Modulation of FLT3 Inhibitor-induced Cytotoxicity in AML by FLT3 Ligand

A Senior Honors Thesis

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By

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ABSTRACT

Acute myeloid leukemia (AML) is a cancer of hematopoietic cells that develops rapidly in the bone marrow and quickly spreads to the blood. According to the American Cancer Society, there will be 11,960 new cases of AML in 2005 and about 9,000 patient deaths as a result. An internal tandem duplication (ITD) in the juxtamembrane domain of the FMS-like tyrosine kinase 3 receptor (FLT3) is present in approximately 25% of AML patients and predicts for poor prognosis. This mutation causes constitutive activation of the FLT3 receptor in the absence of the natural FLT3 ligand (FL). Unlike the wild-type (WT) counterpart, FLT3 ITD confers numerous oncogenic properties, such as uninterrupted proliferation, resistance to apoptosis and a block in differentiation. Selective FLT3 inhibitors have been developed, but clinical trials using these inhibitors as single agents have not been overwhelmingly successful. We hypothesize that one or more factors normally present within the bone marrow may be impacting negatively FLT3 inhibitor efficacy in these early trials. In particular, FL has been shown to promote the expansion of hematopoietic precursors, and is constitutively expressed by most bone marrow stromal tissues. Moreover, overexpression of FL by leukemic blasts has also been reported in AML.

To test our hypothesis, AML cells were treated with the novel FLT3 inhibitor, THRX-165724, in the presence or absence of exogenous FL. After 48 hours, cell viability was determined by MTS cell proliferation assay, and apoptosis was assessed after staining with propidium iodide and analysis of DNA content. Three cell lines were tested, representing the common FLT3 genotypes seen in AML (\(FLT3^{WT/WT}\), \(FLT3^{ITD/WT}\), and \(FLT3^{ITD/-}\)). Molm-13 and MV4-11 cells (both FLT3 ITD+) underwent apoptosis with a 50% inhibitory concentration.
(IC\textsubscript{50}) of approximately 100-120 nM. However, exogenous FL decreased Molm-13 (\textit{FLT3}\textsuperscript{ITD/WT}) sensitivity to FLT3 inhibition 9-fold (p=0.0135), while FL modulation of THRX-165724-induced cytotoxicity of MV4-11 cells (\textit{FLT3}\textsuperscript{ITD/-}) was found not to be significant. No inhibitor-induced cytotoxicity or apoptosis in THP-1 cells (\textit{FLT3}\textsuperscript{WT/WT}) was observed by treatment of up to 5 \(\mu\)M inhibitor, irrespective of presence or absence of FL.

We next examined the downstream pathways (MAPK, STAT5, and AKT) implicated in FLT3 signaling. Cells were treated with THRX-165724, and protein lysates prepared. Using standard Western blotting techniques, we observed that FLT3 signaling along the MAPK pathway was rescued from inhibition by the FLT3 ligand. With combined U0126 (a MAPK inhibitor) and THRX-165724 treatment, reversal of these FL-induced changes in cytotoxicity and apoptosis was seen. These data, if confirmed in a larger study with primary AML samples, may help to provide an explanation for some of the suboptimal clinical results—the majority of FLT3 ITD+ patients identified to date have the \textit{FLT3}\textsuperscript{ITD/WT} genotype, analogous to the Molm-13 cell line.
**Chart 1 - Overall Project**

THP-1 (FLT3\(^{WT/WT}\))  Molm-13 (FLT3\(^{ITD/WT}\))  MV4-11 (FLT3\(^{ITD/}\))

*In vitro* analysis of cells treated with THRX-165724 with or without FL

- pFLT3 levels by western blot
- Cell viability by MTS assay
- Apoptosis by cell cycle analysis

**FL attenuates FLT3 inhibition in a genotype specific manner**

Examination of signaling pathways in Molm13 treated with THRX-165724 with or without FL

- AKT pathway
  - pAKT levels were reduced by FLT3 inhibition, irrespective of the presence of FL
- MAP kinase pathway
  - pERK levels were reduced in cells treated with THRX-165724; the affect was attenuated by FL
- Jak-Stat pathway
  - pSTAT5 levels were reduced by FLT3 inhibition, irrespective of the presence of FL
    - Jak inhibition + FLT3 inhibition does not have any affect beyond that seen by FLT3 inhibition alone

**FL-induced attenuation mediated via MAPK in Molm13 cells**

*In vitro* analysis of Molm-13 treated with THRX-165724 and MEK inhibitor, U0126, with or without FL

- pFLT3 and pMAPK levels by western blot
- Cell viability by MTS assay
- Apoptosis by cell cycle analysis

**Combination treatment partially overcomes FL attenuation effect in Molm-13 cells**
BACKGROUND AND INTRODUCTION

Acute Myeloid Leukemia. Acute myeloid leukemia (AML) is a cancer of the hematopoietic cells that develops rapidly in the bone marrow, quickly spreads to the blood and sometimes to other sites including the spleen, lymph nodes, liver, central nervous system, and testes (1). It has the potential to kill within a matter of weeks or months. Although AML can develop at any age, it is a disease generally of older adults, occurring at the average age of 65 (2). While AML is generally non-hereditary, there are instances where certain families might inherit susceptibility to the disease. Only about 25% of all patients can be expected to achieve complete remission and survive beyond the three-year mark (22). According to the American Cancer Society, there will be 11,960 new cases of AML in 2005 and about 9,000 patient deaths as a result.

The production of blood cells, including most types of immune cells, begins in the bone marrow from a single self-renewing hematopoietic stem cell. The hematopoietic stem cell differentiates along two pathways, giving rise to myeloid and lymphoid lineages (3). The common lymphoid progenitor that is made can further differentiate to become either a T-cell, B-cell, NK cell, or lymphoid dendritic cell. T-cells and B-cells are involved primarily in adaptive immunity. B-cells produce antibodies against foreign antigens that facilitate neutralization and removal of these antigens via various mechanisms, i.e. opsonization or complement-mediated phagocytosis. T-cells recognize antigens presented by the major histocompatibility complex (MHC) on the surface of one’s own cells. Among their functions, T-cells aid in B-cell activation and they mediate lysis of one’s own cells that have been infected by a pathogen or mutagenized. Natural killer (NK) cells guard against certain tumor and virus-infected cells that have ceased to display MHC molecules. NK cells also function in antibody-dependent cell-mediated cytotoxicity, meaning that they can recognize nonspecific Fc regions of antibodies that are bound
to target cells and then facilitate lysis of those targets. Dendritic cells collect antigen by phagocytosis or endocytosis and then process and present antigenic peptides to T-helper cells (T\textsubscript{H}) via MHC class II. In this way, they are the primary activators of naïve T\textsubscript{H} cells. Cancers that develop along the lymphocyte lineage in the bone marrow are called lymphocytic leukemias, while cancers that develop from lymphocytes in the lymph nodes are called lymphomas.

The myeloid lineage gives rise to mononuclear phagocytes, granulocytic cells, Mast cells, erythrocytes, and platelets. Mononuclear phagocytes enter the blood and become monocytes, where they soon enlarge, differentiate, and then migrate to certain tissues as macrophages or myeloid dendritic cells. Macrophages function in phagocytosis and degradation of exogenous antigens and cellular debris. They also serve to present antigens to T\textsubscript{H} cells. Myeloid dendritic cells serve similar functions to that of their lymphoid counterparts. Granulocytic cells are further subdivided into neutrophils, basophils and eosinophils. Neutrophils are the first type of immune cells to arrive at the site of infection. While short-lived, they are actively phagocytic and more potent than macrophages. Eosinophils are also phagocytic and are believed to be specialized to fight against parasitic infections. Basophils reside in the blood and function in Type I Hypersensitivity reactions by binding the Fc regions of IgE antibodies crosslinked to allergens. Basophils then degranulate to release histamine, cytokines, and other mediators of allergic reactions. Mast cells function in IgE-mediated immunity similar to that of basophils except that mast cells reside in the tissues. Erythrocytes, or red blood cells, are the primary cellular constituent of the blood. Their primary function involves the use of the protein hemoglobin that transports oxygen and carbon dioxide. Platelets are small pieces of cells given off by megakaryocytes that coagulate at sites of blood vessel damage. Cancer that develops along the myeloid lineage is known as myeloid leukemia.
Leukemias are further characterized as acute and chronic (1). Acute leukemias occur early in the process of hematopoiesis. They develop rapidly within a patient and kill within a matter of weeks if left untreated. Chronic leukemias originate from progenitor cells that are already partially differentiated. These leukemias develop slowly and less severely; patients can often survive for several years without treatment. Chronic leukemias result in the overproduction of normal or slightly abnormal blood cells, while acute leukemias result in the overproduction of progenitors. These progenitors can further expand and exacerbate the cancer, while the differentiated blood cells that are the result of chronic leukemias live a normal life cycle and generally do not expand.

Acute Myeloid Leukemia (AML) is the most common cancer of the blood, and it is the focus of this study. Conventional treatment for AML patients involves two phases of chemotherapy: induction and consolidation (4). Induction is an intense treatment, usually involving the drugs cytarabine and an anthracyline antibiotic (daunorubicin or idarubicin), that aims at killing off all leukemic cells and forcing the patient into remission. Sometimes several rounds of induction with increasing potency must be undertaken in order to obtain the desired result. Remission is followed up by consolidation therapy, which aims at completely eradicating the cancer or suppressing it. Consolidation therapy can take the form of additional chemotherapy, usually several rounds of cytarabine, or stem cell transplant. Stem cell transplant is often reserved for patients that have relapse/refractory AML, and it involves the use of high-dose chemotherapy that completely destroys all bone marrow cells. New cells are transplanted back into the patient. Stem cell transplant can be allogeneic, meaning from a donor, or autologous, meaning from one’s self. The biggest problem with transplant is the high degree of rejection that occurs in 10-40% of cases, which may result in death of the patient.
**FLT3 and Acute Myeloid Leukemia.** FMS-like Tyrosine Receptor 3 (FLT3) plays a critical role in leukemia. The FLT3 gene is located at chromosome 13q12 and is part of the family of type III receptor tyrosine kinases (RTK) that includes KIT, platelet-derived growth factor, and FMS (4). FLT3 is restricted to CD34+ hematopoietic precursors and a small fraction of CD34− cells destined to become dendritic cells, meaning that FLT3 is expressed on immature progenitors of the myeloid and B-cell lineages (13, 19). FLT3 functioning appears to be redundant with KIT. FLT3 knockout mice exhibit no overt abnormalities, only slight deficiencies in early hematopoietic progenitors.

The FLT3 protein spans the membrane of a cell and contains five immunoglobulin-like domains, a juxtamembrane domain, and an interrupted tyrosine kinase domain that is separated by a kinase insert domain (4). When the growth signal protein, FLT3 ligand (FL), binds to the extracellular domains of two inactive receptors, they aggregate to produce the active dimer form of FLT3. FL is a homodimer of two short chain α-helical bundles (20). FL is constitutively made by human fibroblasts and endothelial cells (3), and it is present in most human tissues, including the bone marrow (13). FLT3 dimerization by FL stabilizes the FLT3 proteins in an ‘open’ conformation, where ATP can bind and phosphorylate the receptor at several locations. Thus, FL binding allows for auto-phosphorylation and activation of the tyrosine kinase domain. This in turn causes the receptor to initiate an intracellular response along multiple pathways. The mechanism of this response is still being determined although there are three likely signaling pathways upon which FLT3 is known to act: the Ras/MAP kinase pathway (17, 18), the JAK/STAT5 pathway (5, 6, 18) and the AKT pathway (11, 12, 17, 18). It is not clear yet how FLT3 might influence these pathways. It is also of note that there appears to be a second form of FLT3 that spans the endoplasmic reticulum (ER) (19). This cytoplasmic form has a lower
molecular weight in comparison to membrane-bound FLT3. It may be that the membrane-bound FLT3 can become internalized and bind to the ER. The role of ER-bound FLT3 is unknown at this point. Unless mentioned, throughout the rest of this paper I will only be referring to the membrane-bound FLT3.

FLT3 is overexpressed in 90% of AML cases and also often in B precursor-cell acute lymphoblastic leukemia (ALL) and in some chronic myelogenous leukemias (CML) (17). FLT3 is maintained in the inactive form within a normal individual, however due to the ubiquitous presence of FL, FLT3 signaling is largely dependent upon levels of FLT3 protein expression.

A common somatic mutation found in 20-25% of AML cases is an internal tandem duplication (ITD) of the juxtamembrane domain (refer to figures 1-3) of FLT3 (4). The ITD originates as an in-frame duplication that includes portions of exons 11 and 12 of the FLT3 gene (5). The duplicated region causes ligand-independent constitutive dimerization of the receptor and an “always active” protein. It is possible that the ITD works by disrupting an autoinhibitory motif within the juxtamembrane domain. A hematopoietic progenitor cell harboring a FLT3 ITD has acquired the ability to divide independently of autoinhibitory controls, even in the absence of FL (4). The presence of a FLT3 ITD has been shown to confer other oncogenic properties, including the repression of myeloid differentiation via downregulation of transcription factors Pu.1 and C/EBPα (5). Also, FLT3 ITD has been linked to the repression of p53 target genes, like GADD45 and BTG-2, which in turn helps to inhibit apoptosis (5).

Other FLT3 mutations have been shown to exist in leukemia. Two particular point mutations (D835 and, less often, I836) within the activation loop of the tyrosine kinase domain have a transforming affect similar to that of the FLT3 ITD and cause aberrant signaling along several pathways (6, 13). Usually, point mutations take the form of an aspartate becoming a
tyrosine. This transformation stabilizes the receptor in the ‘open,’ ATP-binding conformation (13). The prognostic impact of a point mutation is not as evident or well characterized as that of a FLT3 ITD, where a FLT3 ITD is a significant indicator of poor outcomes (13). In total, about a third of all AML cases have a mutated FLT3 receptor.

The prognosis for AML patients with a FLT3 ITD mutation, particularly in those who have lost the WT allele (FLT3ITD/-), is far worse than for patients possessing wild-type (FLT3WT/WT) receptor (4). FLT3 ITD AML patients do not respond well to traditional chemotherapy. In one study, the percentage of AML patients with FLT3 ITD mutations achieving disease-free survival (DFS) after 12 months is only 39%, compared to about 71% of AML patients with only wild-type FLT3 (4). In addition, the median time of survival for FLT3 ITD AML patients is only 11 months in comparison with 46 months for AML patients without a FLT3 ITD.

A number of small molecule pharmacological FLT3 inhibitors (none of which are truly specific for FLT3) have shown the ability to prevent FLT3 autophosphorylation. Several of these are currently undergoing single agent clinical trials in treating FLT3 ITD patients with refractory/relapse AML. While demonstrating anti-leukemic activity, none of these have achieved overwhelming success. For instance, the FLT3 inhibitor PKC412 caused substantial decreases in peripheral and bone marrow blasts in many of the patients in which it was tested; however, this effect was short-lived. The malignancies seemed to be able to overcome FLT3 inhibition within a few months. One possible explanation for these phenomena is that certain clones of the leukemias within particular patients were more dependent upon FLT3 for survival than others. While these clones were suppressed, less dependent clones might have been able to develop in the presence of FLT3 inhibition and, perhaps with the accumulation of additional mutations, move to a FLT3-independent state. A second explanation is that these cancers might
have found a way to become resistant to FLT3 inhibition, despite reliance upon FLT3 signaling. In clinical trials, some patients with FLT3 mutations do not appear to exhibit any noticeable response to FLT3 inhibition (7-8).

It has been suggested that FLT3 genotype might play a role in the observed resistance to FLT3 inhibition. Some studies have shown that a high mutant-to-wild-type (WT) allelic ratio is an indicator of poor outcomes (4, 9). In one study, the percentage of AML patients with hemizygous FLT3 ITD (FLT3^ITD/-) surviving 12 months post diagnosis was 13% in comparison with 72% of patients still possessing a wild-type allele (FLT3^WT/WT or FLT3^ITD/WT) (4).

**Synergism of FL and other hematopoietic growth factors.** Because FL is always present in the bone marrow, whether or not a cell is affected depends on whether it expresses the FLT3 receptor. FL-FLT3 responses are known to have a proliferative effect on early hematopoietic progenitors along the myeloid and lymphoid pathways, in B-cell development, and in development along the granulocyte-monocyte lineage. It has been shown that FL-FLT3 responses have a synergistic or additive effect on proliferation with other cytokines, in particular Stem Cell Factor (SCF), Interleukin 3 (IL-3), and Granulocyte-Monocyte Cell Growth Factor (GM-CSF) (16). These cytokines all play important roles in certain aspects of hematopoiesis.

**The Cell Cycle.** The cell cycle is composed of four phases, going from G1 (Growth 1), to S-phase (Synthesis), to G2 (Growth 2), to Mitosis (cell division). The successful completion of mitosis results in two daughter cells in G1. In addition, cells in the G1 phase can be induced to enter G0, a dormant stage where the cell does not proceed towards cell division, but continues to carry out metabolic activity. In mammalian cells, the ability of extracellular signals to influence the cell cycle is limited to the G1 and the Restriction Point, occurring before the G1/S barrier. After passing into S-phase, cells are obligated to continue the cycle (19). Cells that pass the
Restriction Point within G1 but do not subsequently divide are instead induced to undergo apoptosis.

**MAP kinase pathway.** There are a number of similar cellular pathways grouped under the name MAP kinase (mitogen-activated protein kinase). We are concerned with the ERK1/2 (extracellular signal-regulated kinase) pathway. Constitutive activation of the ERK1/2 MAPK has been associated with numerous types of cancers (10). Briefly, the MAP kinase pathway is initiated when a membrane-bound receptor activates the G-protein Ras by GTP coupling (3). Ras-GTP activates Raf1, which then phosphorylates and activates MEK1. MEK1 phosphorylates ERK1/2 MAPK at serine and threonine residues. MAPK is then able to activate transcription factors, such as cyclin D1, that are associated with G1/S-phase cell cycle progression (10). Several studies have suggested links between FLT3 signaling and MAPK constitutive activation in leukemic lines (5, 6).

The ERK1/2 MAPK plays a broad role in cell growth and proliferation (19). It is able to phosphorylate carbomoyl phosphate synthetase (CPS II), an enzyme that catalyzes the rate-limiting step of pyrimidine nucleotide synthesis. ERK1/2 also enhances the degradation of p27KIP1 in late G1, allowing for the release of cyclinE-CDK2 and entry into S-phase.

**JAK/STAT5 pathway.** Janus Kinase (JAK) family proteins commonly associate with membrane bound receptors (3). In the presence of cytokine, the receptor dimerizes with its counterpart, and the associated JAKs gain tyrosine kinase activity. JAK phosphorylates the receptor, creating docking sites for STAT5 transcription factors (signal transducers and activators of transcription). JAKs then phosphorylate the associated STAT5’s. STAT5’s dissociate and dimerize. The STAT dimer is then able to translocate into the nucleus where it can influence gene transcription.
**AKT pathway.** The AKT pathway has also been implicated in cancer as promoting cell survival and preventing apoptosis (12). FL-induced activation of cells via the FLT3 receptor has been shown to activate AKT, which in turn causes AKT to inactivate downstream targets. Foxo target genes are suppressed, which in turn prevents Foxo-mediated apoptosis. Other targets, such as p27KIP1 and the Bim gene, are up-regulated. In conclusion, it appears as though FLT3 can signal via AKT to promote cell survival and proliferation (12, 17).

**Specific goals.** This study seeks to better understand why patients harboring FLT3 ITD mutations do not respond well to FLT3 inhibition. The effects of FLT3 inhibition by the novel, selective, small molecule inhibitor THRX-165724 will be assessed in a genotype-specific manner on three AML cell lines. THRX-165724 is a heterocyclic, lipophilic compound capable of crossing the cell membrane and inhibiting FLT3, KIT and PDGFRα/β (platelet-derived growth factor receptor). THRX-165724 has a structural mimic of the purine component of adenosine-tri-phosphate (ATP), allowing it to compete for the ATP binding pocket inside the tyrosine kinase domain of FLT3 (13). By excluding ATP, THRX-165724 prevents phosphorylation of the receptor.

Cells will be grown in presence of common components of the bone marrow microenvironment, including FL, SCF, GM-CSF and IL-3; they will be further treated with the FLT3 inhibitor to assess the role of such environmental factors on FLT3 activation. It is possible that certain components within the natural milieu of the bone marrow provide protection for leukemias with FLT3 mutations.

Secondly, the effects of FLT3 inhibition on downstream targets along the MAPK, STAT5, and AKT pathways will be assessed. All three of these pathways have been implicated as possible signaling mechanisms for FLT3 (17). Finally, FLT3 inhibition will be coupled with the
inhibition of targets in these pathways to see if a synergistic effect can be produced. We hope that one or more combination strategies can be shown to have an impact on FLT3-mutated leukemic lines, thus providing a more potent clinical therapy for FLT3-mutated AML patients.
MATERIALS AND METHODS

Cell lines and FLT3 genotype status. Three cell lines, all M5 monocytic AML, were used in the experiments of this study. Molm-13 cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). These cells originally had come from the peripheral blood of a 20-year-old male with refractory AML who died in 1995 (14). They possess a FLT3 ITD as well as a wild-type FLT3 allele (genotype FLT3^{ITD/WT}). They also have a rearranged MLL fusion gene that is commonly seen in AML. They are: CD3-, CD4+, CD13(+), CD14-, CD15+, CD19-, CD33+, HLA-DR-. They are also reverse transcriptase-, EBV-, HBV-, HCV-, HIV-, HTLV-I/II-.

MV411 and THP-1 cells were purchased from the American Type Culture Collection. MV4-11 cells were established from a 10-year-old male who had AML (15). They contain a 4;11 translocation and a FLT3 ITD mutation with loss of WT on the second allele (FLT3^{ITD/-}). They are CD3-, CD4(+), CD5-, CD8-, CD10-, CD13+, CD14-, CD15+, CD19-, CD21-, CD25-, CD33+, CD34-, CD37-, CD138+, HLA-DR+. They are also reverse transcriptase-, EBV-, HBV-, HCV-, HIV-8-, HIV-, HTLV-I/II-.

The cell line THP-1 came from the peripheral blood of a one-year old infant male with monocytic AML (14). They are CD3-, CD4+, CD13+, CD14(+), CD15+, CD19-, CD33(+), CD34-, CD68+, HLA-DR+. They are reverse transcriptase-, EBV-, HBV-, HCV-, HHV-8-, HIV-, HTLV-I/II-. They do not contain a known FLT3 mutation (FLT3^{WT/WT}) and will be used as a negative control in this study.

All cell lines were maintained in RPMI 1640 media with 10% fetal bovine serum (FBS) at 37°C with 5% CO₂, supplemented with antibiotics and antymycotics. Cells were maintained in an active phase of growth and split every 3-4 days and also a day prior to experimentation.
FLT3 genotype status of the cell lines was determined by polymerase chain reaction (PCR) of cDNA with primers that amplify from the middle of exon 11 to the 3’ end of exon 14. To do this, first RNA was extracted from cells using the RNEasy Mini Kit per manufacturer’s protocol (Qiagen, Inc., Valencia, CA) and cDNA was made by a standard method. PCR was carried out with 35 cycles and products were size fractioned through 3% agarose gels with ethidium bromide staining and viewed under UV illumination.

**Reagents.** Recombinant interleukin 3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), and stem cell factor (SCF or KIT Ligand) were all obtained from Stem Cell Technologies. They were utilized at the concentration of 10 ng/mL. Recomiment FL was obtained from Amgen, Inc. and was used at the concentration of 50 ng/mL, unless indicated otherwise. The FLT3 inhibitor THRX-165724 was provided by Theravance, Inc., South San Francisco, CA, and was stored as a 7 mM stock solution in water at 4ºC. The MEK1/2 inhibitor, U0126, was obtained from Calbiochem, dissolved in DMSO and diluted in media to desired concentrations. MEK1/2 is an upstream activator of ERK1/2 MAPK.

**Cell viability by MTS Assay.** The MTS assay (CellTiter 96 AQeuous Non-radioactive Cell Proliferation Assay) for cell viability and proliferation was obtained from Promega, Madison, WI and carried out per manufacturer’s suggested procedure. Briefly, 50,000 cells diluted in media were put into a 96 well plate in triplicate with desired drug/cytokine conditions to make a final volume of 100 uL. Appropriate negative controls were used. DMSO concentrations were kept constant and did not exceed 0.1% of the total volume. Plates were incubated for 48 hours at 37ºC with 5% CO₂. The MTS assay was then performed by adding 20 uL of reagent to wells followed by incubation of 2-4 hours at 37ºC with 5% CO₂ in conditions that were protected from light. The MTS reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-
sulfophenyl)-2H-tetrazolium) is a soluble tetrazolium salt that gets converted into a soluble formazan product by the dehydrogenase enzyme in metabolically active cells. The presence of viable cells thus causes a change in color of the medium from light yellow to dark purple. At the end of incubation, the degree of color change is approximately equivalent to the number of viable cells. Absorbance is then read at 490 nm on a plate reader.

**Cell cycle analysis by flow cytometry.** Flow cytometry was utilized in order to determine the proportions of cells in each stage of the cell cycle and levels of apoptosis following drug treatments. Briefly, 5 x 10^6 cells/mL media were incubated in a similar manner to the MTS assay, with appropriate drug/cytokine conditions for 48 hours and with appropriate negative controls. Following incubation, cells were washed with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol overnight at 4°C. Samples were again washed in PBS and stained at room temperature under light-protective conditions with propidium iodide (PI) (50 ug/mL, Calbiochem) including RNAse A (20 ug/mL, Invitrogen). PI intercalates into double-stranded nucleic acids and fluoresces at 600 nm. RNAse A was used to degrade double-stranded RNA and reduce background RNA levels. Fluorescence was then determined by a Becton-Dickinson Facs Calibur. DNA content was analyzed by Cellquest software.

**Immunoprecipitation and immunoblotting.** 5.0 x 10^6 cells for each condition were diluted in 5 mL media with indicated cytokines/drugs. They were incubated at room temperature for 30 minutes, and then cells were washed in cold PBS. To isolate protein samples, cells were first lysed in RIPA buffer (50 mM Tris at pH 7.5, 1/% NP-40, 150 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), protease inhibitors obtained from Roche, and 1mM activated sodium orthovanadate (NaOV₄) obtained from Upstate Biotechnology. Samples were then snap-frozen at -80°C and centrifuged at 3,000 rpm. Supernatants were removed and stored at -80°C.
In FLT3 immunoprecipitation experiments, lysates were first probed overnight at 4°C with anti-FLT3/FLK2 (Santa Cruz) and Protein A sepharose CL-4B beads (Amersham Biosciences). In theory, FLT3 proteins from the lysates would bind to the antibody-sepharose beads. The beads were subsequently washed in a buffer made up of 10 mM Hepes 7.5 pH, 0.15 M NaCl, 0.2% NP40, 1 mM activated NaOV4 in H2O. They were then boiled for 5 minutes to denature the antibody and remove any bound FLT3.

Lysates were resuspended in sodium dodecyl sulfate (SDS) buffer. SDS is a detergent that equalizes the size/mass ratio for proteins. Proteins were separated on the basis of size using polyacrylamide gel electrophoresis (PAGE) with 4-15% Tris-HCl gels (Immobilon P membranes, Millipore, Bedford, MA). Membranes were then probed with the indicated antibodies. Anti-phosphotyrosine (PY99, Santa Cruz) was used to probe for phosphorylated FLT3. Anti-phospho ERK1/2 (Promega) and anti-p42/44 MAPK (Cell Signaling Technologies) probed for activated and total MAPK, respectively. Anti-phospho AKT and anti-total AKT, anti-phospho STAT5 and anti-total STAT4 did the same to distinguish activated versus total AKT, and active versus total STAT5 (Cell Signaling Technologies). The appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) were used to detect the presence of proteins by the ECL-plus system (Amersham Pharmacia Biotech, United Kingdom).

**Statistical methods.** GraphPad Prism software was used to perform statistical analyses. Where indicated, Tukey’s pairwise comparison test of the means was used with significant differences assessed as p-values ≤ 0.05.
RESULTS

Detection of FLT3 ITD’s by PCR. In order to confirm the FLT3 genotype of the cell lines THP-1, MV4-11 and Molm-13, we isolated RNA and then created cDNA. We then PCR-amplified exons 11-14 (Figure 3). THP-1 showed the expected wild-type band at 456 bp. Molm-13 showed two bands, one that was equivalent to the wild-type and another that was between 460-470 bp. This confirms Molm-13 cells as possessing both a FLT3 ITD and FLT3 wild-type allele. MV4-11 cells gave only a single band between 480-490 bp, indicating the presence of a FLT3 ITD and the loss of the wild-type allele.

THRX-165724 inhibits ligand-induced and constitutive auto-phosphorylation of FLT3.

Figure 6 demonstrates that THRX-165724 is able to inhibit FLT3 ligand-induced activation in THP-1 cells in a dose-dependent manner (IC₅₀ ~ 100 nM). THP-1 cells require FL in order to activate FLT3. We observed a reduction in pFLT3 by THRX-165724 equivalent to levels seen when FL is not added. Molm-13 and MV4-11 cells exhibit constitutive FLT3 activation because of the presence of a FLT3 ITD allele (Figures 9C and 6). This constitutive activation is inhibited by THRX-165724 in a dose-dependent manner (IC₅₀ ~ 100 nM).

THRX-165724 has selective cytotoxicity in AML cells with a FLT3 ITD. Only MV4-11 and Molm-13 cells were found to be sensitive to FLT3 inhibition (IC₅₀s ranged from 100-120 nM) (Figure 7). Cytotoxicity occurred in a THRX-165724 dose-dependent manner. Cytotoxicity in these lines correlated strongly with the level of FLT3 deactivation. We know this because the IC₅₀ of FLT3 dephosphorylation was remarkably similar to the cytotoxic IC₅₀. On the other hand, THP-1 cells did not respond to FLT3 inhibition by THRX-165724 even at doses as large as 10 uM, despite FLT3 deactivation (data not shown).
Addition of FL decreases sensitivity to THRX-165724 in Molm-13 cells in a FL dose-independent manner. Molm-13, THP-1, and MV4-11 cells were treated with increasing doses of THRX-165724 with or without exogenous FL at 50 ng/mL. THP-1 cells exhibited a slight growth response to the addition of FL, but growth of these cells was not affected by THRX-165724 (data not shown). FLT3 phosphorylation in Molm-13 cells assessed by western blot increased ~ 10-fold with the addition of FL, whereas MV4-11 cells were not affected by FL (Figures 9C and 9D). The 10-fold increase in FLT3 activation in Molm-13 cells corresponded with a 9-fold increase in mean cytotoxic IC_{50} from ~ 85 nM to 747 nM (P = 0.0135) (Figures 9A and 10B), and also a 6-fold decrease in THRX-165724-induced apoptosis (P < 0.001) (Figures 10A and 11). In MV4-11 cells, THRX-165724-induced cytotoxicity (Figure 9B) and apoptosis (data not shown) did not change in response to exogenous FL.

To determine whether the concentration of FL mattered, Molm-13 cells were treated with 1000 nM THRX-165724 for 48 hours with increasing doses of FL. The modulation of THRX-165724-induced cytotoxicity by FL did not vary significantly between FL doses ranging from 10 ng/mL to 1000 ng/mL (Figure 8).

Addition of SCF, GM-CSF and IL-3 do not affect THRX-165724 inhibition. The cytokines GM-CSF, IL-3 and SCF (referred to in combination as GIS) were used to treat Molm-13 cells, each at 10 ng/mL, against differing doses of THRX-165724 with or without exogenous FL. GIS alone had a slight but statistically insignificant proliferative effect (Figures 11 and 12). However, the combination of GIS and FL did not reduce THRX-165724-induced cytotoxicity beyond that of FL alone (Figure 11A). Nor did GIS appear to counteract FLT3 inhibition in the same way that FL was able to.
FL-induced attenuation of FLT3 inhibition occurs via the MAP kinase pathway. ERK1/2, STAT5 and AKT phosphorylation were measured by western blot. ERK/12 levels decreased in response to FLT3 inhibition by THRX-165724 in a dose-dependent manner in Molm-13 and MV4-11 cells (Figure 13) and also in THP-1 (data not shown). In fact, there appears to be an almost one-to-one correlation between FLT3 activation and ERK1/2 activation, when comparing western blots (Figures 9C and 9D). Downregulation of MAPK was counteracted by FL in Molm-13 cells but not in MV4-11 or THP-1 cells (THP-1 data not shown). STAT5 and AKT phosphorylation were also decreased by FLT3 inhibition, confirming what had been found in other studies (data not shown). However, deactivation of STAT5 and AKT happened irrespective of presence or absence of FL.

**Combination treatment with THRX-165724 and MEK1/2 inhibitor, U0126, overcomes FL protective effect.** To determine if MAPK inhibition would have an additive or synergistic effect with FLT3 inhibition, we used the MEK1/2 inhibitor, U0126 (5 uM) (Cell Signaling Technologies), in combination with THRX-165724 (1 uM) for 48 hours. MEK1/2 is an upstream activator of MAPK (Figure 5). Treatment of cells with U0126 alone caused a slight, albeit statistically insignificant increase in apoptosis and cytotoxicity. The combination treatment did not affect growth or survival of THP-1 cells (data not shown). MV4-11 cells showed a slight increase in cytotoxicity and apoptosis as a result of combination therapy (data not shown). In Molm-13 cells, the combination treatment had the effect of reversing FL-induced modulation of FLT3 inhibition (Figures 13A, 14 and 15). This reversal was reflected by a reduction in phospho-ERK1/2 levels, an increase in cytotoxicity and an increase in apoptosis to levels observed by that of FLT3 inhibition without FL. The ability of FL to counteract FLT3 inhibition was thus significantly weakened by the combination therapy.
FLT3 inhibition and STAT5 inhibition were coupled in a similar manner to determine if there was a significant additive or synergistic cytotoxic effect. The JAK Inhibitor I (Calbiochem) was not found to have a cytotoxic effect alone upon Molm-13, THP-1 or MV4-11 cells even at concentrations upwards of 0.5 uM, nor did it have an effect on FLT3 inhibition-induced cytotoxicity beyond that of THRX-165724 alone (data not shown).
CONCLUSIONS

The acquisition of a mutation in the gene FLT3 is one of the mechanisms by which AML develops. Cell lines expressing mutated FLT3 appear to have become dependent upon FLT3 signaling for proliferation and survival, while AML cell lines that express only wild-type FLT3 rely upon other parallel mechanisms. The gene FLT3 represents an attractive therapeutic target for the treatment of AML, primarily because its successful inhibition is lethal for FLT3-dependent cell lines and generally not harmful to normal non-malignant cells. The inhibition of the FLT3 gene in normal mice does not negatively affect mouse lifespan or health. Rather it results in mice with subtle deficiencies in B-cell, NK cell and hematopoietic stem cell populations (19).

Researchers are currently developing three strategies towards inhibiting FLT3, which are likely to work synergistically with one another. A recently-explored method involves down-regulation of FLT3 protein expression via short interfering RNA sequences (siRNA) (18). As has already been mentioned, with FL being always present in the bone marrow, FLT3 signaling depends largely upon expression of the receptor. Thus, if it were possible to hinder expression of the FLT3 receptor, this might be an effective treatment for AML. FLT3 siRNA duplexes were shown to induce apoptosis in FLT3-dependent cell lines in vitro. This in turn led to down-regulation of AKT, STAT5, and MAPK. FLT3 siRNA therapy also was shown to have synergistic effects with MLN518, a pharmacological FLT3 and KIT inhibitor. Despite the promise, siRNA therapy has not yet proven to be effective clinically, and it may run into certain limitations, such determining how to get the siRNA duplexes into cells. However, the study does point out the benefits of combination therapy targeting FLT3.
A second method involves the use of an anti-FLT3 antibody that blocks the FL binding site (17). The antibody IMC-EB10 apparently is able not only to inhibit FL-induced phosphorylation but to also inhibit FL-independent phosphorylation of a FLT3 ITD receptor. The result was a decrease in proliferation and an increase in apoptosis of FLT3-dependent cell lines. The anti-FLT3 antibody was also effective as a therapeutic agent \textit{in vivo}, doubling the survival time of NOD SCID mice injected with BaF3-ITD AML cells. Although IMC-EB10 was shown to cross-react with mouse FLT3, it did not have an impact upon murine physiology (histological analysis of bone marrow, brain tissue, spleen, kidney, lung, heart, peripheral blood) or survival, indicating that FLT3 inhibition by this method is generally not harmful to normal populations of cells. A drawback to antibody-mediated inhibition is that the antibody is not readily internalized. Thus, internalized forms of FLT3 escape inhibition, which suggests a possible reason why antibody inhibition only prolonged survival of mice and never actually achieved a cure, if we are to suspect that internalized FLT3 plays a similar role to its membrane-bound counterpart.

The traditional method of targeting FLT3, which we have employed, is to use a pharmacological inhibitor of the receptor. A number of FLT3 inhibitors have been developed with some now undergoing clinical phase I-II trials as single agents (13). Clinically, these inhibitors exhibit short-term (1-3 months) anti-leukemic activity in relapse/refractory FLT3 ITD AML, but they do not demonstrate successful long-term results. It must be of note that certain patients having only wild-type FLT3 have responded to FLT3 inhibition. It is likely that these patients are overexpressing the FLT3 receptor and have become dependent upon its signal. On the other hand, 15-20\% of FLT3$^{\text{ITD/WT}}$ patients do not respond to pharmacologic inhibition. In
In this study, we offer an explanation for why this could be so given that Molm-13 cells were non-responsive to FLT3 inhibition in the presence of FL.

The inhibitor that we used in this study, THRX-165724, works by blocking auto-phosphorylation of the intracellular tyrosine kinase domain. This in turn caused downregulation of STAT5, AKT, and MAPK. THRX-165724 was shown to have selective cytotoxicity for FLT3-dependent cells, especially those containing a FLT3 ITD+. The FLT3\textsuperscript{ITD/WT} cell line Molm-13 appeared to be rescued by the addition of exogenous FL, while FLT3 inhibition of MV4-11 cells (FLT3\textsuperscript{ITD/-}) was unaffected by FL. One possible explanation for these phenomena is that the presence of the wild-type receptor on Molm-13 cells permits for FL to compete against the pharmacologic inhibitor for activation of the receptor, whereas FL does not compete for binding of a FLT3 ITD-ITD receptor. A number of studies have pointed out the significance of losing the wild-type receptor, namely that it causes a more-negative prognosis. We are suggesting that the loss of the wild-type may be a predictor for a better clinical response to FLT3 inhibition. FL-attenuation of FLT3 inhibition happens likely by way of the FLT3 ITD-WT receptor. Because the majority of patients with FLT3 ITD mutations are of the FLT3\textsuperscript{ITD/WT} genotype, this may account for the suboptimal clinical results seen by FLT3 inhibition.

Another possible explanation for differences in FL response is that the size or the nature of the ITD may play a role in allowing FL to continue to influence the receptor (the MV4-11 ITD is slightly larger than the Molm-13 ITD). One cannot expect all mutant FLT3 proteins to behave similarly. It must also be mentioned that the cell lines used in this study are fraught with mutations, any number of which may be influencing our results. A third explanation is that FLT3 has multiple signaling mechanisms (possibly one in the juxtamembrane domain), one of which was able to be rescued by FL in order to activate MAPK.
In this study, we demonstrated that FLT3 signals primarily along the ERK1/2 MAPK pathway in order to cause proliferation and prevent apoptosis. In the presence of a high concentration of FLT3 inhibitor (THRX-165724 ~ 1 uM), the FLT3 receptor appears to be completely overwhelmed to the point where MAPK inhibition has little if any effect. However, FL severely undermined FLT3 inhibition in Molm-13 cells, even though the resulting signal was still weakened. The protective effect caused by FL was reversed when MAPK inhibition was combined with FLT3 inhibition. This indicates that there is a synergistic effect of dual inhibition, especially because MAPK inhibition alone had only a slight effect on proliferation.

There appears to be a competition between inhibition and activation (by FL) signals with the end result being either activation or deactivation of ERK1/2. FLT3 inhibition alone is enough to deactivate ERK1/2, while FL provides enough of an activation signal to keep a cell from undergoing apoptosis. The coupling of FLT3 and MAPK inhibition is strong enough to tip the balance in favor of ERK1/2 deactivation once again.

While this finding certainly suggests a therapeutic advantage to combination therapy, we must also understand that ERK1/2 MAPK is one of the most ubiquitous signal transduction systems (19). MAPK is activated in response to a variety of stimuli and it regulates a number of cellular processes, including the cell cycle at various points. The deactivation of MAPK cannot be achieved in a clinical model without simultaneously causing certain undesirable consequences in nonmalignant cells. While a significant therapeutic window for FLT3 inhibition exists, whether or not a similar window exists for MAPK inhibition has yet to be determined. We demonstrated, however, the possibility of such a window existing in that growth of THP-1 cells (FLT3WT/WT) was unaffected by MAPK downregulation. At least one major pharmaceutical company is working on this problem at the present time. One candidate (CI-1040, a non-ATP
non-ERK competitive inhibitor of MEK1/2) advanced into phase I-II clinical trials (25, 26).
While being well-tolerated, the drug did not cause sufficient anti-tumor activity and has since
been abandoned. A second generation inhibitor, PD 0325901, which showed excellent in vitro
potency (IC50 ~ 1 nM) and oral bioavailability, is now being tested clinically. It is also certainly
plausible that upstream activators of MAPK responsible for transmitting the FLT3 signal could
be targeted for inhibition and achieve the same result we observed in this study without being
toxic to normal cells. With the recent advance in drug development that occurred in the past
decade, it is now becoming possible to target almost any cellular event for inhibition.
Investigations that seek to elucidate the mechanism of FLT3-MAPK interaction are certainly
warranted.

We are confident that the cytokines IL-3, GM-CSF and SCF used in combination do
not play a critical role in FLT3 ITD signaling, nor do they have a significant impact upon FLT3
inhibition. This does not rule out the possibility that other bone marrow stimulating factors may
be influencing FLT3, however these three were the most likely candidates given what is
presently known about hematopoiesis.

**Potential Limitations to FLT3 Inhibition Therapy.** Several studies have demonstrated that
the mutant/wild-type genotypic ratio increases significantly, from 17.9% at diagnosis to 40.5% at
relapse, as assessed by ethidium bromide-stained PCR densitometric analysis (13). Patients with
FLT3 mutations sometimes relapse having gained additional FLT3 mutations and/or having lost
the wild-type allele. Other patients relapse having reverted back to wild-type FLT3.
Additionally, some patients relapse having lost the original FLT3 mutation and having gained
another one.
The loss of the original FLT3 mutation in some relapse patients and the acquisition of new FLT3 mutations suggest that FLT3 mutations may be “late hits” (13). FLT3 mutations may occur only in subclones of the original malignancy, conferring greater growth advantage upon their progeny, while being unnecessary to the overall long-term survival of the cancer. Effective pharmacological targeting of FLT3 may buy patients time by killing off the most malicious AML clones and forcing a pseudo-remission. However, if FLT3 mutations are normally “late-hits”, it is unlikely that FLT3 inhibition will provide us with an effective cure so long as the stem cells remain FLT3-independent.

There is also some evidence indicating that FLT3 mutations may be “early hits”, at least in certain cases. The fact that FLT3 ITD cell lines are killed in response to FLT3 inhibition supports the idea that the FLT3 mutation may have happened early in the development of leukemia. If FLT3 mutations were actually “late-hit mutations,” occurring only in subclones of the original malignancy, the original malignancy would not have been dependent upon FLT3 for survival and proliferation, and would rather have relied upon a parallel mechanism. There had to be a point when these cell lines acquired a dependency upon FLT3 signaling, a type of dependency that is generally not observed in cell lines with normal FLT3. Additionally, studies have been done where murine bone marrow transduced with FLT3 ITD’s was transplanted into NOD/SCID mice, with the result being that the mice developed oligoclonal myeloproliferative disorder (20), but there was no overwhelming evidence of aberrant leukogenesis. At present, the role of FLT3 mutations in the development of leukemia remains unresolved.

**In summary**, we have demonstrated that FLT3 inhibition can be a meaningful treatment of certain subgroups of AML patients, however, effective FLT3 inhibition must first be achieved. Since FL appears to reduce the efficacy of FLT3 inhibitors in FLT3\textsuperscript{ITD/WT} cells via signaling
along the MAPK pathway, combining FLT3 inhibition and MAPK inhibition may have therapeutic advantage in treating this subset of AML. While the FLT3\textsuperscript{ITD-} genotype is a poor prognostic factor clinically, it also may be predictive of a response to FLT3 inhibition. Further studies to confirm these phenomena in primary AML samples are ongoing.
**Figure 1. Hematopoiesis.** This figure depicts the most critical steps of hematopoiesis, starting from the pluripotent, self-renewing hematopoietic stem cell through the myeloid and lymphoid branches. In cases where the cytokines FL, SCF, IL-3 or GM-CSF are known to influence differentiation or growth, they have been included in the figure. This figure was adapted from Goldsby, R., et al.
Figure 2. Wild-type FLT3
Adapted from Levis, M., et al.

Figure 3. PCR products of FLT3 exons 11-14 on ethidium bromide-stained 3% agarose gel.
### Confirming FLT3 Genotype of Cell Lines

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Forward Primer: 1544->1565 (35059->35080)  
5’=>3’ TGTGAGCAGTACTCTAAACAT  

Reverse Primer: 1978<-1999 (36756<-36777)  
5’=>3’ CTTCAGCATTTTGACGGCAAC  

Expected Size of WT FLT3: 456 bp

### FLT3 Sequence

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UTR begins 66612
Figure 4. Wild-type FLT3 activated by FLT3 Ligand. 4.A shows site of THRX-165724 inhibition on Tyrosine Kinase domain. 4.B shows the structure of THRX-165724. 4.C shows the structure of ATP. Notice that THRX-165724 may be acting as a structural mimic to ATP.
Figure 5. Three proposed signaling pathways of FLT3. All of these have been implicated in FLT3 signaling.
• **Figure 6. THRX-165724 inhibits ligand-induced and constitutive FLT3 auto-phosphorylation.** THP-1 cells have only wild-type FLT3 and exhibit phosphorylated FLT3 only in the presence of FL. Phosphorylation is inhibited by THRX-165724 in a dose-dependent manner (IC₅₀ ~ 100 nM). MV411 cells, which contain a FLT3 ITD, show phosphorylated FLT3 in absence of FL. Activation of FLT3 is inhibited by THRX-165724 in a dose-dependent manner (IC₅₀ ~ 100 nM). Molm-13 cells behave similarly to MV4-11 (shown in Figure 9C). Bottom panels show total FLT3 in order to assure equal loading.
Figure 7. FLT3 inhibition by THRX-165724. MV4-11 and Molm-13 cells were treated for 48 hours with different doses of THRX-165724. Cell viability was measured in two independent experiments, each condition done in triplicate, by MTS Assay. Error bars indicate standard deviation.
Figure 8. FL attenuation of FLT3 inhibition with changing doses of FL.
Molm-13 cells were treated with 1000 nM THRX-165742 and variable levels of FL, as indicated, for 48 hours. Cell viability was assessed by MTS Assay with each condition carried out in triplicate. Error bars indicate standard deviation.
Figure 9: Inhibition of FLT3 phosphorylation by THRX-165724 opposed by FL. Figures A and B show the change in mean Optical Density (490 nm) in response to increasing doses of THRX-165724, by the MTS assay, as a fraction of untreated controls, in Molm-13 and MV4-11 cells, respectively. These figures are representative of three independent experiments. Figures C and D show the dose-dependent decrease in phospho-FLT3 in response to FLT3 inhibition in top panels by western blot. The bottom panels demonstrate equal loading of wells.
Figure 10. FL modulation of FLT3 inhibitor-induced apoptosis and cytotoxicity. Figure A shows the effect of FL on THRX-167524 inhibition-induced apoptosis of Molm-13 cells as measured by subG1 fraction. Figure B shows the change in mean cytotoxic IC₅₀’s from FL addition as a result of three independent experiments (from 85 nM to 747 nM).
Figure 11: FL Modulates FLT3 inhibitor-induced apoptosis in Molm1-13 cells. Molm-13 cells treated for 48 hours with or without THRX-165724 at 1000 nM and with FL and/or with the combination of three addition cytokines, labeled as GIS: GM-CSF, IL-3, and SCF. Apoptosis was measured as the change in subG1 fraction by flow cytometry and cell cycle analysis. Data shown in these figures is representative of three independent experiments.
Figure 12. Modulation of FLT3 inhibitor-induced cytotoxicity in Molm-13 cells by FL and three addition cytokines in combination, labeled as GIS (GM-CSF, IL-3 and SCF). Cells were treated for 48 hours, and cytotoxicity was assessed by MTS assay and flow cytometry (Figure A). Apoptosis was assessed by flow cytometry and cell cycle analysis in Figure B. These figures are representative of three independent experiments.
**Figure 13: FL selectively opposes downregulation of the MAPK pathway.** The top panels of Figures A and B show a dose-dependent decrease in phospho-ERK1/2 in response to FLT3 inhibition as assessed by western blot. The bottom panels demonstrate equal loading of wells.
Figure 14: Combination treatment with THRX-165724 & U0126 partially overcomes FL induced protective effects. Molm-13 cells were treated with U0126 (5000 nM) and THRX-165724 (1000 nM) with or without FL and compared against THRX-165724 treatment alone. Mean cytotoxicity and mean apoptosis are shown in Figures A and B, respectively, as the result of three independent experiments.
Figure 15. FL modulates FLT3 inhibitor-induced apoptosis is reversed by combination treatment with MEK1/2 inhibitor, U0126, at 5000 nM. Molm-13 cells were treated for 48 hours in presence of absence of FL, with THRX-165724 (1000 nM) and/or U0126 (5000 nM). This data is representative of three independent experiments.
When do FLT3 Mutations Occur?

Early Hit Model

Normal Stem Cell  $\rightarrow$ FLT3 Mutation  $\rightarrow$ FLT3$^{\text{ITD/WT}}$

Late Hit Model

Normal Stem Cell  $\rightarrow$ Typical AML Mutations  $\rightarrow$ FLT3$^{\text{WT/WT}}$

 FLT3$^{\text{ITD/WT}}$

Figure 16. What stage of leukogenesis do FLT3 mutations occur? This figure shows two proposed models. The Early Hit Model demonstrates that if FLT3 mutations occur early in leukogenesis, all subsequent clones will be FLT3-dependent for survival and proliferation, making FLT3 inhibition an ideal treatment. The Late Hit Model demonstrates a leukemia that is already established before a FLT3 mutation occurs. The FLT3 mutation confers a proliferative advantage on a particular clonal population. While FLT3 inhibition would disable that population, it would not affect other leukemic populations, which would be free to manifest themselves.
Figure 17. MAP kinase effector functions. This figure shows three out of many numerous effector functions that happen as a result of ERK1/2 MAPK activation. First, MAPK phosphorylates Elk. Elk then translocates into the nucleus and stimulates the transcription of Fos and Jun. MAPK also readily translocates into the nucleus, and once there is able to phosphorylate Fos, allowing Fos and Jun to dimerize in order to become the AP-1 heterodimer. AP-1 then upregulates cyclinD1 and p21[CIPI/WAF1] (not shown). MAPK assists with the binding of CDK4/6 and cyclinD1 indirectly by way of upregulating p21[CIPI/WAF1]. CyclinD1-CDK4/6 then phosphorylates pRB (not shown), displacing pRB from the promoters of E2F response genes, allowing E2F to upregulate transcription of cyclinE and cyclinA. This event marks the passage of a cell beyond the Restriction point (denoted R) and out of resting state, which commits a cell to completing a full round of division. CyclinE then binds to CDK2 and p27. MAPK then facilitates degradation of p27, freeing cyclinE-CDK2 complexes. CyclinE expression and activation allows for passage into S-phase. Once in S-phase, cells are no longer subject to external control in regards to division.
Resources


