SIMULTANEOUS INHIBITION OF DRIVER AND EFFECTOR KINASES PROMOTES POTENT GROWTH ARREST OF AML CELLS IN VITRO AND IN VIVO

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Acute myelogenous leukemia (AML) is caused by successive mutations acquired in hematopoietic progenitor cells that lead to the overpopulation of the bone marrow and peripheral blood by immature myeloid cells. The overall survival rate with current therapy is 25%, which decreases steadily with patient age. While numerous genetic alterations occur in AML, mutations to the FMS-like tyrosine kinase 3 (*FLT3*) are the most common, occurring in 30% of patients. Two types of *FLT3* mutations occur in AML: internal tandem duplications (ITDs) and tyrosine kinase domain point mutations. *FLT3* mutations drive AML pathogenesis and numerous small molecule kinase inhibitors have been designed as therapeutics for *FLT3*-mutated AML. However, these FLT3 inhibitors have had limited clinical success owing to lack of potency *in vivo*, toxicity, or short duration of response due to the development of resistance.

Constitutively active FLT3 links to multiple downstream tyrosine kinases that are critical for AML cell survival and proliferation. Gene silencing studies have shown that several of these FLT3-associated kinases are individually crucial for FLT3 oncogenic potential, including SYK, FES, and the myeloid Src-family kinase, HCK. These previous observations raised the hypothesis that a small molecule inhibitor with a selectivity profile targeting FLT3 plus these associated kinases may be a potent AML drug lead with reduced propensity for acquired resistance. To test this hypothesis, we screened a library of N-phenylbenzamide compounds and identified a compound with three-digit nanomolar activity against each of these AML-associated

kinases in vitro. Remarkably, this compound (TL02-59) inhibited FLT3-ITD^{+} cell growth in the picomolar range. Furthermore, TL02-59 demonstrated efficacy against primary AML bone marrow samples and a mouse xenograft model of AML. To explore the full range of targets for TL02-59, we performed a KINOMEscan assay and determined the expression of the TL02-59 target kinases in primary AML bone marrow samples. We discovered that while myeloid Src-family kinases HCK, LYN and FGR are critical to TL02-59 efficacy, inhibition of SYK, FES and the Ser/Thr kinases p38α and TAOK3 also plays a role. Future work will explore the advantages of this potent, multi-targeted kinase inhibitor in combating acquired resistance in AML.

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AKT	V-Akt Murine Thymoma Viral Oncogene Homolog
AML	Acute myelogenous leukemia
APL	Acute promyelocytic leukemia
BLK	B lymphocyte kinase
CN-AML	Cytogenetically normal acute myelogenous leukemia
CR	Complete response
CSC	Cancer stem cell
ELN	European LeukemiaNet
ERK	Extracellular signal-regulated kinases
FAB	French-American-British
FES	Feline sarcoma/Fujinami Avian sarcoma oncogene homolog
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
FL	FLT3 Ligand
FLT3	FMS-like tyrosine kinase 3
FYN	FYN oncogene related to SRC, FGR, YES
HCK	Hematopoietic cell kinase
HiDAC	High dose intermittent ARA-C
HSC	Hematopoietic stem cell

HSCT	Hematopoietic stem cell transplant
ITD	Internal tandem duplication
LCK	Lymphocyte Cell-Specific Protein-Tyrosine Kinase
LSC	Leukemic stem cells
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MDS	Myelodysplastic syndromes
MEK	Mitogen-activated protein kinase kinase
MRD	Minimal Residual Disease
mTOR	Mammalian target of rapamycin
p38α	Mitogen-activated protein kinase 14
PI3K	Phosphoinositide 3-kinase
RAF	V-Raf-1 Murine Leukemia Viral Oncogene Homolog
RAS	Rat sarcoma viral oncogene homolog
SFK	Src-family kinase
SRC	v-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT5	Signal transducer and activator of transcription 5
STK10	Lymphocyte-oriented kinase
SYK	Spleen tyrosine kinase
TAOK3	Thousand And One Amino Acid Protein 3
TKD	Tyrosine kinase domain
WHO	World Health Organization
YES	v-yes-1 Yamaguchi sarcoma viral oncogene homolog

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1.0 INTRODUCTION

1.1 ACUTE MYELOID LEUKEMIA

It is most commonly believed that the first description of a patient with leukemia was made by Alfred Velpeau, a surgeon in Paris at the time. In 1827, he described a patient with swelling of the "abdomen, fever, and weakness" (1). After this patient's death Velpeau discovered an enlarged liver and spleen, as well as "blood like gruel" (2). It was not until 1845 that blood from a leukemia patient was microscopically examined for the first time, by John Hughes Bennett. He determined that the patient had an increase in the number of "colorless corpuscles" in the blood (3). The word "leukemia" was coined by Rudolf Virchow just two years later, in 1847, to describe a disease that filled the blood vessels with a "greenish-yellow-white substance that looked like pus" (4). Numerous other breakthrough discoveries, including establishing that leukemia originates in the bone marrow (5), distinguishing between chronic and acute forms of leukemia (6), and separating myeloid versus lymphatic forms (7) were seminal in beginning the journey to our modern, molecular, understanding of acute myeloid leukemia.

1.1.1 The Origins of Acute Myeloid Leukemia

Numerous lines of evidence have indicated that leukemia arises through a multi-step transformation process beginning with an initiator mutation in a hematopoietic stem cell (HSC),

which ultimately gives rise to a leukemic stem cell (LSC) and eventually the overt disease that is seen clinically.

1.1.1.1 The Multi-Step Model of AML development The theory that AML develops through a multi-step process was originally proposed by Gilliland and Griffin (8). In this paper they separated common AML mutations as either class I or class II, with class I mutations conferring a proliferative or survival advantage and class II mutations impairing differentiation. Under these assumptions class I mutations are mutations such as activated receptor tyrosine kinases or *RAS* mutations and class II mutations are exemplified by fusion proteins containing transcription factors. Significant direct and indirect evidence supports this broad-based separation of mutation types. Under this theory, knocking-in an AML associated mutation (class I or class II) to mouse hematopoietic cells should not cause overt AML, as neither mutation alone is enough to cause disease. In fact, this is what has been documented with several class I and class II mutations (9-15). For example, when FMS-like tyrosine kinase receptor III (*FLT3*) containing an internal tandem duplication (ITD), a class I mutation, was knocked into mice, a fatal myeloproliferative disorder, but not overt AML was the result, with a median survival time of 10 months (16). However, when the *FLT3*-ITD knock-in was combined with a *NUP98-HoxD13* fusion knock-in, a class II mutation, 100% of the mice developed fulminant AML with a mean survival of 95 days (17). Another example, using retroviral gene transfer, demonstrated that the addition of *MLL-SEPT6*, a class II mutation, to hematopoietic stem cells (HSCs) induced a lethal myeloproliferative disease with a long latency but never induced AML. However, with the co-expression of a class I mutation, *FLT3*-ITD, the mice developed AML with complete penetrance (18). These studies provide evidence that at least two mutations are needed for

the clinical development of AML. Further indirect evidence from human patients also supports a two-step model of AML development. *FLT3* mutations and *RAS* mutations, both class I mutations, are rarely identified in the same patient but *FLT3* mutations commonly occur together with gene fusions that disrupt normal differentiation (19-24). More recent genome-wide sequencing studies have discovered novel mutations in AML that do not fit neatly into either the class I or class II mutation families; such as mutations in regulators of DNA methylation and histone modifications (25). These mutations occur in combination with both class I and class II mutations, but tend to be mutually exclusive with mutations in other epigenetic modifiers. This suggests a novel class of mutations that potentially work synergistically with the class I and class II mutations to induce AML (26).

1.1.1.2 The Leukemic Stem Cell The cancer stem cell (CSC) theory, first proposed in 1976, says that most cancers arise from a single cell that through genomic instability gives rise to the later stage tumors seen in the clinic (27). This theory was further expounded upon in two studies that provided evidence for the necessary characteristic of a CSC population; self-renewal and quiescent phenotype (28, 29). These early theories were based on studies in AML showing that only a small fraction of cells are capable of incorporating ^{3}H-thymidine, suggesting that only a small fraction of the bulk tumor cell population is actually moving through the cell cycle (30, 31). Subsequent studies, exploring AML cell growth in methylcellulose, further supported the CSC theory (32, 33). These studies again discovered that only a fraction of bulk tumor cells could expand in methylcellulose, and thus only this population of tumor cells is capable of repopulating the tumor. While a number of studies would further characterize the leukemic stem cell (LSC) and the likelihood of its existence (34-36), it was not until the mid-1990s that the first evidence of a LSC capable of repopulating

an in vivo xenograft model was discovered. These two seminal studies demonstrated the ability of human $CD34^{+}CD38^{-}$ peripheral blood or bone marrow AML cells to repopulate the tumor in a naïve recipient mouse (37, 38). Both studies discovered that the transplanted LSCs reproduced AML in mice that closely recapitulated the clinical characteristics seen in the human patient. In the twenty years since the LSC was identified, numerous studies have further characterized the LSC as sharing many of the same traits of normal HSCs, including their ability to self-renew (39-41) and their quiescent nature (42, 43). Additionally, more recent studies indicate that LSCs share a niche with HSCs, providing the microenvironment necessary for stem cell survival (44). The investigation of LSCs has become extremely important as one study discovered that high leukemic stem cell frequency at diagnosis predicts a poor prognosis (45).

Combining LSCs with the two-hit model discussed in the previous section, it is most commonly thought that the first mutation in AML occurs in an HSC, leading to a pre-leukemia stem cell and eventually a second mutation leads to an LSC (Figure 1) (37, 41). However, several studies have discovered that restricted progenitor cells are capable of receiving an initiator mutation that reactivates self-renewal (46-48). This cell is then capable of obtaining a second mutation to be transformed into an LSC. The quiescent nature of LSCs leads most researchers to believe that an intermediate cell exists before the development of leukemic blasts (41, 49, 50). This cell is a leukemic progenitor cell with significantly increased ability to proliferate into leukemic blasts and a limited ability to self-renew. The blast population contains no potential to self-renew but is able to proliferate excessively.

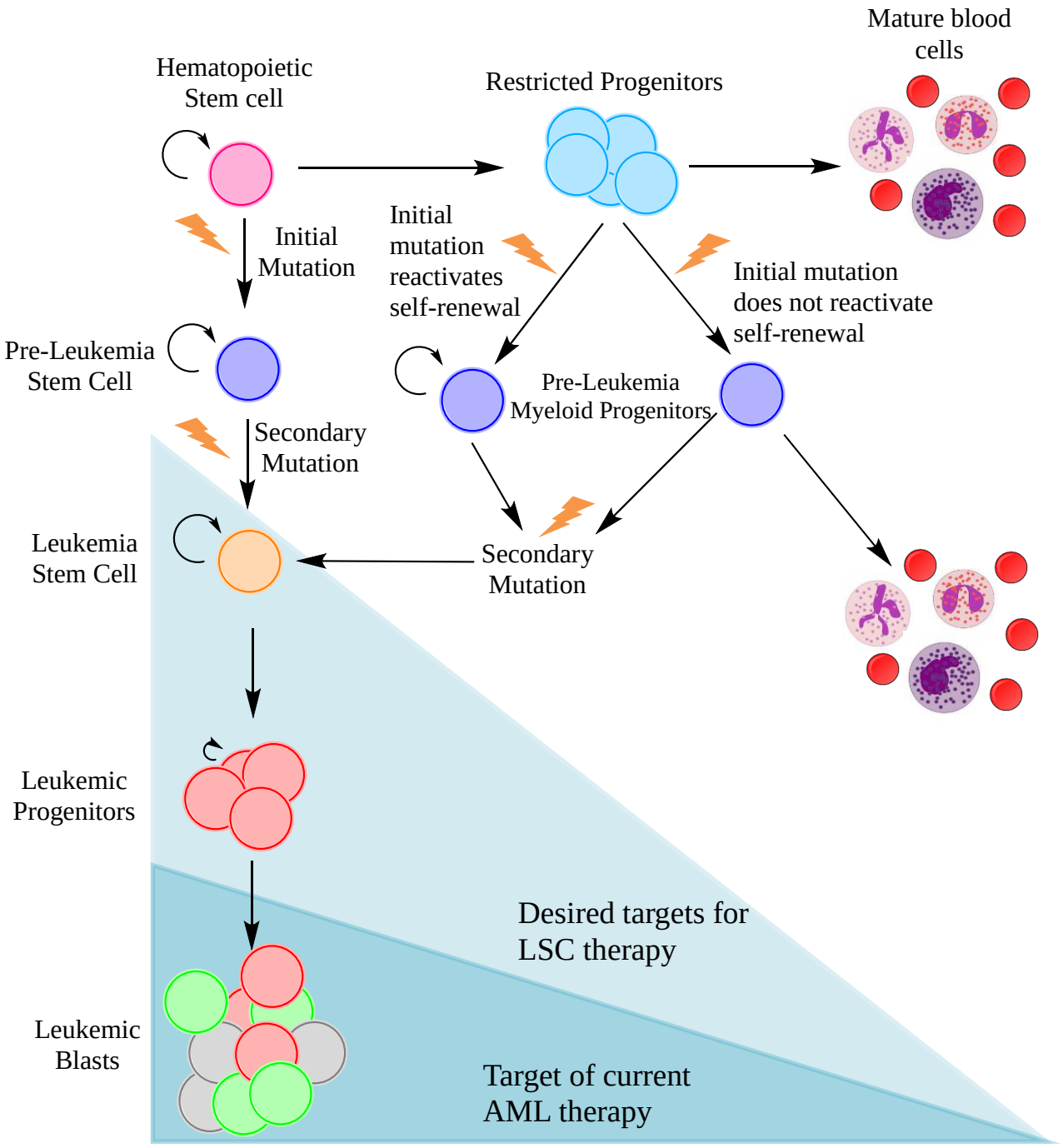


Figure 1: The two step model of AML development

Leukemia develops through a two-step mutation process. Normally original mutations occur in the hematopoietic stem cells and then a secondary mutation leads to the development of an LSC fully capable of self-renewal and proliferation into leukemic blasts. However, it is possible for restricted progenitor cells to acquire the initiating mutation and then a secondary mutation can occur in the same cell leading to an LSC. Current therapy targets the AML blasts but fails at eliminating the leukemic stem cells. Adapted from Roboz et. al. (49).

1.1.2 Common Mutations Occurring in AML

Two large genome-wide sequencing studies of AML discovered that it has one of the lowest median mutation frequencies of all adult cancers (Figure 2) (51, 52). However, significant patient to patient variation existed in both studies. Further sequencing studies have identified a core set of commonly mutated genes in AML. The following sections will briefly discuss each of them and their role in AML pathogenesis.

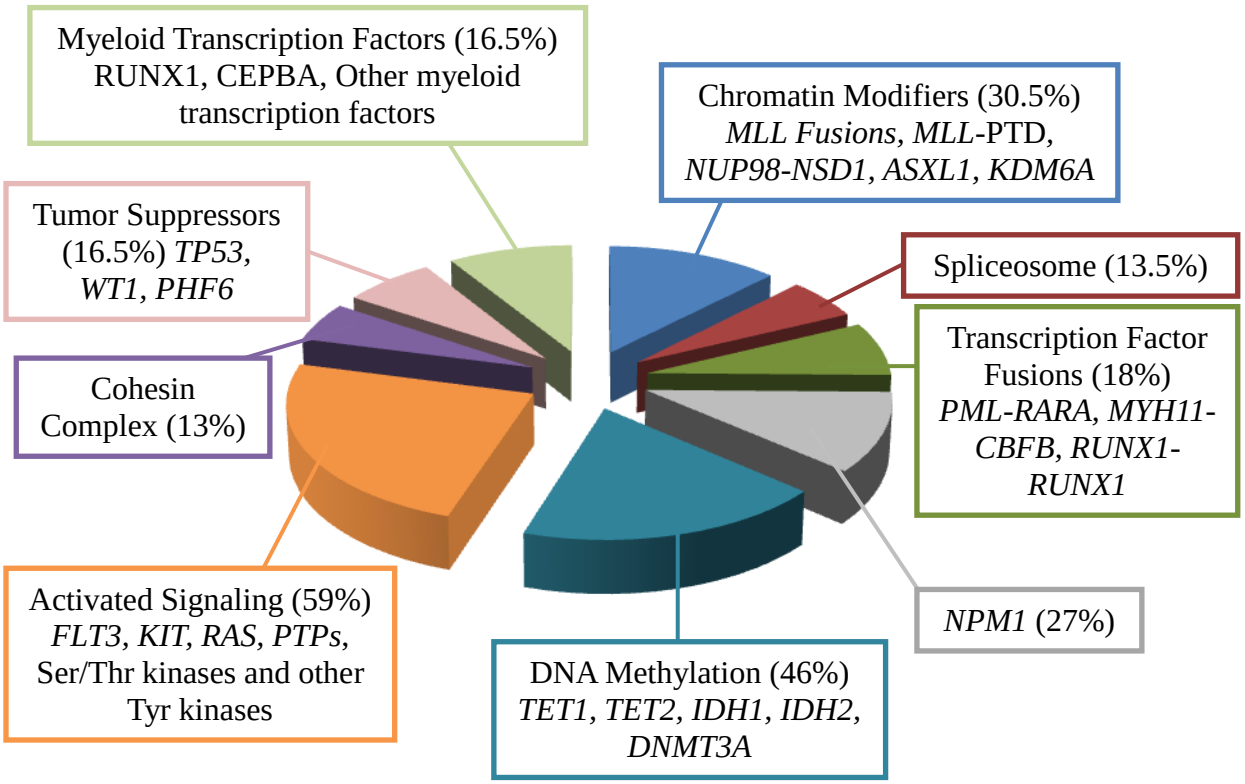


Figure 2: Common mutations in AML.

AML has many commonly occurring mutations and most patients carry more mutations from a number of different protein families. This depicts an overview of how common mutations the frequency of mutations in each family and some of the most common mutations from that family. Mutation data from The Cancer Genome Atlas (51) and figure adapted from Chen et. al. (53)

1.1.2.1 Signaling Pathway Mutations According to The Cancer Genome Atlas, signaling pathway proteins are the most commonly mutated genes in AML (51). Mutations most commonly occur in the, *FLT3*, *c-KIT*, *RAS*, and to a lesser extent Janus Kinase 2 (*JAK2*) genes. *FLT3* is the most commonly mutated gene in AML, and will be discussed in great detail in the following sections.

c-KIT is a receptor tyrosine kinase that under normal circumstances responds to its ligand, stem cell factor (SCF), to regulate the growth and survival of hematopoietic stem cells (54, 55). When mutated in approximately 3-5% of AML cases (56), most commonly by point mutations in the activation loop at D816, c-KIT becomes constitutively active. Oncogenic c-KIT induces activation of the phosphoinositide 3-kinase (PI3K) pathway (57) and activation of signal transducer and activator of transcription 3 (STAT3) leading to upregulation of STAT3 target genes, including, B-cell lymphoma-extra large (BCL-xL) and c-MYC (58). *c-KIT* mutations are most commonly found in conjunction with core binding factor (CBF) mutations (59) and runt-related transcription factor 1 (*RUNX1*) mutations (60). It is clear from the literature that the addition of a *c-KIT* mutation has a negative prognostic impact on CBF- and *RUNX1*-mutated AML (60-64).

RAS mutations, almost exclusively at codons 12 and 13, occur in approximately 20% of AML patients and lead to a constitutively active, GTP-bound form of RAS which activates its downstream effectors such as RAF and MEK (65). *RAS* mutations most commonly occur in AML with inv(16) [CBF fusions to Myosin, Heavy Chain 11 (MYH11)] and AML with inv(3), which leads to the overexpression of the EVI1 transcription factor through the repositioning of a GATA2 enhancer upstream of its promoter (59, 61, 66). Overall, the prognostic impact of *RAS* mutations in AML remains unclear as published studies show *RAS* mutations lead to favorable, unfavorable, or neutral outcomes (67-70).

JAK2, a non-receptor tyrosine kinase normally activated downstream of cytokine receptors (i.e. Interleukin Receptor 3 (IL3R)), is mutated in 1-3% of AML patients (71-73). Mutations occur almost exclusively as phenylalanine substitutions at position V617 in the kinase domain, leading to constitutive activation of the kinase and its downstream effectors; STAT5 and PI3K/AKT (74). *JAK2* mutations do not occur with any particular cytogenetic aberration but they are associated with less differentiated AML (72). Few studies have explored the prognostic impact of *JAK2* mutations; however, one study discovered that upregulation of JAK2 phosphorylation was a predictor of worse response to chemotherapy (75).

1.1.2.2 Mutations in DNA Methylation Proteins Mutations affecting the process of DNA methylation are among the most recent commonly occurring mutations identified in AML. They are characterized by mutations in tet methylcytosine dioxygenase 2 (*TET2*), Isocitrate Dehydrogenase 1/2 (*IDH1/2*), and DNA Methyl Transferase 3a (*DNMT3A*).

TET2 loss of function mutations occur in 7-23% of AML cases (76). *TET2* loss disrupts normal hematopoietic differentiation and provides a proliferative advantage to these cells. Furthermore, these mutations are thought to be early events in progenitor cells and most commonly are identified at diagnosis and relapse. *TET2* mutations are most commonly discovered in cytogenetically normal AML (CN-AML) (77). The prognostic impact of *TET2* mutations is still under investigation. However, it was recently discovered that *TET2* mutations confer shorter event-free survival and shorter disease-free survival in CN-AML (78).

IDH1/2 are NAD^+-dependent enzymes responsible for converting isocitrate dehydrogenase to α-ketoglutarate (79, 80). Gain of function mutations occur exclusively in one of three arginines (IDH1 R132, IDH2 R172, IDH2 R140) that allow these enzymes to convert

α-ketoglutarate to 2-hydroxyglutarate (2-HG) (81, 82). Subsequent studies revealed that *IDH 1/2* mutations are mutually exclusive with *TET2* mutations and AML driven by either of these mutations has a similar global promoter hypermethylation pattern. From these previous studies, it was determined that 2-HG is capable of inhibiting TET2 enzymatic activity (77, 83). *IDH 1/2* mutations induce expression markers of immature cells, with a phenotype nearly identical to *TET2* mutated AML. Mutations have been identified in approximately 10-20% of AML cases, most commonly with mutated *NPM1* but without *FLT3*-ITD (84). Both *IDH1/2* mutations confer a poor prognosis (85-87).

DNMT3A mutations occur in approximately 15-25% of AML patients, most commonly in conjunction with *FLT3* and *NPM1* mutations (88). DNMT3A mutations are either single nucleotide changes at R882 or premature truncation by nonsense or frameshift mutations (89). Normally DNMT3A is responsible for the methylation of CpG motifs in DNA but when mutated it loses enzymatic activity leading to hypomethylation of certain genes. Additionally, since dimerization is essential for DNMT3A function, it is plausible that the mutations are dominant-negative. The role of mutated DNMT3A is not completely understood; however, global methylation data suggest that loss of DNMT3A increases expression of *HOX* genes through promoter hypomethylation (89). Numerous studies have identified the *HOX* genes as crucial for AML development (90). The prognostic impact of *DNMT3A* mutations is not completely understood, but in a particular subgroup of CN-AML patients ($NPM1^{wild\ type}/FLT3^{wild\ type}$) *DNMT3A* mutations confer a significantly worse prognosis (91).

1.1.2.3 Mutations in Chromatin Modifying Proteins The mixed lineage leukemia (*MLL*) mutations or fusions were among the first mutations discovered in AML. More recent work has described mutations in other chromatin modifying proteins such as Additional Sex

Combs Like 1 (*ASXL1*) and Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (*EZH2*).

Over 50 different *MLL* fusion partners have been described in AML but just five fusion partners account for 80% of all *MLL*-bearing leukemias (92, 93). In all fusion proteins, MLL loses its intrinsic H3K4 methyltransferase activity (94) but frequently gains H3K79 methyltransferase activity through an abnormal association with DOTL1 (95). In addition to fusions, *MLL* mutations also occur as partial tandem duplications (PTDs); in-frame duplication of a portion of the MLL protein (96, 97). MLL-PTDs do not perturb the intrinsic methyltransferase activity of MLL but instead increase hematopoietic progenitors in vivo through the induction of *HOX* genes (98). *MLL* mutations occur in approximately 5-10% of all AML cases and are particularly common in infant acute leukemias and secondary leukemia developed after topoisomerase II inhibitor treatment (99). All *MLL* mutations confer a poor prognosis (100, 101).

ASXL1 mutations occur in approximately 10-15% of AML cases and are highly enriched for secondary AML that developed from a myeloproliferative disease (102, 103). Somatic nonsense, missense, frameshift and point mutations have been discovered in *ASXL1*, with frameshifts being the most common (104). How *ASXL1* mutations contribute to leukemogenesis is poorly understood. However, ASXL1, is the DNA-binding component of the polycomb repressor complex 2 (PRC2) which functions to enact H3K27 trimethylation marks (105). Since *ASXL1* mutations typically reduce expression of ASXL1, it is postulated that loss of ASXL1 inhibits recruitment of PRC2 to proper loci in DNA, leading to a loss of H3K27 trimethylation. *ASXL1* mutations are early events and the association with disease outcome is still being debated (106-108).

EZH2 is the methyltransferase enzyme in the PRC2 complex responsible for H3K27 methylation (109). Loss of function mutations in EZH2 decrease global H3K27 methylation (110). Loss of H3K27 methylation potentially causes the aberrant expression of genes critical for leukemogenesis. *EZH2* is mutated in approximately 1-2% of AML patients and no prognostic relevance has been discovered thus far (111).

1.1.2.4 Nucleophosmin Mutations Nucleophosmin (*NPM1*) is mutated in approximately 25-30% of AML patients with enrichment for CN-AML and AML that contains *FLT3*-ITD mutations (112). NPM1 normally shuttles between the nucleus and cytoplasm (113), where it regulates protein aggregation, cell cycle progression, and the ARF-TP53 tumor suppressor pathway (114-116). Most AML- associated *NPM1* mutations cause a frameshift in the C-terminal region of the protein, causing the addition of a nuclear export signal and loss of a nuclear localization signal (117). Ultimately, this results in abnormal cytoplasmic-only localization of NPM1. The exact role of NPM1 in leukemogenesis is unclear but it is associated with the upregulation of stem cells maintenance genes (118). In the absence of *FLT3*-ITD mutations, *NPM1* mutations are a good prognostic indicator, with a five-year survival probability of approximately 60% (119-121).

1.1.2.5 Transcription Factor Mutations Transcription factors are essential for the differentiation of hematopoietic cells so not surprisingly; transcription factor mutations are common in AML. Here I will discuss mutations to runt related transcription factor 1 (*RUNX1*), CCAAT/Enhancer Binding Protein alpha (*CEBPA*), and the retinoic acid receptor alpha (*RARα*).

RUNX1 is the DNA-binding portion of CBF which is intricately involved in the transcriptional programs of hematopoietic differentiation (122). Thus, loss of RUNX1 or impairment of its function prevents differentiation. Chimeric *RUNX1* fusion proteins resulting from chromosomal translocations occur in approximately 10-15% of all AML cases (123). RUNX1 fusion proteins are associated with undifferentiated AML morphologies and commonly occur with trisomy 21 and trisomy 13 (124, 125). Lower complete response rates and an overall worse prognosis are associated with *RUNX1* fusions (126, 127).

The *CEBPA* gene encodes a leucine zipper transcription factor critical for granulocytic differentiation (128-130). *CEBPA* mutations occur in approximately 10-20% of AML patients and occur in parallel with *FLT3*-ITD and *NPM1* mutations (131). Three different varieties of mutations occur in *CEBPA* and typically *CEBPA* mutations are biallelic. Nonsense N-terminal mutations create a dominant-negative truncated isoform that prevents the expression of granulocyte differentiation genes (132, 133). C-terminal point mutations reduce DNA binding and dimerization affinity (134, 135). Finally, other studies identified DNA hypermethylation at the *CEBPA* promoter, eliminating its expression (136). Generally, *CEBPA* mutations are a good prognostic marker and lead to a favorable treatment response (137, 138).

RARα is a vitamin A-responsive nuclear receptor that requires heterodimerization to the RXR nuclear receptor for its function. RARα is essential for myeloid differentiation in the granulocyte/monocyte lineages (139) and loss of RARα results in a differentiation block at the promyelocyte stage (140). Fusion of *RARα* to the promyelocytic leukemia gene (*PML*) from the t(15;17) chromosomal translocation, is the most common cause of acute promyelocytic leukemia (APL) (141, 142). The PML-RARα fusion protein acts like wild-type RARα which has lost the ability to respond to vitamin A (143). APL cases account for 5-10% of all AML patients and has

an excellent prognosis (144). Treatment with all-trans retinoic acid (ATRA) and arsenic-trioxide (ATO) induces the differentiation of PML-RARα expressing cells and gives APL a cure rate of approximately 75-85% (145, 146).

1.1.2.6 Tumor Suppressor Mutations *TP53* mutations are extremely common in solid tumors but only occur in approximately 10% of AML cases (147). Inactivating mutations or loss of a *TP53* allele through imbalanced translocation results in impairment of apoptosis, genome instability, and deficiency in cell cycle arrest (148-150). Commonly, *TP53* mutations are biallelic (151, 152), and are most common in the elderly and patients with complex karyotype AML (153). Poor prognosis and short survival time are typical in patients with *TP53* mutations (154).

Wilms Tumor 1 (*WT1*) encodes a zinc-finger DNA binding protein that is mutated in 5-10% of AML cases (155). Depending on the cellular context, WT1 can be a tumor suppressor or an oncogene obscuring its exact role in leukemogenesis (156). However, two recent studies suggest that loss of WT1 function causes a block in hematopoietic differentiation (157, 158). While the exact prognostic impact of *WT1* mutation remains uncertain, several small studies suggest it promotes induction failure and shorter overall survival (155).

1.1.3 Classification of AML

Three separate methods are used in the clinical classification of AML; the French-American-British (FAB) classification system, the World Health Organization (WHO) classification system, and the European LeukemiaNet Standardized System (ELN). Each is described briefly in the following sections.

1.1.3.1 French-American-British Classification Originally published in 1976, the FAB classification system is the oldest method of classifying AML patients (159). Classification is based entirely on morphological appearances of a Romanowsky-stained bone marrow film. Additionally, cytochemical stains for myeloperoxidase and non-specific esterase are used to distinguish between certain subgroups. At its advent, the FAB system classified AML into six subcategories, M1 through M6 (Table 1). An update to the system in 1985 added the M7 subcategory (160, 161) and a final update in 1991 added an M0 category (162). Furthermore, the FAB classification system set the arbitrary cutoff for AML at >30% blasts in the bone marrow. Anything less than 30% blasts is considered to be a myelodysplastic syndrome (MDS) as opposed to frank leukemia.

The FAB classification system allowed clinicians to determine the prognosis of each subtype. Additionally, with a universal classifications system, clinical trial results could be compared more easily. However, it is obvious with the luxury of hindsight that the FAB classification has its limitations. First, despite the significant histological detail described for each subtype, reproducibility of subtype classification between researchers/clinicians varied significantly (163, 164). Additionally, immunological and genetic studies began to describe subtypes of AML that were not distinguishable in the FAB system. Furthermore, accumulating evidence suggested that the FAB subtypes, except M3 with is caused by the *PML-RARα* fusion 95% of the time (165), did not equate to a particular cytogenetic abnormality, even though some mutations/ translocations are more common in certain subtypes (166). Since response to therapy is largely determined by cytogenetic abnormalities, the FAB system had limited prognostic value, beyond outcomes in the M3 subtype being most favorable, and outcomes in the M1 and M7 subtypes being least favorable (167). The final issue with the FAB classifications system is

how it separates MDS from AML. The 30% cutoff is arbitrary and some patients are likely misclassified (168). Additionally, MDS cases often develop into AML with specific alterations, and no subgroup existed for this type of AML (169). These issues combined with novel genetic data prompted the development of the WHO classification system.

FAB Subtype	Name
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

Table 1: The FAB Classification System

The FAB classification system was the first system used to classify AML and was based entirely on morphological appearance. The eight subgroups are shown in the table and they are divided on extent of differentiation and cell of origin.

1.1.3.2 The World Health Organization (WHO) Classification The WHO classification system was originally published in 2001 (170), updated in 2008 (171) and the further refined in 2016 (172). At its advent, this AML classification system incorporated immunophenotyping and genetic approaches into the already existing morphological system grounded in the FAB approach. In the 2016 edition, AML is divided into four major categories; AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related myeloid neoplasms, and AML not otherwise specified (NOS) (Table 2). Additionally, the WHO classification lowered the blast percentage in the bone marrow necessary for diagnosis of AML to 20%. Furthermore, it allowed for cases that carry particular genetic translocations [i.e. t(15;17)] to be diagnosed as AML regardless of the bone marrow blast percentage.

The WHO classification system is an improvement on the FAB system because it incorporates many recurring cytogenetic abnormalities linked to patient outcome into its diagnosis criteria. Additionally, it adds separate categories for AML caused by previous therapy and AML developing from a myelodysplastic syndrome as these two types of AML carry a particularly poor prognosis. However, current estimates suggest that only about 50% of AML patients can be classified under the first three categories of the WHO classification system (173). This will likely change as novel genetic abnormalities are discovered and validated as prognostically significant in AML. Additionally, further refinement is needed in the AML NOS category because it only uses morphological characteristics to categorize patients, similar to the FAB system, and has little prognostic value (174). Essentially, every AML NOS subgroup has the same outcome. Finally, the WHO classification system fails to incorporate many genetic mutations with prognostic significance, such as the *FLT3*-ITD mutation which is present in more than 20% of AML cases. In the most recent revision, mutations in *NPM1* and *CEBPA* were

added as recurrent abnormalities because they have prognostic significance in CN-AML. However, it is difficult to foresee how this classification system will incorporate genetic mutations with prognostic significance in multiple cytogenetically mutated AML backgrounds.

AML with recurrent genetic abnormalities • AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1 • AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11 • APL with PML-RARA • AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A • AML with t(6;9)(p23;q34.1);DEK-NUP214 • AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM • AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1 • Provisional entity: AML with BCR-ABL1 • AML with mutated NPM1 • AML with biallelic mutations of *CEBPA* • Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes
Therapy related myeloid neoplasms
AML, NOS • AML with minimal differentiation • AML without maturation • AML with maturation • Acute myelomonocytic leukemia • Acute monoblastic/monocytic leukemia • Pure erythroid leukemia • Acute megakaryoblastic leukemia • Acute basophilic leukemia • Acute panmyelosis with myelofibrosis

Table 2: The WHO Classification System.

The WHO classification system uses cytogenetic and genetic data to classify patients. Any patient not classified with a recurrent genetic abnormality, myelodysplastic related change, or therapy related change falls into the AML, NOS category. This category still relies on morphological information to sub-classify patients. NOS: Not otherwise specified

1.1.3.3 European LeukemiaNet (ELN) Classification The ELN was conceived because of the need to stratify patients based on their prognostic outcome (175). Additionally, the ELN allows for the incorporation of genetic mutations with prognostic impact that occur in multiple cytogenetic backgrounds. The ELN classifies patients into four main outcome categories; Favorable, Intermediate-I, Intermediate-II, and Adverse (Table 3).

Numerous studies tested the prognostic significance of the ELN classification system and discovered significantly different outcomes in the four groups, even when patients are subdivided

Genetic Risk Group	Subset
Favorable	• t(8;21)(q22;q22); *RUNX1-RUNX1T1* • inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11* • Mutated *NPM1*without *FLT3*-ITD (normal karyotype) • Mutated *CEBPA* (normal karyotype)
Intermediate-I	• Mutated *NPM1* and *FLT3*-ITD (normal karyotype) • Wild-type *NPM1* and *FLT3*-ITD (normal karyotype) • Wild-type *NPM1* without *FLT3*-ITD (normal karyotype)
Intermediate-II	• t(9;11)(p22;q23); *MLLT3-MLL* • Any cytogenetics not classified as favorable or adverse
Adverse	• inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1* • t(6;9)(p23;q34); *DEK-NUP214* • t(v;11)(v;q23); *MLL* rearranged • Monosomy 5 or del(5q); monosomy 7; abnormal 17p; complex karyotype (≥ 3 abnormalities)

Table 3: The ELN Classification System.

The ELN was designed to classify patients based on clinical outcome. It allows for the design of treatment strategies based on those who are most likely to respond. Studies have determined that these groups have prognostic significance in both younger and older patient groups.

by age (176, 177). One major improvement with this system is the ability to separate the CN-AML patients based on genetic mutations. CN-AML accounts for approximately 40-50% of all AML patients and this group is extremely heterogeneous for genetic mutations and outcomes (178). The ELN uses *NPM1*, *CEBPA*, and *FLT3*-ITD mutations to stratify this group into both the Favorable and Intermediate-I categories, identifying differences in prognosis with in the CN-AML patients. Another unique feature of this system is how easily it can be updated to allow for the addition of novel genetic mutations with prognostic significance. In fact, a number of studies have already suggested that adding other genetic mutations into the ELN further refines the ELN classification system (77, 107).

1.1.4 Clinical Course of AML

Even though AML accounts for only 1.2% of all cancers, it is the most common hematologic malignancy in adults with a median onset age of 67 years (179). It is estimated that in 2016 approximately 20,000 people will be diagnosed with AML and about 10,500 will die from the disease.

1.1.4.1 Risk Factors The majority of AML cases are 'de novo AML' meaning that there is no determinable cause. However, a few risk factors for AML development have been identified. The biggest risk factor for the development of AML is age (180). While AML does occur in children and young adults under the age of 25, the incidence rate rises exponentially with increasing age. Generally, the older a patient is when diagnosed with AML, the worse the prognosis (181).

Several genetic mutations have been linked to a predisposition for developing AML. Studies have identified rare inherited mutations in *RUNX1*, *CEBPA*, or *GATA2* in infants, typically under a year old, who develop AML (182, 183). Additionally, several congenital diseases that affect tumor suppressors or DNA repair proteins (i.e. Li-Fraumeni syndrome or Fanconi anemia) carry a significantly increased risk of developing AML due to the genomic instability associated with these diseases (184, 185). Finally, diseases caused by abnormal chromosome number have an increased risk of developing AML. For example, people with Down syndrome, which is caused by an extra copy of chromosome 21 (trisomy 21), have an increased risk of developing acute megakaryoblastic leukemia, a less common subtype of AML typically caused by *GATA1* mutations (186).

The category of 'secondary leukemia' comprises AML patients whose leukemia developed because of prior exposure to cytotoxic agents or through the continued progression of another disease. A large percentage of secondary leukemia patients are considered to have treatment-related AML. Generally, treatment-related AML is the consequence of prior treatment with alkylating agents or topoisomerase II inhibitors for other cancers. Because patients commonly receive combination therapy, it is difficult to ascertain the causative agent. Treatment with alkylating agents is associated with chromosome 5 and 7 abnormalities (187), while treatment with topoisomerase II inhibitors leads to a higher incidence of *MLL* rearrangements (188). Treatment-related AML has an inferior prognosis with overall survival rates under 10% (189). A very small percentage of patients presenting with secondary leukemia developed it through exposure to environmental carcinogens, most commonly ionizing radiation and benzene (190, 191). The progression of myelodysplastic syndromes (MDS) accounts for the final portion of secondary leukemia. These AML patients originally develop MDS (i.e. polycythemia vera or

idiopathic myelofibrosis) which then evolves into AML (192). Evolution of MDS accounts for 3-4% of all AML cases and as with the therapy-related AML, patients developing AML from MDS have a significantly worse prognosis.

1.1.4.2 Clinical Manifestations The clinical manifestations of AML develop quickly, occurring over a few weeks to a few months as most (193). The most common symptom, anemia, results from the loss of normal bone marrow function, resulting in fatigue, pallor and headache (184). Additionally, patients tend to bruise easily and have evidence of bleeding, commonly noticed as bleeding gums (192). The lack of functioning bone marrow depletes the immune system and AML patients often deal with recurring or severe infections (184). Finally, in rare cases leukemic blasts will infiltrate the skin and lead to 'leukemic cutis', a rash that occurs with raised bumps (192).

In the laboratory, almost all patients present with granulocytopenia even though peripheral white blood cell counts can be highly variable (192). Approximately 25% of patients have high white blood cell counts (>50,000 cells/μL), 50% have white blood cell counts between 5,000 and 50,000 cells/μL, and the last 25% have low white blood cell counts (<5,000 cells/μL) (184). Additionally, as mentioned above, patients often present with anemia and generally are thrombocytopenic. While the peripheral blood can provide crucial information for the diagnosis of AML, a bone marrow aspiration is necessary for confirmation. The bone marrow is typically hypercellular with anywhere from 20% to 100% leukemic blasts.

1.1.4.3 Standard of Therapy Standard therapy for AML is divided into two parts; remission (induction) and postremission therapy (consolidation therapy). The role of remission induction is to induce a complete response (CR) in patients. The postremission

therapy goal is to continue the CR or to bridge over to a bone marrow transplant if that is a practical option.

Induction therapy is commonly given in a 3+7 design regardless of cytogenetic abnormalities or mutations, with the exception of APL (175). This combination chemotherapy regimen consists of continuous cytarabine (100-200 mg/m^2/d) infusion for seven days and either high (60-90 mg/m^2/d) or low (45 mg/m^2/d) dose anthracycline for three days. Both chemotherapeutic agents disrupt DNA synthesis however they employ two different mechanisms. Cytarabine is an antimetabolite that incorporates into DNA while anthracyclines work through DNA intercalation. This treatment regimen was originally designed back in 1973 (194) and has had only minimal changes since that time, despite numerous clinical trials (195-203). The choice between high and low dose anthracycline is typically made based on age, with patients under 65 years old receiving the high-dose regimen and patients over the age of 65 receiving the low dose. Daunorubicin is the most common anthracycline given, however idarubicin (13 mg/m^2/d) can replace the high dose daunorubicin with equal results (204-206). Most patients will achieve a CR from only one round of this treatment regimen; however, a portion of patients will need additional induction cycles in order to achieve CR. In AML, the definition of CR is a patient with <5% blasts in their bone marrow (207). However, several studies have suggested this definition of CR is inadequate and minimal residual disease (MRD) monitoring should consist of more advanced molecular techniques such as PCR to detect translocation breakpoints where feasible (208-210). Overall, approximately 65-80% of patients will achieve CR with this chemotherapy regimen, while approximately 20% will never achieve CR (192).

All AML patients will ultimately relapse without postremission therapy (193). The proper postremission therapy is determined by a patient's cytogenetic profile and can either be chemotherapy, allogeneic hematopoietic stem cell transplantation (HSCT) or rarely autologous HSCT (207). A number of clinical trials have been dedicated to determining which patients will benefit most from each of these possible postremission therapies. Generally, in patients with favorable cytogenetics (based on the ELN categories) there is no difference in survival between chemotherapy or HSCT, and are almost exclusively given chemotherapy (211-215). Postremission chemotherapy typically consists of four cycles of high dose cytarabine (3 g/m^2) given every 12 hours on days 1, 3, 5 (HiDAC) (178, 216, 217). One exception in the favorable group is patients with CBF mutations and concurrent c-KIT mutations (175). While, these patients achieve similar CR rates, they have a substantially increased risk of relapse and thus should be considered for HSCT. The proper postremission therapy for patients stratified to the intermediate I or intermediate II categories is still debated, although recent studies demonstrated increased survival with allogeneic HSCT in these patients (218), particularly those with *FLT3*-ITD mutations (219-221). Generally, these patients still receive HiDAC to bridge them over to HSCT. The unfavorable risk group has a clear advantage for allogenic HSCT (222-224). Normally <15% of these patients will be alive after 5 years but with allogeneic HSCT the 5-year survival rate can be increased to 25% (192). However, it should be noted that unfavorable cytogenetics at diagnosis increases the risk of relapse after allogenic HSCT. Three mechanisms are known to cause relapse after HSCT; failure of response to conditioning regimen, leukemia immune escape, or failure to produce effective graft-versus-leukemia response (225). Finally, for primary refractory patients, allogenic or autologous HSCT is their only option (226, 227); however, outcomes are significantly worse with 3-year survival rates of 19% (228, 229).

Even with current treatment, approximately 50-55% of younger patients and 85% of older patients will eventually relapse, typically in the first 3 years after remission. The most important predictor of response after relapse is the duration of first remission (184, 192). Patients who were in remission for greater than one year are more likely to respond to salvage chemotherapy than patients with a remission of less than one year. While current salvage therapies vary, the main priority is to induce a second CR and bridge over to an allogeneic HSCT (230). For patients unable to receive an allogenic HSCT there are not any more options and palliative care can be offered (193).

As it stands, the standard of therapy in AML has changed little in the last 40 years and current overall survival rates are approximately 25%. Elderly patients (5-year overall survival 5-15%) have a significantly worse prognosis than younger patients (5-year overall survival 35-40%) (231, 232). There is a significant unmet need for novel targeted therapies that not only increase overall survival but limit toxicities associated with cytotoxic chemotherapy especially in older patients. FLT3 small molecule kinase inhibitors are being intensively investigated for their potential as targeted therapy in FLT3-mutated AML and will be discussed in detail in the following sections.

1.2 FLT3 IN NORMAL BIOLOGY AND AML

Originally cloned in 1991 (233, 234), *FLT3* is also known as fetal liver kinase-2 (*FLK-2*) (235) and human stem cell kinase-1 (*STK-1*) (236). FLT3 is a member of the class III receptor tyrosine kinase family, which also contains platelet-derived growth factor receptor (PDFGR), colony-stimulating factor 1 receptor (CSF1R or FMS) and the stem cell factor receptor, c-KIT (237).

This family of receptor tyrosine kinases is well known for its critical role in hematopoietic growth and differentiation.

1.2.1 Structure of FLT3

The *FLT3* gene consisting of 24 exons, encodes a 1000-amino acid protein with a molecular weight of approximately 140 kDa (238). However, upon immunoprecipitation, a 160 kDa band also appears, representing a heavily glycosylated form of FLT3 found on the plasma membrane (239). As is typical of the class III receptor tyrosine kinase family, FLT3 contains an extracellular region consisting of five immunoglobulin-like (Ig-like) domains (240). The three most N-terminal Ig-like domains are involved in FLT3 ligand (FL) binding while the other two are essential for ligand-induced receptor dimerization (241). A single transmembrane domain is followed by the juxtamembrane region. The tyrosine kinase domain of the receptor tyrosine kinase type III family is unique as it contains an 'insert' in the middle of the typical bilobed kinase domain (240).

1.2.2 FLT3 activation and signaling in early hematopoiesis

FLT3 is critical for the growth and survival of $CD34^+$ hematopoietic stem cells and early progenitor cells (242, 243). Interestingly, *Flt3*-knockout mice develop normal hematopoietic cell populations, with the exception of primitive B-lymphoid progenitors (244). However, bone marrow transplanted from *Flt3*$^{-/-}$ mice failed to reconstitute myeloid cells and T-cells in irradiated recipients, supporting a critical role for FLT3 in early hematopoiesis. Beyond the bone marrow, FLT3 is expressed in the liver, thymus, gonads, brain, and placenta (239, 245).

Under normal conditions, receptor tyrosine kinases require ligand binding to promote dimerization and autophosphorylation. In 1993, FLT3 ligand was cloned and proved to be an essential proliferative factor for early progenitor cells (246). During the next couple of years, several groups determined that FL is a homodimeric protein expressed as either soluble or membrane bound versions within the bone marrow microenvironment (247-250). Additionally, FL is capable of inducing progenitor cell proliferation ex vivo (251-253).

FLT3 activation, through binding to FL, stimulates two major downstream signaling pathways critical for proliferation and survival. The phosphoinositide 3-kinase (PI3K) pathway is activated through indirect binding to FLT3, conferred through the adapter proteins SHP-2, CBL, SHC, GAB-2 and GRB2 (254, 255). PI3K activates AKT and mTOR downstream, which in turn increases p70S6K activity and reduces Forkhead transcription factor activity. As a result, pro-apoptotic signaling is reduced, enhancing survival and cell cycle progression. RAS activation is mediated through the guanine nucleotide exchange factor Son of Sevenless (SOS) (256, 257). RAS activation stimulates the MEK/ERK pathway downstream, leading to increased transcriptional activity of CREB. The RAS pathway promotes proliferation upon FL binding. In addition to these two major pathways PLCγ-1 is activated as well as SRC and FYN, both of which physically interact with the active FLT3 receptor (258).

1.2.3 *FLT3* mutations in AML

Approximately 70-100% of AML cases express high levels of FLT3, irrespective of cytogenetics or age (259-261). It should be noted that FLT3 is also expressed at high levels in most cases of B-ALL, T-ALL, and in a small percentage of CML cases (262). It is likely FLT3 promotes survival and proliferation of the leukemic blasts through activation by FL (263). In fact, one

study found that a significant proportion of leukemic blasts express both FLT3 and FL suggesting an autocrine signaling loop (264). Although wild-type FLT3 (FLT3-WT) is highly expressed in multiple types of leukemia, *FLT3* mutations show considerable specificity for AML.

1.2.3.1 FLT3-ITD mutations The internal tandem duplication (ITD) mutation of *FLT3* was originally discovered in 1996 in 5 of 30 patients with AML (265). Subsequent sequence analysis showed that this type of mutation occurs in-frame and was a duplication of a portion of the juxtamembrane region of FLT3. Subsequent studies confirmed these results, and today it is well established that *FLT3*-ITD mutations occur in approximately 25% of all AML cases and most commonly occur in CN-AML (266-269). ITDs typically insert in exons 14 and 15 of *FLT3* and their length can vary significantly, from 3 bps to well over a 100 bps. *FLT3*-ITD mutations commonly occur in only one allele of *FLT3* but interestingly, the *FLT3*-WT allele is lost is some cases of FLT3-ITD$^+$ AML suggesting that loss of the *FLT3*-WT allele may confer a survival advantage (270, 271).

The structural basis for constitutive activation of FLT3 by an ITD was discovered in 2004 with the crystallization of an autoinhibited FLT3-WT kinase domain (272). This study revealed that the juxtamembrane region docks onto the back of the kinase domain and locks it in a downregulated state. Thus, lengthening of this region, via an ITD, could disrupt the autoinhibition caused by the juxtamembrane region resulting in a constitutively active kinase in the absence of FL binding.

FLT3-ITD continuously activates the PI3K and RAS pathways, similar to FLT3-WT, but has gained the ability to activate numerous other downstream signaling pathways as well. The activation of STAT5 is one of the most established downstream effectors of FLT3-ITD (273).

While studies disagree on whether STAT5 binds directly to FLT3 or it is activated by other tyrosine kinases linked to FLT3, it is agreed upon that STAT5 transcriptional regulation is essential for the expression of the anti-apoptotic proteins BCL-xL, PIM kinases, and c-MYC (274-277). In addition, to the activation of STAT5, FLT3-ITD downregulates both CEBPA and PU.1, transcription factors essential for myeloid cell differentiation (278). Other studies have also demonstrated that FLT3-ITD synergizes with WNT signaling through the upregulation of the WNT ligand, Frizzled-4 (279). Finally, FLT3-ITD can increase reactive oxygen species (ROS) in leukemic cells which likely increases genomic instability and lead to further mutations (280).

1.2.3.2 FLT3-TKD mutations In 2001, tyrosine kinase domain (TKD) point mutations were reported in *FLT3*, analogous to mutations previously identified in other receptor tyrosine kinases such as *c-KIT* and *MET* (281). These mutations most commonly occur in the activation loop at amino acid D835. While tyrosine substitutions (D835Y) are the most common mutation, valine, histidine, glutamate, and asparagine mutations also occur. Additionally, in rare cases I836 is mutated, instead of D835, to methionine or asparagine (281, 282). Overall, TKD mutations occur in approximately 5-7% of all AML cases and are most common in CN-AML (283, 284). Additionally, one study concluded that TKD mutations are mutually exclusive with ITD mutations (285).

The activation loop of the FLT3 kinase domain is localized between the N-lobe and C-lobe of the kinase domain, and adopts a specific conformation in the inactive state. However, mutations in the activation loop disrupt this loop packing and prevent inactivation, resulting in constitutive kinase activity (8). FLT3-TKD mutations can transform cells to cytokine-independent growth and activate similar pathways as FLT3-ITD mutations, including PI3K,

RAS/MAPK, and STAT5 (286). However, there are signaling differences. For example, even though studies indicate FLT3-TKD phosphorylates STAT5, one study discovered only weak expression of STAT5 target genes suggesting that FLT3-ITD creates a more robust activation of STAT5 (287). Additionally, CEBPA and PU.1 inactivation are crucial downstream effects of FLT3-ITD signaling (278). However, FLT3-TKD mutants are not capable of inactivating these two transcription factors (287). Another study discovered that FLT3-ITD can activate SRC as an effector kinase but FLT3-TKD was incapable of activating SRC (288). My own work shows that FLT3-ITD, but not FLT3-TKD, activates the FES tyrosine kinase downstream (Appendix A).

Transgenic mouse studies also support differences between *FLT3*-ITD and *FLT3*-TKD mutations. *FLT3*-ITD expression in mice only drives a myeloproliferative neoplasm (MPN), not overt AML (16). The majority of *FLT3*-TKD mice also develop a MPN; however, it is less aggressive than the MPN driven by *FLT3*-ITD, with *FLT3*-TKD mice surviving significantly longer than *FLT3*-ITD expressing mice (289). Interestingly, *FLT3*-TKD mutations developed into more than just an MPN, as some mice developed lymphomas or sarcomas. Additionally, *FLT3*-ITD mice display a loss of long-term hematopoietic stem cells (LT-HSCs), potentially because FLT3-ITD forces these cells to proliferate instead of self-renew (290). However, *FLT3*-TKD mice display no such differences in LT-HSCs (289). Furthermore, *FLT3*-TKD mice display reduced myeloid expansion and similar engraftment ability as *FLT3*-WT expressing mice. Overall, the signaling differences and mouse data present a story where *FLT3*-TKD mutations behave quite differently than *FLT3*-ITD mutations.

1.2.4 Prognostic impact of *FLT3* mutations

Given the differences mentioned above, it is not surprising that patients with *FLT3*-ITD or *FLT3*-TKD mutations have different prognoses. The prognostic impact of *FLT3*-TKD mutations is still controversial, but it is generally agreed that the prognosis is better than a *FLT3*-ITD mutation. Several studies determined that *FLT3*-TKD mutations have similar overall survival and event-free survival as *FLT3*-WT patients (291-293). However, two studies have indicated that *FLT3*-TKD mutations adversely affect overall survival (281, 294). Although the reason for these differences is not understood, one possibility is differences in the patient cohorts. Differences in cytogenetics or patients' ages could confound the effects observed since it is highly unlikely that *FLT3*-TKD mutations occur in isolation.

In contrast to *FLT3*-TKD mutations, *FLT3*-ITD mutations are an independent poor prognostic marker (295-300). ITD mutations are linked to significantly higher white blood cell and blast counts, as well as lower overall survival (271). Interestingly, a hemizygous $FLT3^{ITD/-}$ genotype confers an even worse prognosis than heterozygous $FLT3^{WT/ITD}$ (270). The reason for this is not understood, but it is possible that FLT3-WT can interfere with FLT3-ITD signaling, possibly by maintaining expression of differentiation-related genes. ITDs have limited impact on complete remission rates; however, relapse rates are significantly higher and thus allogeneic HSCT is offered as the best consolidation therapy after remission (263).

The average length of an ITD is approximately 30-50 amino acids. However, as stated earlier, ITDs can vary from 3 amino acids to well over 100 amino acids. Recent studies have begun to explore the possibility that a longer ITD confers a worse prognosis. In support of this hypothesis, two studies determined that patients with long ITDs (>40 amino acids or >48 amino acids) conferred a worse event-free survival and overall survival (301, 302). The structural basis

for this is not understood but one possibility is that a longer ITD provides more binding sites for interacting proteins or potentially allows FLT3 to bind to new effector kinases.

The allelic burden of FLT3 is also being investigated as a prognostic maker in FLT3-ITD$^+$ AML. Most FLT3-ITD$^+$ AML patients carry one *FLT3*-WT allele. However, the expression levels of FLT3-ITD versus FLT3-WT vary significantly between patients. Several studies have discovered that a higher FLT3-ITD allelic burden (ratio > 0.5 of FLT3-ITD/FLT3-WT) conferred significantly worse event-free survival and overall survival (20, 303, 304). However, it should be noted that the ITD always carries a poor prognosis, even if it is short and has a low mutant allele burden. These studies are subdividing the poor prognosis group further to identify more prognostic markers. Finally, one study investigated both mutant allele burden and ITD length (305). They discovered that the prognostic effects of these two makers were additive, but not synergistic.

1.3 TYROSINE KINASE SIGNALING IN FLT3-ITD$^+$ AML

As described previously, FLT3-ITD has many downstream effector kinases. In this section I want to explore a few particular tyrosine kinases that are activated downstream of FLT3-ITD and are crucial for my dissertation research. Additionally, I will provide a brief background on the discovery of these kinases and their normal functions.

1.3.1 Src-Family Kinases (SFKs)

The Src-family is the largest family of non-receptor tyrosine kinases in the human genome. The first family member discovered, c-SRC, (hereafter referred to as 'SRC') is the cellular homolog of the transforming oncogene in Rous Sarcoma Virus (RSV), v-Src. RSV was originally discovered in the early 1900s as a virus capable of inducing rapid tumor growth in chickens (306). However, it was not until the 1970s that v-Src was determined to be the cancer-driving protein expressed by the RSV genome (307). In addition, v-Src was the very first tyrosine kinase discovered; which opened the door to the discovery of many other transforming tyrosine kinase oncogenes and their normal cellular counterparts. Since the discovery of SRC, seven other Src-family kinase (SFK) members have been described in humans: HCK, LYN, FGR, LCK, BLK, YES, and FYN (308). Three of the family members, SRC, YES, and FYN, are expressed in almost all cell types while LYN, HCK and FGR expression is restricted to myeloid cells (309). LCK expression is restricted to T-cells, while BLK displays restricted expression in B-cells. LYN is also expressed in B-cells. Generally, the SFKs facilitate the integration of signals from numerous upstream sources (cytokines, growth factors, cell attachment, others) to signaling pathways downstream. The SFKs are critical for signaling pathways related to cell proliferation, motility, survival, and invasion. A detailed description of individual SFK function is beyond the scope of my dissertation. Additional background information given below is limited to the role of the myeloid SFKs, HCK, LYN, and FGR, normal hematopoiesis and in AML.

HCK, FGR, and LYN have key roles in a number of signaling pathways in myeloid cells, including lipopolysaccharide (LPS) responses (310), FcγR signaling (311, 312), as well as granulocyte-macrophage colony stimulating factor (GM-CSF) (313) and granulocyte colony stimulating factor (G-CSF) (314) signaling. *Hck*, *Fgr*, and *Lyn* triple knock-out mice have greatly

enhanced our understanding of the role of the SFKs in hematopoietic cells. *Hck*$^{-/-}$ and *Fgr*$^{-/-}$ single knockout mice displayed no significant differences from wild-type mice, potentially because of functional overlap with other SFKs (315). However, the *Hck*$^{-/-}$*Fgr*$^{-/-}$ double knockout mice showed significant defects in the innate immune response to the intracellular pathogen, *Listeria monocytogenes*, a significant source of food-borne illness (315). Additionally, while macrophages from these mice displayed no defects, LYN activity was upregulated suggesting compensation for the lack of HCK and FGR (315). Furthermore, integrin signaling in neutrophils was completely blocked in the double knockout mice (316). Finally, *Hck*$^{-/-}$*Fgr*$^{-/-}$ neutrophils have significantly reduced migration (317) and adhesion (318).

Lyn$^{-/-}$ mice present with reduced numbers of recirculating B-cells that is likely the result of reduced B-cell receptor signaling (319). Additionally, *Lyn*$^{-/-}$ mice have a reduced ability to eliminate autoreactive B-cells, resulting in widespread autoimmunity (319). Finally, mast cell function is abrogated in *Lyn*$^{-/-}$ mice due to the signaling function of LYN downstream of the FcεR1 (319). *Hck*$^{-/-}$*Fgr*$^{-/-}$*Lyn*$^{-/-}$ triple-knockout mice display significant defects in FcγR-mediated phagocytosis in macrophages (320, 321). Additionally, innate immune responses were blunted for a number of pathogens (322). Subsequent studies have suggested the innate immune response defects stem from an inability to generate and release pro-inflammatory mediators (323).

The first evidence for HCK, LYN, and FGR involvement in a hematologic malignancy was described for chronic myelogenous leukemia (CML). CML is driven by the reciprocal translocation of chromosome 22 and chromosome 8 that juxtaposes the breakpoint cluster region (*BCR*) and *ABL* tyrosine kinase genes. This translocation creates what is known as the Philadelphia chromosome, named for the city in which it was discovered (324). The

Philadelphia chromosome drives expression of a fusion protein, BCR-ABL, a constitutively active ABL kinase that drives CML pathogenesis. HCK and LYN were originally implicated in CML with the discovery that BCR-ABL binds and activates these kinases (325). Subsequent work revealed that the reciprocal is also true, that HCK and LYN phosphorylate BCR-ABL and as a result may increase its kinase activity and sensitivity to imatinib (326). Imatinib (Gleevec) is a relatively selective, orally active inhibitor of BCR-ABL that was FDA-approved in 2001 as the first-line therapy for CML patients. Additionally, a kinase-dead version of HCK has been shown to suppress the transforming activity of BCR-ABL in a myeloid cell line, suggesting a specific and necessary role for HCK in CML pathogenesis (327). Up-regulation of HCK (328, 329) and LYN (330, 331) expression has also been linked to imatinib resistance in CML in the absence of BCR-ABL mutations. Interestingly, the second tyrosine kinase inhibitor to receive FDA approval for CML therapy was dasatinib (Sprycel). This compound is a potent inhibitor of both ABL and SFKs, which may account for its improved efficacy and reduced resistance potential in CML (332).

Mounting evidence implicates HCK, LYN, and FGR in FLT3-ITD$^+$ AML as well. In 2005, LYN was discovered to be active downstream of FLT3-ITD and a SFK inhibitor, PP1, reduced the growth of FLT3-ITD$^+$ AML cell lines (333). However, subsequent work has established that PP1 is a broad-spectrum tyrosine kinase inhibitor that targets all of the SFKs as well as numerous other kinases (334). Thus PP1 is not useful as a probe compound to specifically implicate LYN kinase activity in FLT3-ITD$^+$ AML, because it also inhibits HCK and FGR among other targets. Subsequent studies discovered that LYN binds to a phosphorylated tyrosine site in FLT3-ITD via its SRC Homology 2 (SH2) domain and that small interfering RNA (siRNA) knockdown of LYN reduces STAT5 phosphorylation (335). Additionally, the

SFK inhibitor, PP2, reduced tumor growth in a xenograft model of FLT3-ITD$^+$ AML. Follow up studies demonstrated that LYN is active in primary AML patient samples and that PP2 inhibits this activation (336). This study also demonstrated that LYN silencing by siRNA inhibits mTOR signaling independent of AKT. Finally, this was the first study to demonstrate that both HCK and FGR are also constitutively active in primary AML patient samples.

Recently a breakthrough discovery occurred when HCK was identified as being differentially expressed in LSCs versus normal HSCs (337). As described earlier, LSCs are notoriously difficult to eliminate because they are chemotherapy-resistant and largely quiescent, making the identification of a targetable kinase an important step in AML therapy. Furthermore, Saito et. al. (338) demonstrated that shRNA knockdown of HCK reduced the growth of FLT3-ITD$^+$ AML cells. Additional work identified an HCK inhibitor, RK-20449 (previously reported in the literature as A-419259), that is efficacious against chemotherapy-resistant primary AML bone marrow samples (338). RK-20449 reduced the growth of patient-derived xenograft models of AML in *Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice. Finally, secondary transplantation experiments demonstrated that RK-20449 treated samples failed to engraft secondary recipients, suggesting the elimination of LSCs in these samples.

FGR is the least well studied of the myeloid-restricted SFKs with respect to its role in AML. Several studies, including my thesis, have identified a dichotomous expression pattern for FGR in AML; it is either expressed at very high levels or very low levels (336, 339). One study showed that knockdown of FGR expression reduces FLT3-ITD AML cell growth and colony formation in soft agar (339). I will note that previous studies used small molecule kinase inhibitors that do not discriminate among SFKs, which suggests that even though a role for FGR was not directly investigated, FGR cannot be ruled out as a contributor to the efficacy of those

compounds. This includes RK-20449, which we found to inhibit not only HCK but also LYN, FGR and FLT3-ITD itself. This issue is described in more detail in Chapter 2.

1.3.2 SYK Tyrosine Kinase

Originally, purified from bovine thymus and porcine spleen, spleen tyrosine kinase (SYK) is expressed at high levels in many hematopoietic cell types (340-342). Numerous studies demonstrated that SYK is responsible for signaling downstream of immune receptors. Integrin signaling is critical for proper adhesion and migration of a number of different cell types, including neutrophils, monocytes, platelets, and osteoblasts (343-347). In each of these cell types, integrin signaling is ablated or reduced in SYK-deficient cells (348). SYK is also critical for pathogen recognition downstream of pattern recognition receptors (349-351). Receptors responsible for the recognition of fungal, bacterial, and viral infection all depend on SYK signaling with one study demonstrating that bacterial infection host defenses are attenuated in *Syk*$^{-/-}$ animals (352). FcR signaling in mast cells and neutrophils is also attenuated with SYK deficiency (353-355), while antibody-dependent cell-mediated cytotoxicity (ADCC) is reduced in natural killer cells lacking SYK (356). Finally, SYK is indispensable for B-cell maturation and B-cell receptor signaling. *Syk*-deficient animals have a complete absence of mature B-cells.

Several studies discovered that the Epidermal Growth Factor Receptor (EGFR) inhibitor, gefitinib, is capable of inducing differentiation in AML cells through a non-EGFR dependent mechanism, as AML cells do not express EGFR (357-359). In 2009, through integration of chemical, proteomic, and genomic screening approaches, SYK was identified as the target of gefitinib in AML cells (360). Further investigation revealed that small hairpin RNA (shRNA) knockdown and small molecule inhibition of SYK can induce differentiation in a diverse set of

AML cell lines. Finally, SYK inhibition was efficacious *in vivo* and against primary AML patient samples. Subsequent studies from the same laboratory revealed that SYK is most active in FLT3-ITD^{+} AML samples and is able to directly bind to FLT3-ITD (361). Furthermore, activation of c-MYC is dependent on SYK activity and overexpression of SYK is capable of inducing resistance to targeted small molecule FLT3-ITD kinase inhibitors. This finding suggests that inhibitors with dual activity against FLT3 and SYK may be less prone to acquired resistance, in a manner analogous to dasatinib in CML. I will return to this theme in detail in the next chapter.

1.3.3 FES Tyrosine Kinase

c-FES (hereafter referred to as 'FES') was originally identified as the cellular homolog of the transforming oncogenes of Fujinami avian sarcoma virus (v-Fps), Gardner-Arnstein feline sarcoma virus (v-Fes), and Snyder-Theilen feline sarcoma virus (v-Fes) (362-366). In 1985 the complete sequence of the human *FES* gene was reported and it was discovered that FES belongs to a unique family of non-receptor protein tyrosine kinases (367). FES is highly expressed in hematopoietic cells (368), particularly those of the myeloid lineage, but it is also expressed at lower levels in endothelial cells, epithelial cells of the breast and gut as well as some neuronal cells (369). FES was originally discovered in terminally differentiated myeloid cells suggesting a role in myeloid differentiation. In support of this hypothesis, ectopic expression of FES in a non-terminally differentiated myeloid cell line, K562, caused the cells to undergo terminal differentiation (370). Furthermore, two studies discovered activation of the essential differentiation transcription factors, CEBPA and PU.1, was driven through FES (371, 372). One study also discovered that FES was essential for the survival of terminally differentiated cells

(373). However, a *Fes*$^{-/-}$ mouse model displayed no defects in myeloid cell differentiation or survival (374, 375). Similar to the SFK knockout mouse models, it is possible that other kinases, especially the closely related FER kinase, compensated for the loss of FES. One interesting phenotype from the *Fes*$^{-/-}$ mouse model was its increased sensitivity to lipopolysaccharide (LPS) (376). FES is abundantly expressed in innate immune cells, particularly neutrophils and macrophages, so Fes potentially regulates inflammation. Subsequent studies revealed an essential role for Fes in macrophage chemotaxis (377). Additionally, FES is implicated in a number of signaling pathways downstream of growth factor and cytokine receptor tyrosine kinases, including GM-CSF, IL-3, IL-4, and IL-6 (378). A possible role for FES in outside of myeloid cells was discovered when an activated form of FES bearing the SRC N-terminal myristoylation signal sequence was introduced into transgenic mice under the control of the natural promoter. These mice developed vascular hyperplasia and multifocal hemangiomas suggesting a role for FES in angiogenesis (379). The role for FES endothelial cell migration and vascular tube formation has been confirmed in several subsequent studies (380).

FES was originally described as a proto-oncogene because of its homology to the cancer-promoting viral oncogenes described above. However, FES mutations discovered in cancer cells to date have been inactivating mutations suggesting a tumor suppressor role for FES. These dichotomous roles are likely caused by tissue specific functions of FES. For example, FES-inactivating mutations, as well as promoter methylation that silences FES expression, were discovered in human colon cancer cell lines and patient samples (381, 382) while pro-tumorigenic functions have been described in leukemia and some brain tumors (383). While FES has potential roles in many cancers, I will focus on the role of FES downstream of FLT3-ITD in AML. FES was originally identified as activated downstream of FLT3-ITD in two FLT3-

ITD positive cell lines, MV4-11 and MOLM-14 (384). Knockdown of FLT3 by siRNA eliminated FES phosphorylation indicating that activation was FLT3-ITD-dependent. Furthermore, expressing exogenous FLT3-ITD in a myeloid cell line that does not normally express FLT3, TF-1, activated endogenous FES. However, FES is active in a *FLT3*-WT cell line, THP-1, and knockdown of FLT3 has no effect on its activity. This suggests that other mechanisms are capable of activating FES. Knockdown of FES by siRNA reduced the growth of both MV4-11 cells and MOLM-14 cells and resulted in impaired cell cycle progression. Additionally, knockdown of FES inhibited signaling downstream of FLT3-ITD. In particular, it reduced STAT5 activation, blocked activation of many PI3K pathway members, and reduced expression of the antiapoptotic protein BCL-xL. Finally, FES is activated in primary AML patient bone marrow samples. These studies suggest that targeted inhibition of FES kinase activity may be of therapeutic benefit in AML.

1.4 TARGETING FLT3 MUTATIONS IN AML

Given the evidence that *FLT3* mutations drive approximately 30% of AML cases, it is not surprising that the development of small molecule inhibitors of FLT3 is of considerable interest for AML therapy. However, unlike imatinib, which revolutionized the treatment of CML, FLT3 inhibitors have had limited clinical success. In this section I will detail the first- and second-generation FLT3 inhibitors tested in clinical trials and explore the reasons for their limited success in the clinic.

1.4.1 First-Generation FLT3 inhibitors

The first-generation FLT3 inhibitors were a group of small molecule receptor tyrosine kinase inhibitors not necessarily developed to inhibit FLT3 specifically. In fact, all of the inhibitors hit a broad range of receptor tyrosine kinases and have also been tested in clinical trials for other solid and liquid tumors.

Sunitinib, a multi-targeted inhibitor of FLT3 and the homologous receptor tyrosine kinases c-KIT, colony stimulating factor 1 (CSF) receptor, PDGFR, GDNF receptor (RET), and vascular endothelial growth factor receptor (VEGFR), is approved for use in metastatic renal cell carcinoma and gastrointestinal stromal tumors (385). Sunitinib inhibits FLT3 in patients with kinase inhibition directly proportional to plasma drug levels (386). In phase I clinical trials, as a single agent, sunitinib induced partial responses in AML patients that lasted between 4-16 weeks (387). Most patients experienced peripheral blast count decreases but no reduction in bone marrow blasts. Single agent phase II clinical trials were never pursued due to the short duration of response and the significant toxicities. However, a phase I/II combination trial with standard therapy (cytarabine plus high dose anthracycline) produced a complete response rate of 53% in FLT3-ITD$^+$ patients (388). Interestingly, the complete response rate for FLT3-TKD$^+$ patients in this study was only marginally higher at 73%, suggesting a potential role for this inhibitor outside of FLT3-ITD$^+$ AML. Several patients were transitioned onto single agent maintenance therapy, with a median response duration of 11 months.

Lestaurtinib (CEP-701), a staurosporine derivative known to target JAK2, VEGFR, and tropomyosin receptor kinase (TRKA) as well as FLT3 has been tested in a number of clinical trials (385). A phase II trial of AML patients refractory to standard treatment resulted in a clinical response (partial or complete response) for 36% of the patients (389). However, as with

sunitinib, the responses were short lived, lasting anywhere from 2 weeks to 3 months. In another phase II clinical trial, older patients deemed not fit for standard therapy received lestaurtinib (390). While some patients exhibited transient peripheral and bone marrow blast reduction, there was no difference between FLT3-ITD and FLT3-WT response rates, suggesting that off-target effects were at least partially responsible for the efficacy. Finally, in a phase III combination clinical trial patients in first relapse received lestaurtinib after chemotherapy (391). No difference in response rates or overall survival was reported. However, a follow up study indicated that as opposed to the other clinical trials, most patients in this study did not achieve FLT3 inhibition (392).

Tandutinib (MLN-518), a targeted inhibitor of FLT3, c-KIT, and PDGFR, was tested in both phase I and phase II clinical trials (385). In both studies, tandutinib had dismal efficacy and produced no complete or partial responses (393). In a combination trial the response rate increased, however a limited number of patients had FLT3 mutations and response was likely due to chemotherapy (394). No further clinical trials were ever pursued with tandutinib.

Midostaurin (PKC-412), another staurosporine derivative, targets FLT3, c-KIT, CSFR1, VEGFR, protein kinase C alpha (PKC-α) and PDGFR (385) and has been tested in phase I, II, and III clinical trials. In a phase II clinical trial, as a single agent, midostaurin reduced peripheral and bone marrow blasts in 71% of FLT3-ITD^{+} patients (395). Median response duration from that clinical trial was 13 weeks. A small combination phase Ib clinical trial in young, newly diagnosed AML patients produced a 92% complete response rate in *FLT3*-mutated AML patients (396). The combination of midostaurin with standard chemotherapy increased disease-free survival and overall survival to a level similar to *FLT3*-WT patients. The results of a combination phase III clinical trial that accrued over 700 patients were reported in 2015 (397).

The addition of midostaurin to induction and consolidation therapy plus use of midostaurin as maintenance therapy increased the median overall survival from 26 months to 75 months. Event-free survival was also increased from a median of 3 months to a median of 8 months. This is the first clinical trial to demonstrate a significant improvement in therapy with the addition of a FLT3 inhibitor.

Sorafenib, a FLT3, c-KIT, RAF, PDGFR, and VEGFR inhibitor currently approved for use in renal cell carcinoma and hepatocellular carcinoma (385), is capable of eliminating peripheral blasts and reducing bone marrow blasts in FLT3-ITD$^+$ patients, with a median response time of 72 days (398). Sorafenib exhibits no efficacy in AML patients with *FLT3*-TKD mutations, however, because these mutants are resistant to the inhibitor. A combination clinical trial of chemotherapy plus sorafenib had a complete response rate of 93% in FLT3-ITD$^+$ patients (399). However, at the follow up time of nine months, half of the patients had relapsed. Another combination clinical trial in elderly patients demonstrated no benefit with the addition of sorafenib (400). However, only a small proportion of the patients were FLT3-ITD$^+$. Several clinical trials demonstrated longer remission and reduced resistance with the use of sorafenib after allogenic HSCT (401-403). This suggests that sorafenib may synergize with the allogeneic hematopoietic stem cell transplant, through an unknown mechanism.

1.4.2 Second-generation FLT3 inhibitors

Two important lessons were learned during clinical trials with the first-generation FLT3 inhibitors. The first is that targeting FLT3 proved more efficacious in *FLT3* mutated patients over *FLT3*-WT patients. Additionally, FLT3 inhibitors proved more effective in patients carrying a high mutant allelic ratio of FLT3. The second is that clinical response closely mirrored

sustained FLT3 inhibition in vivo (404). Whereas the first-generation FLT3 inhibitors were designed to inhibit multiple receptor tyrosine kinases, the second-generation FLT3 inhibitors were purposefully designed for potency and specificity in targeting FLT3. Additionally, some of the inhibitors were purposefully designed to be efficacious against FLT3-TKD mutants.

Quizartinib (AC220) was the first inhibitor purposefully designed to inhibit FLT3, although it also inhibits c-KIT and PDGFR (385). In phase I/II clinical trials in elderly patients with relapsed or refractory disease, quizartinib had a complete response rate of 53% in FLT3-ITD^+ patients (405). To date, this is the greatest single agent efficacy of a FLT3 inhibitor. A similar result was achieved in a phase II clinical trial exploring the efficacy of quizartinib in young relapsed or refractory patients (406). Numerous clinical trials are currently assessing quizartinib as a potential agent for combination with standard chemotherapy or as a single agent in non-relapsed patients. One major side effect of quizartinib is QTc prolongation. This is currently the dose limiting toxicity of this compound.

Crenolanib is in clinical trials for a number of solid tumors due its efficacy against PDGFR. However, recently crenolanib proved to be a potent inhibitor of FLT3. More importantly, crenolanib is capable of inhibiting several FLT3-TKD mutations that are resistant to quizartinib (407). Furthermore, crenolanib exhibits no QTc prolongation and demonstrates selectivity for FLT3 over c-KIT. c-KIT inhibition is thought to be the cause of myelosuppression, a common side effect of treatment with FLT3 inhibitors. Crenolanib could potentially reduce myelosuppression after treatment, leading to faster recovery times for patients. A phase I clinical trial with crenolanib as monotherapy in relapsed or refractory AML demonstrated an overall response rate of 50% (408). Currently, crenolanib is under investigation in two phase II clinical trials.

PLX3397 is a FLT3 inhibitor that exhibits selectivity for FLT3-ITD over FLT3-WT. Additional targets include c-KIT and CSFR1 (409). One advantage of PLX3397 is its activity against a common quizartinib-resistant mutant, FLT3-F691L. However, patients with FLT3-D835 mutations are insensitive to PLX3397. Currently, PLX3397 is in a phase I/II clinical trial and no results have been reported yet.

TTT-3002 is a staurosporine derivative developed in the laboratory of Donald Small. This compound potently targets FLT3 and exhibits picomolar potency against some FLT3-ITD cell lines (410). Additionally, this compound also exhibited potency against primary AML patient samples and *in vivo* models of AML. Finally, TTT-3002 is efficacious against most known FLT3-TKD mutations, including the common quizartinib inhibitor resistant mutations (411). No clinical trials have been started yet for this compound.

Two drugs normally used in the treatment of CML, dasatinib and ponatinib, are currently in clinical trials for use in AML. Ponatinib is efficacious against FLT3-ITD and FLT3-TKD mutations and is being explored against inhibitor resistant mutations of quizartinib (412). Dasatinib does not inhibit FLT3 and likely works through the inhibition of a number of non-receptor tyrosine kinases (413). Both compounds exhibit efficacy against AML cell lines, primary patient samples, and *in vivo* models of AML.

1.4.3 Resistance mechanisms to targeted FLT3 inhibitors

Clinical FLT3-targeted inhibitors are beginning to show efficacy as single agents and in combination therapies. However, response durations are transient, likely because of acquired resistance. Numerous resistance mechanisms have been described and will be detailed below.

1.4.3.1 FLT3-dependent resistance mechanisms The most common resistance mechanism observed with FLT3 inhibitor treatment is selection for inhibitor resistance mutations in *FLT3*. These have been observed for almost all inhibitors and the different chemical classes of inhibitors have unique mutation patterns. For example, resistance to quizartinib, which often overlaps with sorafenib resistance, occurs most commonly through F691L (also F691I), D835Y (also D835V and D835F) and Y842C (Figure 3) (414, 415). However, these FLT3-ITD-TKD mutants are still sensitive to the staurosporine derivatives (416). Fewer studies have identified resistance mutations to the staurosporine derivatives. However, mutations at N676 (most commonly N676D) and G697 (most commonly G697R) confer resistance to midostaurin (Figure 3) (417). It should be noted that newly diagnosed patients with *FLT3*-ITD-TKD mutations have significantly worse outcomes than patients with *FLT3*-ITD alone (418). One potential mechanism for the worse prognosis is the upregulation of anti-apoptotic proteins, such as BCL-xL. Not only would these patients be refractory to FLT3 tyrosine kinase inhibitors, but they are also less sensitive to cytotoxic chemotherapy. This suggests that the development of resistance mutations in the presence of a *FLT3*-ITD mutation may decrease these patients' overall survival.

Another FLT3-dependent mechanism of resistance is the upregulation of FLT3 ligand (FL). As described earlier, most AML blast cells express both FLT3 and FL, suggesting an autocrine loop. While FLT3-ITD does not need FL for activation, it remains highly sensitive to stimulation by FL which leads to proliferation of leukemic blasts (419). In this way FL can interfere with inhibition of FLT3. One *in vitro* study demonstrated reduced potency of FLT3 inhibitors against the FLT3-ITD positive cell line, MOLM-14, in the presence of FL (420). Upregulation of FL is commonly observed after treatment with FLT3 inhibitors. Furthermore, it

is well established that standard chemotherapy induces large upregulation of FL expression (391, 420-422), potentially explaining why FLT3 inhibitors are more efficacious in newly diagnosed patients than relapsed patients. This upregulation of FL is also an issue when trying to determine the best timing for combination therapy.

One final FLT3-dependent mechanism of resistance is the upregulation of FLT3 itself, which could possibly overwhelm the inhibitor at physiological doses (423). One study discovered upregulation of FLT3 surface expression in 13 of 14 patients treated with FLT3 inhibitors. It was noted that no gene duplications were observed.

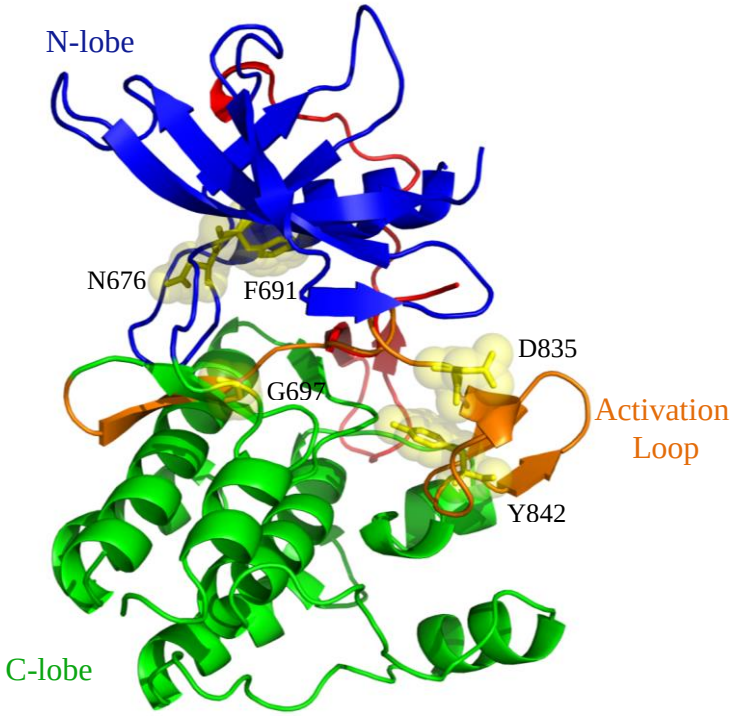


Figure 3: X-Ray crystal structure of FLT3 inhibitor resistant mutations.

The x-ray crystal structure of FLT3 displaying the location of the most common FLT3 inhibitor resistant mutations (PDB ID: 1RJB). Quizartinib and Sorafenib mutations most commonly occur on the activation loop (D835 and Y842) and at the 'gatekeeper' position (F691) while midostaurin resistance occurs in two residues at the back of the ATP binding pocket (N676 and G697).

1.4.3.2 FLT3-independent resistance mechanisms AML blast cells reside in bone marrow niches that are critical for the survival of the blast cells. The bone marrow environment is potentially a physical barrier to some inhibitors, simply not allowing the FLT3 inhibitors to come in contact with the bone marrow AML blasts. Additionally, the niche provides a number of survival signals through physical contact and the release of cytokines (424, 425). Co-culture of FLT3-ITD^{+} cells in the presence of bone marrow stromal cells demonstrated that FLT3 inhibitors fail to inhibit STAT5 signaling in the presence of stroma, even if FLT3 is inhibited. One potential explanation for this discrepancy is that stromal cells can induce the activation of ERK and AKT pathways in the presence of FLT3 tyrosine kinase inhibitors (424).

Similarly, the upregulation of compensatory pathways is observed after prolonged treatment with FLT3 inhibitors. In separate studies, the PI3K/AKT (426), STAT (427), and MEK/ERK (428) pathways have been identified as upregulated and responsible for resistance to FLT3 inhibitors. Furthermore, this upregulation often occurs in the presence of FLT3 inhibition, suggesting selection for alternative driver mutations. These mechanisms are likely connected to the effect of stromal cells mentioned earlier. One study described resistance to FLT3 inhibitors through the development of other driver mutations such as N-RAS-activating mutations (428). Other compensatory mechanisms have been described, as some cell lines express high levels of anti-apoptotic proteins as a resistance mechanism to FLT3 inhibitors (429-431). The driver for this upregulation is unknown.

Leukemic stem cells contain a significant FLT3-independent mechanism of resistance, not only to FLT3 inhibitors but also to chemotherapy. *FLT3* mutations have been discovered in the LSC population, suggesting that mutant-specific FLT3 inhibitors could eliminate LSCs

(432). However, clinical trials have not demonstrated elimination of LSCs in FLT3 inhibitor-treated patients (433). One potential reason is the upregulation of numerous survival pathways in LSCs, which are not dependent on FLT3 oncogenic signaling (433, 434). The elimination of LSCs is one of the most challenging issues currently facing AML therapy.

1.4.3.3 Strategies to overcome resistance to FLT3 targeted inhibitors Several strategies to overcoming resistance to FLT3 inhibitors have been developed or are currently under investigation. The most important FLT3 resistance mechanism to overcome is inhibitor resistance mutations. As mentioned previously, novel FLT3 inhibitors that target these resistance mutations are under development, including crenolanib, PLX3397, ponatinib, and TTT-3002. Another route being explored for the prevention of inhibitor resistance mutations is the development of dual FLT3 inhibitors. For example, a dual FLT3/CDK4 inhibitor (435) and a dual FLT3/AXL inhibitor (436) are currently in early stage clinical trials. Both these inhibitors reduce the rate of resistance when compared to selective FLT3 inhibitors alone. Also under development is a dual FLT3/SYK inhibitor (437). The potential of using these compounds once resistance has arisen or in combination to reduce the rate of resistance holds enormous promise for reducing FLT3 inhibitor resistance mutations.

Combination therapy of a number of different targeted therapies is being explored to find the best approach for patients. As mentioned above, upregulation of compensatory pathways is a common mechanism of resistance to FLT3 inhibitors. However, when FLT3 inhibitors are combined with inhibitors that target the compensatory pathways, the AML blasts become sensitive to treatment again. This has been described for the combination of FLT3 inhibitors with MEK inhibitors (438), PI3K inhibitors (428), JAK inhibitors (439), mTOR inhibitors (440), AKT inhibitors (426), and BH3 mimetics (441). FL upregulation is a problem for the

combination of FLT3 inhibitors with chemotherapy as well as monotherapy with FLT3 inhibitors. Use of FLT3 inhibitors in the presence of neutralizing FL monoclonal antibodies has been suggested as one approach to alleviate this resistance mechanism (442).

The stroma of the bone marrow niche provides numerous survival signals for AML cells. Several studies are exploring options for targeting the stroma in combination with FLT3 targeted inhibitors. The most advanced studies are using CXCR4 inhibitors to inhibit pro-survival signaling through the CXCR4/CXCL12 axis as well as to mobilize AML blasts out of the bone marrow (443). Combination of a CXCR4 inhibitor with sorafenib was more effective at killing AML cells in the bone marrow than sorafenib alone. Importantly, the CXCR4 inhibitor reduced the stromal activation of the PI3K and MEK/ERK pathways. Other stromal targeting agents are in their infancy but studies are exploring the targeting of adhesion molecules (444), hypoxia (445) and osteoblasts (446), which are dysregulated in the AML bone marrow niche.

LSCs are ultimately responsible for the relapse of patients and monotherapy with FLT3 inhibitors has failed to eliminate these cells. However, a recent study discovered that combination of FLT3 inhibitors with all-trans retinoic acid (ATRA) synergistically induced apoptosis in LSCs (447). Bone marrow transplants from combination-treated mice failed to engraft into secondary recipients, suggesting the elimination of LSCs. As described earlier, small molecule inhibition of HCK with the compound A-419259, also eliminated LSCs in a patient-derived AML xenograft model (338). However, at the doses used in that study, A-419259 was also likely to inhibit FLT3 and other Src-family members, suggesting that dual targeting of FLT3 and myeloid SFKs may work synergistically to eliminate LSCs.

1.5 HYPOTHESIS AND SPECIFIC AIMS

1.5.1 Hypothesis

AML is a hematologic malignancy caused by the expansion of immature AML blasts. Treatment outcomes, particularly in the elderly, have improved only minimally in the last 40 years and overall survival is approximately 25%. *FLT3*-ITD mutations occur in approximately 25% of AML patients and have a particularly poor prognosis. Furthermore, *FLT3*-ITD mutations are more common in elderly patients who are ineligible for high-dose chemotherapy because of the significant toxicities associated with the treatment. Novel, less toxic therapies are clearly needed for the treatment of FLT3-ITD$^+$ patients.

Numerous FLT3 inhibitors have been tested in clinical trials or are still under investigation for the treatment of FLT3-ITD$^+$ AML. However, use of FLT3 inhibitors as single agents has produced underwhelming results with only transient clinical responses being observed. The most common mechanism of resistance involves mutations in the kinase domain of FLT3 that prevent drug action. Additionally, compensatory pathway activation either spontaneously or with the assistance of the stromal microenvironment occurs in FLT3 inhibitor-treated patients.

FLT3-ITD is known to activate a number of downstream signaling pathways that contribute to its oncogenesis. The major pathways are the PI3K/AKT pathway and the RAS/MEK/ERK pathway. However, a number of recent studies identified a set of tyrosine kinases that are activated downstream of FLT3-ITD and individually are essential to FLT3-ITD oncogenesis. These kinases are the myeloid-restricted SFKs (HCK/FGR/LYN), SYK, and FES. These tyrosine kinases are essential for the activation of a number of the major pathways

downstream of FLT3-ITD, including the PI3K and RAS/MEK/ERK pathway. Additionally, among the SFKs, HCK has been specifically implicated in LSC survival, making it a particularly attractive target for the eradication of AML.

All of the published data presented here suggests that FLT3-ITD is dependent on a relatively small set of kinases for downstream signaling. Targeting FLT3 plus this set of kinases could improve the potency of a compound against FLT3-ITD$^+$ cells. Additionally, recent reports have demonstrated that dual inhibitors can reduce the rate of resistance, so an inhibitor targeting the complete set of AML-associated tyrosine kinases could potentially provide that advantage as well. Finally, by targeting a set of kinases, multiple pathways are being inhibited at one time, possibly reducing the chance of compensatory pathway activation as a mechanism of resistance. I propose, therefore, that the development of a 'tuned' kinase inhibitor with a selectivity profile favoring FLT3 and its proximal tyrosine kinase signaling partners HCK, FGR, LYN, SYK, and FES would increase the potency against FLT3-ITD$^+$ AML cells and significantly decrease the rate of resistance.

1.5.2 Specific Aims

1.5.2.1 Aim 1: Determine the efficacy of the AML-associated tyrosine kinase inhibitor TL02-59 in both in vitro and in vivo models of AML. FLT3-ITD signaling requires a defined subset of downstream tyrosine kinases to propagate its oncogenic signals. We set out to identify a compound that would inhibit FLT3-ITD, and three of the best characterized AML-associated kinases (HCK, SYK, and FES) to determine if such a compound could be identified and if so whether it displayed significant efficacy against AML cell lines and patient-derived AML bone marrow cells. Using an in vitro kinase assay and recombinant

FLT3-ITD, HCK, FES and SYK, we screened a small collection of N-phenylbenzamide tyrosine kinase inhibitors and identified a compound ('TL02-59') that exhibited three-digit nanomolar potency against all four of these recombinant AML-related kinases. Remarkably, the efficacy against the FLT3-ITD$^+$ cell line, MV4-11, was in the picomolar range. All four kinases are expressed and active in MV4-11 cells, and all were inhibited by TL02-59 treatment, suggesting that the multi-targeted action of the inhibitor is responsible for its potent effect on cell growth. To further explore the efficacy of TL02-59, we obtained 26 primary AML bone marrow samples (half FLT3-ITD$^+$ and half FLT3-WT). TL02-59 displayed a wide range of potencies against the primary samples ranging from 77 nM to over 3 μM. Interestingly, the top four most sensitive samples were FLT3-WT, but expressed very high levels of HCK, FGR and SYK, supporting a role for these kinases as inhibitor targets. Finally, we explored the efficacy of TL02-59 in a mouse xenograft model of AML. MV4-11 cells were engrafted into immunocompromised mice and TL02-59 was give once daily by oral gavage for three weeks. TL02-59 (10 mg/kg) completely eliminated MV4-11 cells from the peripheral blood and spleen, and reduced bone marrow engraftment by 60%. These results were significantly better than those obtained with sorafenib treatment which had no effect on bone marrow or spleen engraftment at the same dose.

1.5.2.2 Aim 2: Investigate the full range of TL02-59 target kinases critical for AML efficacy

The difference in TL02-59 potency against recombinant target kinases in vitro (3-digit nM) and suppression of MV4-11 cell growth (3-digit pM) led us to hypothesize that other targets of TL02-59 might be crucial for its efficacy. To determine the full range of TL02-59 kinase targets important for its anti-AML efficacy, we performed a KINOMEscan assay. We discovered that TL02-59 is remarkably selective, targeting only 24 kinases out of the 456 kinases tested.

which of these 24 kinases were critical in AML, we performed transcriptional analysis on RNA obtained from our 26 primary AML bone marrow specimens for each of the 24 TL02-59 target kinases. This approach identified 15 target kinases that were consistently expressed. The relative expression of each kinase across the 26 AML samples was then correlated with the growth inhibitory IC_{50} value. Remarkably, myeloid SFK expression (FGR, HCK, and LYN) correlated with inhibitor response, as did SYK expression. The fact that all three SFKs correlated with response led us to wonder if inhibition of the SFKs alone could explain TL02-59 efficacy. To explore this possibility, we tested the SFK inhibitor A-419259 against MV4-11 cells and obtained an IC_{50} value about 30-fold higher than TL02-59. This suggested TL02-59 had other important targets that A-419259 did not inhibit. To identify these kinases, we then performed a KINOMEscan assay on A-419259 and explored kinases that TL02-59 inhibited but A-419259 did not. We identified several kinases including SYK as well as the Ser/Thr kinases p38α, TAOK3, and STK10. SYK and p38α have been linked to AML previously but TAOK3 and STK10 have never been explored in this context. To determine if MV4-11 cell growth was dependent on TAOK3 or STK10 expression we performed shRNA knockdown of these two kinases. Knockdown of STK10 exhibited no effect on MV4-11 cell growth. However, knockdown of TAOK3 completely abolished MV4-11 cell growth suggesting that this kinase is important for TL02-59 efficacy. In summary, our studies show that the multi-targeted kinase inhibitor TL02-59 has great promise against AML, especially the subset of cases that express active FLT3 and the AML-associated tyrosine kinases SYK, HCK, FGR, LYN, and possibly TAOK3.

2.0 POTENT INHIBITION OF FLT3-ITD$^+$ AML CELL GROWTH IN VITRO AND IN VIVO BY A SINGLE N-PHENYLBENZAMIDE INHIBITOR TARGETING FLT3, FES, SYK AND MYELOID SRC-FAMILY KINASES

2.1 INTRODUCTION

Acute myelogenous leukemia (AML), the most common form of leukemia in adults, is a hematologic malignancy characterized by the growth of immature myeloid progenitor cells that overpopulate the bone marrow and spill over into the peripheral blood (232, 448). First-line treatment for AML is typically cytotoxic chemotherapy that has remained invariant over the last forty years, despite significant advancements in our molecular understanding of AML (449, 450). As a result, overall survival for most AML patients has remained stagnant, especially for patients over 60 years of age who often present with comorbidities that limit the use of intensive chemotherapy needed to cure the disease (231, 451).

Fms-like tyrosine kinase receptor 3 (*FLT3*) is the most commonly mutated gene in AML, with approximately thirty percent of patients presenting with activating mutations at diagnosis (452, 453). Two types of *FLT3* mutations commonly occur in AML: internal tandem duplications (ITDs) and tyrosine kinase domain point mutations. ITDs are in-frame duplications within the juxtamembrane region of the receptor that interfere with kinase downregulation and occur in approximately 20-25% of patients (454). Point mutations are observed in approximately 5% of patients and often localize to the kinase domain activation loop, inducing an active

conformation in the absence of FLT3 ligand (415). Since constitutively active FLT3 is a common driver of AML oncogenesis, many small molecule FLT3 kinase inhibitors have been developed for clinical use. First-generation FLT3 inhibitors, such as midostaurin, lestaurtinib, and sorafenib, have had somewhat limited efficacy due in part to lack of sustained FLT3 inhibition in vivo and off-target associated toxicity (404). Second-generation FLT3 inhibitors, such as quizartinib and crenolanib, show significantly improved specificity for the FLT3 kinase domain and efficacy as single agents. However, inhibitor resistance, largely through acquired mutations in the kinase domain, has limited the clinical utility of second-generation compounds (385, 421).

A growing number of studies have linked FLT3 signaling in AML to downstream non-receptor tyrosine kinases, including the spleen tyrosine kinase (SYK), the cellular homolog of the feline sarcoma oncogene (FES), as well as several members of the Src kinase family expressed in myeloid cells (HCK, FGR, and LYN). SYK interacts directly with FLT3-ITD and is essential for c-MYC induction downstream (360, 361). Genetic knockdown and pharmacologic inhibition of SYK have both shown efficacy against AML cells both *in vitro* and in a mouse model. The Src-family kinase HCK was found to be highly overexpressed in gene expression profiles of myeloid leukemia stem cells (LSCs) versus normal hematopoietic stem cells (337), suggesting that inhibition of HCK may help eliminate LSC growth. Subsequent work showed that shRNA-knockdown of HCK expression blocked AML cell growth in vitro and that a potent ATP-site inhibitor of HCK (RK-20449; previously reported by us as compound A-419259) (455, 456) dramatically reduced the growth of primary AML cells in engrafted, immunocompromised NOD scid gamma (‘NSG’) mice (338). LYN, another myeloid Src-family member, has been identified as active in a majority of clinical AML isolates and has been linked

to the activation of STAT5 by FLT3-ITD (333, 335, 336). The Src-family kinase FGR is also highly expressed in a subset of FLT3-ITD$^+$ AML samples and selective siRNA knockdown of FGR significantly reduced the growth of primary FLT3-ITD$^+$ AML cells (339). FGR (as well as HCK and LYN) has also been shown to have high kinase activity in patient-derived LSCs (339). Finally, the FES tyrosine kinase, which is also strongly expressed in normal cells of myeloid lineage (457), was reported to be constitutively active in both FLT3-ITD$^+$ AML cell lines and patient samples (384). Selective knockdown of FES using siRNA reduced the growth of FLT3-ITD$^+$ AML cell lines to a similar extent as knockdown of the FLT3-ITD driver oncogene itself (384). Taken together, these studies strongly suggest that active FLT3 cooperates with multiple non-receptor tyrosine kinases in the pathogenesis of AML, and that simultaneous inhibition of FLT3 and these AML-associated kinases may confer additional clinical benefit in patients where these kinases are expressed and active.

Here we report the identification of the N-phenylbenzamide tyrosine kinase inhibitor TL02-59, which inhibits FLT3 as well as each of the AML-associated kinases described above (SYK, FES, HCK, and other Src-family kinases). Remarkably, TL02-59 inhibited the growth of the FLT3-ITD$^+$ cell line MV4-11 with picomolar potency. Each of these AML-associated kinases is expressed and active in this cell line, suggesting that the combined action of TL02-59 against all of these kinases is responsible for its potent anti-proliferative action. We also explored the efficacy of TL02-59 against a panel of 26 patient-derived AML bone marrow samples, and observed a wide range of inhibitory responses spanning nearly two orders of magnitude. KINOMEscan analysis of TL02-59 target specificity followed by kinase gene expression profiling revealed that expression of the Src-family kinases HCK, FGR, and LYN, as well as SYK, were most strongly correlated with the TL02-59 response in primary AML bone

marrow. Several other kinases identified as TL02-59 inhibitor targets by KINOMEscan analysis were also strongly expressed in primary AML cells. One of these kinases, the Ser/Thr kinase TAOK3, was confirmed as a TL02-59 inhibitor target in vitro; shRNA knockdown of TAOK3 resulted in growth suppression of MV4-11 AML cells. Comparative KINOMEscan profiling of TL02-59 vs. A-419259, the previously characterized HCK inhibitor that eliminated AML stem cells in vivo, showed a narrower selectivity profile for A-419259 that included FLT3 and Src-family kinases but not SYK, FES, or TAOK3. Interestingly, TL02-59 was 30-fold more potent than A-419259 against MV4-11 cell proliferation in vitro, strongly suggesting that inhibition of these additional kinases contributes to TL02-59 potency. Finally, TL02-59 efficacy was tested in vivo using immunocompromised mice engrafted with MV4-11 cells. Oral administration of TL02-59 at 10 mg/kg for three weeks completely eliminated MV4-11 cells from the spleen and peripheral blood, while dramatically suppressing bone marrow involvement. Altogether, our results suggest that compounds like TL02-59, capable of simultaneous inhibition of FLT3 and the spectrum of AML-associated kinases defined here, may represent a new generation of targeted therapies for a subset of AML patients. TL02-59 is particularly attractive in this regard, because its narrow specificity profile is very closely tuned to the kinase activities associated with AML, with very few non-AML kinase targets that may hinder clinical development.

2.2 RESULTS

2.2.1 Identification of a small molecule inhibitor of FLT3 and the AML-associated tyrosine kinases SYK, FES and the Src-family kinase HCK

Recent studies have shown that active mutants of FLT3 cooperate with multiple cytoplasmic tyrosine kinases to drive AML pathogenesis, including SYK, FES, and the Src-family kinases HCK, LYN and FGR (see Introduction). Genetic knockdown of each kinase results in growth arrest and apoptosis of FLT3-ITD$^+$ AML cells, suggesting that an inhibitor targeting FLT3 and these AML-associated kinases may potently suppress AML cell growth. To test this idea, we evaluated a collection of ten N-phenylbenzamide kinase inhibitors for activity against recombinant FLT3-ITD, FES, HCK and SYK kinases in vitro. The structures of these compounds are presented in Figure 4A and Table 4. We also determined their growth inhibitory potency towards the FLT3-ITD$^+$ AML cell line, MV4-11, which has been previously shown to express active FES, SYK, HCK and other myeloid Src-family members (337, 338, 360, 361, 384, 458). Three compounds (TL02-59, TL8-133, and TL8-187) inhibited FLT3-ITD, FES, HCK and SYK with three-digit nanomolar potency in vitro (Table 4). Remarkably, each of these compounds also blocked MV4-11 AML cell growth with an IC_{50} value of less than 1 nM, suggesting that simultaneous inhibition of FLT3-ITD and these associated kinases is responsible for their potency. In contrast, compounds that showed lower potency against FLT3-ITD or any of the AML-associated kinases in vitro were also weaker inhibitors of MV4-11 cell growth. For example, compound TL8-143 displayed similar potency against FLT3-ITD and HCK as the top three inhibitors, but about 10-fold lower potency against FES and SYK in vitro. This difference translated into significantly reduced cellular potency against MV4-11 cells, with an IC_{50} value of

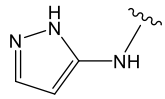
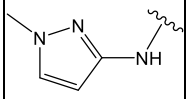
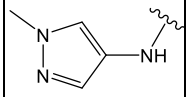
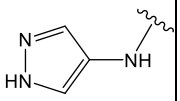
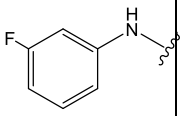
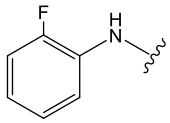
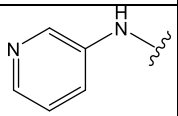
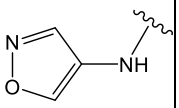
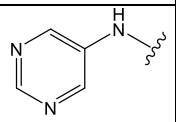
Compound	R-group	MV4-11 cells IC_{50}, nM	In vitro Kinase Assays (IC_{50}, nM)			
			FLT3-ITD	FES	HCK	SYK
TL02-59	–	0.78	440	290	160	470
TL8-133		0.46	490	260	360	400
TL8-187		0.55	710	320	530	670
TL8-130		9.9	490	180	480	600
TL8-142		10.8	700	2500	700	160
TL8-129		27.4	3000	1300	2400	1500
TL8-139		64.7	3000	880	5300	3520
TL8-128		77.1	640	320	730	600
TL9-169		386.5	6400	3000	1410	5300
TL8-143		1567	570	3200	133	5660

Table 4: Screening of TL02-59 and the TL8 analogs.

TL02-59 and the nine analogs shown were evaluated for their growth inhibitory activity in the FLT3-ITD$^+$ AML cell line, MV4-11, using the Cell Titer Blue assay (Promega). Each compound was also tested for inhibitory activity in vitro using recombinant FLT3-ITD, FES, HCK, and SYK kinases and the Z'Lyte kinase assay (Invitrogen). Results from both assays are presented as IC_{50} values (nM). The structures of TL02-59 and the related TL8 scaffold are shown in Figure 4.

1.6 μM (about 2,000-fold less potent than TL02-59). This observation strongly supports the idea that inhibition of AML-associated kinases in addition to the FLT3-ITD driver may be of therapeutic benefit in AML. Subsequent mechanistic studies focused on TL02-59, because of its target kinase spectrum, cellular potency, and favorable pharmacokinetic properties. The synthesis of TL02-59 has been reported previously (analog 8 in Tan, et al.) (459). To determine whether TL02-59 was selective for FLT3-ITD$^+$ AML cells, we examined its inhibitory potency against the THP-1 myeloid leukemia cell line, which expresses wild-type FLT3. TL02-59 had no effect on THP-1 cell proliferation at concentrations as high as 1 μM, with slight growth suppression evident at 3 μM (Fig. 4B and data not shown). TL02-59 also selectively induced apoptosis in MV4-11 cells, without affecting THP-1 cells (Fig. 4C), and was a more potent inducer of apoptosis than tandutinib, a FLT3 inhibitor previously tested in clinical trials against FLT3-ITD$^+$ AML (460).

We next confirmed that each of the TL02-59 target kinases tested in vitro were expressed and active in MV4-11 cells, and that TL02-59 treatment inhibited each of these kinases. MV4-11 cells were treated with 100 nM TL02-59 for six hours, and FLT3-ITD, FES, HCK, and SYK were immunoprecipitated followed by immunoblotting with anti-phosphotyrosine antibodies. As

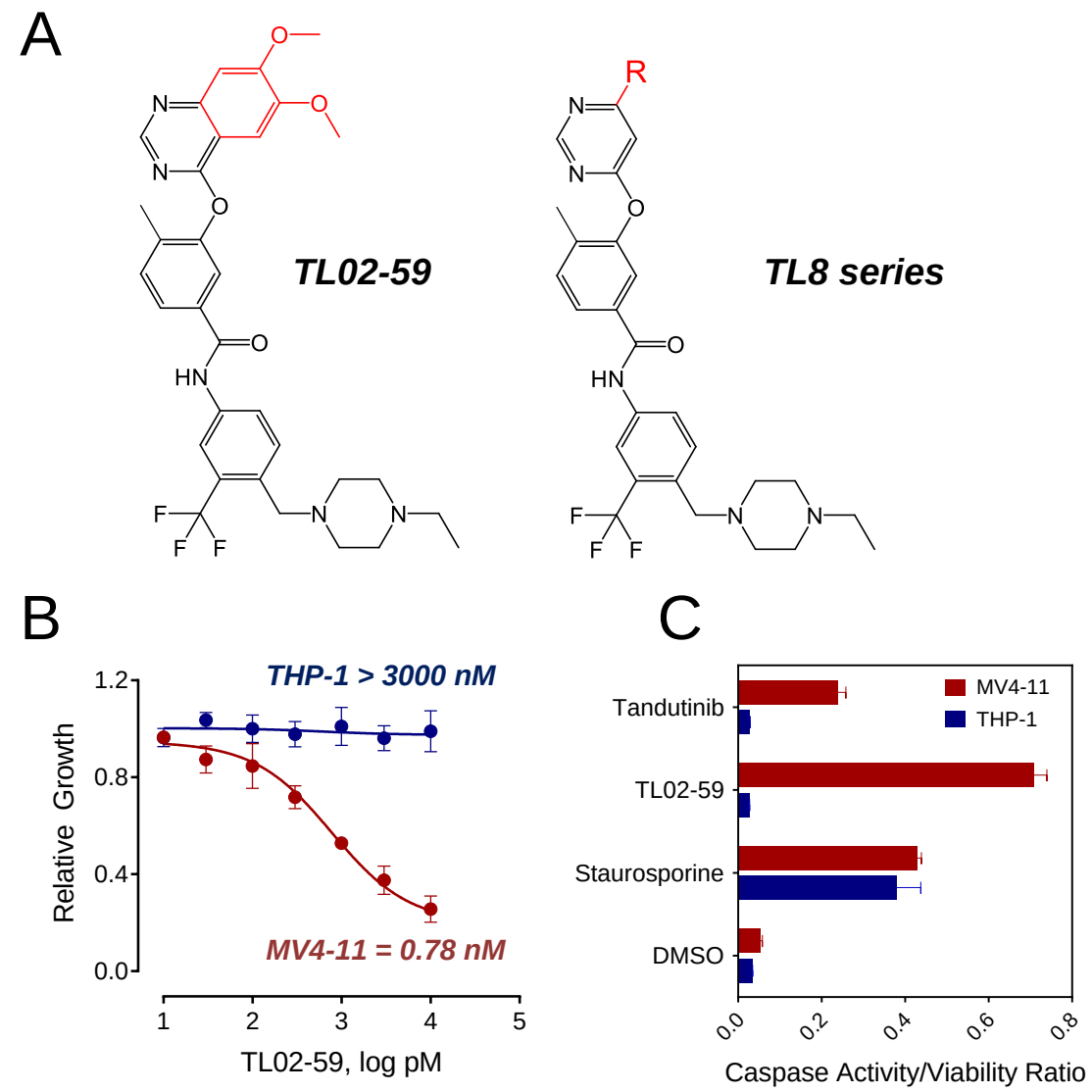

A
TL02-59
TL8 series
R
B
THP-1 > 3000 nM
MV4-11 = 0.78 nM
Relative Growth
TL02-59, log pM
C
MV4-11
THP-1
Tandutinib
TL02-59
Staurosporine
DMSO
Caspase Activity/Viability Ratio
D
TL02-59
pTyr
pY418
pY713
pTyr
FLT3
HCK
FES
SYK

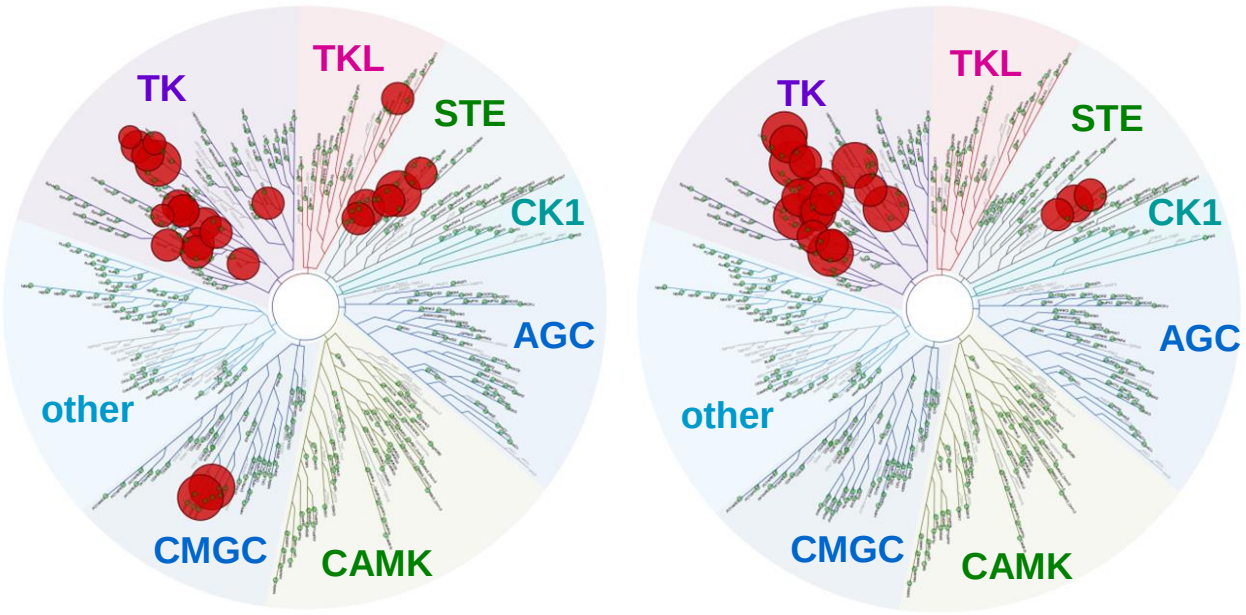
Figure 4: TL02-59 inhibits the growth and induces apoptosis in the FLT3-ITD$^+$ AML cell line, MV4-11.

A) The chemical structure of TL02-59 and the TL8 series N-phenylbenzaminde scaffold; R groups are shown in Table 1. B) Growth inhibition was measured using the Cell Titer Blue viability assay (Promega) by incubating each cell line over a range of TL02-59 concentrations for 72 hours. For THP-1 cells, additional concentrations up to 3.0 μM did not inhibit growth (not shown). C) MV4-11 and THP-1 AML cells were incubated with tandutinib (100 nM), TL02-59 (100 nM), staurosporine (1 μM) or the DMSO carrier solvent for 72 hours. Apoptosis measured using the Apo-ONE Homogeneous Caspase 3/7 Assay (Promega), and cell viability was measure using the Cell Titer Blue assay. Results are present as mean ratios of caspase activity to viability from three independent experiments ± SD. D) Inhibition of endogenous TL02-59 target kinases in MV4-11 cells. Cells were treated with 100 nM TL02-59 for six hours, lysed, and each of kinases shown was immunoprecipitated. For FLT3 and SYK, the immunoprecipitates were blotted with the antiphosphotyrosine antibody, pY99. For HCK and FES, phosphospecific antibodies against the activation loop phosphotyrosine were used (pY418 and pY713, respectively). Aliquots of each immunoprecipiate were also blotted for kinase protein to ensure equal loading.

shown in Figure 4D, all four kinases were present and active in MV4-11 cells, and TL02-59 treatment inhibited their autophosphorylation, consistent with the in vitro kinase assays.

2.2.2 KINOMEscan analysis coupled to primary AML cell kinase expression profiling reveals additional targets for TL02-59

The remarkable potency of TL02-59 toward MV4-11 cells led us to define the complete range of kinase targets for this compound in AML. First, we performed KINOMEscan analysis, a broad spectrum competition binding assay for determining ATP-site inhibitor specificity across almost the entire kinome (461). We profiled TL02-59 against 456 kinases that cover all eight kinase subfamilies at the relatively high concentration of 1 μM, which is about 2- to 6-fold higher than the in vitro IC_{50} values for inhibition of the known target kinases and more than 1,000-fold



TL02-59
456 Assays Tested
24 Interactions Mapped
S-Score = 0.07 (1 μM)

A-419259
468 Assays Tested
19 Interactions Mapped
S-Score = 0.05 (1 μM)

Figure 5: KINOMEscan analysis of TL02-59 and A-419259.

To assess the spectrum of possible kinase targets, TL02-59 and A-419259 were profiled at 1 μM against 456 and 468 kinases, respectively, using the KINOMEscan screening assay (DiscoverRx). Results are presented as circular kinase dendrograms, produced using the program TREEspot (DiscoverRx). The circle size represents the relative binding score for each kinase, with a larger circle size indicating tighter binding. Kinases that are not inhibitor targets are represented by the small green spots. Only kinases with a binding score of < 1% relative to the DMSO control are shown. The specificity (S) score represents the fraction of target kinases within the 1% cutoff relative to the total number screened. The identity of the positive hits from this analysis are shown in Table 7 of the main text.

higher than the IC_{50} value for growth suppression of MV4-11 cells (Table 4). TL02-59 was remarkably selective in the KINOMEscan assay, interacting with only twenty-four kinases out of the 456 tested for a selectivity (S) score of 0.07 (Fig. 5 and Table 7). The majority of the

interacting kinases are members of the tyrosine kinase and tyrosine kinase-like families, and include FLT3 (wild-type and ITD), HCK, SYK, and FES, consistent with our in vitro kinase assay data (Table 4). Other potential tyrosine kinase targets for TL02-59 include the FLT3-related kinase c-KIT and the additional Src-family members LYN, BLK and LCK as well as SRC itself. The remainder of the positive interactions mapped to members of the STE [TAOK2, TAOK3/JIK, SLK, STK10/LOK, MAP4K2) and CMGC (p38α, JNK2) Ser/Thr kinase families. While p38α has been linked to AML in several previous studies (462-465), the remaining Ser/Thr kinases have no known role in AML.

To define the subset of potential target kinases for TL02-59 identified by KINOMEscan that are relevant to AML, we next determined the expression profile of all TL02-59 KINOMEscan hits in a set of bone marrow specimens from 26 AML patients. The clinical characteristics and cytogenetic profiles of our AML patient cohort are presented in Table 5. We first determined the *FLT3* genotype of each patient by PCR of genomic DNA and sequence analysis. Thirteen samples carried an ITD in one *FLT3* allele, while the remaining thirteen patients were wild-type for *FLT3* (Table 6). Using quantitative real-time RT-PCR (qPCR), we next profiled the relative expression of all 24 of the TL02-59 target kinases plus three additional members of the Src-kinase family that were very close to the specificity cut-off in the KINOMEscan analysis (FGR, FYN, YES). A relative expression value was calculated for each kinase as the base 2 antilog of the difference in qPCR C_t value for the GAPDH reference gene and the C_t value for each kinase. Each value was then normalized to the mean expression value for all 27 TL02-59 target kinases analyzed in each individual sample to create an expression profile for each patient (Figure 6). Using this approach, we found that 15 of the 27 target kinases for TL02-59 identified by KINOMEscan analysis showed relative expression values greater than

Patient	Age	Gender	FAB	Cytogenetics	*FLT3* status	% Blasts (PB)	% Blasts (BM)
047	47	F	M4	t(6,9)(p23;34)	ITD	14	59
051	68	M	M2	Normal	WT	54	59
069	30	F	M5a	t(11;19)(q23;p13.3), +19	WT	50	87
078	41	F	M4Eo	-7,inv(16)(p13.1q22)	WT	49	66
079	51	M	NA	Normal	ITD	24	42
080	43	F	NA	t(9;11)(p22;q23)	WT	70	92
093	47	M	M4	inv(16)(p13.1q22)	WT	58	54
104	66	F	M1	Normal	ITD	86	92
175	74	F	NA	Normal	ITD	98	95
194	64	F	M5	t(6,11)(q27;q23)	ITD	20	74
196	71	F	NA	add(12)(p13)	WT	17	46
239	47	M	M4	inv(16)(p13.1q22)	WT	66	71
289	70	M	NA	Normal	ITD	62	70
332	46	M	M4	Normal	ITD	83	86
397	77	M	M4	Normal	WT	18	48
410	54	M	NA	Normal	ITD	74	79
419	72	F	NA	Complex	WT	4	78
451	51	F	NA	Complex	WT	92	90
453	66	M	M5a	t(9;11)(p22;q23)	WT	87	96
454	69	F	NA	Complex	ITD	44	53
465	54	F	NA	+8	WT	25	60
488	82	M	NA	Normal	ITD	21	65
490	65	M	NA	t(2;12)(p13;p13)	ITD	83	96
505	53	F	NA	Normal	WT	88	96
515	48	F	NA	Normal	ITD	90	72
548	69	F	NA	Normal	ITD	86	96

Table 5. Clinical characteristics of primary AML bone marrow samples.

Patient gender, age at diagnosis and French-American-British (FAB) AML classification is shown. *FLT3* status is defined as wild-type (WT) or internal tandem duplication (ITD); N/A, not available. Percent of leukemic blast cells present in peripheral blood (PB) and bone marrow (BM) are also shown. Samples are listed in order of ascending case number.

2 in at least four of the FLT3-ITD$^+$ bone marrow samples (Fig. 6A). All of the kinase targets for TL02-59 previously confirmed by in vitro kinase assay (FLT3, FES, HCK and SYK) were highly expressed in the FLT3-ITD$^+$ samples. Remarkably, a very similar pattern of kinase gene expression was observed in patient AML samples wild-type for FLT3, with the same group of 15 kinases showing relative expression values greater than 2 in at least four samples (Fig. 6B). The most highly expressed kinase in the FLT3-ITD$^+$ samples is FGR, a Src-family kinase with myeloid-restricted expression. High levels of FGR expression were observed in 8 of 13 samples, with much lower expression in the remaining cases. A similar bimodal FGR expression pattern was mirrored in the AML samples wild-type for FLT3, where FGR was among the most highly expressed protein kinases.

We next evaluated whether the pattern of relative TL02-59 target kinase expression in our cohort of primary AML bone marrow samples is representative of the larger AML population. To do this, TL02-59 target kinase gene expression values (determined by RNAseq analysis) from AML specimens with wild-type (n = 111) or ITD (n = 34) forms of *FLT3* were download from the TCGA database (51) and normalized to the average expression across all 27 target kinases for TL02-59 within each sample as per our qPCR data. The average of the normalized expression values for each kinase from the TCGA database were then plotted against the average values obtained for each kinase by qPCR in our AML cohort for the FLT3-ITD and FLT3-WT AML populations (Fig. 7). A strong linear correlation was observed between the two data sets, indicating that the relative TL02-59 target kinase expression pattern observed in our primary AML bone marrow samples is mirrored in this larger population of AML specimens.

We also performed qPCR-based expression profiling for the same set of TL02-59 target kinases using RNA isolated from the FLT3-ITD$^+$ MV4-11 AML cell line, which is remarkably

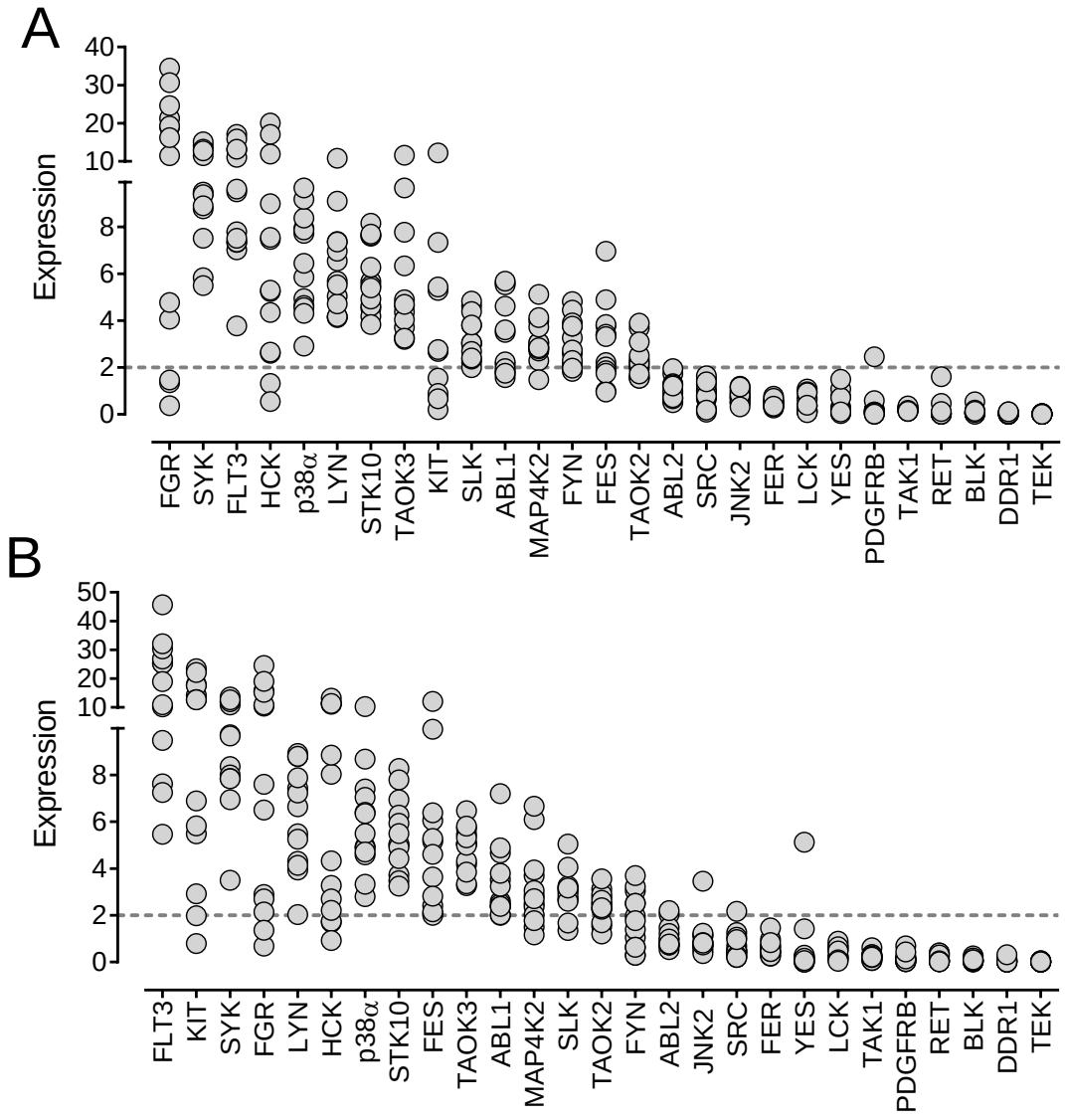


Figure 6: Expression of TL02-59 target kinases in primary AML bone marrow samples.

Expression of the 27 putative TL02-59 target kinases identified by KINOMEscan analysis was determined in 26 primary AML bone marrow samples by qPCR. Relative expression values were calculated as the base 2 antilog of the difference in qPCR C_t values for the GAPDH reference gene and the C_t value for each kinase. The resulting expression values were then plotted as a distribution relative to the mean value for all 27 kinases analyzed in each individual patient sample. All determinations were made on at least two independent RNA samples from each patient. Each patient is represented by a dot. *FLT3* status was determined for each patient by PCR and sequencing (Table 6) and results are grouped as FLT3-ITD (panel A) or FLT3-WT (panel B; n=13 in each case).

sensitive to this multi-targeted kinase inhibitor. The relative expression value for each kinase in MV4-11 cells was then plotted against the average expression value for each kinase across the FLT3-ITD$^+$ primary AML samples (Fig. 8A) and those expressing wild-type FLT3 (Fig. 8B). In both cases, we observed a strong positive correlation between TL02-59 target kinase expression

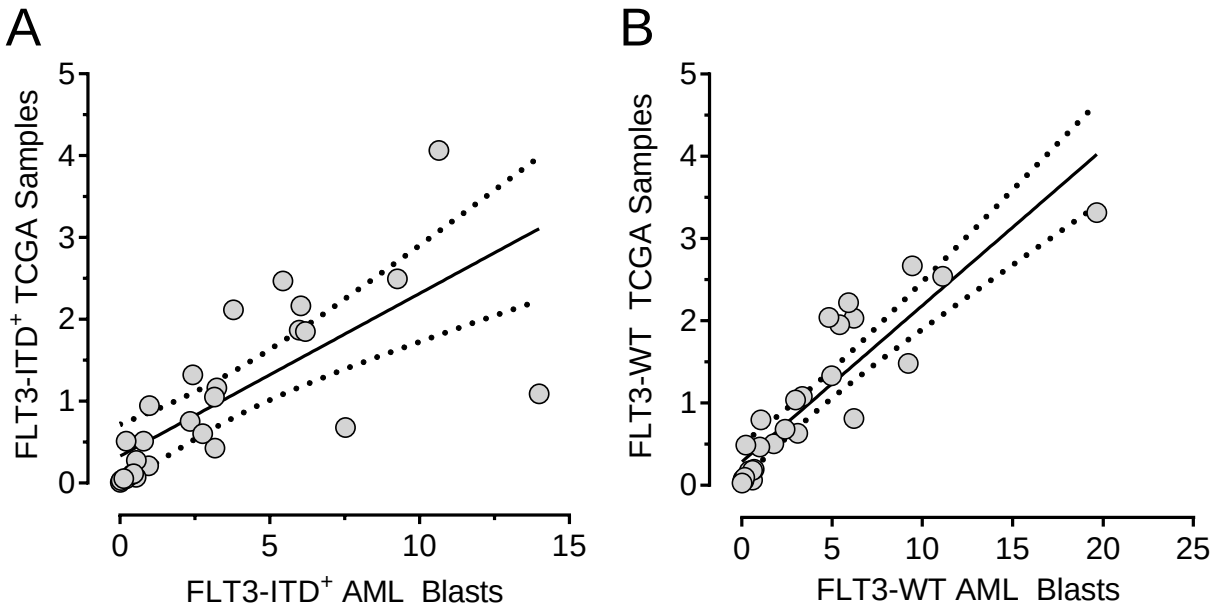


Figure 7: Comparison of TL02-59 target kinase expression in primary AML samples from the TCGA database vs. primary AML bone marrow samples from this study.

Comparison of TL02-59 target kinase expression in primary AML samples from the TCGA database vs. primary AML bone marrow samples from this study. TL02-59 target kinase RNAseq expression data were downloaded as FPKM values (see Garber *et al*. *Nature Methods* 8: 469, 2011) from the TCGA database (see Garber *et al*. *Nature Methods* 8: 469, 2011) and normalized to the average expression across all 27 kinases within each sample. Average values for each kinase from the TCGA database (FLT3-ITD: n = 34, FLT3-WT: n =111) were plotted against the average values obtained for each kinase by qPCR in this study for FLT3-ITD (n = 13; A) and FLT3-WT (n = 12; B) primary AML bone marrow samples. Data were analyzed by linear regression and the best-fit line and 95% confidence intervals are shown; $p < 0.0001$ in both cases.

in MV4-11 cells and primary AML samples, validating MV4-11 cells as a model for testing TL02-59 and the related kinase inhibitors shown in Table 4. One notable outlier in this analysis is FGR, which is strongly expressed in a majority of FLT3-ITD$^+$ primary bone marrow specimens relative to MV4-11 cells. In primary AML samples wild-type for FLT3, both FGR and c-KIT are more highly expressed in primary AML compared to MV4-11 cells.

2.2.3 Sensitivity of primary AML bone marrow samples to TL02-59 treatment correlates with AML tyrosine kinase expression

We next explored the sensitivity of primary AML bone marrow samples to growth arrest in response to TL02-59 treatment. Primary AML bone marrow samples were cultured on mitotically inactive HS-27 feeder fibroblasts in the presence of the myeloid cytokines IL-3, IL-6, and SCF according to the method of Klco et al. (466). This approach allowed all of the primary cells to survive ex vivo and expand in number for about one week. Cultures were then established in the presence of a range of TL02-59 concentrations, and IC_{50} values for growth inhibition were determined using the Cell Titer Blue cell viability assay (see Methods). As shown in Table 6, the primary AML bone marrow samples showed a remarkable range of sensitivity to this kinase inhibitor, ranging from 77 nM to greater than 3000 nM. We then looked for statistical correlations between TL02-59 sensitivity and relative expression values for each of the TL02-59 target kinases present in the AML bone marrow specimens. As shown in Figure 9, relative expression levels of the three myeloid Src-family kinases (FGR, HCK and LYN) showed a significant inverse correlation with the IC_{50} values for TL02-59 across the 26 AML samples. SYK expression also significantly correlated with the inhibitor response, supporting a role for this kinase as a key TL02-59 target kinase as well. FES expression also showed a similar trend

with inhibitor sensitivity that did not reach statistical significance, which may reflect the relatively small patient sample size.

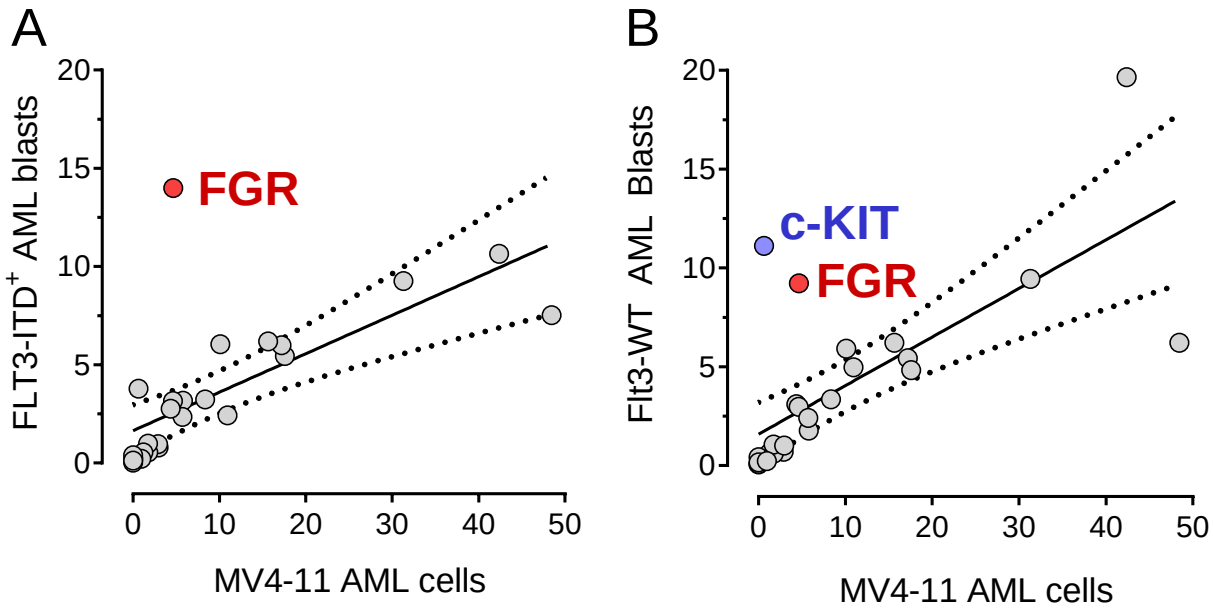


Figure 8: Comparison of TL02-59 target kinase expression in FLT3-ITD$^+$ MV4-11 AML cells vs. primary AML bone marrow samples.

Comparison of TL02-59 target kinase expression in FLT3-ITD$^+$ MV4-11 AML cells vs. primary AML bone marrow samples. The TL02-59 target kinase expression profile in MV4-11 cells was determined by qPCR as described in the Methods section, and the relative expression values were plotted against the average values obtained for each kinase in the FLT3-ITD (n = 13; A) and FLT3-WT (n = 13; B) primary AML bone marrow samples. Data were analyzed by linear regression; the best-fit line and 95% confidence intervals are shown; $p < 0.0001$ in both cases. Outliers included the Src-family kinase FGR, which is strongly expressed in primary AML cells, as well as c-KIT which is strongly expressed in primary cells that are wild-type for FLT3.

2.2.4 Inhibition of Src-family kinases alone does not account for TL02-59 potency

Kinase expression profiling and correlation analyses presented in the previous sections strongly implicate members of the Src kinase family, especially FGR, HCK and LYN, as key targets for TL02-59 action in AML cells. To explore this question directly, we turned to the pyrrolopyrimidine compound A-419259, a potent cell-active inhibitor of HCK and other Src-family kinases (455, 456). Previous studies have shown that treatment with A-419259 (also referenced as RK-20449 in the literature; see Fig. 10A for structure) dramatically reduced the growth of primary AML cells in engrafted immunocompromised mice, an outcome attributed to inhibition of HCK which is highly expressed in AML-derived leukemic stem cells (338). KINOMEscan analysis revealed a target specificity profile for A-419259 even narrower than that of TL02-59, with only 19 interactions observed out of 468 kinases tested for a selectivity score of 0.05 (Fig. 5 and Table 7). In addition to HCK, FGR, and other Src-family kinases, A-419259 also interacted with FLT3-ITD in the KINOMEscan assay, but not with FES or SYK, two other important targets for TL02-59. This observation suggested that A-419259 may serve as a useful tool compound to ask whether inhibition of FLT3-ITD plus Src-family kinases is sufficient to explain the remarkable potency of TL02-59 in MV4-11 cells or whether other kinases play a role. As shown in Figure 10B, MV4-11 cell growth was inhibited by A-419259 with an IC_{50} value of 37 nM, which is about 30-fold higher than that observed for TL02-59. This difference in potency suggests that additional target kinases contribute to TL02-59 efficacy.

To identify additional kinases that contribute to the TL02-59 sensitivity of AML cells, we compared the KINOMEscan profiles for both compounds against each of the TL02-59 target kinases expressed in our primary AML samples. This comparison identified several candidate kinase targets unique to TL02-59 and already implicated in FLT3-ITD$^+$ AML (SYK, FES, and

Patient	*FLT3* Status	ITD sequence	TL02-59 Sensitivity (IC_{50}, nM)
451	wild-type	N/A	77
453	wild-type	N/A	88
196	wild-type	N/A	119
051	wild-type	N/A	185
454	ITD	N.D.	229
488	ITD	GSSDNEYFYVDFREYE	330
332	ITD	YDLKWEVTGSSDNGYFYVDFREYE	403
465	wild-type	N/A	408
079	ITD	YDLKWEVTGSSDNEYFYVDFREYE	477
548	ITD	YDLKWEFPRENLGPYVDFREYE	559
080	wild-type	N/A	635
078	wild-type	N/A	663
239	wild-type	N/A	698
047	ITD	N.D.	701
397	wild-type	N/A	767
194	ITD	N.D.	806
410	ITD	YDLKWEFPRKNEYFYVDFREYE	814
490	ITD	N.D.	891
419	wild-type	N/A	1113
289	ITD	YDRAGSSDNEYFYVDFREYE/ YDLKWEVTGSSDNEYFYADFREYE	1420
515	ITD	YDLKWEFPRENLEQGKQVTGSSDNEYFYVDFREYE	1887
093	wild-type	N/A	2660
505	wild-type	N/A	>3000
175	ITD	YVLKWEFPRGEMVQVTGSSDNEYFYVDFREYE	>3000
104	ITD	VDFREYE	>3000
069	wild-type	N/A	N/A

Table 6: *FLT3* genotype and TL02-59 sensitivity of primary AML bone marrow cells.

Genomic DNA was isolated from 27 AML patients and a portion of the FLT3 kinase domain coding region was amplified by PCR. Thirteen of the samples showed internal tandem duplications (ITDs) within the kinase domain juxtamembrane coding region of one *FLT3* allele. DNA sequence analysis revealed the ITD amino acid sequences of 9 out of 13 samples as shown. Primary cells were then cultured in vitro on fibroblast feeder layers in the presence of IL-3, IL-6, and SCF over a range of TL02-59 concentrations. Viable cell outgrowth was determined by Cell Titer Blue assay and the IC_{50} values shown were calculated by non-linear curve fitting of the resulting concentration-response curves. Samples are listed in ascending order of TL02-59 sensitivity. N/A, not applicable; N.D., ITD present by PCR but sequence could not be determined.

p38α) as well as several others not previously examined in the context of AML (STK10, TAO2/3, MAP4K2 and SLK; Table 7). To validate the KINOMEscan result, we performed *in vitro* kinase assays with recombinant SYK, FES, TAOK3, p38α, as well as FGR. As shown in Figure11, SYK, FES, TAOK3 and p38α were all inhibited by TL02-59 in the mid-nanomolar range, with much lower sensitivity to A-419259. These findings suggest that in addition to FLT3 and myeloid Src-family kinases, inhibition of FES, SYK, p38α and TAOK3 may also contribute to TL02-59 efficacy in MV4-11 cells and in primary AML bone marrow. We also tested both kinase inhibitors against FGR, the kinase most highly correlated with TL02-59 sensitivity in our primary AML bone marrow samples. To our surprise, FGR was inhibited by both compounds in the low picomolar range, identifying it as the most sensitive kinase tested *in vitro*. Note that previous studies have shown that the Ser/Thr kinase MAP4K2 is potently inhibited by TL02-59 as well, suggesting that inhibition of this kinase may also contribute to AML sensitivity to this inhibitor (459). KINOMEscan and AML kinase expression profiling data presented above implicated the Ser/Thr kinases TAOK3 and STK10 as contributors to the AML phenotype and as potential TL02-59 inhibitor targets. To independently explore the role of TAOK3 and STK10 in

AML, we performed lentiviral shRNA knockdown studies targeting each of these kinases in MV4-11 cells. We also targeted FGR by this approach, because of its high-level of expression in a subset of primary AML bone marrow cells. HCK was included as a positive control, because previous studies have shown that knockdown of HCK expression reduces the growth of MV4-11

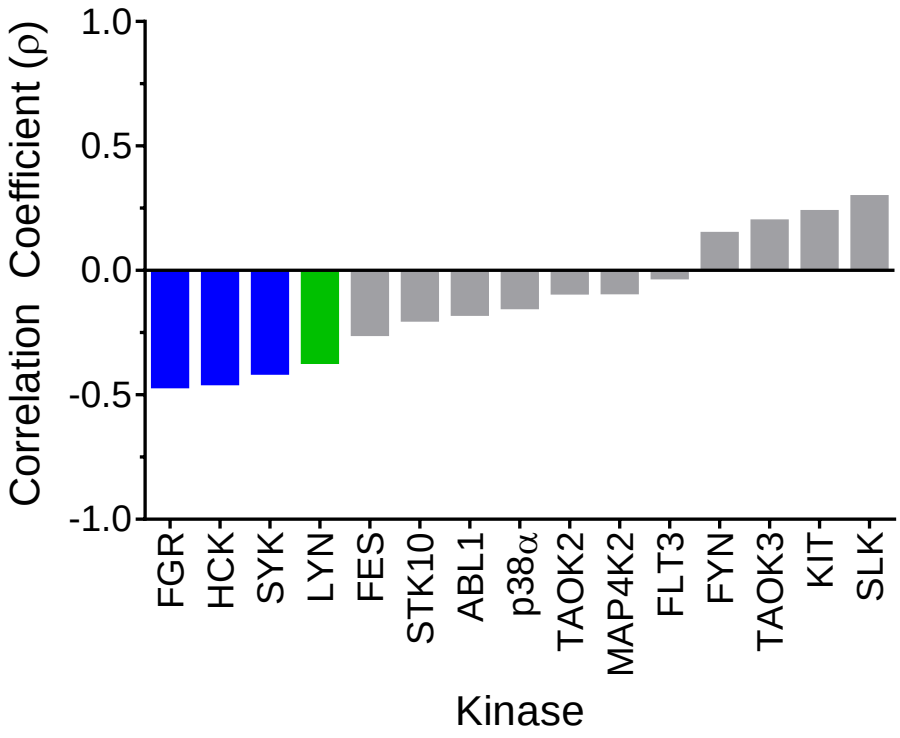


Figure 9: Myeloid-restricted Src-family kinases and SYK significantly correlate with TL02-59 sensitivity in patient AML bone marrow samples.

Relative TL02-59 target kinase expression (from Figure 6) was correlated with TL02-59 IC_{50} values determined for each of the primary AML bone marrow samples (Table 6). Spearman's correlations were calculated for each kinase across all of the AML samples, and the resulting correlation coefficients are plotted in ascending order. Blue bars represent kinases that show a significant negative correlation with TL02-59 IC_{50} values with $p < 0.05$; green bars represent $p < 0.1$. Grey bars represent kinases that did not reach statistical significance

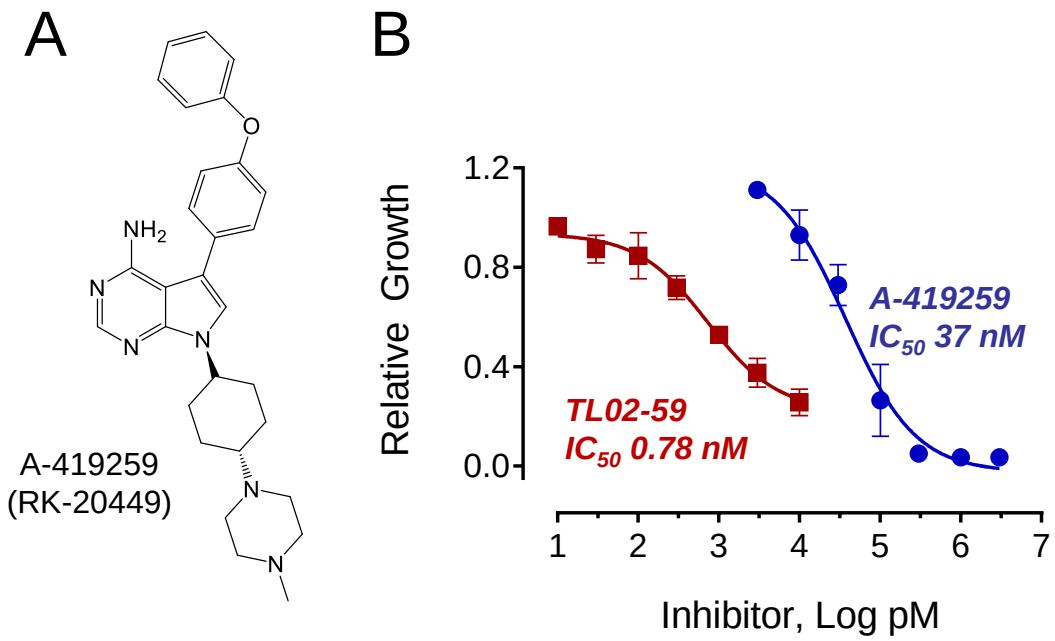


Figure 10: The Src-family kinase inhibitor A-419259 is less potent than TL02-59 against MV4-11 AML cells.

A) Chemical structure of the pyrrolopyrimidine Src-family kinase inhibitor A-419259, also known as RK-20449. B) MV4-11 cells were incubated over the range of inhibitor concentrations shown for 72 hours and viability was assessed by Cell Titer Blue assay. Curves were fit by non-linear regression and yielded the IC_{50} values shown.

cells (337, 338). For these experiments, MV4-11 cells were infected with equivalent amounts of virus and growth was monitored over the course of six days (Fig. 12A and B). Cells infected with virus expressing GFP served as the reference control. The efficacy of target kinase knockdown was determined in parallel for each infected cell population by qPCR (Fig. 12C). As shown in Figure 12A, both lentiviral shRNAs targeting FGR resulted in significant growth suppression, consistent with the ~ 50% reduction in FGR mRNA levels observed by qPCR (Fig. 12C). For HCK, efficient lentiviral knockdown was achieved with one of the two shRNAs tested and this also resulted in significant growth arrest. A similar result was observed with TAOK3, in which more efficient shRNA knockdown resulted in almost complete growth suppression, while

TL02-59 Kinase Target	KINOMEscan Percent residual binding at 1.0 μM inhibitor	
	TL02-59	A-419259
FGR	2.3	0.85
SYK	1.2	100
FLT3	0.6	0.65
HCK	0.3	1.9
p38α	0	100
LYN	1	1.7
STK10	0	20
TAOK3	0.3	100
c-KIT	2.2	0.15
SLK	0.8	100
ABL1	0.2	0.25
MAP4K2	0.25	98
FYN	23	5.1
FES	0.75	100
TAOK2	0.1	85
SRC	1	0.1
YES	5.8	0

Table 7: TL02-59 target kinases expressed in MV4-11 AML cells and primary FLT3-ITD$^+$ AML blasts show differential sensitivity to A-419259.

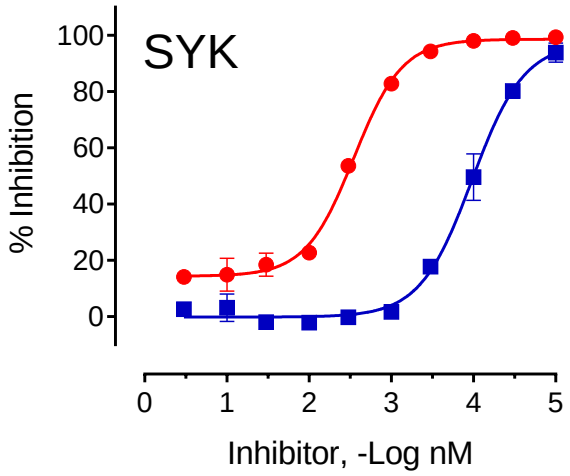
Putative TL02-59 kinase inhibitor targets identified in the KINOMEscan assay and expressed in primary FLT3-ITD$^+$ AML blasts are listed, together with their responses in the KINOMEscan assay. Values represent percent of residual kinase binding to the immobilized probe at an inhibitor concentration 1.0 μM (e.g. a value of zero equals 100% probe displacement, while a value of 100 equals no binding of test compound to the target kinase). Kinases are shown in rank order of their relative expression in FLT3-ITD$^+$ primary AML bone marrow samples. The highlighted rows show at least 80-fold selectivity for TL02-59 vs. A-419259.

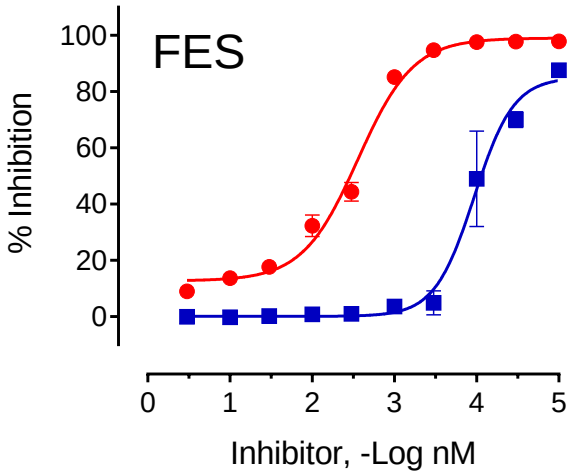
less efficient knockdown was without effect. Finally, a single shRNA targeting STK10 resulted in an 85% suppression of mRNA levels for this kinase, but did not significantly influence cell growth. Altogether, these results link TAOK3 to AML pathogenesis for the first time, and suggest that inhibition of TAOK3 kinase activity contributes to the efficacy of TL02-59 in MV4-11 cells as well as primary AML bone marrow cells, in which it is consistently expressed at relatively high levels.

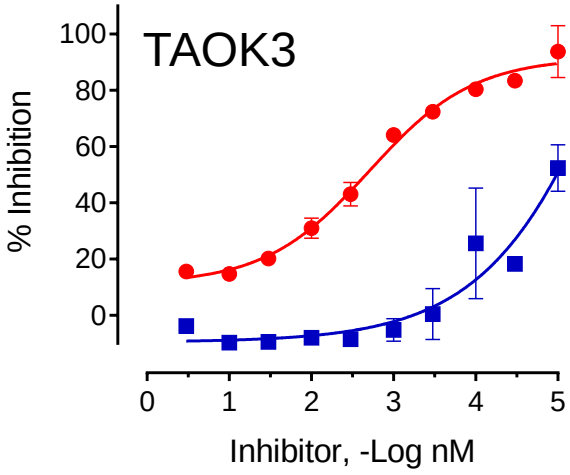
2.2.5 TL02-59 is an orally active inhibitor of AML in vivo

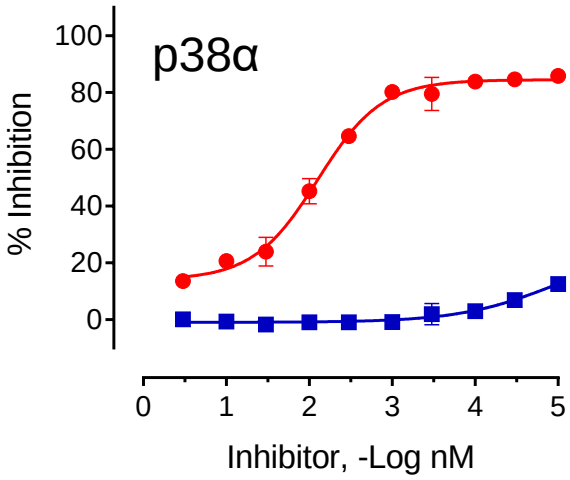
Studies presented above demonstrate that TL02-59 is an effective inhibitor of AML-derived cell lines and primary bone marrow samples, and that its kinase inhibitor target profile closely matches the spectrum of kinases commonly expressed in many AML patients. Pharmacokinetic studies in mice demonstrated the TL02-59 is orally bioavailable and displays a plasma half-life of about 6 hours, making it an attractive candidate for in vivo evaluation (data not shown). To test the efficacy of TL02-59 *in vivo*, we turned to an MV4-11 AML cell xenograft model based on NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) immunocompromised mice. A recent study demonstrated that tail vein injection of NSG mice with MV4-11 cells leads to rapid engraftment of the bone marrow and spleen, providing a rigorous model to test TL02-59 efficacy in vivo (466).

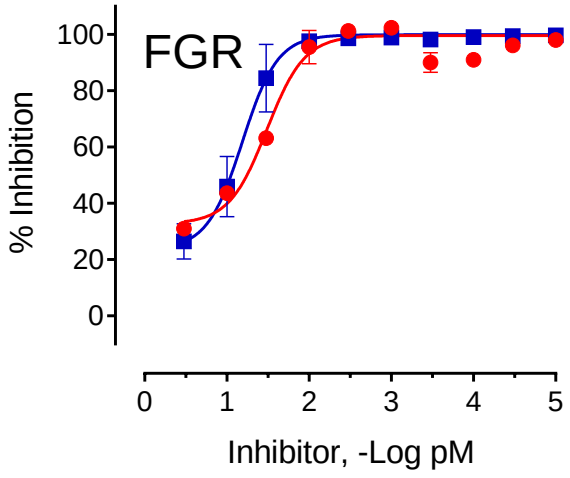
For our studies, cohorts of NSG mice were injected with MV4-11 cells through the tail vein, and allowed to engraft for two weeks. After the engraftment period, mice were treated daily by oral gavage with TL02-59 at 1 and 10 mg/kg, the FDA-approved FLT3 inhibitor sorafenib (10 mg/kg) as a basis of comparison, or vehicle alone for three weeks. At the end of the study, the presence of MV4-11 cells in the bone marrow, spleen, and peripheral blood was











Kinase	TL02-59 (IC_{50} nM)	A-419259 (IC_{50} nM)
SYK	351	9624
FES	360	9349
TAOK3	508.7	>100000
p38α	126	>100000
FGR	0.031	0.015

Figure 11: A subset of TL02-59 target kinases show differential sensitivity to TL02-59 and A-419259 *in vitro.*

Recombinant FGR, SYK, TAOK3, p38α, and FES were tested for inhibitor sensitivity in the Z'LYTE in vitro kinase assay with the ATP concentration set to the K_m value for each kinase. IC_{50} values (nM) were calculated by non-linear regression analysis of the resulting dose-response curves using GraphPad Prism, and are summarized in the table.

assessed by flow cytometry using antibodies specific for human $CD45^+$ and $CD33^+$ both of which are expressed on the surface of MV4-11 cells (Figs. 13A and B). Three weeks of TL02-59 treatment at 1 mg/kg reduced bone marrow engraftment of MV4-11 cells by 20%, reduced spleen engraftment by half while suppressing levels of leukemia cells in the peripheral blood by 70%. At 10 mg/kg, TL02-59 treatment resulted in a 60% reduction in bone marrow engraftment of MV4-11 cells, with complete eradication of leukemic cells from the spleen and peripheral blood. On the other hand, three weeks of sorafenib treatment at 10 mg/kg had no effect on either bone marrow or spleen engraftment of MV4-11 cells, although a significant reduction of leukemic cell counts in the peripheral blood was observed. Histopathological examination of both the spleen and the bone marrow from each of the treatment groups supports the flow cytometry results. Thin sections from all five groups were stained with antibodies to human CD45 and are presented in Figure 13C. Bone marrow sections from mice receiving MV4-11 cells alone show heavy infiltration of MV4-11 cells. Treatment with TL02-59 at the higher dose restored the normal bone marrow architecture, although some MV4-11 cells are still evident. Spleen sections from mice treated with TL02-59 at 10 mg/kg were completely devoid of MV4-11 cells. In contrast, spleen and bone marrow sections from animals treated with sorafenib at 10 mg/kg still stained strongly for MV4-11 cells in a manner indistinguishable from the vehicle controls

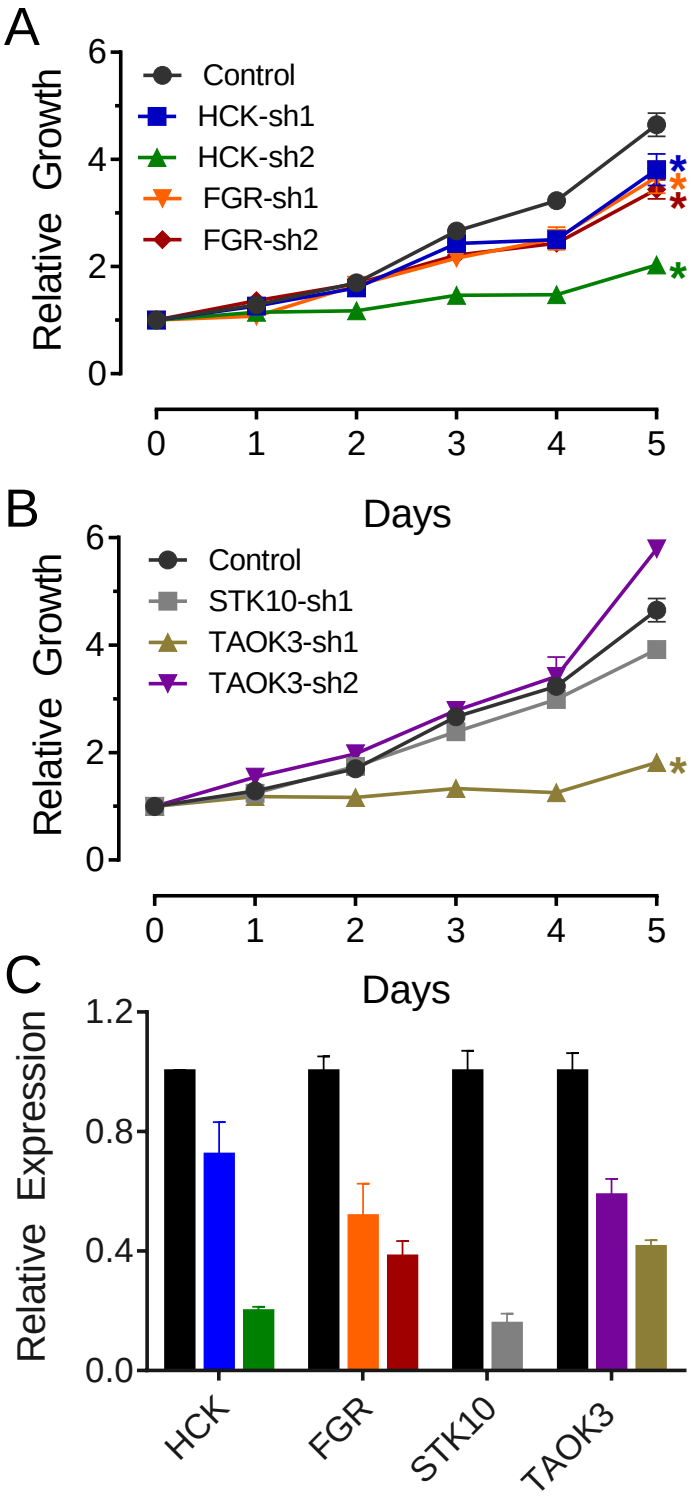

A
Relative Growth
Control
HCK-sh1
HCK-sh2
FGR-sh1
FGR-sh2
Days
B
Relative Growth
Control
STK10-sh1
TAOK3-sh1
TAOK3-sh2
Days
C
Relative Expression
HCK
FGR
STK10
TAOK3

Figure 12: shRNA knockdown of TL02-59 target kinases reduces the growth of MV4-11 cells.

MV4-11 cells were infected with lentiviruses containing shRNAs targeting HCK, FGR (panel A), STK10 or TAOK3 (panel B) and cell growth was measured daily for the next six days using the Cell Titer Blue assay. Control cells were infected with a GFP lentivirus as a marker for infection efficiency. Asterisks indicate significant differences ($p < 0.05$). C) RNA was isolated 72 hours post-infection and relative kinase expression levels were determined by qPCR to determine the effectiveness of the shRNA knockdown relative to the GFP controls. The entire study was performed in duplicate, with each data point measured in triplicate. Mean values are shown ± SD.

2.3 DISCUSSION

Here we report the identification of a unique N-phenylbenzamide tyrosine kinase inhibitor, TL02-59, with potent growth inhibitory activity against AML both in vitro and in vivo. By combining KINOMEscan analysis, in vitro kinase assays and kinase gene expression profiling of primary AML bone marrow samples, we defined a consistent pattern of AML-associated kinases as key kinase targets for this inhibitor. In addition to FLT3, these target kinases include SYK, the myeloid Src-family kinases FGR, HCK and LYN, as well as FES. Several Ser/Thr kinases were also linked to TL02-59 action, including p38α and TAOK3; the latter kinase has not been previously linked to AML. TL02-59 is orally bioavailable and induced substantial reductions in splenic and bone marrow engraftment of human FLT3-ITD$^+$ AML cells in NSG mice in response to short-term drug exposure with limited toxicity, identifying this multi-kinase inhibitor as a promising scaffold for clinical development.

TL02-59 showed picomolar inhibitory activity against the FLT3-ITD$^+$ AML cell line MV4-11 in vitro, consistent with our observation that these cells express active FLT3 as well as all of the AML-associated TL02-59 kinase targets found in primary AML cells. In primary AML bone

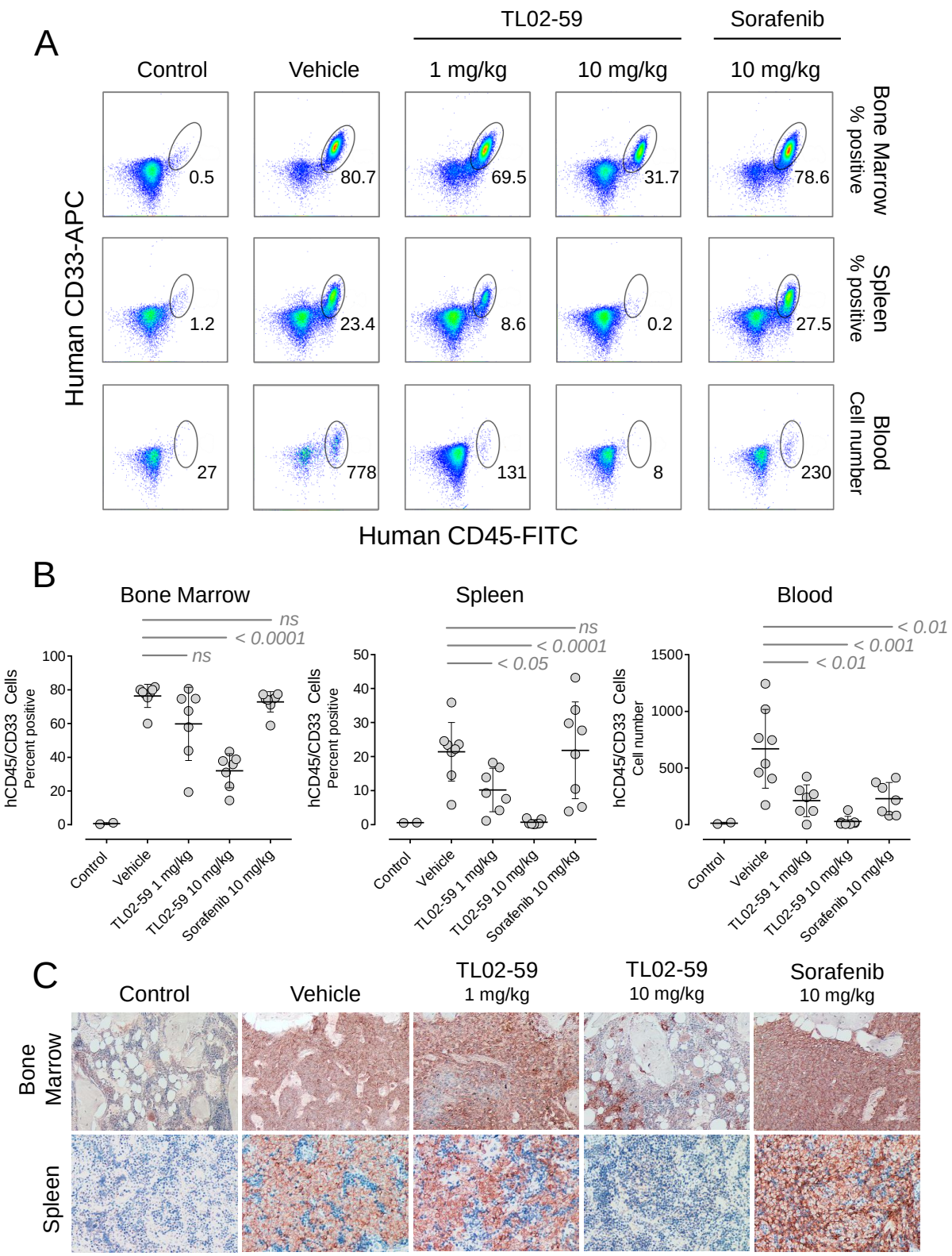

A
TL02-59
Sorafenib
Control
Vehicle
1 mg/kg
10 mg/kg
10 mg/kg
Bone Marrow % positive
Spleen % positive
Blood Cell number
Human CD33-APC
Human CD45-FITC
0.5
80.7
69.5
31.7
78.6
1.2
23.4
8.6
0.2
27.5
27
778
131
8
230
B
Bone Marrow
Spleen
Blood
hCD45/CD33 Cells Percent positive
hCD45/CD33 Cells Cell number
ns
< 0.0001
ns
ns
< 0.0001
< 0.05
< 0.01
< 0.001
< 0.01
Control
Vehicle
TL02-59 1 mg/kg
TL02-59 10 mg/kg
Sorafenib 10 mg/kg
C
Control
Vehicle
TL02-59 1 mg/kg
TL02-59 10 mg/kg
Sorafenib 10 mg/kg
Bone Marrow
Spleen

Figure 13: TL02-59 reduces MV4-11 cell engraftment in an AML mouse model.

Human FLT3-ITD$^+$ MV4-11 AML cells were injected into the tail vein of immunocompromised (NSG) mice and allowed to engraft for two weeks. Mice were then treated daily by oral gavage with TL02-59 (1 and 10 mg/kg), sorafenib (10 mg/kg), or vehicle. Two mice were not engrafted with MV4-11 cells and received no treatment for use as baseline controls. Following three weeks of treatment, the animals were sacrificed and the presence of MV4-11 cells in the bone marrow, spleen and peripheral blood were assayed by flow cytometry. A) Representative flow cytometry diagrams for a single mouse within each treatment group. For the bone marrow and spleen, the inset number represents the percentage of human CD45$^+$/CD33$^+$ MV4-11 leukemia cells present. For whole blood, the number of MV4-11 cells present per 25 μL of blood is shown. B) Summary of all results, where each dot represents a single mouse. For the bone marrow and spleen samples each mouse is plotted as the percentage of human CD45$^+$/CD33$^+$ cells present, while blood samples are represented as the number of MV4-11 cells found per 25 μL. Horizontal bars indicate the mean value in each group +/- SD. Statistical significance was determined by pairwise Student's t-test, with p values indicated for significantly different groups; *ns*, not significant. C) Representative thin sections of spleen and bone marrow from all five treatment groups. MV4-11 AML cells were visualized in the sections by immunohistochemistry with an antibody specific for human CD45.

marrow samples, however, we observed a wide range of inhibitor responses that were not linked to FLT3 mutational status. In fact, the four most sensitive primary AML bone marrow samples were wild-type for FLT3. However, these TL02-59 sensitive cells expressed the highest overall levels of HCK, FGR, SYK, FES, and p38α, all of which have been independently linked to AML, as well as TAOK3, which we validate here as a TL02-59 target by shRNA silencing. In contrast, primary AML bone marrow samples that were not sensitive to TL02-59 treatment in vitro expressed low levels of the TL02-59 target kinases, regardless of their FLT3 mutational status. These observations suggest that the simultaneous action of TL02-59 against this group of AML-related target kinases is responsible for its remarkably potent action in MV4-11 cells as well as the most sensitive primary AML bone marrow specimens. By extension, simple qPCR-

based analysis of the TL02-59 target kinase expression pattern such as the one presented here may be a powerful prognostic tool for patient responses to this inhibitor. Furthermore, our results predict that TL02-59 may be efficacious against AML samples with diverse cytogenetic backgrounds, provided that they express this key group of AML-associated kinase activities.

While the majority of the primary AML bone marrow samples exhibited growth arrest in response to TL02-59 treatment in vitro, their sensitivity to this inhibitor was less than that of the FLT3-ITD$^+$ AML cell line, MV4-11. This difference in sensitivity can be attributed to several important differences between the cells and the methods used to culture them in vitro. First, while MV4-11 cells express the complete spectrum of known kinase targets for TL02-59, the pattern of AML-related kinase expression varies greatly among the primary AML samples (Fig. 6). Primary AML cells often carry additional genetic changes that contribute to the leukemic phenotype but are not necessarily linked to TL02-59-sensitive kinase pathways (25, 26). Second, primary AML bone marrow samples require culture on fibroblast feeder cells in the presence of a cocktail of hematopoietic cytokines, while MV4-11 cells do not. These culture conditions, which mimic the bone marrow niche to some extent, provide additional growth and survival signals not present in the MV4-11 cell cultures. Indeed, culture of MV4-11 cells on fibroblast feeders plus cytokines reduces the TL02-59 IC_{50} value by about 10-fold, supporting this idea (data not shown).

Several previous studies have investigated the role of Src-family kinases in AML, with a particular emphasis on HCK. Important clues pointing to a role for HCK in AML pathogenesis originally came from comparative gene expression profiling, which showed that HCK is highly overrepresented in primary AML stem cells relative to normal hematopoietic stem cells (337). More recently, selective shRNA-knockdown of HCK was shown to block AML cell growth in

vitro (338). Furthermore, the potent ATP-site inhibitor A-419259 (referred to as RK-20449 in this prior work) dramatically reduced the growth of primary AML cells in engrafted NSG mice, including AML cells derived from patients with acquired resistance to conventional AML chemotherapy (338). These results validate HCK as a kinase inhibitor target in AML, and suggest that compounds with activity against HCK may help to eliminate the leukemia stem cell population and reduce the risk of relapse. KINOMEscan data presented here show that A-419259 also has the potential to inhibit FLT3 as well as other members of the Src kinase family expressed in primary AML bone marrow including FGR, FYN and LYN. Indeed, in vitro kinase assays showed that A-419259 (as well as TL02-59) is a picomolar inhibitor of FGR (Fig. 11). Thus the efficacy of A-419259 in the patient-derived AML xenograft mouse model (338) is likely to result from inhibition of multiple kinase targets, not just HCK. Interestingly, A-419259 was less potent then TL02-59 against MV4-11 AML cell proliferation in vitro, suggesting that extension of the selectivity profile to include FES, SYK as well as the Ser/Thr kinases p38α and TAOK3, adds additional efficacy to the multi-targeted kinase inhibitor approach.

Our observation that the Ser/Thr kinase TAOK3 is a target for TL02-59 and that shRNA knockdown of this kinase induces MV4-11 cell growth arrest implicates this kinase in AML for the first time. Previous studies have linked TAOK3 to stress-activated protein kinase pathways, providing a possible link to other kinases implicated in AML. TAOK3 may be important for the activation of p38α following genotoxic insult, because siRNA knockdown of TAOK3 prevented activation of p38α and the downstream anti-apoptotic stress response following DNA damage (467). Inhibition of the p38α stress response may also bolster cellular sensitivity to other chemotherapeutic agents used in AML. For example, inhibition of p38α increases the cellular response to both all-trans retinoic acid (462) and arsenic trioxide (468, 469). TAOK3 has also

been linked to the inhibition of JNK, which promotes a pro-apoptotic response to genotoxic stress (470-472); inhibition of TAOK3 may therefore enhance the pro-apoptotic signal from JNK. Interestingly, RNAi screens for modulators of TRAIL-induced apoptosis (473) and chemoresistance (474) both identified TAOK3 as a negative regulator of apoptosis; in both studies, RNAi knockdown of TAOK3 increased apoptosis. These findings are consistent with our observation that shRNA-mediated knockdown of TAOK3 by more than 50% resulted in growth arrest of MV4-11 cells, and suggest that inhibition of TAOK3 by TL02-59 may contribute to the efficacy of this inhibitor. These observations raise the possibility that TL02-59 may synergize with existing AML drugs to induce apoptosis.

While many FLT3 inhibitors have been tested in clinical trials, none have produced durable responses as monotherapy for AML. One important example is quizartinib (also known as AC220), a very potent and selective FLT3 inhibitor that was among the first to show efficacy as a single agent against FLT3-ITD$^+$ AML (404, 475). While quizartinib often induces rapid remission in FLT3-ITD$^+$ AML, its use has been beset by acquired drug resistance, commonly in the form of FLT3 kinase domain mutations (404, 415, 476-478). Unlike TL02-59, we found that quizartinib does not inhibit other AML-associated kinases including SYK, FES, HCK, or FGR in vitro; the same is true for other FLT3 inhibitors tested in the clinic, including sorafenib, sunitinib, and tandutinib (data not shown). The activity of TL02-59 against virtually all known AML-related kinases within a relatively narrow overall specificity profile is a unique and important feature of TL02-59. In this way, TL02-59 may display the appropriately tuned 'polypharmacology' (479) required for a kinase inhibitor drug with maximal efficacy against AML. Finally, TL02-59 (or related compounds with similar specificity profiles) may induce sustained responses in vivo and exhibit a reduced propensity for acquired resistance due to

simultaneous inhibition of multiple AML kinase pathways. Future work will address the molecular mechanisms of resistance for TL02-59 vs. quizartinib both in vitro and in vivo.

2.4 MATERIALS AND METHODS

2.4.1 Cell Culture, Reagents, and Antibodies

The human AML cell lines MV4-11 (CRL-9591) and THP-1 (TIB-202) were obtained from the American Type Culture Collection (ATCC) and were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Life Technologies), 100 units/ml of penicillin, 100 μg/ml of streptomycin sulfate, 0.25 μg/ml of amphotericin B (antibiotic-antimycotic, Life Technologies). Human 293T and Hs27 (CRL-1634) cells were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotic-antimycotic.

Tandutinib and staurosporine were obtained from LC Laboratories. Sorafenib was purchased from ChemieTek and A-419259 was obtained from Sigma-Aldrich. Custom synthesis of TL02-59 was performed by A Chemtek, Inc. (Worcester, MA).

Primary antibodies used in this study were obtained from Santa Cruz Biotechnology (HCK, M-28; HcCK, N-30; pY99 anti-phosphoyrosine, SC-7020; FES, N-19) and Cell Signaling Technologies (FLT3, 3462S; SYK, 2712S). The activation loop antiphosphotyrosine antibody for Src-family kinases (pY418) used in this study was obtained from Invitrogen and the FES activation loop phosphospecific antibody (pY713) was previously developed by our group (480).

Alkaline phosphatase-linked secondary antibodies used in this study were purchased from Southern Biotech.

2.4.2 Growth Inhibition and Apoptosis Assays

Cells were seeded at a density of 100,000 cells per 1.0 ml in 48-well culture plates. All compounds were made up as concentrated (1000x) stocks in DMSO and diluted 1/1000 when added to the cells. Cell viability was assessed 72 hours later using the CellTiter-Blue Cell Viability assay (Promega) according to the manufacturer's protocol. Fluorescence intensity was measured using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices) with an excitation wavelength of 560 nM excitation and emission wavelength of 590 nm. Apoptosis was assayed as caspase activation under the same cell culture conditions using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) and the manufacturer's protocol. Fluorescence intensity was measured at an excitation wavelength of 485 nM and an emission wavelength of 585 nM. Where applicable, IC_{50} values were determined by non-linear regression analysis of concentration-response curves using the GraphPad PRISM software package (version 6.0).

2.4.3 Immunoprecipitation and Western Blotting

MV4-11 cells (2.5×10^6 cells in 5 mL) were cultured in the presence of kinase inhibitors or DMSO for 6 hours prior to lysis by sonication in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) supplemented with 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 units/ml Benzonase (Novagen), and Protease Inhibitor Set III (Calbiochem). Lysate protein concentrations were determined using

the Pierce Coomassie Plus assay reagent (Thermo Fisher). FLT3, HCK, FES, and SYK were immunoprecipitated from 1 mg of cell lysate with 2 µg of antibody and 25 µL of protein G-Sepharose beads (Invitrogen) overnight at 4 ^{o}C. Immunoprecipitates were collected by microcentrifugation and washed three times by resuspension in 1.0 ml of lysis buffer. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes and probed with the indicated antibodies followed by appropriate alkaline phosphatase-linked secondary antibodies. CDP-Star Western Blot Chemiluminescence Reagent (Perkin-Elmer) was used for detection.

2.4.4 In vitro kinase assays

Recombinant protein kinase assays were performed using the FRET-based Z'-LYTE Kinase Assay according to the manufacturer's instructions (Thermo Fisher Scientific). Recombinant FLT3-ITD, SYK, TAOK3, p38α, and FGR were purchased from Life Technologies, while recombinant FES (expressed in *E. coli*) and HCK (expressed in Sf9 insect cells) were purified as reported elsewhere (481, 482). The Tyr-2 peptide substrate was used for FLT3-ITD, SYK, FES, HCK, and FGR while the Ser/Thr 7 peptide was used for TAOK3 and the Ser/Thr 4 peptide was used for p38α. Kinase titration experiments were performed first to determine the kinase concentration required for approximately 90% phosphorylation of the peptide substrate with the ATP concentration set to the K_m value for each kinase. For inhibitor experiments, kinases were preincubated with inhibitors for 30 minutes, followed by addition of ATP and peptide substrate for 1 hour. Reactions were quenched by addition of development reagent, followed by incubation for an additional hour prior to fluorescence measurements on a SprectraMax M5 Multi-Mode Microplate Reader (Molecular Devices). Fluorophores were excited at a wavelength

of 400 nm; coumarin fluorescence and the fluorescein FRET signal were monitored at 445 nm and 520 nm respectively. Data were then analyzed as the emission ratio of the coumarin to FRET signals; note that unphosphorylated peptide substrate remaining in the reaction is selectively cleaved by a protease present in the development reagent. Data are then corrected for normalized control reactions run in the absence of ATP and normalized to the emission ratio obtained with a stoichiometrically phosphorylated control peptide. IC_{50} values were calculated using the non-linear regression analysis of the resulting concentration response curves fit by the GraphPad PRISM software (version 6.0).

2.4.5 RNA Isolation, cDNA preparation, qPCR

Total RNA was isolated from cell lines and primary AML bone marrow samples using the RNeasy Plus Mini Kit (Qiagen) according to manufacturer's instructions. cDNA was then prepared from 1.0 µg of RNA using the RETROscript Reverse Transcription Kit (Ambion) according to the manufacturer's instructions. Each 20 µL RT reaction was then diluted 10-fold with water of reaction mix, and 8 µL was used for each real-time quantitative PCR assay using SYBR Green detection (Qiagen) and gene-specific QuantiTect primers (Qiagen). The C_t values for each kinase was corrected for the corresponding GAPDH C_t value (ΔC_t) and then converted to a relative expression value by calculating the base 2 antilog. The relative expression value for each kinase within a given sample was then normalized to the average expression value for all TL02-59 target kinases examined within each sample to establish a relative kinase expression profile.

2.4.6 Lentivirus Production and shRNA Knockdown

Human 293T cells were co-transfected with MISSION shRNA-containing lentiviral vectors (Sigma-Aldrich) and MISSION Lentiviral Packaging Mix using X-tremeGENE 9 DNA Transfection Reagent (Roche) according to the protocol provided with the lentiviral packaging mix. A GFP lentiviral expression vector (Sigma-Aldrich) was used as a positive control for infection. All lentiviral supernatants were titered using the HIV-1 p24 Gag AlphaLISA Detection Kit (Perkin-Elmer) according to the manufacturer's instructions. Equal amounts of each virus (250 ng p24 equivalents) were used to infect MV4-11 cells (2 x 10^5 in one mL of culture medium) in the presence of 8 µg/mL of polybrene in a 48-well plate. Cells were centrifuged at 1350 x g for 4 hours to enhance infection efficiency and then incubated at 37 ^{o}C overnight. The next day the medium was replaced and cell growth was measured daily using the CellTiter Blue Assay. Three days after infection, the extent of kinase knockdown was determined by qPCR relative to cells infected with the GFP control virus. Kinase Ct values were corrected for GAPDH expression and the effect of knockdown on kinase expression is expressed relative to values from cells infected with the control GFP virus as normalizer ($\Delta\Delta C_t$ method).

2.4.7 Culture of Primary AML Bone Marrow Samples

Hs27 fibroblasts were mitotically inactivated by treating a confluent monolayer of cells in a 10 cm tissue culture dish with 10 µg/mL of mitomycin C (MMC; Sigma) for 6 hours and cryopreserved until use. De-identified primary human AML bone marrow samples were obtained from the Health Sciences Tissue Bank at the University of Pittsburgh as cryopreserved specimens. Primary AML cells were cultured using the method of Klco, et al. (483) as follows:

MMC-treated Hs27 cells (5 x 10^5) were plated in each well of a 6-well plate and allowed to attach overnight. Primary AML samples were thawed quickly, mixed with 10 mL of PBS, centrifuged at 1000 x g for 3 minutes and then resuspended in Iscove's modified Dulbecco's medium supplemented with 15% FBS, antibiotic-antimycotic, 2 mM L-glutamine, and human SCF (50 ng/mL), IL-3 (10 ng/mL), and IL-6 (20 ng/mL) (cytokines from Peprotech). The primary AML bone marrow samples were then gently plated on the MMC-treated Hs27 fibroblast feeder layers. After one day in culture in a 6-well plate, the primary AML bone marrow samples were transferred to fresh feeder layers on 48-well plates. Test compounds were then added and cultures were incubated for an additional 72 h prior to Cell Titer Blue assay as described above.

The *FLT3* genotype of each primary AML bone marrow sample was determined as follows. Genomic DNA (gDNA) was extracted from frozen bone marrow cells using the PureLink Genomic DNA Mini Kit (ThermoFisher) according to the manufacturer's instructions. *FLT3* exons 14-15, where ITD mutations common occur, were then amplified by PCR using the following primers: forward: 5'-GCAATTTAGGTATGAAAGCCAGC and reverse: 5'-CTTTCAGCATTTTGACGGCAACC. PCR reactions (15 µL) were run in DreamTaq Green Buffer (ThermoFisher) and contained 100 ng gDNA, 0.2 mM dNTPs, 1 µM of each primer, and 1.25 U DreamTaq polymerase. Denaturing, annealing, and extension steps were performed at 95 ^oC for 30 s, 56 ^oC for 60 s, and 72 ^oC for 30 s, respectively, for 35 cycles. Reactions also included an initial 9-minute denaturation step at 95 ^oC and a final 10-minute extension step at 72 ^oC. The resulting PCR products were run on 3% agarose gels to confirm the presence or absence of an ITD. Samples containing ITDs were purified using the Gel DNA Recovery Kit (Zymogen)

and sequenced. Genomic DNA was isolated from THP-1 cells (wild-type *FLT3*) and MV4-11 cells (FLT3-ITD^{+}), and included in negative and positive control reactions, respectively.

2.4.8 Mouse AML Xenograft Model

Prkdc^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice were obtained from Jackson Laboratories and injected with human MV4-11 AML cells (10^7) suspended in PBS via the tail vein. Control mice received a tail vein injection of PBS only. All mice were left for 14 days to allow for AML cell engraftment to spleen and bone marrow. After 14 days the mice received daily treatments of vehicle, 1 mg/kg TL02-59, 10 mg/kg TL02-59, or 10 mg/kg sorafenib for 21 days by oral gavage. All compounds were suspended in 90% saline (Fisher), 5% solutol HS-15 and 5% N-methyl-2-pyrrolidone (Sigma), followed by vortexing and sonication to make 1 mg/mL stock solutions. TL02-59 fully dissolves in this combination of solvents while sorafenib remains as a suspension. At the end of the study, all mice were sacrificed and blood, bone marrow and the spleen were collected. Histology was performed on sections of the spleen and trimmed femurs after decalcification in 10% formic acid.

2.4.9 Flow Cytometry

Bone marrow, spleen, and blood samples from control and drug-treated mice were analyzed for the presence of human AML cells by flow cytometry following staining with human CD45-FITC (Clone HI30, BD Biosciences), mouse CD45-PE (Clone 30-F11, BD Biosciences), and human CD33-APC (Clone WM53, BD Biosciences) antibodies according to the manufacturer's instructions. Red blood cells present in the bone marrow and spleen were lysed in 10 mM

$KHCO_3$, pH 7.4, 155 mM NH_4Cl, and 130 μM EDTA prior to antibody staining in FACS buffer (PBS, pH 7.5, 3% FBS, 0.02% NaN_3, 1 mM EDTA). Twenty-five μL of blood from each animal was also stained with the three antibodies and then fixed and lysed using a fix/lyse solution (BD Biosciences). Cells were then analyzed on a BD Accuri C6 Flow cytometer and resulting data were evaluated using FlowJo software.

3.0 OVERALL DISCUSSION

3.1 SUMMARY OF FINDINGS AND SIGNIFICANCE

Despite unprecedented knowledge of the cytogenetic abnormalities and genetic mutations that drive AML, the overall survival rate is only twenty-five percent, with elderly patients faring worse than younger patients. Furthermore, standard therapy for all AML patients, with the exception of APL patients, is still high dose cytotoxic chemotherapy which most elderly patients are ineligible to receive. The standard chemotherapy treatment used today was originally described in 1973 and has undergone only minor changes since that time. Relapse rates in the first three years of diagnosis are around fifty percent and for most patients, hematopoietic stem cell transplant offers the only hope of a cure.

AML has one of the lowest median mutation rates of all human cancers and a significant number of recurrent mutations have been identified through genome-wide sequencing studies. To date, the most commonly mutated protein in AML is the FLT3 receptor tyrosine kinase which is mutated in approximately thirty percent of AML cases. In about twenty-five percent of patients, *FLT3* is mutated through an ITD, which produces a constitutively active kinase that activates a number of signaling pathways involved in cell survival and proliferation. The activation of many of these pathways is co-dependent on a small number of essential cytoplasmic tyrosine kinases, including HCK, FGR, LYN, SYK and FES.

The development of clinically active FLT3 inhibitors is of major interest for the treatment of AML cases with activating mutations in this receptor tyrosine kinase. However, FLT3 inhibitors tested in clinical trials thus far have had limited success, especially as single agents. The first-generation inhibitors were designed to target a number of receptor tyrosine kinases and not FLT3 specifically, resulting in lower binding affinities and toxicities due to off-target effects. Second-generation inhibitors have had significantly more success as single agents due to improved affinity and specificity for FLT3. However, this specificity left these inhibitors vulnerable to the development of resistance, most commonly through inhibitor resistance mutations in the tyrosine kinase domain.

Since the identification of the clinical utility of imatinib, most drug discovery has focused on a single drug targeting a single kinase. This selectivity is supposed to limit off-target toxicities due to inhibition of multiple kinases. However, these inhibitors are often subject to the development of resistance, particularly in cancer treatment (484). Furthermore, due to targeting a single kinase, upregulation of collateral signaling pathways occurs as an alternative mechanism of resistance. The combination of highly selective inhibitors has emerged as a possible mechanism to reduce resistance and increase potency. However, combination of toxicities, interactions between compounds, and the difficulties in finding proper dosing regimen are all potential pitfalls of using combination therapies (485). To bypass these pitfalls, the concept of polypharmacology has recently emerged in drug discovery. In this case, a single inhibitor is purposefully designed to target a small set of kinases. This eliminates the need for combination therapies, reduces the rate of resistance, increases potency, and some studies have indicated that activity at multiple therapeutic targets can reduce toxicities (486). Notably, many kinase inhibitors on the market inhibitor multiple targets, and in some cases these ‘off-target’ effects are

critical to their efficacy. However, the main difficulty with polypharmacology is identifying the proper combination of targets to increase efficacy and reduce toxicities.

Due to the lack of efficacy and the quick development of resistance of the clinical FLT3 inhibitors, we decided to take a 'polypharmacological' approach to treating FLT3-ITD^{+} AML by targeting FLT3 and its best defined downstream kinase partners; HCK, SYK and FES. In the first Aim of my thesis we screened a small library of compounds and identified TL02-59, a kinase inhibitor with efficacy against FLT3, HCK, SYK, and FES. The potency of TL02-59 led us to wonder which targets are most critical for efficacy and whether any novel targets could be discovered. Therefore, in the second Aim of my thesis we performed kinome-wide profiling of TL02-59 and explored expression of the target kinases in primary AML bone marrow samples.

3.1.1 Polypharmacology screening approach yields a highly active AML-associated tyrosine kinase inhibitor, TL02-59

To test our polypharmacology hypothesis, we screened a small library of N-phenylbenzamide compounds and identified TL02-59, a compound with three-digit nanomolar activity against FLT3, HCK, SYK and FES in vitro. To further explore the efficacy of this compound in cells, we tested it against and THP-1 and MV4-11 cells. TL02-59 was effective in the picomolar range against MV4-11 cells but had no effect on THP-1 cells. The fact that the cell line potency is lower than the in vitro kinase potency suggests that the cellular potency results from the combination of targeting all four kinases. Notably, all four kinases are expressed and active in MV4-11 cells, and treatment with TL02-59 at just 100 nM inhibits all four kinases. Furthermore, the lack of potency against THP-1 cells suggests that a kinase fingerprint of therapeutic response

exists with this compound and that, at least in cell lines, the potency we observed in MV4-11 cells was not due to toxicity.

To validate TL02-59 in a more AML-relevant context we tested the potency of the compound against a panel of 26 primary AML bone marrow samples: 13 with *FLT3*-ITD mutations and 13 with wild-type *FLT3*. We discovered a range of potencies for growth inhibition of these samples, from 77 nM to greater than 3 μM. The range of potencies did not surprise us as the samples varied in cytogenetic abnormalities, chromosomal changes, age and genetic alterations. However, the one surprise from this experiment was that the four most sensitive samples were *FLT3*-WT. I will describe in section 3.1.2 one potential explanation for why these samples were so sensitive, but it suggests that TL02-59 could be applicable to other subsets of patients, not just FLT3-ITD^{+} patients. Further genomic sequencing of these samples to determine concurrent genetic mutations could allow us to further define the subgroup of patients that would respond.

Finally, we tested TL02-59 efficacy in vivo using a mouse xenograft model of AML. Oral TL02-59 at 10 mg/kg once per day for just three weeks completely eliminated xenografted human MV4-11 AML cells from the peripheral blood and spleen. Additionally, we observed a sixty percent reduction in bone marrow involvement after only three weeks of treatment. These data demonstrate that TL02-59 is orally bioavailable and efficacious in this mouse model. Furthermore, TL02-59 is capable of substantially reducing bone marrow engraftment as a single agent. Future experiments will address whether higher doses or a longer treatment schedule will result in complete eradication of the engrafted AML cells. We also need to follow the survival of the mice following cessation of drug treatment, and look for leukemia relapse of drug-resistant cells. Notably, a subset of the mice exhibited potential toxicity to TL02-59, as displayed by

weight loss. However, in most cases cutting the dose in half for a day or two allowed the mice to regain weight and then continue on the normal dosing schedule. We delivered TL02-59 once daily by oral gavage so one possibility to eliminate this weight loss issue is to determine a dosing schedule that allows the mice a couple of off days. This would likely reduce toxicity associated with daily dosing. Even though we noticed some weight related toxicity, upon gross examination neither the liver nor the kidneys displayed any abnormalities, suggesting that weight loss was not an effect of toxicity to either of those organs.

In summary, taking a polypharmacological approach we identified a highly active compound with efficacy in vitro, in cell lines, in primary AML bone marrow samples, and in an in vivo model of AML. While many dual FLT3 kinase inhibitors are under development, this is the first kinase inhibitor to our knowledge designed specifically to target FLT3 and three linked tyrosine kinases; HCK, SYK and FES. This inhibitor displays greater activity in cells than in targeting either of the kinases in isolation, suggesting that the potency comes from inhibiting the combination of kinases. A kinase inhibitor targeting multiple AML-associated kinases holds the potential to significantly reduce the rate of acquired resistance as it would be necessary for multiple kinases to develop inhibitor resistance mutations. Furthermore, since a multitude of signaling pathways are presumably inhibited by this compound, the ability for AML to upregulate collateral pathways will be diminished. As mentioned earlier, one mechanism of resistance to FLT3 inhibitor is upregulation of collateral signaling pathways, particularly the PI3K/AKT and the MEK/ERK pathways. Furthermore, upregulation of anti-apoptotic proteins is also seen. Knockdown of FES and LYN inhibits some PI3K pathway members, FES knockdown reduces BCL-xL expression and SYK is implicated in the activation of ERK downstream of FLT3. Assuming that each of these tyrosine kinases helps to activate bypass pathways in FLT3

resistant cells, inhibition with TL02-59 could reduce this mechanism of resistance. Finally, the odds of each kinase being expressed and active in cells other than AML blasts is low, thus TL02-59 holds the potential to significantly limit treatment related toxicity.

3.1.2 Expression and identification of TL02-59 target kinases in AML

The potency of TL02-59 against MV4-11 cells pushed us to investigate the full range of TL02-59 target kinases. We hypothesized that inhibition of other kinases beyond FLT3, HCK, SYK and FES were contributing to TL02-59 efficacy. To begin to explore other targets, we performed a KINOMEscan assay and discovered that TL02-59 was remarkably selective, targeting only 23 kinases out of the 456 screened. Included in those twenty-seven kinases were all four of the AML-associated kinases we used to identify TL02-59. After obtaining 26 primary AML bone marrow samples, we performed quantitative real-time RNA expression analysis for each of the 27 TL02-59 target kinases. As mentioned in section 3.1.1, half of the primary AML bone marrow samples were *FLT3*-WT and the other half were *FLT3*-ITD^{+}. Surprisingly, the expression pattern of all 27 kinases was almost identical between the *FLT3*-WT samples and *FLT3*-ITD samples. In each case the same 15 kinases were constitutively expressed. This suggests that although these AML samples are driven by different mechanisms, a shared set of kinases contributes to oncogenesis. Using IC_{50} values from the TL02-59 growth inhibition curves described in section 3.1.1 we correlated expression of each kinase with response across all 26 samples. We discovered that relative expression of HCK, FGR, LYN and SYK positively correlated with TL02-59 response. Interestingly, each of the four AML samples that were most sensitive to TL02-59 expressed high levels these four kinases, and were wild-type for *FLT3*. Notably, they also expressed high levels of FLT3, even though it did not correlate with response.

These data suggest that response to TL02-59 is determined by expression of a set of kinases and not expression of one particular kinase, validating the polypharmacology approach to tyrosine kinase inhibition in AML. Furthermore, since the expression of each kinase is important for efficacy, predicting response to TL02-59 would involve a model that incorporates the expression of each of these kinases. Finally, I will note that one Src-family kinase, YES, showed a negative correlation with TL02-59 response even though it was not very highly expressed in any of the samples. This suggests that YES may actually play an opposing role to the other SFKs in AML blasts, raising the possibility that inhibition of some kinases may be detrimental to efficacy. For example, YES may contribute to growth suppression, to balance the growth and survival signals of other kinases, or fulfill a role in differentiation. Support for this idea comes from previous work in our group, where SRC and YES were shown to have opposing roles in embryonic stem cell growth and differentiation, despite their close phylogenic relationship (487, 488).

Given that three members of the Src kinase family, HCK, LYN and FGR, are correlated with response to TL02-59 we wanted to determine if inhibition of SFK activity alone accounted for the potency of TL02-59. To investigate this hypothesis, we tested A-419259, a well-known pyrrolopyrimidine pan-SFK inhibitor, against MV4-11 cell proliferation and determined an IC_{50} value of 37 nM. That potency is approximately thirty-fold lower than what we observed for TL02-59 in this cell line, suggesting that TL02-59 inhibits targets beyond the SFKs that are critical to potency. To identify other kinases that may represent TL02-59 targets in AML, we performed KINOMEscan analysis on A-419259 and discovered it was even more selective than TL02-59, targeting just 19 kinases out of 468 tested. Upon comparing KINOMEscan profiles, we identified four kinases, SYK, p38α, TAOK3 and STK10, which were inhibited by TL02-59 but not A-419259 and constitutively expressed in primary AML bone marrow cells. While SYK

and p38α have defined roles in AML pathogenesis, neither TAOK3 nor STK10 has been examined in the context of AML. To investigate their role in MV4-11 cell growth, we performed shRNA knockdown of both kinases in MV4-11 cells. While knocking down STK10 had no effect on MV4-11 cells, knockdown of TAOK3 completely inhibited cell growth. These data suggest that inhibition of some Ser/Thr kinases, not just tyrosine kinases, contributes to TL02-59 potency. Among the few studies that have investigated TAOK3, two functions have been discovered: inhibition of JNK and activation of p38α in response to genotoxic stresses. JNK signaling is known to be pro-apoptotic, so inhibiting TAOK3 could increase apoptotic signaling by relieving JNK inhibition in AML cells. Furthermore, p38α signaling in response to genotoxic stresses is known to induce pro-survival signaling which is blocked directly by TL02-59. In either case, inhibition of TAOK3 could reduce anti-apoptotic signaling, pushing the cells toward apoptosis. This raises the intriguing possibility that TL02-59 could synergize with existing chemotherapy agents used in AML to induce apoptosis. As it becomes increasingly clear that combination therapy is more likely to be efficacious in AML, combining multitargeted kinase inhibitors with existing chemotherapy drugs is likely to be advantageous (388, 397, 399). A related advantage is the potential for the addition of a potent multi-targeted compound to reduce the necessary chemotherapy dose. This would reduce toxicities related to treatment.

In summary, we explored the full range of kinase targets for TL02-59 in AML and discovered that expression HCK, LYN, FGR and SYK were critical for efficacy. Furthermore, comparative KINOMEscan analysis with A-419259 suggested that FES as well as the Ser/Thr kinases p38α and TAOK3 are also important targets for TL02-59. Our discovery that the expression of these TL02-59 target kinases is remarkably consistent across a broad array of AML genotypes suggests that TL02-59 could be therapeutic for a broad spectrum of AML patients.

Furthermore, HCK was recently identified as overexpressed in LSCs versus HSCs suggesting it is a potential target for LSC therapy. Additionally, one study also identified LYN as being expressed and active in the LSC compartment. Since TL02-59 efficacy correlates most closely with HCK and LYN expression, it is possible that TL02-59 is capable of eliminating LSCs, the main cause of relapse in AML patients. Finally, given the role of TAOK3 in genotoxic stress signaling and apoptosis, TL02-59 seems likely to synergize with existing chemotherapeutic agents used in AML treatment.

3.2 FUTURE DIRECTIONS

3.2.1 Determine efficacy of TL02-59 in FLT3 inhibitor-resistant cells

Clinical trials of FLT3 inhibitors have largely produced underwhelming results. Despite high hopes, single agent therapy has produced low response rates and quick relapse rates. Quizartinib is the most efficacious FLT3 inhibitor to date, with a 53% response rate as a single agent, but responses are limited in duration from a few weeks to a few months due to the development of resistance. The main cause of resistance is tyrosine kinase domain mutations that inhibit quizartinib binding. Most commonly these mutations occur as D835Y, Y842C or F691L mutations. Midostaurin is a staurosporine derivative that again produces only transient clinical responses. Inhibitor resistance mutations to midostaurin occur most commonly as N676D, F691I, and G697R mutations.

One potential advantage of the polypharmacology of TL02-59 is that it is not dependent on one single kinase for its potency. While we provided evidence to suggest the dependency of

TL02-59 on multiple kinases, we never directly assessed the potency of TL02-59 in the presence of inhibitor resistance mutations. As mentioned above, FLT3 inhibitor resistance mutations pose a significant clinical challenge so I propose that future studies investigate the efficacy of TL02-59 against cells expressing inhibitor resistance mutations. One caveat to these experiments is that the cell line we choose must express HCK, FGR, LYN, SYK, and FES; otherwise we will simply be assessing the ability of TL02-59 to inhibit FLT3 inhibitor resistance mutations and not addressing the combination of kinases. The one cell line that meets these criteria is MV4-11 cells. Using CRISPR/Cas9 it would be possible to make each of the FLT3 inhibitor resistant mutations mentioned above. I hypothesize that TL02-59 would lose some potency as inhibition of each kinase is important for its overall effect. However, TL02-59 should still be a potent inhibitor since it could inhibit all of its other targets. Subsequent experiments could investigate the role of inhibitor resistant mutations in each of the other AML-associated kinases, HCK, FGR, LYN, SYK, and FES. Again, I hypothesize that in each case the TL02-59 would lose some potency but still effectively inhibit MV4-11 cells since it can still inhibit its other known targets. While FLT3 inhibitor resistant mutations would be focused on those that are clinically identified, no clinically relevant inhibitor resistant mutations have been identified in the FLT3-associated kinases. To identify potential inhibitor resistant mutations, we could model TL02-59 binding into the kinase domains of each of the kinases and determine critical residues for binding. These residues would then have to be tested empirically through the purification and subsequent in vitro testing of kinase domains containing the inhibitor resistant mutations.

A complimentary unbiased approach for identifying the rate of resistance and resistance mechanisms to TL02-59 would be to grow MV4-11 cells in the presence of sub-lethal doses of TL02-59. The dosage could continually be increased by small increments selecting for the

inhibitor resistant clones. At the conclusion of this experiment, RNAseq could be performed on the resistant clones to identify resistance mechanisms, whether it is upregulation of a distinctive pathway or acquisitions of inhibitor resistant mutations. This experiment would also determine the rate of resistance against TL02-59. I hypothesize that if done alongside quizartinib, TL02-59 would display a reduced rate of resistance compared to quizartinib, due to the multi-targeted nature of the compound. Furthermore, while quizartinib resistance would appear in the form of FLT3 inhibitor resistant mutations, the mechanism(s) for TL02-59 resistance would be more diverse as the compound is not dependent on one target for efficacy.

3.2.2 Determine potency of TL02-59 against LSCs in vitro and in vivo

Elimination of LSCs remains a clinical challenge to AML therapy, as LSCs are the main cause of relapse in patients. Elimination of LSCs would significantly reduce relapse rates of AML, which are approximately 50-70%. Furthermore, LSCs are one of the reasons bone marrow transplants fail, suggesting that elimination of LSCs could increase the effectiveness of bone marrow transplants. Recently, HCK was identified as differentially expressed in LSCs versus HSCs, suggesting it may provide a viable target for anti-LSC therapy. A known inhibitor of HCK, A-419259, reduced the LSC population in a bone marrow transplant model of AML. Furthermore, LYN was also identified as expressed and active in the LSC compartment. In this thesis I identified TL02-59 as a more potent inhibitor than A-412959 yet it maintains potent activity against HCK and all other myeloid Src-family kinases. If TL02-59 is capable of eliminating LSCs, it could not only treat AML but also significantly reduce relapse rates.

LSCs are often defined by their cell surface markers and most commonly associated with the $Lin^{-}CD34^{+}CD38^{-}$ population. To begin testing the efficacy of TL02-59 against LSCs, Lin^{-}

$CD34^{+}CD38^{-}$ cells could be sorted out of primary AML patient samples, and cultured in vitro using established methods. Simple experiments determining LSC survival in the presence of TL02-59 could then be performed as a critical first step. It would also be very interesting to repeat TL02-59 target kinase expression profiling on LSCs derived from patient bone marrow, to determine if they are similar to those already reported here for unfractionated bone marrow specimens. While LSC assays would provide proof-of-concept for TL02-59 action in vitro, the ultimate determination of LSC elimination would need to occur in patient derived xenograft (PDX) models of AML. PDX models require the xenotransplantation of primary AML samples into immunocompromised mice; which proliferate and recapitulate the malignancy observed in the AML source. PDX models of AML would be treated with TL02-59 for a specified period of time. Then the same number of viable $hCD45^{+}$ bone marrow cells from the primary recipient would be transplanted into a naïve secondary recipient mouse. The ability of the transplanted cells to engraft the secondary recipient would determine if LSCs are eliminated by TL02-59 treatment.

HCK and LYN are both critical targets of TL02-59 and are expressed and active in LSCs, suggesting that TL02-59 has the potential to eliminate LSCs. Elimination of LSCs would significantly reduce the rate of relapse in patients and improve clinical outcomes. If TL02-59 is capable of eliminating LSCs it would provide a very strong rationale for further clinical development of this compound.

3.3 CONCLUDING REMARKS

Significant breakthroughs in our understanding of AML have occurred over the last 40 years. However, only limited changes have been made to standard of care treatment for AML and these changes have resulted in only minimal outcome improvement. Outcomes for AML remain dismal compared to other types of leukemia which have made significant strides in treatment response rates. The overall survival rate for AML is approximately 25%. Many targeted treatment options are currently undergoing evaluation in clinical trials, including FLT3 inhibitors. It was initially hoped that FLT3 inhibitors would revolutionize treatment of AML the same way that imatinib revolutionized CML treatment. However, first-generation FLT3 inhibitors have not produced durable clinical responses in patients and have been beset with toxicity issues. Second-generation FLT3 inhibitors have significantly improved clinical responses, with approximately half of patients achieving a clinical response. However, the duration of response is limited by the development of resistance. Furthermore, LSCs still remain a significant challenge to any AML therapy and ultimately are the cause of relapse in patients. Treatment of FLT3 still holds major promise for therapy in AML; however, resistance must be reduced or eliminated. In this context, we set out to identify a potent inhibitor of FLT3-ITD$^+$ AML that is not solely dependent on FLT3 inhibition for its activity. We identified TL02-59, a potent inhibitor of FLT3-ITD$^+$ cell growth in vitro and in vivo. The mechanism of action for TL02-59 appears to be driven not only through FLT3-ITD inhibition but also through inhibition of the SFKs, SYK, FES, and some Ser/Thr kinases. Given that potency depends on the inhibition of multiple kinases, TL02-59 holds the potential to reduce the rate of resistance. Furthermore, TL02-59 might also inhibit cells resistant to current FLT3 inhibitors which only depend on FLT3 inhibition. Notably, we identified few differences in the expression of TL02-59

target kinases in FLT3-WT versus FLT3-ITD bone marrow samples, including samples with very complex cytogenetic profiles, suggesting that TL02-59 will potentially be clinically active beyond the FLT3-ITD^{+} subset of patients. Finally, two targets of TL02-59 have been identified as critical for LSCs survival in other work (HCK and LYN), suggesting that TL02-59 could eliminate LSCs as well as bulk AML blasts. The data described in this study and the potential for reduced resistance and LSC therapy support further clinical development of TL02-59 for the treatment of AML.

APPENDIX A

DUAL INHIBITION OF FES AND FLT3 TYROSINE KINASE ACTIVITIES POTENTLY BLOCKS AML CELL PROLIFERATION

A.1 INTRODUCTION

Acute myelogenous leukemia (AML) is the most common hematologic malignancy in adults (207). The usual standard of care for AML is cytotoxic chemotherapy that has changed little in the last 40 years, leading to a stagnant overall survival rate of approximately 25% (232, 448). While numerous cytogenetic abnormalities and mutations have been identified in AML, the receptor tyrosine kinase FMS-like tyrosine kinase 3 (*FLT3*) is mutated in approximately 30% of all AML cases (8, 422). *FLT3* mutations occur as internal tandem duplications (ITDs), in-frame duplications of varying length within the juxtamembrane region, or as tyrosine kinase domain (TKD) point mutations, most commonly at position D835 within the activation loop of the kinase (231, 421). Both types of mutations result in a constitutively active kinase that drives AML pathogenesis. *FLT3*-ITD mutations in particular are associated with a poor prognosis relative to other forms of AML (385, 450).

The FES tyrosine kinase belongs to a unique family of tyrosine kinases and is highly expressed in hematopoietic cells, particularly in the myeloid lineage (378, 383). Originally

identified as the cellular homolog of the transforming oncogene present in several avian and feline sarcoma viruses, FES kinase activity is tightly regulated in cells (489). FES normally functions as a signaling mediator downstream of growth factor, cytokine and immune cell receptors and is involved in hematopoietic cell growth, survival and differentiation as well as innate immune responses (490).

Voisset and colleagues have implicated FES as an important downstream signaling partner for FLT3-ITD$^+$ AML (384). They discovered that FES was constitutively active in two FLT3-ITD$^+$ AML cell lines, MV4-11 and MOLM-14, as well as in primary AML bone marrow samples. Knockdown of FLT3 in both cell lines significantly reduced FES kinase activity and decreased FLT3-ITD$^+$ AML cell growth to a similar extent as knockdown of FLT3-ITD itself. Furthermore, the activity of many of the oncogenic signaling mediators for FLT3-ITD, particularly STAT5 and PI3K, were also substantially decreased in FES-knockdown AML cells. Finally, treatment of primary AML patient samples with the FLT3 inhibitor, SU5416, reduced both FLT3 and FES activation. These data strongly suggest that FES is essential for the activation of signaling pathways downstream of FLT3-ITD, and that inhibition of FES kinase activity may be therapeutically beneficial in AML. In the present study, we explored the role of FES kinase activity in AML cell growth using a panel of ATP-site inhibitors selective for FES, selective for FLT3, or with dual activity for FES and FLT3. Our results show that inhibition of FES kinase activity alone is sufficient to block AML cell growth, and that inhibitors with dual activity against both FLT3-ITD and FES are even more active, with IC50 values in the low nM range.

A.2 RESULTS

To test the hypothesis that inhibition of FES kinase activity may be of therapeutic relevance to AML, we first investigated the effects of FES kinase inhibitors from three distinct chemical classes (Figure 14A) previously identified by our lab (481) on the growth of AML cell lines either wild-type for *FLT3* (THP-1) or with an ITD (MV4-11). All of the FES kinase inhibitors tested blocked the growth of the MV4-11 cells in a concentration-dependent manner, while having minimal effects on the growth of THP-1 cells (Figure 14B). However, inhibitors from the diaminopyrimidine (TAE-684) and pyrrolopyridine (HG7-92-01) classes were significantly more potent, with IC_{50} values of 90 and 36 nM, respectively, than the two pyrazolopyrimidines (WZ4-49-1 and WZ4-49-8), which yielded IC_{50} values in the single-digit micromolar range. Notably, both TAE-684 and HG7-92-01 were significantly more potent than the FLT3-selective inhibitor, tandutinib, in MV4-11 cells.

Next, we investigated whether FES inhibitors were able to induce programmed cell death in FLT3-ITD$^+$ AML cells. MV4-11 and THP-1 cells were treated with each inhibitor at a concentration of 1.0 µM, and induction of apoptosis was assayed as Caspase 3/7 activity three days later. All four compounds induced significant apoptosis under these conditions in MV4-11 cells but not in THP-1 cells, which is consistent with the observation that FES is constitutively active solely in MV4-11 cells (384) (Figure 14C). However, both TAE-684 and HG7-92-01 induced significantly more apoptosis under these conditions than the two pyrazolopyrimidines.

Data presented so far show that all four FES inhibitors were able to induce growth arrest and apoptosis in FLT3-ITD$^+$ AML cells. However, the differences in potency across the compounds suggested that inhibition of other kinase targets, including FLT3-ITD itself, may be account for the higher potency of the diaminopyrimidine and pyrrolopyridine inhibitors.

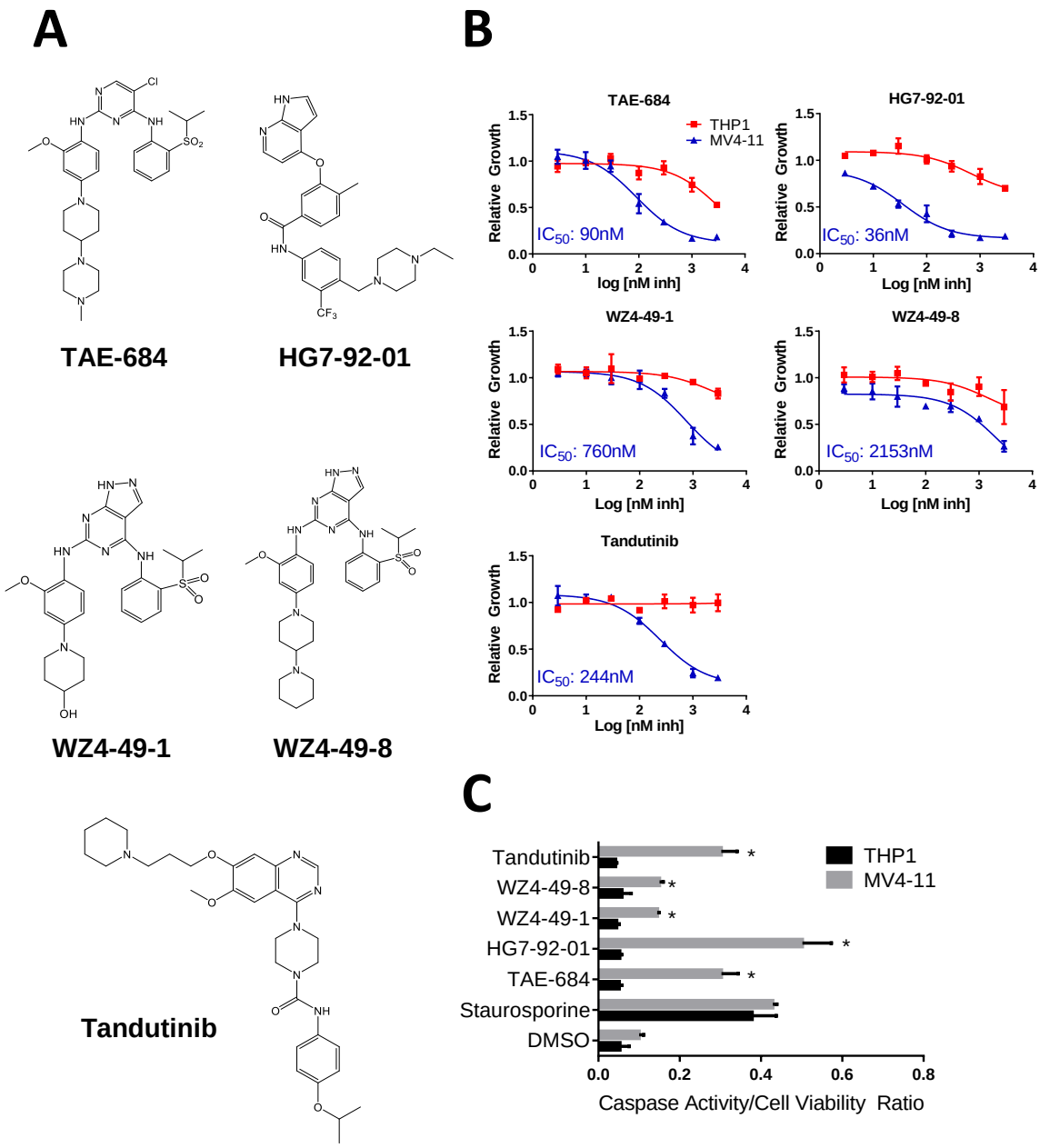


Figure 14: FES tyrosine kinase inhibitors induce growth arrest and apoptosis in the FLT3-ITD$^+$ AML cell line, MV4-11.

A) Chemical structures of the tyrosine kinase inhibitors used in this study. B) Dose-response curves of MV4-11 and THP-1 AML cells treated with varying doses of the inhibitors for 72 hours. Cell viability was measured using the Cell Titer Blue Assay (Promega). C) Apoptosis was measured after 72 hours of treatment with each of the inhibitors at 1 μM using the Apo-ONE Caspase 3/7 Assay (Promega). All experiments were performed in triplicate. Asterisks indicate statistical significance with $p < 0.05$.

Previous KINOMEscan analysis of the kinome-wide target specificity profiles of these four compounds support this idea (491). KINOMEscan data show that the more potent compounds, TAE-684 and HG7-92-01, bind to a wider range of kinases (including FLT3) compared with the two less potent pyrazolopyrimidines, which are more selective for FES and do not bind to FLT3. To compare the inhibitory profiles of each compound for FES and FLT3 directly, we performed in vitro kinase assays using recombinant purified full-length FES and FLT3 kinase domains (wild-type, and the AML-associated mutants, FLT3-ITD and FLT3-D835Y). As shown in Table 8, the most potent inhibitors of MV4-11 cell growth and survival inhibited both FES and FLT3 with similar potencies. For example, the pyrrolopyridine analog, HG7-92-01, inhibited both FES and wild-type FLT3 in the 500 nM range, and also potently inhibited MV4-11 cell growth (IC_{50} of 36 nM). This compound is also a potent inhibitor of the FLT3-ITD kinase domains, with an IC_{50} value of 149 nM. A very similar inhibitory profile was also observed for TAE-684 with FES, wild-type FLT3, and FLT3-ITD. In contrast, the pyrazolopyrimidine analog, WZ4-49-8, inhibited both forms of FES in the 700 nM range, but was much weaker against the wild-type and ITD forms of FLT3 (about 20-fold lower potency). This difference may explain the lower potency of WZ4-49-8 for inhibition of MV4-11 cell growth (IC_{50} value of 2.1 μM). The closely related pyrazolopyrimidine analog, WZ4-49-1, showed a similar inhibitory profile as WZ4-49-8, although the difference in potency between full-length FES and FLT3-ITD was only about 3-fold. This compound produced an IC_{50} value for MV4-11 growth inhibition of about 800 nM, suggesting that it may target not only FES but also FLT3 to some extent.

We next investigated whether transformation with FLT3-ITD is directly responsible for activation of FES in a myeloid cell background. For these studies, we used the human myeloid leukemia cell line TF-1, which expresses endogenous FES proteins but not FLT3. These cells

are dependent on GM-CSF for growth and survival, and have been previously shown to undergo transformation to a cytokine-independent phenotype following introduction of another transforming oncogene BCR-ABL (492). To determine if TF-1 cells were also susceptible to transformation by active mutants of *FLT3* associated with AML, we transduced TF-1 cells with retroviral vectors for wild-type *FLT3*, a *FLT3*-ITD mutant identical to the one found in MV4-11 cells, and an active *FLT3* point mutant, D835Y. As shown in Figure 15A, TF-1 cells transduced with the ITD and D835Y mutants of *FLT3* underwent transformation to cytokine independent

Inhibitors		MV4-11 Cells (IC_{50}, nM)	In vitro Kinase Assay (IC_{50}, nM)			
Compound	**Class**		**FES**	**FLT3-WT**	**FLT3-ITD**	**FLT3-D835Y**
TAE-684	Diaminopyrimidine	90	569	536	120	218
HG7-92-01	Pyrrolopyridine	36	527	536	149	3,646
WZ4-49-1	Pyrazolopyrimidine	760	494	3,138	1,390	642
WZ4-49-8	Pyrazolopyrimidine	2153	706	13,997	14,345	2,347
Tandutinib	Piperazinylquinazoline	244	>100,000	580	788	7,370

Table 8: In vitro inhibitor specificity profiles with recombinant FES and FLT3 kinase domains.

In vitro kinase assays were performed with recombinant full-length FES as well as wild-type (WT), ITD and D835Y forms of the FLT3 kinase domain (Z'-Lyte assay; Life Technologies). Each inhibitor was tested in triplicate over a range of concentrations with the ATP concentration set to the K_m value for each kinase. IC_{50} values were calculated by non-linear regression analysis of each of the dose-response curves. Also included in the table are the IC50 values for inhibition of MV4-11 AML cell growth, calculated from the dose-response curves shown in Figure 1. In vitro kinase data were produced by Dr. Sabine Hellwig, a former postdoctoral fellow in our group.

growth, while cells expressing wild-type *FLT3* did not.

We next explored the expression and activity of both FLT3 and FES kinases in control and *FLT3*-transformed TF-1 cell populations. For these experiments, we immunoprecipitated each kinase protein, and then assessed activity by immunoblotting FES with antibodies for the activation loop phosphotyrosine (pTyr713) and FLT3 with general antiphosphotyrosine antibodies. As shown in Figure 15B, FLT3 kinase protein was readily detected in the three TF-1 cell populations transduced with *FLT3* retroviruses, but was absent in the parental TF-1 cells as expected. However, only the ITD and D835Y forms of FLT3 showed reactivity with the antiphosphotyrosine antibody, indicating that they are constitutively active. These findings are consistent with the GM-CSF-independent outgrowth of these two cell populations. Endogenous FES kinase protein was also readily detected in all four cell populations. Interestingly, endogenous FES was active only in TF-1 cells transformed with *FLT3*-ITD, and not in the *FLT3*-D835Y cells or either of the control cell populations. This observation suggests important structural differences between these two active forms of FLT3 in terms of downstream effector kinase coupling (see Discussion).

We next investigated whether transformation of TF-1 cells with active forms of FLT3 resulted in sensitization to growth suppression by the panel FES inhibitors. Results obtained with TF-1 cells expressing FLT3-ITD recapitulated the effects seen in the FLT3-ITD$^+$ AML cell line, MV4-11. As shown in Figure 16, TAE-684 and HG7-92-01 treatment resulted in potent inhibition of TF-1/FLT3-ITD cell growth, with IC_{50} values of 160 and 59 nM, respectively. The pyrazolopyrimidine FES inhibitors, WZ4-49-1 and WZ4-49-8, which showed lower activity against FLT3-ITD than FES in vitro, inhibited TF-1/FLT3-ITD cell growth with IC_{50} values of

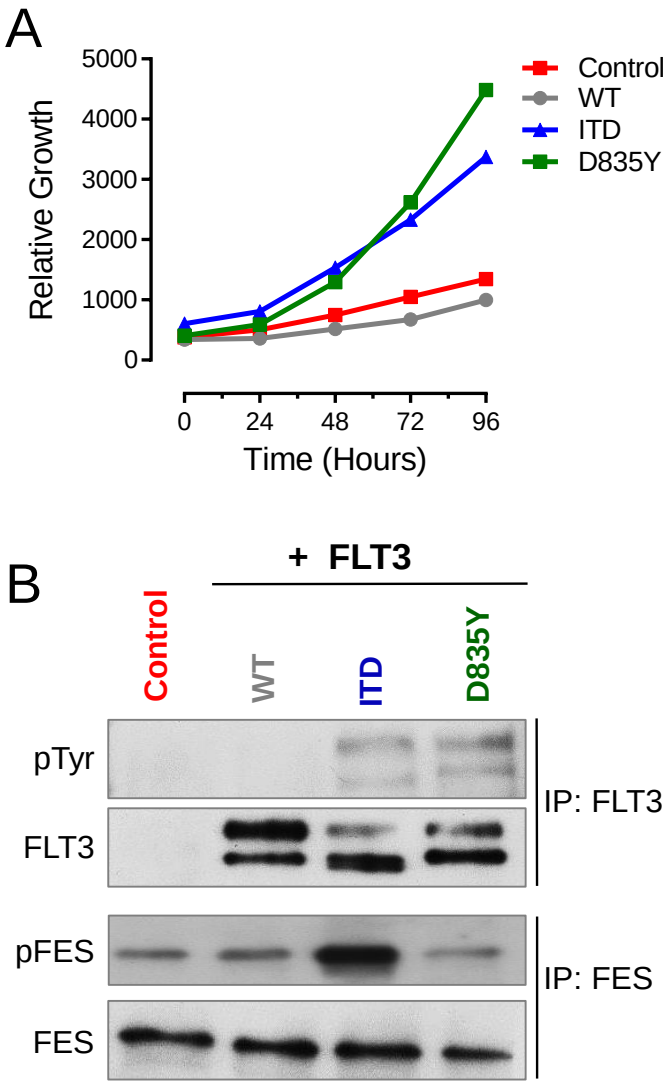


Figure 15: Active mutants of *FLT3* (FLT3-ITD and FLT3-D835Y) transform human TF-1 cells to cytokine independent growth.

A) The human myeloid TF-1 cells were transduced with recombinant retroviruses carrying wild-type (WT), ITD, or D835Y forms of *FLT3*. Each of the TF-1 cell populations, along with parental TF-1 cells, were cultured in the absence of GM-CSF, and viable cell outgrowth was monitored by Cell Titer Blue assay (Promega) over the course of 96 hours. The mean fluorescence values from triplicate cultures are shown. B) FES and FLT3 kinase proteins were immunoprecipitated from each of the cell populations shown in A, followed by immunoblotting with antibodies to phosphotyrosine (pTyr), the FES activation loop phosphotyrosine (pFES), as well as the FLT3 and FES proteins. This experiment was performed in triplicate with comparable results, and a representative example is shown.

cells expressing wild-type *FLT3* or the parental cell line. Comparison of the inhibitor selectivity profiles against the FES and FLT3-ITD kinases in vitro (Table 8) provides insight regarding the role of each kinase as an inhibitor target in TF-1/FLT3-ITD cells. For example, TAE-684 and HG7-92-01 both potently inhibited the growth of TF-1/FLT3-ITD cells, with IC50 values of 160 and 59 nM, respectively. These values are lower than the individual IC50 values obtained for either kinase in vitro (see Table 8), suggesting that dual inhibition of both FES and FLT3-ITD may be responsible for their potent growth inhibitory effects. On the other hand, WZ4-49-1 and WZ4-49-8 block TF-1/FLT3-ITD cell growth with IC50 values of 465 and 816 nM, respectively, values almost identical to those observed with FES in vitro (494 and 706 nM respectively). In contrast, these two compounds are much less potent against FLT3-ITD in vitro (1390 and 14345 nM, respectively). Taken together, these results suggest that suppression of TF-1/FLT3-ITD cell growth by these two pyrazolopyrimidines is the result of selective FES inhibition. Finally, tandutinib inhibited TF-1/FLT3-ITD cell growth and FLT3-ITD kinase activity in vitro with very similar potencies (825 vs. 788 nM, respectively), suggesting that tandutinib works primarily via FLT3-ITD. Note that tandutinib had no effect on FES kinase activity in vitro, even at concentrations as high as 100 μM. In a final series of experiments, we explored the sensitivity of TF-1 cells transformed with the *FLT3* kinase domain point mutant D835Y, in which FES activity is not enhanced. In these cells, TAE-684 treatment resulted in potent growth suppression, with an IC50 value close to 150 nM (Figure 16). This value is virtually identical to that obtained with the TF-1/FLT3-ITD cells, suggesting that the cellular activity of TAE-684 may result primarily from inhibition of the FLT3 mutants. HG7-92-01, on the other hand, had very little activity against TF-1/FLT3-D835Y cells, which is consistent with the reduced sensitivity of this compound to FLT3-D835Y in vitro in comparison to FLT3-ITD (Table 8). This observation,

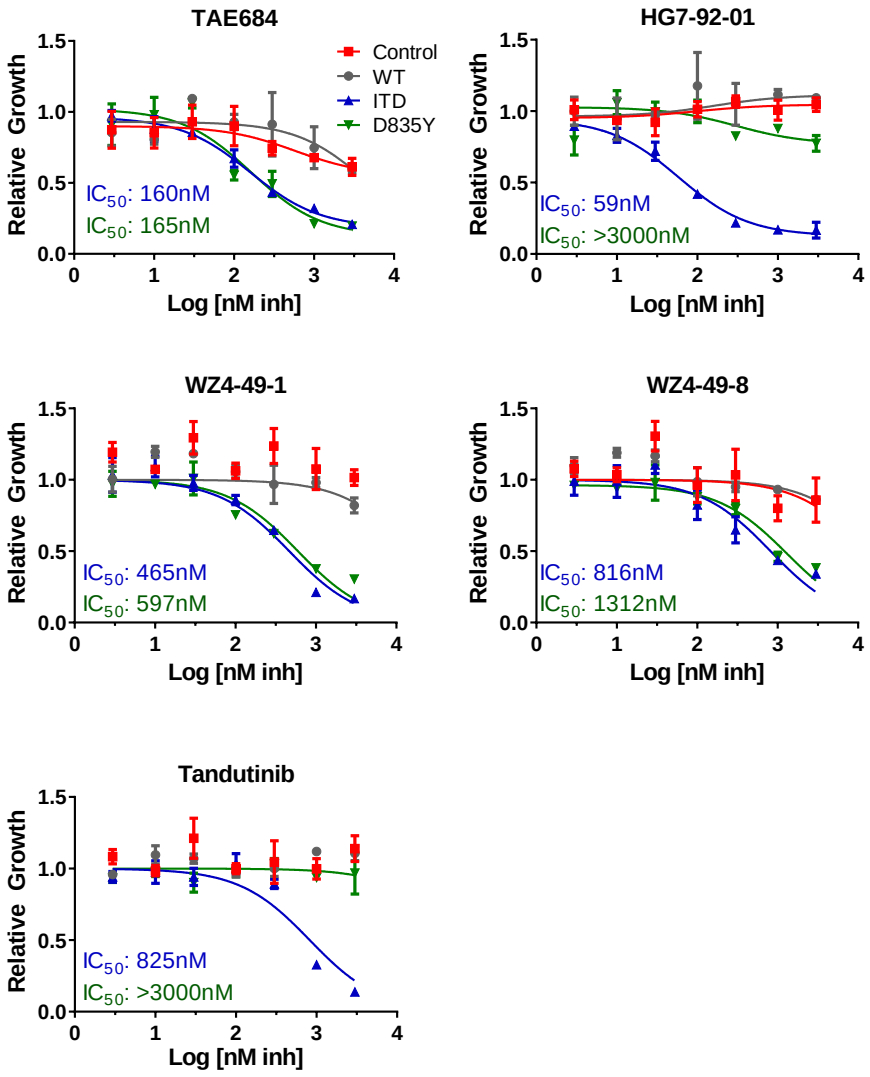


Figure 16: Inhibitor sensitivity of TF-1 cells transformed with wild-type (WT) and AML-associated mutants of *FLT3*.

TF-1 cells expressing WT, ITD, and D835Y forms of *FLT3* were incubated with the kinase inhibitors shown over a range of concentrations for 72 hours. Cell viability was determined using the Cell Titer Blue Assay for triplicate cultures at each drug concentration. Raw fluorescence data were corrected for background and then normalized to cultures grown in the presence of the DMSO carrier solvent alone (0.1%). Results are presented as the mean normalized growth ± S.D. Curves were fit by non-linear regression analysis to estimate the IC_{50} values shown; blue, TF-1/FLT3-ITD cells; green, TF-1/FLT3-D835Y cells.

plus the finding that FLT3-D835Y is unable to activate FES as a secondary inhibitor target, likely explains its lack of activity in TF-1/FLT3-D835Y cells. Both of the pyrazolopyrimidines, WZ4-49-1 and WZ4-49-8, inhibited TF-1/FLT3-D835Y cell growth with IC50 values of 597 and 1312 nM, respectively. Interestingly, in vitro kinase assays show that the D835Y mutation sensitizes FLT3 to inhibition by both of these compounds relative to FLT3-ITD, suggesting that these compounds directly inhibit FLT3-D835Y in cells.

A.3 DISCUSSION

Previous studies have shown the FES tyrosine kinase is constitutively active in FLT3-ITD$^+$ AML cells, and that RNAi knockdown of FES expression causes growth arrest and apoptosis. In the present study, we tested the efficacy small molecule inhibitors of FES kinase activity against FLT3-ITD$^+$ AML cells. Our results show that direct inhibition of FES kinase activity by two selective pyrazolopyrimidine compounds resulted in growth suppression in MV4-11 cells, which are derived from a FLT3-ITD$^+$ patient, and in TF-1 myeloid cells transformed by retroviral transduction of *FLT3*-ITD. Furthermore, inhibitors with activity against both FES and FLT3-ITD kinases in vitro blocked FLT3-ITD$^+$ AML cell growth in the low nanomolar range, suggesting that dual inhibition of this key AML driver mutation and the proximal effector kinase FES provides added benefit in this type of AML. Indeed, these dual inhibitors, TAE-684 and HG7-92-01, were about 8- and 20-fold more potent than tandutinib, which inhibits FLT3-ITD but not FES.

One unexpected discovery from our study was the observation that the AML-associated FLT3 point mutant, D835Y, did not activate FES in TF-1 cells transformed with this mutant.

This observation is consistent with earlier work showing important differences in the downstream kinases and signaling pathways activated by FLT3-ITD vs. FLT3-D835Y. For example, SRC kinase is activated by FLT3-ITD but not by FLT3-D835Y in 32D/FLT3-ITD expressing cells (288). Furthermore, siRNA knockdown of SRC eliminated STAT5 activity in these cells. Treatment with the SFK inhibitor, dasatinib, reduced STAT5 activation in MV4-11 cells and reduced the growth of primary AML patient samples. Additionally, FLT3-ITD signaling suppresses the expression of two transcription factors essential for myeloid cell differentiation, while the D835Y does not (278). The mechanism of this suppression is unknown but RNA levels are suppressed in FLT3-ITD expressing cells. These differences in signaling may help to explain increased disease severity observed in *FLT3*-ITD vs. *FLT3*-D835Y knock-in mouse models and by extension the worse prognosis for patients with *FLT3*-ITD vs. D835Y (289).

Consideration of differences in pyrazolopyrimidine inhibitor potency between the two model systems used in our study (MV4-11 vs. TF-1/FLT3-ITD cells) provides some additional mechanistic insight regarding inhibitor target kinase selection in vivo. For example, the potency of both pyrazolopyrimidines, WZ4-49-1 and WZ4-49-8, was about two-fold higher in TF-1/FLT3-ITD cells compared to MV4-11 cells. One possible explanation for this difference is that in MV4-11 cells, FLT3-ITD activates not only FES, but also SYK and several myeloid members of the Src kinase family (HCK, LYN, and FGR). Activation of these alternative kinase pathways may help to overcome the effect of FES inhibition, resulting in reduced sensitivity to the inhibitor. Note that previous KINOMEscan data showed that WZ4-49-8 does not bind to HCK, LYN or SYK (481) strongly suggesting that its growth suppressive activities in MV4-11 cells are primarily due to inhibition of FES and not these other AML-associated kinases. The

kinase target specificity profile of the structurally related compound, WZ4-49-1, is likely to be quite similar. On the other hand, TF-1/FLT3-ITD cells do not express HCK, LYN or FGR (data not shown), potentially making FLT3-ITD oncogenic signaling more dependent on FES and thus more sensitive to these FES-selective inhibitors.

Our study also revealed an unexpected sensitizing effect of the FLT3-D835Y mutation towards the two pyrazolopyrimidine inhibitors. In vitro kinase assays showed that FLT3-D835Y was about 5-fold more sensitive to both WZ4-49-1 and WZ4-49-8 compared to wild-type FLT3. This result is consistent with previous KINOMEscan analysis of WZ4-49-8 target specificity, in which no displacement activity was observed with the wild-type or ITD form of FLT3, while the D835Y and D835H both showed partial responses (481). This observation is consistent with their growth suppressive activities in TF-1/FLT3-D835Y cells, where these compounds inhibit cell proliferation with IC_{50} values that closely mirror their potency against the FLT3-D835Y kinase domain in vitro. In contrast, the D835Y mutation results in strong resistance to tandutinib in vitro, and this compound had no effect on TF-1/FLT3-D835Y cell proliferation.

In summary, these studies provide the first evidence that ATP-site inhibitors with selectivity for FES kinase activity cause growth arrest and induce apoptosis in AML cells. These results validate FES as a viable inhibitor target for drug development in AML. Furthermore, compounds with dual activity against both FES and FLT3-ITD induce very strong apoptotic responses in FLT3-ITD$^+$ AML cells, suggesting that this therapeutic approach may be valuable in primary FLT3-ITD$^+$ AML as well.

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