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Review

The molecular mechanisms behind activation of FLT3 in acute myeloid leukemia and resistance to therapy by selective inhibitors

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ABSTRACT

Acute myeloid leukemia is an aggressive cancer, which, in spite of increasingly better understanding of its genetic background remains difficult to treat. Mutations in the FLT3 gene are observed in ≈30% of the patients. Most of these mutations are internal tandem duplications (ITDs) of a sequence within the protein coding region, an activation mechanism that is almost non-existent with other genes and cancers. As patients each carry their own unique set of mutations, it is challenging to understand how ITDs activate the protein, and ascertain the risk for each individual patient. Available treatment options are limited due to development of drug resistance. Here, recent studies are reviewed that help to better understand the molecular mechanism behind activation of the FLT3 protein due to mutations. It is argued that difference in mutation sequences and especially location might be coupled to prognosis. When it comes to FLT3 inhibitors, key differences between them can be attributed to the mode of inhibition (type-1 and type-2 inhibitors), effective inhibitory coefficient in the blood plasma and off-target binding. Accounting for the position and length of insertions may in the future be used to predict prognosis and rationalise treatment. Development of new inhibitors must take into account the potential for resistance mutations. Inhibitors aimed at multiple specific targets are currently being developed. These, and as well as combination therapies will hopefully lead to longer periods during which targeted FLT3 therapy will remain effective.

1. Introduction

FMS-related tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase that plays a role in haematopoiesis. Overactivity of FLT3 appears to be a driving force in acute myeloid leukemia (AML). Indeed, about 30% of the patients carry activating mutations in FLT3 (for recent reviews about the role of FLT3 in AML from a clinical standpoint, see [1,2,3]). There are two types of such mutations. The most common type involves insertions of a sequence from the protein coding part of FLT3 in tandem, so that a part of the protein sequence is repeated. Such insertions are called "internal tandem duplications" (ITDs). They can be of various lengths; around 10–15 amino acids (AAs) is typical [4], but some are only few AAs in length, whereas insertions were reported that are over 60AAs long. ITDs are observed in about 25% of all AML patients. About 7–8% of the patients carry mutations in the kinase domain (KD) of FLT3. At present, high allelic ratio (>50%) of FLT3-ITD is seen as conferring an adverse risk for AML prognosis in children [5] and adult patients under 60 years of age [6]. The importance of FLT3-KD mutations for the clinical outcome is still a matter of debate. While it is clear that such mutations lead to an over-active protein, it has not been demonstrated that their presence is associated with worse prognosis. The discovery and clinical approval of several FLT3 inhibitors in the recent years is important in the context of AML therapy, but has not yet shown to be a game changer, in particular because resistance has quickly emerged against such therapies. Understanding of the molecular biology of FLT3, and in particular which signalling networks it affects and whether the tumour becomes dependent on them is clearly of high interest in both adult and childhood AML. Furthermore, studying the protein's biophysics and conformational landscape is needed for the development of better therapies and for diagnosis, e.g., foreseeing the risks associated with the various FLT3 mutations.

2. FLT3 - gene and protein structure

FLT3 is one of 58 known human receptor tyrosine kinases (RTKs) [7]. As RTKs participate in cellular growth, motility and differentiation, many are recognised for their role in promoting tumour growth and metastasis [8]. As an RTK, FLT3 has an extracellular receptor part, a

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short α-helical transmembrane part, and an intracellular part. The receptor part includes five immunoglobulin-like (IGL) domains. Under normal conditions, FLT3 is activated by the binding of a hematopoietic growth factor, FLT3-ligand, to IGL domains D2 and D3 (Fig. 1A); a dimer of ligands bind to the receptor which then dimerises itself. This dimerisation of FLT3 transforms the signal to the intracellular part, where the FLT3 KDs bind to and phosphorylate each other.

The intra-cellular part of the protein is comprised of a short unstructured linker (residues 564–571), a juxtamembrane domain (JM, residues 572–603), and a KD. A short hinge region connects the JM and KD domains. The KD is divided into two subdomains (KD1 and KD2) with an unstructured kinase insertion domain between these; Fig. 1B. The JM domain interacts with a regulatory feature called helix *αC. Helix* αC needs to move in order to make the protein active, which cannot be done when the JM domain is in place. Activation therefore requires a motion of the JM-domain together with two other regulatory parts: the phosphate binding loop (P-loop, which moves only slightly) and the activation loop (A-loop) that has to undergo a larger transition (Fig. 1C).

The 100 k base long FLT3 gene is located on chromosome 13, map 13q12.2. The gene includes 24 exons. The translation starts at exon 1 and ends at exon 24. The first 13 exons encode the extracellular domain. The TM domain is encoded by exon 13, the JM domain by exons 13–14, and the KD by exons 14–23. ITDs can replicate several exons starting from exon 13. Activating mutations in the KD typically involve exon 20. Interestingly, the 5'UTR of the FLT3 gene has several matches to the 3'UTR of the human RAS-related RAP1B (Reqseq sequence NM_015646.6, percent identity 88%, query cover 21%), which is highly expressed in squamous carcinomas [9].

3. Internal tandem duplications - a peculiar mode of protein activation

ITDs were first reported in 1996 [14]. Screening for mRNA of FLT3 in acute lymphoblastic leukemia (ALL) and AML patients, the authors observed longer transcripts, where sequences were duplicated in tandem, repeating some parts of the JM region and, in some cases, also downstream regions in the protein. It has since been established that these mutations make FLT3 constitutively active even in the absence of the FLT3 ligand [15]. The presence of FLT3-ITD is now used in the clinic for estimating patients' risk and for deciding on therapy, since these mutations confer sensitivity of the tumour to FLT3 inhibitors [1].

Single point mutations and to a lesser extent insertions and deletions are common modes of evolutionary processes that drive both organism survival and genetic (or genetically-associated) diseases. Such mutations occur in FLT3 and many other protein kinases, where they can make the protein more active or resistant to drug therapy in cancers [16,17]. Gene duplications have also occurred many times throughout the evolution; these make it possible for proteins to evolve in a parallel fashion. However, mutations that include tandem duplications within the same gene are rare. ITDs are almost exclusively discussed within the concept of FLT3, and few other examples exist. ITDs within the BCOR gene are associated with clear-cell sarcoma of the kidney, a rare paediatric cancer [18]. Another, recently reported example, was the case of a single patient suffering from colorectal carcinoma that had an early ITD mutation in NRAS [19].

3.1. Characteristics of ITDs

In our study of insertions in FLT3 [4], we characterised those insertions and found the following features:

1. The median length depended on the point of insertion. Insertions that occur at the JM-S and JM-Z regions were numerous but shorter (median length 12–13 AAs). The length increased when the insertions happen in the hinge, KD and P-loop domains.
2. Specific residues are hot-spots for insertions, i.e., insertions are more common in those residues. These include residues Tyr^{597}-Asp^{600} of the JM-Z domain, Phe^{605} and Pro^{606} of the hinge region and Phe^{612} of the KD.
3. Aromatic and acidic residues (Tyr, Asp, Glu, Phe and Trp) are most common in those repeats.

These characteristics appear to be inter-dependent, i.e., the location and position of the insertion hot-spots are preferred partly because these lead to over-abundance of acidic and aromatic residues. These, in turn, modify the protein's electrostatic surface and likely make it more prone to dimerise and become active.

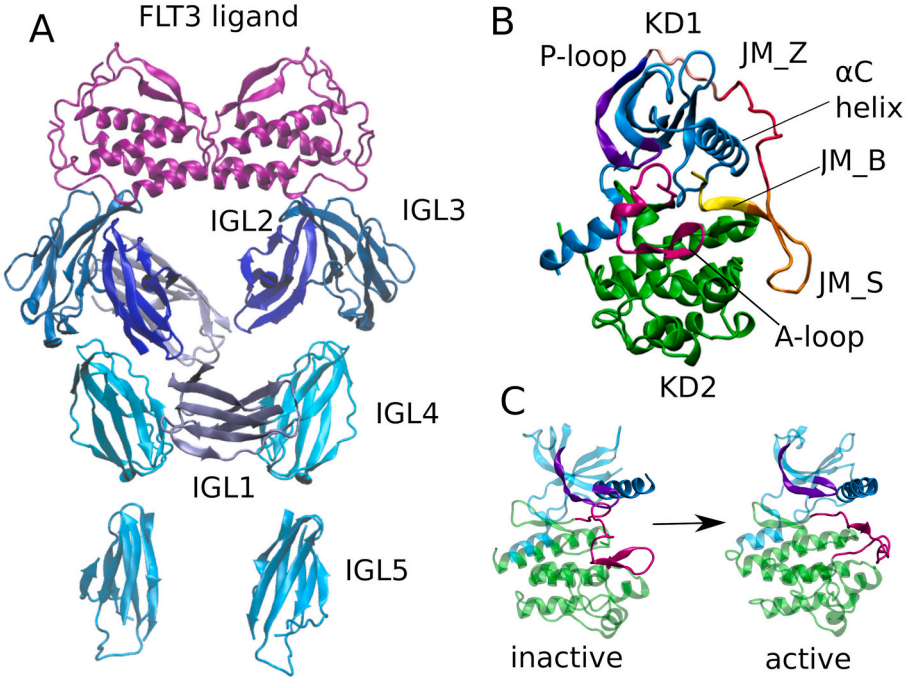

Fig. 1. Structure of FLT3. (A) The extracellular domain, shown as a dimer and bound to FLT3 ligand (also as a dimer), with the five IGL-domains indicated [10]. The FLT3 ligand is shown in purple; the IGL domains in different shades of blue. (B) Inactive FLT3 with the JM domain [11]. (C) The activation of FLT3, which depends on transitions of the P-loop, helix αC and most prominently activation loop [12,13]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. The mechanism of FLT3 activation by ITD

As ITDs are most common in the JM domain, and as they commonly amplify Tyr residues within this domain (Tyr^{589}, Tyr^{591}, Tyr^{597} and Tyr^{599}), it might be assumed that the insertions lead to additional phosphorylation of these residues, which in turn weakens the interactions between the JM domain and the KD. Indeed, it has been shown that the presence of residues Tyr^{589} and Tyr^{591} is crucial for oncogenic transformation [20]. However, mutating the Tyr residues to Phe did not alter the ability of an ITD mutant to activate the protein [21]. This reveals that phosphorylation is not the main event that drives activation when an ITD is present. To explain why different ITDs lead to activation of the protein, we utilised a widely used computational technique, molecular dynamics simulations [22,23]. Such simulations can be used to explore the conformational space of a protein, which can then be compared to a reference (e.g., wild-type versus mutant). Studying FLT3-ITDs, the simulations revealed that the ITDs confer a larger conformational freedom, especially in the JM region [24,4]. Interestingly, deletions in the JM region occur in AML (though very rarely), and activate the protein in a similar fashion [25].

The mechanism of FLT3 activation by ITDs can be explained as follows. The insertions increase the flexibility of the JM domain which more easily detaches from the protein. In addition, it is likely that the acidic and aromatic residues that are added increase the affinity of one FLT3 monomer to another [4], which is necessary for cross-phosphorylation.

3.3. Connection between the insertion location, length and protein activation

Although it has been suggested that longer insertions lead to worse prognosis in AML [26], this view has been challenged [27], and the current AML guidelines do not suggest such connection [6]. On the other hand, it is well established that not all ITDs are born equal in their biological (and thereby medical) effects [28]. Two studies where bioinformatics analysis, accounting for the length and location of the mutations in multiple samples was used, came to the conclusion that mutations that reach into the JM-domain are those that lead to worse prognosis, not necessarily those that are longer [29,4]. In this context, it should be mentioned that the JM domain of FLT3 is in fact shorter than that of other type 3 RTKs. Thus, it is not the longer JM domain per se that is pathogenic. In light of the proposed mechanism for activation, mutations that lead to further increase in the plasticity of the JM domain are those that are more effective [24,4].

4. FLT3 inhibitors

4.1. Types of tyrosine kinase inhibitors

The catalytic domains of protein kinases have a remarkable structural similarity, making it difficult to develop specific kinase inhibitors. This is often seen as a limitation, since specificity is important to avoid toxicities and side effects; many protein kinases are housekeeping genes that are required for healthy cellular activity. Nausea and vomiting, diarrhoea, rash and cardiac toxicities (such as QT prolongation) are side effects that are often observed with patients treated by tyrosine kinase inhibitors (TKIs), with individual inhibitors leading to other or additional side effects. The side effects that are common to different TKI likely depend on inhibition of housekeeping processes. Of note, while developing specific inhibitors is often seen as a key to success in TKI development, multi-kinase inhibitors can have their advantages as inhibition of several signal transduction networks is sometimes desired to achieve better efficacy and reduce the risk for resistance.

Most TKIs belong to one of three classes - type 1 inhibitors, type 2 inhibitors and covalent inhibitors. Such inhibitors bind to the ATP binding pocket of the target enzyme, with additional interactions with side pockets that lead to better affinity and specificity [30]. Type 1 inhibitors bind preferentially to the active conformation of the enzyme. Since the active conformation is more similar between different protein kinases, it is often more difficult to develop specific type 1 inhibitors. On the other hand, such inhibitors are often more robust against resistance mutations. Type 2 inhibitors bind to an inactive conformation of the protein, which is somewhat more divergent between different protein kinases. Both types of inhibitors are competitive inhibitors and their binding is transient. Covalent inhibitors bind covalently to the active site of the protein, most often to a cysteine residue. The binding is often stronger and less transient. Two drawbacks of such inhibitors are that there is a risk for interactions with other cysteine residues (covalent off-target binding, i.e., binding to proteins other than the molecular drug target) and that a single mutation in the cysteine residue (for example, EGFR C797S against a covalent EGFR inhibitor [31]) often renders them useless as they can no longer covalently bind to their target. The binding of three inhibitors, one of each type, to FLT3 is shown in Fig. 2. As shown in the figure, differences in size and interactions will also affect the potential for developing mutations that confer drug resistance. Moreover, inhibitors that interact with their target mainly through hydrogen-bonds to the backbone of residues and van der Waals interactions are less prone to resistance mutations, as shown for CDK4/6 inhibitors [32].

4.2. FLT3 inhibitor affinities: IC_{50} in culture medium and in blood plasma

Multiple inhibitors of FLT3 have been reported that have IC_{50} values <100 nM when their effect is measured on the growth of cells in culture media. In practice, the inhibitors should inhibit the protein in the blood, and hence absorption, distribution, excretion and particularly binding to plasma proteins can reduce their effect. In particular, many FLT3 inhibitors strongly bind to plasma proteins, which significantly limits their efficacy. For this reason, there exist inhibitors with low IC_{50} values such as lestaurtinib ($IC_{50} = 3$ nM) that do not achieve high potency in the clinic [33]. Even midostaurin, an FDA approved FLT3 inhibitor, has a much reduced affinity to FLT3 in the plasma [33,34] (it is however active as a multikinase inhibitor, see below).

5. Resistance to AML therapy targeted at FLT3

5.1. Resistance mutations

Resistance mutations are the primary mechanism of resistance against FLT3 inhibitors. The main types of FLT3 resistance mutations are the gatekeeper mutation F691L, which leads to resistance against all of the inhibitors that are currently in clinical use and the activating KD mutations D835V/Y/F and Y842C (in some cases also Y842H) that lead to resistance against type 2 inhibitors. Other mutations have also been reported, but are presumably less common. The positions of these residues is shown in Fig. 2. Consult Table 1 for a list of mutations that lead to resistance against the different inhibitors.

5.1.1. Why these mutations cause resistance

Mutations that lead to resistance reduce the efficacy of a drug, which in studying cancer therapies is normally measured by estimating the drug concentration which reduces the proliferation of cancer cells by 50% (growth IC_{50}, gIC_{50}). There are different mechanisms by which this can happen, and understanding *how* a certain mutation leads to resistance is important to the development of new drugs. Studies on the mechanism of resistance against BCR-ABL inhibitors in chronic myeloid leukemia (CML) [16,40] and EGFR in non-small cell lung caner (NSCLC) [41] have contributed much to our knowledge of the subject. Three mechanism are effective in FLT3: mutations that directly interfere with drug binding, mutations that stabilise the active conformation, and mutations that make the protein more active than wild-type. These mechanisms are not necessarily mutually-exclusive.

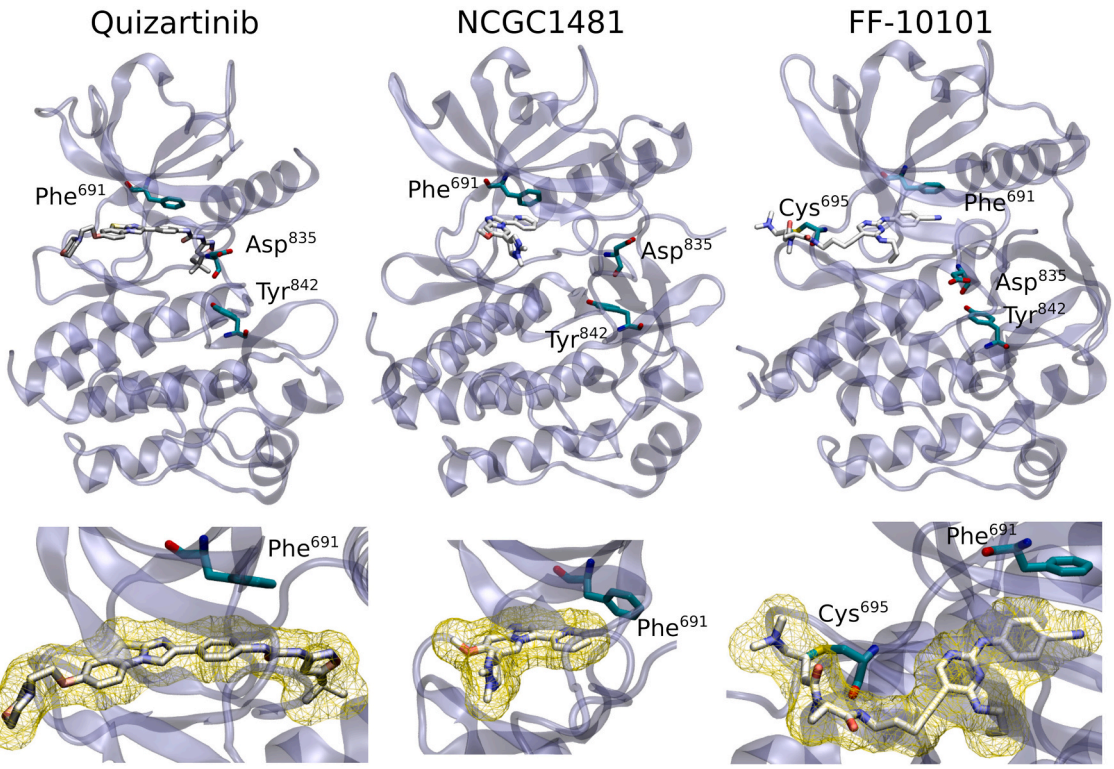

Fig. 2. Inhibitor binding to FLT3. Quizartinib (left) is a type 2 highly specific inhibitor and binds to FLT3 through multiple interactions. The gatekeeper residue Phe^{691} sits on top of the inhibitor, and computational studies reveals interactions between the residue and quizartinib [35,13]. Mutations in Residues Asp^{835} and Tyr^{842} lead to resistance but not due to interference with protein-drug binding but because they stabilise the active state which type 2 inhibitors do not bind. NCGC1481 (middle) is a smaller type 1 inhibitor. As a type 1 inhibitor, mutations in KD residues Asp^{835} and Tyr^{842} do not affect binding. Owing to its small size, there are no interactions between Phe^{691} and the inhibitor and hence it is not sensitive to the F691L gatekeeper mutation. FF-10101 (right) is a covalent inhibitor. It binds covalently to residue Cys^{695} and is not affected by F691L or KD mutations. Mutations at Cys^{695} will reduce its affinity but were hitherto not reported. The figure was prepared with VMD [36] using crystal structures downloaded from the protein data bank with codes 4XUF [37], 6IL3 [38] and 5X02 [39].

Table 1
Mutations in FLT3 that lead to inhibitor resistance. Mutations that were not described in patients are not included.

Inhibitor	Mutations
Midostaurin	N676K, F691L
Gilteritinib	F691L[a]
Quizartinib	F691L, D835F/V/Y, Y842C/H
Sorafenib	F691L, D835F/V/Y, Y842C/H
Crenolanib	F691L

[a] D698N leads to marked resistance but has not yet been identified in patients.

5.1.1.1. Mutations that directly interfere with drug binding. Such mutations are in principle most easy to explain, as they occur in residues that directly interact with the drug. F691L is a prominent example in FLT3 resistance. Mutations in residues that are in the same position as Phe^{691} (gatekeeper mutations) are common in kinases and become prevalent in tumour clones almost exclusively only when inhibitors are present. Although in principle it is easy to see why mutations of this type lead to resistance, the consequences of such mutations may involve contributions from several types of bonds, and interfere not only with the enthalpy of drug binding but also with the entropy [42]. In addition, it may not always be visible from a static crystallographic structure that a certain residue indeed interacts with the drug, requiring methods that can follow on protein dynamics (e.g., nuclear magnetic resonance, MD simulations, and enhanced crystallographic methods [43]).

5.1.1.2. Mutations that stabilise the active conformation. These mutations shift the equilibrium between the inactive (*I*) and active (*A*) states of the kinase $I \rightleftharpoons A$ towards the active state, thereby making the mutant form less sensitive to type 2 inhibitors. Such mutations lead to drug resistance but do not perturb protein-drug interactions directly. The KD mutations Y842H and D835F were suggested early on to kinetically stabilise the active state [35]. Other mutations in these residues are likely to employ a similar mechanism.

5.1.1.3. Mutations that make the mutant more active. With or without modifying the equilibrium between the active and inactive states, some mutations make the enzyme more active, increasing the velocity of the reaction, affinity to a substrate or both, and eventually leading to a higher catalytic efficiency [44]. In FLT3, the M664I mutation, that lead to resistance against the inhibitor pexidartinib, seems to employ such mechanism [13]. The mutation does not commonly occur when other inhibitors are used, perhaps since other mechanisms of resistance are more efficient in those cases. Nevertheless, this is a common mechanism with other kinases and inhibitors and may be shown to be operative against FLT3 inhibitors other than pexidartinib.

5.1.2. How resistance mutations evolve

AML is a clonal disease, where the tumour is heterogeneous and tumour cells of different origins (clones) compete for survival [17]. In this context, pre-existing mutations that lead to resistance (if such exist) have an advantage as they increase the fitness of the tumour. However, according to the "nearly neutral" theory [45], that is widely accepted in contemporary molecular evolution, mutations that drive the evolutionary process are either neutral (providing no benefit to the fitness but do not reduce the benefit either) or slightly deleterious (have a slight negative impact on the fitness). Mutations that increase the fitness of the organism are much less common and typically occur on the background

of other mutations. Applying this concept, it is important to consider that the tumour has no predetermined mechanism to mutate the genes and residues that matter for its survival. Once therapy is introduced, there is a strong shift in the conditions that determine the fitness, since tumour cells that are not resistant are wiped out. At this point, cells that confer resistance, by any mechanism, grow faster than other cells. New mutations (de novo resistance mutations) arise in the cells all the time and most, as stated, do not lead to any benefit in terms of cell proliferation. Anti-cancer treatment however drastically modifies the cellular environment. Mutations that are normally neutral or even slightly deleterious may suddenly become favourable simply because they lead to drug resistance. Of note, in the context of treating cancer, it is less relevant if the mutations are novel or not [46], and more important to differentiate between resistance mutations and other forms of adaptation that leads to drug resistance.

5.2. Other means of resistance

In case of resistance against FLT3 inhibitors, it is important to understand what mechanism led to resistance, as this might impact any potential treatment. To avoid resistance mutations, the best strategy is to use drugs with a narrower resistance profile (e.g., gilteritinib) or combination therapy [47,48,49,50,51]. Although both approaches have their limitations [16,52], in terms of risk for more aggressive mutations and toxicity (for combination therapy) they generally lead to superior results. Lowering the dose of an effective drug in the hope that some cells will be killed but the evolutionary pressure would not lead to development of resistance has been suggested before [53], but does not seem to be a viable approach in many cancers [46], as the growth of the tumour means that more mutations emerge. In fact, computational models show that in the absence of better solutions, high doses of a single drug may be the most viable strategy to postpone resistance in AML [51]. Of note, adaptive therapy, where the dose of the drug is adjusted according to the response of the tumour (and may be lowered) has been suggested following evolutionary modelling (see e.g. [54,55]). Such approach, and more recent strategies where drugs are modified after initial (successful) application [56] may have their merits especially given the potential toxicity of many drugs. Evaluating treatment strategies must however be carefully tailored to the specific cancer considering the availability and toxicity of drugs and have yet to be studied in $FLT3^{+}$-AML, where finding viable, long-term treatment is still a difficult challenge [2].

5.2.1. Overexpression of the FLT3 ligand

FLT3-ITD mutations make the protein active without requirement for binding to the FLT3 ligand (FL). In vitro, the efficacy of FLT3 inhibitors is normally measured against cells that display FLT3-ITD mutants, and is similar between cell lines that are homogeneous to such mutants (MOLM-14 and MV4/11) but often lower for cell lines that express wtFLT3. Owing to the heterogeneity in the tumours, it may be assumed that some tumour cells in patients do not express FLT3-ITD mutants, and still depend on FL for activation of FLT3. In such cases, overexpression of FL makes a larger share of the FLT3 enzymes in their active state and ready to phosphorylate downstream targets. Moreover, in the case of type 2 inhibitors, once the $I \rightleftharpoons A$ equilibrium is shifted towards the active state the inhibitors will not bind the protein, leading to resistance. In type 1 inhibitors, such mutations do not lead to resistance, but overexpression of FL does, especially if the inhibitors are not as active against wtFLT3 as they are against FLT3-ITD. In practice, overexpression of FL is a prominent source of resistance to FLT3 inhibitors [57]. Gilteritinib might be less sensitive to such resistance as it is a type 1 inhibitor with high affinity to wtFLT3.

5.2.2. Bypass signalling pathways

Activation of FLT3 leads to cell proliferation and loss of apoptosis. When FLT3 is inhibited, other proteins or pathways take over the role of FLT3. These can be proteins that are primarily downstream of FLT3, such as PI3K, AKT and mTOR, or pathways that take over instead - Pim1, RAS/RAF and factors emitted by the bone marrow stroma (stromal cytokines and chemokines) [58]. Such adaptation mechanisms can emerge as a mechanism of resistance against any FLT3 inhibitor in principle. However, some inhibitors might be partially effective against kinases that are important even for resistance. Inhibition of c-kit, for example, might be beneficial for AML therapy [59], as it is involved in several pathways that are important for proliferation. Quizartinib, midostaurin and crenolanib inhibit c-kit. Identifying signalling pathways that are of importance for resistance might offer targets for combination therapies [50,58,60].

5.2.3. Activating mutations in other genes

Gain-of-function mutations in genes other than FLT3 may make tumour cells resistant to FLT3 inhibitors as they adopt a new phenotype. In a recent study, patients were followed after treatment failure with different FLT3 inhibitors [61]. The most common mutations involved IDH1, NRAS, the WT1 transcription factor and TP53.

Isocitrate dehydrogenase (IDH) enzymes catalyse decarboxylation of isocitrate to α-ketoglutarate, α-KG. Mutations in Arg residues of the two enzyme isoforms, IDH1 and IDH2, lead to conversion of α- KG to R-2-hydroxyglutarate (R2-HG). R2-HG acts as an oncometabolite, which inhibits multiple enzymes and confers an oncogenic phenotype through various mechanisms [62]. Mutations in IDH1 and IDH2 are shown in many cancers, including AML. In AML, such mutations are less common than FLT3 mutations, and sometimes occur together with FLT3-ITD. There is one clinically-approved IDH1 inhibitor (ivosidenib), another approved IDH2 inhibitor (enasidenib) and several other inhibitors under development [63].

NRAS is a protein of the RAS family that plays a role in multiple cancers. RAS proteins are commonly mutated in cancers and notoriously difficult to inhibit by drugs. TP53 is the most commonly mutated protein in human cancers and generally implies increased risk in AML [64]. Another protein, Wilms Tumour 1 (WT1) is often overexpressed in AML. WT1 mutants are also observed in AML patients, and are believed to affect DNA regulation in a mechanism that is related to that of mutant IDH proteins [65]. Interestingly, WT1 mutations often lead to a disfunctional protein, i.e., both loss-of- function mutations and overexpression of WT1 are associated with AML [66]. Overall, activating mutations in genes other than FLT3 are a significant challenge and one of the reasons than monotherapy against FLT3 is not likely to emerge as a treatment with long duration.

5.3. Specific FLT3 inhibitors that are used in the clinic

5.3.1. Midostaurin

Midostaurin (Rydapt®) is approved for newly diagnosed $FLT3^{+}$-AML patients, where it is used together with chemotherapy. Originally a natural compound isolated from bacteria (*Streptomyces staurosporeus*), midostaurin is a multikinase inhibitor with high activity against FLT3-ITD, FLT3 with KD mutations, CDK1, c-kit and many other kinases. It is active even against wild type (wt) FLT3, though it is about 10 times more potent against KD and ITD mutants [67]. Activating KD mutations (D835X, Y842C) do not lead to resistance against midostaurin, Other mutations, in particular F691L, termed “gatekeeper mutation” due to its position at the entry to the ATP binding pocket, significantly increase the drug's IC_{50} towards FLT3 [68,69]. However, patients that relapse on chemotherapy + midostaurin may still benefit from newer inhibitors, suggesting that midostaurin's effects may be due to inhibiting other targets than FLT3 and that multiple midostaurin resistance mechanisms exist, of which mutations in FLT3 are not a major contributor.

5.3.2. Gilteritinib

Gilteritinib (Xosapta®) is a small molecule used since 2018 for treatment of relapsed or refractory (R/R) $FLT3^{+}$-AML. As a type 1 inhibitor, gilteritinib is effective against FLT3/ITD, wtFLT3 and FLT3 with

KD mutations [70], with the highest affinities to FLT3/ITD without additional KD mutations. Unfortunately, other mutations in the FLT3 gene, including F691L, Y693C, G697S, and D698N lead to drug resistance even against gilteritinib [71]. Higher doses of the drug may overcome such resistance [72]. As often is the case, the inhibitor is active against multiple kinases at therapeutic doses including AXL, LTK and others, but is not considered a multikinase inhibitor like midostaurin, and its mechanism of action for AML is FLT3/ITD inhibition. Although toxicities are not uncommon, the drug is tolerated better than chemotherapy [72]. One of the most serious complications with gilteritinib is differentiation syndrome [73], a biological response to the sudden maturation of many tumour cells; this occurs also in AML patients treated with inhibitors of another kinase (IDH inhibitors) and can be seen as a consequence of the therapy rather then the drug's chemistry.

5.3.3. Quizartinib

Quizartinib (Vanflyta®, AC220) is a type 2 FLT3 inhibitor, approved in Japan for a similar indication as gilteritinib. Being a type 2 inhibitor, it is more specific than gilteritinib [74]. On the other hand, there are multiple resistance mutations that limit the efficacy of the drug, including F691L and mutations in residues Asp^{835} and Tyr^{842}. Moreover, in spite of its overall high specificity, quizartinib inhibits c-kit (unlike gilteritinib) which may be one reason for bone marrow toxicities that occur upon treatment [75] (although inhibiting c-kit might be desired for additional therapeutic effect). Drug- induced QT-prolongation, a common side effect of many drugs that can have life-threatening complications, was also observed in patients. As a consequence of these toxicities, use of higher doses to counter resistance mutations is risky, and the drug is not approved for use in the EU or US.

5.4. Inhibitors in clinical trials

5.4.1. Sorafenib

Sorafenib is a type 2 multikinase inhibitor with high affinity towards FLT3. It is approved to use for different cancers, but not AML, for which it is used off-label in the US. Initially effective, its benefits appear to be short-termed as it is sensitive to resistance mutations [76] in similarity to other type 2 inhibitors (KD mutations and F691L). Recent results with adult patients were somewhat inconclusive, showing improvement in some factors but not in overall survival [77]. In contrast, sorafenib might be useful to treat paediatric AML [78].

5.4.2. Crenolanib

Crenolanib is a second generation type 1 FLT3 inhibitor. It is considered specific, with high affinity mainly to FLT3 and the structurally related kinases PDGFRα and PDGFRβ, but also inhibits c-kit and many other targets [74]. As a type 1 inhibitor it is active against FLT3-KD mutants but the F691L mutation make FLT3 resistant to crenolanib. Recent data suggested that crenolanib is also useful for patients suffering from paediatric AML [79], in whom it was well tolerated.

5.4.3. FF-10101-01

FF-10101-01 is a covalent FLT3 inhibitor that has shown to be effective even against the F691L mutation. The results of an initial, first-in-human study has recently been reported [80]. The main reported toxicity was differentiation syndrome. No new resistance mutations were reported in the small cohort of patients that were studied. The C695S mutation increases the IC_{50} by about 30-fold [39], but such or other C695 mutations have not been reported in patients. A search of the COSMIC database (date: 13.08.2021) confirms that no such mutations were reported, which gives additional hope for an inhibitor with reduced resistance.

6. Strategies to overcome resistance to FLT3 inhibitors in AML

6.1. Pan-FLT3 inhibitors

Pan-FLT3 inhibitors are chemical agents that can inhibit FTL3/ITD, wtFLT3 and most or all resistant mutations. Lessons from inhibitor development against Abl1 (for CML) and EGFR (for NSCLC) revealed that inhibitors with a much better resistance profile can be developed. From a theoretical point of view, given the stochastic nature of the evolution of resistance mutations, it is beneficial to have inhibitors that are less sensitive to resistance mutations even if it means that the evolutionary pressure on the tumour becomes very high (which carries a risk for developing a more aggressive phenotype). However, the success or failure of such inhibitors depends on other aspects. One of these is the inhibitory profile (as shown e.g., for midostaurin - it is not highly specific yet it is effective). Another aspect is tolerability - a pan-resistant inhibitor with more toxicities might not be as useful as a moderate one that is better tolerated.

At present, gilteritinib is the inhibitor which is the least susceptible to resistance mutations, although it is sensitive to the gateway mutation F691L (Table 1) and to other resistance mechanisms. FF-10101 is able to inhibit the gatekeeper mutant F691L mutant, but needs to be further studied in the clinic, including mutational screening. Pexidartinib is an approved kinase inhibitor that can inhibit FLT3/F691L but is sensitive to multiple other mutants [81] and is thus not a pan-resistant inhibitor. Experiments have shown that rotating between pexidartinib and quizartinib, in the hope of delaying the emergence of resistance mutations that limit the efficacy of the drugs is not a viable approach [51]. Further pan-FLT3 inhibitors are being developed; such inhibitors may not cure AML but can help patients stay on remission following more aggressive treatment, especially if they also have a favourable safety profile.

6.2. Dual inhibitors

Advancements in medicinal chemistry and biochemistry enable in some cases the development of inhibitors against two interesting molecular targets at once. Recognising the role of FLT3 and CDK6 in AML, an inhibitor was developed that could inhibit both targets [82]. Unfortunately, this inhibitor, FLX925, showed rather modest activity at the tolerated doses, while higher doses were prohibited owing to toxicity [83]. Despite this, there is hope that dual inhibitors might be useful and multiple such inhibitors are or have been developed, with efficacies against FLT3-ITD and JAK2, MEK, Pim, tubulin and others [84,85,86].

6.3. Combination therapy

Combination therapy is widely used for AML, especially with younger patients and during induction. At present, the common approach is to combine conventional chemotherapy or hypomethylating agents (HMA) with midostaurin. Moreover, multiple trials have been initiated where specific FLT3 inhibitors are used together with chemotherapy, HMA, or other specific inhibitors not targeting FLT3 [87,88]. It is thus expected that treatment algorithms will involve combinatorial therapies already in the near future.

7. Conclusions

FLT3 in AML is activated by ITD mutations or activating mutations in the KD, where the former are more common and lead to worse prognosis on the one hand and sensitivity to inhibitors on the other. Conversely, KD mutations may lead to resistance against FLT3 inhibitors and often occur on the background of FLT3-ITD. ITD mutations activate the protein by modifying its internal dynamics, leading to dislocation of the JM-domain and likely also increase the chance for dimerisation and cross-phosphorylation. Each FLT3 mutation is practically unique, but some characteristics have emerged following bioinformatic studies, and point

out that insertions that reach into the JM domain appear to be more active. Such mutations are therefore more likely to carry risk, irrespective of their length. Pin-pointing mutations that lead to worse prognosis may be possible in the future, by examining where the ITD repeat starts and identifying positions that lead to more active protein. FLT3 inhibitors are effective, though subject to resistance, often (but not always) due to mutations in the FLT3 gene. Type 1 inhibitors such as gilteritinib tend to be more robust against mutants. These are however often less specific than type 2 inhibitors such as quizartinib (which, in spite of its higher specificity, does not seem to have a favourable toxicity profile). FLT3 inhibitors are being developed with the aim of overcoming resistance mutations. Based on lessons from other targets, this approach might be useful to postpone the emergence of resistance. However, owing to many other resistance mechanisms against FLT3 inhibitors, it is likely that multi-kinase inhibitors or combination therapy will be required to sustain therapeutic effects.

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Declaration of Competing Interest

The author declares no conflict of interest.

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