AFTER CHRONIC MYELOGENOUS LEUKEMIA: TYROSINE KINASE INHIBITORS IN OTHER HEMATOLOGIC MALIGNANCIES

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ABSTRACT
Tyrosine kinases phosphorylate proteins on tyrosine residues, producing a biological signal that influences many aspects of cellular function including cell growth, proliferation, differentiation and death. Constitutive or unregulated activity through mutation or overexpression of these enzymes is a common pathologic feature in many acute and chronic leukemias. Inhibition of tyrosine kinases represents a strategy to disrupt signaling pathways that promote neoplastic growth and survival in hematologic malignancies, and likely in other neoplasias as well. This review will focus on tyrosine kinases that have been implicated in the pathogenesis of hematologic diseases other than chronic myelogenous leukemia (CML) and will discuss the evidence for the use of small molecules to target these kinases.
INTRODUCTION

Tyrosine kinases are enzymes that transfer phosphate from ATP to tyrosine residues in specific substrate proteins. There are approximately 100 tyrosine kinases in mammalian cells, which can be divided into two large subfamilies, receptor and non-receptor tyrosine kinases. Receptor tyrosine kinases are highly conserved transmembrane enzymes that respond to ligand binding at the cell surface and transmit messages throughout the cell via phosphorylation of target proteins. Non-receptor tyrosine kinases may be found in the cytoplasm or nucleus, and like receptor tyrosine kinases, are also often involved in the regulation of differentiation, growth and cell death.

Abnormal activation of tyrosine kinases or the signaling pathways they control is thought to play a critical role in the neoplastic process of many human malignancies (Table 1). In normal cells, ligand binding tightly regulates kinase activation. However, mutation or aberrant expression may result in constitutive activation of kinase activity, setting off an intracellular cascade of signaling events that may promote unregulated cell growth or other features of transformed cells. Mutations resulting in constitutive autophosphorylation due to conformational change or overexpression due to gene duplication are likely to be pathophysiologically central to the neoplastic clone. Overexpression due to upregulation at the RNA or protein synthesis level could still be potentially worthy of targeting, but perhaps less so than mutations. Selective inhibition of these activated tyrosine kinases by small molecule inhibitors represents a rational strategy to disrupt signaling pathways that promote neoplastic growth and survival.

Therapy with imatinib mesylate (Gleevec or formerly STI571) in chronic myelogenous leukemia (CML) exemplifies the paradigm of successful targeted therapy through tyrosine kinase inhibition. Imatinib binds to the ATP binding site in the kinase domain of the BCR/ABL tyrosine kinase, thus preventing ATP binding and activation of the kinase. Therapy with imatinib results in durable and complete cytogenetic response in the early stages of CML. However, in accelerated phase and blast crisis CML (whether lymphoid or myeloid) as well as Philadelphia chromosome positive acute lymphoblastic leukemia (ALL), response rates are not as impressive and are short-lived. This review will focus on the use of tyrosine kinase inhibitors in other hematologic malignancies and highlight the difficulties inherent in this therapeutic strategy.
MUTATED TYROSINE KINASES IN CHRONIC MYELOPROLIFERATIVE DISORDERS

As noted above, the successful targeting of BCR/ABL by imatinib has intensified the search for tyrosine kinases that play similar pivotal roles in the molecular pathogenesis of other malignancies. Chromosomal translocations that generate fusion genes involving tyrosine kinase receptors have been identified in a small fraction of myeloproliferative disorders. These fusion genes represent ideal targets for tyrosine kinase inhibition, because of their presumed central role in the neoplastic process of these disorders.

PDGFR-β

Platelet derived growth factor receptor beta (PDGFR-β) belongs to the type III receptor tyrosine kinase family characterized by a transmembrane domain, a juxtamembrane domain, a split kinase domain containing a kinase insert region and a C-terminal tail (Figure 1). Ligand binding to the receptor initiates the signaling cascade by inducing receptor dimerization, leading to activation of the kinase domain, and resulting in autophosphorylation. The autophosphorylated tyrosine residues then act as intracellular docking sites for second messengers involved in mitogenesis, cytoskeletal rearrangements and chemotaxis.

PDGFR-β rearrangements are associated with chronic myelomonocytic leukemia (CMML), a myeloproliferative disorder characterized by dysplastic monocytosis, bone marrow fibrosis, eosinophilia and progression to AML. In 1994, Golub et al. demonstrated that the chromosomal abnormality t(5;12)(q31-q33;p13) found in rare cases of CMML resulted in a fusion gene linking TEL (ETV6), a transcription factor, with PDGFR-β on chromosome 5. Since the initial description, multiple PDGFR-β rearrangements have been identified which characterize other cases of CMML (Table 2). These chromosomal rearrangements result in fusion proteins such that PDGFR-β activity is ligand independent and constitutively activated. The breakpoint of the PDGFR-β contains the transmembrane and intracellular domains of the kinase, but the fusion gene partner replaces its extracellular ligand-binding domain and likely functions to induce ligand-independent dimerization, and ultimately, constitutive activation of the kinase. The fusion protein ETV6-PDGFR-β alone causes hematopoietic cell lines to become
growth factor independent as well as cause a CMML-like disorder in transgenic mice, implicating it in the molecular pathogenesis of the disorder.

Imatinib inhibits PDGFR-β suggesting it is a rational therapy for patients with myeloproliferative disorders associated with activated PDGFR-β receptors. In preclinical models, imatinib inhibited cell lines expressing the ETV6- PDGFR-β, as well as the RAB5- PDGFR-β fusion proteins. In mouse models of CMML transformed by ETV6-PDGFR-β, imatinib treatment resulted in statistically significant prolonged survival compared to controls.

Given these results, imatinib was studied in patients with PDGFR-β translocations. In an updated report by Apperley, et al., nine patients with chromosomal translocations involving PDGFR-β (5q33) were treated with 200-800 mg of imatinib daily. Five of the nine patients had the ETV6- PDGFR-β fusion gene. All patients had leukocytosis and eosinophilia. At a median follow-up of 14 months, all patients responded rapidly. All but one patient had a complete cytogenetic response and two patients attained a complete molecular remission as defined by polymerase chain reaction (PCR) negativity for the ETV6-PDGFR-β transcript. Similarly, a patient with CMML and the RAB5- PDGFR-β fusion gene also responded to imatinib treatment following relapse after stem cell transplantation. In contrast, imatinib has not been shown to be effective in patients with CMML without a PDGFR-β gene rearrangement.

PDGFR-α

In the above report by Apperley et al., eosinophilia was a prominent clinical feature in patients with PDGFR-β gene rearrangements. The authors speculated that PDGFR-β activation is linked to the eosinophilia seen in these patients. DeAngelo and colleagues, treated 16 patients with HES, a rare disorder characterized by sustained overproduction of eosinophils in the bone marrow, peripheral eosinophilia, and tissue infiltration with resultant organ damage, of whom 11 had symptomatic disease and were treated with imatinib 100 mg to 400 mg a day. Nine patients had normal cytogenetics, one patient had a t(1;4)(q44;q12) and one patient had multiple cytogenetic abnormalities. Ten of 11 patients achieved a complete hematologic remission after a median treatment duration of 4 weeks, one of which was transient. This patient was later unresponsive to escalated doses of imatinib. These observations led Cools, Gilliland and their colleagues to closely
examine known imatinib targets for possible involvement in the pathogenesis of HES. One patient in this clinical series was noted to have a t(1;4) chromosomal translocation. This focused attention on the PDGFR-α locus; known to be located at chromosome 4q12. In an elegant example of molecular detective work, Cools and Gilliland found that the kinase domain of PFGFR-α was fused to a previously uncharacterized gene also found on chromosome four, and subsequently named FIP1-like1 (FIP1L1). Further analysis of patient samples found that 9 of 16 patients contained the same novel fusion gene, eight of whom were men. Interestingly, this fusion gene is not the result of a chromosomal translocation, but rather an interstitial deletion.

Sequence analysis of the PDGFR-α kinase domain in the patient with clinical resistance to imatinib demonstrated that the fusion protein had acquired a substitution of isoleucine for tyrosine at codon 674 (T674I) in the ATP-binding region, an analogous position to the common T315I mutation found in BCR/ABL that confers clinical resistance to imatinib. This verifies that the fusion gene is the target for imatinib in HES. The IC50 of imatinib in BA/F3 cell lines transformed with the novel fusion gene is 3.2 nM, thus FIP1L1-PDGFR-α is more sensitive to imatinib than BCR/ABL, explaining why patients with HES responded to imatinib at doses of 100 mg/day, well below the established 400 mg/day used in CML. Only 60% of the patients who responded to imatinib in this series had the novel fusion gene, suggesting that the remaining 40% of responders possibly contain another mutated tyrosine kinase(s) yet to be identified.

An interesting molecular overlap has been identified between the rare disorders of HES and systemic mast cell disease (SMCD). The clinical observation that patients with HES who respond to imatinib tend to have elevated tryptase levels, combined with the evidence that three patients with SMCD and a peripheral eosinophilia had complete responses to imatinib lead investigators to search for evidence of the FIP1L1-PDGFRα fusion gene in these patients. Using fluorescent in situ hybridization (FISH), the novel fusion gene was found all three responding patients. Thus, in this fraction of patients with SMCD, the therapeutic target of imatinib may not be related to c-KIT as previously thought, but rather to FIP1L1-PDGFRα.
OTHER MUTATED KINASES IN CHRONIC MYELOPROLIFERATIVE DISORDERS

Classic translocations resulting in novel fusion genes have been identified in several chronic myeloproliferative disorders and may be additional potential targets for small molecule tyrosine kinase inhibitors. The 8p myeloproliferative syndrome, a rare myeloproliferative disorder with associated eosinophilia that rapidly transforms into acute leukemia has been linked to translocations involving chromosome 8. The classic translocation, t(8;13)(p11;q12) which was first described in this disorder, juxtaposes the fibroblast–growth factor receptor (FGFR) to a zinc finger motif, generating a fusion protein with constitutive activity. In addition, other fusion proteins such as TEL-ABL, or TEL-JAK2, or BCR-JAK2, are also potential targets for tyrosine kinase inhibition. In one brief report, a patient with an aggressive myeloproliferative disorder characterized by a t(9:12) translocation (TEL-ABL) was treated with imatinib 600 mg daily and had a considerable, although transient, clinical response to this treatment. Therefore, patients with disorders characterized by these rare translocations may derive benefit from treatment with small molecule inhibitors and this is an active area of investigation.

MUTATED TYROSINE KINASES IN ACUTE LEUKEMIAS

FLT3

Recently, the Fms-like tyrosine kinase 3 (FLT3) receptor has been identified as a potential therapeutic target in AML. The FLT3 receptor also belongs to the type III class of receptor tyrosine kinases (figure 1), and was cloned in 1991. It plays an important role in normal hematopoiesis as well as leukemogenesis. FLT3 is expressed on stem cell progenitors as well as in 70-100% of AML, but is uncommon in B-cell ALL, T-cell ALL, and CML. Its endogenous ligand is FLT3 ligand, a growth factor for immature myeloid cells and stem cells.

Mutations in FLT3 were first reported in 1996 when internal tandem duplications (ITDs), repeats of 5 – 40 or more amino acids in the juxtamembrane region were discovered in patients with AML. Subsequent studies have demonstrated that these FLT3-ITDs are found in approximately 25% of all cases of AML, 3-5% of myelodysplastic syndromes and infrequently in ALL. FLT3 mutations are more
common in AML patients with normal cytogenetics and t(15;17). The mutation is associated with increased peripheral blood leukocyte counts and a worse prognosis compared to patients without the mutation. In patients with t(15;17), FLT3 mutations are associated with a higher white blood cell count and an increased induction death rate and therefore a lower complete remission rate, but no increase in relapse rate. The loss of the wild type FLT3 allele in conjunction with a FLT3-ITD has been shown to confer an even poorer prognosis.

Mutations within the FLT3 activation loop of the kinase have also been identified. Approximately 7% of AML patients have a substitution of aspartic acid at codon 835, most commonly for a tyrosine residue (D835Y), but other substitutions have been reported. In two patients, a 6 base-pair insertion between codons 840 and 841 in the activation loop of FLT3 has been reported. Interestingly, although infants with mixed lineage leukemia (MLL) do not have FLT3-ITD mutations, a significant fraction has point mutations in the activation loop. These mutations may be at either codon 835 or 836. Infants with MLL gene translocation also tend to express wild-type FLT3 receptors at exceptionally high levels.

The result of either an FLT3-ITD mutation or an activating loop mutation is constitutive activation of the kinase. When wild type and mutant FLT3 genes are transfected into IL-3-dependent cell lines, including 32D and BA/F3 cells, the mutant FLT3-transfected cells (FLT3-ITD) become growth factor independent. In contrast, the wild-type FLT3-transfected cells have minimal proliferation despite stimulation with FLT3 ligand. Furthermore, the cells expressing mutant FLT3-ITD demonstrate constitutive activation of STAT5 and MAP kinases, the signaling pathways of FLT3. When FLT3-ITD mutants identical to those identified from primary human leukemia samples were transduced into primary mouse bone marrow cells using a retrovirus, a myeloproliferative disorder but not overt leukemia developed. This suggests that FLT3-ITD is sufficient to induce a proliferative signal similar to that of BCR/ABL in chronic phase CML but not sufficient to cause acute leukemia unless paired with other cellular events. The need for a second cooperating mutation has been confirmed in a murine model using PML-RARA transgenic mice transduced with an activated FLT3 allele. Introduction of the mutated FLT3 allele into the background of the PML-RARA mouse
resulted in a more rapid transformation to leukemia, with a range of 62-299 days versus 8.5 months in mice without the mutated FLT3 allele. 62

These preclinical studies demonstrated that the FLT3-ITD mutation is a potential therapeutic target in AML. The first studies to validate this concept used the compounds herbimycin a, AC1296 and AG1295. 63-65 These compounds inhibited FLT3-ITD transformed cells, and prolonged the development of leukemia phenotypes in mice with FLT3-ITD induced myeloproliferative disorders.

Newer FLT3 inhibitors have been developed which have shown promise in preclinical models and have moved onto clinical trials in humans. At least four compounds are currently under development (Table 3). All have been found to be active in preclinical in vitro and animal models of FLT3-ITD disease. 66-70 CEP-701 (Cephalon, Inc., West Chester, PA) is a novel indolocarbazole derivative, which inhibits the autophosphorylation of wild type and constitutively activated FLT3 in vitro with an IC50 of 2-3 nM. 71 Results of the phase I/II trial using single agent CEP-701 in patients with relapsed, refractory or poor risk AML and activating FLT3 mutations have recently been reported. 71 The first three patients were treated at 40 mg by mouth twice a day, but ex-vivo analysis showed incomplete inhibition of FLT3 autophosphorylation and no response was seen. The next 14 patients were treated at 60 mg twice a day, 3 of whom were escalated to 80 mg twice a day. Of these 14 patients, 4 had a decrease in peripheral blood leukemic blasts to <5%, with improvement in absolute neutrophil counts and one patient had a decrease in bone marrow blasts to <5%. Grade 3 and 4 toxicities included febrile neutropenia, which occurred in 11 patients. More common less severe, grade 1 and 2 side effects included nausea, emesis and fatigue.

MLN518 (CT53518) is a piperazinyl quinazoline. In a phase I trial, 40 patients with AML and/or myelodysplasia were treated with escalating doses of the compound. 72 The dose-limiting toxicity of MLN518 was reversible, generalized weakness, which occurred in 3/9 patients treated at doses of 525 mg or more. This toxicity correlated with plasma concentrations >2000 nM, well above the level associated with inhibition of FLT3 autophosphorylation. Stabilization of peripheral blood counts, for > 5 months was seen in 2 patients with wild-type FLT3. In one patient with a FLT3-ITD, the bone marrow blast count decreased from 80% to 15% with reductions of the peripheral blood
blast count over the first 288 days of therapy. The phase II study of this drug will evaluate response at the maximum tolerated dose of 525 mg twice-daily dose in patients with relapsed or refractory AML with confirmed FLT3-ITD. Of note, unlike CEP-701, MLN518 is not active against the D835Y mutation in the activation loop of FLT3.\(^{67}\)

PKC412 (Novartis, Pharmaceuticals, Basel, Switzerland), an N-benzolystaurosporine, originally developed as a vascular endothelial growth factor receptor (VEGFR) and protein kinase C (PKC) inhibitor, is one of the more developed FLT3 inhibitors. A phase I trial of PKC412 in patients with advanced solid malignancies showed it to be a well tolerated oral therapy. The most frequent treatment related toxicities were nausea, vomiting, fatigue and diarrhea.\(^{73}\) A phase II trial of PKC412 at 75 mg by mouth three times a day was undertaken in patients with AML that expressed either a FLT3-ITD or an activation loop mutation.\(^{74,75}\) Patients had to have relapsed or refractory disease or not be candidates for cytotoxic chemotherapy. Of the first 14 patients treated, 12 had a >50% reduction in peripheral blasts compared to baseline including two patients who cleared blasts by day 29. Five patients had reduction in bone marrow blasts by >50%, of whom one had <5% blasts with normal peripheral counts on day 96 of treatment. Furthermore, a decrease in FLT3 autophosphorylation relative to total FLT3 protein occurred in 75% of patients’ blast samples obtained 24 hours after the start of PKC 412 compared to baseline, indicating the target was inhibited. A phase II trial was also undertaken in patients with wild type FLT3 and no significant responses beyond transient hematologic improvement were seen.\(^{76}\)

SU5416, SU5614 and SU11248 (Sugen, San Francisco, Ca), which are indolinones, also have FLT3 inhibitor activity whereas SU6668 has no significant effect on FLT3 (IC\(_{50}\) >50 µM).\(^{69,77}\) A phase I study of SU11248 has been completed in patients with AML.\(^{78}\) Five patients had FLT3 gene mutations (3 ITD and 2 activating loop mutations). The investigators reported a decrease in peripheral blast counts in some patients following a single dose of SU11248, however data on response rates are not yet available.

The initial results of these early FLT3 inhibitor studies indicate that these compounds have biologic activity against AML. However, few patients have achieved a complete or durable remission with single agent therapy. Diverse factors may contribute
to the rather modest activity of FLT3 inhibitors in AML. Incomplete kinase inhibition has been seen in some cases. However, primary resistance was also observed in the presence of complete kinase inhibition, perhaps because FLT3 mutations are a late event in the pathogenesis of AML, and thus may not be essential to leukemogenesis per se. This is in contrast to BCR-ABL, which is thought to initiate CML. In addition, the patients studied were heavily pretreated. In this setting, a low response rate is not unexpected, in analogy to myeloid blast crisis of CML, where patients pretreated for blastic transformation responded less well to imatinib than patients without prior therapy. Nonetheless, similar to imatinib, these agents are well tolerated with little side effects and their toxicity profiles are well suited to combination with cytotoxic chemotherapy. To have a significant clinical impact, it is apparent that FLT3 inhibitors will need to be combined with chemotherapy or even other targeted therapy, much in the way that ATRA is for acute promyelocytic leukemia.

c-KIT

c-KIT is another receptor tyrosine kinase in the type-III subfamily (Figure 1). It is expressed on hematopoietic progenitor cells, mast cells, germ cells, and the pacemaker cells of the gut. c-KIT is expressed in a variety of human malignancies and is mutated and constitutively activated in gastrointestinal stromal cell tumor (GIST), mastocytosis/mast cell leukemia and acute myelogenous leukemia. Activating mutations can occur in many different exons of the c-KIT gene and activation of signaling pathways leads to proliferation, differentiation, migration and survival of hematopoietic stem cells, mast cells, melanocytes and germ cells.

Valine substituted for aspartic acid at codon 816 (D816V mutation) in the activation loop of the kinase catalytic domain is the most common activating mutation in c-KIT (figure 1). It is predominantly found in systemic mastocytosis or mast cell leukemia, but has also been detected in patients with myeloproliferative disorders and some cases of AML. This mutation results in a 10-fold increase in the specific activity and a nine-fold increase in adenosine triphosphate (ATP) affinity. Although imatinib inhibits wild type c- KIT, the D816V mutation is resistant to imatinib. This is
likely related to the increased affinity of the mutated c-KIT for ATP or to conformational changes in the activation loop of the receptor that inhibits binding of imatinib.

Recently, investigators demonstrated that PKC412 inhibits BA/F3 cell lines stably transformed by the c-KIT mutations, D816Y and D816V, with an IC$_{50}$ of 20 and 30 nM, respectively.$^{83}$ These results were extended clinically when a 48-year-old woman with mast cell leukemia and a D816V mutation underwent treatment with PKC412. Treatment with PKC412 100 mg twice daily resulted in clinical improvement, with a reduction of mast cells, and a decrease in myeloblasts from 5-10% in the marrow at diagnosis to <5% after two months of therapy. Further follow-up and larger studies are warranted to determine the true efficacy of PKC412 or other tyrosine kinase inhibitors in diseases characterized by D816V c-KIT mutations.

Two isoforms of mutated c-KIT have been identified in AML, both associated with the cytogenetic abnormalities of t(8;21) or inversion (16).$^{80,84}$ One involves Aspartate 816 previously mentioned; the other involves a highly conserved aspartic acid residue at position 419, located in the extracellular domain (Figure 1).$^{80}$ The D816 mutation is identical to that in mastocytosis and thus is not amenable to targeted therapy with imatinib. It is unclear if the other mutation is amenable to targeting with imatinib, as it has not been reported to be an activating mutation.$^{80}$ Targeting of c-KIT mutations in AML is further inhibited by the fact that less than 8% of AML patients have c-KIT mutations.$^{80}$

**OVEREXPRESSION OF TYROSINE KINASE AS TARGET**

**c-KIT**

Although c-KIT is rarely mutated, it is expressed in approximately 60-80% of AML, and “overexpressed” in a fraction of those cases.$^{85-87}$ The addition of stem cell factor (SCF), the endogenous ligand for c-KIT results in the proliferation of KIT-positive AML blast cell lines such as MO7E.$^{88}$ Since a significant percentage of AML patients overexpress wild-type c-KIT, and imatinib has been shown to be selective inhibitor of c-KIT, the use of imatinib in c-KIT positive AML has been proposed. The rationale for treatment is based on the hypothesis that high levels of receptor *in vivo* might confer a growth advantage in the marrow because of the presence of abundant KIT ligand present in stromal cells. In a phase II pilot study, 21 patients with relapsed, or refractory c-KIT
positive AML were treated with imatinib 600 mg a day.\textsuperscript{89} Five responses were seen, 2 patients had a complete hematologic remission, 2 had partial responses, and one patient had no evidence of leukemia. Western blotting of primary blasts taken from patients confirmed that c-KIT was activated, but mutational analysis did not identify any previously identified c-KIT mutations. Furthermore, activity of imatinib did not correlate with decreased c-KIT phosphorylation. Therefore, it is difficult to determine the cause for the modest activity of imatinib in this disease.

As noted, the small molecules SU5416 and SU6668 inhibit c-KIT, as well as VEGFR-2, FGFR, FLT-3 and PDGFR.\textsuperscript{90} In preclinical models using M07E cells, a human myeloid leukemia cell line, SU5416 and SU6668 inhibited tyrosine autophosphorylation of c-KIT in a dose dependent manner. In addition, when used on leukemic blasts from c-KIT positive patients, both compounds inhibited stem cell factor induced phosphorylation of c-KIT and induced apoptosis.\textsuperscript{90} SU5416 has been tested in patients with refractory c-KIT positive AML in Europe.\textsuperscript{91,92} In this trial 43 patients with AML whose leukemic blasts overexpressed c-KIT were treated with twice weekly infusions of 145 mg/m\textsuperscript{2} of SU5416. Of 25 evaluable patients, only one patient had a complete response, defined as <5% blasts in the bone marrow, absence of circulating blasts in the peripheral blood, but without normalization of blood counts and 7 patients had a PR (defined as reduction of blasts in blood and/or bone marrow by at least 50%) which lasted 1-5 months. None of the patients that responded had FLT3–ITD’s but the presence of FLT3 activating loop mutations was not evaluated and targeting of c-KIT was not demonstrated in ancillary studies.

**VEGF**

Similar to c-KIT in AML, the vascular endothelial growth factor receptors (VEGFR) are tyrosine kinases that are overexpressed in hematologic malignancies. Their ligand, VEGF is responsible for many endothelial cell functions, which are regulated through the receptors: VEGFR-1 (Flt-1), VEGFR-2 and VEGFR-3 (Flt-4).\textsuperscript{93} VEGF has been implicated in tumor angiogenesis and may play a role in the pathophysiology of hematologic malignancies by regulation of bone marrow angiogenesis through autocrine and paracrine mechanisms. Murine models using homozygous gene knockouts suggest differential roles for VEGFR-1 and VEGFR-2; VEGFR-2 expression is important in
angiogenesis and vasculogenesis whereas VEGFR-1 may be more important for endothelial organization and remodeling.\textsuperscript{94,95}

Microvessel density is an indirect way of measuring angiogenesis and is elevated in the bone marrow of patients with MDS and AML, and decreases following chemotherapy in responsive disease.\textsuperscript{96,97} Immunohistochemical analysis of bone marrow biopsies reveal that changes in microvessel density parallels changes in VEGF and VEGFR-2 expression in patients with AML implicating VEGF signaling in angiogenesis.\textsuperscript{98} Furthermore, cellular VEGF levels appear to correlate inversely to survival in patients with AML.\textsuperscript{99} As a result, a number of receptor tyrosine kinase inhibitors are being used in hematologic malignancies to block angiogenesis through VEGF inhibition. These include SU5416, SU11248, PKC412, PTK787, and others.\textsuperscript{100,101}

In the large European study using SU5416 in patients with c-KIT positive AML, correlative studies were performed to assay VEGF inhibition.\textsuperscript{92} The investigators evaluated VEGF mRNA expression, measured by polymerase chain PCR and found that patients who responded to SU5416 expressed high levels of VEGF mRNA prior to treatment. In contrast, the non-responders did not have high VEGF expression prior to treatment. Decreased microvessel density in the bone marrow of responders correlated with VEGF expression. Though these results are intriguing, it remains unclear whether the clinical activity seen with this compound is due to VEGF or c-KIT or FLT3 inhibition, or if these results are an epiphenomenon.

Furthermore, large studies have not been able to demonstrate a clear relationship between changes in microvessel density and clinical response\textsuperscript{102} and the best assay for measuring changes in angiogenesis remains unclear. Recently, circulating endothelial cells (CEC) have been shown to be a promising, noninvasive surrogate marker for angiogenesis in preclinical studies using murine models of cancer, as well as in several clinical studies of angiogenesis inhibitors.\textsuperscript{103-105}

PROBLEMS IN THE DEVELOPMENT OF TYROSINE KINASE INHIBITORS AS THERAPEUTIC AGENTS

The most important issue pertaining to tyrosine kinase inhibition is whether a putative target is relevant in the pathogenesis of the disease in question. Mutation independent overexpression, as seen with VEGF and c-KIT, may well represent an
epiphenomenon, and may not be important in the neoplastic pathophysiology. As a result, inhibition of the overexpressed target might have little clinical benefit. Furthermore, a mutation may be a later mutational event in the pathophysiology of the disease and inhibition of this ‘secondary’ target may only inhibit the proliferative thrust, but not delete the leukemic clone. Ancillary studies from the clinical trial of CEP-701 demonstrated that despite >85% FLT3 inhibition in two patients there was no clinical impact on disease.\textsuperscript{71} Other studies suggest that FLT3 mutational status changes from diagnosis to relapse and different FLT3 mutation clones may be dominant from one stage of disease to another.\textsuperscript{106,107} If a FLT3-ITD is a later event, then there will be FLT3 negative, or wild type leukemic clones, and thus FLT3 inhibition may preferentially select for these clones causing resistance. Key to further development of these inhibitors will be the identification and verification of their therapeutic targets given their broad specificity.

Resistance remains one of the greatest challenges facing the development of tyrosine kinase inhibitors. Imatinib resistance in CML serves as a cautionary note for other drugs under development. The mechanism of action of these small molecule inhibitors are thought to be similar; blocking of ATP binding to the kinase domain.\textsuperscript{108,109} Thus, mechanisms of imatinib resistance in CML may be analogous to resistance to other small molecule inhibitors. In CML point mutations in BCR/ABL have been identified at the time of resistance that are not always detectable in samples taken prior to the initiation of therapy.\textsuperscript{31,110-113} These point mutations predominantly occur in the kinase domain, and more than a dozen different mutations have been reported.\textsuperscript{110-112,114} This same mechanism of resistance has been demonstrated in HES as well as with FLT3 inhibitor resistance, \textit{in vitro}.\textsuperscript{115} Data are emerging from the FLT3 inhibitor clinical trials, which will help to determine clearly the mechanisms of resistance to these small molecules.

Strategies to overcome resistance may include the combination of small molecules with chemotherapy or even other signal transduction inhibitors. For example, the development of FLT3 inhibitors in AML will probably require a combination of small molecules with chemotherapy for full activity. Preclinical studies demonstrate that the schedule of administration and drug combinations are important factors for efficacy.\textsuperscript{116}
Giving a tyrosine kinase inhibitor prior to chemotherapy could prevent DNA synthesis, thereby making the leukemic blasts relatively resistant to cell cycle active chemotherapy agents. In addition, inhibitors with different binding specificities could be combined. *In vitro* studies have demonstrated that different FLT3 mutations have varying sensitivities to the different FLT3 inhibitors.\textsuperscript{117} This suggests that molecular analysis of relapse samples may be useful in determining which inhibitor to use or how best to combine inhibitors.

CONCLUSION

Tyrosine kinase inhibition as a therapeutic strategy has been proven successful by the treatment of chronic phase CML with imatinib. This rationale has been translated successfully to other chronic hematologic malignancies such as CMML and HES that may arise from a mutation of a single tyrosine kinase. This strategy is more complicated when extended to advanced diseases such as AML. In some cases, tyrosine kinases are not mutated but overexpressed resulting in increased autocrine signaling through these receptors. Targeting this mechanism of signal transduction activation has not resulted in overwhelming benefit or response, as exemplified by c-KIT, wild type FLT3 and VEGF inhibition. Furthermore, if a FLT3 inhibitor works it will be important to determine if efficacy is only due to inhibition of activating FLT3 mutations or due to inhibition of other targets. Ancillary studies may help to determine which targets are inhibited, but there is no substitute for clinical trials in this regard. Furthermore, given the low frequency of complete remissions with single agent therapy, FLT-3 inhibitors will need to be combined with cytoreductive chemotherapy. As the use of tyrosine kinases in chronic and acute leukemias has expanded, new targets such as the FIP1L1-PDGFR-\textalpha rearrangement in HES and SMCD have been discovered. The search is on to identify other novel targets. Molecularly targeted therapy remains a promising area of development that will continue to expand, as we understand more about the molecular pathogenesis of hematologic malignancies.
Table 1. Mutated Tyrosine Kinases in Human Malignancies

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<td>Gastric Carcinoma</td>
</tr>
<tr>
<td>FLT3</td>
<td>AML</td>
</tr>
<tr>
<td></td>
<td>MDS</td>
</tr>
<tr>
<td></td>
<td>ALL</td>
</tr>
<tr>
<td>PDGFR-α</td>
<td>Hypereosinophilic syndrome</td>
</tr>
<tr>
<td></td>
<td>Systemic Mast Cell Disease</td>
</tr>
<tr>
<td></td>
<td>GIST</td>
</tr>
<tr>
<td>c-KIT</td>
<td>AML</td>
</tr>
<tr>
<td></td>
<td>Mastocytosis/Mast Cell Leukemia</td>
</tr>
<tr>
<td></td>
<td>GIST</td>
</tr>
<tr>
<td>c-met</td>
<td>Lung Cancer</td>
</tr>
<tr>
<td>c-fms</td>
<td>AML, MDS</td>
</tr>
<tr>
<td>FGFR</td>
<td>MPD/AML</td>
</tr>
</tbody>
</table>

Abbreviations: CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CNNL, chronic myelomonocytic
leukemia; MEN2, multiple endocrine neoplasia 2 syndrome; MDS, myelodysplasia; GIST, gastro-intestinal stromal cell tumor; MPD, myeloproliferative disorder.
Table 2: Fusion Proteins and Cytogenetic Abnormalities involving PDGFRβ

<table>
<thead>
<tr>
<th>CYTOGENETIC ABNORMALITY</th>
<th>TYROSINE KINASE FUSION PROTEIN</th>
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<tbody>
<tr>
<td>t(5;12)(q33;q13)(^{12})</td>
<td>TEL-PDGFRβ</td>
</tr>
<tr>
<td>t(5;7)(q33;q11)(^{13})</td>
<td>HIP1-PDGFRβ</td>
</tr>
<tr>
<td>t(5;10)(q33;q21)(^{14,15})</td>
<td>H4-PDGFRβ</td>
</tr>
<tr>
<td>t(5;17)(q33;p13)(^{16})</td>
<td>RAB5-PDGFRβ</td>
</tr>
<tr>
<td>t(5;14)(q33;p32)(^{17})</td>
<td>CEV14-PDGFRβ</td>
</tr>
<tr>
<td>t(5;14) (q33; q24)(^{18})</td>
<td>NIN- PDGFRβ</td>
</tr>
<tr>
<td>t(1;5) (q 23; q33)(^{19})</td>
<td>Myomegalin-PDGFRβ</td>
</tr>
<tr>
<td>t(5;17) (q33; p11.2)(^{20})</td>
<td>HCMOGT- PDGFRβ</td>
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<tr>
<td>Tyrosine Kinase Inhibitor</td>
<td>Class</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------</td>
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<tr>
<td>PKC41268,73,75,76</td>
<td>Benzoylstaurosporine</td>
</tr>
<tr>
<td>CEP-70166,71,116,144,145</td>
<td>Indolocarbazole</td>
</tr>
<tr>
<td>CT35318 67,72,146</td>
<td>Piperazinyl quinazoline</td>
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<tr>
<td>SU541677,90,92,147</td>
<td>Indolinone</td>
</tr>
<tr>
<td>SU1124890,78</td>
<td>Indolinone</td>
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† Receptor activity in descending order of potency
‡ FLT3 autophosphorylation in vitro
* Cardiac toxicity observed in AML patients with prior anthracycline use
Figure 1: Structure of Type III Receptor Tyrosine Kinases and Common Mutations

Legend: Schematic depicting structure of receptor tyrosine kinase type III family on left, showing five immunoglobulin-like ligand-binding domains; JM, juxtamembrane domain; and two split kinase domains. FLT3 receptor has mutations identified in the JM; as well as in the second split kinase domain at codon 835. C-KIT has a mutation in the immunoglobulin extracellular domain at a highly conserved Aspartate residue at codon 419 as well as in the split kinase domain at codon 816, which is frequently seen in mast-cell leukemia or mastocytosis.
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