Targeting FLT3 Kinase in Acute Myelogenous Leukemia: Progress, Perils, and Prospects

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Abstract: Activating mutations of the FLT3 receptor tyrosine kinase are the most common recurring genetic abnormality in acute myelogenous leukemia (AM). Inhibition of FLT3 kinase activity by small molecule inhibitors has been proposed as a novel therapeutic approach AML. The pre-clinical and clinical development of candidate FLT3 inhibitors will be reviewed.

Keywords: FLT3, tyrosine kinase, kinase inhibitor, AML, KIT, PDGFR

INTRODUCTION

Acute myelogenous leukemia (AML) is the most common type of acute leukemia in adults. Patients typically respond to initial treatment with anthracycline and cytosine arabanoside-based induction chemotherapy, but most patients ultimately relapse and die of refractory disease. The prognosis is worse in elderly patients who typically experience substantially greater treatment-related toxicities with few durable remissions and are rarely cured. In addition, the elderly are usually not candidates for more intensive therapy using stem cell transplantation. Although advances in supportive care have improved treatment-related mortality, the overall cure rate in AML has not improved significantly in the last decade. This plateau has lead to a search for newer therapies that might target fundamental molecular abnormalities in AML cells [1-4].

MOLECULAR ABNORMALITIES IN AML

Our understanding of the genetic abnormalities underlying AML has increased significantly over the last two decades. This progress has been aided by the relative ease of obtaining AML cells from the blood and/or bone marrow for study, cloning of hematopoietic growth factors and receptors, and advances in techniques for the detection and characterization of genomic abnormalities including gene deletions, chromosomal translocations and point mutations. A striking feature of leukemia in general, and AML in particular, are recurrent non-random cytogenetic abnormalities, particularly chromosomal translocations. Human leukemias are associated with more than 300 recurring chromosomal translocations. As of 2003, more than 100 of these translocations have been cloned [5, 6].

Almost invariably, chromosomal translocations associated with AML target transcription factors or components of the transcriptional activation machinery. These include retinoic acid receptor alpha (RARα), HOX family members, core binding factor (CBF) components, MLL, CPB, and p300. Disruption of these genes results in altered protein-protein interactions and changes in the program of gene transcription that lead to impaired hematopoietic cell differentiation [5, 6].

Several lines of evidence suggest that such translocations are necessary but not sufficient to cause AML. For example, individuals with familial platelet disorder with propensity to develop AML (FPD/AML), have an autosomal dominantly inherited syndrome due to inheritance of a single copy of a dominant negative AML1 gene that inactivates the wild-type allele. Affected individuals have qualitative and quantitative platelet disorders, progressive dysplasia and pancytopenia that worsens with increasing age, and a marked increase in risk of AML. AML in such individuals develops with a long latency and is invariably associated with additional cytogenetic or genomic abnormalities[7]. Likewise, the AML that develops in mice with targeted disruption of CBF (e.g. AML1/ETO) is invariably associated with additional mutations. Similar results are obtained in murine models of AML associated with the PML/RARα or MLL/CBP fusion genes [8-13].

In the past decade, a number of recurrent mutations that increase the proliferation of AML cells have been identified. These include point mutations of NRAS and KRAS as well as varied mechanisms for the activation of non-receptor and receptor tyrosine kinases (RTKs). Constitutive tyrosine kinase activation can result from chromosomal translocations generating fusion proteins with one or more N-terminal dimerization/oligomerization motifs and a C-terminal tyrosine kinase domain. Alternatively, mutation of critical regulatory regions of RTKs can result in constitutive kinase activation [6, 14-17].

Recent evidence suggests a "two mutation class" model of AML (Fig. **1**). As discussed above, one class of mutation (usually involving transcription factors) acts primarily to impair hematopoietic differentiation. Acting alone, these abnormalities lead to ineffective hematopoiesis and varying degrees of bone marrow failure. The second class of mutations result in increased proliferation and/or survival but does not substantially affect differentiation. Acting alone, mutations of this second type lead to a

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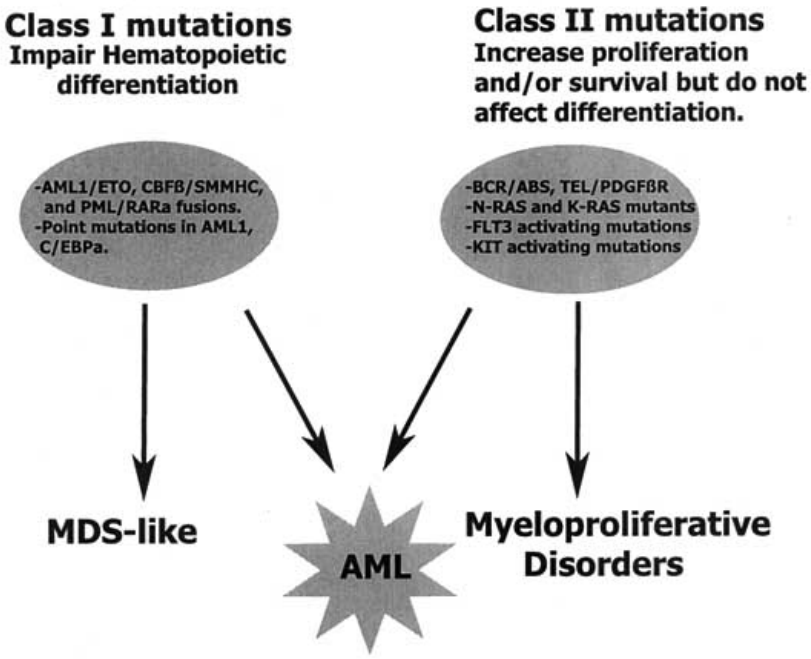
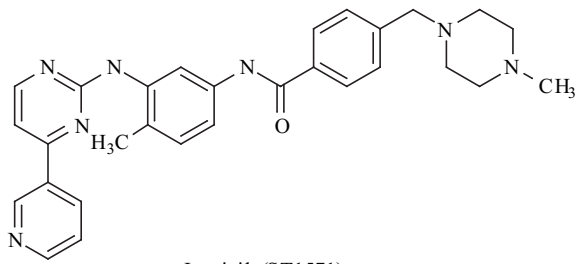


Fig. (1). Multistep molecular pathogenesis of AML. Class I mutations, such as translocations involving RARα or AML1, primarily impair hematopoietic differentiation with increasing cellular proliferation. In isolation, these mutations result in bone marrow failure/myelodysplastic syndromes. Class II mutations, such as activating mutations of NRAS or FLT3, primarily increase hematopoietic cell proliferation without impairing cellular differentiation. In isolation, acqusition of a class II mutation in a hematopoietic stem cell would result in a myeloproliferative syndrome (e.g. CML). Sequential acquisition of a class I mutation followed by a class II mutation results in AML. This figure is adapted from a model proposed by Kelly and Gilliland [6].

myeloproliferative disorder (e.g. Chronic Myelogenous Leukemia (CML)). Serial acquisition of a mutation from both classes results in AML, which is characterized by increased proliferation of poorly differentiated myeloblasts. Based on this model, it is predicted that mutations that impair differentiation usually arise first, as most cases of AML do not have an antecedent myeloproliferative phase. Indeed, in many instances a myelodysplastic syndrome or bone marrow failure syndrome precedes the development of overt AML [6, 18].

The recent success of imatinib mesylate (Gleevec, Glivec, formerly STI571, see Fig. **(2)**) in treating CML has lead to enthusiasm for the development of targeted therapy for AML [19, 20]. Logically, such therapies should be most efficacious if the target is a critical molecular mechanism initiating or maintaining the leukemic state. Although the weight of evidence suggests that translocations involving transcriptional machinery are the initiating event in many cases of AML, in most instances it is not yet possible to effectively target these abnormalities. The lone exception has been the development of all trans-retinoic acid (ATRA) therapy in a subtype of AML known as acute progranulocytic leukemia (APL) [21, 22]. New approaches to selective inhibition of specific histone deacetylases and disruption of pathological transcription factor complexes are urgently needed [23, 24]. In contrast, substantial progress has been made in the development of specific tyrosine kinase inhibitors.



Imatinib (ST1571)

Fig. (2). Chemical Structure of imatinib mesylate (Gleevec, formerly STI571).

STRATEGIES TO INHIBIT TYROSINE KINASES

There are two broad strategies for inhibiting tyrosine kinase activity: 1) inhibition of protein expression; and 2) inhibition of enzymatic activity (Table 1). While methods to inhibit kinase expression have been demonstrated in the laboratory, this strategy has yet to be translated successfully to the clinic (with the exception of monoclonal antibodies, which in some cases may induce degradation of the targeted kinase) [25]. Likewise, it has proven difficult to develop drugs that specifically inhibit the binding of signaling substrates to the catalytic domains of tyrosine kinases, even though this approach offers potential selectivity due to substantial sequence divergence in this domain of tyrosine kinases. Interfering with substrate binding theoretically could allow for the development of inhibitors that block the binding of proteins required for "oncogenic" signaling while

still allowing binding of proteins required for "normal" signal transduction through other pathways [26, 27].

Table 1. Therapeutic Approaches to Inhibiting Tyrosine Kinases in Human Cancers

Strategies to Inhibit the Expression of Tyrosine Kinases
Nucleic acids
Anti-sense oligonucleotides
Interfering RNAs
Monoclonal antibodies
Inhibitors of molecular chaperones (e.g. HSP90)
Strategies to Inhibit the Activity of Tyrosine Kinases
Monoclonal antibodies
Soluble Receptor (to bind ligand)
Monomeric ligand (to block dimerization of receptor tyrosine kinases)
Small molecule ATP antagonists
Small molecule inhibitors of protein substrate binding

Two methods for inhibiting tyrosine kinases have achieved clinical success. These are RTK-specific monoclonal antibodies and small molecule inhibitors that block the binding of ATP to tyrosine kinases. Trastuzumab (Herceptin^{TM}), a monoclonal antibody to the RTK HER2 is FDA approved for the treatment of breast carcinomas that over-express HER2 [28]. The mechanisms of action of trastuzumab are not fully understood but appear to include: 1) accelerated degradation of HER2 leading to lowered cellular HER2 expression; 2) inhibition of the activation of HER2 by interference of the heterodimerization of HER2 with other HER family members (e.g. HER1); and 3) targeting the immune system to HER2 over-expressing cells [25]. Although it is not clear what the relative contributions of these or other mechanisms are to clinical efficacy, it has been suggested that immune targeting may be the major mechanism of action of trastuzumab [29]. Another example of a monoclonal antibody-based approach to kinase inhibition is cetuximab (C225), an antibody that targets the EGF receptor, which is being tested against a variety of carcinomas [30]. Because monoclonal antibodies are most effective against extra-cellular epitopes, they are better suited for targeting RTKs than NRTKs.

Small molecule antagonists of ATP binding to tyrosine kinases have thus far proven to be the most effective drugs for inhibiting tyrosine kinase activity. All tyrosine kinases share a similar enzymatic activity – transfer of a phosphate group from ATP to a tyrosine residue – and are therefore, highly homologous in their kinase domains. Despite the high degree of conservation of the ATP binding domain in all protein kinases, there are variations in the protein conformations around the ATP binding pocket that have allowed the development of inhibitors with surprising specificity and affinity. The specificity is achieved in part through interaction of the inhibitors with amino acids that are different than those directly involved in binding ATP [26]. The remainder of this review will focus on the pre-clinical and clinical development of small molecule inhibitors of FLT3 kinase for the treatment of AML. These efforts offer a paradigm for future development of tyrosine kinase inhibitors (TKIs) for novel targets in AML and other human cancers.

FLT3: BACKGROUND

FLT3 is a member of the type III RTK family that includes KIT, CSF1R, PDGFRA, and PDGFRB. All of these growth factor receptors have conserved structural features including an extracellular ligand binding domain with five immunoglobulin like domains, a single trans-membrane domain, and a catalytic domain that is split into two portions separated by a hydrophilic kinase insert. Consistent with this conserved protein structure, the genes share a common genomic organization. The kinase domains of the family members are highly conserved, whereas there is substantial sequence divergence in the extracellular and kinase insert regions [31, 32].

Human FLT3 has 993 amino acids and a predicted molecular mass of 110 kiloDaltons. The cell-associated protein is expressed in two forms: a fully glycosylated and processed form with a molecular mass of 155 kiloDaltons and a partially glycosylated/processed form with a molecular mass of 135 kiloDaltons [31]. FLT3 is widely expressed by lymphohematopoietic tissues but not in most non-hematopoietic tissues except for the gonads, brain, and placenta [31, 33]. High-level expression of FLT3 protein is confined to the $CD34^+$ cell compartment (bone marrow 60-80% positive, umbilical cord blood 90%]. CFU-GM, HPP-CFC and B-cell precursors express FLT3. In contrast, FLT3 is not expressed by mature B or T lymphocytes, monocytes, eosinophils, granulocytes, BFU-E or nucleated red blood cells[34].

FLT3 ligand (FLT3L), the cognate ligand for FLT3, is a type I trans-membrane protein with a four helix bundle structure. FLT3L, like CSF-1 (ligand for CSF1R, also known as M-CSF) and SLF (ligand for KIT), is expressed in various membrane-bound and soluble isoforms. These isoforms are generated by variable RNA splicing and/or proteolytic cleavage of membrane-bound precursors. Both soluble and membrane-bound FLT3L isoforms have biologic activity. However, the relative biological significance of the various isoforms has not been determined. Normal serum levels of FLT3L in humans are < 100 pg/ml. Levels do not increase in patients with isolated anemia, but are markedly elevated in patients with pancytopenia due to aplastic anemia or chemotherapy- or radiation-induced myelosuppression [35, 36].

Binding of homodimeric FLT3L to FLT3 results in receptor dimerization, activation of tyrosine kinase activity, tyrosylphosphorylation in trans and binding of signal transduction effectors (Fig. **3**). FLT3L activation of human FLT3 results in direct association of FLT3 with Grb2 and SOCS1. Downstream signal transduction pathways are activated as evidenced by the phosphorylation of STAT5A, CBL, CBLB, SHC, SHIP, SHP2, GAB1, GAB2, and MAP kinase. Unlike murine FLT3, human FLT3 does not contain a potential SH2-domain binding site for the p85 subunit of PI3K in the carboxyterminal region of FLT3. Instead, PI3K does not directly associate with human FLT3

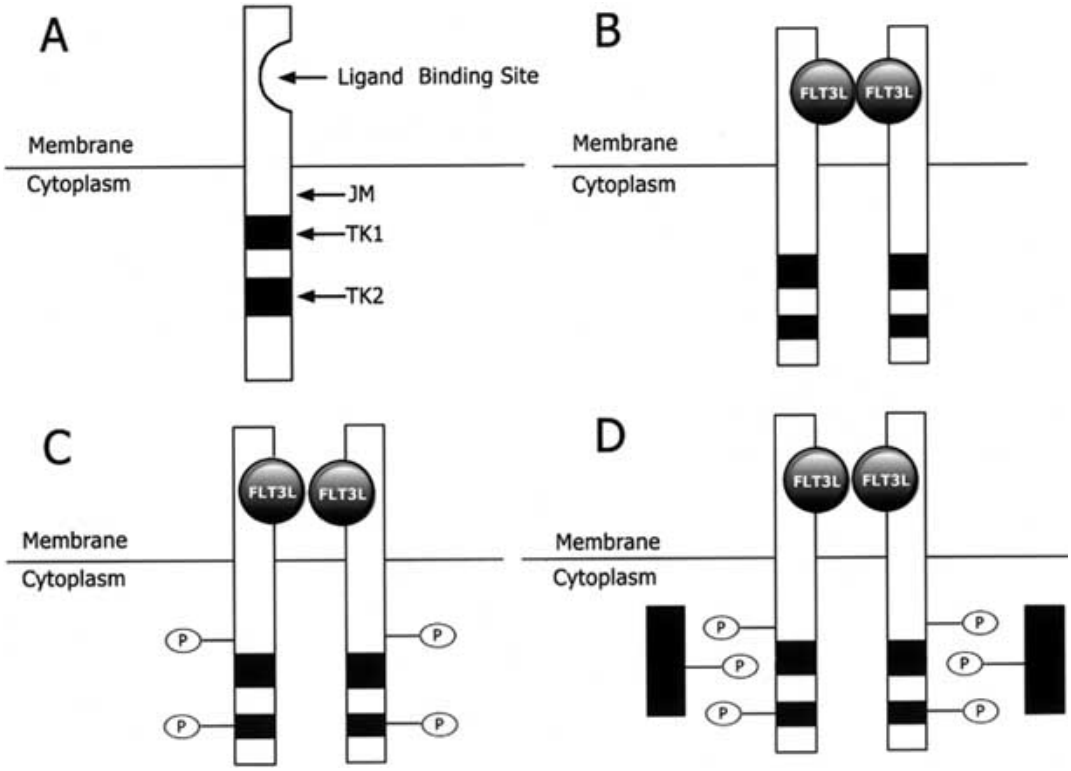


Fig. (3). Ligand induced activation of FLT3 tyrosine kinase activity. (A) Native FLT3 is expressed as a trans-membrane protein. The cytoplasmic domain contains the tyrosine kinase portion of the protein (split into 2 domains, TK1 and TK2, as indicated). An arrow indicates the location of the juxtamembrane domain (JM). (B) Binding of homodimeric FLT3L, results in physical interaction of two FLT3 proteins. (C) Ligand-induced dimerization activates the cytoplasmic tyrosine kinase domains, resulting in transphosphorylation (P) of selected tyrosine residues. (D) Substrates and adapter proteins bind to phosphorylated tyrosine residues and in some cases are directly phosphorylated by FLT3.

but is indirectly associated via interaction with a complex containing GAB1, SHP2, CBL, SHIP and CBLB [37-40]. STAT5A appears to be critical for FLT3 signal transduction as FLT3L is unable to stimulate proliferation of hematopoietic progenitors from STAT5A -/- mice. In contrast, FLT3L potently stimulates the proliferation of hematopoietic progenitors from STAT5B -/- mice [41].

FLT3L has potent effects on the proliferation and differentiation of hematopoietic progenitors. Similar to SLF, FLT3L has little *in vitro* colony-stimulating activity as a single agent, but strongly synergizes with a variety of other hematopoietic growth factors when present as single cytokines or as part of growth factor combinations. Growth factors that synergize with FLT3L include SLF, IL-3, IL-7, GM-CSF, IL-6, M-CSF and IL-11[36, 42-44]. The biological activity of FLT3L is somewhat restricted—for example, FLT3L has little effect in stimulating erythroid, megakaryocytic, mast cell or eosinophil precursors [45-48]. In contrast, FLT3L potently stimulates the growth of B-cell and granulomonocytic precursors[49, 50]. The effects of FLT3L on hematopoiesis appear to be largely confined to the $CD34^{+}CD38^{-}$ fraction of bone marrow or umbilical cord blood [51,52].

FLT3L also strongly stimulates the proliferation and differentiation of dendritic cell (DC) precursors *in vitro*. DCs are efficient antigen-presenting cells for T cells and are being actively investigated as a component of cancer immunotherapy protocols [53-55]. Parenteral treatment of mice with FLT3L increases the absolute number of DC in the spleen, lymph node and peripheral blood compartments by 17-, 4-, and 6-fold, respectively. FLT3L-stimulated DC are functionally active, as assessed by their ability to act as antigen presenting cells *in vitro* and to prime an antigen-specific immune response *in vivo*. FLT3L acts synergistically with GM-CSF and IL-4 in inducing DC differentiation *in vitro* and *in vivo* [56, 57].

ANIMAL MODELS OF FLT3 OR FLT3L DEFICIENCY

In considering targeted inhibition of a kinase (or of any protein) it is relevant to examine the phenotype of animals with absolute or relative deficiency of the proposed target. Indeed, Zambrowicz and Sands have recently reviewed the 100 best-selling drugs and concluded that in most cases the knockout phenotype correlated well with drug efficacy [58]. Mice nullizygous for FLT3 or FLT3L have been generated and phenotypically characterized. Somewhat surprisingly, FLT3 "knockout" mice are viable with no overt generalized marrow failure state. Specifically, such mice have no gross abnormalities of bone marrow or spleen cellularity, peripheral blood counts, or hematopoietic cell morphology.

The frequency of myeloid bone marrow progenitors in the bone marrow of FLT3 -/- mice is the same as in FLT3 +/- mice. Although splenic and thymic cell and bone marrow differentiated B cells were normal in FLT3 -/- there were 25-50% reductions in pro- and pre-B –cell populations. Thus, although FLT3 is not required for steady-state lymphohematopoiesis, deficiency does lead to a sub-clinical defect in B lymphopoiesis. FLT3 -/- mice do have a defect involving hematopoietic stem cells. In competitive repopulation experiments, stem cells from FLT3 -/- mice transplanted into irradiated recipients do not effectively reconstitute the hematopoietic system, especially T-lymphoid lineage. Mice homozygous for FLT3-/- and partial but not complete KIT kinase deficiency (W/W^v) had reduced hematopoiesis and a life expectancy of 6 weeks [59].

McKenna *et al.* characterized the phenotype of mice nullizygous for FLT3L (FLT3L -/-). These mice had reduced leukocyte cellularity in the bone marrow, peripheral nodes and spleen. In contrast, thymic cellularity, hematocrit, and platelet count were not affected. Bone marrow CFU-GM and B-CFU were reduced approximately 40% and 90%, respectively. DC from spleen, thymus and lymph nodes were reduced approximately 10-fold. An unexpected finding in the FLTL -/- mice was an 80% reduction in NK cells. Despite these defects in lymphohematopoiesis, the animals were viable, fertile and appeared healthy without any apparent increase in infections (at least when maintained in a pathogen-free animal facility) [60].

FLT3L is the only known ligand for FLT3 and there is no evidence that FLT3L binds to other receptors. Therefore, the phenotype of mice nullizygous for either the receptor or the ligand should be identical. Currently, it is not clear why the FLT3 -/- and FLT3L -/- have marked phenotypic differences. However, strain effects can sometimes affect the phenotype of targeted gene disruption and may explain the observed differences [61, 62]. Alternatively, FLT3L may have an additional, unidentified receptor in addition to FLT3.

The existing mouse models of FLT3 kinase deficiency suggest that targeted inhibition of FLT3 kinase may have some effects on steady state hematopoiesis and possibly more profound effects on B-cell and DC physiology. Given the potential importance of DC function to immune system activity against AML, it will be important to carefully characterize the immune cell number and function in treated animals. In particular, these studies might be even more important in the case of inhibitors that target both KIT and FLT3 [59, 60, 63].

THE ROLE OF FLT3 IN AML

Consistent with the normal role of FLT3 in regulating the proliferation of primitive hematopoietic progenitors, FLT3 is expressed by myeloblasts in 70-100% of cases of AML. In addition, FLT3L stimulates the *in vitro* growth of myeloblasts in the majority of cases of AML in a dose-dependent manner. FLT3L is also synergistic with a variety of hematopoietic growth factors. A number of leukemic cell lines express both FLT3 and FLT3L, suggesting the possibility of autocrine stimulation in some cases of AML [46, 64-67].

One of the more compelling developments in AML biology in the last decade has been the discovery of genomic mutations of FLT3. To date, two different classes of FLT3 mutations have been reported: 1) internal tandem duplications (ITD) of the FLT3 juxtamembrane domain (JM); and 2) point mutation or small in-frame deletions or insertions of the activation loop[17, 68].

Nakao *et al.* originally described FLT3-ITD mutations in 1996, and their observations have been confirmed and extended in studies of over 3000 patients [17, 68-72]. Combining these results, a FLT3-ITD mutation was identified in 644 out of 2836 patients (22.7%) [69-71]. FLT3-ITD mutations consist of in-frame duplication of a portion of the JM domain encoded by exon 14 of FLT3. In general, the mutations are clustered in a tyrosine-rich stretch of the protein that includes tyrosine residues encoded by codons 589 and 599. The length of the ITD is highly variable and ranges from 3 to >180 bases. The location of FLT3-ITD mutations in relationship to the structural features of the protein are depicted in Fig. **(4)** [17, 68-72]. In contrast to similar mutations of KIT or PDGFRA found in human Gastrointestinal Stromal Tumors, point mutations and in-frame deletions of FLT3 have not been reported in primary AML specimens [73, 74]. The prognostic significance of FLT3-ITD mutations will be discussed below. FLT3-ITD mutations are found in a lower frequency in myelodysplasia and secondary AML. FLT3-ITD mutations occur less frequently in pediatric AML (10-15% of cases). FLT3-ITD mutations are very rare in acute lymphoblastic leukemia and have not been found in CML (chronic phase or blast crisis), lymphomas, CLL, Multiple Myeloma, or a variety of solid tumors (including epithelial, neural, germ cell tumor, and endocrine neoplasms) [75-78]. Thus, a "pure" inhibitor of FLT3 kinase would potentially be therapeutically useful only for the treatment of AML and rare cases of ALL.

In most studies, AML with a FLT3-ITD mutation is associated with higher peripheral white blood cell counts and a greater bone marrow blast percentage. The frequency of FLT3-ITD mutations is not uniform among different AML FAB classification groups. FLT3-ITDs are more common in FAB M3 (especially M3v), M1, M5b, and M4 and are less commonly found in M4eo, M6, or M7. FLT3 mutations are also non-randomly distributed between cytogenetic groups and are more frequent in patients with normal cytogenetics or t [15;17]. FLT3-ITD mutations are less commonly found in AML with t[8;21], inv[16], or adverse risk karyotypes (≥ 3 abnormalities, deletion of 5q, -5, -7, abn(3q)) [69-72, 79, 80].

The relationship between the presence or absence of a FLT3-ITD mutation and clinical outcome has been examined in a number of studies. In most reports, the presence of a FLT3-ITD does not affect the probability of obtaining a complete hematologic remission with induction chemotherapy treatment. However, in most studies, event-free and overall survival is significantly shorter in cases of AML with FLT3-ITD. In a minority of patients of AML patients the malignant cells lack expression of wild-type FLT3 mRNA and protein [15, 69-71, 79-84]. It has recently been suggested that absence of expression of wild-type FLT3 (FLT3-ITD/-) confers an especially bad prognosis that may be responsible for most of the negative prognosis of the total

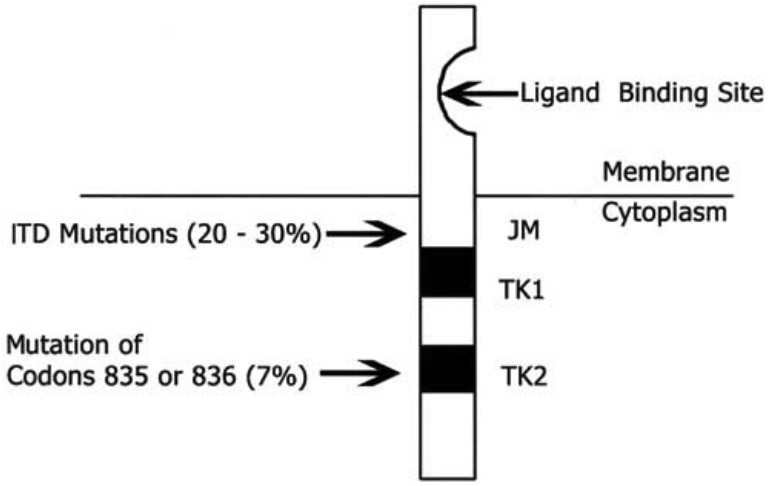


Fig. (4). Oncogenic mutations of FLT3 involve the JM and TK2 domains. FLT3-ITD mutations involve the JM domain and are found in 20-30% of cases of AML. FLT3-AL mutations most commonly involve codons 835 or 836 (TK2) domain and are found in 7% of cases of AML.

FLT3-ITD group. This hypothesis will require further testing as most studies have not been reported on the presence or absence of FLT3-WT expression by myeloblasts [69, 71, 81].

Missense mutations of the activation loop (FLT3-AL) are also found in AML. The activating nature of such mutations were first reported by Fenski *et al.* who used site directed mutagenesis to generate a murine D838V mutation. These investigators generated this mutant based on the known association of similar mutations involving the homologous KIT residue in human AML and mast cell disorders. The murine D838V isoform was constitutively activated and converted 32D cells to factor independent growth [85]. Subsequently, FLT3-AL mutations involving the same conserved aspartic acid (residue 835 in human FLT3) were reported in human AML [68, 86]. The most common FLT3-AL mutations are mis-sense mutations of codon 835, but deletion of isoleucine 836 and short insertions in this region of FLT3 have also been reported (Fig. **4**). FLT3-AL mutations are found in approximately 7% of AML and much less frequently in myelodysplasia and ALL. In a minority of cases, AML cells may have both the FLT3-ITD and FLT3-AL mutation. The prognostic significance of isolated FLT3-AL mutations remains unclear, as most series have had too few patients to allow adequately powered statistical testing. Like FLT3-ITD, however, these mutations appear to significantly increase WBC count and may be associated with shorter disease-free and overall survival [68, 69, 86].

BIOLOGIC ACTIVITIES OF MUTANT FLT3

Animal models of FLT3-ITD have been generated and used to test the role of mutated FLT3 in the pathogenesis of AML. In the first model, reported by Kelly et al., bone marrow was transduced with a retrovirus encoding cDNAs for either human FLT3-ITD or wild-type human FLT3. Transduced cells were used to transplant lethally irradiated syngeneic mice. Mice transplanted with marrow transduced with either wild-type FLT3 or control vector encoding EGFP engrafted normally and had normal survival with a follow up of more than 200 days. In contrast, mice receiving transplants with FLT3-ITD transduced marrow developed a lethal myeloproliferative disease with a median latency of 40-50 days. Affected mice had marked peripheral blood leukocytosis, due almost entirely to an increase in neutrophils, as well as extra-medullary hematopoiesis. Almost identical results were obtained using a different AML-derived FLT3-ITD. FLT3-ITD in this murine bone marrow transplant model induced an oligoclonal myeloproliferative disease but not AML [87]. Interestingly, use of the same model system in two additional strains of mice did not result in any myeloproliferative disorder but instead in T cell lymphoblastic leukemia/lymphoma that developed with a long latency. Thus, genetic background can modify the response to FLT3-ITD [88]. These results are consistent with other models of murine myeloproliferative disorders induced by transplantation of marrow transduced with oncogenic tyrosine kinase (e.g. BCR-ABL) [89].

These findings were extended by the same group of investigators in a murine model of APL. Human APL is always associated with chromosomal translocations generating fusion proteins involving RARα. Expression of the PML-RARα fusion protein, which results from the balanced reciprocal translocation t [15;17](q22;q11), under the control of the cathepsin G promoter in transgenic mice caused a nonfatal myeloproliferative syndrome in all such mice; a minority developed APL after a long latency period. FLT3-ITD is extremely common in human APL and has been proposed as a candidate second genetic event in the development of APL (Fig. **1**) [70]. To test this hypothesis, bone marrow from transgenic PML-RARα mice were transduced with FLT3-ITD and transplanted into syngeneic mice as described above. Mice transplanted with FLT-ITD transduced marrow developed an APL-like leukemia with a short latency and complete penetrance. The leukemia that developed in FLT3-ITD marrow transplanted mice was similar to the long latency APL seen in a minority of PML-RARα transgenic mice. Importantly, APL cells from the

FLT3-ITD/PML-RARα mice remained responsive to treatment with ATRA. The results suggested a cooperative rather than additive effect of the two oncoproteins. Thus, these data are supportive of the "two-mutation" model of AML depicted in Fig. **(1)**. It is noteworthy that clonal karyotypic abnormalities were present in each case of FLT-3 ITD/ PML-RARα leukemia suggesting that additional genetic effects may either 1) be required for complete transformation; or 2) provide a significant growth or survival advantage to cells expressing both FLT3-ITD and PML-RARα.

MECHANISMS OF FLT3 ACTIVATION

Understanding the molecular mechanisms of FLT3 activating mutations is relevant to the development of potent and specific FLT3 TKIs. Indeed, if various FLT3 activating mutations significantly alter the kinase structure it may not be possible to target all mutations with a single inhibitor. However, such structural differences might allow the development of inhibitors that selectively target mutant but not wild-type kinases. Alternatively, if oncoproteins kinases have a different dependence upon activation of downstream pathways than the wild-type kinase, it may be possible to selectively target these pathways and block oncogenic- but not normal-signaling. In the case of FLT3, data are just emerging on the mechanisms by which FLT3-ITD mutations constitutively activate kinase activity. Kiyoi *et al.* demonstrated that an AML-associated FLT3-ITD mutation, a non-ITD insertion and deletions of 1-11 codons in the JM region all resulted in constitutive kinase activity and transformed 32D cells to factor-independence. In addition, human leukemia cell lines with point mutation of the FLT3 JM have been reported. Thus, at least *in vitro*, a wide variety of mutations of the FLT3 JM can induce constitutive kinase activity. To date, however, deletion or point mutation of the FLT3 JM has not been reported in primary human AML specimens [90, 91].

As the FLT3 JM has a number of tyrosine residues that are reduplicated in FLT3-ITD mutation, it was hypothesized that constitutive kinase activation was dependent on these reduplicated tyrosines. However, mutagenesis of all JM tyrosines in FLT3-ITD to phenylalanine neither reduces autophosphorylation of FLT3-ITD nor abrogates the ability of the oncoprotein to transform 32D cells to factor-independent growth. In contrast, mutagenesis of all JM tyrosines to phenylalanine in wild-type FLT3 blocks ligand-induced phosphorylation and the ability of FLT3L to replace IL-3 as survival growth factor [90, 92].

In most cases of AML with FLT3-ITD mutations, the malignant cells express both FLT3-ITD and FLT3-WT (heterozygous). In a minority of cases only the FLT3-ITD mRNA/protein is expressed, either due to gain of the mutant allele (homozygous) or loss of the wild-type allele (hemizygous). Failure to express wild-type FLT3 appears to confer a worse prognosis compared to cases of AML heterozygous for FLT3-ITD, as discussed above [69, 81].

Congruent with the normal mechanism of ligand-induced activation of FLT3-WT, all tested FLT3-ITD isoforms constitutively homodimerize. In addition, FLT3-ITD can heterodimerize with FLT3-WT, resulting in phosphorylation of tyrosine residues on both FLT3-WT and FLT3-ITD isoforms. *In vitro* experiments have been performed, in which FLT3-WT is co-expressed with either FLT3-WT lacking the entire kinase domain or with FLT3-ITD with a deleted kinase domain. Cells co-expressing full-length FLT3-WT and a truncated FLT3-ITD lacking a kinase domain had constitutive activation of FLT3 and were converted to growth-factor independence. In contrast, cells expressing both full-length FLT3-WT and FLT3-WT with a truncated kinase domain did not grow in the absence of exogenous growth factor. Although, heterodimerization of FLT3-WT and FLT3-ITD resulted in kinase activation, it is not clear whether such a complex results in qualitative or quantitative differences in signaling compared with a FLT3-ITD homodimer [90].

Based on the above results, and data from KIT and Ephrin B2 receptors, the following model of FLT3 activation by ITD mutations can be proposed. The JM domains of these RTKs normally adopt a helical conformation that makes numerous contacts with α helix C in the N-terminal kinase lobe and also with residues in the C-terminal lobe. These interactions, which are stabilized by JM tyrosine residues, distort the ATP binding pocket and also prevent the activation loop from assuming the active conformation. Phosphorylation of the JM tyrosine residues changes the JM conformation and removes these inhibitory constraints. FLT3-ITD mutations appear to serve the same purpose, disrupting the normal inhibitory conformation of the JM domain. Although point mutations or deletions of FLT3 JM can also activate FLT3, these types of mutations are rare in AML and this observation might provide insight into the genomic mechanism that generates ITD mutations in AML stem cells [93-96].

The mechanism of action of FLT3-AL mutations is somewhat less clear. In FLT3-WT, the AL is capable of rotating to assume either an inactive conformation or an active conformation. In the inactive conformation, access of ATP to the ATP binding pocket is blocked, as is substrate binding. Normally, phosphorylation of JM and AL tyrosine residues is required to stabilize the activation loop in the open/active conformation [97, 98]. FLT3-AL mutations (e.g. D835V) are predicted to "lock" the protein into the active conformation [99]. In addition, such mutations may alter kinase substrate specificity and increase ATP affinity. At the current time it is not clear whether FLT3-AL mutations require homo- or hetero-dimerization for activation [95, 100].

PRE-CLINICAL STUDIES OF FLT3 TKI

In identifying potential FLT3 TKIs, there are a number of systems available for screening candidate compounds—each has its advantages and disadvantages. In addition, compounds of interest can be further validated by use of more complex, clinically relevant systems, such as animal models. Recombinant protein, usually limited to the kinase domain of RTKs, can be used in automated high throughput screening assays. However, the effect of a compound on such a recombinant protein may be misleading in that the full-length protein, expressed in a living cell, may have a greater or lesser sensitivity. Such differences may be due to poorly

soluble compounds with limited cellular uptake and/or protein conformation differences between the recombinant and native protein [101, 102].

Expression of activated tyrosine kinases in the IL-3-dependent murine cell line BaF3 is a widely used method for testing the inhibitory properties of a compound against a kinase of interest. Indeed, BaF3 cells can be converted to factor-independence by expression of FLT3-ITD or FLT3-AL mutant isoforms. This model system provides a versatile platform for testing of potential FLT3 inhibitors against various classes of FLT3 mutation. Novel insertion or point mutations discovered from genotypic studies of the FLT3 gene in AML specimens can be synthetically generated using site-directed mutagenesis of FLT3 cDNA and expressed in BaF3 cells, making them IL-3 independent. In testing a potential inhibitor of FLT3 kinase activity in such cells, the addition of IL-3 can serve as an internal control for drug specificity. If growth inhibition by the compound is rescued by IL-3, the effect was likely due to FLT3 inhibition; if not, the compound may have non-specific cellular toxicity [91, 103-105].

Although the use of BaF3 cell-based models is useful in screening compounds for activity against mutant FLT3 isoforms, the model has several shortcomings. First, it is based on the use of murine factor-independent cell lines. Although the human and murine FLT3 proteins are highly conserved, there are a number of potentially compounding biologic differences in downstream signaling pathways such as activation of PI3 kinase [106]. Thus, the expression of human or murine FLT3 in a murine cellular context may not be completely representative of the biology of human FLT3 in a human leukemic cell. In addition, the expression of activated FLT3 renders such cells artificially dependent upon this receptor tyrosine kinase for survival. In human AML the genetic background is more complicated and includes other molecular abnormalities, such as loss of functional activity of individual transcription factors (e.g. AML1), RAS mutations, loss of functional activity of p53 and/or other tumor suppressor genes [15].

Recently, a number of naturally occurring human leukemia cell lines with FLT3-ITD mutations have been identified. Potent FLT3 inhibitors induce apoptosis in these lines despite the presence of other complex somatic genomic alterations. These results suggest that targeted inhibition of FLT3 *in vivo* may be efficacious in AML. In contrast, FLT3 inhibitors tested to date have not had much biologic effect on leukemic cell lines (AML, ALL, CML, etc) lacking FLT3-ITD mutations, irrespective of expression of wild-type FLT3. The activity of some of the FLT3 inhibitors discussed below has been validated using primary human AML specimens [91, 105, 107].

Finally, candidate FLT3 inhibitors can be tested in one of several described *in vivo* murine systems. These *in vivo* models allow assessment of pharmacokinetic and pharmacodynamic properties of a given compound as well as traditional endpoints of tumor growth rate and animal survival. Three general model systems have been used: 1) xenografts of FLT3-ITD BaF3 cells in immunodeficient mice; 2) xenografts of human leukemia cell lines in immunodeficient mice; and 3) bone marrow transplant models of FLT3-ITD dependent myeloproliferative disease or leukemia. As noted above, xenograft models of BaF3 FLT3-ITD may not be completely reflective of AML associated with FLT3-ITD, as the engineered cells are artificially dependent upon FLT3-ITD kinase activity for survival [103, 108, 109].

Bicyclic quinoxalines (aka tyrphostins) were the first class of compounds identified as having inhibitory activity against FLT3 kinase (Fig. **(6)**, Table 2). In contrast, the structurally related compounds AG490, AG1478, and AG1879 had no specific activity against FLT3 (IC_{50} > 10 μM) [110, 111]. The potential clinical activity of both AG1295 and AG1296 is limited by their poor solubility. The tricyclic quinoxalines AGL2033 and AGL2043 have been recently reported as having increased solubility and retained potency against FLT3 and may be more suitable for clinical development [112]. To date, no studies have been published concerning the *in vivo* efficacy of bi- or tri-cyclic quinoxalines in inhibiting FLT3 kinase.

Recently, FLT3 inhibitors with more favorable "drug-like" properties have been developed from a variety of chemical families including indolinones, indolocarbazoles,

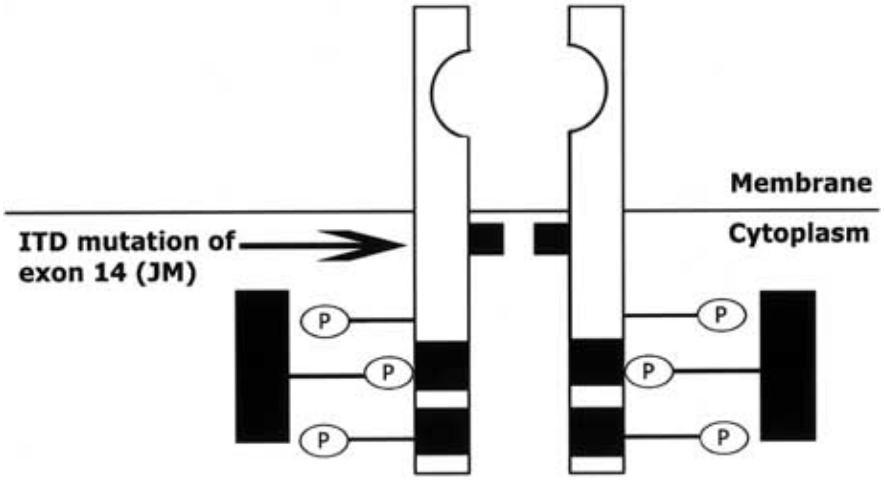


Fig. (5). FLT3-ITD mutations result in ligand-independent FLT3 dimerization and activation of kinase enzymatic activity. FLT3 activation is followed by receptor autophosphorylation and binding of substrate and adapter proteins. In some cases, FLT3 directly phosphorylates these FLT3-associated proteins.

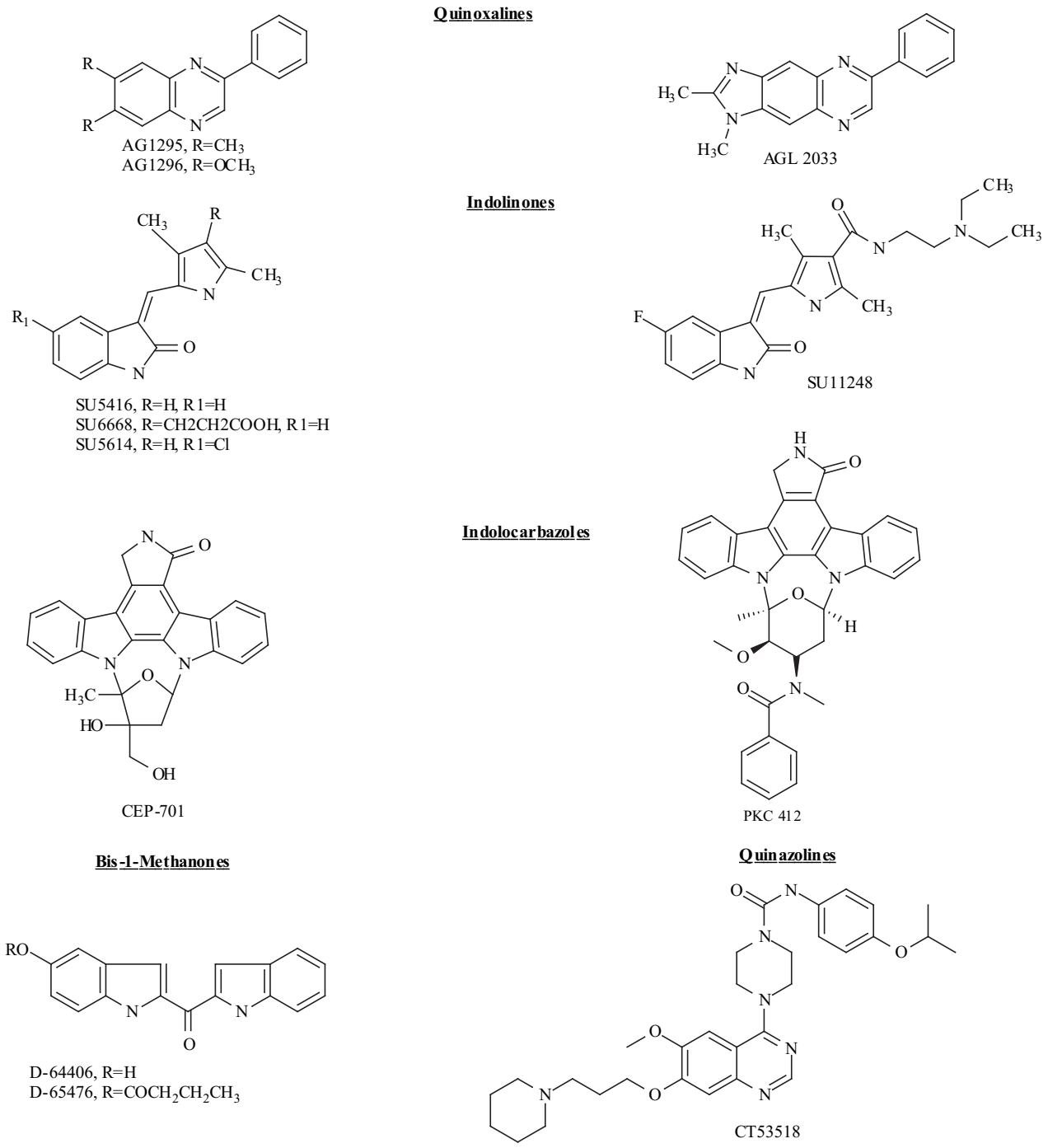


Fig. (6). Chemical structure of representative FLT3 TKIs. Compounds of interest are grouped by chemical class, and the biological activities of these compounds are discussed in the text and listed in tables 2-4.

quinoxalines, and Bis(1H-2-indolyl)-1-methanones. The chemical structures of these inhibitors are depicted in Fig. **(6)** and their biological activities are summarized in tables 2 and 3. A number of these compounds are in clinical development for targeted inhibition of FLT3 in AML and will be discussed further below. However, a few summary comments about the development of these inhibitors will be made here. Given that a pure FLT3 TKI might potentially be useful only in the subset of AML with FLT3 mutations, it is not surprising that all of the clinically studied inhibitors have emerged from programs attempting to develop kinase inhibitors to another target. In most cases, FLT3 TKI have surfaced during screening efforts to develop potent inhibitors to PDGFR, a closely related member of the type IIII RTK family. In the case of SU5416, SU11248, CEP-701, and PKC412, FLT3 was not one of the initial kinases screened and the activity of these compounds against FLT3 was identified only after phase I/II trials had already been completed. As a consequence there is little published data on systematic determination of the structure-activity relationship (SAR) of FLT3 TKIs and the feasibility of developing "pure" FLT3 inhibitors without activity against other type III RTK family members [104, 105, 108, 113-116].

Table 2. Pre-Clinical Profiles of Selected FLT3TKIs. The chemical structures of these compounds are shown in Fig. **(6)**. The potency of the compounds against FLT3 and the related type III RTKs, KIT and PDGFRB are listed. The potency against the type III RTKs is based on inhibition of receptor autophosphorylation in a cellular based assay rather than *in vitro* activity against recombinant kinase. Available data on other known kinase targets and compounds selectivity are also listed—these values were obtained using different methods (recombinant kinase vs. cellular based assays) and may not be directly comparable from one compound to another.

Compound	Chemical Class	IC_{50} FLT3 (μM)[a]	IC_{50} PDGFRB/KIT (μM)[a]	IC_{50} Other Targets (μM)	Specificity (μM)	Comments	References
AG1295 AG1296	Bicyclic Quinoxaline	0.3-1.0	0.4/1.0	NI	EGFR> 100 FGFR 12.3 KDR > 10	Poor solubility	[110,111,147]
AGL2033	Tricyclic Quinoxaline	1.0	0.7/1.0	NI	SRC > 30 EGFR> 30 PKB/AKT >30 IGF-1R > 30	Poor solubility	[112]
D-64406	Bis(1H-2-indolyl)-1-methanone	0.2	0.2/ND	NI	FGFR1 >10 EGFR > 10 SRC > 10 PKC > 10		[102,148]
D-65476	Bis(1H-2-indolyl)-1-methanone	0.2	1.1/ND	NI	ND		[102,148]
SU5416	Indolinone	0.1	37.9/ 0.01	KDR 1.3 FGFR1 4.2	EGFR >100 IGFR1R 19.5 ZAP70 20.7	Poor solubility	[101,105]
SU5614	Indolinone	0.01	0.13/ 0.05	KDR 0.5 FGFR1 3.1	EGFR >100 IGFR1R 6.7 ZAP70 2.7		[101,105]
SU11248	Indolinone	0.25	0.01/ND	KDR 0.01 SRC 0.1 ABL 0.8	EGFR >10 CDK2>10 MET 4.0 IGFR-1 2.4	Improved solubility	[109,149]
PKC412	Indolocarbazole	0.001	0.08/0.3	KDR 1.0 PKC 0.5	IGF1R > 100 InsR >100 EGFR > 100 ABL > 100	AKA CGP41251	
CEP-701	Indolocarbazole	0.002	1.0 /1.0	TRKA/B/C 0.003 VEGFR2 0.065 PKC 0.2	EGFR>1 InsR>1	Pan-TRK inhibitor	[108,150,151]
CT53518	Quinazoline	0.22	0.20/0.17	CSF-1R 3.4	EGFR, FGFR1, InsR, SRC, ABL, PKC all >30	AKA MLN518	[103]

Legend: a: Receptor autophosphorylation in cellular based assay
ND: Not determined
NI : None identified to date

However, there is some published data on the SAR of compounds related to CT53518 (Fig. **(7)** and Table 4). The SAR of this pharmacophore demonstrates the feasibility of developing KIT/PDGFR inhibitors that lack substantial activity against FLT3. However, as the purpose of the SAR was to maintain/optimize activity against PDGFR, insufficient data are reported to determine the feasibility of using this pharmacophore to develop FLT3 inhibitors with lesser activity against KIT and/or PDGFRB. In the case of indolinones, it should be noted that SU5416 is active against KIT and FLT3 but not PDGFR, whereas the closely related compound SU5614 is active against all three kinases (Table 2, Fig. **(3)**) [101, 105]. Although not developed with this goal in mind, some indolocarbazole compounds have been developed that are substantially more active against wild-type and mutant FLT3 kinase than against PDGFR or KIT. For example, PKC412 is 80-300-fold and CEP-701 is 500-fold more active against FLT3 than against either KIT or PDGFR [104, 108, 117].

It is clear that small differences in target protein structure among these highly related kinases can be exploited to influence TKI spectrum of activity. For example, imatinib is a potent inhibitor of KIT and PDGFR kinases but has no useful activity against FLT3 kinase (IC_{50} > 10 μM) [118, 119]. Imatinib forms a critical O-HN hydrogen bond with T315 of ABL and is predicted to form a similar bond with the homologous T681 in PDGFRB. Somatic mutation of T315 to isoleucine in CML cells results in resistance to

Table 3. FLT3 TKIs that Have Been Used in Human Clinical Trials. The *in vitro* and animal model activity of the various compounds and the stage of clinical development is listed above. Chemical structures of these compounds are depicted in Figure 6, and additional biochemical properties are listed in table 2.

Compound	Pharmaceutical Company	IC_{50} FLT3-ITD mutation (nM)	IC_{50} FLT3-AL mutation (nM)	Animal Model Activity	Formulation	Status of Clinical Development	References
CEP-701	Cephalon, Inc.; West Chester, PA	5	5	Yes: xenografts of Baf3 TEL-FLT3-ITD	Oral	Phase II (FLT3-ITD + AML)	[108]
CT53518	Millennium Pharmaceuticals; Cambridge, MA	50	NR	Yes-xenografts of BaF3 FLT3-ITD; bone marrow transplant model of FLT3-ITD induced myeloproliferative disorder	Oral	Phase I (refractory AML and high risk MDS)	[103]
PKC412	Novartis Pharma, AG; Basel, Switzerland	10	10	Yes: bone marrow transplant model of FLT3-ITD induced myeloproliferative disorder	Oral	Phase II (FLT3-ITD + AML) Phase III planned	[104,133]
SU5416	SUGEN, Inc.; South San Francisco, CA	100	NR	NR	Intravenous	Phase II (Refractory KIT+ AML)	[105,152]
SU11248	SUGEN, Inc.; South San Francisco, CA	50	Varies by D835 substitution (range 30-300 nM)	Yes; xenografts of human AML cell line with FLT3-ITD: both subcutaneous tumor and bone marrow transplant models	Oral	Phase I (Refractory AML)	[109,135, 136]

NR: Not reported.

imatinib resistance [120]. FLT3 has a phenylalanine residue at the homologous position (codon 691) that is predicted to sterically clash with imatinib binding. Mutation of FLT3 F691 to threonine results in sensitivity to imatinib. Conversely, the kinase activity of the mutant PDGFRB T681F is insensitive to imatinib. These data clearly indicate that somatic mutation of targeted FLT3 kinases can result in insensitivity to small molecule inhibitors without loss of intrinsic kinase activity [121]. Such a phenomenon has been observed in CML with acquired resistance to imatinib [120, 122, 123].

Another critical determinant of TKI activity/specificity is the conformation of the kinase AL [121, 123-126]. Therefore, it would be predicted that FLT3 inhibitors with different modes of binding to FLT3 should have varying potency against FLT3-AL mutations (e.g. D835Y). For example, PKC412 and CEP-701 have equal potency against FLT3-ITD and FLT3-AL mutations. SU11248 has variable activity against FLT3-AL mutations depending upon the exact type of mutation. The activity of CT53518 against FLT3-AL mutants has not been reported [103, 104, 108, 109].

PRELIMINARY RESULTS FROM PHASE I/II TRIALS OF FLT3 TKIs

SU5416 was the first FLT3 inhibitor to be used in a phase II AML trial. However, at the time the trial was designed and conducted, the activity of SU5416 against FLT3 kinase had not been discovered [105, 127] A phase II trial of SU5416 in patients with KIT-positive AML was conducted in Europe with the intent of targeting KIT and/or VEGFR2 kinase activity. In a preliminary report of 14 patients treated twice weekly with intravenous145 mg/m^2, early progression was observed in 6, while 5 of 8 patients who received the full four-week course showed a partial response. One patient continued to respond after more than 3 months of therapy. Whether any of these patients had FLT3 mutations was not addressed in the report [128]. In addition, a patient in second relapse of KIT-positive AML was treated with SU5416 on a compassionate use protocol. The patient had normalization of bone marrow by morphology and flow cytometry, increased neutrophil, red cell and platelet counts, and achieved a hematological complete remission (with the exception of a platelet count that only rose to 50-80 x10^9/L). The patient developed a third relapse 9 months after initiation of SU5416. These data indicate that SU5416 has clinical activity in AML but the mechanism of action remains unclear. It is possible that the observed responses were related in part to anti-angiogenic effects of SU5416 rather than its effects on KIT and/or another kinase [129].

Two other FLT3 TKIs, PKC412 and CEP-701, were also previously used in phase I trials of patient with solid tumors and were well tolerated as oral medications. In a phase I trial of CEP-701, the most common drug-related side effects were gastrointestinal symptoms (nausea, vomiting, diarrhea, pain,

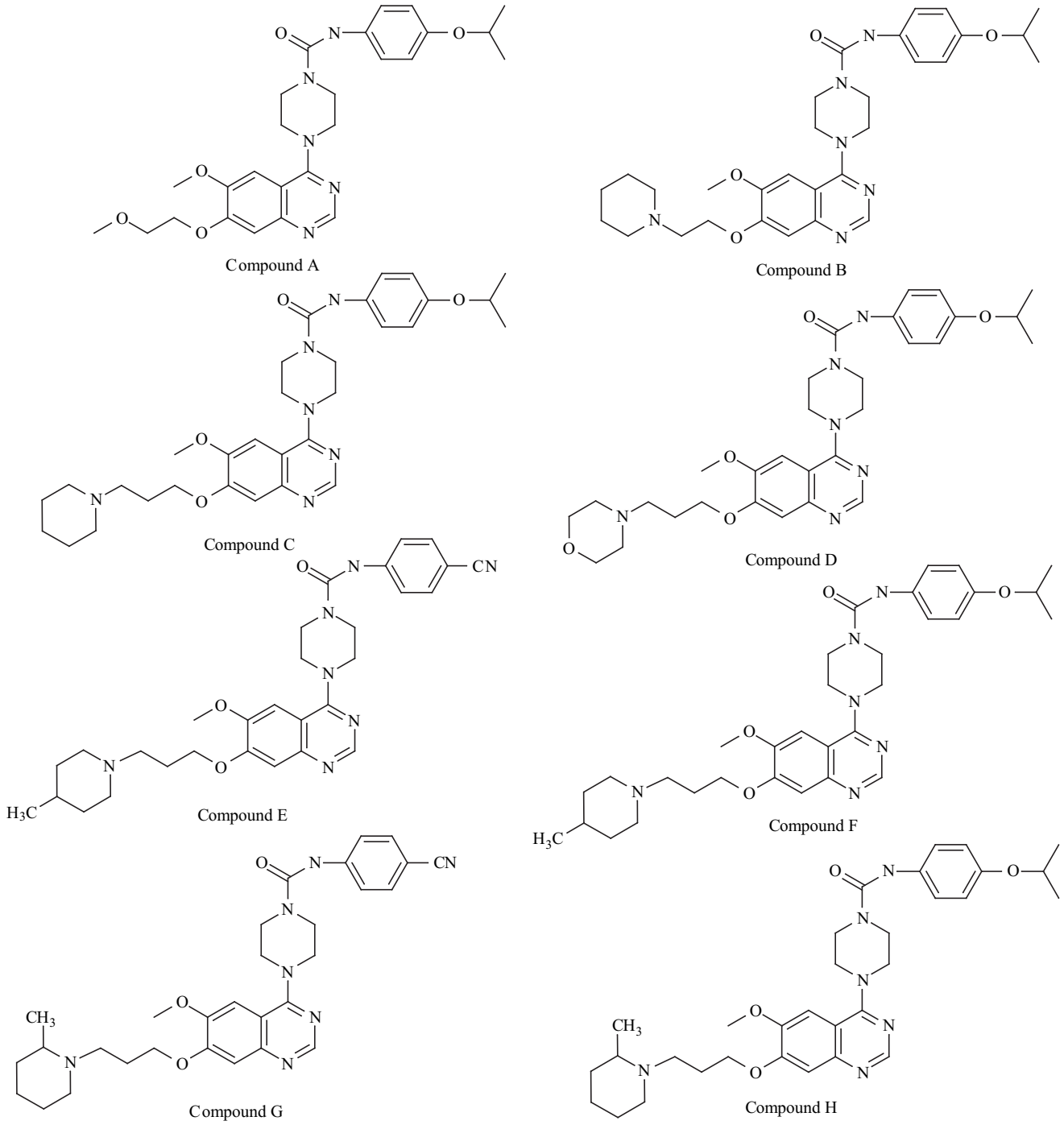


Fig. (7). SAR of representative quinazoline compounds. Chemical structures of compounds of interest are shown. The biochemical potency of these compounds against the kinase activity of type III RTKs is listed in table 4. [153,154].

dyspepsia). Other toxicities included moderate muscle cramps, paresthesias and mild fatigue. Importantly, no myelosuppression was observed. All patients taking 160 mg bid discontinued treatment because of gastrointestinal symptoms. The oral dose of 40 mg bid was particularly well-tolerated during chronic administration [130].

A phase II trial of CEP-701 is in progress for patients with refractory or relapsed AML expressing FLT3 mutations. Dosing was initiated at 40 mg bid, but based on variable plasma levels and incomplete inhibition of FLT3 phosphorylation, the trial was amended to allow a starting dose of 60 mg bid for one month with further escalation to 80 mg bid for the second month. CEP-701 was well tolerated with minimal side effects, and A decreases in peripheral blast counts in several patients and a complete marrow response were reported in abstract form at the 2002 American Society of Hematology meetings. As of February of 2003, this trial is continuing to enroll patients [131].

In a phase I study of oral PKC412 in patients with solid tumors, nausea, vomiting, fatigue and diarrhea were identified as dose limiting toxicities (DLTs). Patients treated with ≥ 100 mg/day had significant reductions in circulating

Table 4. SAR of Representative Quinazoline Compounds in Relationship to Activity Against the Kinase Activity of Type III RTKs. The chemical structures of these compounds are shown in Fig. (**7**) [153,154]. In this study, compounds that potently inhibit FLT3 kinase activity have equal or greater potency against KIT and PDGFRB (e.g. C and D). In contrast, compounds can potently inhibit KIT and PDGFRB with substantially less activity against FLT3 (e.g. E and G). Compound G is the most selective type III RTK TKI and potently inhibits KIT with 25- and 1465-fold less activity against PDGFRB and FLT3, respectively. None of the tested compounds has substantial activity against CSF1R. Compound C (CT53518) is currently being tested in a phase I trial of patients with refractory leukemia [134]. Additional information on CT53518 is included in tables 2 and 3.

Compound	IC_{50} FLT3 (μM)	IC_{50} KIT (μM)	IC_{50} PDGFRB (μM)	IC_{50} CSF1R (μM)
A	0.45	0.19	0.20	5.46
B	0.07	0.11	0.24	3.43
C (CT53518)	0.22	0.17	0.20	3.43
D	0.04	0.06	0.10	1.40
E	1.91	0.004	0.073	NR
F	0.13	0.021	0.039	NR
G	8.79	0.006	0.150	NR
H	0.20	0.016	0.074	NR

NR: Not reported

lymphocyte and monocyte counts but only 2 out of 32 patients developed myelosuppression (both grade 2). A dose of 150 mg per day was identified as suitable for future phase II studies. Plasma levels of 02.-0.7 μM PKC412 were obtained at the top dose levels (150-300 mg per day). Due to avid binding of this drug by the plasma protein α-1-acidic glycoprotein, it is unclear what concentrations of free PKC412 were obtained in this study. However, pharmacodynamic effects on suppression of cytokine release and inhibition of MAPK activation in peripheral lymphocytes were observed [116, 117, 132].

A phase II trial of PKC412 for patients with relapsed or refractory AML associated with FLT3-ITD or FLT3-AL (D835Y) mutations has been reported in abstract form. Patients were treated with 225 mg PKC412 per day as monotherapy. At this dose, PKC412 was well tolerated with few side effects (other than grade 1 nausea) that were felt to be drug-related. However, some adverse events that were felt to be due to underlying disease (e.g. neutropenic fever) may actually have been drug-related. This caveat applies to all such trials in refractory AML. Most patients treated with PKC412 experienced reductions in peripheral blood blast counts, with some having reductions in marrow blast counts as well. However, none of the patients had a confirmed complete response [133]. A randomized phase III trial comparing chemotherapy alone vs. chemotherapy + PKC412 is planned.

CT53518 is currently being studied (February, 2003) in a phase I trial of patients with refractory or relapsed AML or high-risk myelodysplastic syndrome. FLT3 mutation is not required for trial entry but will be retrospectively determined in all enrolled patients. In a preliminary report, oral dosing of 50-150 mg bid of CT53518 was well tolerated and demonstrated favorable pharmacokinetic properties. Several patients had 40-50% decreases in peripheral blast counts but no complete or partial marrow responses were reported. Pharmacodynamic properties of CT53518 have not yet been reported. As of February 2003, this trial remains open to enrollment and higher doses of CT53518 will be studied [134].

SU11248 has also been tested in a phase I trial of patients with advanced AML. FLT3 mutation status was determined retrospectively in all patients. Dosing commenced at 25, 50, 75, and 100 mg once daily for two weeks followed by a 2-week washout period. Patients without grade 3 DLT and at least stable disease were permitted to repeat dosing in additional cycles. Grade 3-4 asthenia was identified as the DLT at the 75 and 100 mg doses. As these toxicities were not observed in other FLT3 inhibitor trials, it is presumed that they are due to inhibition of one or more other SU11248-sensitive kinases. Most patients with circulating peripheral blood blasts had a >50% decrease in blood blast counts as their best response. At the 100 mg dose, 3/10 patients had a complete remission except for platelets < 100 x 10^9/L. These responses were not sustained during the drug washout period. As of February 2003, this trial was closed to enrollment. The future clinical development of this drug in AML is uncertain because of the inability to dose continuously at an effective dose for FLT3 inhibition without DLT [135, 136].

INTERPRETATION OF CLINICAL RESULTS USING FLT3 TKIs

Based on these four trials, it appears that inhibition of FLT3 kinase is safe and well tolerated, even using inhibitors that also target KIT and/or PDGFRA/B. Although careful immunologic studies have not been performed, none of these inhibitors appeared to increase infection frequency or spectrum beyond that typically seen in patients with refractory AML. Importantly, none of the agents appeared to cause significant myelosuppression.

In all of these studies, there was some evidence of clinical activity; particularly in decreasing peripheral blood blast counts. In contrast, decreases in bone marrow blast count were less frequently noted, even in patients with >90% clearance of blood myeloblasts. There are several potential explanations for this result. With the exception of SU11248, a pharmacodynamic relationship between plasma drug level, degree of FLT3 inhibition, and hematologic response has not been determined. Therefore, dosing may have been inadequate in some of the trials.

The SU11248 trial incorporated a novel single dose study design followed by continuous dosing in patients who elected to continue therapy. Patients were dosed with escalating single doses of SU11248 with detailed PK/PD measurements over the following 48 hours. After the washout period, patients were entered into the continuous dosing trial. Enrollment in the single dose study was not mandatory for entry into the extended dosing trial, and as a consequence the doses in the single dose and extended dosing trial did not have a fixed relationship. Using this design, strong (≥ 50% decrease) modulation of FLT3 phosphorylation was reliably seen using a single dose of 200 mg or more. This trial design should be strongly considered in future trials of targeted agents in leukemia [135, 136].

In addition to PK/PD concerns, there may be underlying biological reasons for the differential activity of these agents against circulating versus marrow resident myeloblasts. In pre-clinical models, FLT3 AML cells can be partially or completely rescued from the effects of FLT3 kinase inhibition by stimulation via alternative growth factor receptors [104, 105]. In the case of human AML, it seems likely that circulating blasts are more dependent upon FLT3 signaling than blasts residing in the marrow where they will receive supplemental growth signals (growth factors, adhesion molecules) from neighboring leukemia and/or stromal cells [137]. These effects may have been underestimated in mouse xenograft models, in which species differences in ligands and receptor pairs may have partially or completely blocked such "escape" mechanisms. It is therefore, possible that FLT3 inhibitors may induce apoptosis in the peripheral blood leukemia compartment but might only inhibit proliferation in the bone marrow compartment.

FUTURE PERILS AND PROSPECTS IN THE DEVELOPMENT OF FLT3 INHIBITORS

Although the preliminary results are encouraging, there are some perils in the development of FLT3 inhibitors for the treatment of AML. The biggest hurdle at the current time relates to the biology of the AML stem cell pool. In the proposed model of the molecular pathogenesis of AML (Fig. **1**), class II mutations (those enhancing proliferation) cooperate with class I mutations (those blocking differentiation) to produce AML. Indeed, the class I mutations may provide selective pressure for acquisition of such mutations as proliferation and/or progenitor survival is impaired as a consequence of the class I mutation (e.g. AML1-ETO). In the case of AML1-ETO it appears that alternative class II mutations, such as NRAS, FLT3 or KIT mutations can all provide escape from the anti-proliferative effects of the translocation [15, 16, 71, 138]. This raises the possibility that some or perhaps most cases of AML have a polyclonal population of different class II mutations that arose independently. In such a setting, one clone might predominate but the other clones could be selected for by use of a targeted agent. If this model were correct, one would predict that treatment of FLT3-ITD AML might result in clearance of the FLT3-ITD clone but emergence of clones with alternative class II mutations, such as activating point mutations of NRAS. Even using conventional chemotherapy treatments, some relapsed AML patients have no evidence of their original FLT3-ITD mutation and express either wild-type FLT3 or a different FLT3-ITD. Thus, in assessing the clinical effects of FLT3 inhibitors it will be crucial to monitor cells for emergence of alternative leukemic clones (e.g. RAS mutations) [138, 138, 139].

Another barrier to FLT3 inhibitor therapy, especially monotherapy, is the possibility that acquisition of additional somatic mutations in the FLT3 kinase domain may induce drug resistance. Pre-clinical development of FLT3 inhibitors has indicated that small changes in drug or protein structure can profoundly influence selectivity and potency. Certainly in the case of Ph^+ ALL and CML blast crisis, rapid development of imatinib resistance as a consequence of ABL kinase domain mutation has been observed. In CML, BCR-ABL is the initiating molecular event and cells remain dependent upon kinase activity even in advanced disease [20, 120, 123, 140, 141]. In the case of FLT3 inhibitors, it will be important to determine if clinical resistance develops due to FLT3 mutation or due to selection of clones with alternative oncogenic events, such as RAS mutation [138]. If secondary mutations of FLT3 are the dominant mechanism of acquired drug resistance, this will actually serve to validate FLT3 mutation as a critical event in leukemogenesis.

The experience with imatinib in advanced CML and Ph^+ ALL suggests that monotherapy with a FLT3 inhibitor in AML may result in substantial clinical activity that is limited by a rapid development of resistance due to the inherent genomic stability of AML cells. The optimal use of such agents will probably require moving such inhibitors into front line therapy in combination with standard chemotherapy agents. *In vitro* and *in vivo* experiments suggest additive to synergistic activity when combining FLT3 inhibitors with anthracyclines, cytosine arabanoside, or ATRA [13, 142]. Given the high complete remission rates seen in AML with standard therapy, the major clinical benefit of adding FLT3 inhibitors to standard treatment may be to increase remission duration and cure rates [143, 144]. The use of these endpoints will require comparison of FLT3 inhibitor with standard chemotherapy versus standard chemotherapy in adequately powered randomized phase III trials.

The move towards combination therapy using chemotherapy and a FLT3 inhibitor is analogous to the clinical development of ATRA in APL. Treatment with ATRA alone as a single agent results in early remission but unacceptably high relapse rates. However, the use of ATRA in combination with traditional chemotherapy agents produces the highest cure rates of any FAB subtype of AML [22, 145, 146].

CONCLUSIONS

Rapid progress has been made in the development of potent and relatively selective FLT3 TKIs. A number of these compounds have advanced to phase I/II trials, where they have proven to be safe and well tolerated with some encouraging evidence of clinical activity. These results allow us to be optimistic about the prospects for continued clinical development of this class of agents. The ultimate clinical potential of FLT3 TKIs remains uncertain and will be determined in large part by the fundamental biology of AML rather than drug pharmacology.

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