35% of all adult AML. However, in pediatric AML, only 5–15% harbor a FLT mutation[31,25,29] and these mutations have been shown to be strong, independent predictors of poor clinical outcome.[31,32] ITD mutations are associated with leukocytosis and increased blast counts,[29] and the presence of a FLT3-ITD mutation is a poor prognostic marker for overall survival (OS), relapse free

survival (RFS) and event-free survival (EFS).[24] However, the association of FLT3-TKD mutations and prognosis is not clear and often dependent on co-occurring mutations and cytogenetic changes,[9,24] and the association of TKD mutations with leukocytosis is rarely observed.[33]

FLT3 small molecule inhibitors

Due to FLT3 dysregulation in AML[34] and a high frequency of FLT3 mutations,[35] the oncogene has been investigated as a potential drug target in AML.[1,7] FLT3 small molecule inhibitors interact at the kinase domain of the FLT3 receptor to prevent autophosphorylation and downstream signaling.[5,36] Small molecule FLT3 inhibitors are categorized based on their distinct inhibitory mechanism and are divided into two types: type-I and type-II. Type-I inhibitors bind to the ATP-binding site of the active enzyme (DFG_{in} conformation, Fig. 3A) and type-II inhibitors interact with the hydrophobic pocket adjacent to the ATP binding site, which is accessible when the enzyme is inactive (DFG_{out} conformation, Fig. 3B). D835 is the most common FLT3-TKD mutation, and this mutation stabilizes FLT3 in the active conformation. This stabilization event attenuates inhibition by type-II inhibitors, as the TK domain is energetically favored to adopt the DFG_{in} conformation. These mutations do not typically impair binding of type-I inhibitors to the FLT3 kinase domain, as these inhibitors bind to the active conformation of the enzyme.[37] Type-I inhibitors include midostaurin, lestaurtinib, gilteritinib, crenolanib, sunitinib, and pacritinib while type-II inhibitors consist of tandutinib, sorafenib, and quizartinib. Additionally, FLT3 small molecule inhibitors can be further divided into reversible and irreversible inhibitors. This categorization is based on formation of a covalent bond with C695 on FLT3 (Fig. 3C).[38] The classifications of FLT3 inhibitors is outlined in Table 1.
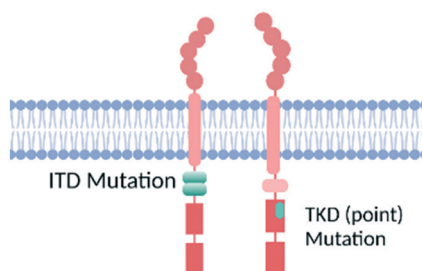

Fig. 2 FLT3 mutations found in AML. FLT3-ITD mutations are present in approximately 25% of AML, while FLT3-TKD point mutations are present in approximately 5% of AML. These mutations promote ligand-independent activation of FLT3, which stimulates continuous expression of cell survival and proliferation genes. Both overexpression of and mutations within FLT3 have been associated with poor prognosis in hematological malignancies.
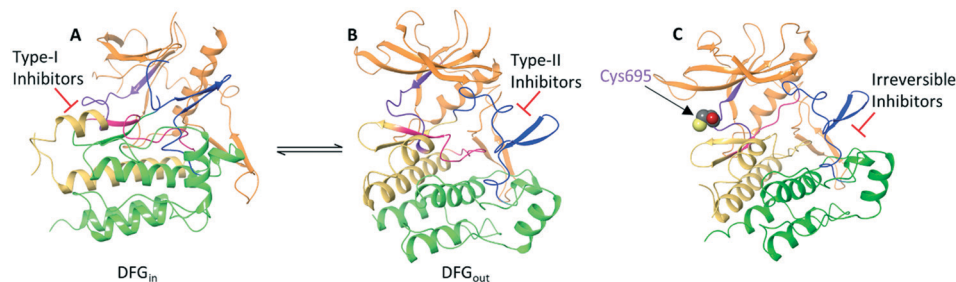
Fig. 3 (A) The active conformation of the FLT3 kinase (DFG$_{in}$, PDB ID: 6JQR). Type-I inhibitors bind to the active conformation and are ATP competitive. (B) The inactive conformation of FLT3 kinase (DFG$_{out}$, PDB ID: 4XUF). Type-II inhibitors bind to the inactive conformation and are ATP non-competitive. (C) Irreversible inhibitors form a covalent bond with C695 on the hinge of the FLT3 kinase. Irreversible inhibitors are ATP non-competitive. The N-lobe is orange, the C-lobe is green, the activation loop is blue, the hinge is purple, and the DFG motif is fuchsia.

Staurosporine analogs: midostaurin

Midostaurin (*N*-benzoyl staurosporine, PKC412, Novartis) was the first FDA-approved small molecule inhibitor for AML, receiving approval in 2017. Midostaurin was investigated as a derivative to the alkaloid staurosporine (from the bacteria *Streptomyces staurosporeus*) with the aim of improving selectivity against protein kinase C.[39–41] Midostaurin (**1c**) was synthesized by acylation of staurosporine (**1a**) with benzoyl chloride (**1b**) in chloroform in the presence of *N,N*-diisopropylethylamine (Scheme 1). Midostaurin was successfully synthesized in 1986 (ref. 40) and was shown to be active against solid tumors including colorectal cancer, gastric cancer, lung cancer, melanoma, glioblastoma, and breast cancer.[42–44] It was later demonstrated that midostaurin was active against tyrosine kinases including vascular endothelial growth factor (VEGFR)-2, platelet-derived growth factor receptor (PDGFR) α and β, c-KIT, and FLT3.[39,41,45]

Midostaurin was first evaluated against chronic lymphocytic leukemia and melanoma as a monotherapy and also in solid tumors as a single agent or in combination with chemotherapy irrespective of tumor genotype.[46–49] These clinical studies failed to demonstrate efficacy and further clinical development in these areas was abandoned. Midostaurin was then evaluated as an angiogenesis inhibitor for the treatment of diabetic retinopathy, but failed to demonstrate clinical efficacy.[50] While clinical efficacy was never achieved, these early trials provided key pharmacokinetic and safety data. The identification of additional targets led to trials where the focus shifted to study inhibition of FLT3 in AML and c-KIT in systemic mastocytosis (SM).

Midostaurin was first studied in 32 patients with advanced solid tumors in a phase I trial.[49] Patients received doses of 12.5–300 mg daily in 28 day cycles. This study revealed that a dose of 150 mg per day or less was well-tolerated. The most common toxicities were nausea, vomiting, diarrhea, and fatigue. Circulating lymphocyte and monocyte levels were significantly reduced in patients who received ≥100 mg per day,[49] which suggested midostaurin impairs myeloid and lymphoid hematopoietic lineages and could potentially be effective in certain hematological malignancies. The identification of additional targets in hematological malignancies suggested midostaurin could be used against FLT3-driven disease. Midostaurin was reported to induce G1 arrest and apoptosis in Ba/F3 cell lines with FLT3 activating mutations at an IC$_{50}$ of less than 10 nM.[51,52] Furthermore, midostaurin was able to prevent leukemia progression in BALB/c mice with FLT3-ITD-induced leukemia.[51] Midostaurin was computationally modeled in the FLT3 active site to gain insight into the receptor/drug complex (Fig. 4). The modeling suggests that midostaurin hydrogen bonds with C694 and E692 at the hinge region of FLT3.

The combination of midostaurin with dacinostat (LAQ824), a histone deacetylase inhibitor, demonstrated synergism against AML expressing mutant FLT3.[53] In addition, midostaurin together with conventional leukemic

Table 1 Classification of FLT3 inhibitors. *denotes clinical approval for AML

ATP competitive	ATP non-competitive	
Type-I inhibitors	Type-II inhibitors	Irreversible inhibitors
Midostaurin* (Staurosporine analogues)	Tandutinib	FF-10101
Lestaurtinib (Staurosporine analogues)	Sorafenib	
Gilteritinib*	Quizartinib*	
Crenolanib		
Sunitinib		
Pacritinib		
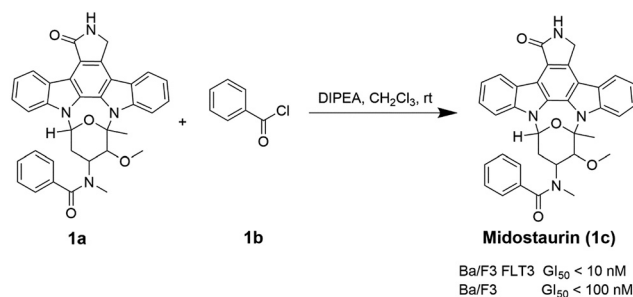
Scheme 1 Synthetic route to access midostaurin from staurosporine (**1a**).
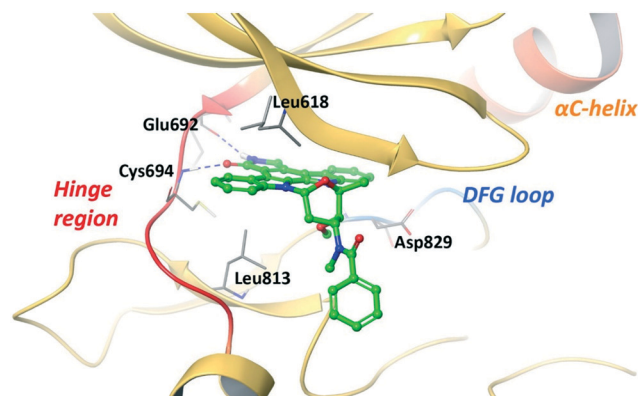
Fig. 4 Molecular modeling of midostaurin in the FLT3 active site. Midostaurin is a type-I inhibitor that is ATP competitive and binds to the active (DFG$_{in}$) conformation of the kinase. At the FLT3 hinge region, midostaurin hydrogen bonds with C694 and E692. The hinge region, αC-helix, and DFG loop are illustrated in red, orange, and blue, respectively.

agents such as cytarabine, doxorubicin, and idarubicin showed synergistic effects against FLT3-mutated AML compared to AML with wild type FLT3.[54] Preclinical clinical studies demonstrating the safety and pharmacokinetics of midostaurin propelled the agent for new clinical investigation against AML in a phase II trial.

In phase II clinical trials, midostaurin was administered to 20 patients with relapsed/refractory AML with a FLT3 mutation or high-grade MDS that were ineligible for chemotherapy.[55] Patients were given 75 mg of midostaurin orally three time a day. For the majority of patients, the drug was well tolerated. However, in a rare occurrence, three patients developed fatal pulmonary toxicity during treatment. One of the fatalities was unrelated to drug administration and the etiology of the other two deaths was unclear, suggesting midostaurin-induced pulmonary toxicity does not have a clear mechanism and is likely influenced by other comorbidities of the patient. The most common toxicities were nausea, vomiting, diarrhea, and fatigue. Grade 1 or 2 nausea and vomiting occurred in 13 patients (65%). The clinical trial demonstrated encouraging results as the peripheral blast count and bone marrow blast count decreased by 50% in 70% and 30% of patients with AML and MDS. Unfortunately, none of the patients attained complete remission (CR), although one patient had less than 5% marrow blasts and normal peripheral blood counts with moderate marrow hypocellularity at 10% and was documented as a near-complete remission.

A phase IIB trial commenced with midostaurin as a single agent and included 95 patients with AML or MDS with either wild-type or mutated FLT3 (NCT00045942).[56] The patients randomly received oral midostaurin at 50 or 100 mg twice daily, and 70% of patients with FLT3 mutations and 42% of patients with wild type FLT3 showed 50% reduction in blasts. Like the prior study, no patients attained a CR and only one patient experienced a partial remission. Midostaurin was well

tolerated at both doses with no differences in toxicity, but results from phase II trials suggested midostaurin did not induce a robust antileukemic response as a monotherapy.

Pre-clinical studies with midostaurin demonstrated synergy with chemotherapy, and this led to clinical studies of midostaurin in combination with chemotherapy.[57–59] In a phase I/II trial, midostaurin co-administered with 5-azacitidine (hypomethylating agent) was investigated in 54 patients with AML and high-risk MDS (NCT01202877).[57] After 12 weeks, the overall response rate (ORR) was 26%. One patient achieved a CR, six achieved a CR with incomplete bone marrow recovery, six a morphologic leukemia-free status, and one a partial remission. Twenty-five out of 54 patients (53%) had a >50% reduction in bone marrow blasts.

Another phase Ib trial evaluated midostaurin in combination with chemotherapy in previously untreated AML patients aged 18–60 (NCT00651261).[59] This trial was designed to study the safety, efficacy, and pharmacokinetics of combining midostaurin with an established AML chemotherapy regime consisting of daunorubicin and cytarabine followed by high-dose cytarabine. Patients received either 50 or 100 mg of oral midostaurin twice daily. Gastrointestinal adverse effects (grade 3/4) occurred at the 100 mg dose while no grade 3/4 gastrointestinal toxicities were seen in the 50 mg dosage group. The CR rate for 50 mg was 80% (74% for FLT3-wild type and 92% for FLT3 mutant). The OS probabilities of patients at 1 and 2 years were 85% and 62% in patients with FLT3-mutated AML and 78% and 52% in patients with FLT3-wild type AML. The promising results of this trial led to a phase III RATIFY trial.

The phase III RATIFY trial was a multinational, double-blind, placebo-controlled randomized trial to determine the benefit of addition of midostaurin on days 8–21 to standard chemotherapy in treatment-naïve patients with FLT3 mutations (NCT00651261).[58] Over 3200 patients aged 19 to 60 were screened for FLT3-mutated AML, which identified 717 eligible patients for the trial.[58] The median OS and median EFS for patients receiving midostaurin were 74.7 and 8.2 months whereas the OS and EFS for placebo group were 25.6 and 3.0 months. The results from the RATIFY trial led to approval of midostaurin in adults with newly diagnosed AML with a FLT3 mutation in the United States, European Union, and other countries.[60,61]

Staurosporine analogs: lestaurtinib

Lestaurtinib (CEP-701, Cephalon), an analogue of staurosporine, is derived from **K252a**, a bacterial fermentation product isolated from *Nocardiopsis* (Scheme 2).[62] Initially reported to have activity against tropomyosin receptor kinase A (TRKA, IC$_{50}$ < 25 nM), lestaurtinib (**2**) was found to be active against additional RTKs including FLT3 (IC$_{50}$ = 3 nM)[63] and janus kinase 2 (JAK2, IC$_{50}$ = 1 nM).[63–65] Interestingly, lestaurtinib inhibited autophosphorylation of both wild-type and mutant FLT3 *in vitro*.
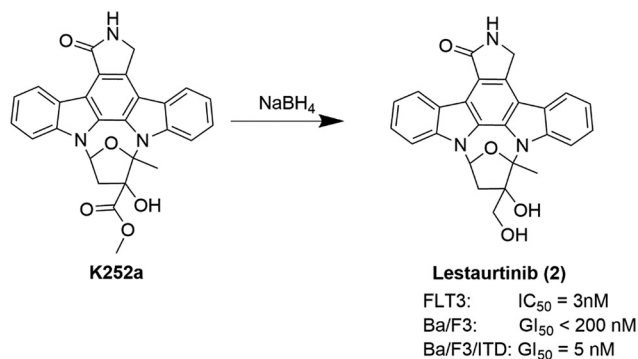
Scheme 2 Synthetic route to access lestaurtinib from **K252a**.

The selectivity of lestaurtinib was evaluated by comparing effects on parental Ba/F3 cells to Ba/F3-ITD cells. While lestaurtinib inhibited the proliferation of Ba/F3-ITD cells in a dose-dependent manner with a GI_{50} of approximately 5 nM, the inhibition of parental Ba/F3 cells did not occur until concentrations above 200 nM.[63] Molecular modeling of lestaurtinib within the FLT3 tyrosine kinase domain shows formation of hydrogen bonds with C694 and E692 at the hinge region (Fig. 5). A study using a mouse model with FLT3-ITD leukemia demonstrated that lestaurtinib inhibited autophosphorylation of FLT3 and prolonged survival.[63]

Lestaurtinib was evaluated in a phase I/II trial as salvage treatment in 14 patients with relapsed, refractory, or poor-risk FLT3-mutated AML.[66] An oral dose of 60 mg twice daily was well-tolerated with mild toxicities. A greater than 50% reduction in peripheral blood blasts was observed in 5 out of 14 patients. One patient had a CR with a reduction in blood blasts greater than 95%. Following this initial study, a phase II study was conducted in untreated adults with FLT3-mutated and FLT3 wild-type AML who were not considered eligible for induction chemotherapy.[67] An initial dose of 60 mg twice daily for 8 days followed by escalation to 80 mg twice
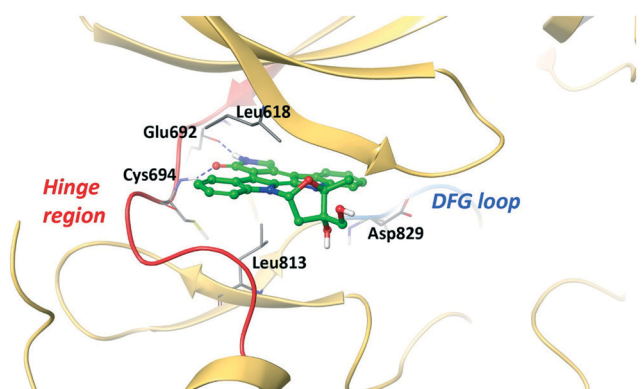

Fig. 5 Binding pose of lestaurtinib in the FLT3 tyrosine kinase domain. Lestaurtinib is a type-I inhibitor that is ATP competitive and binds to the active (DFG$_{in}$) conformation of the kinase. The hinge region and DFG loop are illustrated in red and blue, respectively.

daily was reported to be well tolerated. Three out of 5 patients with mutated FLT3 and 5 out of 22 patients with wild type FLT3 experienced a transient reduction in blast counts. However, another phase II trial demonstrated that lestaurtinib treatment after chemotherapy did not improve response rate nor prolonged survival compared to current standard of care. Similarly, another clinical trial showed that adding lestaurtinib to chemotherapy yielded no overall clinical benefit.[68] Following poor efficacy results from clinical trials, the clinical development of lestaurtinib was discontinued.

Type-I inhibitors: gilteritinib

Gilteritinib (ASP2215, Astellas Pharma Inc.) is a diamino heterocyclic carboxamide developed by Astellas Pharma, Inc. The synthesis of gilteritinib (**3e**) is outlined in Scheme 3.[69] Gilteritinib was developed as a dual FLT3/AXL inhibitor (FLT3 IC_{50} = 0.29 nM and AXL IC_{50} = 0.73 nM),[70] and was approved by the FDA in 2018 for relapsed or refractory AML with a FLT3 mutation. Gilteritinib inhibits the growth of Ba/F3 cells transfected with FLT3-ITD and FLT3-D835Y at a GI_{50} of 1.8 nM and 1.6 nM, respectively.[70] Gilteritinib is a type-I inhibitor and selectively inhibits FLT3 and also AXL, since AXL facilitates FLT3 activation and FLT3 inhibitor resistance. A co-crystal structure of FLT3 and gilteritinib shows that the molecule binds the active conformation of the FLT3 tyrosine kinase (DFG$_{in}$) (Fig. 6). Additionally, gilteritinib was shown to engage in a hydrophobic interaction with the F691 gate keeper residue suggesting gatekeeper TKD mutations confer resistance to gilteritinib, which has been supported enzymatically.[70]

A phase I/II trial of gilteritinib in adults with relapsed or refractory AML was initiated (NCT02014558).[71] The primary goal of this study was to assess safety and tolerability of gilteritinib and to determine the maximum tolerated dose (MTD). Gilteritinib was well tolerated, and the MTD was determined to be 300 mg/day. A starting dose of 120 mg per day achieved uniform target inhibition and a high proportion of patients who were within this cohort were able to achieve an overall response, which warranted a recommended starting dose of 120 mg per day for further clinical studies. Dose-limiting toxicities (DLT) occurred at 450 mg per day and included grade 3 diarrhea and elevated aspartate aminotransferase levels. Common adverse effects included diarrhea, fatigue, and elevated liver enzymes, which were reversible with reduction or discontinuation of treatment.[71] The most common grade 3/4 adverse events were febrile neutropenia, anemia, thrombocytopenia, sepsis, and pneumonia. Out of 249 patients receiving gilteritinib, 8% achieved a complete remission, 4% achieved a complete remission with incomplete platelet recovery, 18% achieved a complete remission with incomplete hematological recovery, and 10% achieved a partial remission (ORR was 40%).

Subsequently, a phase III trial was conducted with 371 patients with relapsed or refractory AML with FLT3-ITD or a
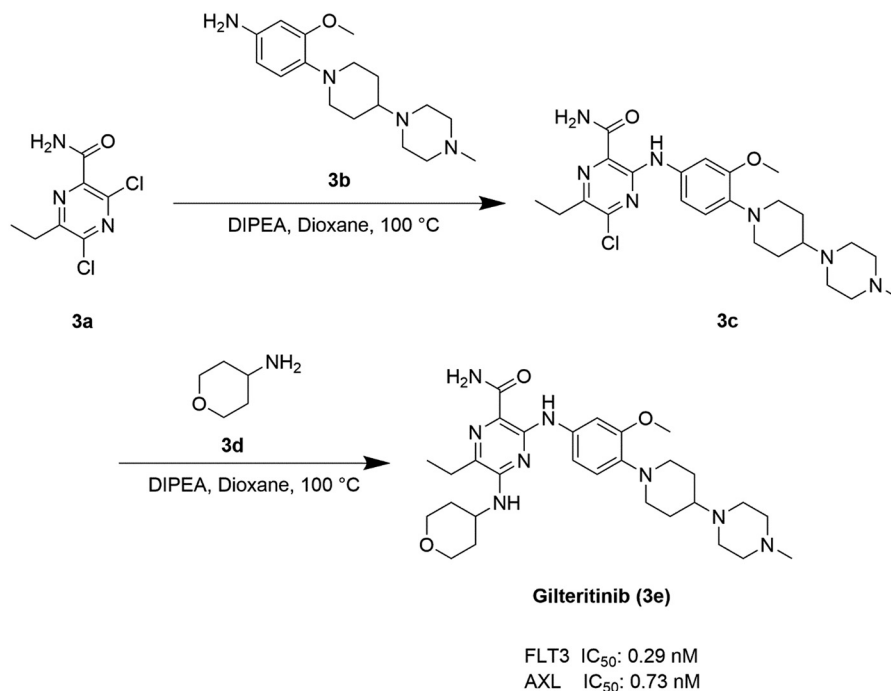
Scheme 3 Synthetic route to access gilteritinib.

TKD mutation who were randomly assigned to receive gilteritinib or salvage chemotherapy (ADMIRAL trial, NCT02421939).[72] The overall survival in patients receiving gilteritinib was significantly higher compared to salvage chemotherapy (9.3 vs. 5.6 months). The median event-free survival for patients receiving gilteritinib or chemotherapy was 2.8 months and 0.7 months. Adverse events of grade 3 or higher occurred less frequently in the gilteritinib cohort compared to the chemotherapy cohort. The safety and efficacy data obtained from the ADMIRAL trial led to approval of gilteritinib by the FDA in 2018.

Fig. 6 Co-crystal structure of gilteritinib bound to the FLT3 tyrosine kinase domain (PDB ID: 6JQR).[70] Gilteritinib is a type-I inhibitor that is ATP competitive and binds to the active (DFG$_{in}$) conformation of the kinase. The hinge region, αC-helix, and DFG loop are illustrated in red, orange, and blue, respectively.

Type-I inhibitors: crenolanib

Crenolanib (CP868596, Arog Pharmaceuticals), a benzamidine quinoline derivative, was originally developed as a selective PDGFR-β inhibitor and was also found to bind to class III RTKs including FLT3.[73] The synthesis of crenolanib (**4h**) is outlined in Scheme 4.[74] Crenolanib is a type-I inhibitor with activity against FLT3-ITD, FLT3-D835Y, and FLT3-F691 mutations[75,76] and exhibits activity against various FLT3 mutations in Ba/F3 transfected cells (Table 2).[75] Mutations at the F691 gate-keeper residue confer resistance to crenolanib.

Crenolanib has been shown to bind the active conformation (DFG$_{in}$) of the FLT3 kinase (Fig. 7). At the hinge region of FLT3, crenolanib forms a hydrogen bond with the amide backbone, which is a typical interaction of kinase inhibitors that bind to a kinase active site.[73] Crenolanib also interacts with the activation loop via a hydrogen bond.[73] Crenolanib delayed the growth of an MV4-11 xenograft mouse model and combination with sorafenib resulted in a significant decrease in leukemic burden and prolonged survival.[76] Crenolanib has also been reported to act synergistically with FLT3-chimeric antigen receptor (CAR) T-cells, suggesting CAR T-cell immunotherapy combined with small molecule inhibition may improve clinical response in AML.[77]

Because of promising pre-clinical data, crenolanib was progressed into clinical studies to determine safety and tolerability with standard induction chemotherapy in patients with newly diagnosed FLT3 mutant AML (NCT02283177).[78] Out of 22 patients, 19 had a FLT3-ITD mutation while the
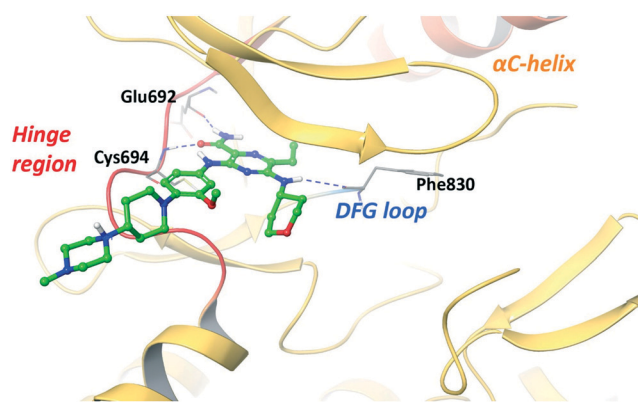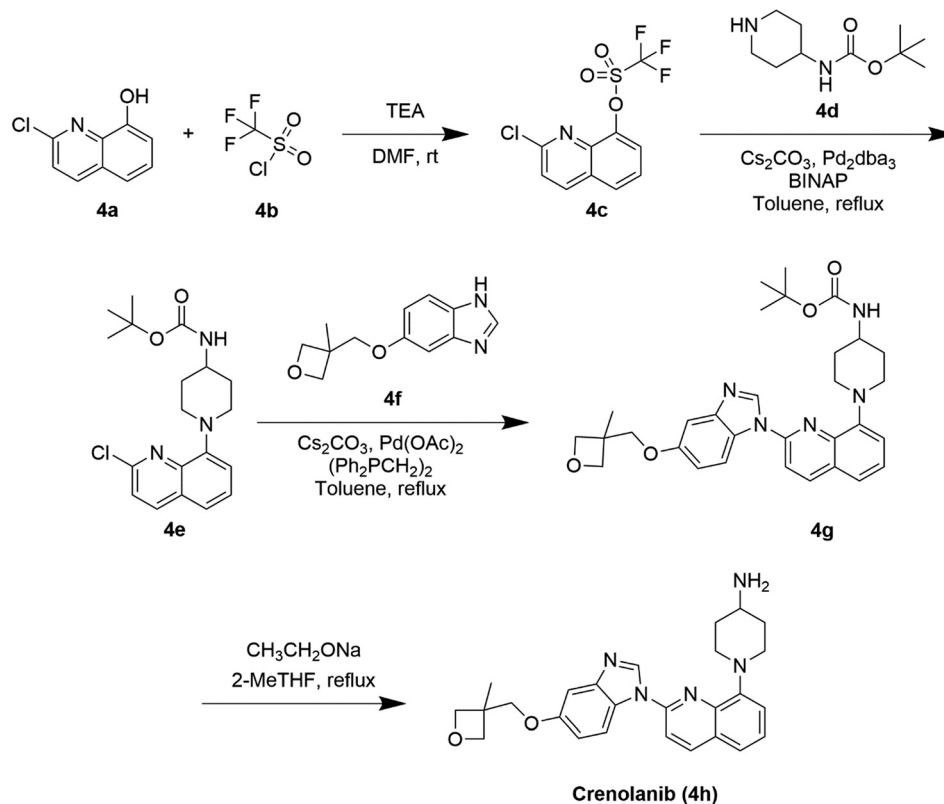
Scheme 4 Synthetic route to access crenolanib.

remaining 3 had a FLT3-D835 mutation and 88% of patients achieved a complete remission in the trial. Crenolanib is currently in phase III clinical trials to investigate the efficacy in combination with chemotherapy compared to chemotherapy alone in patients with relapsed/refractory mutated AML. Another phase III trial has been initiated investigating crenolanib against midostaurin following induction and consolidation chemotherapy in patients with newly diagnosed FLT3 positive AML.

Type-I inhibitors: sunitinib

Sunitinib (SU11248, Pfizer Inc.), an indolinone derivative, is a multikinase inhibitor with activity against RET, VEGFRs, KIT, FLT3, and CSF-1R.[79] Sunitinib is currently approved for the treatment of advanced renal cell carcinoma, gastrointestinal

stromal tumors, and pancreatic neuroendocrine tumors. In preclinical studies, sunitinib exhibited dose-dependent efficacy against a FLT3-ITD xenograft model and a bone marrow engraftment model.[80] Sunitinib is equally effective against Ba/F3 cell lines expressing ITD and TKD FLT3 mutations.[81] A docking study of sunitinib with FLT3 suggests sunitinib binds to the active conformation (DFG$_{in}$) of the kinase (Fig. 8). When bound to FLT3, sunitinib interacts with C694 at the hinge region and D829 at the DFG motif via a

Table 2 GI$_{50}$ of crenolanib against various FLT3 mutations expressed by the Ba/F3 cell line[75]

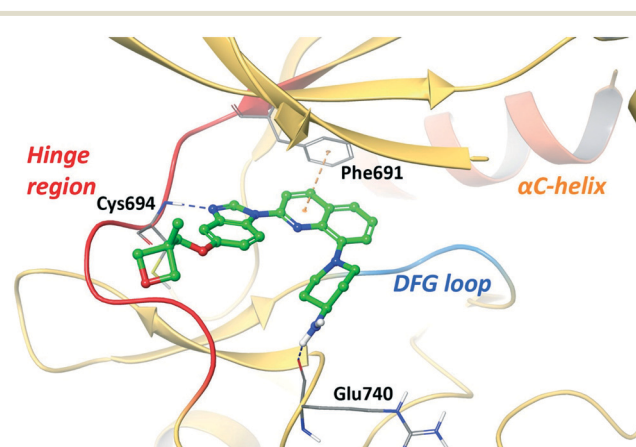| Ba/F3 cell line	GI$_{50}$ nM
FLT3-ITD	1.3
FLT3-ITD-D835Y	8.7
FLT3-WT-D835Y	6.9
FLT3-WT-D835F	6.5
FLT3-WT-D835H	19.38
FLT3-WT-D835N	4.3
FLT3-WT-D835V	2.3
FLT3-ITD-F691L	67.8

Fig. 7 Molecular modeling of crenolanib in the FLT3 active site.[73] Crenolanib is a type-I inhibitor that is ATP competitive and binds to the active (DFG$_{in}$) conformation of the kinase. The hinge region, αC-helix, and DFG loop are illustrated in red, orange, and blue, respectively.

Fig. 8 Docking study of sunitinib bound to the FLT3 tyrosine kinase domain.[81] Sunitinib is a type-I inhibitor that is ATP competitive and binds to the active (DFG$_{in}$) conformation of the kinase. The hinge region, αC-helix, and DFG loop are illustrated in red, orange, and blue, respectively.
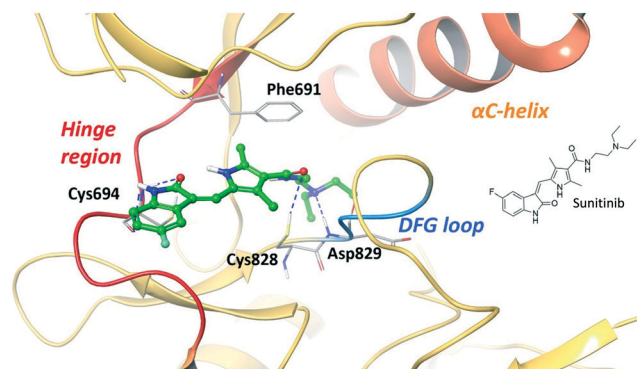
Table 3 IC$_{50}$ of pacritinib against various kinases

Kinase	IC$_{50}$ (nM)
JAK2	6.0
FLT3	6.4
FLT3-ITD	12.0
FLT3-D385Y	18.3
JAK3	18.3
IRAK-1 (IL-1 receptor kinase)	13.6
FMS	39.5

hydrogen bonding. Sunitinib retains activity against the F691L gatekeeper mutation, which is supported by modeling studies as sunitinib does not interact with the F691 gatekeeper residue.[81,82]

Sunitinib was tested in patients with refractory or resistant AML but only elicited short-term, partial remissions.[83] In a phase I/II study of sunitinib in combination with induction and consolidation chemotherapy, 50% of patients with FLT3-ITD and 38% of patients with FLT3-TKD achieved a complete remission. During the study, dose-limiting toxicities were experienced by three patients, which necessitated a dose reduction. Sunitinib is not approved for AML or any other cancer expressing a FLT3 mutation.

Type-I inhibitors: pacritinib

Pacritinib (SB1518, S*BIO Pte. Ltd.) is an aminopyrimidine macrocycle with equal potency against JAK2 and FLT3 kinases.[84–86] The lead compound **5a** was discovered through an in house screen against various kinase targets.[86] FLT3 mutations impose geometric constraints in the active site and pacritinib was designed as a constrained macrocycle to accommodate these constraints. To accomplish this, the open end of ring A was connected to the open end of ring C to form a macrocycle without compromising binding of the molecule to the kinase hinge region. The linker Z was

designed with hydrophilic atoms to improve interaction at the solvent front of the active site (Scheme 5).[86] Pacritinib is active against JAK2, FLT3, and IRAK-1 but not against JAK1.[86,87] The IC$_{50}$ of pacritinib against various kinases are listed in Table 3.[87]

Pacritinib was found to induce apoptosis and cell cycle arrest in addition to blockade of proliferation of wild type FLT3 and mutant FLT3.[88] Pre-clinical studies of pacritinib demonstrated that the inhibitor was active against FLT3-ITD (GI$_{50}$ = 133 nM) and FLT3-TKD (GI$_{50}$ < 434 nM) expressing Ba/F3 cells. Further evaluation of pacritinib showed that it was active against various FLT3-ITD AML cell lines including MV4-11 (GI$_{50}$ = 33 nM), MOLM13 (GI$_{50}$ = 73 nM), and FLT3 inhibitor-resistant MOLM13 (GI$_{50}$ = 173 nM).

A phase I/II study with pacritinib was designed to evaluated efficacy against myelofibrosis and advanced myeloid malignancies and also to determine the maximum tolerated dose and safety profile (NCT00719836).[89] The study demonstrated that a dose as high as 500 mg per day was tolerated.[89] From the study, the clinical beneficial rate was approximately 43% in patients with AML, but a long-term assessment was not completed because the study was terminated prematurely due to financial reasons.[89] Another phase I clinical trial was conducted to evaluate the pharmacokinetic and toxicity profile of pacritinib in combination with cytarabine and doxorubicin or decitabine in adults with FLT3-ITD AML (NCT02323607).[85] A total of 13 patients were included in the study; however, three patients were not evaluated either due to death (1 out of 13) or discontinuation of therapy (2 out of 13). The study concluded that pacritinib was well tolerated at a dose of 100 mg twice daily in combination with chemotherapy but failed to reach FDA approval.[85]
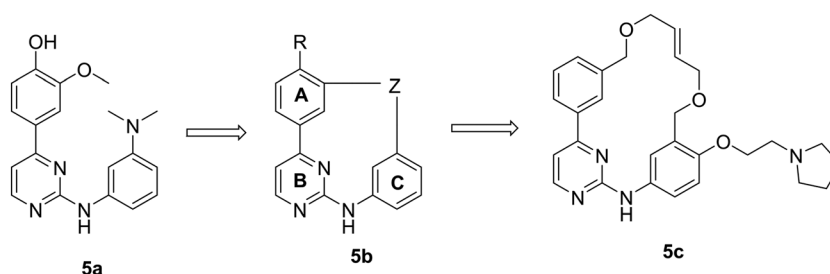
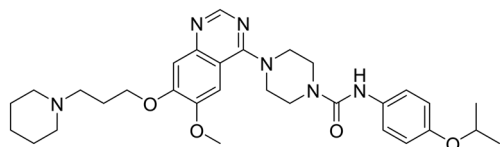**Scheme 5** Hit to lead strategy to develop the constrained macrocycle, pacritinib (**5c**).

Fig. 9 Molecular structure of tandutinib.

Type-II inhibitors: tandutinib

Tandutinib (MLN518/CT53518) is a quinazoline derivative with activity against type III RTKs such as FLT3, PDGFR, and KIT (Fig. 9).[90,91] Tandutinib inhibited IL-3 dependent growth and FLT3-ITD autophosphorylation in cell lines expressing a FLT3-ITD mutation.[92] Following pre-clinical studies, a phase I study of tandutinib among 40 patients with AML or MDS was initiated.[93] The drug was administered from 50 mg up to 700 mg twice daily and dose limiting toxicities occurred at doses higher than 525 mg twice daily. DLTs were reversible, but further analysis showed that tandutinib caused QT prolongation, which could cause a severe cardiac event. From the results of the phase I trial, it was also not clear if tandutinib could elicit a preliminary anti-leukemic response. Tandutinib was progressed into a phase II trial to further evaluate efficacy but was terminated due to adverse events.
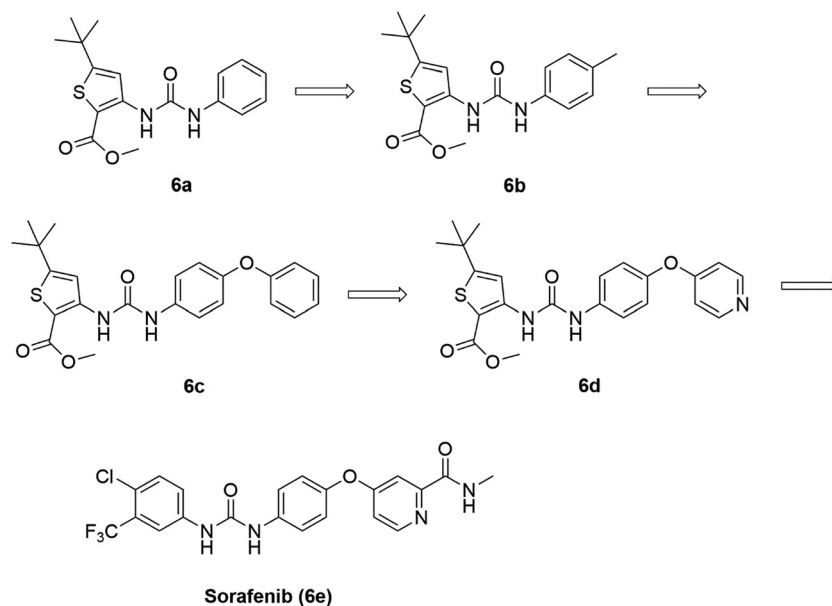
Type-II inhibitors: sorafenib

Sorafenib (Nexavar, Bayer Pharmaceuticals), a multikinase inhibitor, was initially developed as a Raf1 targeting drug to block the RAS–RAF–MEK–ERK cell-survival pathway. It has since been found to inhibit numerous RTKs such as FLT3, RET, KIT, VEGFR-1/2/3, and PDGFR-β.[94] Sorafenib was discovered from a high throughput screen against Raf1 that led to the discovery a 3-thienyl urea (**6a**) compound (Scheme 6). Subsequent optimization of the hit compound led to the discovery of sorafenib.[95] Sorafenib is currently approved for the treatment of hepatocellular carcinoma, advanced renal cell carcinoma, and metastatic thyroid carcinoma refractory to radioactive iodine treatment.[96,97]

Preclinical studies have shown sorafenib inhibits FLT3-ITD mutated AML cells *in vitro* and *in vivo*.[98–100] The study of antileukemic effects of sorafenib against Ba/F3 cell lines expressing mutant ITD, D835Y, or D835G revealed that sorafenib was more effective against FLT3-ITD and D835G mutations compared to D835Y or wild type.[101] Modeling of sorafenib with FLT3 suggests that sorafenib interacts with C694 at the hinge region and D829 of the DFG motif (Fig. 10). Sorafenib forms an integral interaction with the F691 gatekeeper residue and exhibits a loss in activity against F691 gatekeeper mutants, which is typical for type-II inhibitors.[81]

A phase I study with sorafenib caused a reduction in leukemic blast counts in patients with FLT3-ITD mutations suggesting efficacy of sorafenib in patients with an ITD mutation.[101] Another phase I/II study was conducted to determine safety and tolerability of the combination of sorafenib, cytarabine, and idarubicin in patients with AML (NCT00542971).[102] The regime was well-tolerated and the combination of drugs was deemed safe. Seventy five percent of patients achieved a CR, 93% of patients with FLT3 mutation achieved a CR with incomplete platelet recovery, whereas only 66% of patients with wild type FLT3 achieved a CR with incomplete recovery. The study revealed achieving a CR is greater for FLT3 mutated patients than wild type. However, another study of sorafenib with standard induction and consolidation therapy among elderly patients (>60 years) did not improve OS and was declared not beneficial

Scheme 6 Development of sorafenib (**6e**) from 3-thienyl urea (**6a**).
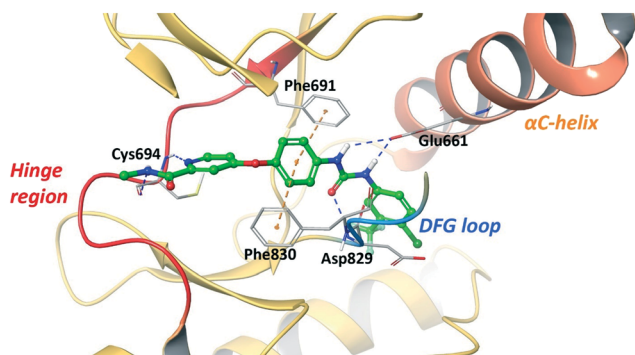
Fig. 10 Sorafenib docked into the FLT3 kinase domain.[81] Sorafenib is a type-II inhibitor that is ATP non-competitive and binds to the inactive (DFG$_{out}$) conformation of the kinase. The hinge region, αC-helix, and DFG loop are illustrated in red, orange, and blue, respectively.

(NCT00373373).[103] Higher treatment-related mortality and a lower CR was seen in the sorafenib cohort. Interestingly, in another randomized, double-blind, placebo-controlled phase II trial in patients younger than 60 years, addition of sorafenib to standard induction chemotherapy significantly prolonged event-free survival and relapse-free survival (SORAML trial, NCT00893373).[104] However, the addition of sorafenib also increased drug-induced toxicity as grade 3/4 adverse events were more common in patients receiving sorafenib.

In phase II and phase III trials adults with FLT3-ITD AML were randomized to receive sorafenib maintenance therapy following allogeneic hematopoietic stem cell transplantation (allo-HSCT, NCT02474290).[105,106] Both studies independently demonstrated that sorafenib maintenance after allo-HSCT prevented disease relapse in AML patients with a FLT3-ITD mutation. However, the mechanism as to how sorafenib produced this activity and appropriate duration for maintenance therapy remains unknown.

A combination study of sorafenib with decitabine, a DNA hypomethylating agent, has been evaluated preclinically, which led to the initiation of a phase I study.[107] The combination was well tolerated where 4 of 5 patients with relapsed/refractory AML achieved a complete response with incomplete count recovery. Despite exhibiting promising clinical efficacy, sorafenib has not been approved to treat FTL3-driven disease.

Type-II inhibitors: quizartinib

Quizartinib (AC220, Daiichi Sankyo) is a bis-aryl urea that was designed to be a FLT3 inhibitor, and the synthesis is outlined in Scheme 7. The identification of quizartinib (**7g**) started from urea derivative (**7h**), which was screened against a kinase library and exhibited affinity for FLT3. Further evaluation revealed the that amino-carbonyl derivative (**7j**) at the *para*-position had better cellular activity than carboxamide derivatives (**7i**). Further SAR studies of the amide–urea series led to identification of AB530 (**7k**). Removal of amide group and introduction of an aliphatic, basic amine led to the discovery of quizartinib (**7g**) (Scheme 8).[108]

Quizartinib has high selectivity and sensitivity to FLT3 and received approval in Japan for the treatment of relapsed/refractory AML with a FLT3-ITD mutation but has yet to
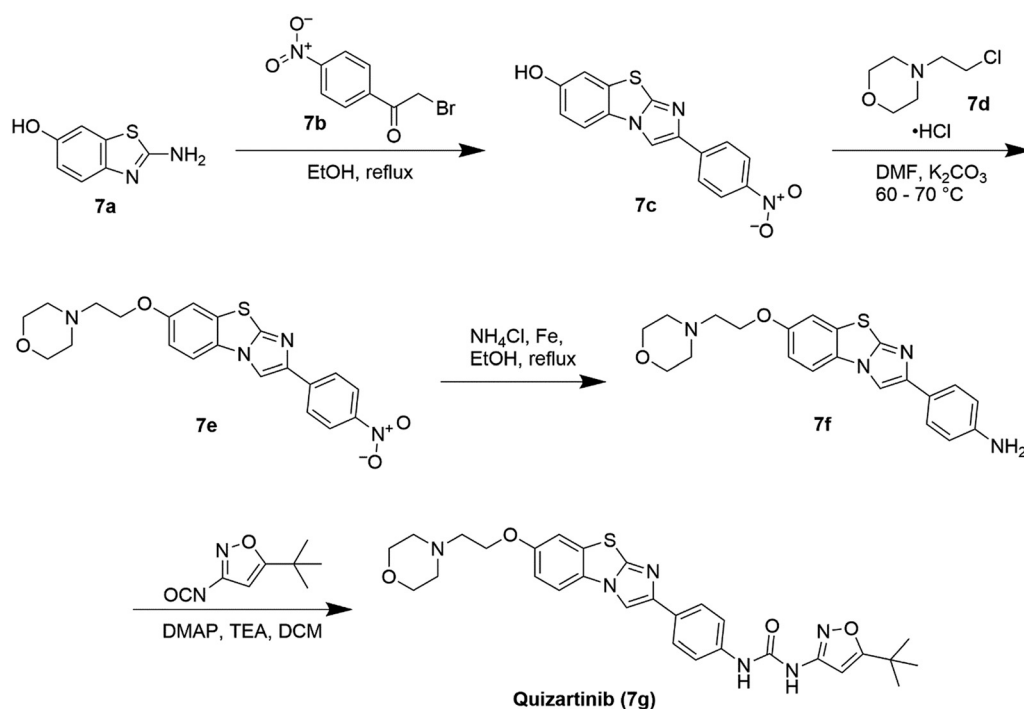
Scheme 7 Synthesis of quizartinib (**7g**).
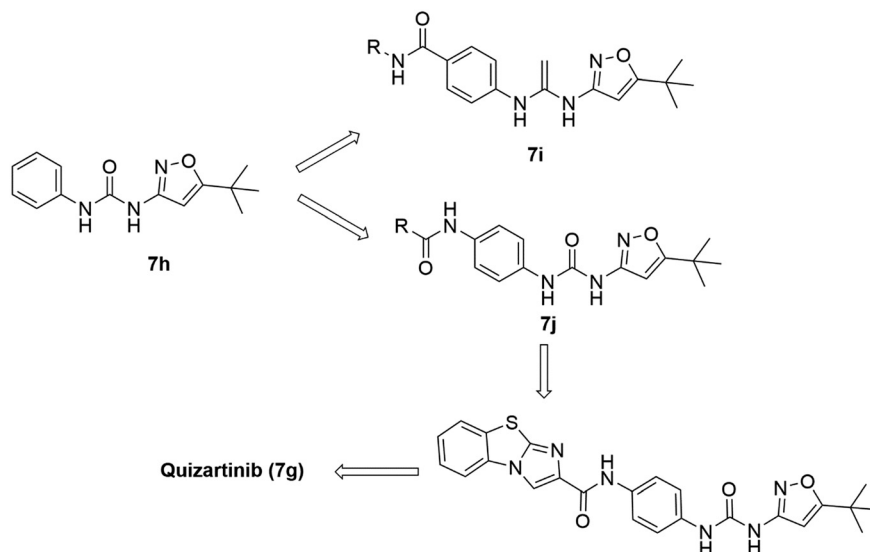
Scheme 8   Discovery of quizartinib (**7g**).

receive approval elsewhere. Quizartinib is active against FLT3-ITD but does not retain similar activity against FLT3-TKD mutations. *In vivo* and *in vitro* studies have shown that quizartinib is active against FLT3 wild type and ITD.[108–110] The IC$_{50}$ of quizartinib against the FLT3-ITD cell line MV4-11 is 0.56 nM.[108] Quizartinib binds to the FLT3 kinase in the inactive conformation (DFG$_{out}$) as shown in Fig. 11.[111]

Following preclinical studies, a phase I dose escalation trial was completed to assess safety and tolerability of quizartinib among 76 patients irrespective of FLT3-ITD status (NCT00462761).[112] The maximum tolerated dose was determined to be 200 mg per day, and QT prolongation was identified as a dose-limiting toxicity. This study did show higher overall response rates in patients with FLT3-ITD mutations compared to those with wild type FLT3 at 53% *vs.* 14%. A subsequent phase II trial evaluated the efficacy and safety of quizartinib as a single agent in patients with

relapsed/refractory AML (NCT00989261).[113,114] The results confirmed the clinical efficacy of quizartinib in refractory/relapsed AML as 46–56% of patients with a FLT3-ITD mutation achieved complete remission compared to 30–36% of patients with wild type FLT3. A phase III randomized trial evaluated efficacy of quizartinib against salvage chemotherapy among patients with refractory/relapse AML with a FLT3-ITD mutation (QuANTUM-R, NCT02039726).[115] Overall survival for quizartinib was longer than chemotherapy at 6.2 months *vs.* 4.7 months. The most common drug-related toxicities were febrile neutropenia, sepsis or septic shock, and QT prolongation. For the chemotherapy cohort toxicities were febrile neutropenia, sepsis or septic shock, pneumonia, and pyrexia.

The clinical efficacy of quizartinib combined with a manageable safety profile led to approval of quizartinib in Japan. Based on the same findings, a new drug approval (NDA) for quizartinib was filed by Daiichi Sankyo with the FDA. The NDA was rejected by the FDA in 2019 because of inconsistencies in the clinical trial results and concerns from a lack of improvement in event-free survival and cardiac-related adverse events.[116]
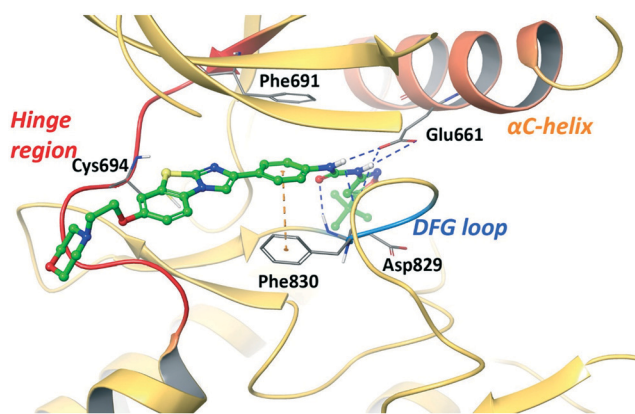
Irreversible FLT3 inhibitor: FF-10101

FF-10101 (**7j**) is a novel FLT3 inhibitor designed and developed by FUJIFILM Corporation. The synthesis is outlined in Scheme 9. The inhibitor binds selectively to FLT3 by forming a covalent bond to cysteine 695 irrespective of the kinase conformation state. The covalent bond formation between FF-10101 and FLT3 was shown by X-ray analysis of a co-crystal structure of FF-10101 bound to the FLT3 kinase domain (Fig. 12).[38] In addition to the covalent bond with C695 at the hinge region, FF-10101 interacts with E692 and K644 *via* hydrogen bonds.
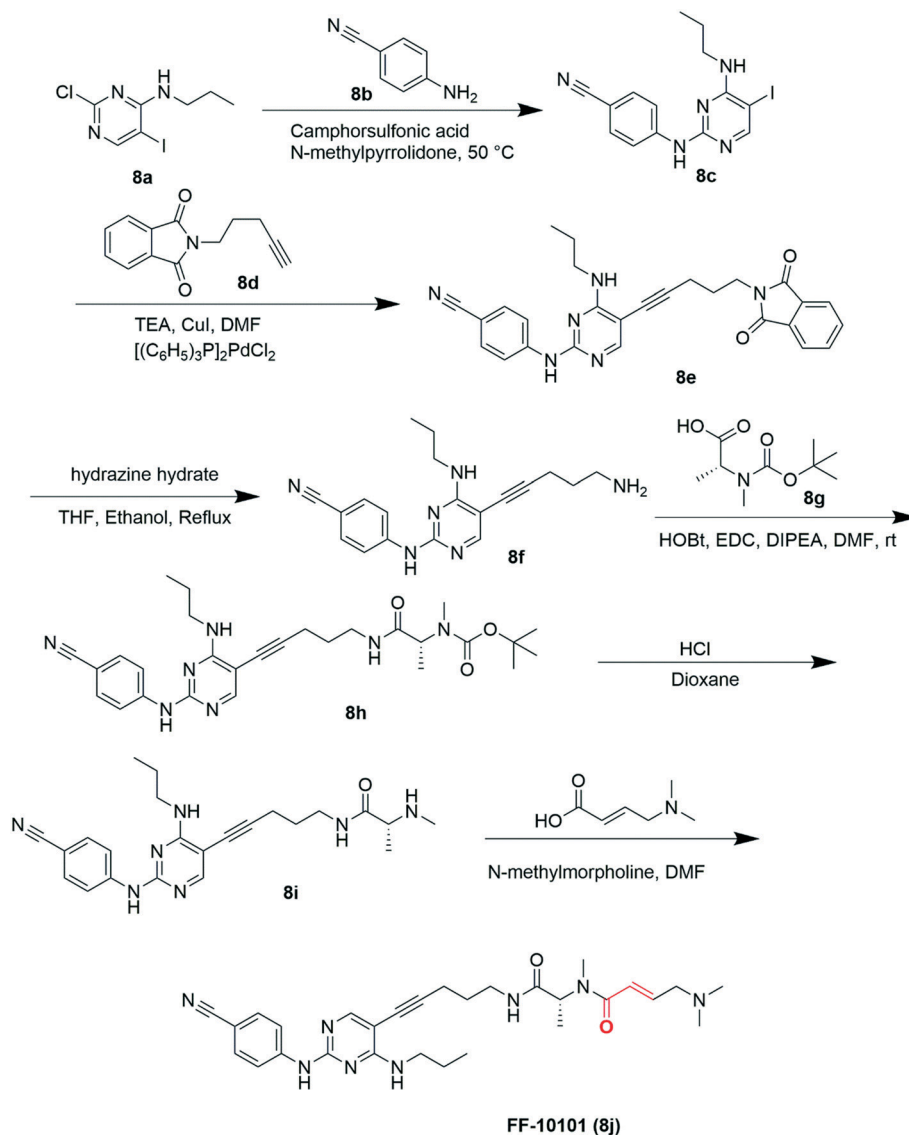

Fig. 11   Co-crystal structure of quizartinib bound to the kinase domain of FLT3 (PDB ID: 4XUF).[111] Quizartinib is a type-II inhibitor that is ATP non-competitive and binds to the inactive (DFG$_{out}$) conformation of the kinase. The hinge region, αC-helix, and DFG loop are illustrated in red, orange, and blue, respectively.

Scheme 9 Synthesis of FF-10101 (**8j**). The α,β-unsaturated carbonyl that forms a covalent adduct with FLT3 is highlighted in red.

Preclinical studies of FF-10101 have shown high efficacy against AML cell lines harboring FLT3 mutations including quizartinib-resistant mutations. The growth inhibitory profile of FF-10101 against human AML cell lines are listed in the Table 4.[38]

Similarly, the growth inhibitory profile of FF-10101 against various types of FLT3 mutant-expressing 32D cells are listed in Table 5.[38] Clinical trials with FF-10101 are ongoing.

Resistance to FLT3 inhibitors

Small molecule kinase inhibitors targeting FLT3 have exhibited immense promise for the treatment of AML. Despite numerous small molecule kinase inhibitors and a number of clinical trials, prolonged efficacy from the inhibition of FLT3 has remained a challenge due to the development of resistance from secondary mutations and other FLT3 activating pathways (Fig. 13).[117]

One of the most common forms of acquiring drug resistance are on-target secondary point mutations in the kinase domain of FLT3. Mutations may occur at the activation loop (*e.g.*, D385) or gatekeeper region (*e.g.*, F691). Secondary TKD mutations lead to a change in the conformational state of the kinase, thereby negatively altering binding kinetics of small molecules. For instance, mutations at the activation loop can cause resistance to type-II inhibitors by energetically favoring the active conformation of the kinase. While mutations at the gatekeeper region can cause resistance to both type-I and -II inhibitors. In another instance, after treatment with quizartinib, simultaneous double-mutants have been identified at both the activation loop and gatekeeper region, which can exacerbate drug resistance.[118] Drug resistance to midostaurin has also been identified and is caused by point mutations at position 676 (N676K) in the FLT3 kinase domain.[55] Since many FLT3 inhibitors are selective against wild type FLT3, the presence
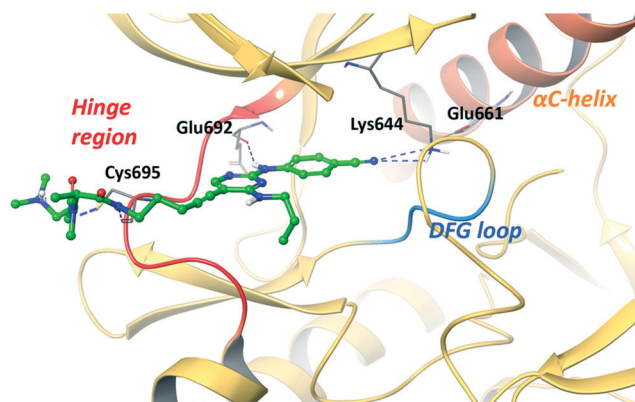
Fig. 12 Co-crystal structure of FF-10101 bound to the FLT3 tyrosine kinase domain (PDB ID: 5X02).[38] FF-10101 is an irreversible, ATP non-competitive inhibitor of FLT3. C695 of the FLT3 hinge region forms a covalent adduct with the α,β-unsaturated carbonyl on FF-10101. The hinge region, αC-helix, and DFG loop are illustrated in red, orange, and blue, respectively.

of wild-type FLT3 in patients with a FLT3-ITD mutation can confer resistance to FLT3 inhibitors as well.[119]

Beyond on-target mutations, the microenvironment can also influence treatment durability as high amounts of FLT3 ligand in the bone marrow microenvironment during induction and consolidation therapy can result in persistent activation of the FLT3/MAPK pathway, which ultimately signals leukemic blast for continuous survival.[120] The bone marrow microenvironment may also influence the sensitivity of leukemic cells to inhibitors. For instance, CYP3A4 expression by bone marrow stromal cells can increase metabolism of inhibitors promoting resistance.[121] Interestingly, resistance to FLT3 inhibitors can also arise from activation of downstream signaling pathways independent of FLT3. Growth factors within the bone marrow microenvironment are likely to contribute to resistance by activating FLT3-independent pathways. For example, fibroblast growth factor 2 (FGF2) is highly expressed by bone marrow and is known to activate fibroblast growth factor receptor 1 (FGFR1) present on AML cells to activate downstream MAPK signaling independent of FLT3.[122] Prolonged exposure to FLT3 inhibitors is likely to select for FLT3-resistant clones that can activate downstream signaling pathways independent of FLT3.[123] For instance, a study in patients who relapsed after treatment with gilteritinib

Table 4 Cell growth inhibitory profile of FF-10101 against various cell lines[38]

Cell lines	GI_{50}, nM
MV4–11	0.83
MOLM-13	1.1
MOLM-14	1.5
Kasumi-1	26
EOL-1	72
THP-1	>1000
K562	>1000

Table 5 Cell growth inhibitory profile of FF-10101 against various FLT3 mutations[38]

32D cell lines	GI_{50}, nM
FLT3-ITD	1.9
FLT3-ITD-D835Y	0.81
FLT3-ITD-Y842C	3.5
FLT3-ITD-Y842H	5.3
FLT3-ITD-F691L	10
FLT3-D835Y	1.1

showed that 12.2% harbored an activating mutation in the RAS/MAPK pathway that bypassed FLT3 inhibition. Furthermore, 24% had additional FLT3-mutations together with RAS/MAPK activating mutations.[124] The microenvironment can also influence FLT3 inhibitors through expression of P-glycoprotein (P-gp). P-gps are overexpressed in leukemic cells and the efflux pump decreases sensitivity of AML to FLT3 inhibitors.[125]

One strategy to overcome resistance to small molecule inhibitors is combination therapy. Numerous clinical trials with combination therapies have been conducted with treatments that target complimentary signaling pathways or treatments that inhibit key signaling pathways with different mechanisms of action. Combination therapies can be helpful in improving response rates as well as prolonging remission in AML patients with FLT3 mutations. Combination therapy has been tested with FLT3 inhibitors in combination with established chemotherapy modalities to target complimentary pathways. This includes combination with bortezomib (proteasome inhibitor), atezolizumab (anti-PD-L1 antibody), venetoclax (Bcl-2 inhibitor), vorinostat (HDAC inhibitor), and omacetaxine (STAT inhibitor).[126]

In a trial assessing the efficacy of sorafenib in combination with omacetaxine, thirty-nine patients with relapsed or refractory FLT3-ITD-AML and 5 newly diagnosed patients were recruited.[127] Four of the newly diagnosed cohort achieved a CR and 1 achieved complete remission with incomplete hematological recovery (CRi). Among the 39 relapsed or refractory patients, 71% achieved CR/CRi. In another study, sorafenib was evaluated in patients with poor-risk AML in combination with bortezomib and vorinostat.[128] The first dosage group evaluated sorafenib and vorinostat, and the second dosage group studied the addition of bortezomib to the previous combination. With the first dosage group, 44% of patients achieved partial remission and 6% achieved complete remission. In the second dosage group, 7% achieved CR, 7% achieved CRi, and 14% achieved PR. In another combination trial, the safety and efficacy of gilteritinib in combination with induction and consolidation chemotherapy in newly diagnosed AML was evaluated,[129] and the overall survival was 35.8 months. In FLT3-mutated patients, duration of CRc and disease-free survival were 14.1 and 15.2 months. In FLT3-ITD patient achieving CRc, 70% of patients receiving ≥120 mg of gilteritinib achieved mutational clearance.
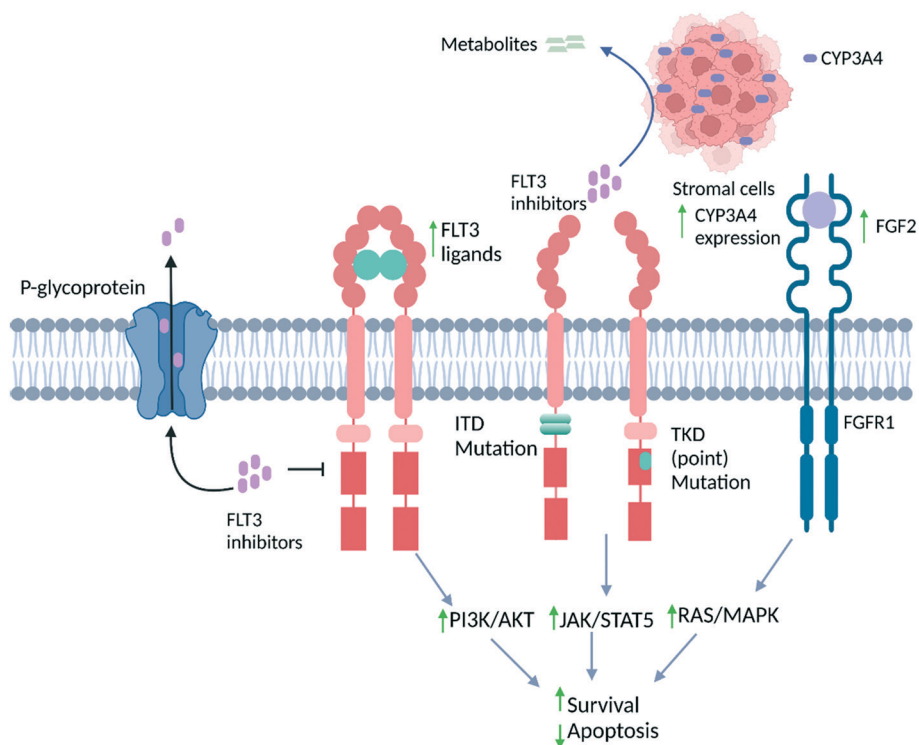
Fig. 13 Mechanisms of resistance to FLT3 inhibitors. The most common resistance mechanisms to FLT3 inhibitors are the selection for drug resistant FLT3 mutations or an increase in FLT3 WT expression. The microenvironment can also promote resistance through activation of alternate cell-survival pathways, increasing drug metabolism by CYP3A4 in stromal cells, and increasing drug efflux. Resistance is also observed through ligand-independent activation of downstream cell survival pathways such as Ras or AKT. One of the most effective clinical strategies to combat resistance mechanisms is the use of combination therapy by selecting treatments that target complimentary signaling pathways or treatments that inhibit key signaling pathways with different mechanisms of action.

Conclusion

Despite the development of numerous FLT3 inhibitors, AML continues to be one of the most difficult-to-treat hematological malignancies with less than 30% of patients surviving five years or more. Several small molecule inhibitors have been approved to treat AML. Midostaurin was the first FLT3 inhibitor to be approved by the FDA in 2017 for the treatment of FLT3-mutated AML. Gilteritinib was then approved the following year for treatment of patients with relapsed or refractory AML with a FLT3-mutation. Quizartinib has only been approved in Japan for the treatment of patients with FLT3-ITD-positive relapsed or refractory AML.

Additional FLT3 small molecule inhibitors are being evaluated both clinically and pre-clinically. Resistance is a constant issue and is a significant hindrance in the successful development and clinical evaluation of these new inhibitors. To this end, inhibitors targeting multiple FLT3 mutations have been developed to mitigate on-target resistance. However, mutations at the gatekeeper residue F691L continue to be a difficult mutation to drug. Mutations that lower drug affinity to FLT3 lead to shorter clinical responses and ultimately a faster relapse. FF-10101, a novel FLT3 inhibitor that covalently binds to a cysteine moiety at C695, is a covalent inhibitor that has been developed to

mitigate on-target resistance mechanisms. Preclinical studies have shown that this inhibitor is not rendered ineffective despite the presence of a F691L mutation, which suggests irreversible inhibitors could be beneficial in drug-resistant mutations. However, covalent inhibitors for other kinase targets, such as afatinib for EGFR, exhibit resistance when the amino acid necessary to form the covalent adduct mutates in the active site.[130] This is a possible resistance mechanism that could occur during FF-10101 treatment so even covalent inhibitors of FLT3 may lose efficacy through on-target mutations.

In lieu of overcoming on-target resistance to improve clinical outcome, clinical trials utilizing combination therapy targeting multiple signaling pathways have been conducted with high success. A thorough understanding of the mechanisms of resistance to FLT3 inhibitors could provide insight into the design of clinical trials with combination therapy to target common resistance mechanisms. In addition, development of multitargeted FLT3 inhibitors that can also target complementary signaling pathways could extend efficacy of treatment.

The heterogeneity within AML is a constant threat that supplies additional mutations when a new therapy is used. The new therapy places a selection pressure on the tumor that promotes the growth of treatment-resistant clones.[131–133]

The most successful way to combat these new mutations is to complete clinical trials with new therapies or combination therapies and then determine mechanisms of resistance that occur at relapse. If the mechanisms are tractable drug targets, new therapies can be developed to target these resistance mechanisms. If the resistance mechanisms are targetable with known drugs, new drug combinations can be evaluated in clinical trials. The identification and development of new therapies to target these new resistance mechanisms is critical to improve the duration of remission. This process is iterative and incremental but necessary for the development of new strategies to better combat AML.

Author contributions

B. A. and B. F. contributed to conceptualization of this review article. B. A. and B. F. wrote the initial manuscript. All authors contributed to analysis and discussion of the article.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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