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Mechanisms of resistance to FLT3 inhibitors

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Abstract

The success of the small molecule tyrosine kinase receptor inhibitor (TKI) imatinib mesylate (Gleevec) in the treatment of chronic myeloid leukemia (CML) constitutes an eminent paradigm shift advocating the rational design of cancer therapeutics specifically targeting the transformation events that drive tumorigenicity. In acute myeloid leukemias (AMLs), the most frequent identified transforming events are activating mutations in the FLT3 receptor tyrosine kinase that constitutively activate survival and proliferation pathways. FLT3 TKIs that are in various phases of clinical trials are showing some initial promise. However, primary and secondary acquired resistance stands to severely compromise long-term and durable efficacy of these inhibitors as a therapeutic strategy. Here, we discuss the mechanisms of resistance to FLT3 inhibitors and possible strategies to overcome resistance through closer examination of the events of leukemogenesis and design of combination therapy.

Keywords

AML; Combination therapy; Multitargeted agents; NF- κ B inhibition; Leukemic stem cells; PKC412; MLN518; SU-5416; SU11248; KW-2449; AC220; CEP701

1. Introduction

Several critical discoveries have identified the FMS-like receptor tyrosine kinase (FLT3) as an attractive target for small molecule receptor inhibitor-based therapy. First, FLT3 is shown to be commonly overexpressed in most B lineage acute lymphocytic leukemia (ALL), acute myeloid leukemias (AMLs), in subsets of T-cell ALL and chronic myeloid leukemias (CML) in blast crisis (Carow et al., 1996). Additionally, upregulation of FLT3 and its ligand (FL) by the leukemic cells creates an autocrine signaling loop which stimulates proliferation of both cell lines and primary AML patient samples (Zheng et al., 2004b). FLT3 is also an attractive

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target because it is normally expressed by primitive hematopoietic stem/progenitor cells (HSPCs) and is lost upon differentiation (Gotze et al., 1998). But perhaps the most important finding was the discovery that FLT3 activating mutations are present in about 1/3 of AML patients (Small, 2006).

The activating mutations in FLT3 can be classified into two major groups: (i) internal tandem duplications (ITDs) of the juxtamembrane domain (exons 14 and 15) that interfere with its normal negative regulatory role (Griffith et al., 2004; Levis and Small, 2003; Nakao et al., 1996) and (ii) activation loop mutations of the kinase domain (TKDs) that result in a constitutively open ATP binding pocket. Most of the activation loop mutations result in changes to aspartic acid 835 or isoleucine 836 (Abu-Duhier et al., 2001; Frohling et al., 2002; Griffith et al., 2004; Grundler et al., 2003; Thiede et al., 2002; Yamamoto et al., 2001). Regardless of within which domain the mutation lies, the constitutively activated receptor loses its dependence on its ligand and activates downstream signaling pathways which lead to increased proliferation and survival (Gilliland and Griffin, 2002), as well as contribute to a block in differentiation (Zheng et al., 2004a; Zheng, 2005). Gene expression studies of leukemic cell lines and primary samples harboring FLT3 mutations have identified a number of proteins and pathways that may contribute to leukemogenesis (Choudhary et al., 2005) including the PI3 Kinase/AKT, RAS/MAPK, and STAT5a pathways (Small, 2006).

The potential for tyrosine kinase inhibitors to have a significant impact on the disease has been demonstrated in recent years in CML. The use of imatinib, which specifically targets the BCR-ABL protein in CML, induces complete hematological remission in patients in the chronic phase of CML, CMML and CEL (Druker et al., 2001a,b). A recent follow-up study showed an 89% 5-year event free survival for CML patients (Druker et al., 2006). Imatinib's success serves as a proof of principle that TKIs can indeed elicit significant clinical responses. Furthermore, targeted TKIs are in general more easily tolerated and less toxic than cytotoxic chemotherapies. Thus, in AML, given the frequency of dependence on FLT3 signaling, there is a clear rationale for the development of FLT3 inhibitors.

The many FLT3 inhibitors currently under investigation are described in Table 1. Some of these have selectivity for FLT3 while others affect a broad range of kinases. Several reviews of the current status of these trials, as well as general FLT3 inhibition strategies are available (Knapper, 2007; Levis and Small, 2003; Tam and Gilliland, 2008; Tickenbrock et al., 2006).

For the most part, preclinical and early clinical studies using FLT3 inhibitors such as PKC412, MLN518, SU-5416, SU11248, KW-2449, AC220 and CEP701 as monotherapy show some biological activity (Cortes et al., 2007a,b; D. Deangelo et al., 2006; D.J. DeAngelo et al., 2006; Fiedler et al., 2003, 2005; Giles et al., 2003; O'Farrell et al., 2003; Smith et al., 2004; Stone et al., 2002). However, most of the responses consisted of clearance of peripheral blasts and less frequently, major reductions in bone marrow blasts (Grundler et al., 2003; Knapper et al., 2006a; R.M. Stone et al., 2005; Weisberg et al., 2002). These responses tended to be transient, lasting weeks to months, followed by progressive disease. Besides the inherent heterogeneity of AML, the major emerging challenge to FLT3 targeted therapy therefore is resistance to treatment with TKIs. Recognizing and addressing the

mechanisms that give rise to TKI resistance will help to provide more rational design strategies for the development of new agents and lead to more effective treatment.

2. Mechanisms of resistance

2.1. Pharmacokinetic and pharmacodynamic barriers

The efficacy of compounds that compete with ATP for the binding pocket of the kinase domain of FLT3 is variable and, in part, may depend on pharmacokinetic and pharmacodynamic barriers to which all small molecule inhibitors are subject. These include rapid and/or induction of first pass metabolism, solubility and protein binding characteristics of the inhibitors. One example is the induced metabolism of the potent FLT3 inhibitor PKC412 into metabolites that have reduced activity against FLT3 and a broader kinase profile has been seen in AML patients on trials with this drug (Levis et al., 2005c). Many of these hydrophobic drugs are strongly bound by alpha-1 acid glycoprotein resulting in greatly decreased free levels of the drug being available to bind to target. CEP701, for example, appears to be >99% protein bound in human plasma. Much of the heterogeneity in response will be dependent on patient metabolism and genetic variation. Low intracellular concentrations of the drug may also be attributed to active transport by ABC multi-drug transporters that increase the efflux of small hydrophobic molecules. Although no significant correlation between FLT3 mutations and high levels of *MDR1* mRNA has been found (Galimberti et al., 2003), a positive correlation between MRP1 expression and FLT3 mutations has been observed, which could provide a resistance pathway in FLT3-positive blast cells (Schaich et al., 2005). Pharmacokinetic failure to sustain continued target inhibition was recently shown in a phase I trial of KW-2449, which stresses the importance to confirm *in vivo* target inhibition (Pratz et al., 2009).

Upregulation and overexpression of FLT3 itself in leukemic cells may also play a role in the transient responses. FLT3 expression in patients in a UK CEP701 phase II study actually showed increased FLT3 expression on blast surfaces in 13 of 14 patients after treatment with the inhibitor (Knapper et al., 2006a). This could provide a mechanism by which the receptor can out-compete its inhibitor at the physiologically relevant range. Currently, there is no evidence to suggest that the FLT3 gene itself has been duplicated at any significant frequency in patient samples, though it is in the SEM-K2 human leukemia cell line.

Additionally, the microenvironmental niches in which the leukemia blasts reside may also play a role in resistance. In the stromal cell-dense bone marrow, inhibitors could be hampered not only by relative inaccessibility of the leukemic cells but also by the secretion of factors by stromal support cells that could rescue cells from inhibitor-induced apoptosis are well-recognized mechanisms contributing to drug inefficacy (Tortora et al., 2007; Tam and Gilliland, 2008).

2.2. Drug-binding resistance mutations

The short-lived response to FLT3 inhibitors suggests that resistance occurs quickly. This is most likely due to the selection of pre-existing populations harboring mutations that allow them to escape inhibitor cytotoxicity. Selection of clones with mutations that prevent the

binding of imatinib to the ATP binding pocket of BCR–ABL have been the most frequently observed cause of acquired resistance to imatinib in CML patients (Gorre et al., 2001; Wadleigh et al., 2005). To date more than 100 resistance mutations have been uncovered in these patients (Branford et al., 2002, 2003; Corbin et al., 2003; Gorre et al., 2001; Hochhaus et al., 2002; Shah et al., 2002; Burchert, 2007). Single amino acid residue changes found in FLT3–TKDs dramatically alter the structure and activation status of the activation loop, locking the receptor in a constitutively open conformation. A number of point mutations in the activation loop of the kinase domain change the structure and binding characteristics of FLT3 TKI dramatically. In fact, inhibitors that are usually potent against the ITD mutation often do not inhibit some or all of the kinase domain mutations. The presence of kinase domain mutations in FLT3–ITD patient cells would also clearly further prevent inhibitors from binding the ATP binding pocket. The first clinical resistance mutation was identified in a FLT3–ITD patient who had relapsed after treatment with PKC412 (Heidel et al., 2006). A single amino acid substitution at position 676 (N676K) within the kinase domain destabilized the conformation of the hinge segment that normally hydrogen bonds with the lactam ring of PKC412, thus preventing binding of the inhibitor (Heidel et al., 2006). It would not be surprising if additional mutations in the kinase domain region of FLT3–ITD patients are found in the future.

Prior to its discovery in patients, an *in vitro* random mutagenesis screen of a FLT3-ITD expressed in Ba/F3 cells followed by drug selection had already predicted that the N676K substitution would confer specific resistance to PKC412 (Cools et al., 2004, 2005). This same screen also predicted other mutations within FLT3/ITDs that would abrogate PKC412 binding. These positions (Ala-627, Phe 691, and Gly-697) conferred variable resistance, not only to PKC412 but also to SU5614 and K-252a. In particular, the G697 mutation conferred high-level resistance to all of the experimental FLT3 inhibitors tested (Cools et al., 2004). Though these mutations have not yet been found in patients resistant to FLT3 inhibitors, they represent potentially serious challenges to the efficacy of PKC412 and possibly other FLT3 TKIs currently in clinical trials.

2.3. Alternate activation of survival and proliferation pathways and FLT3 independence

Alternate mechanisms by which AML may escape FLT3 inhibitor therapy are through events that remove the oncogenic dependence of the transformed cells on FLT3 signaling. This phenomenon has already been observed with CML patients on imatinib. Several groups have reported imatinib-resistant CML that lack point mutations in ABL kinase domain (e.g., T315I) and instead have overexpression of genes in alternate pathways (e.g., LYN kinase) (Agaram et al., 2008; Donato et al., 2003; Kancha et al., 2008; Miething et al., 2007; Pocaly et al., 2008; Sherbenou et al., 2007; Wu et al., 2008). Others have also reported not only the absence of the BCR–ABL kinase domain mutations before and after imatinib treatment, but also a lack of overexpression of BCR–ABL (Agarwal et al., 2008; Pocaly et al., 2008; Ren, 2005; Sherbenou et al., 2007; Wei et al., 2006). In imatinib-resistant CML, activation of *Kras* (Agarwal et al., 2008) and SRC family kinases (Wu et al., 2008) as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion (Donato et al., 2003; L. Wang et al., 2007; Y. Wang et al., 2007) has been well documented. These

alternative pathways render leukemic cells resistant to imatinib treatment as the leukemia resorts to compensatory pathways for its survival.

In primary AML blast samples treated with the FLT3 inhibitor agents, CEP701 and PKC412, it is evident that FLT3 may not provide the sole survival signal for the leukemic cell (Knapper et al., 2006b). Responses to both inhibitors were highly heterogeneous between samples. Additionally, there was significant overlap between FLT3 wild type and mutant FLT3 samples with regard to cytotoxicity to FLT3 inhibitors. Even when complete inhibition of FLT3 was achieved, STAT5a and MAPK activity persisted in some samples, indicating that these resistant blasts are dependent on proliferation and survival pathways independent of FLT3 signaling (Knapper et al., 2006b) (see Fig. 1).

The idea that additional genetic alterations are acquired with time and confer resistance has also been demonstrated in a FLT3-ITD background *in vitro*. Primary AML samples harboring the ITD mutation displayed constitutive activity of anti-apoptotic pathways of MAP kinases, Akt, NF- κ B and STAT proteins are constitutively activated in primary AML samples in the presence of FLT3 inhibitors (Siendones et al., 2007). Furthermore, prolonged treatment with FLT3 inhibitors selects for resistant clones which most often have no secondary mutations in FLT3. FLT3 in these resistant clones remains inhibited by known FLT3 tyrosine kinase inhibitors, but the cells continue to show downstream activation of signaling pathways including PI3K/Akt and RAS/MAPK as well as expression of other genes involved in FLT3-mediated transformation. Inhibition of these downstream pathways restored the sensitivity of these resistant cell lines to FLT3 inhibitors (Piloto et al., 2007).

Subsequently, activating *N-Ras* mutations were discovered in two of these resistant cell lines as a compensatory, parallel pathway that has been selected for under the pressure of inhibition of FLT3 (Piloto et al., 2007). The *N-Ras* activating mutations provide the cells with an alternative to FLT3 signaling by constitutive activation of survival and proliferation signaling pathways. The mechanisms by which the downstream pathways are activated in the other resistant cell lines are still unknown. It would not be surprising if more of these alternate pathways are uncovered. Indeed, another group has reported upregulation of the anti-apoptotic protein BCL2 in a cell line model with activating *FLT3* mutations (Kohl et al., 2007). Sensitivity to FLT3 inhibition was restored when these cells were treated with a BH3 mimetic, ABT-737 (Zhang et al., 2007). In addition in primary blast samples, the level of BCL2 protein was high and did not change with phosphorylation status of FLT3, indicating yet another parallel and compensatory pathway that could lead to resistance. The combination of ABT-737 with FLT3 inhibitor treatment synergized to induce apoptosis in primary blast samples.

Hence, while targeting of FLT3 alone may have short-term positive effects, its long-term, efficacy may be compromised by the selection of clones that are independent of FLT3 signaling.

2.4. Leukemic stem cells (LSCs)

Increasing evidence suggests that resistance to anticancer treatment may arise from small populations of stem cells that escape inhibitor killing (Jordan and Guzman, 2004,

Kavalerchik et al., 2008; Krause and van etten, 2007; Stubbs and Armstrong, 2007). Treatments that target the bulk tumor may not have a major effect on the stem cell population because of additional survival pathways utilized by these cells. This population can then repopulate the bulk tumor and/or acquire additional mutations that confer survival and proliferative advantages which may even make them resistant to inhibitor treatment. For example, some *ABL* kinase point mutations that confer resistance to inhibitor therapy are present even before inhibitor treatment is started in CML patients (Hochhaus et al., 2002; Kavalerchik et al., 2008; Ren, 2005; Roche-Lestienne et al., 2002; Shah et al., 2002). Such *a priori* mutations may remove the leukemic cell's dependence on FLT3 signaling and diminish the efficacy of any FLT3 inhibitors. The stage at which these mutations take place, whether it occurs in HSCs, the earliest progenitors or more differentiated progeny, is of importance when considering the stem cell contribution to disease. In AML, when and where the mutation in *FLT3* takes place is still an unresolved issue. Approximately 5% of MDS patients display *FLT3* mutations, which is evidence for the mutation occurring relatively early in the process of transformation. On the other hand, the acquisition of *FLT3* mutations is commonly found as part of the transformation from MDS into AML, indicating that it may be a relatively late, contributing "hit" as well (Cloos et al., 2006; Shih et al., 2002).

FLT3 mutations have been identified in the CD34+/CD38-leukemia initiating stem cells in a majority of samples from patients with FLT3-ITD AML (Levis et al., 2005a). Additionally, in a high percentage of FLT3-ITD patients, the ITD mutation is detectable at relapse (Shih et al., 2002). Thus, current data suggests that these mutations are present in the LSCs responsible for disease propagation in most patients. However, in about 15% of patients the ITD mutation is no longer present at relapse, supporting the idea that the mutation occurred in a subclone of the leukemia and not in the LSC (Shih et al., 2004). Treatment with FLT3 inhibitors in these patients might help to eliminate this subclone but would be expected to leave the LSC responsible for disease progression, relatively unaffected. Without a doubt, elucidating the stage(s) at which resistance mutations are acquired will be important as it will dictate the type and timing of treatment (Levis and Small, 2003).

3. Future directions in overcoming drug resistance

FLT3 signaling plays a major role in the leukemogenesis of AML. However, unlike the BCR-ABL fusion, which appears to be the single, causative driving force behind CML (a myeloproliferative disease), in AML, *FLT3* mutations are just one of several "hits" that lead to a fully transformed disease state. The "oncogene dependence" that is seen in CML is not fully recapitulated in FLT3-ITD AML. In mice, *FLT3-ITD* mutations cause a myeloproliferative disease (Kelly et al., 2002b), but alone are insufficient to cause AML. However, when combined with other genetic alterations, such as PML-RARa, NU98-Hox, AML1-ETO, MLL-ENL, MLL-SEPT6, fully transformed acute leukemia develops (Kelly et al., 2002a). The opposite has also been shown: the inversion of chromosome 16 (inv(16) p13q22), which fuses the core binding factor beta subunit to the smooth muscle myosin heavy chain, alone does not lead to AML. Only when secondary mutations, specifically *FLT3-ITDs*, are introduced, do animals in these models progress to AML (Kim et al., 2008). Thus, *FLT3* mutant AML is more analogous to blast crisis CML in which additional

mutations that cooperate with BCR–ABL signaling have been acquired and lead to full transformation to acute leukemia. These other hits may activate alternate pathways, making FLT3 inhibition alone insufficient. There are however several strategies that may be able to overcome the emergence of resistance.

3.1. Monitoring of FLT3 inhibition and tailored therapies

Dysregulated FLT3 signaling is clearly an important event which provides an excellent molecular target in a large number of AML patients. There is enough clinical data to suggest that while targeting the receptor by itself is not sufficient to achieve complete remissions or cures, combination with other modalities, such as chemotherapy, may yield favorable clinical outcomes (Levis et al., 2004, 2005b, Möllgård et al., 2008). Because of the heterogeneity of AML, future therapies would optimally be individualized. In patients where blasts are dependent on FLT3 signaling, levels of FLT3 inhibition should be assayed over time, as inadequate inhibition is a clear mechanism that would result in lack of efficacy (Levis et al., 2006). By having this information, appropriate doses of the inhibitor can be determined to guarantee that inhibition is maintained throughout the treatment course. Furthermore, specific typing by sequencing of the FLT3 activating mutation is also crucial in deciding the appropriate treatment, as some kinase domain mutations are inherently resistant to some of the FLT3 inhibitors currently in clinical trials.

In vitro experiments have suggested that the mutant FLT3 receptor type and level have important implications in the response that might be expected with the FLT3 inhibitor lestaurtinib (CEP701) in clinical trials. In these studies, FLT3–ITD harboring AMLs were more sensitive to inhibitor treatment than FLT3–WT and FLT3–TKD blasts (Scholl et al., 2007). FLT3 inhibition alone, however, may have only a limited short-term effect as selection of resistant clones with secondary mutations appears to be a common, rapidly occurring phenomenon.

3.2. TKI and chemotherapy combination treatment

Combining FLT3 TKI with chemotherapy is a current strategy that combines the most active cytotoxic chemotherapy agents in AML with a targeted approach. Combinations of PKC412 with cytarabine (Ara-C) and daunorubicin showed that the effects on FLT3–ITD positive patients with combination therapy were mostly *additive* in both patient samples and cell lines (Möllgård et al., 2008). A similar study with PKC412, cytarabine and daunorubicin induction and high-dose cytarabine consolidation had been conducted as well (R.M. Stone et al., 2005). The effects of PKC412 in combination with cell cycle specific drugs may also depend on the *level* of mutant FLT3. One study suggests that PKC412 induces apoptosis instead in G2 arrest in cell lines that do not express mutant FLT3 (Odgerel et al., 2008). *In vitro*, it was observed that CEP701 and chemotherapeutic agents have a synergistic cytotoxic effect on FLT3–ITD cells. The studies also suggest the chronological sequence by which FLT3 inhibitors should be administered, specifically after chemotherapy, to avoid unwanted toxicity (Levis et al., 2004). SU11248 has also been administered with cytarabine and daunorubicin (Yee et al., 2002). This approach may also prove effective by decreasing the pool of cells from which resistant clones might arise and clinical trials of CEP701 and combination chemotherapy are underway.

3.3. Targeting multiple pathways

It is feasible that treatment with several different FLT3 inhibitors given concurrently could improve killing and prevent the selection of resistant clones with secondary *FLT3* mutations. However, this strategy is likely to induce additional toxicities as all of the inhibitors in clinical trials also have off-target effects, and thus unintended signal transduction pathways are likely to be affected. It also would not have any impact on the mechanism by which most resistant clones escape killing i.e. mutations outside of FLT3 that lead to alternative activation of downstream pathways. Therefore, instead of developing inhibitors solely to FLT3 and its activating mutations, therapies that target both FLT3 and alternate activation of downstream pathways (such as MAPK, AKT, and STAT5a), might yield the most significant clinical responses (Fig. 1). One study combining treatment of PKC412 with rapamycin, which targets mTOR (Jiang and Liu, 2008), showed cytotoxicity against both cells expressing PKC412-sensitive and resistant FLT3 mutants. The proposed mechanism by which this occurs is through the inhibition of phosphorylation of 4E-BP1, thus inhibiting translation. The addition of a MAPKK inhibitor along with rapamycin resulted in even further efficacy (Mohi et al., 2004). The combination of FLT3 inhibition with SU11657 together with all-trans retinoic acid (ATRA) showed a strong *in vivo* response in a PML–RARA positive APL mouse model (Sohal et al., 2003). This reinforces the observation that multiple events contribute to cause progression of cancer, all of which may serve as potential targets in combination therapy (Broxterman and Georgopapadakou, 2005; Fojo, 2007).

Though initially counter-intuitive, the use of multi-targeted TKIs may also prove effective. The clinical testing of inhibitors such as sorafenib, which besides targeting FLT3 also targets c-Raf, VEGF family receptors, platelet derived growth factor receptors (PDGFR) and c-KIT (Adnane et al., 2005; Wilhelm et al., 2004) against FLT3–ITD harboring AML cells (Zhang et al., 2008) is further facilitated by its already approved use by the FDA in renal cell and hepatocellular carcinomas. Currently, a phase I trial of sorafenib in refractory AML patients is underway (Quintás-Cardama et al., 2007). Furthermore, the combination of the FLT3 inhibitor, sunitinib, with MAPK inhibition by the MEK1/2 kinase inhibitor AZD6244 in FLT3-positive cell lines synergistically inhibited the phosphorylation of ERK1/2 and p70S6K, effectors critical to cell proliferation and survival (Nishioka et al., 2008). Newer generation compounds such as SU14813 are showing promise as inhibitors against FLT3–ITD AMLs (Patyna et al., 2006; L. Wang et al., 2007; Y. Wang et al., 2007). ABT869 is another example of a multi-targeted kinase inhibitor with efficacy against mutant FLT3 harboring AML (Shankar et al., 2007) and has been shown to inhibit tumor growth *in vivo* while also decreasing the activation of VEGF (Zhou et al., 2008). ABT869 was also recently shown to promote apoptosis in AML cell lines with FLT3–ITD mutations by upregulation of proapoptotic proteins such as Bax, Bid and Bak (Zhou et al., 2008).

The downstream pathway of NF- κ B signaling may be another target. The activation of NF- κ B by overexpression of FLT3 has been reported (Takahashi et al., 2005) and is consistent with the observation of constitutive NF- κ B DNA binding activity in AML, presumably mediated by Ras/PI3K pathways (Birkenkamp et al., 2004). Since the ITD and TKD mutations of FLT3 both activate these same pathways, targeting NF- κ B might increase efficacy, especially in light of incomplete FLT3 inhibition. Treatment with NF- κ B inhibitors

preferentially induces apoptosis of AML blasts, as compared to normal CD34⁺ hematopoietic cells, thus creating a therapeutic window (Frelin et al., 2005). A dual inhibitor of both I κ B kinase 2 and FLT3, AS602868, has been shown to trigger leukemic cell death *in vitro* (Griessinger et al., 2007). Inhibitors with multiple target specificities may actually be desirable, as they can serve to block multiple pathways important for the leukemic cell to continue to survive and proliferate. In addition, targeting NF- κ B may also be an attractive strategy for its preferential targeting of LSCs and is discussed later.

Another downstream pathway that has potential for targeting is the β -catenin pathway. Recent investigations have shown that Wnt/ β -catenin signaling and its subsequent downstream transcription activities were higher in FLT3-ITD mutant AML cells than in wild-type cells (Tickenbrock et al., 2005). It has been suggested that activated FLT3 tyrosine phosphorylates β -catenin, directly causing its nuclear localization and increased transcriptional activity (Kajiguchi et al., 2007). Thus, targeting β -catenin signaling could prove crucial for the effective inhibition of pathways that leukemic cells utilize for continued survival and proliferation.

As the above studies indicate, the pathways that lead a tumor's abnormal survival and proliferative capacity are attractive targets for combination therapy. The inhibition of AML cell lines with the FLT3 inhibitor SU14813 led to the down-regulation of cyclin D2 and D3 and dephosphorylation of pRb. The resulting G1 cell-cycle arrest suggests that the cyclinD-Cdk4/6 complex is a downstream target of FLT3 signaling and thus an exploitable target for inhibition. In the same report, the use of a single-agent inhibitor of Cdk4/6 caused sustained cell cycle arrest in FLT3-ITD samples and prolonged survival in an *in vivo* model (L. Wang et al., 2007; Y. Wang et al., 2007). Though Cdk4/6 inhibition could be reversed by reactivation of Cdk2, this study shows that targeting of cell cycle components could be a viable strategy.

Recently, the pro-apoptotic agent LBW242 was shown to increase killing of both PKC412-sensitive and resistant cell lines when combined with PKC412 or standard cytotoxic agents (doxorubicin and Ara-C) (Weisberg et al., 2007). The synergy of PKC412 and LBW242 was further demonstrated by *in vivo* imaging of FLT3-ITD AML engrafted NCr-nude mice that showed a lower level of tumor burden when compared to single-agent treatment alone. Thus, because AML blasts are inherently resistant to apoptosis, inhibitors of IAPs (inhibitors of apoptosis proteins) are attractive potential targets for combination therapy (Weisberg et al., 2007).

Farnesyl-transferase inhibitor therapy strategies remain an area of interest despite limited responses in monotherapy trials. These inhibitors interfere with the final isoprenylation step that tethers Ras to the plasma membrane and offers another, though less specific, strategy to interfere with the activation of downstream FLT3 pathway signaling. PKC412 in combination with Lonafarnib, a farnesyl-transferase inhibitor, was shown to have additive/synergistic effects in both FLT3-ITD positive and negative cell lines (Möllgård et al., 2008).

For mutant FLT3 driven AML, the targeting of heat shock proteins in combination with FLT3 inhibitors may also prove to be fruitful. In the case of AML, FLT3-ITD requires

Hsp90. Therefore, disrupting the proper folding of mutant FLT3 represents another way of inhibiting FLT3 signaling. Herbimycin was found to disrupt the Hsp90–FLT3–ITD interaction and inhibits tumor growth *in vitro* (Zhao et al., 2000). Treatment with another Hsp90 inhibitor, the geldanamycin analog 17-AAG, combined with PKC412 was shown to be effective in FLT3–ITD leukemias (George et al., 2004). Hsp90 inhibitor development has recently been reviewed (Barginear et al., 2008).

As suggested by the efficacy of combining FLT3 inhibitors with inhibitors to other targets in leukemia, there is a varying degree of dependency on FLT3 signaling which can be pre-existing or acquired. For example, we have observed that the increased sensitivity of AML blasts to FLT3 inhibitors in relapsing patients correlates with an increase in the allelic ratio of mutant to wild-type FLT3-ITD. Additional targets may activate downstream signaling pathways similar to FLT3; for instance we observed selection of RAS mutations in FLT3 mutant cell lines grown under increasing concentrations of FLT3 TKI. Alternatively, these targets may lead to survival and proliferation of blasts through completely novel pathways and mechanisms. Once these pathways and their mechanisms of activation are elucidated, they can become molecular targets for the development of novel combination treatment with FLT3 TKI in FLT3 mutant leukemia, potentially improving the long-term survival of AML patients as well as providing us with a closer glimpse of the biology and the events that drive leukemogenesis.

3.4. Monoclonal antibodies to FLT3

Another prospective options for the treatment of AML dependent on FLT3 signaling include anti-FLT3 monoclonal antibody treatment. Monoclonal antibodies may give more specific inhibition while also avoiding the same mechanisms of acquired resistance seen with small molecule-based therapy. The antibodies IMC–EB10 and IMC–NC7 prevent FLT3 from binding its ligand and EB10 was able to induce ADCC in TKI resistant cell lines *in vivo*, regardless of the phosphorylation status of FLT3 (Piloto et al., 2007).

3.5. Targeting LSCs

As the evidence for the role of a cancer stem cell compartment accumulates, the properties of LSCs and their roles in tumor initiation and at relapse have become of intense interest (Guzman and Jordan, 2004; Krause and van etten, 2007; Stubbs and Armstrong, 2007). The close link of AML to a stem cell origin and elucidation of a specific leukemic stem cell phenotype of surface markers may facilitate the targeting of this subpopulation of cells. Besides using cell surface markers to possibly one day sort between normal and leukemic progenitors (Van Rhenen et al., 2007), other molecular differences such as higher than normal expression of anti-apoptotic proteins (such as interferon regulatory factor 1 (IRF1), death associated protein kinase 1 (DAPK-1), and BCL2) can be exploited as future targets (Guzman and Jordan, 2004). Targeting of LSCs may also be aided by the observation that NF- κ B is abnormally activated in AML–LSCs. Inhibiting the degradation of IK β seems to reduce engraftment in NOD-SCID mice (Guzman et al., 2007). Early *in vitro* experiments have demonstrated that treatment of AML cells and normal CD34+ cells with the compound AS602868, an inhibitor of IKK2 kinase, resulted in decreased clonogenic potential of the AML cells while having minimal effects on normal hematopoiesis (Griessinger et al., 2008).

In vivo re-colonization experiments in NOD-SCID mice with treated AML cells showed a strong decrease in engraftment versus treated CD34+ normal cells (Griessinger et al., 2008). Though serial transplantation experiments need to be undertaken, this early evidence suggests that targeting LSCs by NF- κ B inhibition is an attractive possibility. In the future, therapeutic strategies that incorporate both the targeting of the bulk tumor and the LSC population will likely increase the rate of tumor remission while decreasing the likelihood of relapse. Targeting pathways, such as Wnt/ β -catenin, Hox, Notch and Hedgehog, that are important in HSCs but may be aberrant in LSCs will no doubt be of importance. Evidence is already demonstrating the importance of these pathways in leukemogenesis, particularly in BCR-ABL CML (Dierks et al., 2008; Hu et al., 2008; Sengupta et al., 2007).

4. Conclusions

The response of FLT3 mutant AML patients to FLT3 inhibitors has been much more limited compared to the response of CML patients to BCR-ABL inhibitors. While it is clear that aberrant FLT3 signaling is a major transforming event and plays an important role in leukemogenesis, fully transformed AML has additional “hits” that make sole FLT3 inhibition inadequate. This is in contrast to chronic phase CML, which is not a true leukemia but a myeloproliferative disorder more dependent on a single genomic alteration. In light of the acquisition of secondary mutations, novel FLT3 inhibitors, such as NVP-AST487 (Weisberg et al., 2008) are being developed. However, the emergence of resistance resulting from FLT3 signaling independence suggests that FLT3 inhibitors as monotherapy may not be the most realistic long-term treatment strategy. Instead, elucidation of the various events that cause full transformation and a shift toward combination therapies are likely to help to overcome resistance and improve long-term survival of patients with AML.

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Fig. 1.

Major mechanisms and future targets for AML resistant to therapy with FLT3 TKIs. (1) Normally, ITD mutations in the juxtamembrane domain and point mutations in the tyrosine kinase domains (TKD) lock the receptor into an active conformation. Constitutively activated FLT3 activates downstream signaling pathways including PI3K/Akt, MAPKK and STAT5a which lead to continued cell proliferation and survival. (2) Alternate upstream mechanisms that result in activation of the same downstream pathways activated by FLT3 that would not be inhibited by FLT3 inhibitors (e.g., *N-Ras*). (3) Alternate pathways completely exclusive of FLT3 and downstream FLT3 effector pathways that also lead to transcription of products that allow for continued cell proliferation and survival (e.g., BCL2, NF- κ B, etc.). (4) Drug efflux and other mechanisms that reduce overall intracellular drug concentrations. (5) The microenvironmental niche of blasts, first pass drug induction/

metabolism, high drug-protein binding resulting in lower free drug levels. (6) Upregulation/ increased expression of FLT3 receptor and FL ligand can lead to increased signaling.

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Table 1

Clinical trials of FLT3 TKIs.

Compound name	Trade name	Chemical class	Targets	Phase(s) of trial	Reference(s)
PKC412	Midostaurin	Staurosporine	FLT3, PKC, VEGF KIT, PDGFR	Phase I/IB/II	R.M. Stone et al. (2005) R.M. Stone et al. (2005)
CEP701	Lestaurtinib	Indolocarbazole	FLT3, TrkA, VEGFR2 PKC	Phase I/II/III	Smith et al. (2004) Levis et al. (2005b)
MLN518	Tandutinib	Quinazoline	FLT3, PDGFR	Phase I/II	D. Deangelo et al. (2006) D.J. DeAngelo et al. (2006)
SU-5416	Semaxinib	Indolinone	FLT3, KIT, PDGFR VEGFR	Phase II	Giles et al. (2003)
SU11248	Sunitinib	Indolinone	FLT3, VEGFR PDGFRb, KIT	Phase I	O'Farrell et al. (2003)
BAY-43-9006	Sorafenib	Bi-aryl urea	PDGFR, VEGFR Raf kinase	Phase I	Quintás-Cardama et al. (2007)
KW-2449	–	NA	FLT3, Abl, VEGFR FGFR1, Aurora A	Phase I	Cortes et al. (2007b) Pratz et al. (2009)
AC220	–	NA	FLT3, KIT, CSF1R/FMS RET, PDGFRa/b	Phase I	Cortes et al. (2007a)