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REVIEW

Therapeutic Targeting of FLT3 in Acute Myeloid Leukemia: Current Status and Novel Approaches

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Abstract: FMS-like tyrosine kinase 3 (FLT3) is mutated in approximately 30% of acute myeloid leukemia (AML) patients. The presence of FLT3-ITD (internal tandem duplication, 20–25%) mutation and, to a lesser extent, FLT3-TKD (tyrosine kinase domain, 5–10%) mutation is associated with poorer diagnosis and therapy response since the leukemic cells become hyperproliferative and resistant to apoptosis after continuous activation of FLT3 signaling. Targeting FLT3 has been the focus of many pre-clinical and clinical studies. Hence, many small-molecule FLT3 inhibitors (FLT3is) have been developed, some of which are approved such as midostaurin and gilteritinib to be used in different clinical settings, either in combination with chemotherapy or alone. However, many questions regarding the best treatment strategy remain to be answered. On the other hand, various FLT3-dependent and -independent resistance mechanisms could be evolved during FLT3i therapy which limit their clinical impact. Therefore, identifying molecular mechanisms of resistance and developing novel strategies to overcome this obstacle is a current interest in the field. In this review, recent studies of approved FLT3i and knowledge about major resistance mechanisms of clinically approved FLT3i's will be discussed together with novel treatment approaches such as designing novel FLT3i and dual FLT3i and combination strategies including approved FLT3i plus small-molecule agents targeting altered molecules in the resistant cells to abrogate resistance. Moreover, how to choose an appropriate FLT3i for the patients will be summarized based on what is currently known from available clinical data. In addition, strategies beyond FLT3i's including immunotherapeutics, small-molecule FLT3 degraders, and flavonoids will be summarized to highlight potential alternatives in FLT3-mutated AML therapy.

Keywords: AML, FLT3-ITD, FLT3 inhibitor, FLT3i resistance, targeted therapy, flavonoid

Introduction

Acute myeloid leukemia (AML) is an aggressive disease characterized by the accumulation of abnormal hematopoietic precursors, which are overproliferative with blocked differentiation and suppressed apoptosis in the bone marrow and peripheral blood.[1] Although AML is genotypically and phenotypically heterogeneous, various chromosomal abnormalities and gene mutations have been identified, which are crucial to determine AML classification, risk groups, and treatment strategies.[2]

FMS-like tyrosine kinase 3 (FLT3) gene encodes for a receptor tyrosine kinase (RTK), which is mainly expressed on immature hematopoietic progenitors and hematopoietic stem cells (HSCs). Its expression is reduced when the cells complete the differentiation process.[3] FLT3 signaling is initiated when FLT3 ligand (FLT3 L) binds to FLT3, inducing FLT3 dimerization and activation via autophosphorylation at tyrosine residues. PI3K/AKT, MAPK, and JAK2/STAT5 are the activated downstream signaling pathways, which lead to cell proliferation and suppression of apoptosis.[4]

Activating mutations in FLT3 account for 30% of all AML cases, which are FLT3 internal tandem duplication (ITD) and FLT3 tyrosine kinase domain (TKD) mutations. FLT3-ITD is observed in 20–25% of newly diagnosed AML cases while FLT3-TKD mutations represent 5–10% of all cases.[5] FLT3 receptor is continuously activated as a result of these mutations irrespective of the presence of FLT3 L, leading to increased cell proliferation and decreased cell apoptosis.[1,5] In addition to PI3K/AKT and MAPK signaling pathways, STAT5 pathway is found to be

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continuously activated in the presence of FLT3-ITD.[6] Clinical impacts of FLT3-ITD mutations are associated with higher relapse rate, decreased overall survival (OS) rate, poorer treatment response and shorter disease-free survival (DFS) compared to patients with wild-type FLT3 (WT-FLT3) while adverse clinical outcomes of FLT3-TKD mutations are controversial.[7]

Identifying the roles of FLT3 mutations in disease pathogenesis and clinical outcomes has made it a therapeutic target, resulting in the development of FLT3i with different specificity and potency.[8] Although some of these inhibitors, midostaurin and gilteritinib, have been clinically used in combination therapies or alone, respectively, for FLT3-mutated AML, responses are short-lived and patients relapse due to the emergence of resistance.[9] Therefore, enlightening primary or secondary resistance mechanisms and designing novel modalities to overcome resistance are urgently needed to maximize the benefits of FLT3i. In addition to FLT3i, novel targeted therapies are currently at the stage of pre-clinical and early clinical investigation, which include novel FLT3i, dual FLT3i, FLT3 targeted CAR T cell therapy and FLT3-specific antibodies.[10] Additionally, novel immunotargets have been identified with therapeutic potential. Moreover, there are studies investigating the effects of small-molecule FLT3 degraders such as HSP90 and proteasome inhibitors on FLT3 positive AML. Activities of flavonoids or their synthetic analogs on FLT3 positive AML could lead to the discovery or development of novel FLT3i or to their implementation as integrative medicine or nutraceuticals into FLT3 AML therapy.[11] It seems to be the most rational strategy to combine approved FLT3i with the modulators of altered intracellular targets, resulting in the discovery of novel targets and therapeutics.

In this review, we aim to expand on the body of the literature in the management of FLT3 AML and provide an update on newly reported knowledge by specifically focusing on targeted FLT3 therapies including clinically approved FLT3is, novel FLT3i, combination approaches, immunotherapeutics, small-molecule FLT3 degraders and flavonoids. Additionally, mechanisms observed in clinical or experimental resistance to approved FLT3is will be discussed together with potential solutions to reverse the resistant phenotype.

FLT3 Structure

The *FLT3* gene, encoding FMS-like tyrosine kinase 3 transmembrane receptor, is located on chromosome 13q12, containing 24 exons and 993 amino acid residues.[5,12,13] FLT3, also known as fetal liver kinase 2 or human stem cell kinase-1, belongs to the type III RTK family, which also includes FMS, KIT, and PDGFR kinases that share strong sequence similarities.[13] FLT3 receptor is mainly expressed on HSCs, multipotent progenitors, common myeloid and lymphoid progenitor cells, and mature dendritic cells.[5,14] The expression of FLT3 is lost or reduced once the cells differentiate into mature lymphoid or myeloid cells.[15–17]

After translation of the receptor as a 110 KDa protein, it is sent to the endoplasmic reticulum (ER) to be transformed into 130 KDa N-glycosylated immature protein that is rich in mannose. Subsequently, it is further processed in the Golgi apparatus (GA) to become a mature 160 KDa protein which will be then directed to the cell surface.[18,19] The final form of FLT3 consists of 4 different domains: an extracellular domain containing 5 immunoglobulin-like subdomains, a transmembrane domain, an intracellular juxtamembrane (JM) domain, and an intracellular C-terminal domain, comprising 2 tyrosine kinase subdomains; tyrosine kinase domain 1 and 2 (TKD1 and TKD2) connected by an activation loop (A-loop) (Figure 1).[5]

FLT3 L is an extracellular ligand produced by a wide range of cells including lymphocytes, HSCs, and bone marrow stromal cells.[1,14,20] FLT3 L is found as membrane-bound or in soluble form.[20] The concentration of soluble FLT3 L is generally low, however, can increase exponentially due to aplasia causing only necessary activation of FLT3 via the negative-feedback mechanism.[21] Binding of FLT3 L to the extracellular domain of FLT3 causes structural changes including dimerization of the monomeric receptor. The JM domain has an inhibitory function on the kinase domain. Upon binding, the JM domain changes conformation to make the kinase domain accessible for ATP binding, which eventually leads to autophosphorylation of several tyrosine residues and activation of the receptor.[12,22,23] Activation of the receptor further activates the downstream signaling pathways such as PI3K and RAS cascades, resulting in hematopoietic cell maturation and proliferation.[23] Thus, the function of FLT3 L is to act as a growth factor to stimulate myelopoiesis.[24]

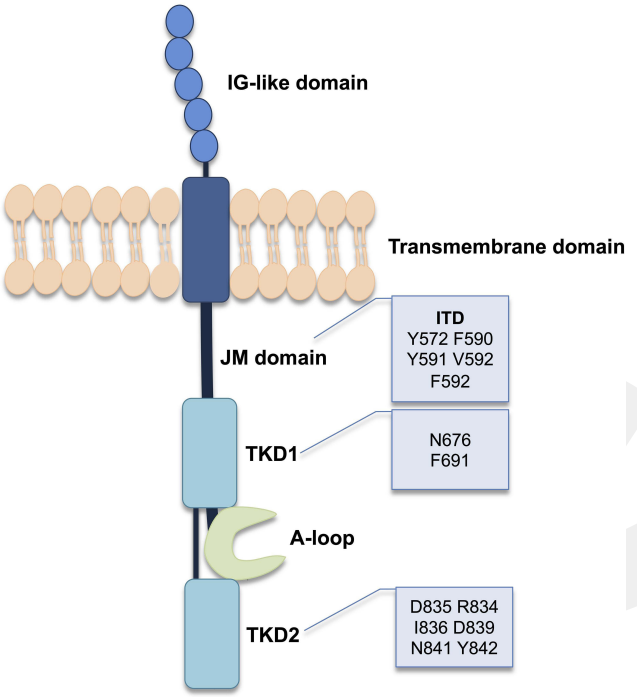

Figure 1 FLT3 structure and common FLT3 mutations.[1,42]
Notes: Data from the references: ©2012 Grafone et al, Licensee PAGE Press, Italy. Grafone T, Palmisano M, Nicci C, Storti S. An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment. Oncol Rev. 2012;6(1):e8-e8 under Creative Commons CC BY-NC 3.0.[1] ©2018 Staudt et al, Licensee MDPI, Basel, Switzerland. Staudt D, Murray HC, McLachlan T, et al, Targeting oncogenic signaling in mutant FLT3 acute myeloid leukemia: the path to least resistance. International Journal of Molecular Sciences. 2018;19(10):3198 under Creative Commons CC BY 4.0.[42]

FLT3 Mutations

FLT3 mutations are the most frequently identified mutations in AML patients.[25] Of all cases in AML, FLT3-ITD and FLT3-TKD account for approximately 20–25% and 5–10%, respectively.[5] The receptor, which stays in a monomeric form in WT-FLT3, becomes dimerized independent of the FLT3 L binding in mutated FLT3.[5,26] Therefore, mutations in the receptor cause activation of the tyrosine kinase even in the absence of the ligand resulting in aberrant proliferation of the malignant cells.[26]

FLT3-ITD mutations are in frame gain-of-function mutations occurring in the JM domain which, in fact, is responsible for the inhibition of the receptor activation through the inhibition of the kinase domain; therefore, the mutation constitutively activates the TKD action.[27–29] Patients with FLT3-ITD mutations show increased relapse and decreased OS.[27] FLT3-ITD occurs as a duplication of a fragment that varies in length and position. The length of the fragment is evidently negatively correlated with the OS rate.[30,31] FLT3-TKD mutations are generally single amino acid mutations such as substitution, deletion, and insertion in the A-loop of the TKD, causing loss of auto-inhibition.[21,32,33] The most common point mutations in FLT3-TKD are substitution of aspartic acid of TKD2 with tyrosine or histidine at residue 835 and substitution of asparagine or phenylalanine of TKD1 at residues of 676 and 691, respectively (Figure 1).[32,33]

FLT3 Signaling

In WT-FLT3, several signaling pathways are activated to regulate the proliferation, differentiation, and apoptosis of the HSC.[20] Upon binding of FLT3 L, trans-autophosphorylation of tyrosine residues in FLT3 is followed by binding of adaptor proteins including SHP2, GRB2, and SRC family kinases, leading to activation of mainly PI3K/Akt/mTOR and RAS/MEK/ERK pathways.[34–38] FLT3-ITD and FLT3-TKD activate similar pathways with WT-FLT3. However, FLT3-ITD also induces JAK/STAT signaling through STAT5A phosphorylation.[39] FLT3-TKD shows increased activation of SHP1 and SHP2, of which SHP1 is a negative reGulatory phosphatase for the JAK signaling pathway.[40,41] Therefore, only low levels of JAK2 and STAT3 activation are observed in FLT3-TKD.[42] Moreover, FLT3-ITD mutations can decrease the expression of c/EBPalpha and PU.1 which are associated with the differentiation of myeloid cells.[39] In contrast, FLT3-TKD mutations do not suppress the c/EBPalpha and PU.1.[39,43] (Figure 2).

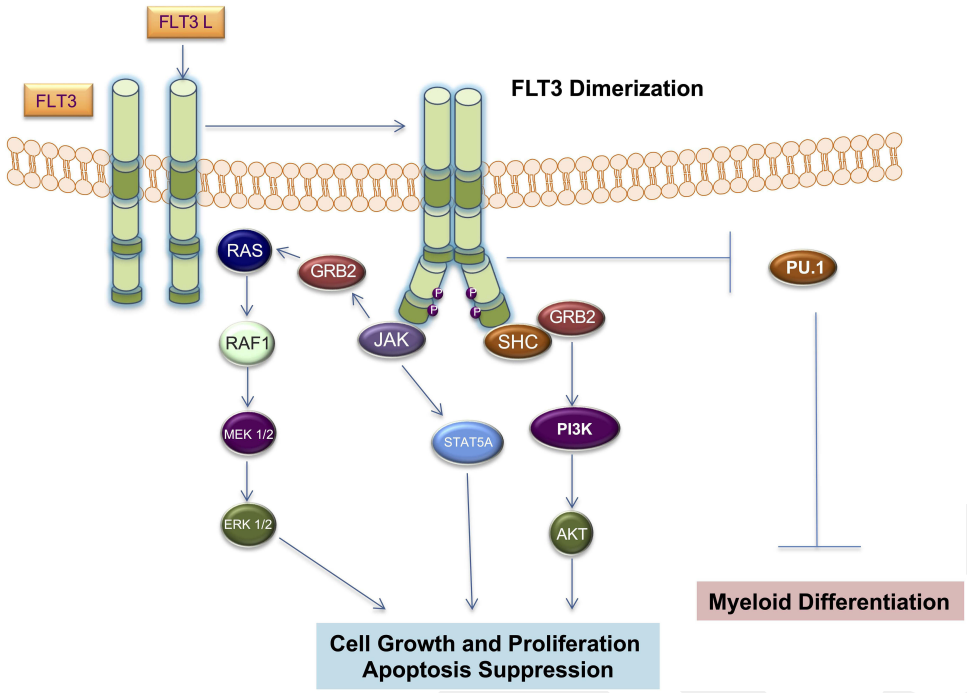

Figure 2 FLT3-ITD signal transduction.[1,9]

Notes: Data from the references: ©2012 Grafone et al, Licensee PAGE Press, Italy. Grafone T, Palmisano M, Nicci C, Storti S. An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment. Oncol Rev. 2012;6(1):e8-e8 under Creative Commons CC BY-NC 3.0.[1] ©2017 Hospital et al, Licensee Dove Medical Press Limited. Hospital MA, Green AS, Maciel TT, et al, FLT3 inhibitors: clinical potential in acute myeloid leukemia. Onco Targets Ther. 2017;10:607-615 under Creative Commons CC BY-NC 3.0.[9]

Leukemogenesis in FLT3 Positive AML

The two-hit model of leukemogenesis suggests that class I and class II mutations must occur together. Class I mutations activate the proliferative pathways and class II mutations impair hematopoietic differentiation in AML.[44,45] Along with K/NRAS, TP53, and c-KIT mutations, FLT3 mutations are classified as class I mutations. It is still controversial whether an FLT3 mutation is a passenger or driver mutation even though it seems to be an early event in disease development.[46,47] Isolated FLT3-ITD mutations, when injected into transgenic mice were not sufficient to induce leukemia, suggesting that FLT3 mutation is a passenger mutation.[48] However, the high incidence rate of FLT3 mutations and emergence of additional mutations along with FLT3 leading to FLT3i resistance suggests that FLT3 mutations are driver mutations.[49–51] 75% of the FLT3-ITD positive patients still have FLT3-ITD mutation after relapse, suggesting that FLT3 mutation is a driver mutation[52] and the FLT3 mutation induced shifting from the pre-leukemic state to leukemia.[53] Several important genes including MYC, GAB2, and DNMT3A have been identified to cooperate with FLT3 in promoting leukemogenesis.

MYC family genes are regulated by upstream FLT3-ITD signaling and contribute to leukemogenesis. In an FLT3-ITD mouse model, simultaneous upregulation of MYC genes and downregulation of the MYC antagonists, the MXD genes was observed, which could explain the expansion of leukemic progenitors.[54] c-MYC reportedly increased the expression and stability of SIRT1 deacetylase in FLT3-ITD AML, resulting in the maintenance of leukemic stem cells (LSC).[55] Telomerase reverse transcriptase (hTERT) upregulated in FLT3-ITD AML cells in a c-MYC dependent manner and inhibition of FLT3-ITD caused transcriptional repression of c-MYC.[56] FLT3-ITD induced c-MYC, which increased the transcription of alternative nonhomologous end-joining (NHEJ) pathway, leading to genomic instability.[57]

Gab2, an amplifier protein in signaling pathways, is identified as an essential signaling molecule for leukemic transformation in FLT3-ITD AML. Bone marrow cells from Gab2-deficient and -proficient or -haploinsufficient mice were transfected with FLT3-ITD. FLT3-ITD infected cells survived when Gab2 is functional; however, FLT3-ITD infected cells could not transform in the absence of Gab2.[58] In a recent study, Gab2 deficiency was shown to prevent FLT3-ITD AML development in an FLT3-ITD knock-in, DNMT3A haploinsufficient mouse model.[59]

FLT3-ITD knock-in and DNMT3A (a DNA methyltransferase) knock-out mice model had reduced disease onset and shortened survival as compared to either mutation alone, proving the role of DNMT3A mutations in driving leukemogenesis via enhancing self-renewal capacity of long-term-HSCs (LC-HSCs). The loss of a single allele of DNMT3A was sufficient to shorten survival and induce leukemia development.[60]

Prognostic and Clinical Impact of FLT3 Mutations

AML patients with an FLT3 mutation have poor prognosis compared to patients with WT-FLT3. The response rates of AML patients with or without an FLT3 mutation are similar; however, patients with an FLT3 mutation are more likely to experience relapse.[61,62] Even when FLT3 mutation is not detected, the receptor can still be overexpressed, resulting in the survival and proliferation of the leukemic cell.[13,24] Furthermore, chemotherapy can induce aplasia and stimulate FLT3 L production which eventually leads to recovery and expansion of the AML cells.[24]

The association of the FLT3-TKD mutation with the prognosis of AML is still not clear, although FLT3-TKD mutation increases the kinase activity.[2,32,33] On the other hand, FLT3-ITD mutation alone is an adverse prognostic marker. The presence of FLT3-ITD is associated with high leukemic burden, poor OS, and early relapse.[21,63] The prognostic impact of FLT3-ITD depends on the mutant-to-WT allelic ratio (AR), mutation insertion site, the length of the ITD duplication, karyotype, and the co-existing mutations.[2,64–66] While FLT3 mutations contribute to the uncontrolled proliferation of leukemic cells, other mutations such as DNMT3A, NPM1, and IDH1/2 cause impaired differentiation and self-renewal.[5,67,68]

One of the most common co-mutations in FLT3-ITD AML occurs in DNMT3A. The presence of a DNMT3A co-mutation was required to consider FLT3-ITD as an adverse prognostic indicator. There was no significant difference in OS between FLT3-ITD positive and FLT3-ITD negative patients in the absence of DNMT3A co-mutation. Patients with FLT3-ITD and DNMT3A mutation had shorter OS compared to patients with either FLT3 mutation or DNMT3A mutation. After post-induction therapy, the patients with DNMT3A and FLT3-ITD co-mutation showed the highest rate of relapse.[69]

The nucleophosmin 1 (NPM1) gene encodes a multi-functional protein and is mostly mutated in FLT3-ITD AML. NPM1 mutations could be useful to determine the prognosis, which is dependent on the presence or absence of FLT3-ITD and FLT3-ITD AR.[70] Based on the risk stratification organized by the European LeukemiaNet (ELN) and the National Comprehensive Cancer Network (NCCN), the presence of WT-NPM1 and FLT3-ITD mutation is considered as adverse risk. High FLT3-ITD AR and co-existing NPM1 mutation are classified as intermediate risk while low FLT3-ITD AR and NPM1 mutation are categorized as favorable.[65,71] The effects of age and gender on NPM1 and FLT3-ITD mutations were investigated in 1570 patients younger than 75 years old. Females had more FLT3-ITD and/or NPM1 mutations compared to males. More males were double negative. FLT3-ITD caused poor survival in younger patients while NPM1 was related to good survival in older patients. FLT3-ITD/NPM1 double mutant patients' survival was less related to age.[72] In a study, patients having FLT3-ITD, DNMT3A and NPM1 triple mutations were significantly younger than patients having single or double mutations. Most of the triple-mutated patients were women having heavy disease burden and high white blood cell and bone marrow blast counts. Patients carrying triple gene mutations had the shortest OS followed by DNMT3A alone, FLT3-ITD/DNMT3A double mutation. The response to treatment was the best in patients with DNMT3A/NPM1 double mutation. Patients having triple mutation and FLT3-ITD alone showed no response to treatment. However, patients with either NPM1 mutation alone or FLT3-ITD alone had longer OS.[73]

Other co-mutations such as IDH1/2, CEBPA, and ASXL1 could affect the prognosis of AML patients. The prognostic analysis of isocitrate dehydrogenase (IDH1 and IDH2) mutations in newly diagnosed FLT3-ITD AML patients showed that double-mutated patients had higher white blood cell counts, increased peripheral and bone marrow blast percentages, a higher frequency of NPM1 mutations and a lower frequency of DNMT3A. There was no significant difference in OS between double-mutated and FLT3-ITD patients.[74] In a recent study, CR did not differ in registered AML patients regardless of their molecular abnormalities including FLT3-ITD.[75]

AML Patients with additional Sex Comb-like 1 (ASXL1) and FLT3-ITD mutations had poor prognosis with a shorter OS, EFS,[76] and RFS in Egyptian AML patients.[77]

The prognostic effect of CCAAT/enhancer binding protein A (CEBPA) involved in the proliferation and differentiation of myeloid progenitor cells depends on whether patients carry double or single mutated CEBPA. Single mutated CEBPA was seen more commonly in FLT3-ITD mutated AML patients although relatively less percentages of double

mutated CEBPA were also observed.[78] The effect of CEBPA mutation in patients with R/R *FLT3*-ITD-positive AML treated with quizartinib or SC was analyzed and CEBPA mutations were associated with high CRc rates and relatively long median OS, regardless of the treatment protocol.[79]

The prognostic significance of mutations in R/R FLT3-ITD AML was evaluated compared to the mutational status of newly diagnosed FLT3-ITD AML patients. The frequency of FLT3-ITD mutation increased while that of CEBPA biallelic (double) mutation decreased. NPM1, FLT3-ITD, and DNMT3A triple mutations were only found in the relapse group although their co-existence was detected in newly diagnosed, relapsed, and refractory patients. Refractory patients with NPM1 and FLT3-ITD co-mutation experienced shorter OS than the patients with FLT3-ITD mutation alone or WT-NPM1.[80]

Single, double and triple mutations of FLT3-ITD, NPM1, and DNMT3A were more prevalent in females. The allelic ratio of FLT3-ITD was not found to be different among the sexes. FLT3-ITD mutated female population had significantly poor outcomes. FLT3-ITD patients younger than 60 years had poor OS compared to older patients. Considering the sex and age, the female and younger population had poor OS, while there was not any significant difference in OS between the young and old male patients.[81]

In a recently published study, 2017 ELN risk classification has been revisited based on the current understanding of the roles of molecular targets in AML. The updated version included AML with FLT3-ITD in the intermediate risk group, irrespective of the AR or co-presence of NPM1 mutation. The absence of FLT3-ITD with WT NPM1 and the presence of mutated NPM1 with FLT3-ITD are categorized as the intermediate risk group.[82]

In conclusion, deciphering the mutation spectrum of FLT3-ITD AML could lead to an in-depth understanding of the pathogenesis and refine the prognostic classification of the disease. It is also possible to follow up the treatment response of the patients based on the mutational analysis, which could help reorganizing the treatment in case of relapse.

Clinically Approved FLT3 Inhibitors in Therapy

FLT3 has become an attractive target in AML given the correlations between its mutated forms and disease development, poor prognosis, high mortality rates, and insufficient therapy response.[23] FLT3is have been the focus of small molecule drugs against FLT3 mutations some of which have been approved for clinical settings with favorable outcomes.[30] FLT3 is are tyrosine kinase inhibitors (TKI) categorized as first- and next-generation inhibitors based on their specific ability to inhibit FLT3 and associated downstream signaling pathways.[23,30] Apart from this broad classification, both first and next-generation inhibitors could be either type I or type II inhibitors in relation to their effectiveness against both FLT3-ITD and TKD mutations or only FLT3-ITD mutation, respectively.[23,30,83] Type I inhibitors can bind to both active and inactive conformations of the FLT3 receptor since they bind to the FLT3 gatekeeper domain or the ATP binding pocket. Type II inhibitors bind adjacent to ATP binding domain located in the hydrophobic region when the receptor is in its inactive conformation.[83] In this review, we have specifically focused on the FLT3is approved for the clinical settings (Table 1) and we will summarize the clinical trials of these FLT3is involved in novel combination studies (Table 2).

Table 1 Summary of the Clinical Trials Leading to Approval of FLT3 Inhibitors

FLT3i	Generation/ Type	Patient Group	Study	Clinical Approval
Midostaurin	First/I	Newly diagnosed FLT3-mutated AML in combination with chemotherapy	RATIFY trial (NCT00651261)	FDA approval, 2017
Sorafenib	First/II	R/R FLT3-ITD AML patients in combination with 5-azacitidine	Phase II (NCT01254890) Phase II (NCT02196857)	National Comprehensive Cancer Network (NCCN), 2019
Gilterinitib	Next/I	R/R FLT3-ITD AML patients	ADMIRAL trial (NCT02421939)	FDA approval, 2018
Quizartinib	Next/II	R/R FLT3-ITD AML	QuANTUM-R trial, Phase III (NCT02039726)	Only in Japan, 2019

First-Generation FLT3 Inhibitors

The first-generation inhibitors show non-specific activity for FLT3 and inhibit other kinases such as KIT, PDGFR, VEGFR, RAS/RAF/MEK, and JAK kinases, hence having more off-target effects.[23,30] Off-target activities could result in

Table 2 Selected Clinical Trials of Approved FLT3i in Different Therapeutic Settings

Phase- Clinical Trial Number	Therapeutic Approach Including FLT3i	Patient Group	Response	Status
Phase III NCT02752035	Gilteritinib + Azacitidine	Newly diagnosed AML with FLT3 mutation and not eligible for induction chemotherapy	No significant **OS** with improved **CR** rates	Active, not recruiting
Phase II NCT01253070	Sorafenib + chemotherapy (daunorubicin hydrochloride and cytarabine)	AML patients (≥60 years of age) with FLT3-ITD or -TKD mutations and have not received chemotherapy	**CR after induction therapy** FLT3-ITD: 74% FLT3-TKD: 73% **1-year OS** FLT3-ITD: 62% FLT3-TKD: 71% **1-year DFS** FLT3-ITD: 52% FLT3-TKD:36% **1-year EFS** FLT3-ITD: 39% FLT3-TKD: 27%	Active, not recruiting
Phase I/II NCT01892371	Quizartinib + Azacitidine or Cytarabine	Refractory or relapsed MDS, CMML or AML patients with or without FLT3 mutation	**CRc** 64% with azacitidine 29% with low-dose cytarabine **RFS** 5.8 with azacitidine and 6.2 months with low-dose cytarabine **OS** 12.8 with azacitidine and 4 months with low-dose cytarabine	Active, not recruiting
Phase III NCT04174612	Midostaurin + Daunorubicin + Cytarabine	Newly diagnosed AML patients with FLT3 mutation between the age of 18 and 65	No results posted	Recruiting
Phase III NCT03182244	Gilteritinib vs salvage chemotherapy	Patients with relapsed or refractory AML with FLT3 mutation after first line therapy	No results posted	Active, not recruiting
Phase I NCT05024552	Gilteritinib + Vyxeos (cytarabine/ daunorubicin liposomal complex)	Patients with relapsed or refractory AML with FLT3 mutation	No results posted	Recruiting
Phase II NCT03135054	Quizartinib + Omacetaxine Mepesuccinate (Homoharringtonine) Injection	Newly diagnosed or relapsed/ refractory AML patients with FLT3-ITD mutation	No results posted	Active, not recruiting
Phase II NCT03170895	Sorafenib + Omacetaxine Mepesuccinate Injection	Newly diagnosed or relapsed/ refractory AML patients with FLT3-ITD mutation	**CR/CRi rate** for R/R patients: 72% **LFS and OS**: 5.6 and 10.9 months	Completed
Phase II NCT03622541	Sorafenib	Relapsed or refractory AML patients with FLT3-ITD mutation	No results posted	Completed

(*Continued*)

Table 2 (Continued).

Phase- Clinical Trial Number	Therapeutic Approach Including FLT3i	Patient Group	Response	Status
Phase I/II NCT03730012	Gilteritinib + Atezolizumab (PD-L1 mAb)	Relapsed or refractory AML patients with FLT3 mutation	**CRc** Gilteritinib 120 mg + Atezolizumab 420 mg: 33.3% Gilteritinib 120 mg + Atezolizumab 840 mg:12.5% **CR** Gilteritinib 120 mg + Atezolizumab 420 mg:33.3% Gilteritinib 120 mg + Atezolizumab 840 mg: 0%	Completed
Phase I/II NCT04240002	Gilteritinib + Fludarabine + Cytarabine + G-CSF	Children and young adults with relapsed or refractory FLT3-ITD+ AML	No results posted	Recruiting
Phase II NCT02927262	Gilteritinib	Patients with FLT3-ITD or FLT3-TKD mutated AML	**RFS** up to 2 years - 24.02 months **EFS** up to 2 years - 16.06 months	Active, not recruiting
Phase I NCT00943943	G-CSF + Plerixafor (CXCR4 inhibitor) + Sorafenib	Relapsed or refractory AML patients with FLT3 mutation	Response rate 36%	Completed
Phase III NCT02421939	Gilteritinib	Relapsed or refractory AML patients with FLT3 mutation	**CR** Gilteritinib: 21.1% Salvage Chemotherapy: 10.5% **CR/CRh** Gilteritinib: 34% Salvage Chemotherapy: 15.3% **OS** (months) Gilteritinib: 9.3 Salvage Chemotherapy: 5.6 **EFS** (months) Gilteritinib:2.8 Salvage Chemotherapy: 0.7	Active, not recruiting
Phase II NCT02196857	Azacitidine + Sorafenib	AML and high risk MDS patients with FLT3-ITD mutation	**CR**: 26% **OS**: 8.3 months **RFS**: 7.1 months	Completed
Phase III NCT04027309	Gilteritinib or midostaurin	AML or MDS patients with excess of blasts-2 and FLT3 mutation	No results posted	Recruiting
Phase II NCT02984995	Quizartinib	Relapsed or refractory AML patients with FLT3-ITD mutation	**CRc** 20mg/Day Quizartinib: 33.3% 30mg/Day Quizartinib: 56.5% **OS** (weeks) 20mg/Day Quizartinib: NA 30mg/Day Quizartinib: 34.1 **EFS** (weeks) 20mg/Day Quizartinib: 0.1 30mg/Day Quizartinib: 12.7 **LFS** (weeks) 20mg/Day Quizartinib: NA 30mg/Day Quizartinib: 16.1	Completed

(*Continued*)

Table 2 (Continued).

Phase- Clinical Trial Number	Therapeutic Approach Including FLT3i	Patient Group	Response	Status
Phase I/II NCT05010122	Decitabine and Cedazuridine + Gilteritinib + Venetoclax	Newly diagnosed, relapsed or refractory AML or high risk MDS patients with FLT3 mutation	No results posted	Recruiting
Phase I/II NCT04140487	Azacitidine + Gilteritinib + Venetoclax	Relapsed or refractory AML, CMML or MDS/MPN patients with FLT3 mutation	No results posted	Recruiting
Phase I/II NCT03793478	Quizartinib + Intrathecal (IT) triple chemotherapy prophylaxis + Fludarabine + Cytarabine + Etoposide	Relapsed or refractory AML patients (1 month to 25 years of age) with FLT3-ITD mutation	No results posted	Recruiting
Phase III NCT02997202	Gilteritinib	AML patients with FLT3-ITD mutation in CR1	No results posted	Active, not recruiting
Phase II NCT03836209	Daunorubicin + Cytarabine + Gilteritinib or Midostaurin	AML patients with FLT3 mutation	No results posted	Recruiting
Phase III NCT02039726	Quizartinib	AML patients with FLT3-ITD mutation	**OS** (months) Quizartinib: 6.2 Salvage Chemotherapy: 4.7 **EFS** (months) Quizartinib: 1.4 Salvage Chemotherapy: 0.9 months	Completed
Phase I/II NCT04687761	Quizartinib + Venetoclax + Azacitidine or Cytarabine	Newly diagnosed AML patients (≥60 years of age)	No results posted	Recruiting
Phase I/II NCT03661307	Quizartinib + Decitabine + Venetoclax	Untreated or relapsed AML patients or high risk MDS	No results posted	Recruiting
Phase I NCT04496999	Midostaurin + HDM201 (MDM2 inhibitor)	Relapsed or refractory AML patients with FLT3 mutation and WT-TP53	No results posted	Recruiting
Phase I/II NCT04385290	Midostaurin + Daunorubicin + Cytarabine+ Gemtuzumab Ozogamicin	Newly diagnosed AML patients	No results posted	Active, not recruiting
Phase I/II NCT01254890	Azacitidin + Sorafenib	Refractory AML, CMML or MDS patients and relapsed AML patients	**CR**: 16% **OS**: 6.2 months **EFS**: 3.8 months	Completed
Phase III NCT00651261	Midostaurin + Cytarabine + Daunorubicin + Dexamethasone Acetate	Newly diagnosed AML patients with FLT3 mutation	**OS** (months) Midostaurin: 74.7 Placebo: 25.6 **CR** Midostaurin: 58.9% Placebo: 53.5% **EFS** (months) Midostaurin: 8.2 Placebo: 3.0	Active, not recruiting

Abbreviations: CR, complete remission; CRc, composite complete remission; CR/CRh, complete remission and complete remission with partial hematological recovery; OS, overall survival; DFS, disease-free survival; EFS, event-free survival; RFS, relapse-free survival; LFS, leukemia-free survival; G-CSF, granulocyte colony-stimulating factor.

decreased clinical efficacy in FLT3-mutated AML with high allelic burden while resulting in increased toxicity profile and clinical benefit for WT-FLT3 AML.[84] First-generation inhibitors include midostaurin and sorafenib, which are also type I and type II inhibitors, respectively.[23,30,83,84]

Midostaurin is active against both FLT3-ITD and TKD mutations and showed limited and transient activity in early clinical phase trials when used alone. In a clinical setting including relapsed/refractory (R/R) FLT3-mutated AML patients, midostaurin induced a 50% reduction in peripheral and bone marrow blasts.[85] Another milestone study investigated the effects of midostaurin in AML patients carrying WT-FLT3 or mutated FLT3. Reduction in peripheral and bone marrow blasts was 71% in patients with mutant FLT3 and 42% in patients with WT-FLT3.[86] Overall, midostaurin could not induce a complete remission (CR) and its efficacy remained limited to blast reduction due to activation of alternative survival-promoting pathways, protection of the leukemic clones in stem-cell niche and limited concentration of free midostaurin in patient's plasma.[85,86]

However, its combination with several cytotoxic anti-leukemic agents including cytarabine, doxorubicin, azacitidine, or daunorubicin resulted in promising outcomes in in vitro models of FLT3 positive AML leading to its investigation in the clinic for combination therapy.[87,88] Newly diagnosed younger patients with FLT3 positive AML were treated with 50 mg midostaurin/twice a day in combination with standard chemotherapy for 14 days, resulting in high CR and OS rates.[47] The addition of midostaurin into standard care chemotherapy dramatically changed the course of the disease based on the results of RATIFY trial in which registered therapy-naive patients with FLT3 mutations (FLT3-ITD and FLT3-TKD) showed increased OS rates. The presence of either ITD or TKD and the ITD AR status was not distinctive factors on patients' conditions. US Food and Drug Administration (FDA) approved midostaurin in 2017 to be used in combination with standard cytarabine and daunorubicin induction therapy and cytarabine consolidation therapy in newly diagnosed young (18–59 years) FLT3-mutated AML patients based on the findings of RATIFY trial.[89] Further analysis from RATIFY trial investigated the suitability of midostaurin as a maintenance therapy while the patients were in the first CR after intensive cytarabine consolidation therapy and the analysis showed that the added effect of midostaurin during maintenance therapy was not conclusive even though overall relapses were reduced.[90] The impact of midostaurin in patients having only FLT3-TKD mutations in RATIFY trial was significantly higher in terms of 5-year event-free survival (EFS) rate (45.2%) compared to the placebo arm (30.1%) while OS was similar.[91]

A recent study investigated the roles of midostaurin in patients' survival who were initially treated with midostaurin plus intensive chemotherapy and then referred to allo-SCT in the first CR. Midostaurin therapy improved OS specifically in patients with high AR and only midostaurin therapy and allo-SCT in first CR were found as positive predictors for OS.[92] The effect of midostaurin to prevent relapse in FLT3-ITD carrying patients (18–70 years old) subjected to allo-SCT showed that midostaurin plus standard chemotherapy could not improve relapse-free survival (RFS) (89%) significantly as compared to only chemotherapy arm (76%), concluding the addition of midostaurin as maintenance therapy following allo-SCT could be only beneficial for some patients with FLT3-ITD AML.[93] Midostaurin with standard chemotherapy for older FLT3-ITD AML patients (18–70 years) was shown to be safely effective as induction therapy. Allo-SCT in the first CR after midostaurin plus chemotherapy was highly effective, irrespective of age. Maintenance with midostaurin could only be beneficial for some patients after high-dose consolidation chemotherapy or allo-SCT.[94]

Sorafenib is a first-generation, type II FLT3i whose safety and efficacy was established in newly diagnosed FLT3 mutant AML in combination with standard anthracycline/cytarabine induction therapy.[95] The randomized SORAML trial in 2015 showed that sorafenib improved EFS and RFS and did not cause a change in OS in registered AML patients regardless of the FLT3 status compared to the placebo group.[96] However, these results were updated in 2017 with increased OS.[97] In a recent study, 99 patients with newly diagnosed FLT3-ITD AML were registered for sorafenib+intensive chemotherapy or placebo and EFS was not different between the two groups. However, the sorafenib group had improved OS.[98] Two separate clinical trials including R/R FLT3-mutated AML patients and untreated older patients not fit for intensive chemotherapy showed the efficacy and safety of sorafenib in combination with hypomethylating agent (HMA) 5--azacitidine,[99,100] which resulted in the inclusion of sorafenib+azacitidine for R/R FLT3-ITD AML patients' treatment by the recommendation of NCCN.[101] Sorafenib has been widely investigated in FLT3 mutant AML patients eligible for allo-SCT. Sorafenib increased OS as maintenance therapy after allo-SCT in FLT3 mutant AML patients.[102] The SORMAIN study including 83 FLT3-ITD patients in CR after allo-SCT investigated the addition of sorafenib as maintenance therapy

compared to the placebo group, resulting in a higher probability of 24-month OS in the sorafenib group.[103] FLT3-ITD AML patients who underwent allo-SCT were divided randomly into sorafenib maintenance (400 mg orally twice daily) or control arms, concluding that sorafenib maintenance therapy decreased relapse.[104]

Next-Generation FLT3 Inhibitors

Next-generation FLT3 inhibitors are more specific and potent for FLT3 with fewer off-target effects compared to first-generation inhibitors. They could also have the capacity to induce myeloid differentiation and show greater clinical activity as monotherapeutic agents.[24]

Gilteritinib is a next-generation, type I FLT3i with more potency against mutated FLT3 among other TKIs even though it shows some activity for other kinases such as AXL.[105] In the dose-escalation/expansion trial, 20–450 mg gilteritinib in an FLT3-mutated R/R AML patient population was studied and it was shown that its plasma concentration is higher since it is less bound to plasma protein and it inhibits the phosphorylation of FLT3 more than 85% at very low concentrations (CHRYSALIS trial).[106] A landmark Phase III study called ADMIRAL investigated the effects of 120 mg gilteritinib in R/R FLT3-mutated AML patients compared to salvage chemotherapy (SC). The percentage of gilteritinib-treated patients who achieved CR with full or partial hematologic recovery (34%) was higher compared to the chemotherapy given group (15.3%) and OS was 9.3 months vs 5.6 months, respectively. The OS outcomes of ITD or TKD mutated patients were similar (9.3 vs 8 months, respectively).[107] The results of this study were proof for the FDA approval of gilteritinib monotherapy in 2018 for R/R FLT3-mutated AML patients in the US. The long-term effects and safety of gilteritinib were analyzed for the patients enrolled and survived in the ADMIRAL trial, showing that 49 patients in the gilteritinib arm were alive for more than 2 years. Twenty-six patients treated with gilteritinib were alive without relapse; 18 gilteritinib given patients underwent transplantation and 16 patients were treated with gilteritinib as post-HSCT maintenance therapy. Thus, continued and post-HSCT gilteritinib maintenance therapy resulted in sustained remission with a stable safety profile.[108] There are several ongoing clinical trials investigating the potential of gilteritinib versus placebo as maintenance therapy after consolidation (NCT02927262) or after allo-HCT in patients with FLT3-ITD mutations (NCT02997202). The combination of venetoclax and gilteritinib resulted in high mCRc (modified CRc) and FLT3 molecular response rates regardless of prior FLT3 inhibitor exposure. However, there is a need to determine proper doses to overcome myelosuppression.[109] A randomized Phase II trial of gilteritinib versus midostaurin in combination with induction and consolidation chemotherapy is also currently recruiting (NCT03836209). The phase III LACEWING trial (NCT02752035) was designed to compare gilteritinib plus azacitidine with azacitidine alone in newly diagnosed older (65–86 years old) FLT3-mutated AML patients who were ineligible for intensive induction chemotherapy. The results showed that the gilteritinib plus azacitidine combination was safe for registered patients. CR rates were improved although there were no significant OS differences.[110] However, this trial has been terminated as a result of interim analysis by an independent group of reviewers, which was due to the lack of a statistically significant increase in OS.[111]

Quizartinib is a next-generation, type II FLT3i active against FLT3 and, to a lesser extent (around 10-fold less), KIT, CSF1R, PDGFR, and RET kinases.[112] Two sequential phase II studies including R/R FLT3-mutated AML patients treated with quizartinib monotherapy resulted in significant bone marrow remissions; however, adverse cardiac signals raised concerns about its safety even when used in lower concentrations.[113,114] Recently, quizartinib monotherapy and SC were compared in a phase III study (QUANTUM-R, NCT02039726) with R/R FLT3-mutated AML patients. Quizartinib increased OS (6.2 months) as compared to SC (4.7 months).[115] 32% of patients in quizartinib arm could proceed to an allo-SCT (11% of patients in SC). 62% of the patients on the quizartinib arm who received allo-SCT received post-SCT quizartinib maintenance. Quizartinib is not approved by FDA in the US due to cardiac toxicities and strong myeloid suppression based on the results of QUANTUM-R; however, it is approved in Japan as monotherapy in R/R FLT3-ITD AML. A post hoc analysis of the patients on the quizartinib arm and SC therapy arm who referred to allo-SCT in QuANTUM-R showed that continuation of quizartinib after HSCT was tolerable, with no new safety signals.[116] Patients with R/R FLT3 AML were treated with quizartinib plus azacitidine or low-dose cytarabine (NCT01892371). CRc rates were 64% with azacitidine and 29% with low-dose cytarabine, RFS 5.8 and 6.2 months, and OS 12.8 and 4 months, respectively. 28% of the patients from the quizartinib plus azacitidine arm underwent an allo-SCT compared to only 6% of patients from the quizartinib plus low-dose cytarabine arm.[117] There is an ongoing phase I/II trial (NCT04687761)

including the combination of low-dose cytarabine or azacitidine + venetoclax + quizartinib in newly diagnosed AML patients. Recently, the incorporation of quizartinib into 7+3 regime and continuation therapy of newly diagnosed FLT3-ITD AML patients aged up to 75 years old increased OS compared to those with SC.[118]

How to Choose an Approved FLT3i for the Patient

Clinical decisions on a specific FLT3i for a specific group of patients such as how to choose a proper concentration of FLT3i in combination approaches, and when to use FLT3i (as a frontline or maintenance therapy), before or allo-SCT should be carefully considered based on the presence of simultaneous myeloid neoplasm-related mutations, type of FLT3 mutation, FLT3-ITD insertion size and position, the use of prior FLT3i, the toxicity profile of FLT3i, patients' age and overall health condition and eligibility for allo-SCT.[83]

There are recent studies investigating the impact of concurrent mutations, FLT3-ITD AR, and insertion size on the therapeutic outcome of FLT3i. An analysis of RATIFY study related to the prognostic impact of FLT3-ITD insertion site and the presence of NPM1 mutation highlighted that the presence of NPM1 mutation was correlated with the presence of insertion site in only JM domain. Insertion site in only TKD1 was evaluated as a negative prognostic factor. Midostaurin was effective for the patients carrying insertion site in only JM domain following allo-SCT in the first CR.[119] Subsequent analysis of the patients with only FLT3-TKD mutations in RATIFY trial showed that the co-existence of NPM1 mutations or core binding factor (CBF)-rearrangements were considered as favorable prognostic factors in terms of midostaurin treatment.[91] FLT3-ITD AR, ITD length, and the expression of hepatic leukemia factor (HLF) were checked to understand differential responses to FLT3i. High AR samples showed increased sensitivity compared to low AR samples while no association was found between ITD length and FLT3i response. RNA seq analysis displayed that there was a correlation between high AR and high HLF expression, which could determine FLT3i response.[120] FLT3-ITD AR, FLT3-ITD length, or multiple FLT3-ITD mutations did not have any adverse effects on survival outcomes with gilteritinib; however, the presence of DNMT3A/NPM1 double mutations resulted in the most favorable outcomes in patients received gilteritinib.[121]

Prior FLT3i use or sequential FLT3i exposure could be a factor to decide which FLT3i therapy could be chosen. Sorafenib and midostaurin treated R/R FLT3-mutated AML patients involved in the CHRYSALIS and ADMIRAL trials were compared with those without prior FLT3i use after treated with gilteritinib. Similar high composite CR (CRc) rates were obtained irrespective of prior FLT3i use; however, remission duration was shorter in the prior FLT3i exposure group.[122] The R/R FLT3-mutated patients previously treated with midostaurin plus intensive induction therapy showed 58% CR rate after gilteritinib treatment. However, the presence of NRAS, KRAS, and PTPN11 MAPK pathway activating mutations (known to cause gilteritinib resistance) lowered CRc and OS rates as compared to the patients without these mutations.[123] In a retrospective study, 239 FLT3-mutated AML patients were exposed to sequential FLT3i and CRc rates dropped progressively and CRc rates were higher in the patients treated with sequential FLT3i exposure compared to those of FLT3i-monotherapy.[124]

The older or unfit adults who are not eligible for intensive chemotherapy have benefited from the combinations of FLT3i with low-intensity chemotherapy. Sorafenib or midostaurin plus azacitidine was found to be safe and feasible for those patients.[99] It was shown that naive FLT3-mutated patients had the greatest benefits from the combination of midostaurin and 5-azacitidine.[125] The recent study with the data from the previous clinical trial (NCT02993523) including the naive and ineligible patients (age ≥75 years and/or with comorbidities) treated with venetoclax plus azacitidine or placebo plus azacitidine groups analyzed the impact of FLT3 mutation on therapy outcomes. CRc rates were 67% and 36% and median OS was 12.5 and 8.6 months for FLT3-mutated patients in venetoclax plus azacitidine and azacitidine groups, respectively. Patients with FLT3 mutations and WT-FLT3 had similar outcomes when treated with venetoclax plus azacitidine.[126] Low-dose cytarabine with or without quizartinib in older FLT3-mutated AML patients not fit for intensive chemotherapy had improved survival. Median OS was 13.7 months compared with 4.2 months with low-dose cytarabine alone.[127] Based on the overall opinion from the mentioned studies, a new study analyzed the effects of triplet combination including HMA +venetoclax+FLT3i on older/unfit patients as frontline therapy, resulting in higher CR (67% vs 32% in triplet arm compared to doublet arm (HMA plus FLT3i).[128] A phase II trial including 13 newly diagnosed patients (older than 60 years) or 12 R/R (older than 18 years) FLT3-mutated AML patients (without prior venetoclax exposure, some with prior FLT3i use and allo-

SCT), decitabine was combined with venetoclax and an FLT3i (10 patients with gilteritinib, 10 sorafenib, and 5 midostaurin). This study revealed that even FLT3-mutated patients with prior FLT3i treatment achieved durable remissions and underwent allo-SCT consolidation after low-intensity triplet therapy.[129] In conclusion, the combination of low-intensity therapy (±low dose HMA or chemotherapy±venetoclax) with FLT3i could be the best option for the patients with FLT3-mutated AML who are not eligible for intensive chemotherapy even though the combination of FLT3i with intensive chemotherapy in fit/young patients could be the preferred option.[130]

All mentioned key FLT3i trials in this review highlighted the impact of FLT3i in the context of allo-SCT. The presence of an FLT3i arm could make it possible to proceed to an allo-SCT either as consolidation or maintenance therapy even for older/unfit patients. Among the aforementioned FLT3is, sorafenib seems to be the most promising one as post-allo-SCT maintenance therapy in FLT3-mutated patients.[103,108,116]

The safety profile should be carefully monitored to decide on the proper FLT3i alone or in combination protocols. Common adverse effects of midostaurin include QTc prolongation, skin rash, and myelosuppression, which are all manageable as compared to chemotherapy except for skin rash. However, its interaction with azole derivatives has been shown to cause serious side effects such as an unpredictable increase in plasma dose level and pulmonary toxicity, which should be kept in mind while evaluating the patients' need for azoles. Gilteritinib has a good safety profile with mild to severe alanine transaminase (ALT) and aspartate transaminase (AST) levels, QTc prolongation, and myelosuppression, which could be manageable with temporary drug suspension and dose reductions. Quizartinib also caused mild myelosuppression, gastrointestinal side effects, and QTc prolongation, which could need drug suspension and dose reductions.[107,115,131]

Development of Resistance Toward FLT3 Inhibitors: Possible Mechanisms and Overcoming Strategies

Primary and secondary resistance against FLT3i in mono- and combination therapies remains a significant obstacle for successful and long-lived remission. Primary resistance (innate resistance) restricts the efficacy of FLT3i at initial administration, whilst secondary resistance (acquired resistance) emerges during continuous exposure to FLT3i during treatment cycles, resulting in R/R disease.[132] While primary resistance is rarely observed in FLT3-mutated AML patients, the likelihood of secondary resistance development towards FLT3i seems a major contribution. Mechanisms involved in resistance could be heterogenous and grouped as FLT3-dependent and FLT3-independent resistance mechanisms, which are not required to be equally or mutually present.[132,133]

FLT3-Dependent Resistance Mechanisms

The presence or emergence of FLT3-TKD or FLT3-ITD-TKD (compound) mutations before or during FLT3i treatment causes the development of resistance against FLT3i therapy. The gatekeeper mutation, F691L, in the TKD is the common one that led to resistance against all clinically used FLT3is.[132,133] Gilteritinib was shown not to be effective in patients carrying F691L mutation who enrolled in the ADMIRAL study.[121] While the majority of FLT3 inhibitors are effective against FLT3-ITD, especially type II inhibitors are ineffective against TKD mutations. Therefore, secondary point mutations occurring in FLT3-ITD during treatment might play a role in gaining resistance, thus rendering their original effect. Quizartinib-treated FLT3-ITD AML patients developed resistance due to the emergence of mutations at D835 and Y842 residues as well as F691 in the TKD.[51] FLT3-ITD AML patients developed resistance against midostaurin via having N676K mutation in TKD.[134] Association of these mutations with resistance development could be explained by their effects on drug binding, stabilization of active receptor conformation, and receptor activity or activation of downstream signaling pathways.[135] For instance, F691L mutation prevents the binding of the drug to the receptor by assumably affecting several bonds, since the motions of the inhibitor are in correlation with those of the Phe 691 residue.[136,137] An integrated computational approach investigated the possible mechanism of quizartinib resistance via F691 gatekeeper mutation by comparing the receptor–inhibitor interactions between FLT3 kinase domain (wild-type or F691L) and quizartinib or PLX3397 (its activity is not affected by F691L mutation). When quizartinib was bound to FLT3-FL691L, the conformational change of αC-helix and A-loop of the FLT3 protein could be induced, rendering functional receptor structure. Additionally, quizartinib dissociated more easily from FLT3-F691L than from FLT3-WT,

which made quizartinib stay shorter in the mutated receptor. In contrast, there was no significant difference between the dissociation processes of WT-FLT3 and FLT3-F691L from PLX3397.[138] D835F and Y842H mutations in TKD were thought to make quizartinib and sorafenib ineffective.[51,139] In a particular study, multiple simulations of WT- and mutant (D835F, Y842H) FLT3 in drug-bound, drug-free, inactive or active forms were investigated.[137] These mutations might shift the equilibrium towards the active state of the receptor without affecting drug–receptor interactions directly based on the results of fully atomistic molecular dynamics (MD) simulations of FLT3.[137] M664I, D835N, and Y842S mutants in FLT3-TKD were highly active with enhanced autophosphorylation capacity and quizartinib-bound inactive molecules had many conformational alterations resulting in ineffective inhibition.[140] FLT3-N676K mutation resulted in increased surface expression in Ba/F3 cells transfected with FLT3 N676K mutant compared to D835Y and ITD, but it was similar to WT-FLT3.[141] It was found that FLT3-N676K mutation induced the phosphorylation of FLT3, MAPK, and AKT strongly compared to FLT3-ITD mutation.[141,142] Leukemic cells carrying the FLT3-N676K mutant in the absence of an ITD mutation were highly sensitive to FLT3 inhibitors such as quizartinib and sorafenib.[142,143] Therefore, this particular mutation seems to have a leukemogenic potential based on in vitro cell lines and in vivo mice studies. On the other hand, the FLT3-ITD-N676K compound mutation was predicted to inhibit the auto-inhibitory function of FLT3 by reducing the stability of the JM domain, resulting in midostaurin resistance.[134] A novel gatekeeper mutation, N701K was detected in gilteritinib-resistant FLT3-ITD cell lines by sterically interfering with the binding of gilteritinib.[144]

The modulation of FLT3-ITD localization is considered as one of the on-target mechanisms behind FLT3i resistance. WT-FLT3 is mainly localized to the cell membrane while FLT3-ITD localization is mainly restricted to ER/GA due to the impaired post-translational glycosylation of FLT3-ITD caused by its constitutive kinase activity.[145] The intracellular localization of FLT3-ITD activates predominantly STAT5 signaling while the one localized to the cell membrane predominantly activates AKT and MEK.[43] Therefore, FLT3-ITD localized to both ER and cell membrane cooperates in cellular transformation. The impairment of FLT3-ITD maturation via inhibiting N-glycosylation could be effective and synergistic with FLT3i. Tunicamycin, an N-glycosylation inhibitor, inhibited the proliferation and induced apoptosis of FLT3-ITD expressing human and murine cell lines via partly inhibiting FLT3-ITD activated AKT and ERK signaling and its combination with quizartinib showed synergistic effects.[146] A recent study also revealed the importance of different localization of FLT3-ITD mutant variants associated with the FLT3i resistance, which could be overcome by combining the inhibitors of N-glycosylation with distinct downstream signaling pathways' inhibitors.[147] FLT3-ITD was found to be S-palmitoylated by the palmitoyl acyltransferase ZDHHC6, which could be responsible for its localization to ER. The disruption of S-palmitoylation localized FLT3-ITD to the plasma membrane and activated AKT and ERK in addition to STAT5 and induced the progression of leukemia in a mice model. Inhibition of FLT3-ITD palmitoylation synergized with gilteritinib, which impaired the growth of primary FLT3-ITD$^+$ AML cells.[148]

Surface expression of FLT3 due to its lower turnover rate and increased half-life was detected in MOLM-13 FLT3-ITD AML cells with acquired midostaurin resistance.[149] Majority of FLT3-mutated AML has both WT-FLT3 and mutant-FLT3 expression and this heterogenicity could be responsible for limited response to FLT3i. In a study, the effects of quizartinib and sorafenib were decreased in 32D cells co-expressing WT- and FLT3-ITD as compared to 32D cells with only FLT3-ITD expression,[150] which is partially explained by the activation of WT-FLT3 by FLT3 ligand secreted by stromal cells.

FLT3-Independent Resistance Mechanisms

Additional mutations in the FLT3 receptor account for a small proportion of resistant cases. Studies elucidating FLT3-independent resistance mechanisms such as the impact of the tumor microenvironment, metabolism of FLT3i, and modulation of alternative intracellular signaling pathways have been paid attention.[151]

Bone marrow stromal cells with elevated CYP3A4 expression, a cytochrome P450 enzyme, impaired the activity of sorafenib, quizartinib, and gilteritinib in FLT3-ITD-positive AML by reducing the plasma concentrations of the inhibitors.[152] Another resistance-causing mechanism in the bone marrow microenvironment is the release of fibroblast growth factor 2 (FGF2) from the stromal cells, which caused quizartinib resistance in patients.[153] Pim kinase-2 overexpression was detected in sorafenib-resistant FLT3-ITD AML patients compared to untreated samples at the time of diagnosis, which resulted in sequestration of anti-apoptotic BAD in the cytoplasm and suppression of apoptosis.[154]

AXL kinase expression and activation increased in midostaurin and quizartinib-resistant primary blasts from FLT3-ITD AML patients by constantly activating the RAS/MAPK and PI3K/AKT/mTOR pathways.[155] In in vitro models of midostaurin and sorafenib resistance, cytokine CCL5 was found to be elevated, which also increased phosphorylated AKT and STAT5 levels.[156] Also, midostaurin-resistant blast cells from the patient showed increased CCL5 transcript levels. Activating JAK mutations were identified in both in vitro models (cell lines) and patient samples of FLT3-ITD AML with midostaurin or sorafenib resistance. JAK1 V658F mutation specifically was found to activate CSF2RB-JAK-STAT5 pathway to eliminate the effects of FLT3i.[157]

In a recent study, it was shown that actin filaments, one of the cytoskeletal components, are remodeled in a RAC-1-dependent manner, causing the development of midostaurin resistance in FLT3-ITD AML cell lines and primary patient samples. Anti-apoptotic BCL-2 expression and activity increased as a result of RAC-1 activation.[158] Early resistance and late resistance mechanisms were well defined in gilteritinib-resistant cell lines and patient samples by using whole-exome sequencing, CRISPR-Cas9, metabolomics, proteomics, and pharmacologic approaches.[159] Early resistant cells were protected by the bone marrow microenvironment to evolve into late resistant cells with newly gained intrinsic alterations. Late resistant cells were derived from the subclones already carrying NRAS mutations. Aurora kinase B was activated in early resistant cells while both early and late resistance cells underwent metabolic reprogramming. Activating mutations in RAS/MAPK pathway were also shown in gilteritinib-resistant FLT3-ITD AML patients using targeted-next generation sequencing (NGS).[160] NGS data from bone marrow samples of FLT3-ITD AML patients collected after type I or type II FLT3i treatment (secondary resistance group) showed mutational differences at relapse.[161] Detected mutations against FLT3i were found in epigenetic modifiers such as DNMT3A and RAS/MAPK pathway in addition to FLT3-D835 mutation. The most common secondary mutation was in RAS/MAPK pathway against type I FLT3i while FLT3-D835 was the most emerged mutation against type II FLT3i.[161] FLT3-ITD cell lines with acquired sorafenib resistance and primary patient samples from sorafenib-resistant FLT3-ITD AML showed activation of PI3K/mTOR pathway.[162] Clonal evaluation of midostaurin resistance in patients in RATIFY trial revealed different molecular profiles at the time of diagnosis and relapse. Midostaurin resistance emerged due to acquired mutations in signaling pathways such as MAPK while they became FLT3-ITD negative based on whole genome sequencing.[163] Transcriptome analysis of the samples from patients with FLT3-ITD/D835 mutations in comparison to those with FLT3-ITD only revealed the overexpression of anti-apoptotic BCL2A1 in FLT3-ITD/D835 patient samples, which was associated with decreased quizartinib sensitivity. The combination of quizartinib with venetoclax specifically in FLT3-ITD/D835 cell lines showed that the presence of this compound mutation could be responsible for quizartinib and venetoclax resistance.[164] MCL-1, an anti-apoptotic BCL-2 family member was shown to be upregulated in FLT3-ITD AML through STAT5 activation,[165] and responsible for leukemia relapse, poor therapeutic outcomes, and venetoclax resistance.[166–168] Hence, several MCL-1 inhibitors such as VU661013, S63845, and MIK665 have been developed. MCL-1 inhibition together with BCL-2 inhibition could show synergistic effects and overcome venetoclax resistance.[168,169] There are recruiting clinical trials using the combination of S64315 with venetoclax (NCT03672695) and the combination of a novel BCL-2 inhibitor S65487/VOB560 with an MCL-1 inhibitor MIK665 (NCT04702425) in R/R FLT3 AML. In FLT3-ITD AML models, multikinase inhibitor olverembatinib (HQP1351) induced apoptosis via MCL-1 downregulation which was enhanced in the presence of BCL-2 inhibitor lisaftoclax (APG-2575). The elevated expression of the TEK-family kinase, BMX, in gilteritinib-unresponsive patients was detected using single-cell RNA sequencing, which mediated gilteritinib resistance via upregulation of cell-cycle, DNA/RNA metabolic processes, and protein translation.[170]

Overall, these studies underlined the importance of differences in resistance profiles of type I and type II inhibitors to design an appropriate treatment strategy after identifying the genetic makeup of each patient.

Approaches to Overcome FLT3i Resistance

Common strategies to overcome FLT3i resistance could be discussed under two general groups, including the development of novel FLT3is and dual-inhibitors and combinational approaches with the inhibitors of altered signaling molecules, anti-apoptotic molecules, epigenetic targets, or other molecular targets (Figure 3). Ongoing clinical trials of novel treatment strategies in FLT3 positive AML including novel FLT3is, dual FLT3 inhibitors, FLT3 antibody, CAR-T cell therapy or novel targeted therapies beyond FLT3is are summarized in Table 3.

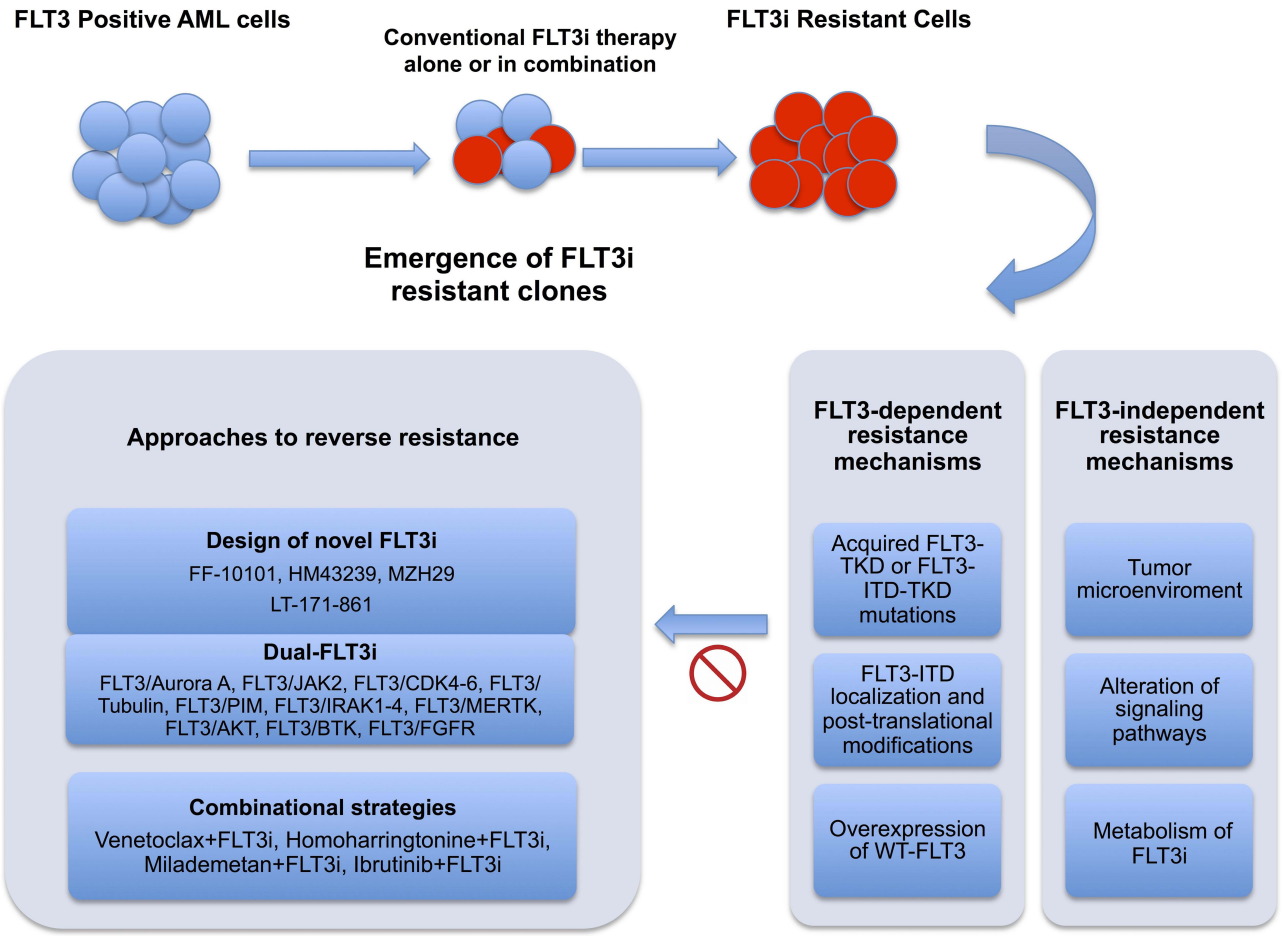

Figure 3 Selected FLT3i resistance mechanisms and strategies to overcome resistance.

Designing Novel FLT3i

Secondary mutations against type II FLT3is tend to occur on FLT3 itself while FLT3-independent resistance commonly occurs against type I FLT3is based on the results from previously discussed pre-clinical and clinical studies. However, the F691L mutation is still responsible for resistance to all clinically approved FLT3is. Hence, the development of novel FLT3is could be a possible way to overcome such resistance.

FF-10101 is a newly designed type I FLT3i, which binds covalently to C695 residue on FLT3 in an irreversible manner. It binds to both active and inactive FLT3 and is active against various FLT3 mutations, including FLT3-ITD, D835, Y842, and even F691L mutations.[171] FF-10101 is recently shown to reduce bone marrow stromal cell-mediated protection of FLT3-ITD AML observed in resistance against other FLT3is such as quizartinib.[172] FF-10101 is currently evaluated in phase I/IIa studies to identify its pharmacokinetics, toxicity profile, and safety in R/R AML (NCT03194685). HM43239, a novel reversible small-molecule type I FLT3 inhibitor with activity against WT-FLT3, FLT3-ITD, FLT3-TKD, and also FLT3 ITD/TKD double mutations showed in vitro (WT-FLT3 and mutant FLT3 cell lines) and in vivo (FLT3 ITD/TKD double mutated xenograft mouse model) efficacy via inhibiting STAT5, ERK, SYN, JAK1/2, and TAK1 kinases, which resulted in a currently recruiting phase I/II clinical trial (NCT03850574) to evaluate its appropriate doses and safety in R/R FLT3 AML.[173] MZH29, a novel FLT3i, showed inhibitory activity against WT, FLT3-ITD, FLT3-D835H/Y/V, and FLT3-K663Q mutants and FLT3-ITD/F691L double mutation.[174] LT-171-861, a novel FLT3i, bound to FLT3 strongly and inhibited the growth of FLT3 mutant cell lines such as FLT3-D835Y, FLT3-ITD-N676D, FLT3-ITD-D835Y, FLT3-ITD-F691L, FLT3-ITD-Y842C and AML blasts from patients with FLT3-ITD. In vivo effects also led to tumor regression and prolonged survival.[175]

Table 3 Ongoing Clinical Trials of Novel Treatment Approaches in FLT3 Positive AML

Therapeutic Approach	Patient Group	Phase	Clinical Trial Number
Azacitidine plus Venetoclax (BCL-2 inhibitor)	Previously untreated AML patients who are unsuitable for treatment	Phase III	NCT02993523
S64315 (MCL-1 inhibitor) + Venetoclax	Relapsed or refractory AML patients	Phase I	NCT03672695
VOB560 (BCL-2 inhibitor) + MIK665 (MCL-1 inhibitor)	Relapsed or refractory patients with NHL, MM and AML	Phase I	NCT04702425
FF-10101-01 (novel FLT3i)	Patients with refractory or relapsed AML and who are not eligible for any treatment	Phase I/II	NCT03194685
HM43239 (novel FLT3i)	Refractory or relapsed AML patients	Phase I/II	NCT03850574
SEL24/MEN1703 (Dual FLT3/PIM kinase inhibitor)	Refractory or relapsed AML patients who cannot receive any approved treatment	Phase I/II	NCT03008187
CG-806 (Luxeptinib) (Dual FLT3/BTK kinase inhibitor)	Patients with refractory or relapsed AML or high risk MDS and intolerant of chemotherapy or transplantation	Phase I	NCT04477291
MAX-40279-01 (FGFR/FLT3 dual inhibitor)	Relapsed or refractory AML patients	Phase I	NCT04187495
Ibrutinib (BTK inhibitor) with chemotherapy in the absence or presence of an FLT3i	Relapsed or refractory AML patients with FLT3 mutation	Phase II/III	NCT03642236
IMC-EB10 (anti-FLT3 antibody)	Relapsed or refractory AML patients	Phase I	NCT00887926
FLYSYN (Fc-optimized FLT3 antibody)	AML patients with minimal residual disease	Phase I/II	NCT02789254
AMG 553 (FLT3 CAR-T)	Relapsed or refractory AML patients with FLT3 mutation	Phase I	NCT03904069

Abbreviations: NHL, non-Hodgkin lymphoma; MM, multiple myeloma; MDS, myelodysplastic syndrome.

Rationalized design of dual inhibitors targeting both FLT3 and another target such as cyclin-dependent kinase 4/6 (CDK4/6), JAK, Aurora A, tubulin, and PIM is thought to reverse resistance by simultaneously inhibiting two or more signaling pathways.[176] CCT24571, a dual FLT3/Aurora A inhibitor showed cytotoxic and apoptotic effects on FLT3-ITD carrying MOLM-13 and MV4-11 AML cells and reversed D835Y resistance in vitro.[177] Another FLT3/Aurora A inhibitor, CCT241736, inhibited tumor growth of FLT3-ITD and FLT3-ITD-TKD human tumor xenograft models and also showed efficacy in primary patient samples with quizartinib resistance.[178] AMG925, a novel dual inhibitor of FLT3/CDK4-6 induced apoptosis in both WT and mutant FLT3 AML cell lines and primary blasts.[179] KX2-391 is a recently identified dual FLT3/Tubulin inhibitor with promising resistance-reversion activity against FLT3-ITD and FLT3-ITD-D835/F691 mutation in in vitro cell lines, a murine model, and patient blasts.[180] Inhibition of JAK2 and FLT3 at the same time could be a possible strategy to eradicate resistant cells with FLT3 mutations. Momelotinib, a potential dual FLT3/JAK2 inhibitor, gave better responses against D835, D839, and Y842 mutations and growth factor-mediated resistance in mouse and human primary cells expressing FLT3-ITD.[181] The dual JAK/FLT3 inhibitor pacritinib in combination with chemotherapy showed clinical activity in FLT3-ITD and FLT3-TKD AML patients.[182] Dual targeting of FLT3 and PIM kinase by SEL24 showed anti-proliferative and apoptotic activities against MOLM-13, MV4-11 FLT3-ITD positive cells, and primary FLT3-ITD cells compared to FLT3 inhibitor or PIM kinase inhibitor alone. Its activity was not lowered by FTL3-ITD, certain FLT3-TKD, or FLT3-ITD-TKD mutations.[183] A dose escalation trial for SEL24 (NCT03008187) is recruiting in R/R FLT3 AML with no available treatment strategy.

Innate immune pathway activation via the interleukin-1 receptor-associated kinase 1 and 4 (IRAK1/4) complex contributed to adaptive quizartinib resistance in FLT3-mutant AML cells via restoring RAS/MAPK signaling along with NF-κB, which could be overcome by a small molecule-dual inhibitor, NCGC1481, targeting both FLT3 and IRAK1/4

kinases.[184] MRX-2843, a dual MERTK/FLT3 inhibitor, showed activity against quizartinib-resistant FLT3-ITD-D835 or F691 mutants and prolonged survival in xenograft models of quizartinib-resistant AML.[185] A674563, a dual inhibitor targeting both FLT3-ITD and AKT was active against FLT3-D835Y mutant-expressing cells and could overcome FLT3 ligand-induced drug resistance.[186] CG-806, dual BTK (Bruton's tyrosine kinase)/FLT3 inhibitor is being evaluated in a trial (NCT04477291) including R/R patients or ineligible patients for other treatments. FGFR/FLT3 dual inhibitor, MAX-040279, is under evaluation in a Phase I (NCT04187495) study for R/R FLT3 AML due to the role of FGF2/FGFR signaling in FLT3i resistance.

To sum up, there are many newly designed FLT3is or dual inhibitors to reduce drug resistance and increase responses. However, most novel FLT3is and dual-target inhibitors are only tested in pre-clinical studies at present or some of them are undergoing early-stage of phase studies.

Novel FLT3i Combinatorial Treatment Approaches

Apart from combinations of FLT3i with conventional chemotherapy as mentioned in critical trials, inhibiting altered signaling pathway molecules or other players could be synergistically effective in FLT3i-resistant FLT3-ITD AML.

MEK inhibitor trametinib combined with midostaurin had a synergistic effect to overcome midostaurin resistance in FLT3 mutated AML.[187] The combination of venetoclax, a BCL-2 inhibitor, with midostaurin or gilteritinib showed synergism in MOLM-13 and MV4-11 FLT3-ITD AML cell lines.[188] MOLM-13 xenograft treated with gilteritinib plus venetoclax had improved survival compared to gilteritinib alone. Simultaneous downregulation of MCL-1 by midostaurin or gilteritinib and inhibition of BCL-2 by venetoclax made BIM free, resulting in synergistic induction of apoptosis in FLT3-ITD AML cell lines and patient samples.[189] Cotreatment with venetoclax and quizartinib had greater anti-leukemic activity in pre-clinical models of FLT3-ITD AML and a patient-derived FLT3-ITD AML xenograft model.[190] There is a recently completed Phase IB/Phase II trial investigating the side effects and appropriate dose of venetoclax in combination with quizartinib in R/R FLT3-ITD AML patients (NCT03735875). Triple combination including decitabine (DNA methyltransferase inhibitor) + venetoclax + quizartinib was shown to be highly active in R/R FLT3-ITD mutated AML patients, with CR rates of 69% and the median OS of 7.1 months, which is under clinical phase I/II study (NCT03661307).[191] The combination of gilteritinib with venetoclax specifically in FLT3-ITD/D835 cell lines had synergistic effects in contrast to quizartinib plus venetoclax via downregulating MCL-1.[164] Homoharringtonine, as a STAT inhibitor, was evaluated in vitro and in vivo in combination with sorafenib, quizartinib, and gilteritinib in FLT3-ITD AML. Sorafenib in combination with low-dose homoharringtonine induced synergism in an R/R FLT3-AML patient, which is evaluated in phase II trial (NCT03170895) in R/R FLT3-ITD AML.[192] Homoharringtonine plus quizartinib triggered apoptosis via upregulating BIM and BAX and downregulating MCL-1 in FLT3-ITD AML cell lines and increased OS in mice model.[193] The combination of homoharringtonine with gilteritinib also decreased MCL-1 by UBE2L6-mediated proteasomal degradation.[194] There is a phase II trial (NCT03135054) evaluating if quizartinib plus omacetaxine mepesuccinate (homoharringtonine) results in durable CRc in patients with newly diagnosed or R/R AML carrying FLT3-ITD. Activating JAK mutation, JAK1 V658F, was related to midostaurin resistance and combination of JAK1/2 inhibitor, ruxolitinib, with midostaurin was able to sensitize FLT3-ITD AML resistant cells to midostaurin.[157] Inhibition of RAC-1 or BCL-2 using pharmacological inhibitors together with midostaurin overcame midostaurin resistance.[158] BTK is identified as a novel target in FLT3-ITD AML patient blasts and cell lines. Inhibition of BTK by ibrutinib blocked the survival and proliferation of FLT3-ITD primary AML blasts and AML cell lines by inhibiting MAPK, AKT, and STAT5.[195] Ibrutinib might specifically target FLT3-ITD in addition to BTK via decreasing FLT3-ITD autophosphorylation. c-MYC expression and STAT5 phosphorylation were also decreased in response to ibrutinib in FLT3-ITD AML cell lines and it showed synergistic or additive effects in combination with FLT3i.[196,197] There is a phase II/III trial (NCT03642236) accepting registration to investigate the efficacy and safety of the combination of BTK inhibitor, ibrutinib with chemotherapy with/without FLT3 inhibitor in refractory/relapsed FLT3 mutant AML. The combination of MDM2, a negative regulator of the tumor suppressor p53, inhibitor milademetan with quizartinib showed synergistic apoptotic effects in FLT3-ITD positive and p53 WT AML cell lines and murine cell lines with FLT3-ITD +F691L and D835Y mutations via suppression of MCL-1 and upregulation of p53, PUMA and p21. In vivo mice model treated with this combination had prolonged survival.[198] Milademetan (MDM2 inhibitor) plus quizartinib combination

study in FLT3-ITD mutant AML patients is recently completed (NCT03552029, results not shared yet). MDM2 inhibitor NVP-HDM201 and midostaurin combination showed synergistic effects in AML cells with high FLT3-ITD AR and WT TP53 and NPM1,[199] which is under a phase I trial (NCT04496999). The combination of gilteritinib with a pharmaceutical inhibitor of the NFKB family inhibited the secretion of tumor-promoting cytokines from gilteritinib-treated leukemic blasts.[200] Apoptosis induced in FLT3-ITD AML cell lines and primary patient samples treated with gilteritinib in combination with CUDC-907, a dual inhibitor of PI3K and histone deacetylases via FLT3 downregulation, inhibition of MAPK/ERK and JAK/STAT pathways, reduction of MCL-1 and c-MYC and induction of BIM.[201] Protein arginine methyltransferase 1 (PRMT1) was upregulated and defined as an important target involved in the maintenance of FLT3-ITD$^+$ AML. PRMT1 methylates FLT3-ITD at R972/973 residues which enhanced Y969 phosphorylation to recruit downstream SH2 domain-containing adaptor molecules. Thus, R972/973 methylation promoted STAT5 and AKT phosphorylation. Inhibition of PRMT1 using MS023 in combination with quizartinib enhanced the elimination of FLT3-ITD cells.[202] Translation initiation factor eIF4a was inhibited with rohinitib (RHT) via downregulation of transcription factor heat shock factor 1 (HSF1) in FLT3-ITD AML cells, resulting in an anti-leukemic effect. Knockdown of HSF1 sensitized FLT3-mutant AML cells with both ITD and TKD mutations to clinical FLT3i.[203]

Targeted FLT3 AML Therapeutics Beyond FLT3 Inhibitors

Even though there are small FLT3is with FDA approval such as midostaurin and gilteritinib, they have their own limitations for effective treatment such as drug resistance, toxicities, limited and short-lived responses. Therefore, there is still a need to search for novel treatment modalities for FLT3-ITD AML including immunotherapy, small-molecule FLT3 degraders, and flavonoids.

FLT3 Antibodies

Monoclonal antibodies (mAb) with high specificity and affinity for FLT3 have been developed and their effects have been evaluated in pre-clinical and early clinical phase studies. IMC-EB10, a human anti-FLT3 antibody, targeted both WT-FLT3 and FLT3-ITD mutant in a ligand-dependent (via blocking the binding of FLT3 L to FLT3) and -independent manner and inhibited downstream signaling pathways such as MAPK and AKT in both cell lines and mice model.[204] IMC-NC7 is another human anti-FLT3 antibody sharing similar working mechanism with IMC-EB10.[205] In this case, IMC-EB10 initiated antibody-dependent cell-mediated cell toxicity in FLT3 expressing cells and decreased engraftment of FLT3 expressing cells into non-obese diabetic/severe combined immunodeficient mice more effectively.[205] Although a phase I study (NCT00887926) was initiated based on this result, it is recently terminated due to lack of efficacy in 26 registered R/R AML patients. 4G8SDIEM is the first reported Fc-optimized antibody targeting FLT3 and induced cellular toxicity on both FLT3 expressing cell lines and AML blasts,[206] which led to the current phase I/II clinical trial (NCT02789254) for AML patients with minimal residual disease (MRD). To target a wider population of AML patients, an immunoglobulin G-based bispecific antibody (7370) with an affinity for both FLT3 and CD3 has been developed recently.[207] It was shown to have a long half-life and target FLT3 irrespective of mutation profile in AML blasts and it gave promising results in cynomolgus monkeys via inducing depletion of peripheral FLT3$^+$ dendritic cells and bone marrow FLT3$^+$ stem cells and progenitors. CLN-049, a CD4$^+$ and CD8$^+$ T cell activating bispecific antibody targeting FLT3 and CD3 is just developed.[208] In mouse xenograft models established using human leukemic cell lines and patient-derived AML blasts, CLN-049 was highly active.

T-Cell Based Approaches

Autologous T cells could be modified and given back to the patient to target FLT3-ITD with increased specificity for cancer cell killing. The most common strategy is chimeric antigen receptor (CAR) T cell therapy for FLT3 positive AML.

CARs have an extracellular domain to recognize cancer-specific antigens and an intracellular signaling domain for T cell proliferation and activation to kill cancer cells.[209] T cells from healthy donors were engineered to recognize FLT3 with CD28 co-stimulatory signaling domain and CD3ζ activation domain.[210] Its cytotoxicity was evaluated in FLT3 expressing cell lines including FLT3-ITD positive MOLM-13 and MV4-11 cells and WT-FLT3 expressing cells EOL1. FLT3 CAR T cells killed the FLT3 expressing cells and secreted IFN-γ and IL-2. Similar results were also obtained using

FLT3 expressing AML blasts. In vivo xenograft mice models established via engraftment of MOLM-13 cells and FLT3 positive AML patient blasts, FLT3 CAR T cells showed anti-leukemic activity with 100% survival rate compared to the controls.[210] There is a phase I study evaluating the safety, tolerability, and efficacy of FLT3 CAR T cell therapy (AMG 553) in FLT3 R/R AML (NCT03904069).[211]

FLT3 L CAR T cells were constructed using FLT3 L as an extracellular recognizing domain with 4–1BB and CD3ζ intracellular signaling domains.[212] FLT3 L CAR T cells were co-cultured with FLT3 positive cell lines and patient blasts and showed cytotoxicity via secreting IFN-γ and TNF alpha. In vivo xenograft model treated with FLT3 L CAR T cells had prolonged survival. Newly designed FLT3 L CAR T cells with a combined ICOS and 4–1BB co-stimulatory domain and a CD3ζ activating domain were effective against WT-FLT3 carrying THP-1 cells.[213] The combination of FLT3 CAR T cell therapy with FLT3i, midostaurin or quizartinib, showed promising results.[214] These FLT3is increased surface expression of FLT3, then FLT3 CAR T cells recognized MOLM-13 and MV4-11 FLT3-ITD cells effectively and improved response rate in mice model. There is a pre-clinical study investigating the effect of allogeneic FLT3 CAR T cell therapy with a rituximab on-off switch mechanism to eliminate some challenges observed in autologous therapy such as limited or nonfunctional peripheral T cells in patients after treatment, which renders effective production of patient-based T cells for CAR T therapy.[215] This approach resulted in promising results in vitro and in vivo, however, there was also the elimination of human HSC and progenitor cells leading to myelotoxicity. In this case, rituximab switch was useful to remove FLT3 CAR T cells after depleting AML blasts and allowing bone marrow to recover. The synergistic effect of dual-target FLT3 single-chain fragment variable (scFv)/NKG2D (Natural killer group 2 member D ligands) CAR T cells with gilteritinib associated with the lysis of R/R FLT3-ITD AML cell lines and mouse model. Gilteritinib increased the efficacy of bispecific CAR T cells via increasing the expression of NKG2DL.[216]

Novel Immunotargets in FLT3 AML

Higher expression of the IL3 receptor α-chain (CD123) and MIC-2 (CD99) in combination with the IL2 receptor α-chain (CD25) within the $CD34^+$ cell population was detected in minor FLT3-ITD subclones at diagnosis and also in 83% of cases with FLT3-ITD relapse. The presence of the CD34/CD25/CD123/$CD99^+$ population was significantly associated with ITD mutation in the FLT3 gene, which could be the LSCs.[217,218] A higher FLT3-ITD load was observed within CD34/CD123/CD25/$CD99^+$LSCs. Treatment with an anti-CD99 mAb was cytotoxic on LSCs in two patients, whereas there was no effect on $CD34^+$cells from healthy donors.[219] Anti-CD99 mAb showed more apoptotic effects on FLT3-ITD AML patient samples and cell lines compared to those expressing WT-FLT3 via upregulating both intrinsic and extrinsic pathways of apoptosis with a specific emphasis on the p53-mediated pathway. CD99 targeting also reduced glycolysis and mitochondrial respiration.[220] Targeting CD33 with gemtuzumab ozogamicin (GO), a calicheamicin-conjugated mAb, in combination with induction chemotherapy in pediatric patients with high FLT3-ITD AR reduced relapse.[221] An active clinical trial (NCT04385290) is investigating the safety and efficacy of midostaurin plus GO as frontline therapy in newly diagnosed FLT3 mutated patients. The role of programmed cell death 1 (PD-1) and PD-1 ligand (PD-L1) is not clear for AML; however, the high expression of PD-L1 could be responsible for worse prognosis in NPM1/FLT3 double mutant AML patients.[222] A clinical study of atezolizumab, PD-L1 targeting mAb in combination with gilteritinib in R/R FLT3 mutated AML is recently completed (NCT03730012). CSF2RB was phosphorylated directly by FLT3-ITD via direct interaction and its knockdown increased sensitivity against midostaurin via decreasing STAT5 phosphorylation, hence, targeting CSF2RB-FLT3-ITD interaction using small peptides could be a strategy to improve FLT3i' efficacy.[223]

Small-Molecule FLT3 Degraders

The first report about the degradation of FLT3 was the inhibition of HSP90, a molecular chaperone, in FLT3-ITD expressing leukemia cells, which proved FLT3-ITD as a client kinase for HSP90.[224] HSP90 inhibitor 17-AAG was cytotoxic to primary AML cells carrying FLT3 mutants, but not for WT-FLT3 via inhibiting JAK/STAT, MAPK, and PI3K/AKT pathways and 17-AGG dissociated FLT3-ITD from HSP90, hence inducing FLT3-ITD degradation.[225] The patients with FLT3-ITD expression also had high HSP90 levels to stabilize FLT3-ITD. HSP90 inhibition had a stronger pro-apoptotic effect on FLT3-ITD AML cells compared to those with WT-FLT3.[226] Inhibition of HSP90 in FLT3i resistant FLT3-D835Y and several FLT3-ITD/TKD mutants by HSP90 inhibitors geldanamycin 17-AAG or 17-DMAG

resulted in the degradation of mutant FLT3 in addition to FLT3-ITD and inhibition of STAT5 and ERK1/2.[227] c-Cbl and Cbl-b are reported as E3 ubiquitin ligases for FLT3-ITD and are involved in the degradation process via the ubiquitin-proteasomal pathway induced by 17-AAG.[228] A loss-of-function mutation in the E3 ligase domain of c-Cbl was found to eliminate its degradative function in a mice model established using HCS with FLT3-ITD mutation and c-Cbl mutation since mice developed AML.[229] The combination of 17-AAG and midostaurin showed high activity against AML cells with FLT3 mutations through downregulating FLT-3, *p*-FLT-3, *p*-AKT, *p*-ERK1/2, and *p*-STAT5 and inducing apoptosis.[230] Ba/F3 cells expressing FLT3-ITD+D835V cells were very sensitive to HSP90 inhibitors which resulted in degradation via autophagy. Quizartinib-resistant MV4-11 cells with FLT3-ITD+D835H and FLT3-ITD+D835V were also sensitive to HSP90 inhibitors.[231] USP10 was identified as an FLT3-specific deubiquitinase to stabilize FLT3, hence its inhibition by small USP10 inhibitors gave promising results in cell lines, patient samples, and a mouse model of FLT3-ITD AML.[232] A novel USP10, Wu-5, induced both WT-FLT3 and FLT3-ITD degradation and induced apoptosis.[233] HSP70 could be a new therapeutic target that was shown to interact with FLT3-ITD, leading to its stabilization. The inhibition of HSP70 by QL47 induced degradation in both FLT3-ITD and drug-resistant mutants including F691L, N676D, and D835Y.[234]

Proteasome inhibitor, bortezomib triggered apoptosis specifically in FLT3-ITD AML cell lines and patient samples compared to WT-FLT3 AML cells by down-regulating PI3K/AKT, STAT5, and MAPK/ERK and the degradation of FLT3-ITD was related to autophagy. Bortezomib treatment overcame acquired quizartinib resistance in MOLM-14 FLT3-ITD-D835Y double mutant.[235] Recently, proteaphagy, a degradation system activated after proteasome inhibition was identified in FLT3-ITD AML cells after bortezomib treatment via activation of autophagy, which suggested the inhibition of proteasome together with autophagy (using bafilomycin A) could be synergistic.[236] In a mouse model of FLT3-ITD AML, arsenic trioxide (ATO) induced autophagic degradation of FLT3-ITD, which resulted in decreases in leukemic burden.[237] Treatment with ATO resulted in the degradation of FLT3-ITD via partly decreasing its interaction with USP10, causing poly-ubiquitination and proteasomal degradation. ATO showed synergistic effects with sorafenib and quizartinib via inhibiting FLT3 autophosphorylation and downstream STAT5, AKT, and ERK signaling pathways.[238]

Polyphenols from green tea, (–)-epigallocatechin-3-gallate, (–)-epigallocatechin, and (–)-epicatechin-3-gallate, inhibited the proliferation and suppressed the FLT3 expression in FLT3 mutated cells which eventually led to inhibition of the downstream pathways such as PI3K, MAPK, and STAT5.[239] The reason behind the FLT3 suppression was the disruption of the interaction between of HSP90 and FLT3-ITD, resulting in degradation of FLT3-ITD. These flavonoids also induced apoptosis in FLT3 mutated cells synergistically in combination with midostaurin.[239] PROTAC, proteolysis targeting chimera, are bifunctional small molecules designed to bind target protein and E3 ubiquitin ligase simultaneously, which induce target protein ubiquitylation and then degradation by the proteasome.[240] Several PROTAC molecules were synthesized based on the binding model of dovitinib and FLT3 among which molecules 101 and 102 showed anti-proliferative effects against MOLM-13 and MV4-11 cells and induced the degradation of FLT3-ITD.[241] A promising PROTAC PF15 was synthesized recently and degraded FLT3 and inhibited downstream STAT5 signaling.[242] All degraders showed efficacy in vivo models. Quizartinib was converted into a PROTAC with more selectivity and enhanced apoptotic activity via FLT3-ITD degradation in both in vitro and in vivo models although its kinase inhibitory function was partly abolished.[243]

Flavonoids in FLT3 AML

Flavonoids are natural products commonly found in plants.[11] These compounds work as anti-oxidant, anti-bacterial, anti-viral, anti-inflammatory, and anti-cancer agents.[244] Only flavones and flavonols are suggested to be anti-cancer agents among flavonoids due to their ability to induce mitochondria-mediated apoptosis and suppress multiple signaling pathways including MAPK, PI3K, and NF-κB.[11] Hispidulin, luteolin, acacetin, and eupatin, subgroups of flavones and flavonols, are shown to have inhibitory properties towards FLT3.[245,246]

Natural chalcones were more potent toward FLT3-ITD cell lines and inhibited cell growth. All chalcones inhibited the FLT3 and reduced the phosphorylation of downstream pathways including STAT5 and ERK.[247]

O-methylated flavonol, a precursor of fisetin, inhibited the activity of FLT3-ITD and FLT3-D835Y kinases.[244] Moreover, FLT3-ITD MOLM-13 and MV4-11 cells, when treated with this flavonol, arrested cell cycle at the G0/G1 phase and activated apoptotic proteins such as PARP, caspase 3, and BAX.[244]

Isoliquiritigenin extracted from licorice root also demonstrated anti-cancer activity by targeting FLT3. The proliferation of FLT3-ITD AML MOLM-13 and MV4-11 cells was selectively inhibited by isoliquiritigenin. In vivo studies on isoliquiritigenin revealed that it inhibited tumor growth in the MV4-11 xenograft mouse model and extended the survival time. The inhibitory activity of isoliquiritigenin towards resistant FLT3-ITD/F691L cell line makes it a promising natural compound in FLT3-ITD mutant AML.[248]

Resveratrol, an anti-carcinogenic plant-derived polyphenol was shown to inhibit the proliferation of FLT3-ITD mutated MV4-11 and MOLM-13 cells via modulation of sphingolipid metabolism enzymes including anti-apoptotic sphingosine kinase-1 and glucosylceramide synthase and apoptotic serine palmitoyltransferase.[249,250] Although the studies investigating the roles of flavonoids in FLT3 positive AML are limited, natural products or their derivatives seem to possess the potential to develop new therapeutic agents targeting FLT3 or to be used as an integrative medicine in combination with FLT3i.

Conclusion

The discovery of FLT3 and its mutations has dramatically changed the AML course and treatment. The patients with FLT3 mutations have better options to be treated due to the approval of FLT3i in the clinical settings, which should be carefully selected based on patients' current health condition, previous treatment history, genetic characterization, and eligibility for induction, consolidation or maintenance therapy as discussed extensively. On the other hand, the emergence of primary and mainly secondary resistance is a major limiting factor for effective FLT3i treatment, therefore understanding the detailed molecular mechanisms behind resistance will obviously increase the design of novel FLT3i or dual FLT3i and combinational treatment strategies. Additionally, emerging treatments involve immunotherapeutics such as FLT3 targeted antibodies, FLT3-specific T cell therapy, and novel potential immunotargets such as CD99 and PD-L1 and small molecule FLT3 degraders including HSP90 and proteasome inhibitors and PROTACs which have resulted in some initial success in pre-clinical and early phase clinical studies. However, more mechanistic studies or larger randomized trials are still needed to prove the advantages of these novel treatment modalities over approved treatments. Their specificity, safety profile, and appropriate dose should be carefully discussed based on the data which will be accumulating in the future. It is also inevitable that the development of novel therapeutics after identifying specific molecular targets in FLT3-ITD AML will be shaping the treatment strategies. To be noted, flavonoids are being investigated for their potential as adjuvants in FLT3-mutated AML with increasing anti-leukemic potentials. Based on all the studies investigating the targeting of FLT3, it would be possible to find a more specific and durable FLT3-ITD therapy approach even for a specific group of patients in the near future most probably in combination with already approved therapies.

Abbreviations

FLT3, FMS-like tyrosine kinase 3; AML, acute myeloid leukemia; ITD, internal tandem duplication; TKD, tyrosine kinase domain; FLT3i, FLT3 inhibitor; WT-FLT3, wild-type FLT3; RTK, receptor tyrosine kinase; HSC, hematopoietic stem cell; LSC, leukemic stem cell; LC-HSC, long-term hematopoietic stem cell; GA, Golgi apparatus; ER, endoplasmic reticulum; A-loop, activation loop; FLT3 L, FLT3 ligand; OS, overall survival; DFS, disease free survival; JM, juxtamembrane domain; TKI, tyrosine kinase inhibitor; R/R, relapsed/refractory; CR, complete remission; CRc, composite complete remission; mCRc, modified composite complete remission; EFS, event-free survival; RFS, relapse-free survival; CDK4/6, cyclin-dependent kinase 4/6; MRD, minimal residual disease; CAR, chimeric antigen receptor; hTERT, telomerase reverse transcriptase; NHEJ, nonhomologous end-joining; NPM1, nucleophosmin 1; IDH, isocitrate dehydrogenase; ASXL1, additional Sex Comb-like 1;CEBPA, CCAAT/enhancer binding protein A; HMA, hypomethylating agent; SC, salvage chemotherapy; CBF, core binding factor; HLF, hepatic leukemia factor; ALT, alanine transaminase; AST, aspartate transaminase; FGF2, fibroblast growth factor 2; NGS, next generation sequencing; IRAK1/4, interleukin-1 receptor-associated kinase 1 and 4; HSF1, heat shock factor 1; mAb, monoclonal antibodies;

scFv, single-chain fragment variable; NKG2D, Natural killer group 2 member D ligands; GO, gemtuzumab ozogamicin; PD-1, programmed cell death 1; ATO, arsenic trioxide.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. Aysun Adan supervised the study.

Disclosure

The authors report no conflicts of interest in this work.

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