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Mechanisms of resistance to FLT3 inhibitors and the role of the bone marrow microenvironment

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Synopsis

The presence of FLT3 mutations in AML carries a particularly poor prognosis making the development of FLT3 inhibitors an imperative goal for these patients. The last decade has seen an abundance of clinical trials using these drugs alone or in combination with chemotherapy. This culminated with the imminent approval by the FDA of FLT3 inhibitors for the treatment of AML. Unfortunately, the initial success stories have been rapidly followed by the emergence of clinical resistance. While novel FLT3 inhibitors are actively being developed, studies into mechanisms of resistance to these drugs raise hope of new strategies to prevent emergence of resistance and eliminate minimal residual disease in this AML.

Keywords

FLT3 inhibitors; FLT3-ITD; AML; stem cell niche

Introduction

FLT3 mutated AML represents about a third of all cases of newly diagnosed AML[1]. Two classes of mutations are frequently found: activation loop or tyrosine kinase domain mutations (TKD, about 5-10% of patients) and in-frame, internal tandem duplication (ITD, about 23% of patients). While the prognostic impact of de novo FLT3-TKD mutations is usually minimal, the presence of FLT3-ITD mutations confers poor prognosis in AML[2-4]

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and the frequency of the mutated allele (allelic ratio)[4] as well as the length of the tandem repeat (ITD length) correlate with worse outcome[5].

FLT3 is a class III receptor tyrosine kinase that dimerizes upon ligand binding and undergoes auto-phosphorylation to initiate multiple intracellular signaling programs[6]. These pathways, including PI3K/AKT, Jak/STAT and Ras/MAPK, transduce signals resulting in survival and proliferation of target cells (see Figure 1).

During normal hematopoiesis, FLT3 is expressed in early progenitor cells and the receptor is down-regulated while cells differentiate down the myeloid lineage. Patients with FLT3 mutated AML not only have constitutively active signaling but given the lack of differentiation, these AML blasts continue to express high levels of this mutated protein in addition to the wild type FLT3 receptor[7].

This sustained survival and proliferation signal is the hallmark of FLT3 mutated AML. The clinical presentation of these patients is dominated by hyper-leukocytosis, myelo/monoblastic differentiation and usually de novo (as opposed to secondary) AML. Compared to other poor prognostic factors in AML, the presence of a FLT3 ITD mutation does not have a major impact on achieving remission post-induction chemotherapy, but the remission is characteristically short-lived and relapse often occurs during cycles of consolidation (sometimes while an allogeneic donor search is underway). There is a consensus that prompt blood or marrow transplantation in first remission can improve the outcome in this disease even in the absence of FLT3 inhibitors. Nevertheless, the high rate of relapse even post-transplantation makes this approach alone suboptimal.

In the setting of relapsed disease, the FLT3-ITD mutation is often present at a high allelic ratio[8], and the mutated receptor in the malignant clone renders this disease resistant to chemotherapy and addicted to signaling downstream of this RTK and thus, sensitive to FLT3 inhibitors. Thus, the clinical development of small molecule inhibitors that target mutant FLT3 is an active area of research. More than 60 clinical trials are either open or completed testing different FLT3 inhibitors as single agents or in combination with other therapeutic approaches in AML (clinicaltrials.gov). Full updates on the clinical development of these strategies can be found in this issue by Garcia J and Stone R. Here, we will focus our attention on mechanisms of resistance to FLT3 inhibitors and strategies to overcome such resistance and achieve cure in FLT3 mutated AML.

Mechanisms that allow survival of FLT3 mutated AML cells during treatment with FLT3 inhibitors

Wisdom gathered from over seven decades of antimicrobial use to treat infections tells us that resistance is either acquired via genetic adaptation or due to already-present clones that are selected under survival pressure. In AML, resistance to chemotherapy can take either of the two forms. Elegant genetic studies have shown that AML at diagnosis is a polyclonal disease, while relapsed AML is usually more oligoclonal[9]. Most of the time the relapsed clone can be retrospectively found at presentation but at much lower frequency. This appears to be the case with FLT3 mutated AML relapsing after induction chemotherapy. Most of the

initial malignant subclones are sensitive to treatment and the patient achieves a clean complete remission. Nevertheless some clones survive chemotherapy and are responsible for disease relapse. At relapse, the mutant allelic ratio is often higher than what was seen at diagnosis.

On the other hand, it is probable that the development of resistance to FLT3 inhibitors is at least in part dependent on some genetic or epigenetic events. In order for these genetic and epigenetic events to take place, some FLT3 mutated cells will need to have survived the initial treatment.

Initial studies using FLT3 inhibitors demonstrated clearance of circulating FLT3-ITD blasts, but there was little to no effect on bone marrow blasts[10]. More potent and selective FLT3 inhibitors are effective at differentiating most bone marrow blasts[11] but since these drugs cannot eliminate minimal residual disease as single agents, some leukemia cells, perhaps residing in the stem cell niche, must survive the treatment with the inhibitor.

This early evidence pointed towards stromal mediated mechanisms of survival. To this end, co-culture of FLT3-ITD AML cells with bone marrow mesenchymal stroma also protects the blasts from Quizartinib[12] as well as Fl-700[13]. In these settings, soluble as well as membrane bound cytokines that appear to play a major role include CXCRL12, angiopoietins, as well as VEGF/EGF/IGF and G-CSF/GM-CSF/TNF[12,13]. Similarly, patients with FLT3-ITD AML treated with Quizartinib develop high levels of stromal-derived FGF2 in bone marrow mesenchymal cells and signaling downstream of FGFR1 maintain active RAS/MAPK signaling in FLT3-ITD blasts treated with Quizartinib[14]. Most patients with FLT3-ITD continue to have a wildtype allele of FLT3. This receptor is rather resistant to FLT3 inhibitors but sensitive to FLT3 ligand. Since high levels of FLT3 ligand are found in the bone marrow microenvironment during induction therapy, ligand induced activation of WT FLT3 – MAPK pathway may provide survival signals to leukemic blats even in the presence of effective TKI treatment[14,15].

Potent FLT3-ITD inhibition results in apoptosis of AML blasts in the absence of stroma. The presence of bone marrow mesenchymal stroma rescues the blasts from apoptosis through ERK mediated signaling but not STAT5[12]. In this context, inhibition of FLT3-ITD induces G1 arrest, maybe via downregulation of cyclin D2/Cyclin D3 and subsequent dephosphorylation of Rb[16]. Individual FLT3 inhibitors may have differential effects, for instance, contact with niche cells expand FLT3-ITD blasts treated with SU5615 but not Sorafenib[17].

Complete and sustained inhibition of FLT3-ITD is paramount for successful elimination of the malignant clone. Initial studies with Midostaurin clearly demonstrated the impact of hepatic drug metabolism and CYP3A4 activity in particular of this drug plasma PK's. More so, PD studies have shown wide variations between systemic concentrations and plasma inhibitory activity of various TKIs, likely due to unique protein binding affinities of specific drugs. PD-directed dose escalation clinical studies have mitigated these limitations for the most part and resulted in improved efficacy in clearing not only circulating blasts but also most bone marrow disease. Recent work proposes the existence of unique niches in the bone

marrow, some of which are true biochemical sanctuaries where local drug levels may be significantly different from systemic plasma drug levels[18]. To this end, bone marrow mesenchymal stroma expresses similar levels of drug metabolizing enzymes compared to hepatocytes. They are able to metabolize CYP3A4 substrates creating potential biochemical spaces where FLT3 inhibitors achieve levels inadequate for potent inhibition of FLT3-ITD.

Emergence of resistance

Initially, survival in the setting of TKI therapy probably happens in remote and unique niches within the bone marrow, and thus relies on the presence of a minute population of leukemia stem cells. The emergence of clinical resistance is most likely preceded by cell intrinsic events that allow the new clone to leave the “nest” and dominate the organism. Accumulating knowledge from studying the emergence of resistance to imatinib in CML point towards two types of resistance: a) mutation in the target receptor or b) activation of alternative pathways that by-pass the mutant receptor.

a) Mutations in the target receptor

A variety of FLT3 mutations that could confer resistance to FLT3 inhibitors have been predicted based on *in vitro* models of resistance. Some of these predicted mutations have been confirmed in patients relapsing with FLT3 mutated disease during treatment with FLT3 inhibitors.

Most FLT3 inhibitors are active against FLT3-ITD but have limited activity against TKD mutants. Even though a TKD mutation may have only minimal prognostic value when present at diagnosis, the appearance of point mutations in the FLT3 receptor is a major mechanism of resistance to FLT3 inhibitors. These point mutant can develop either in cis or in trans and newer FLT3 inhibitors have various degrees of activity against individual mutants[19].

It is important to recognize that depending on the domain used to bind the receptor, FLT3 inhibitors can be segregated in two classes: type I inhibitors, like CEP-701, PKC-412 and crenolanib, bind to the “gatekeeper” domain adjacent to the activation loop or the ATP-binding domain; type II inhibitors, like Sorafenib, Quizartinib and MLN518, directly bind the ATP-binding domain. As expected, point mutants conferring resistance to one TKI show cross resistance within the class. To this end, patients with FLT3-ITD that relapse while treated with Quizartinib, if they are found to have point mutations in the activation loop (most frequent D835) or “gatekeeping” domain (i.e. F691) usually show resistance to Sorafenib, another type II TKI. Interestingly, these cells remain sensitive to type I TKIs such as PKC412 and crenolanib[20]. Similarly, some TKIs, like the type I inhibitor TTT-3002 demonstrate preclinical potential to target both type of mutations[21].

b) Activation of alternative signaling pathways

While intensively studied, the accumulation of additional mutations in the FLT3 receptor represents a minority of cases developing resistance to FLT3 inhibitors. In a small study following 60 patients with FLT3-ITD alone treated with single agent TKI, two thirds of patients progressed on FLT3 inhibitor treatment even though they showed no additional

mutations in FLT3 wt allele or FLT3-ITD. Only 22% of patients acquired additional mutations, all of them D835 or I836[22]. Thus, alternative mechanisms of resistance, independent of FLT3 receptor, must be playing a major role and recent studies have uncovered some of these pathways.

Generally, these pathways either provided survival signals independent of FLT3-ITD or they change the transcriptional factor network of the leukemic cell to a state where FLT3 signaling can be replaced by activation of other RTKs.

As mention FLT3-ITD can activate signaling cascades downstream of JAK/STAT, PI3K/AKT and MAPK pathways. Since blasts become addicted to this constitutively active signaling, FLT3 inhibitors induce rapid apoptosis. While microenvironmental factors may rescue these cells in the stem cell niche, development of cell intrinsic mechanisms that can protect these cells from apoptosis coincide with development of resistance to TKIs. FLT3-ITD changes the balance between anti-apoptotic proteins such as Bcl2/Bcl_{XL} and pro-apoptotic BAD. Sustained activation of phospho-STAT5 by FLT3-ITD signaling, for instance, activates Pim kinases which in turn, by phosphorylating BAD, sequesters these proteins in the cytoplasm and allows anti-apoptotic activities of Bcl2 and Bcl_{XL}[23,24]. Inhibition of FLT3-ITD results in rapid loss of phospho-STAT5 and downregulation of Pim-1[23]. Cells resistant to FLT3-inhibitors show sustained activity of Pim-1[23] or Pim-2[25,26] and high levels of phospho-BAD and thus, protection from apoptosis. Thus, combined inhibitions of FLT3-ITD and Pim1[27] or Pim-2[26] are synergistic in inducing apoptosis in mutant blasts. Similarly, high levels of Bcl2 can also confer resistance to FLT3 inhibitors. In these settings the use of Bcl2 inhibitors such as ABT-737 (85) rescues FLT3 inhibitor – induced apoptosis of mutated cells. Interestingly, FLT3-ITD/TKD mutants that show sustained activation of phospho-STAT5 also exhibit elevated levels of anti-apoptotic signals mediated by Bcl_{XL}[28]. In these models, inhibition of the mTOR pathway can rescue the sensitivity of these cells to both FLT3 inhibitors and anthracyclines[28]. Similarly, cells resistant to Sorafenib continue to have an active mTOR/PI3K/Akt pathway even in the presence of effective FLT3 inhibition[29,30], and mTOR inhibitors can re-sensitize the blasts to TKI[29]. Some FLT3-ITD point mutations (D627E) can induce expression of Mcl-1 (a Bcl-2 family member) independent of kinase activity via a conformational change that favors Grb-2 docking[31]. Since Mcl-1, in addition to its anti-apoptotic roles, also impacts mitochondrial morphology and function[32], it is not surprising that Sorafenib resistant cells adopt an abnormal mitochondrial respiratory chain and rely mostly on glycolysis for their energy demands[33]. Thus, glycolytic inhibitors like 2-deoxyglucose can re-sensitize cells to Sorafenib[33]. Of note, a major limitation to a predominantly glycolytic metabolism is a sustained drop in intracellular pH. Consistent with this concept, FLT3-ITD cells developing resistance to Sorafenib also upregulated tescalcin, a type I Na/H exchange channel. Downregulation of this protein or inhibition via amiloride reduced leukemia initiation in xenograft models of Sorafenib resistant FLT3-ITD AML[34].

Maintaining an active MAPK/ERK pathway either by expression of constitutively Axl-1[35] or acquiring activating mutations in NRAS[30] has also been shown to be potential mechanisms of resistance to FLT3 inhibitors.

Epigenetic events, particularly methylation of target genes have been proposed as potential mechanisms of resistance to FLT3 inhibitors. To this end, methylation of SHP-1[36] and silencing of SOCS proteins[37] both negative regulators of JAK/STAT pathway have been implicated in resistance to FLT3-inhibitors. Treatment with DNMT inhibitors not only rescues expression of these proteins but also re-sensitizes the cells to the TKI[36].

Strategies to prevent development of resistance or to sensitize cells to FLT3 inhibitors

Initial agents used to treat patients with FLT3-ITD had broader activity against multiple receptor tyrosine kinases but also less-than-ideal pharmacologic properties. Recently developed inhibitors are more specific and very potent. More targeted agents have fewer side effects and thus, higher doses can be used. On the other hand, agents that inhibit multiple RTKs may prevent emergence of resistance via these mechanisms. Plasma Inhibitory assay (PIA) directed studies have overcome some of the primary failure seen with single agent FLT3-inhibitors and helped optimize dosage for newer drugs.

Given the type of secondary mutations that can arise in the FLT3 receptor during treatment with FLT3 inhibitors, it was suggested that switching to a different class of inhibitor may prove beneficial in these patients. Ponatinib for instance is effective against gate keeper mutations (F691) that arise during treatment with Quizartinib but not against activation loop mutations (D835)[38]. In the same study, SAR302503, a dual Jak2/FLT3 inhibitor was highly effective in vitro against both types of mutants. On the same note, Crenolanib, a type I inhibitor is effective against FLT3-ITD expressing cells that became resistant to Sorafenib via accumulation of mutations in both activation loop[39,40] and gate keeper mutations[40]. Similarly, G-749 a FLT3 inhibitor active against multiple activation loop as well as gate keeper mutations has shown great efficiency in xenograft models of FLT3-ITD AML that would be otherwise resistant to Quizartinib or PKC412[41].

Though single agent FLT3 inhibitors can induce remission, the complete eradication of disease relies on combination therapy. There are a number of preclinical studies that investigate the efficiency of concomitant targeting of FLT3 signaling as well as pathways implicated in resistance. For instance, targeting the ERK/MAPK pathway either by inhibition of upstream RTK such as Axl1[35], inhibition of MEK[42], MERTK[43] or NRas[30] with small molecules showed promising activity in preclinical models including xenografts of FLT3-ITD AML. This approach of multi kinase inhibition can likely by-pass the stromal protection against FLT3 inhibitors and decrease emergence of resistance[12]. To this end, a promising strategy is targeting Pim kinases (known to be upregulated in response to cytokines produced by the mesenchymal stroma). The combination of Pim1/Pim2 inhibitors with FLT3 inhibitors is active against FLT3-ITD AML in preclinical models[26]. Additionally, agents that target anti-apoptotic mechanisms important in FLT3-ITD signaling have shown activity in AML blasts in combination with FLT3 inhibitors. To this end, a dual inhibitor of Akt/FLT3-ITD, A674563, can overcome FLT3 ligand induced drug resistance *in vitro* and in xenograft models[44]. Similarly, mTOR inhibition can sensitize cells to FLT3 inhibition[28] likely via targeting signaling downstream of PI3K[29]. An alternative approach that showed

preclinical activity is to directly target Bcl2. In this regard, ABT-737 has shown synergistic effects with FLT3 inhibitors against FLT3-ITD AML[45]. Corroborated with preliminary data coming from clinical studies using Bcl2 inhibitors agents in other AML subtypes, it may be of interest to study the effects of FLT3 inhibitors in combination with Bcl2 inhibitors in clinical studies.

As mentioned above, a potential mechanism that is associated with resistance to FLT3 inhibitors relies on CDK4/6 activity and their impact on either cyclin D2/cyclin D3 or direct transcriptional activation of FLT3 and Pim kinases. Targeting this mechanism with either the dual CDK4/FLT3-ITD inhibitor, AMG925[46,47] or by adding Palbociclib[48], a CDK6 inhibitor showed promise to sensitize resistant cells to FLT3 inhibitors.

Since the survival of FLT3-ITD leukemia cells depends on absolute levels of FLT3-ITD, decreasing oncoprotein stability using Hsp90 inhibitors[49] or activating autophagy via proteasome inhibitors like Bortezomib[50] can also resensitize resistant FLT3-ITD AML cells to TKI. Lastly, in a small case series, concomitant treatment with CsA benefitted patients with FLT3-ITD AML who were being treated with FLT3 inhibitors, perhaps via inhibition of NFATc1[51]. This mechanism of sensitization will need to be compared in larger patient studies before definitive conclusions can be drawn.

Our approach to resistant FLT3-ITD AML

In spite of all the potentially available strategies to overcome resistance to TKIs, the treatment of patients with FLT3-ITD AML relapsing while on FLT3 inhibitors remains a major clinical challenge. Our current strategy relies on enrollment in a clinical trial if available. If not available, one of the most promising approaches in our clinic is the use of the combination of FLT3 inhibitors (e.g., sorafenib) with hypomethylating agents. We prefer 5-azacitidine[52,53] but other groups have shown similar results with decitabine. In patients receiving sorafenib and 5-azacitidine the leukemia undergoes differentiation. There is a gradual decrease of bone marrow blasts to the point where after three cycles 40-50% of these patients have achieved a morphological remission. To date, it remains unclear how 5-azacitidine or decitabine sensitizes FLT3-ITD cells to TKI but mechanisms may include re-expression of methylated genes such as SOCS1, SOCS2, SOCS3[37] or SHP-1[36] or even tumor de-bulking without the associated increased in FLT3 ligand seen with classical chemotherapy[53]. Lastly, it was suggested that DNMT inhibitors may sensitize cells to FLT3 inhibitors via their pro-differentiation effects[52]. Treatment with 5-azacitidine for instance, decreases total FLT3-ITD as cells differentiate and thus, make them more sensitive to a FLT3 inhibitor. To this end, differentiation agents such as homoharringtonine[54] or all-trans retinoic acid[55] have been shown to synergize with FLT3 inhibitors in inducing apoptosis in FLT3 mutated AML. To what extent these are viable approaches to not only control the bulk of the tumor but eliminate MRD and prevent resistance remains to be tested in clinical studies. Moreover, recent evidence suggests that some bone marrow niches may inactivate retinoids and thus, protect malignant cells from differentiation[56-58].

For this reason, patients with FLT3-ITD that achieve remission after treatment with FLT3 inhibitor plus 5-azacitidine still go on to receive allogeneic transplantation in our center. In

addition, it is our experience that treatment with a FLT3 inhibitor post-transplant helps maintain disease burden to undetectable levels. This may be mediated via both a direct effect on the leukemia clone as well as potential immune modulatory effect of FLT3 inhibitors. Nevertheless, many of these patients do experience various degrees of graft vs host symptoms. To what extent this approach will translate into a viable clinical option is currently being investigated in a BMT CTN clinical trial.

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Key points

1. Pharmacokinetics and pharmacodynamics don't always correlate and pharmacodynamics is a better predictor of efficacy when using FLT3 inhibitors.
2. FLT3 inhibitors are potent in clearing circulating blasts yet this does not always translate into similar effects on bone marrow blasts.
3. Single agent FLT3 inhibitors can induce remission without cure in FLT3-ITD AML but to eliminate the last bastion of minimal residual disease additional interventions are required.
4. Resistance to FLT3 inhibitors may be facilitated by signals from the microenvironment that allow survival of AML cells in the presence of FLT3 inhibitors.
5. Accumulation of additional mutations in the FLT3-ITD AML clone allows for emergence of clones with cell intrinsic resistance to FLT3 inhibitors.
6. Effective and complete elimination of the FLT3-ITD AML clones may be possible via a coordinated approach that targets not only signaling downstream of FLT3-ITD but also the microenvironment-dependent mechanisms of resistance and activate a potent and sustained immune response.

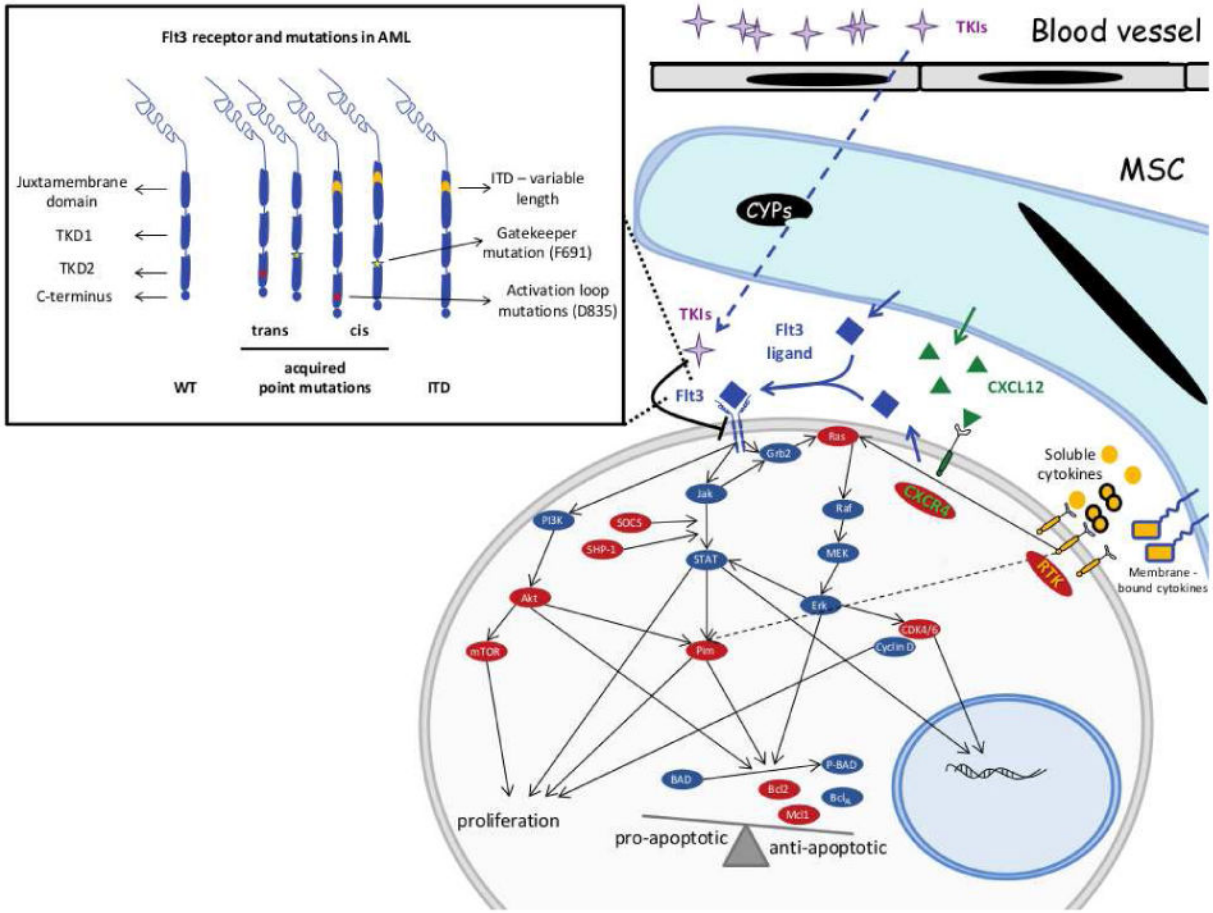

Figure 1. FLT3 signaling and mechanisms of resistance to FLT3 inhibitors

Wild type as well as FLT3 mutated receptor signal via Jak/STAT, PI3K/Akt and Ras/MAPK to provide anti-apoptotic as well as proliferative signaling to the leukemic blasts. Mechanisms that maintain these pathways active in the presence of FLT3 inhibitors create the conditions for the development of resistance. Combining FLT3 inhibitors with inhibitors of these pathways hold the promise of preventing development of resistance. Potential targets are highlighted in red. Point mutations in the FLT3 receptor are detailed in the rectangular insert.