MUTATIONEN DES FLT3 GENES IN AKUTER MYELOIDER LEUKÄMIE

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MUTATIONS OF THE FLT3 GENE IN ACUTE MYELOID LEUKEMIA

Contents

1. ABSTRACT (English)

Activating mutations in the juxtamembrane domain (FLT3-length mutations, FLT3-LM) and in the protein tyrosine kinase domain (TKD) of FLT3 (FLT3-TKD) represent the most frequent genetic alterations in acute myeloid leukemia and define a distinct molecular entity in AML.

In this work two new mutations in FLT3 gene are described: the first is a 6-bp insertion on the activation loop of FLT3 between codons 840 and 841 of FLT3 (FLT3-840GS) in two unrelated patients with AML and the second is a single and identical T→C nucleotide exchange in both the MM6 and MM1 cell lines resulting in a V592A mutation.
In functional analyses we could show that these mutants are hyperphosphorylated on tyrosine and confer interleukin-3 independent growth to Ba/F3 cells, which can be inhibited by a specific FLT3 PTK inhibitor.

These results show for the first time that in addition to known mutations in the JM and the catalytic domain, further activating mutations exist in the FLT3 gene.

Furthermore we could show that distinct activating FLT3-TKD mutations at position D835 mediate primary resistance to FLT3 PTK inhibitors in FLT3 transformed cell lines. In the presence of increasing concentrations of the FLT3 PTK inhibitor SU5614 inhibitor resistant Ba/F3 FLT3-internal tandem duplication (ITD) cell lines (Ba/F3 FLT3-ITD-R1-4) were generated and characterized by a 7-26 fold higher IC_{50} to SU5614 compared to the parental ITD cells. The detailed molecular characterization of ITD-R1-4 cells demonstrated that specific TKD mutations (D835N and Y842H) on the ITD background were acquired during selection with SU5614. Introduction of these “duale” ITD-TKD, but not single D835N or Y842H FLT3 mutants in Ba/F3 cells restored the FLT3 -inhibitor resistant phenotype.

These data show that pre-existing or acquired mutations in the PTK domain of FLT3 can induce drug resistance to FLT3 PTK inhibitors *in vitro*. These findings provide a molecular basis for the evaluation of clinical resistance to FLT3 PTK inhibitors in patients with AML.

2. ZUSAMMENFASSUNG

Aktivierende Mutationen in der juxtamembranösen Region (FLT3-Längenmutationen, FLT3-LM; FLT3- Interne Tandemduplikation, FLT3-ITD) und der Tyrosinkinase-Domäne (FLT3-TKD, FLT3-D835) von FLT3 stellen die häufigsten genetischen Alterationen in der AML dar und definieren eine klinisch-prognostische-Subgruppe in der AML.

In der vorliegenden Arbeit wurden zwei neue Mutationen in FLT3 identifiziert und charakterisiert. Die erste Mutation (FLT3-840GS) wurde in zwei Patientenproben nachgewiesen. Moleculargenetisch stellte diese eine Längenmutation in Exon 20 dar, und war durch eine 6 bp-grosse Insertion bzw. Glycin+Serin Insertion in der Aktivierungsschleife der katalytischen Domäne zwischen den Kodons 840 und 841 nahe der Aktivierungsschleife verursacht. Die zweite Mutation, eine aktivierende Punktmutation in der JM-Region von FLT3 (FLT3-V592A) wurde in den AML-Zelllinien MonoMac 6 und MonoMac 1 identifiziert. Die funktionelle Analyse ergab, dass diese Mutanten eine konstitutive Tyrosinphosphorylierung aufweisen und zu einem IL-3 unabhängigem Wachstum in Ba/F3 Zellen führen, welches durch einen spezifischen Proteintyrosinekinase (PTK) Inhibitor gehemmt werden konnte.
Diese Ergebnisse zeigen, dass neben den bisher beschriebenen noch weitere aktivierende Mutationen in der JM- und der katalytischen Domäne in FLT3 Gen existieren.

Die weiteren Untersuchungen zeigten, dass verschiedene FLT3-TKD (D835) Mutationen eine deutlich unterschiedliche Empfindlichkeit gegenüber selektiven FLT3 PTK Inhibitoren aufwiesen. Weiter wurde gezeigt, dass durch kontinuierliche Exposition von FLT3-ITD transformierten Leukämiezellen mit dem FLT3 PTK Inhibitor SU5614 *in vitro* resistente Mutanten generiert werden können. Die molekulare und funktionelle Charakterisierung dieser SU5614-resistenten Zelllinien (Ba/F3 FLT3-ITD-R1-4) ergab, dass spezifische Mutationen in der TKD-Domäne ursächlich für die Inhibitorresistenz verantwortlich waren. Die FLT3-ITD-R1-4 Zellen wiesen somit Doppelmutationen im FLT3-Gen (LM + TKD) auf und waren durch eine 7-26-fach höhere IC_{50} gegenüber dem Inhibitor gekennzeichnet. Solche Doppelmutanten von

FLT3 wurden mittels *in- vitro*- Mutagenese generiert und rekapitulieren in Ba/F3 Zellen den SU5614-resistenten Phänotyp.

Diese Ergebnisse zeigen, dass prä-existierende oder erworbene FLT3-TKD Mutationen Resistenzen gegenüber FLT3-PTK Inhibitoren *in vitro* induzieren können. Diese Befunde stellen die molekulare Basis für zelluläre Resistenzen gegenüber FLT3-PTK Inhibitoren bei Patienten mit AML dar.

3. INTRODUCTION

Acute myeloid leukemia (AML) is the most common form of acute leukemia occurring in adults, comprising approximately 80 to 85% of cases of acute leukemia diagnosed in individuals greater than 20 years of age. Currently, more than 80% of young adults and 60% of all patients can achieve complete remissions.
Due to the identification of leukemic fusion genes encoded by balanced chromosomal translocations, the pathophysiology of certain AML subtypes has been identified. Prognostic factors and disease heterogeneity can be defined using molecular markers and provide the potential for individualizing of antileukemic therapy in patients with AML.

3.1 Acute myeloid leukemia (AML)

The pathophisiology of AML can be explained by acquired genetic changes in bone marrow stem cells that cause a complete or partial block in normal hematopoietic stem cell maturation. The genetic changes may involve mutations that lead to activation of growth-promoting proto-oncogenes, inactivation of tumor suppressor genes, or alterations of transcription factors.

Although the acquired genetic lesions that lead to leukemia are being rapidly defined, the causative agent or environmental factor can be identified for a small fraction of patients with AML. Nonetheless, leukemias clearly occur with increased frequency after military or therapeutic radiation exposure, after certain types of chemotherapy and with heavy and continuous occupational exposure to benzene.

There have been a number of large, survey-type epidemiologic studies that have attempted to link a variety of environmental exposures to leukaemia incidence. But, as in many epidemiologic studies, it is difficult to quantify the degree of exposure to various environmental insults. There may be a small increased risk associated with cigarette smoking, whereas exposure to electromagnetic radiation seems an unlikely cause of AML. However, occupational exposures, particularly to

benzene or petrochemicals have been implicated in the development of AML. Alkylating agents used in Hodgkin's disease, multiple myeloma, ovarian cancer, and colon cancer have been associated with loss of chromosome 5 and 7, whereas tenoposide use in childhood acute lymphoblastic leukaemia (ALL) and high-dose anthracycline/cyclophosphamide combinations have caused alterations of chromosome 11q23. With the few exceptions noted above, however, there is no clear relationship between environmental exposures and the occurrence of acute leukemia.

3.1.1 Classification of AML

In 1976, a group of morphologists from France, the United States, and Great Britain (FAB) suggested a classification system designed to define distinct subtypes of AML and ALL (M0-M7) (Table 1).

FAB-Typ	Monoclonals for precursor cells			Myeloid markers			Monocyte markers			Cytogenetics
	Tdt	HLA-DR	CD34 (My10)	CD13 (My7)	CD33 (My9)	CD15 (Leu Mi)	CD11 (My01)	CD14 (My4)	Other	
M0 (minimally differentiated AML)	±	+	+	+	±	±	-	-		11q13
M1 (myeloid leukemia without maturation)	±	+	+	+	+	-	±	-		-5,-7,-17 del 3p+21,+8
M2 (myeloid leukemia with maturation)	-	+	-	+	+	+	±	-		t(8;21) del 3p or inv 3 -5,-7 t(6;9),+8
M3 (promyelocytic APL)	-	-	-	+	+	±	-	-		t (15;17)
M4 (myelomonocytic)	-	+	-	+	+	+	+	+		inv (16) or -16q t (16;16)
M5 (monocytic leukemia)	-	+	-	±	+	+	+	+		occ t(8;21), -5,-7 t(6,9) t(9;11)(p21;p23)+8
M6 (erythroid)	-	±	-	±	±	-	±	-	Glyco-protein A	-5q,-5,-7,-3,+8
M7 (megacaryocytic)	-	+	+	-	±	-	-	-	Platelet glyco-protein	inv or del 3 +8,+21

Table 1: FAB classification of AML.
Tdt-terminal deoxinucleotide transferase, HLA-DR-human leukocyte antigen D-related (from ''Cancer Medicine'')

The diagnosis of AML requires that myeloblasts constitute 20% (based on a recent World Health Organization classification system) or more of bone marrow cells or circulating white blood cells. Neoplastic promyelocytes, monoblasts, or promonocytes and megakaryoblasts are included in this calculation and their presence defines the various subtypes of AML. Monoclonal antibodies directed against antigen groups termed cluster designation (CD) considered to be restricted to cells committed to myeloid differentiation are also helpful. Antibodies against CD11b, CD13, CD14 and CD33 are used most commonly.

The new WHO classification retains the morphologic subgroups of the FAB system, but has created new categories which recognize the importance of certain cytogenetic translocations as predictors of response to therapy (Table 2).

WHO classification for AML

AML with recurrent cytogenetic translocations
AML with t(8;21), (q22;q22)
AML with t(15;17),(q22;q11-12)+ variants=APL
AML with abnormal bone marrow eosinophils, inv (16)(p13;q22) or t(16;16)
AML with 11q23 (MLL) abnormalities
AML with multilineage dysplasia
With prior myelodysplastic syndrom (MDS)
Without prior MDS
AML and MDS, therapy-related
Alkylating agent-related
Epipodophyllotoxin-related (some may be lymphoid)
Other
Acute biphenotypic leukemias

Table 2: WHO classification of AML (from ‘‘Cancer Medicine’’)

3.2 Receptor tyrosine kinases

3.2.1 Signaling through receptor tyrosine kinases

The extracellular signal proteins that act through receptor tyrosine kinases consist of a large variety of secreted growth factors and hormones. The human genome as currently sequenced, is thought to contain 90 tyrosine kinase genes, of which 58 are of the receptor type. Receptor tyrosine kinases can be classified into more than 16 structural families, each dedicated to its complementary family of protein ligands.

In all cases, the binding of the signal protein to the ligand-binding domain on the outside of the cell activates the intracellular tyrosine kinase domain (Figure 1). Once activated, the kinase domain transfers a phosphate group from ATP to selected tyrosine side chains, both on the receptor proteins themselves and on intracellular signaling proteins that subsequently bind to the phosphorylated receptor. The tyrosine phosphorylated receptor serves as a docking site for an array of intracellular signalling molecules, including the GTPase-activating protein (GAP), the p85 subunit of phosphatidyl-inositol 3'-kinase (PI3K), phospholipase C-γ (PLC-γ), the protein tyrosine phosphatase SHP1, Grb2 and Src-like non-receptor kinases; (Rosnet, Buhring et al. 1996)Porter and Vaillancourt, 1998). These activated proteins then initiate serine/threonine phosphorylation cascades resulting in activation of transcription factors that determine a variety of cell responses, including cell maintenance, mitogenesis, migration and differentiation (Claesson-Welsh, 1994). All RTKs contain an evolutionary conserved kinase domain, and the available crystal structures of RTK kinase domain exhibit a conserved structure (Hubbard 1994, Mohammadi 1996, McTigue 1999). Crystallografic studies of the insulin receptor (IR), solved in both active and inactive conformations, reveal a twofold mechanism of activation (Hubbard 1994, Hubbard 1997). In the inactive conformation, the activation loop of IR occupies the active site, blocking substrate access and/or ATP binding. Upon phosphorylation of tyrosine residues within the activation loop, structural changes occur that reposition the activation loop away from the active site, resulting in an active conformation. Mutations both in the activation loop and in other regions of the kinase domain have been identified in several RTKs, which most probably stabilize an active kinase conformation.

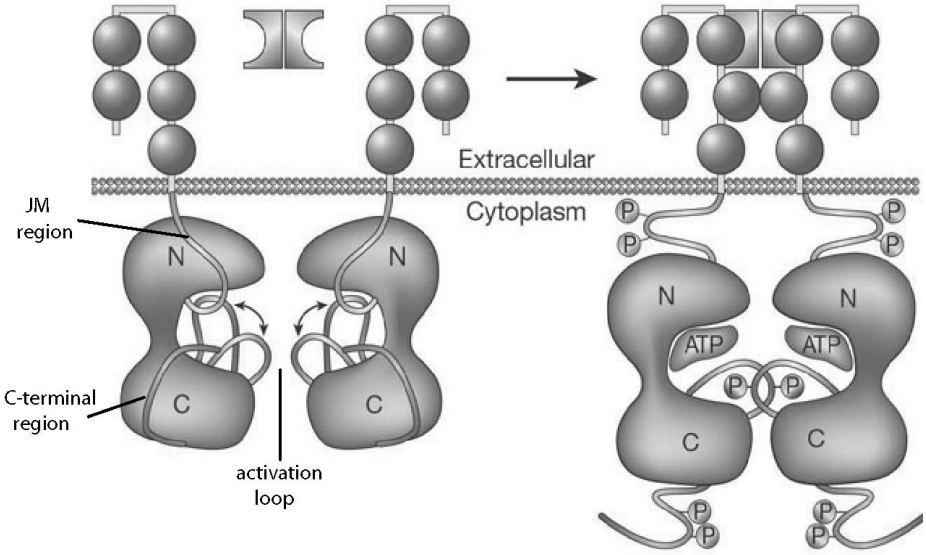


Figure 1 Protein tyrosine kinase activation mechanisms.
Left: inactive conformation of RPTK. Right: ligand-induced receptor dimerization and tyrosine autophosphorylation (From P.Blume-Jensen and T.Hunter, Nature 411:355-365, 2001).

3.2.2 Class III receptor tyrosine kinases: role in leukemogenesis

The class III RTKs, which include FMS, KIT, FLT3, PDGFRα and PDGFRβ play an important role in normal hematopoiesis (with the exception of PDGFR). FMS, the receptor for the macrophage colony-stimulating factor (M-CSF), is crucial for the growth and differentiation of the monocyte-macrophage-osteoclast lineage (Sherr, 1990). FLT3 and KIT are both required for the survival, proliferation and differentiation of hematopoietic progenitor cells, while c-kit is also important for the growth of mast cells, melanocytes, primordial germ cells and interstitial cells of Cajal. (Drexler 1996; Lyman and Jacobsen 1998)The hematopoietic functions of PDGFRβ are less well defined, although the receptor and its ligand probably play a significant role in megekaryocytopoiesis (Yang, 1997).

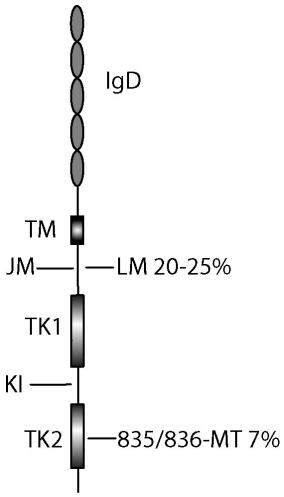
The class III RTKs have recently been linked to the pathogenesis of an increasing number of hematological malignancies. KIT mutations, for example, have been shown to be causative in adult-type mastocytosis, as well as being associated with acute myeloid leukaemia and sinonasal lymphomas. Indeed, Longley et al (Longley, Reguera et al. 2001)have proposed a classification of mast cell disease

based on the nature of the c-kit mutations. FLT3 is a gene most commonly mutated in AML and the presence of FLT3-ITD appears to be a second strongest independent prognostic factor in the disease after cytogenetic. (Kiyoi, Naoe et al. 1999) The role of PDGFRβ-fusion genes in bcr-abl-negative chronic myeloproliferative disorders is still unfolding, but at least eight partner genes have been identified.

3.2.3 Mutations of FLT3 in AML

Recent advances in genetics have shown that not only chromosome abnormalities but also molecular alterations are useful to characterize and subclassify acute myeloid leukemia (AML). For example, partial tandem duplication within the MLL gene (MLL-PTD) has been shown to define a subgroup of AML patients with unfavourable clinical outcome. (Schnittger, Kinkelin et al. 2000)

FLT3 (*fms*-like tyrosine kinase-3; STK1, human stem cell tyrosine kinase-1 or FLK-2, fetal liver kinase-2) is a receptor tyrosine kinase (RTK), which is known to play an important role in normal hematopoiesis and leukemogenesis. (Matthews, Jordan et al. 1991; Rosnet, Marchetto et al. 1991)FLT3 has strong sequence similarities to other members of the class III RTK receptor family. This protein family include FLT3, FMS, platelet-derived growth factor receptor (PDGFR), and KIT and characterized by an extracellular domain comprised of 5 immunoglobulinelike (IG-like) domains and by a cytoplasmic domain with a split tyrosine kinase motif (Figure 2).



<u>Figure 2:</u> FLT3 receptor.

IgD- immunoglobuline domain; JM- juxtamembrane domain; KI-kinase insert; TK1, TK2-tyrosine kinase 1and 2 domains; LM-length mutation; MT-mutation

FLT3 is expressed in a variety of human and murine cell lines of both myeloid and B-lymphoid lineage. In normal bone marrow, expression appears to be restricted to early progenitors, including CD34+ cells with high levels of expression of CD117 (C-KIT). FLT3 is also expressed at high levels in a spectrum of hematologic malignancies including 70% to 100% of acute myelogenous leukemia (AML) of all FAB subtypes, B-precursor cell acute lymphoblastic leukemia (ALL), a fraction of T-cell ALL, and chronic myelogenous leukemia (CML) in lymphoid blast crisis. These data indicate that FLT3 expression may play a role in the survival or proliferation of leukemic blasts. (Carow, Levenstein et al. 1996; Drexler 1996)

Both FLT3 overexpression and activating mutations in the FLT3 gene can be found in patients with AML. 20-25% of patients with AML carry activating FLT3-LM in the juxtamembrane domain. (Nakao, Yokota et al. 1996; Kottaridis, Gale et al. 2001; Reilly 2002; Schnittger, Schoch et al. 2002)In another 7% mutations at codons 835/836 in the activation loop of FLT3 can be detected (FLT3 tyrosine kinase mutations, FLT3-TKD).; (Abu-Duhier, Goodeve et al. 2001; Thiede, Steudel et al. 2001; Yamamoto, Kiyoi et al. 2001; Thiede, Steudel et al. 2002)FLT3-LM have been detected in all FAB subtypes of AML, with the highest reported frequency in the M3 subtype, and less frequently in the M2 subtype. FLT3-LM are associated with leukocytosis and poor prognosis in most, (Kiyoi, Naoe et al. 1999; Xu, Taki et al. 1999; Rombouts, Blokland et al. 2000; Thiede, Steudel et al. 2001; Whitman, Archer et al. 2001; Yamamoto, Kiyoi et al. 2001)but not all studies. (Schnittger, Schoch et al. 2002; Thiede, Steudel et al. 2002)

Recent data indicate that FLT3-LM are not present in systemic mast cell disease nor in a spectrum of solid tumors. FLT3-LM have not been detected in normal hematopoietic cells, including cord blood and bone marrow cells in which there are high levels of expression of FLT3. In addition to length mutations in one allele of FLT3 several studies have demonstrated biallelic mutations in FLT3, as well as patients in whom the residual wild-type allele is lost. (Kottaridis, Gale et al. 2001;

Whitman, Archer et al. 2001; Schnittger, Schoch et al. 2002; Thiede, Steudel et al. 2002)

FLT3-LM and FLT3-TKD mutants are constitutively autophosphorylated on tyrosine residues, causing activation of signal transducer and activator of transcription (STAT)-5 and mitogen-activated protein (MAP) kinases. (Hayakawa, Towatari et al. 2000; Mizuki, Fenski et al. 2000; Tse, Mukherjee et al. 2000; Tse, Novelli et al. 2001; Spiekermann, Dirschinger et al. 2003)In addition, transduction of FLT3-ITD and TKD mutants in murine IL-3-dependent cell lines, such as Ba/F3 and 32D induces IL-3 independent growth (Fenski, Flesch et al. 2000; Hayakawa, Towatari et al. 2000; Mizuki, Fenski et al. 2000; Tse, Mukherjee et al. 2000; Yamamoto, Kiyoi et al. 2001)*in vitro* . Injection of 32D or Ba/F3 cells stably transfected with constitutively activated FLT3 into syngeneic recipient mice results in the development of a leukemic phenotype. (Mizuki, Fenski et al. 2000) Furthermore, retroviral transduction of FLT3-ITD constructs in primary murine bone marrow cells induces a myeloproliferative phenotype in a mouse bone marrow transplantant model. (Kelly, Liu et al. 2002)These data demonstrate that, although FLT3-ITDs have been associated primarily with AML in humans, FLT3-ITD alone are not sufficient to induce AML in primary hematopoietic cells. Furthermore, a kinase-dead mutant of FLT3 in the context of FLT3-ITD abrogates the myeloproliferative disease, indicating an absolute requirement of FLT3 kinase activity for the myeloproliferative phenotype in this model. No difference in biologic activity of FLT3-ITD mutants have been found in cell culture or murine models, despite considerable variation in repeat length that ranges from several to more than 50 amino acids.

Thus, both LM and TKD mutations in the FLT3 gene result in constitutive activation of the kinase activity and downstream targets including STAT5, RAS/MAPK, and PI3K/AKT. Expression of FLT3 activating mutations in primary hematopoietic cells results in a myeloproliferative phenotype, and indicates, that additional mutations are required for development of AML.

3.3 RTK inhibitors

Although activating FLT3 mutations seem not be sufficient to cause an AML phenotype they represent a potential therapeutical target. Targeted inhibition of aberrant kinase signalling can be an effective therapeutic intervention in hematologic malignancies, as evidenced by hematologic and cytogenetic responses in chronic myelogenous leukemia (CML) and CML blast crisis patients treated with the BCR-ABL kinase inhibitor imatinib mesylat (STI571, Gleevec®). (Druker, Sawyers et al. 2001; Druker, Talpaz et al. 2001; Savage and Antman 2002)An analogous kinase inhibitor strategy might have therapeutical potential in AML patients with activating mutations in the FLT3 gene.

In the past decade, many laboratories embarked on projects aimed at generating compounds that specifically inhibit the activity of the signalling cascades triggered by tyrosine kinases. Compounds with selective activity to class III RTK *in vitro* include AG1295, (Levis, Tse et al. 2001)SU5416, (Fong, Shawver et al. 1999; Spiekermann, Faber et al. 2002)SU5614 (Yee, O'Farrell et al. 2002; Spiekermann, Dirschinger et al. 2003)and CT53518. (Kelly, Yu et al. 2002)Three compounds (CEP-701, SU11248 and PKC412) with *in vivo* activity are currently evaluated in phase I/II clinical trials in patients with AML and have shown promising results. (Foran, O'Farrell et al. 2002; Foran, Paquette et al. 2002; Smith, Levis et al. 2002; Stone, Klimek et al. 2002)

The PTK inhibitor imatinib mesylate has high clinical activity in BCR-ABL-positive CML in chronic phase and blast crisis as well as in BCR-ABL-positive acute lymphoblastic leukemia and gastrointestinal stromal tumors (GISTs) carrying activating KIT mutations. (Druker, Talpaz et al. 2001; van Oosterom, Judson et al. 2001; Kantarjian, Sawyers et al. 2002)However, small molecule protein tyrosine kinase inhibitors may lead to drug resistance. (Sawyers 2001)Recent studies have indicated that resistance to imatinib may be multifactorial. (Mahon, Deininger et al. 2000; Weisberg and Griffin 2000)In certain BCR-ABL-positive cell lines, resistance was associated with amplification of the fusion gene. (le Coutre, Tassi et al. 2000; Mahon, Deininger et al. 2000; Weisberg and Griffin 2000)Additionally, overexpression of the alpha 1 acid glycoprotein was detected in some resistant

cells. (Gambacorti-Passerini, Barni et al. 2000)At least ten different mutations in the ABL gene that induce imatinib resistance *in vitro* were found in patients with BCR-ABL-positive CML and ALL. (Gorre, Mohammed et al. 2001; Barthe, Gharbi et al. 2002; Branford, Rudzki et al. 2002; Hofmann, Jones et al. 2002; Roumiantsev, Shah et al. 2002; Shah, Nicoll et al. 2002; von Bubnoff, Schneller et al. 2002)Although FLT3 PTK-inhibitors have shown promising results in phase I/II clinical studies in patients with AML, it is unknown whether similar mechanisms of resistance exist for FLT3 PTK inhibitors.

Previously, our group and others have demonstrated that the small molecule PTK inhibitor SU5614 is a selective inhibitor of FLT3 that induces growth arrest and apoptosis in FLT3 transformed cells and AML-derived cell lines expressing a constitutively activated FLT3. (Yee, O'Farrell et al. 2002; Spiekermann, Dirschinger et al. 2003)

4. THE GOAL OF STUDY

FLT3 is known to be the most mutated gene in AML. FLT3-LM were found in 20-25% of patients with AML and 7% of patients carry FLT3-TKD mutations. The high incidence of FLT3 mutations in AML makes it an attractive target for therapeutic interventions. The important questions here are, whether other FLT3 mutations can also be found in patients with AML and whether the kind of mutation in FLT3 might be important for the sensitivity to PTK inhibitors.

It was recently shown that conformational changes in the activation loop of KIT by the D816V mutation led to resistance of this mutant to PTK inhibitor imatinib. Therefore, we wanted to determine, whether FLT3-ITD and different point mutations in TKD of FLT3 may differ in their sensitivity to FLT3 PTK inhibitors.

The development of drug resistance is major problem of chemotherapy in patients with AML. Previous studies on the BCR-ABL PTK inhibitor imatinib have shown that drug resistance may be multifactorial and include: overexpression of the fusion gene, development of additional mutations and overexpression of the alpha 1 acid glycoprotein. Although FLT3 PTK inhibitors have shown promising results in phase I/II clinical studies in patients with AML, the similar mechanisms of drug resistance might occur. In this study Ba/F3 FLT3-ITD transformed cells were used as a model system and were incubated in the presence of the increasing concentration of the FLT3 PTK inhibitor. Furthermore, these cells were used to characterize potential mechanisms of drug resistance *in vitro*.

It was the aim of this study to detect and characterize new mutations in the FLT3 gene and answer the questions whether FLT3-TKD mutations can confer primary resistance to PTK inhibitors and whether the secondary resistance to these inhibitors can develop in FLT3-ITD transformed hematopoietic cell lines.

5. MATERIALS AND METHODS

5.1. Chemicals

* Corrosive; ** Toxic; *** Comburant

Acetic acid (Merck, Darmstadt, Germany) *

Acrylamide/Bisacrylamide 40% (Roth, Karlsruhe, Germany) **

Agarose (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Ammonium peroxidisulfate (APS) (Bio-rad, Munich, Germany)

Aprotinin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Bovine serumalbumin (BSA) (Fluka, Buch, CH)

Ethanol (Merck, Darmstadt, Germany) ***

Glycerin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

HEPES (N-(2-Hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Leupeptin A (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Methanol (Merck, Darmstadt, Germany) ***

Pepstatin A (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) **

Ponceaus (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Protein G-sepharose (monoclonal) (Amersham, Schweden)

Protein A-sepharose (polyclonal) (Upstate, USA)

Sodium Vanadate (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Sodiumdodecylsulfat (SDS) (Bio-rad, Munich, Germany)

Tetramethylethylendiamine (TEMED) (Serva, Heidelberg, Germany)

Tris(hydroxymethyl)aminomethane (Tris) (Merck, Darmstadt, Germany)

Trypan blue (Serva, Heidelberg, Germany) **

Tween 20 (Merk, Darmstadt, Germany)

5.2 Kits

Annexin V/Propidium Iodide (PI) kit (Alexis, Lausen, Switzerland)

Bio-rad Protein estimation kit (Bio-rad, Munich, Germany)

Detection system ECL® (enhanced chemiluminescence) (Amersham Pharmacia, Freiburg, Germany)
Qiagen Plasmid Maxi Kit (Qiagen, Germany)
RNeasy Mini Kit (Qiagen, Germany)
QIAprep-spin Miniprep Kit (Qiagen, Germany)
Omniscript™ Reverse Transcriptase Kit (Qiagen, Germany)

5.3 Laboratory equipment

Blotting chamber (Bio-rad, Munich, Germany)
Cell culture CO_2 incubator (Haereus, Rodenbach, Germany)
Cell culture hood (Bio Flow Technik, Meckenhein, Germany)
Centrifuges ROTIXA/P (Hettich, Tuttlingen, Germany)
Developing machine M35X-OMAT Processor (Kodak AG, Stuttgart, Germany)
Eppendorf ultracentrifuge 2K15 (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)
FACSscan (Beckton Dickinson, Mountain View, CA)
Fridge (4°C, -20°C) (Siemens AG, Germany)
Fridge (-80°C) UF80-450S (Colora Messtechnik GmBH, Lorch, Germany)
Gel electrophoresis systems (Bio-rad, Munich, Germany)
Heating block BT 130-2 (HLC, Haep Labor Consult, Bovenenden, Germany)
Liquid nitrogen tank (Cryoson, Schöllkrippen, Germany)
Microscope (Carl Zeiss Jena, Germany)
Nitrocellulose membranes (Sartorius, Goettingen, Germany)
pH-meter 766 (VWR International, Ismaning, Germany)
Rotor Ti 75 (Beckman, Palo Alto, CA)
Shaker (Edmund Bühler, Tübingen, Germany)
Spectophotometer Smartspec ™ 3000 (Bio-rad, Munich, Germany)
Ultracentrifuge L7-65 (Beckman, Palo Alto, CA)
Vortex (Cenco, Breda, the Netherlands)
Water bath (HAAKE, Karlsruhe, Germany)

5.4 Cells

Suspension cells

Cell type	Description	Origin
Ba/F3	Mouse pro B cells, IL-3 dependent murine pro B cell line, established from peripheral blood BALB/c mouse	DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany)
MM1 and MM6	Human acute monocytic leukemia, established from peripheral blood	DSMZ

Adherent cells

Cell type	Description	Origin
293	Human embryonic kidney, adherent fibroblastoid cells	DSMZ

5.5 Materials used for the cell culture

5.5.1 Cell culture media and other reagents for cell culture

RPMI 1640 (Life Technologies Invitrogen, GIBCO BRL, Kalsruhe, Germany)

10% FCS (Fetal calf serum) (Biochrom AG seromed, Berlin, Germany)

DMSO (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Penicillin/streptomycin (GIBCO BRL, Kalsruhe, Germany)

L-glutamine (GIBCO BRL, Kalsruhe, Germany)

Phosphate Buffer Saline (PBS) (GIBCO BRL, Kalsruhe, Germany)

Trypan Blue (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)

Trypsin-EDTA (Life Technologies Invitrogen, GIBCO BRL, Kalsruhe, Germany)

5.5.2 Culture vessels and plastic ware

Culture flasks and plates

Container	**Surface area (cm^2)**	**Medium required (ml)**	**Cell volume of inoculum**	**Company**
96-well flat plate	0,3	0,1	< $1x10^4$	Nunc, Wiesbaden, Germany
96-well round plate	0,3	0,1	< $1x10^4$	Nunc, Wiesbaden, Germany
50 ml flask	25	5	2 $x10^5$	Greiner Labortechnik, Frickenhausen, Germany
250 ml flask	75	15	6 $x10^5$	Greiner Labortechnik, Frickenhausen, Germany
600 ml flask	125	30	2 $x10^6$	Greiner Labortechnik, Frickenhausen, Germany

Other plastic material

Centrifuge vials (15-50 ml) (Sarstedt, Nümbrecht, Germany)

Eppendorf cups (0,5-1 ml) (Eppendorf, Hamburg, Germany)

Freezing tubes (Nunc, Wiesbaden, Germany)

Micropipettes, Pipettes (Gilson, Langenfeld, Germany)

Parafilm M (American National Can, Greenwich, USA)

5.6 Antibodies for Western Blot

Anti-rabbit-HRPO (horse radish peroxidase) IgG sc-2025 (Santa Cruz Biotechnology, Santa Cruz, California, USA)

Anti-mouse-HRPO IgG sc-2027

FLT3/Flk-2 (S-18) rabbit polyclonal sc-480

β-Aktin mouse monoclonal

Phospho-STAT5 (Tyr 694) rabbit polyclonal (Cell Signalling Technologies, New England Biolabs GmbH, Germany, Frankfut am Main)

STAT5 rabbit polyclonal

Phospho-p44/45 (Thr 202/Tyr 204) rabbit polyclonal

P44/45 MAPK (ERK1/2) kinase antibody rabbit polyclonal

5.7 Software

Cellquest software (Bekton Dickison, Mountain View, CA)

Microsoft Word, Excel, Powerpoint, Photoshop

Winmdi 3.2

BioEdit

Adobe Illustrator

Adobe Photoshop

Tina 2.09

5.8 Cell culture methods

5.8.1 Cell culture techniques

The mammalians cells grow attached to a substratum, like for example plastic or in suspension in plastic flasks. The plastic ware is obtained in sterile wraps from commercial suppliers and is specially prepared for use in cell culture (see Table in materials). All these tissue culture flasks have caps with filters which allow gaseous exchange, allowing maintenance of correct pH (which is monitored by the colour of the phenol red present in the medium) and the right percentage of CO_2 (5%). The optimal atmosphere conditions are allowed by CO_2 incubator, which automatically control temperature and pCO_2; it operates with a try of water on the base in an attempt to maintain more than 98% relative humidity. Temperatures of the incubator was set at 37°C and regurarly controlled. The use of plastic pipettes and special medium (very low endotoxin) was necessary to work in an endotoxin free condition.

5.8.2 Cell quantification and evaluation of viability

An efficient way of counting cells and at same time evaluates the percentage of viable cells is the technique of “dye exclusion”: this test is based on the concept that viable cells do not take up some dyes, whereas dead cells are permeable to these dyes. Trypan blue is the most commonly used dye, but have the disadvantage of staining soluble proteins. In the cell culture however some miss-leading situations such as recent trypsinization and freezing and thawing in presence of dymethylsulphoxide (DMSO) may lead to membrane leakeness.

From each suspension cells an aliquot of 10 µl was harvested, mixed with 90 µl of Tryplan Blue (ratio cells:trypan blue=1:1) and counted on a counting slide under the microscope. The mean of at least three counts of viable cells (not stained with tripan blue)/quadrant was considered and multiplied to the magnitude (10^4) and the dilution factor.

5.8.3 Storage of cells

In order to minimize the cellular injury induced by freezing and thawing procedures (intracellular ice crystals and osmotic effects), a cryoprotective agents such as dimethyl sulphoxide (DMSO) or glycerol are added. A variable number of suspension cells between 2-10 x 10^6 are spin down and resuspended in a 500 µl 10% DMSO solution (DMSO diluted in fetal calf serum). Freezing ampoules are cooled up before and every step is performed on ice. While short-term preservation of cell lines using mechanical freezers (-80°C) is possible, storage in liquid nitrogen (-196°C) or its vapour (-120°C) is much preferred. Rapid thawing of cell suspension is essential for optimal recovery.

5.8.4 Proliferation assay

Base culture medium for suspension cells (Ba/F3, MM1, MM6) was RPMI 1640 supplemented with 10 % FCS, 1% penicillin and streptomycin + 1% L-glutamine. Both FCS as well as the antibiotics penicillin and streptomycin are aliquoted and stored at – 20°C. Serum contains complement which may interfere in virus or cytotoxity assays; FCS is therefore inactivated by heating it to 56°C for 45 minutes. Serum supplies many essential factors as adhesion promoting components, nutrients and trace minerals, transport proteins (albumin, ferritin) and

other proteins. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% humidity.

Cells were seeded at a density of $4x10^4$/ml growth medium in the presence or absence of IL-3 and inhibitors or cytostatics as indicated. Viable cells were counted at 24, 48 and 72 hours in a standard hemacytometer after staining with trypan blue. The IC_{50} was defined as the concentration of inhibitor at which 50% of cells were viable compared to cells grown in the absence of inhibitor.

5.8.5 Transfection of the 293 cells

The cells were seeded at a density $5x10^5$/ml. Transient transfections were then carried out using the calcium-phosphate co-precipitation method with a total 6µg DNA per 6-well (mix: 54 µl 250mM $CaCl_2$, 54 µl HBS, pH=7.8, 6 µg DNA). 18 hours after transfection 3 ml fresh medium was added, the cells were allowed to grow for another 30h and the retroviral supernatant was used for transduction of Ba/F3 cells.

5.8.6 Stable transduction of Ba/F3 cells

$2x10^5$/ml Ba/F3 cells were seeded in 200µl of growth medium RPMI-1640 and subsequently transduced once with 200µl of retroviral supernatant in the presence of polybrene (8µg/ml). After 4-12 hours the 1 ml of medium was added to avoid the toxic action of the polzbrene on the cells. The cells were sorted after 48-72 hours.

5.8.7 Subculturing of adherent cells

293 cells were maintained in Dulbecco's modified Eagle Medium (DMEM; PAN, Germany) with 10% FBS. Cells growing as monolayers on plastic surfaces are held together and to the substratum by mucoproteins and sometimes by collagens; in addition, many cell monolayers require divalent cations (Ca^{2+}, Mg^{2+}) for their integrity. Thus for releasing cells from monolayers various protease solutions are used, sometimes in association with chelating agent. Therefore Trypsin 0,5 EDTA was used for subculturing of adherent cell line with following protocol: when cells were seen to be confluent (in a 75 cm^2 flask, after 2 day of culture) old medium was washed out, cells were washed with PBS and incubated with 3 ml of Trypsin-EDTA for 5-10 minutes in the incubator; prolonged exposure to trypsin should be

avoided as this damages the cells; therefore to inactive it, medium containing serum, which contain a natural trypsin inhibitor, was added. Cells were than splittet (subcultured) in a split ratio of 1:3 (from one 75 cm^2 flask, three 225 cm^2 flasks).

5.8.8 Cell sorting

The FACS-Ventage system equipped with a Turbo-Sort device (Becton Dickinson, San Jose, CA) was used to highly purify EGFP/EYFP-positive pool cells 48 hours after infection. For this purpose, the cells were washed with PBS and then diluted in medium containing 1µg/ml propidium jodid.

5.8.9 FACS analysis

5.8.9.1 Expression of CD135 by flow cytometry

Ba/F3 cells were incubated for 30 minutes on ice with a mouse phycoerythrin (PE) labeled isotype-matched control antibody (Becton Dickinson) or CD135-PE (Becton Dickinson) antibody. Viable cells were analyzed using a FacsCalibur flow cytometer (Beckton Dickinson, San Jose, CA).

5.8.9.2 Apoptosis analysis

Assessment of apoptotic cells was carried out by annexin V/7-amino-actinomycin D (7-AAD) staining as recommended by the manufacturer (annexin V-phycoerythrin (PE) apoptosis detection kit, Becton Dickinson, Heidelberg, Germany) using a FacsCalibur flow cytometer (Beckton Dickinson, San Jose, CA).

5.8.10 Development of SU5614 resistant Ba/F3-FLT3-ITD cell lines

Ba/F3 cells carrying the FLT3-ITD (W51) mutation were cultured in the presence of increasing concentrations of SU5614 (0.2 µM to 0.8µM) over a period of at least three months. Cells were initially cultured in the presence of 0.2 µM SU5614, with culture medium changes every 3-4 days, for a total 27 days. Cells were then cultured in 0.4µM SU5614 for an additional 35 days, and finally cells were grown in medium containing 0.8µM SU5614. Then cells were analyzed for drug resistance and further characterized.

5.9 BIOCHEMICAL METHODS

5.9.3 Buffers and solutions

Lysis buffer	5 ml 1M HEPES pH 7,5 3 ml 5M NaCl 0.5 ml 200 mM EGTA 20 ml 50% glycerol 1 ml Triton X-100 0.42 g NaF 0.45g$Na_2P_2O_7x10H_2O$(tetra-Natriumdiphosphatdekahydrat as buffer) add 100 ml aqua dest. 50 µl Aprotinin (200x) 100 µl 0.1 M PMSF (350 mg/20 ml EtOH) 100 µl 0.1 M Orthovanadat (183 g/10ml aqua dest)
10xNET	1.5 M NaCl 0.05 M EDTA pH 8 0.5 M Tris pH 7,5 0.5% Triton X-100
1x G-NET	2.5 g gelatin 100 ml 10xNET 900 ml H_2O
10xTBS	10mM Tris pH 8 150 mM NaCl
1xTBST	100 ml 10xTBS 0.2 % Tween 20 Water to 1L

4XHNTG	200 mM HEPES pH 7,5 600 mM NaCl 0.4% Triton X-100 40% glycerol
Stripping buffer	800 ml 3xTBS 0.2% Tween 20 H_20 to 1L
Laemmli-Buffer	187,5 mM Tris 6% SDS 30% glycerine bromphenolblue
Laemmli-Dithiothreitol (DTT) buffer	0.6 g of DTT 2.5 ml of Laemmli buffer

All of chemicals were obtained from Sigma or Merck (Germany).

5.9.1 Cell lysis

Cells were incubated in lysis buffer for 30 minutes at 4°C. Whole cell lysates containing 100 µg protein as determined by the Bradford method were denatured by boiling for 5 min in SDS sample buffer, loaded into one lane and separated by 10% SDS-PAGE.

5.9.2 SDS-Polyacrylamidgelelectrophoresis PAGE

To separate proteins according to their molecular weight (1D electrophoresis) protein samples (whole cell lysates) are first denaturized by boiling at 95°c for 5 minutes in a SDS buffer; 40 to 100 µg of sample are loaded into a SDS gel and let run at 150 volts in a running buffer to separate electrophoretically. Proteins with different molecular weight and load are run differently. A marker with well-characterized proteins allows identifying the molecular weight of unknown proteins. Gel must be fresh prepared; gel solution solidify and polimerize quickly (5-10 minutes) after giving APS and Temed. Gels can be kept in a humified ambience for 1-2 days.

5.9.4 Western Blot analysis

After electrophoresis, proteins can be transferred to nitrocellulose membranes in a blotting puffer for 1hour at 75 volts and probed with appropriate Ab. To see all proteins transferred, the membrane can be stained shortly with Ponceau staining, that is a transient red staining which bind every protein. Before antibody incubation, the membrane has to be blocked with 0.25% gelatine in NET buffer (1xG-NET) for 1 hour and incubated overnight with appropriate antibodies diluted 1:1000. The membrane is washed 3 times for 15 minutes in TBST and then incubated for 1 hour at room temperature in horseradisch peroxidase (HRP) conjugated antibody diluted in the G-Net solution. Membrane was washed 3 times for 15 minutes. Detection of proteins/antibodies complexes was achieved by the ECL (Enhanced Luminol Reagent) system. This western blot chemiluminescence reagent is a non-radioactive light emitting system which detects proteins immobilized on a membrane, from the oxidation of luminol, which results in light emission at a wave length of 428 nm, captured by a autoradiography film.

5.9.5 Protein quantification

To estimate the protein concentration Bradford assay was used: the Bradford assay are colorimetric assay based on the differential color change of a dye in response to various protein concentration. The Bradford assay has the advantage of having slight chemical interferences, of beeing sensitive and rapid (only one reagent).

Material and Method	**Chemical interference**	**Company, reagents**	**Technique procedure**
Bio-rad protein assay (Bradford)	slight	- one bottle of dye reagent (dye, phosphoric acid and methanol) stored at 4°C	- add standard, sample, blank 0,8 ml - add 0,2 ml dye reagent concentrate - after 5 minutes measure **OD =595**

Biorad purchase most of the reagents; required are also a spectophotometer and cuvettes for measurements. For the protein standard lyophilized preparations of bovine serum albumin are rehydrated in NaOH, diluted, aliquoted and stored at –80°C.

5.10 Immunological methods

5.10.1 Immunoprecipitation

The cells ($3x10^7$/ml) were lysed using lysis buffer. The sepharose (protein A: polyclonal rabbit, IgG_2 or protein G: IgG_1) was washed with prechilled 1x HNTG and diluted same volume of 1x HNTG, mixed by vortexing and stored at 4°C. To isolate antigen on antibody bead the following steps were performed: 30 µl sepharose, 15 µl antibody and 300 µl extracted cells were mixed and incubated on rotation wheel for 4 hours. Than the probes were washed to remove unbound proteins, then 20 µl Laemmli buffer were added and the samples were boiled at 100°C heating block for 8 min.

5.11 Molecular biology methods

5.11.1 Buffers and solutions

1x TBE	100 ml 5x TBE add 500 ml aqua dest.
0.5 M EDTA	18.61g EDTA 100 ml aqua dest pH 8.0
5xKCM	5ml 3M KCl 4.5 ml 1M $CaCl_2$ 7.5 ml 1M $MgCl_2$ 13 ml H_2O
LB-medium	25g Luria Broth Base add 1L H_2O
LB-plates	37g Luria agar add 1L H_2O
LB-medium/LB agar	Powder obtained from Gibco BRL

All of chemicals were obtained from Sigma (Germany), Gibco BRL(Germany) and Merck (Germany). Enzymes were obtained from NEB (Germany).

5.11.2 Polyacrylamide gel electrophoresis

Polyacrylamide gels provide somewhat better resolution as well as significantly higher capacity.

8% polyacrylamide gel:

13.3 ml acrylamide
6.25 ml bisacrylamide
5 ml 10xTBE
25.5 ml aqua dest
250 µl APS
40 µl TEMED

The gel solution was prepared in flask, poured between the gel and left to polymerize for 30 min. After the polymerization was complete, the comb was removed. The lower reservoir of the electrophoresis tank was filled with 1xTBE buffer. The gel plates were clamped to the top of the electrophoresis tank and the upper reservoir was filled with 1xTBE. DC power supply was used to pre-run and warm the gel for 30 min at 5 V/cm (constant voltage). After that 5xloading buffer was added to DNA samples and molecular weight markers (to 1x final) and the probes were loaded on the gel. The gel was run at 5V/cm, and then stained for 5-10 min in 0.5 µg/ml ethidium bromide. The DNA was detected using UV transilluminator.

5.11.3 Digestion of DNA with restriction enzyme

0.5-1 µl Restriction enzyme (10-20 U/ul)
2 µl 10xRestriction enzyme buffer (Reb)
0.2 µl (1mg/ml) Bovine Serum Albumin (BSA, DNase free)
to 20 µl water

The DNA was added and the mix was incubated at the appropriate temperature for required period of time (typically 2-3 hours).

5.11.4 Procedure for agarose gel electrophoresis

The correct amount of powdered agarose (Gibco BRL, Germany) was added to a measured quantity of electrophoresis buffer (1xTBE), than the mix was heated slurry in a microwave oven until agarose was dissolved. After cooling of this

solution to 50°C, the warm solution was poured into an. After the gel was completely set (ca. 30-40 min at room temperature) 1xTBE buffer was added to cover the gel to a depth of ca. 1 mm. The samples were mixed with 6xloading buffer (Promega) and loaded into the slots of the submerged gel. After running the gel was incubated for 15 min in ethidium bromide solution [0,5 ug/ml] (Roth,Germany) and DNA was detected using UV lamp.

5.11.5 Polymerase chain reaction (PCR)

10xPCR Buffer, dNTP mix, primer solutions, and 25 mM MgCl2 were thawed on the ice. A master mix was prepared from 10 µl 10xPCR buffer, 10 µl 25mM $MgCl_2$, 2µl dNTPs and primers. Appropriate volumes of the master mix were dispensed into PCR tubes and template DNA was added. The PCR tubes were placed in the thermal cycler and programm was started.

PCR-Programm (standard):

94°C 2min	
94°C 1min	
58°C 1min	35-40 cycles
72°C 1min	
72°C 7min	
4°C ∞	

5.11.6 Preparation of the competent E.coli

Over-night culture of E.coli was prepared, than the cells were transplanted in LB broth and grown up to OD_{600} = 0.3-0.4 (over night). After that the cells were centrifuged at 4000-5000 rpm for 10 min at 4°C and the pellet was resuspended in 15ml ice-cold TSB-medium. 100µl aliquots of cells were transferred in Eppendorf-tubes and frozen down immediately in a dry-ice for 1 hour. The aliquots were stored at -80°C.

5.11.7 Transformation of competent E.coli

The cells were thawed on the ice and 25µl of the cells were transferred in a tube. After that 1-10 µl DNA (0.1-1 µl) were added and also 5µl 5xKCM and water to 50 µl. The mix was incubated on the ice for 20min and than for 10 min at room temperature. After addition of 250 µl of LB medium, the tubes were incubated at 37C for one hour. After incubation 150 µl of the cells were spread on LB plate with appropriate antibiotics and the plates were incubated at 37° overnight.

5.11.8 DNA purification

5.11.8.1 Purification of genomic DNA

(DNeasy protocol for cultured animal cells, QIAGEN)

The appropriate number of cells (max. $5x10^6$) was centrifuged for 5 min at 300xg. The pellet was resuspended in 200 µl PBS. 20 µl proteinase K and 200 µl Buffer AL were added to the sample, mixed by vortexing and incubated at 70°C for 10 min. 200 µl ethanol (96-100%) were added to the sample. The mixture was placed into the DNeasy spin column placed in a 2 ml collection tube and centrifuged at 6000xg for 1 min. The DNeasy spin column was placed in a 2 ml collection tube, 500 µl Buffer AW1 were added and centrifuged at 6000xg for 1 min. Than 500 µl Buffer AW2 were added and centrifuged for 3 min at a full speed to dry the DNeasy membrane. After that the DNeasy spin column was placed in a clean 1,5 ml or 2 ml microcentrifuge tube, 200 µl Buffer AE (elution buffer) were added directly onto the DNeasy membrane. After incubation at RT for 1 min the DNA was eluted.

5.11.8.2 Mini-preparation

(QIAprep Spin Miniprep Kit Protocol)

This protocol was designed for purification of up to 20µl of high-copy plasmid DNA from 5 ml overnight cultures of E.coli in LB (Luria-Bretani) medium.

Pelleted bacterial cells were resuspend in 250µl of Buffer P1 and transferred to a microfuge tube. 250 µl of Buffer P2 were added. 350 µl of Buffer N3 were added and tubes were centrifuged for 10 min. The supernatants were applied to the QIAprep column by pipetting and centrifuged for 30 sec. QIAprep spin column were washed by adding 0.75 ml of Buffer PE and centrifuged for 30 sec and then

for an additional 1 min to remove residual wash buffer. Further QIAprep spin column were placed in a clean 1.5-ml microfuge tubes. To eluate DNA, 50µl of Buffer EB (10mM Tris-Cl, pH 8.5) were added and centrifuged for 1 min.

5.11.8.3 Maxi-preparation

(using the EndoFree Plasmid Maxi Kit from QIAGEN)

A single colony was picked from a freshly streaked selective plate and incubated first in 2-5 ml LB medium containing the appropriate selective antibiotic for 1 hour, then in 100 ml for additional 12-16 hours at 37C with vigorous shaking (300rpm).

After that the bacterial cells were harvested by centrifugation at 6000xg for 15 min at 4°C and resuspended in 10 ml Buffer P1. Then10 ml Buffer P2 were added and incubated at room temperature for 5 min. After that 10 ml chilled Buffer P3 were added to the lysate and whole mix was poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for 10 min.

After filtration of the cell lysate into a 50 ml tube, 2.5 ml Buffer ER were added and incubated on ice for 30 min. A QIAGEN-tip 500 were equalibrated by applying 10 ml Buffer QBT. After that the filtered lysate was placed to the QIAGEN-tip and the QIAGEN-tip were washed with2x30 ml QC buffer. DNA elution was performed with 15 ml Buffer QN. DNA was precipitated by adding 10.5 ml room-temperature isopropanol and centrifuged at 15,000xg for 30 min at 4C. DNA pellet was washed with 5 ml of endotoxin-free, room-temperature 70% ethanol and centrifuged at 15,000xg for 10 min. The pellet was air-dried for 5-10 min, and redissolved in a suitable volume of endotoxin-free Buffer TE.

5.11.9 RNA isolation, RNeasy Mini Protocol for isolation of total RNA from animal cells.

RNeasy kit (Qiagen, Hilden, Germany)

The cells (no more than $1x10^6$) were harvested. 350 µl Buffer RLT (lysis buffer) were added to the cells. 1 volume (350 or 600 µl) of 70% ethanol to the homogenized lysate was added. 700 µl of each sample were placed into each RNeasy column placed in a 2 ml collection tube and centrifuged for 15s at 8000xg. 700 µl Buffer RW1 were added to the RNeasy column and centrifuged for 15s at 8000xg. The RNeasy column was transferred into a new 2 ml collection tube. 500 µl Buffer RPE were placed onto the RNeasy column and centrifuged for 15s at

8000xg. After that another 500 µl Buffer RPE were placed to the RNeasy column and centrifuged for 2 min at 8000xg to dry the RNeasy silica-gel membrane.
To elute, the RNeasy column were transferred to a new 1.5 ml collection tube, 30 µl RNase-free water was added directly onto RNeasy silica-gel membrane and centrifuged for 1 min at 8000xg .

5.11.10 Reverse transcription RNA to cDNA

(using Dilute QIAGEN kit Omniscript Reverse Transcriptase, Hilden, Germany)
The template RNA solution was thawed on ice. The primer solutions, 10xBuffer RT, dNTP Mix, and RNase-free water were thawed at room temperature and than stored on ice immediately after thawing. Each solution was mixed by vortexing, and centrifuged briefly to collect residual liquid from the sides of the tubes. RNase inhibitor was diluted to a final concentration of 10 units/µl in ice-cold 1x Buffer RT, mixed carefully by vortexing for no more than 5 sec, and centrifuged briefly to collect residual liquid from the sides of the tubes. Fresh master mix was prepared (2µl 10x RT Buffer, 2 µl dNTP Mix, 2µl Oligo-dT primer, 1µl RNase inhibitor, 1µl Omniscript Reverse transcriptase and water variable). After that template RNA were added to the individual tubes containing the master mix and incubated for 60min at 37°C. For analysis of shorter cDNAs by PCR or other downstream enzymatic applications, Omniscript Reverse Transcriptase was inactivated by heating the reaction mixture to 93°C for 5 min followed by rapid cooling on ice.

5.11.11 In vitro mutagenesis

QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA)

Mutagenic primer design:

The primers were designed between 25 and 45 bases in length, with a melting temperature of ≥75°C.
Tm=81.5=0.41(%GC)-675/N-%mismatch

- N is the primer length in bases
- Values for %GC and %mismatch are whole numbers

The desired mutation was closed to the middle of the primer with 10-15 bases of template-complementary sequence on both sides.

Optimum primers had a minimum GC content 40% and terminated on one or more C and G bases at the 3'-end.

Mutagenesis reaction:

10xPCR Buffer, dNTP mix, primer solutions, and 25 mM MgCl2 were thawed. A master mix (35µl H_2O, 5µl 10xPCR buffer, 3µl Quick Solution, primers) was prepared. After that appropriate volumes were dispensed into PCR tubes. Then template DNA and 1µl dNTPs were added to the individual tubes, containing the master mix. After that the PCR tubes were placed in the thermal cycler and the program was started.

Cycling parametrers (standard):

94°C 2min
80°C 1min - hot start

94°C 1min
56°C 1min 4 cycles
72°C 1min

94°C 1min
58°C 1min 14 cycles
72°C 1min

72°C 7min
4°C ∞

5.12 Primer

Mutagenesis Primer :

FLT3 V592A

5’-CAGGTGACCGGCTCCTCAGATAATGAGTACTTCTAC**GCT**GATTTC-3’

FLT3 840GS

5’- GATATCATGAGTGATTCC**GGATCC**AACTATGTTGTCAGG -3’

FLT3 D835H

5’- GACTTTGGATTGGCTCGA**CAT**ATCATGAGTGATTCCAACC -3’

FLT3 D835Q

5’- GACTTTGGATTGGCTCGA**CAG**ATCATGAGTGATTCCAACC -3’

FLT3 D835V

5’-GACTTTGGATTGGCTCGA**GTT**ATCATGAGTGATTCCAACC -3’

FLT3 D835Y

5’-GACTTTGGATTGGCTCGA**TAT**ATCATGAGTGATTCCAACC -3’

FLT3 D835N

5’- GACTTTGGATTGGCTCGA**AAT**ATCATGAGTGATTCCAACC -3’

FLT3 Y842H

5’-GAGTGATTCCAAC**CAT**GTTGTCAGGGGCAATG-3’

Primers for FLT3 sequencing:

pMSCV	5’- CCCTTGAACCTCCTCGTTCG-3’	1-260bp of FLT3 cDNA
FLT3 2	5’-AAGACCTCGGGTGTGCGTTG-3’	260-600
FLT3 3	5’-ACGCCCTGGTCTGCATATC-3’	600-940

FLT3 843F	5’- CGGGCTCACCTGGGAATTAG-3’	940-1280
FLT3 5	5’-TTTGCAATCATAAGCACCAGC- 3’	1280-1620
FLT3 6	5’-ATACAATTCCCTTGGCACATC-3’	1620-1960
FLT3 7	5’-AACGGAGTCTCAATCCAGG-3’	1960-2300
FLT3 2205F	5’-CAGCATGCCTGGTTCAAGAG-3’	2300-2640
FLT3 9	5’-AGGCATCTACACCATTAAGAG-3’	2640-2980

5.13 Plasmids

FLT3-WT FLT3-W51	Provided by G.Gilliland, Howard Hughes Medical School and Institute and Bringham and Women’s Hospital Harvard Institute of Medicine, Harvard Medical School, Boston, MA
FLT3 D835H/Q/V/Y, FLT3-V592A, FLT3-840GS	Prepared in K.Spiekermann group, CCG-Leukemia, GSF National Research Center for Environment and Health, Munich, Germany
MSCV-IRES-EYFP/EGFP	Retroviral expression vector that contains an IRES site and allows translation of EYFP and the construct from the same transcript. Provided by R.K. Humphries, The Terry Fox Laboratory, Vancouver, University of British Columbia, Canada

6. RESULTS

6.1 FLT3-TKD and FLT3-LM in patients with AML

Bone marrow or blood samples from 60 adult patients with newly diagnosed and untreated AML were analysed. All were diagnosed as having AML according to standard French-American-British (FAB) and WHO criteria and were referred to our clinic for central cytomorphologic and cytogenetic diagnostics. The studies abide by the rules of the local internal review board and the tenets of the revised Helsinki protocol. Genomic DNA from the patients was screened for activating FLT3-LM and TKD-mutations to investigate the frequency of these mutations in AML (summarized in Table 3).

FAB subtype	No. of patients	TKD	FLT3-LM	TKD or FLT3-LM
M0	2	0	1	1
M1	17	2	8	10
M2	11	0	6	6
M3	2	0	2	2
M4	17	2	6	8
M5	8	2	3	5
Unknown	3	0	1	1
Total	60	6 (10.0%)	27 (45%)	33/60 (55%)

Table 3: Frequency of FLT3-TKD and FLT3-LM in patients with AML
The frequency of FLT3-TKD mutations (column 3) and FLT3-LM (column 4) mutations is given in different morphologically defined AML-subgroups according to the FAB-classification. The percentage of patients carrying either the FLT3-TKD mutation or the FLT3-LM is listed in column 5.

The loss of the EcoRV restriction site at codon 835/836 by mutations (Yamamoto, Kiyoi et al. 2001)and the previously described PCR-based assay for detection of the FLT3-LM (Kiyoi, Naoe et al. 1999)was used for a mutation screen. Abnormal restriction profiles at codon D835/836 were found in 6/60 (10%) of AML patients. The presence of a TKD mutation was confirmed in all cases by nucleotide sequencing. The mutations found at D835 were genetically heterogeneous and resulted in substitution of D835 by glutamine (n=2), tyrosine (n=1), valine (n=1), alanine (n=1) and histidine (n=1). When related to the FLT3 length mutations

which were found in 27/60 (45%) of patients, both genetic alterations occurred independently and none of the patients carried both mutations.

6.2 A new and recurrent mutation of FLT3: FLT3-840GS

6.2.1 Detection of the FLT3-LM in exon 20

We used a PCR based method to screen for mutations of codons D835/836 in patients with AML. After gel electrophoresis of the amplification products from exon 20 we observed an additional PCR fragment which was slightly larger than the PCR product of the wildtype allele in two patients (Figure 3).

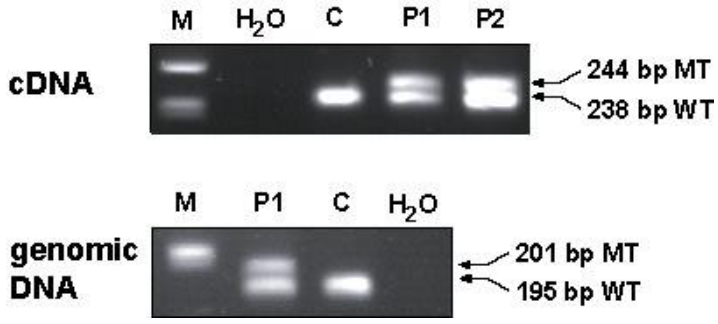


Figure 3: Detection of the FLT3-LM in exon 20 in AML after agarose gel electrophoresis.
Detection of the length mutation in exon 20 after conventional agarose gel electrophoresis of PCR-products from cDNA and genomic DNA. M: Molecular weight standard, H_20: water control, C: Patient without exon 20 mutation, P1/2: patient 1/2 with the length mutation in exon 20.

RT-PCR was repeated more than ten times with different aliquots of RNA as well as cDNA and always gave the same results. Sequencing revealed an insertion of 6 nucleotides between the codons S840 and N841 just five amino acids (AA) downstream of D835. These nucleotides generated a BamHI restriction site which was confirmed by BamHI digestion and gel electrophorese. For this purpose, the TKD2 of FLT3 was amplified by PCR, than PCR product was digested with BamHI and detected on the gel (Figure 4). The DNA from FLT3-WT was used as a control.

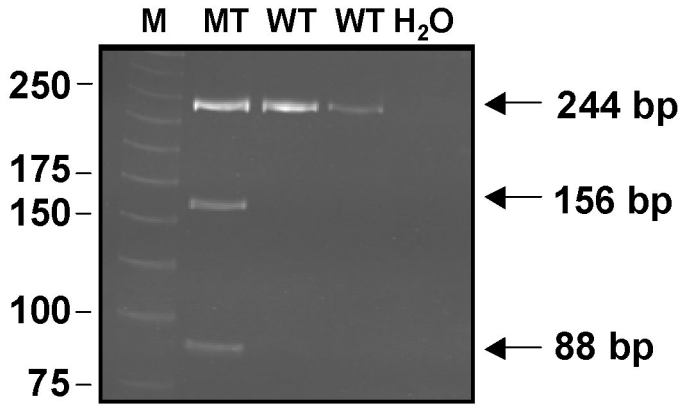


Figure 4: Confirmation of the mutation by *Bam*HI digestion after polyacrylamide gel electrophoresis.
M: molecular weight standard, MT: patient with mutation, WT: two patients without mutation, H_20: water control. The PCR products were digested with BamHI and detected after polyacrylamid gel electrophoresis.

The Figure 5 shows the schematic presentation of the FLT3-840GS mutation.

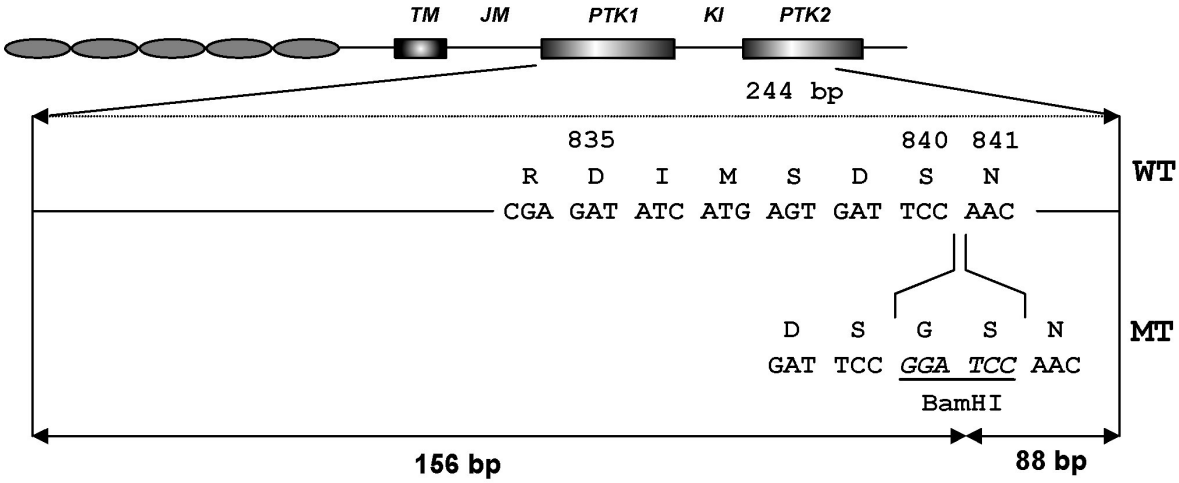


Figure 5: Schematic presentation of the FLT3-840GS mutation.
WT- FLT3 wild type; MT- mutation; TM- transmembrane domain; JM-juxtamembrane domain; PTK1 and 2 – protein tyrosine kinase domain 1 and 2, respectively; KI- kinase insert.

For patient 1 a DNA sample was available and the mutation could be shown also at the genomic level by PCR, confirming the presence of the mutation (Figure 4). The respective mutation was not detected in further 357 unselected AML pts.

Additional screening for frequent genetic mutations in these two patients showed no further activating mutations of FLT3, NRAS, KIT or MLL (Table 4).

case	sex/ age	FAB	Molecular markers not altered
1	F/69	M0/ M1	FLT3-LM FLT3D835 CKITD816 NRAS MLL-PTD
2	M/76	M6	FLT3-LM FLT3D835 CKITD816 NRAS MLL-PTD

Table 4: Clinical data and alterations of other molecular markers in the two patients carrying the FLT3-840GS mutation. FLT3-LM, length mutations in the JM-region of FLT3; FLT3D835, mutations in codons 835/836; KIT D816, point mutations in codon 816; NRAS, activating point mutations in codons 12, 13 and 61; MLL-PTD, MLL-partial tandem duplication.

6.2.2 Generation of FLT3-840GS Ba/F3 cell lines

To analyze the transforming potential of the FLT3-840GS mutant, Ba/F3 FLT3-840GS, FLT3-WT, FLT3-ITD and mock-expressing cells were generated. Ba/F3 cells were transduced with the pMSCV-IRES-EYFP retroviral expression vector that contains an IRES site and allows translation of EYFP and the FLT3 construct from the same transcript. Transduced EYFP-positive cells were sorted by FACS on the basis of EYFP fluorescence and were expanded in the presence of IL-3. As a positive control we generated Ba/F3 cells expressing the FLT3-ITD (W51) mutant. (Spiekermann, Dirschinger et al. 2003)

As shown in Figure 6, the FLT3-840GS expressing Ba/F3 cells grew factor-independently, whereas mock-expressing cells were unable to proliferate in the absence of IL-3.

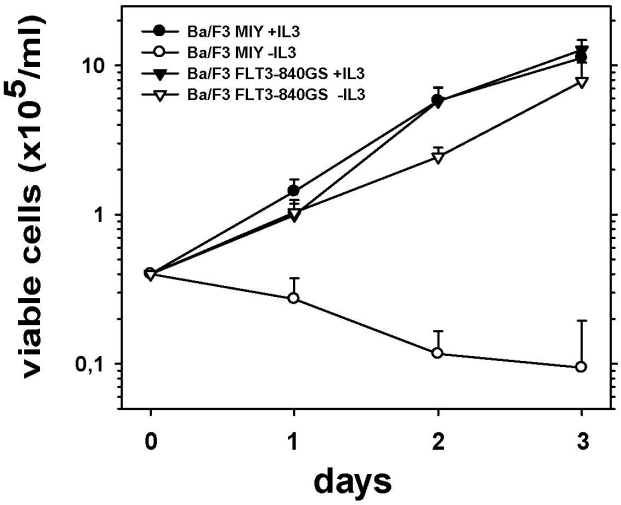


Figure 6: Transforming potential of the FLT3-840GS mutant.
Ba/F3 cells transduced with either pMSCV-EYFP-IRES-FLT3-840GS or empty vector (pMSCV-EYFP-IRES, mock) were grown in the absence or presence of IL-3 as indicated. Results represent means ± SD of three independent experiments.

6.2.3 FLT3 expression and hyperphosphorylation in FLT3-840GS transformed Ba/F3 cells.

To confirm the identical expression level of all FLT3 constructs flow cytometry analysis was performed. For this purpose, FLT3WT, FLT3ITD, FLT3-840GS or empty vector expressing Ba/F3 cells were incubated with a mouse isotype-matched control antibody (open histograms) or CD135-phycoerythrin (filled histograms) antibody. Viable cells were analyzed using a FacsCalibur flow cytometer (Figure 7).

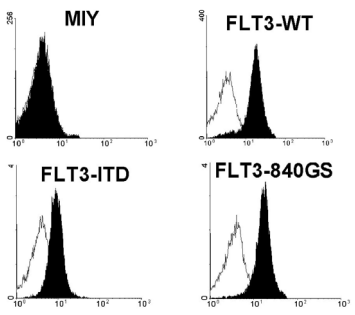


Figure 7: FLT3 expression in Ba/F3 MIY (vector), FLT3-WT, FLT3-ITD or FLT3-840GS expressing cells.

Given the profound biological differences between the FLT3-WT and FLT3-MT (TKD or LM) constructs, we next analyzed the FLT3 receptor activation. Lysates from Ba/F3 cells were immunoprecipitated with αFLT3-antibody and analyzed by Western blot using an antiphosphotyrosine (αPY) antibody, stripped and reblotted with αFLT3 antibody. Our data show, that the FLT3-840GS and the FLT3-ITD receptors were hyperphosphorylated in contrast to the FLT3-WT receptor (Figure 8).

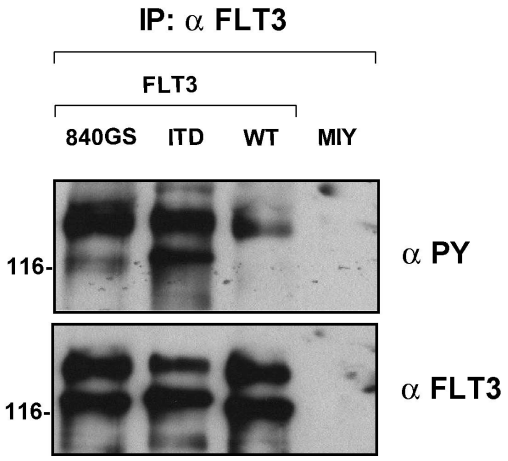


Figure 8: The FLT3-840GS mutant receptor is hyperphosphorylated in Ba/F3 cells.

6.2.4 Transforming potential of the FLT3-840GS mutant

The detailed analysis of the growth characteristics of FLT3-840GS Ba/F3 cells showed a significantly slower growth rate compared to the FLT3ITD expressing cells. To analyze, whether Ba/F3 cells expressing FLT3-840GS can achieve a maximum proliferation rate, the cells were grown for 72h in the absence or presence of different concentrations of IL-3 as indicated. Ba/F3 cells expressing FLT3-WT or FLT3-ITD were used as a control. The growth of FLT3ITD expressing Ba/F3 cells at 72h was defined as 100%. Our data clearly show that for maximal proliferation, the FLT3-840GS expressing cells required additional IL-3 at concentrations as low as 0.1 ng/ml (Figure 9).

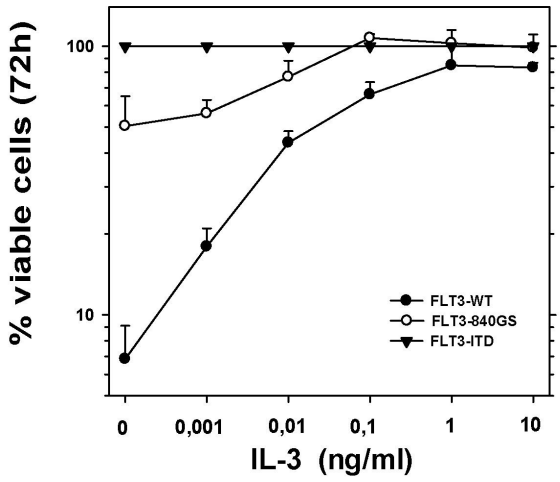


Figure 9: For maximal proliferation the FLT3-840GS expressing cells require additional IL-3.

Results represent means ± SEM of three independent experiments.

6.2.5 FLT3-840GS activates the MAPK-STAT signalling pathway of FLT3

To identify common signalling pathways that specifically transduce the transforming signals of the FLT3-840GS- mutated receptor, we analyzed the activity of this mutant to activate STAT5 and MAPK. Using phosphospecific antibodies, we found that FLT3-840GS induced a strong activation of MAPK (Figure 10A), but only a weak activation of STAT5 (Figure 10B).

6.2.6 Sensitivity of the new mutation FLT3-840GS to the selective PTK inhibitor SU5614

We next asked whether the FLT3-840GS mutant is sensitive to the growth inhibitory activity of SU5614, a FLT3 PTK inhibitor. (Spiekermann, Dirschinger et al. 2003) Our results clearly show that SU5614 induced a growth inhibition of the FLT3-840GS as well as of the FLT3ITD transformed Ba/F3 lines in the absence, but not in the presence of IL-3 (Figure 11).

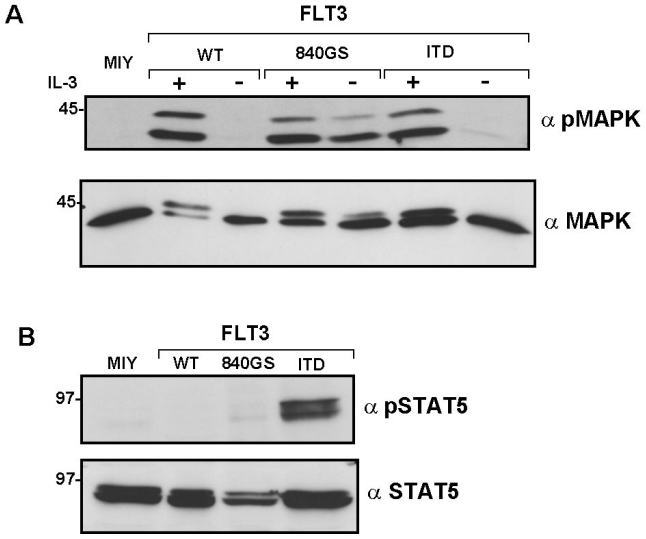


Figure 10: Activation of FLT3 downstream targets (STAT5 and MAPK) in Ba/F3 cells transformed with FLT3-ITD and FLT3-840GS mutants.
A. Lysates were prepared from Ba/F3 cells expressing mock, FLT3-WT, FLT3-ITD, FLT3-840GS constructs after IL-3 stimulation (for 5 min, RT) and without stimulation (+ and -). Immunoblotting of lysates was performed with anti-pMAPK and anti-MAPK antibodies.
B. Lysates were prepared from Ba/F3 cells expressing mock, FLT3-WT, FLT3-ITD, FLT3-840GS constructs. Immunoblotting of lysates was performed with anti-pSTAT5 and anti-STAT5 antibodies.

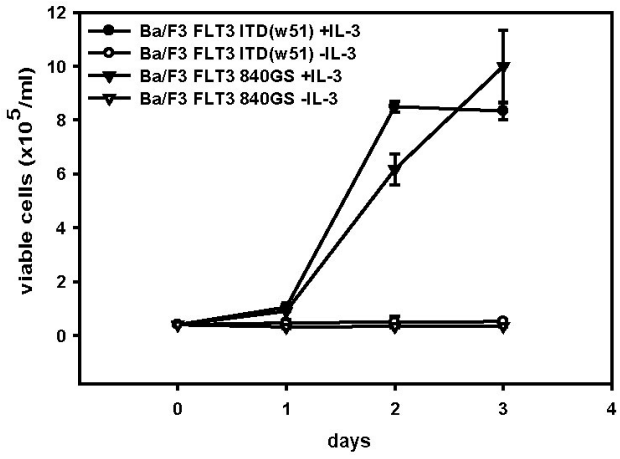


Figure 11: Sensitivity of the FLT3-840GS mutant to the growth inhibitory activity of the FLT3 PTK inhibitor SU5614
Dose-responses curves of the inhibitory activity of SU5614 in Ba/F3 FLT3-ITD and FLT3-840GS cells after 72h of incubation. Ba/F3 cells expressing different FLT3-constructs were seeded at a density of $4x10^4$ cells/ml in the absence or presence of different concentrations of SU5614. Viable cells were counted after 72 h by trypan blue exclusion.

6.3 A new point mutation in the juxtamembrane region of the FLT3: FLT3-V592A

6.3.1 MM1 and MM6 cell lines

MonoMac1 and MonoMac6 cell lines were established from the peripheral blood of a 64-year-old man with leukemia (AML FAB M5) (DSMZ). Previous studies have shown that these cell lines express an autophosphorylated FLT3 receptor sensitive to SU5614. (Spiekermann, Dirschinger et al. 2003)

To further clarify the mechanisms of constitutive FLT3 activation in MM6 and MM1 cells, the complete FLT3 cDNA was sequenced. Both MM1 and MM6 cells carry a point mutation in the JM region, which result in the substitution of valine by alanine at position 592 (V592A) (Figure 12).

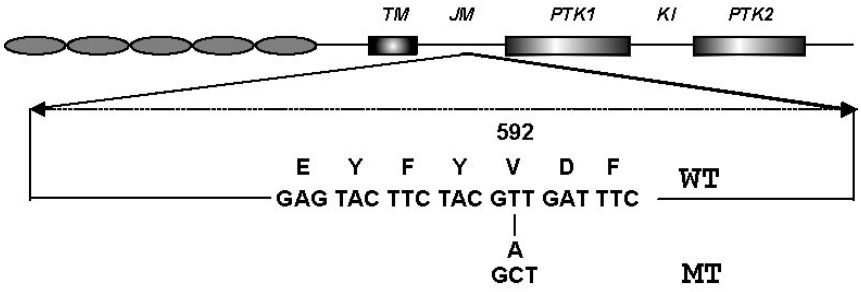


Figure 12: Schematic presentation of the FLT3-V592A mutation.

6.3.2 Generation of FLT3-V592A expressing Ba/F3 cells

To analyze the transforming potential of the FLT3-V592A mutation, Ba/F3 FLT3-V592A cells were generated. Overexpression of this mutant in IL-3-dependent Ba/F3 cell lines resulted in factor-independent growth, thereby showing the transforming activity of this mutant (Figure 13). Compared with FLT3-ITD-expressing cells, FLT3-V592A-Ba/F3 cells showed a slightly slower growth rate.

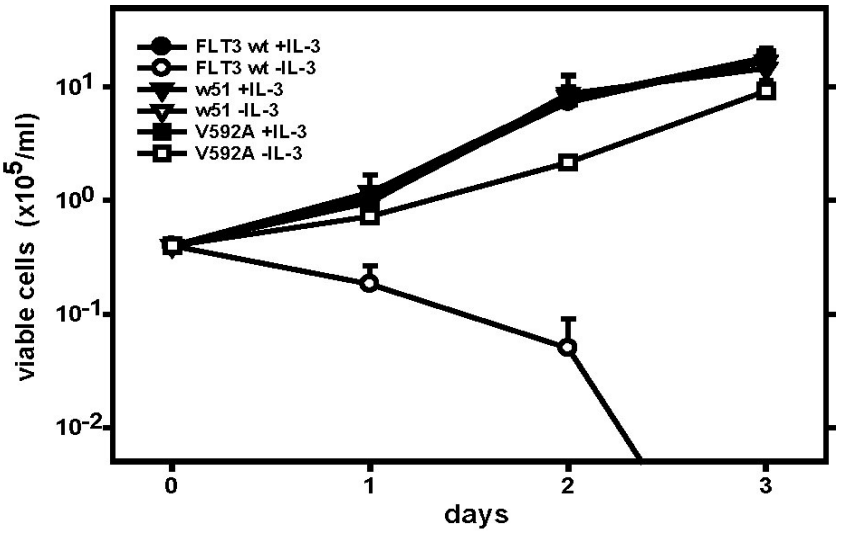


Figure 13: Ba/F3 cells transduced with either the pMSCV-EYFP-IRES-FLT3-WT/ITD or V592A construct were grown in the absence or presence of IL-3 as indicated. Results represent means ± SD of three independent experiments.

6.3.3 Sensitivity of the new mutation FLT3-V592A to the selective PTK inhibitor SU5614

The selective PTK inhibitor SU5614 has been reported to inhibit the growth of FLT3-ITD and FLT3-D835Y carrying Ba/F3 cells. (Yee, O'Farrell et al. 2002; Spiekermann, Dirschinger et al. 2003)To analyze the sensitivity of the FLT3-V592A mutant to SU5614, FLT3-V592A expressing Ba/F3 cells were cultured in the presence of different concentrations of SU5614 for 72 hours. FLT3-V592A and FLT3-ITD showed a similar and high sensitivity to SU5614 (IC_{50} =0.1) (data not shown).

6.3.4 Importance of the Y589/591 tyrosine residues in FLT3-V592A signalling

FLT3 is constitutively activated by the substitution of Ala for Val at codon 592 in the juxtamembrane domain, and this mutation confers a factor independent growth on IL-3-dependent cell line. To examine how FLT3-V592A yields oncogenic signal

transduction, we constructed the expression vector pMSCV-YEFP carrying FLT3 mutants that encode the Y→F substituted FLT3-V592A or FLT3-D835Y. To examine further the effect of Y→F substitution at codons 589 and 591 on the factor-independent growth by FLT3-V592A and FLT3-D835Y, these constructs were transfected into IL-3 dependent Ba/F3 cell lines. As controls, Ba/F3 cells were also transfected with the expression vector carrying FLT3-V592A and FLT3-D835Y.

As shown in Figure 14A, Ba/F3 FLT3-D835Y-Y589/591F cells showed factor-independent growth at an almost similar level to Ba/F3 FLT3-D835Y cells.

Ba/F3 FLT3-V592A Y589/591F cells also proliferated in a factor-independent manner, but their magnitude of proliferation was lower than that of Ba/F3 FLT3-V592A cells (Figure 14B).

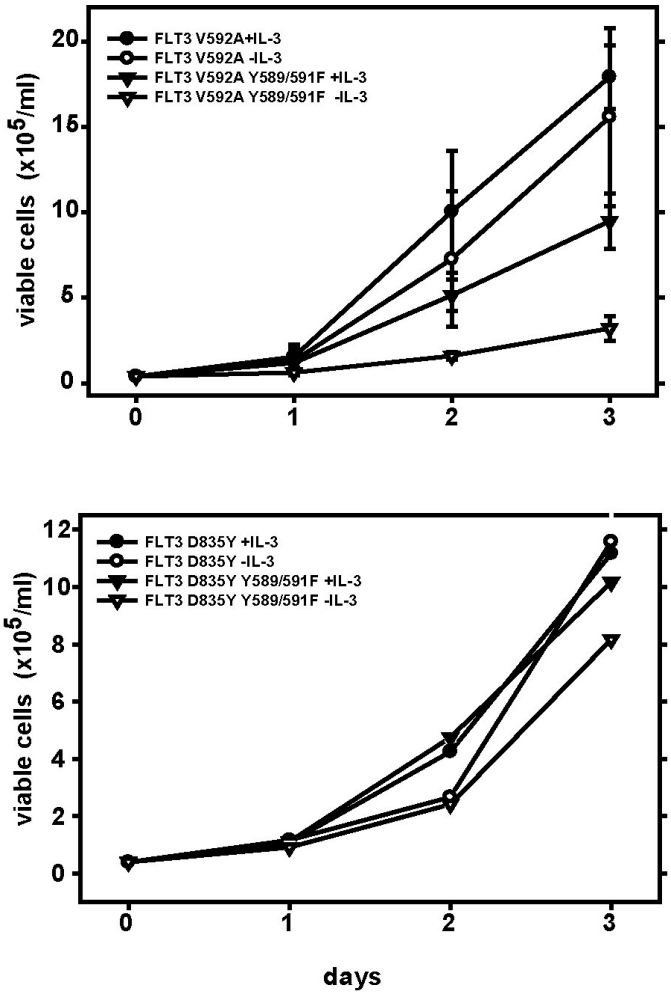


Figure 14: Ba/F3 cells transduced with either pMSCV-EYFP-IRES-FLT3-D835Y, D835Y-Y589/591F (**A**) or V592A, V592A-Y589/591F (**B**) were grown in the absence or presence of IL-3 as indicated. Results represent means ± SD of three independent experiments.

These findings indicate that tyrosines at codons 589 and 591 are important for full transformation activity of the FLT3-V592A, but not of the FLT3-D835Y mutant receptor.

6.4 Sensitivity of the different FLT3-TKD point mutations to the PTK inhibitor SU5614

6.4.1 The FLT3-TKD mutants D835Y/V/H/Q induce IL-3 independent growth in Ba/F3 cells

In order to analyze the transforming potential of FLT3-TKD mutants in IL-3 dependent Ba/F3 cells, we generated FLT3-D835 Y/V/H/Q expressing cell lines. Ba/F3 cells were transduced with the pMSCV-IRES-EYFP retroviral expression vector that contains an IRES site and allows translation of EYFP and the FLT3 construct from the same transcript. Transduced EYFP-positive cells were sorted by FACS on the basis of EYFP fluorescence and were expanded in the presence of IL-3. Identical expression level of all FLT3 constructs was confirmed by flow cytometry and by Western blot after immunoprecipitation (IP) with a FLT3 specific antibody (data not shown). As a positive control we generated Ba/F3 cells expressing the FLT3-ITD (W51) mutant. (Spiekermann, Dirschinger et al. 2003)
As described previously, the FLT3-ITD, but not the FLT3-WT construct conferred IL-3 independent growth. All FLT3-D835 mutant-transduced Ba/F3 cell lines were able to grow in the absence of IL-3 at a growth rate comparable to that of the FLT3-ITD expressing cells (Figure 15).

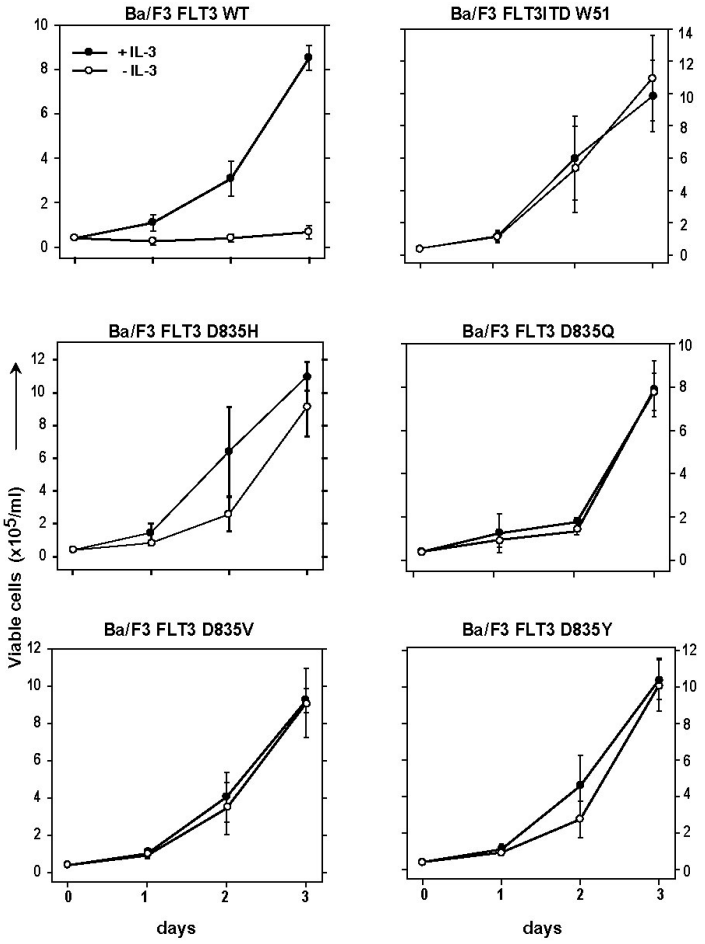


Figure 15: Ba/F3 cells transduced with either pMSCV-EYFP-IRES-FLT3-WT/ITD or D835H/Q/V/Y were grown in the absence or presence of IL-3 as indicated. Results represent means ± SD of three independent experiments.

6.4.2 FLT3-TKD mutants activate the STAT5 and MAPK signaling pathways

We and others have previously shown that FLT3-ITD and TKD mutants induce a constitutive STAT5 and MAPK activation in Ba/F3 or 32D cells. (Hayakawa, Towatari et al. 2000; Mizuki, Fenski et al. 2000; Tse, Mukherjee et al. 2000; Tse, Novelli et al. 2001; Spiekermann, Dirschinger et al. 2003)To characterize the signaling properties of FLT3-TKD mutants, we analyzed lysates from Ba/F3 cells expressing the FLT3-WT, -ITD or -D835Y/V/H/Q constructs. Using an anti-phosphotyrosine antibody, we could clearly show that the FLT3-D835Y/V/H/Q-transduced Ba/F3 cells express a hyperphosphorylated FLT3 receptor (data not

shown). Furthermore, we analyzed the ability of FLT3-TKD mutants to activate STAT5 and MAPK, two known downstream targets of FLT3-ITD mutants. For this purpose, crude lysates from Ba/F3 cells expressing the FLT3-D835Y/V/H/Q mutants were analyzed by phosphospecific antibodies against STAT5 and MAPK. As shown in Figure 16 all FLT3-D835 mutants induced a strong constitutive activation of STAT5 and MAPK at levels comparable to the FLT3-ITD construct.

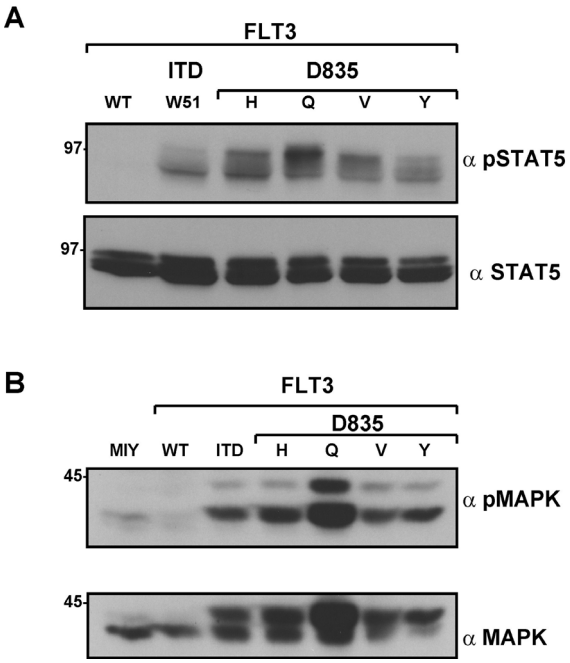


Figure 16: Activation of FLT3 downstream targets (STAT5 and MAPK) in Ba/F3 cells transformed with FLT3-ITD and TKD mutants
Lysates were prepared from Ba/F3 cells expressing the FLT3-WT, FLT3-ITD, FLT3-D835H/Q/V/Y constructs. Immunoblotting of lysates was performed with anti-pSTAT5 and anti-STAT5 (**A**), anti-pMAPK and anti-MAPK antibodies (**B**).

These data clearly demonstrate that all FLT3-TKD mutants analyzed in our study have transforming potential and induce a hyperphosphorylation of FLT3. STAT5 and MAPK represent common downstream targets of the constitutively active FLT3-ITD and FLT3-TKD-mutant receptors.

6.4.3 FLT3-TKD mutants differ significantly in their sensitivity to the growth inhibitory activity of the FLT3 PTK inhibitor SU5614

We and others have previously reported that the PTK inhibitor SU5614 inhibits the growth of FLT3-ITD and FLT3-D835Y carrying Ba/F3 cells. (Yee, O'Farrell et al. 2002; Spiekermann, Dirschinger et al. 2003)To analyze the sensitivity of different FLT3-D835 mutants to SU5614 we cultured FLT3- D835Y/V/H/Q-expressing Ba/F3 cells in the presence of different concentrations of SU5614 for 72 hours. Ba/F3 cells expressing different FLT3-constructs were seeded at a density of $4x10^4$ cells/ml in the absence or presence of different concentrations of SU5614. Viable cells were counted after 72 h by trypan blue exclusion. As shown in Figure 17A, FLT3-TKD mutants differ significantly in their sensitivity to the inhibitor. FLT3-D835Y and FLT3-ITD cells show a similar and high sensitivity to SU5614 (IC_{50}=0.2μM and 0.1μM respectively).

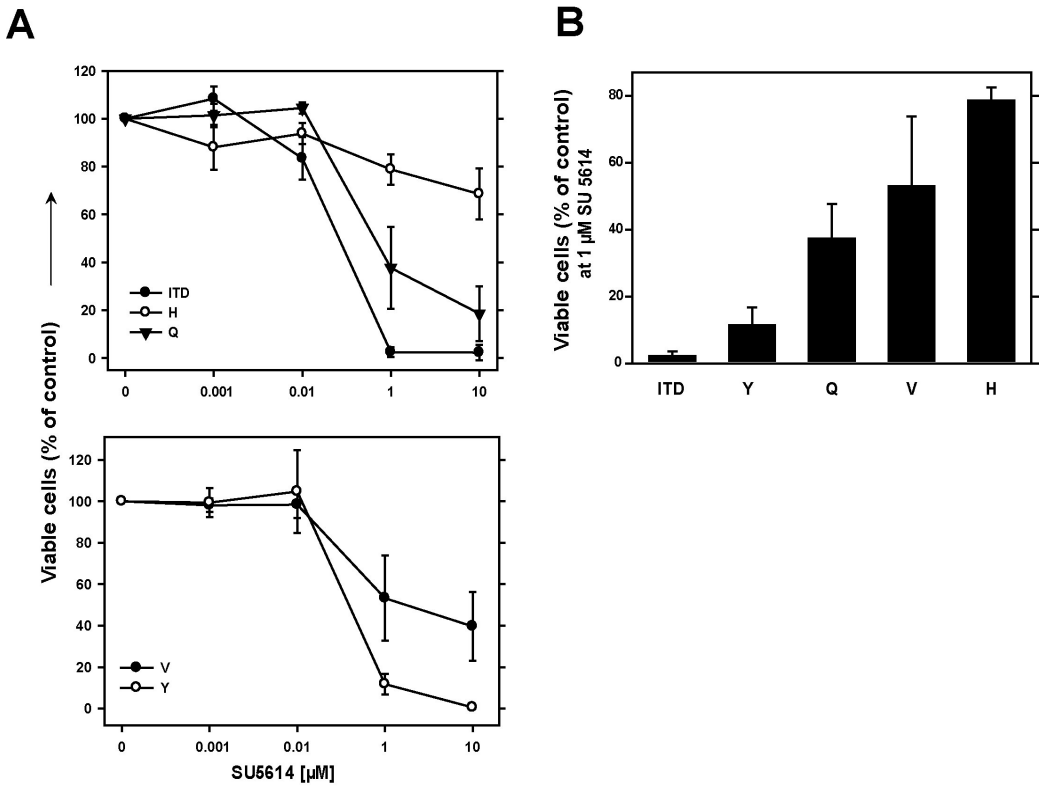


Figure 17: FLT3-TKD mutants differ significantly in their sensitivity to the growth inhibitory activity of the FLT3 PTK inhibitor SU5614
A. Dose-responses curves of the inhibitory activity of SU5614 in Ba/F3 FLT3-ITD and FLT3-TKD cells after 72h of incubation. The growth of cells that were incubated without inhibitor was defined as 100%. Values represent means and standard errors from three independent experiments. **B.** Sensitivity of FLT3-ITD and TKD mutants to SU5614. Values represent means and standard errors from three independent experiments.

In contrast, the FLT3-D835Q and D835V mutants were significantly less sensitive to the inhibitor (IC_{50}=0.4 and 1μM respectively), and the FLT3-D835H was the most resistant mutant (IC_{50}>10μM). At concentrations of 1μM of inhibitor FLT3-ITD cells showed a viability of 3% compared to 10% (FLT3-D835Y), 38% (FLT3-D835Q), 52% (FLT3-D835V) and 78% (FLT3-D835H) after 72 hours of incubation (Figure 17B).
These data demonstrate that FLT3-TKD mutants differ significantly in their sensitivity to the FLT3 PTK inhibitor SU5614 in the following order: D835Y > D835Q > D835V > D835H.

6.4.4 SU5614 induces apoptosis in FLT3-ITD and FLT3-D835Y, but not in FLT3-D835H transformed cells

To further characterize the mechanisms of primary resistance of the FLT3-D835H mutant to SU5614, we analyzed induction of apoptosis after inhibitor treatment of FLT3-ITD, D835Y and D835H transformed Ba/F3 cells with SU5614.
Figure 18A (upper panel) shows that FLT3-ITD transformed cells underwent rapid apoptotic cell death after exposure to increasing concentration of SU5614 after 24 hours as measured by the expression of annexin V/7-AAD. FLT3-D835Y cells show the same level of sensitivity to SU5614 (IC_{50} = 0.1μM) (Figure 18A, middle panel), but not the FLT3-D835H cells (Figure 18A, bottom panel). In the presence of 5μM of SU5614 FLT3-D835H cells show only 18% apoptotic cells in comparison to FLT3-ITD (63%) and FLT3-D835Y cells (60%). A representative experiment is shown in Figure 18B and clearly demonstrates that SU5614 at a concentration of 5μM induces apoptosis in 11.6% of D835H (lowest panel) cells compared to 60.2% and 78.3% in FLT3-D835Y (middle panel) and FLT3-ITD (upper panel) expressing Ba/F3 cells, respectively.

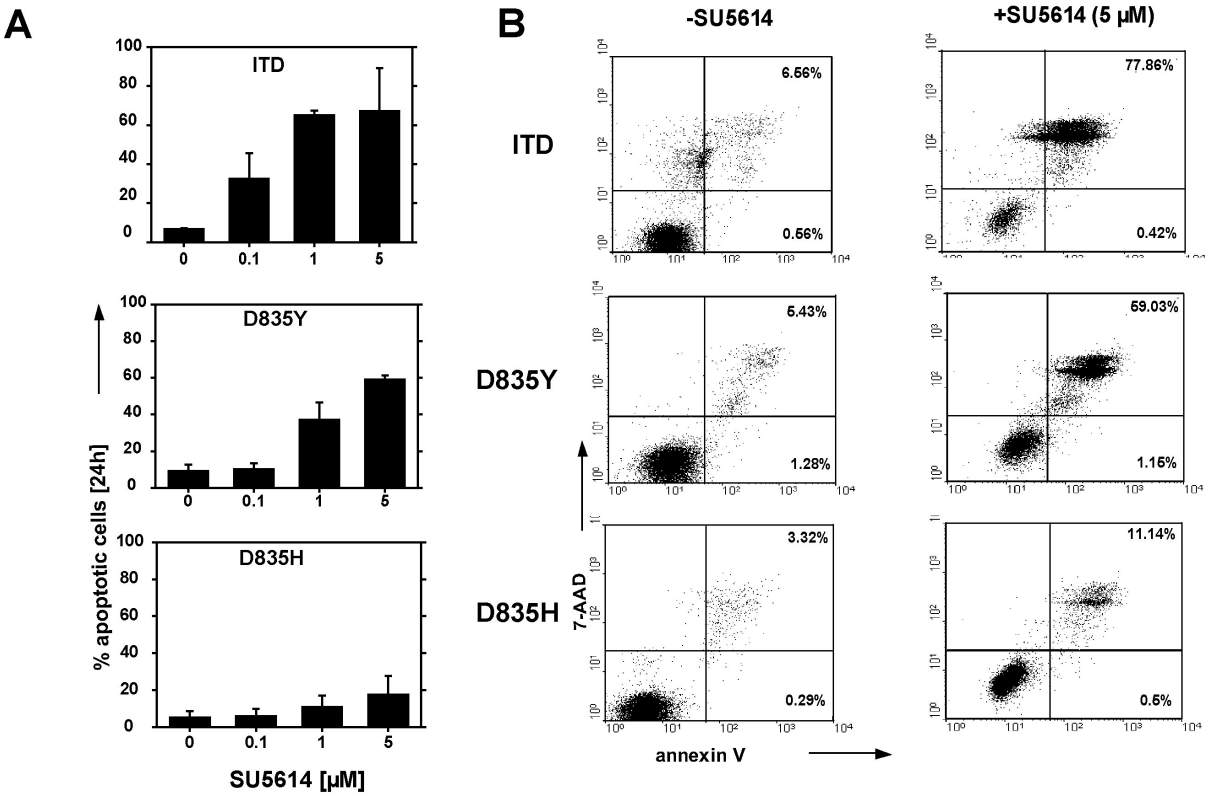


Figure 18: SU5614 induces apoptosis in FLT3-ITD and FLT3-D835Q/V/Y, but not in FLT3-D835H cells.
A. and B. Ba/F3 cells transduced with the FLT3-ITD, FLT3-D835Y or FLT3-D835H constructs were incubated with different concentrations of SU5614 for 24 hours and were analyzed by flow cytometry after staining with annexin V-PE and 7-AAD. Representative dot plots from one out of three independently performed experiments are shown.

6.4.5 The FLT3 PTK inhibitor SU5614 downregulates autophosphorylation of the FLT3-ITD, but not the FLT3-D835H receptor mutant

It was recently shown that SU5614 inhibits phosphorylation of the FLT3-ITD receptor and downregulates STAT5 and MAPK phosphorylation. (Yee, O'Farrell et al. 2002; Spiekermann, Dirschinger et al. 2003)To confirm the resistance of FLT3-D835H cells to SU5614 on the level of individual signaling pathways we analyzed the activation of FLT3, MAPK and STAT5 after incubation with SU5614.

For this purpose, 293T cells were transiently transfected with either the FLT3-ITD or the D835H construct. Lysates were prepared after incubation of the cells with SU5614 (0, 1, and 10µM) for 4 hours and immunoprecipitated using a polyclonal anti-FLT3 antibody. The phosphorylation of FLT3 was analyzed by Western blot analysis using a monoclonal anti-phosphotyrosine (α-PY) antibody. As shown in

Figure 19A, SU5614 induced an efficient dephosphorylation of the FLT3-ITD receptor at a concentration of 1μM (38.5% of control, defined as the FLT3 phosphorylation in the absence of SU5614 compared to basal levels of FLT3 measured by densitometry). In contrast, the D835H mutant was still strongly hyperphosphorylated (92.7%) in the presence of 1μM SU5614. At concentrations of 10μM SU5614 the FLT3-ITD construct was almost completely dephosphorylated (7.4%), whereas a significant autophosphorylation was still detectable in FLT3-D835H transfected cells (46.4%).

We further analyzed the effects of SU5614 on the activation of two important downstream signalling pathways activated by FLT3-LM/TKD mutants. In the presence of 1μM of SU5614 STAT5 phosphorylation in Ba/F3 FLT3-ITD cells was almost completely inhibited (Figure 19B). In contrast, in Ba/F3 FLT3-D835H expressing cells STAT5 was only slightly dephosphorylated in the presence of this concentration of inhibitor. The incubation of the cells with 10μM of SU5614 produced an almost complete inhibition of STAT5 phosphorylation in both Ba/F3 FLT3-ITD and D835H transduced cell lines.

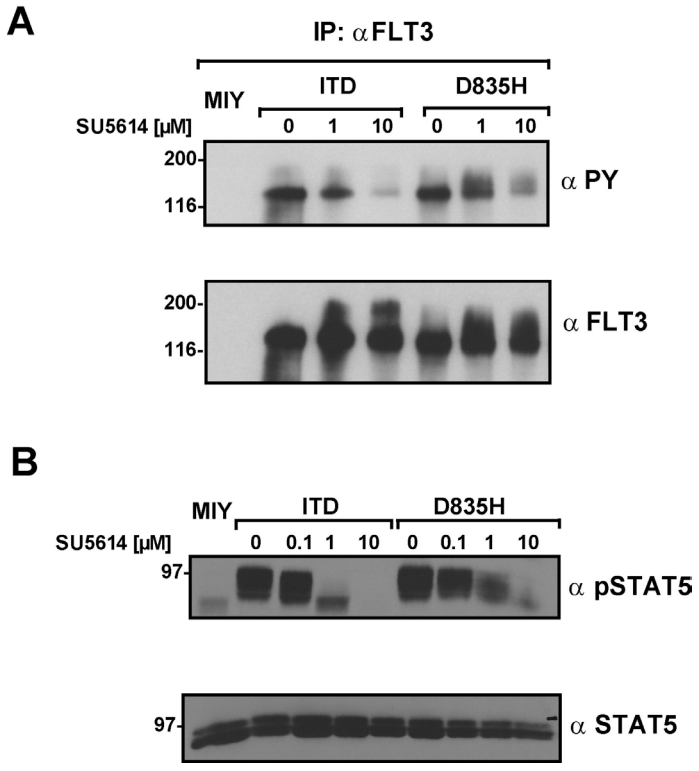


Figure 19: The FLT3 PTK inhibitor SU5614 downregulates autophosphorylation of the FLT3-ITD, but not FLT3-D835H receptor mutants

A. Tyrosine phosphorylation of FLT3 was determined by Western blot analysis using a monoclonal anti-PY antibody and identical loading was confirmed by reblotting with a polyclonal anti-FLT3 antibody. **B.** The expression and phosphorylation of STAT5 in Ba/F3 MIY, FLT3-ITD and FLT3-D835H expressing cells treated with 0.1, 1 and 10µM of SU5614 or untreated cells was determined by Western blot analysis.

These results confirm at a molecular level the different sensitivity of FLT3-TKD mutants to FLT3 PTK inhibitors.

6.5 Mechanisms of drug resistance to SU5614

6.5.1 Generation of SU5614-resistant Ba/F3 FLT3-ITD cells

FLT3 PTK inhibitors are now being evaluated in clinical phase I/II studies in patients with AML (see above), but it is unknown to what extent clinical resistance will develop and influence the clinical efficiency of these inhibitors.

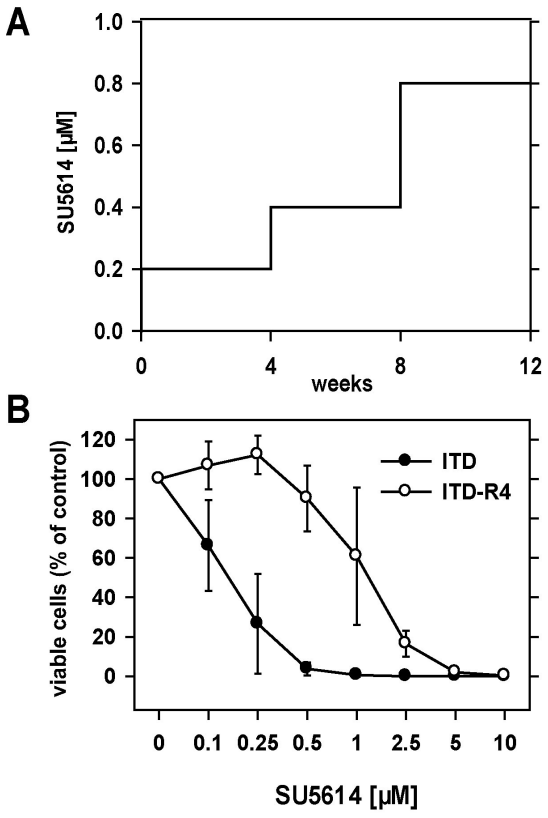


Figure 20: Generation of SU5614-resistent cell lines.
A. The polyclonal Ba/F3 FLT3-ITD cell line was cultivated in RPMI-1640 medium containing 10% FBS in the presence of increasing concentrations of SU5614 **B.** The Ba/F3 FLT3-ITD-R4 cells were seeded at a density of $4x10^4$ cells/ml in the absence and presence of different concentrations of SU5614 and viable cells were counted after 72 hours by trypan blue exclusion.

To address this important issue, FLT3-ITD-W51 expressing polyclonal Ba/F3 cells were cultivated either in the presence of increasing concentrations of SU5614 (0.2µM to 0.8µM) or in the absence of inhibitor (control cells) for a time period of 3 months (Figure 20A). After a cultivation period of 2 weeks at a concentration of 0.2µM SU5614 two independent cell lines were obtained that were each split in two sublines (ITD-R1+R2 and ITD-R3+R4, respectively). In contrast to the control cell line all four cell lines (ITD-R1-R4) growing in the presence of inhibitor developed a partial resistance to SU5614 and showed an $IC_{50,}$ which was at least 7-fold higher than the IC_{50} of control Ba/F3 FLT3-ITD cells (Table 5).

A representative example is shown in Figure 20B: Ba/F3 FLT3-ITD-R4 cells were cultured for 72 hours in the presence of increasing concentration of SU5614. The IC_{50} for SU5614 was 0.2µM in the control ITD cell line and 1.3 µM in ITD-R4 cells. At high concentrations of 10µM of inhibitor the ITD-R4 line was still sensitive to the growth inhibitory activity of SU5614 showing that the proliferation of these cells was still dependent on the presence of the activating FLT3 mutant.

cell line	IC50		FLT3 expression		FLT3-TKD mutation
	SU 5614 [µM]	AraC [µg/ml]	FACS [MCF]	WB [% of ITD native]	
ITD native	0.2	0.06	14.1	100	-
ITD-R1	4.5	0.03	50.9	725	Y842H
ITD-R2	5.2	0.08	41.2	918	Y842H
ITD-R3	2.1	n.d.	63.3	1016	D835N
ITD-R4	1.3	0.04	54.4	1186	D835N

Table 5: Functional and molecular characterization of Ba/F3 FLT3-ITD-R1-4 cells.

The IC_{50} of SU5614 and Ara-C in ITD-R1-4 cells was determined as described in Figure 20. FLT3 expression was measured by FACS and the mean channel fluorescence (MCF) was calculated. The expression of FLT3 (WB) was determined by Western blot analysis using an anti-FLT3 antibody. The blot was stripped and reprobed with an anti-β-actin antibody. The results were quantified by densitometry according to the FLT3-ITD/β-actin

ratio of parental cells that was set to 100%. FLT3-TKD mutation indicates the presence (Y842H and D835N) (or absence (-)) of point mutations in tyrosine kinase domain (TKD).

6.5.2 Activation of STAT5 and MAPK in FLT3-ITD-R1-4 cell lines in response to SU5614

To confirm the resistance to SU5614 at a molecular level, we analyzed two important downstream targets of FLT3: STAT5 and MAPK. For this purpose FLT3-ITD-R2 and R4 cell lines were incubated with different concentrations of SU5614 (0, 1 and 10µM) for 4 hours. As shown in Figure 21, SU5614 efficiently induces dephosphorylation of STAT5 and MAPK at a concentration of 1µM in Ba/F3 FLT3-ITD cells. In contrast to the parental cell line, STAT5 and MAPK were still phosphorylated in the FLT3-ITD-R2 and R4 cells even in presence of 10µM SU5614. These results are in good agreement with the IC_{50} of these cell lines in cell proliferation assays showing a 4 fold higher IC_{50} of ITD-R2 compared to ITD-R4 cells (see Table 5).

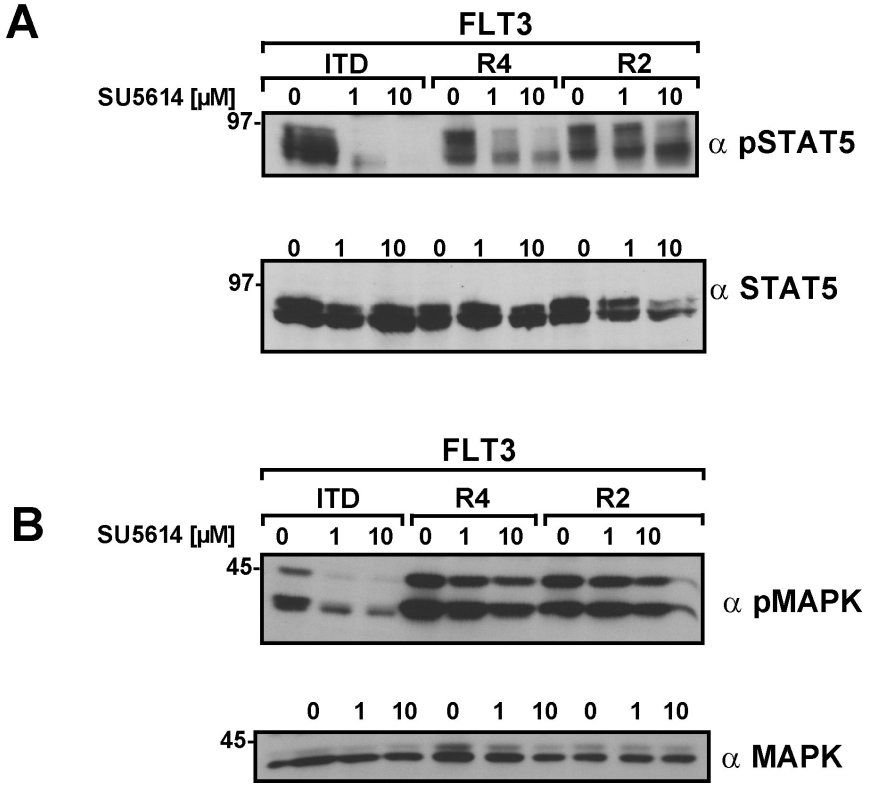


Figure 21: Activation of STAT5 and MAPK in FLT3-ITD-R1-4 cell lines in response to SU5614.

The phosphorylation status of STAT5 and MAPK in extracts of Ba/F3 FLT3-ITD and FLT3-ITD-R4/2 cells treated with 1 and 10 µM of SU5614 was determined by Western-Blot analysis using the polyclonal anti-pSTAT5 (**A.**) and anti-pMAPK antibodies (**B.**). Expression of STAT5 and MAPK in the same lysates was analyzed by immunoblotting with polyclonal anti-STAT5 and anti-MAPK antibodies.

6.5.3 Ba/F3 FLT3-ITD-R1-4 cells are resistant to the FLT3 PTK inhibitor AG1295, but not to Genistein and Ara-C

To further characterize the SU5614-resistant cell lines and analyze whether these cells developed a specific resistance to FLT3 PTK inhibitors, we cultivated FLT3-ITD-R1-4 cells in the presence of two different PTK inhibitors: either AG1295, that has inhibitory activity to class III PTK (Levis, Tse et al. 2001) or the broad spectrum PTK inhibitor Genistein. (Akiyama, Ishida et al. 1987) We could demonstrate that all SU5614-resistant cell lines developed also resistance to the FLT3 selective PTK inhibitor AG1295 (Figure 22A,B), but not to Genistein (Figure 22C).

Further we investigated whether the ITD-R1-4 cells developed an unspecific resistance to apoptotic cell death induced by cytotoxic drugs. For this purpose we exposed these cells to different concentrations of Ara-C (cytosine arabinoside, 0-5 µg/ml), a deoxycytidine analogue that is the most effective cytotoxic agent in the treatment of AML. We found that FLT3-ITD-R1-4 mutants are sensitive to Ara-C at a level comparable to that seen in the parental FLT3-ITD line (Figure 22B, summarized in Table 5).

These data clearly show that the ITD-R1-4 cell lines are resistant to the growth inhibitor activity of the FLT3 PTK inhibitor AG1295, but not to unselective PTK inhibitors or the cytotoxic agent Ara-C.

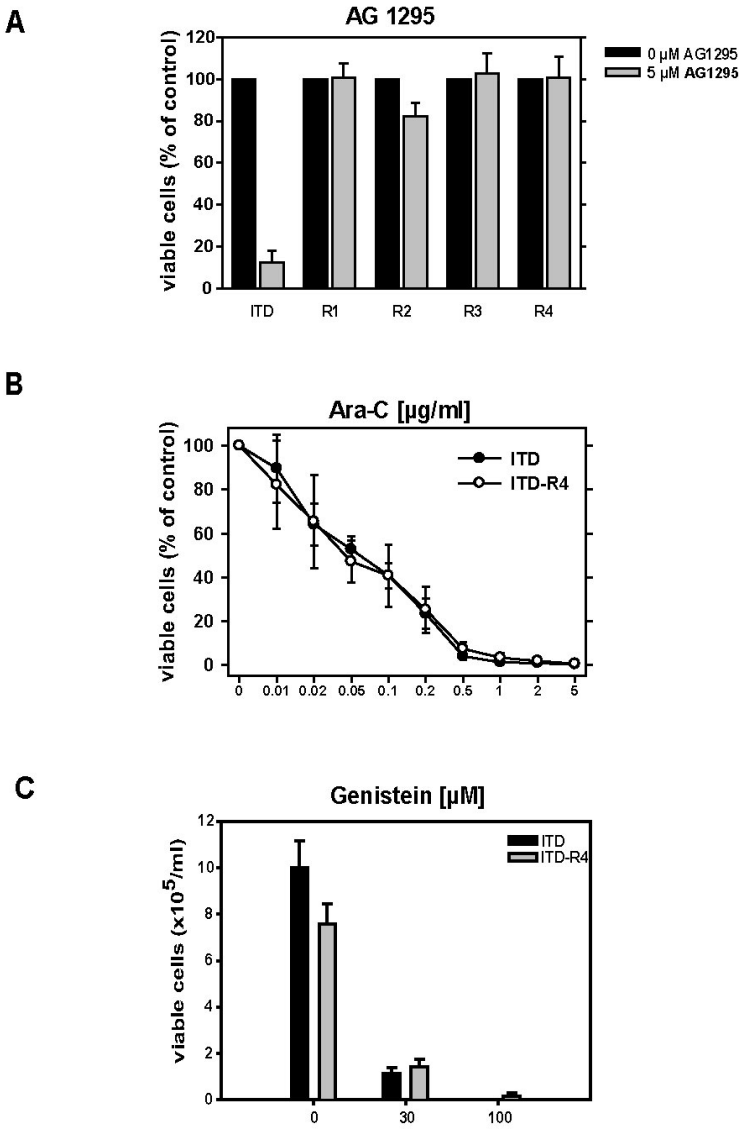


Figure 22: Ba/F3 ITD-R1-4 cells are resistant to AG1295, but not to Genistein and Ara-C.
A. Ba/F3 FLT3-ITD-R1-4 cells were seeded in the absence or presence of 5µM AG1295 and counted after 48 hours by trypan blue exclusion. **B.** Parental Ba/F3 FLT3-ITD and FLT3-ITD-R4 cells were cultured for 48 hours in the absence of IL-3 with different concentrations of Ara-C. **C.** Parental Ba/F3 FLT3-ITD and FLT3-ITD-R4 cells were cultured for 72 hours in the absence of IL-3 with different concentrations of Genistein (as indicated).

6.5.4 Expression of FLT3 protein in resistant cell lines

Overexpression of the target kinase is a well known mechanism of PTK-inhibitor-resistance. (le Coutre, Tassi et al. 2000; Mahon, Deininger et al. 2000; Weisberg and Griffin 2000)To further evaluate the mechanisms of SU5614 resistance in the ITD-R1-4 cell lines we analyzed FLT3 surface expression by flow cytometry using

a PE-labelled monoclonal anti-FLT3 antibody. All resistant cell lines expressed significantly higher levels of FLT3 in comparison to the parental FLT3-ITD cell line (Figure 23, Table 5).

These data were confirmed by Western blot analyses using a polyclonal antibody directed against the interkinase domain of FLT3. Identical protein loading in all lanes was confirmed by immunoblotting using an anti-ß-actin antibody. FLT3 expression was significantly higher in ITD-R4- (1285%), ITD-R3- (1016%), ITD-R2- (918%) and ITD-R1-cells (725%) compared to FLT3 levels in the parental FLT3-ITD cell line (100%).

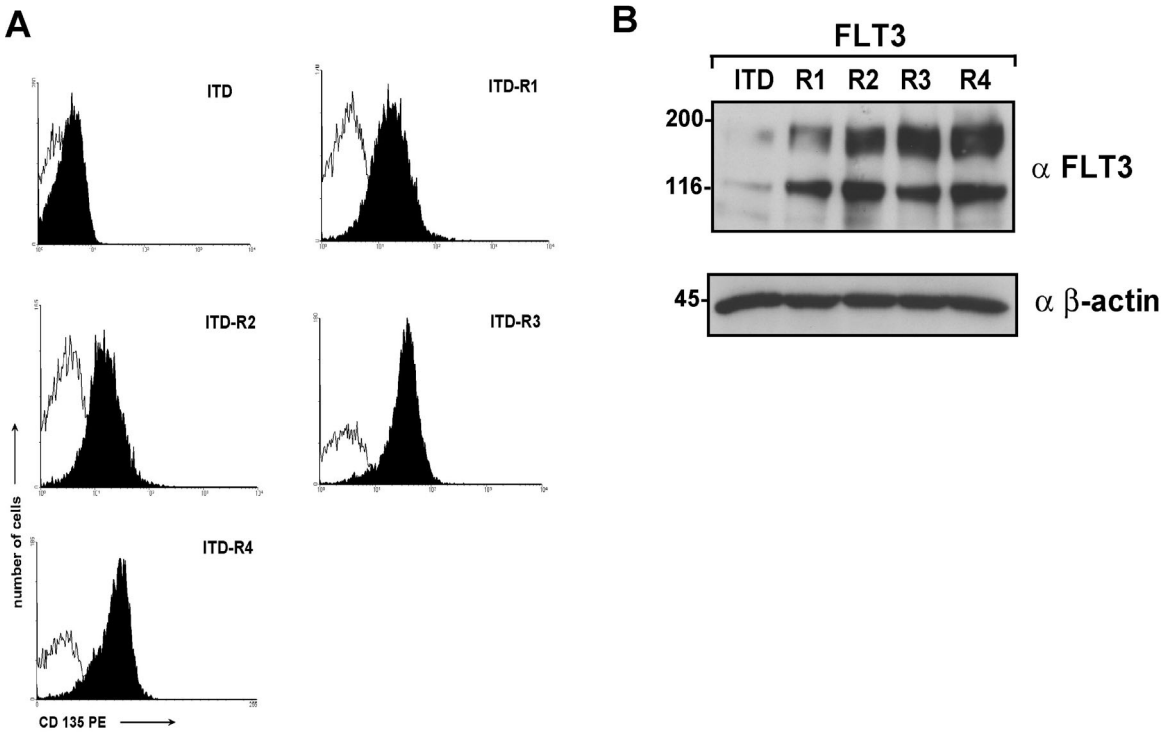


Figure 23: Expression of FLT3 in SU5614-resistent cell lines in comparison to parental FLT3-ITD cells.
A. The expression of FLT3 in Ba/F3 FLT3-ITD-R1-4 was analyzed by FACS analysis. Open histograms represent isotype control (PE-labelled control antibody); filled histograms show fluorescence intensity of CD135. **B.** FLT3 expression was determined by immunoblotting using a polyclonal anti-FLT3 antibody.

6.5.5 The SU5614 resistant ITD-R1-4 cell lines acquired distinct FLT3-TKD mutations

Another mechanism of clinical resistance to PTK inhibitors represents the acquisition of distinct mutations in the ATP-binding site and the kinase activation loop. This phenomenon was first described in patients with CML who developed clinical resistance to imatinib and were found to carry imatinib-resistant point mutants of BCR-ABL in their leukemic blasts. (Gorre, Mohammed et al. 2001; Branford, Rudzki et al. 2002; Hofmann, Jones et al. 2002; Roumiantsev, Shah et al. 2002; Shah, Nicoll et al. 2002; von Bubnoff, Schneller et al. 2002)We hypothesized that similar mechanisms might also be responsible for resistance to FLT3 PTK-inhibitors in SU5614-resistant FLT3-ITD cell lines. For this purpose we analyzed the known hot spot regions for activating mutations in the FLT3 gene found in patients with AML: the juxtamembrane region (JM) and the activation loop of the kinase domain. The JM region was screened for mutations using the previously published primers 11F and 12R and subsequent visualization of the PCR-products in agarose gels. (Kiyoi, Naoe et al. 1997; Schnittger, Schoch et al. 2001)The ITD-R1-4 lines expressed the parental ITD (W51) mutation, as shown by an identical size of the PCR product compared with the control cell line (Figure 24A).

To screen for mutations in the TKD domain we used melting curve analyses after amplification of a 244 base pair fragment by real-time PCR as described previously. (Schnittger, Schoch et al. 2001; Schnittger, Boell et al. 2002)The design of the hybridization probe allows detection of the point mutations and insertions/deletions ranging from amino acid 825 to 839 in the TKD domain of FLT3. The real-time PCR analysis demonstrated abnormal melting curves for the ITD-R3 and R4 mutants using either cDNA or genomic DNA as a template. Nucleotide sequencing of genomic DNA from these cells confirmed a single and identical G→A nucleotide (nt) exchange in both the ITD-R3 and ITD-R4 cell lines resulting in a D835N mutation. Sequential analysis of the FLT3-ITD-R4 cell line at 12 weeks and 18 weeks during selection with SU5614 indicated that the ratio of D835N/D835 DNA continuously increased over time (Figure 24B). The melting curve analyses shown in Figure 9B clearly demonstrates that the D835N mutant allele accounted for approximately 50% of DNA after 12 weeks of selection and increased to 100% of total DNA after continuous exposure to SU5614 for another 6 weeks. These results were confirmed by direct nucleotide sequencing of the DNA templates used for melting curve analysis (data not shown).

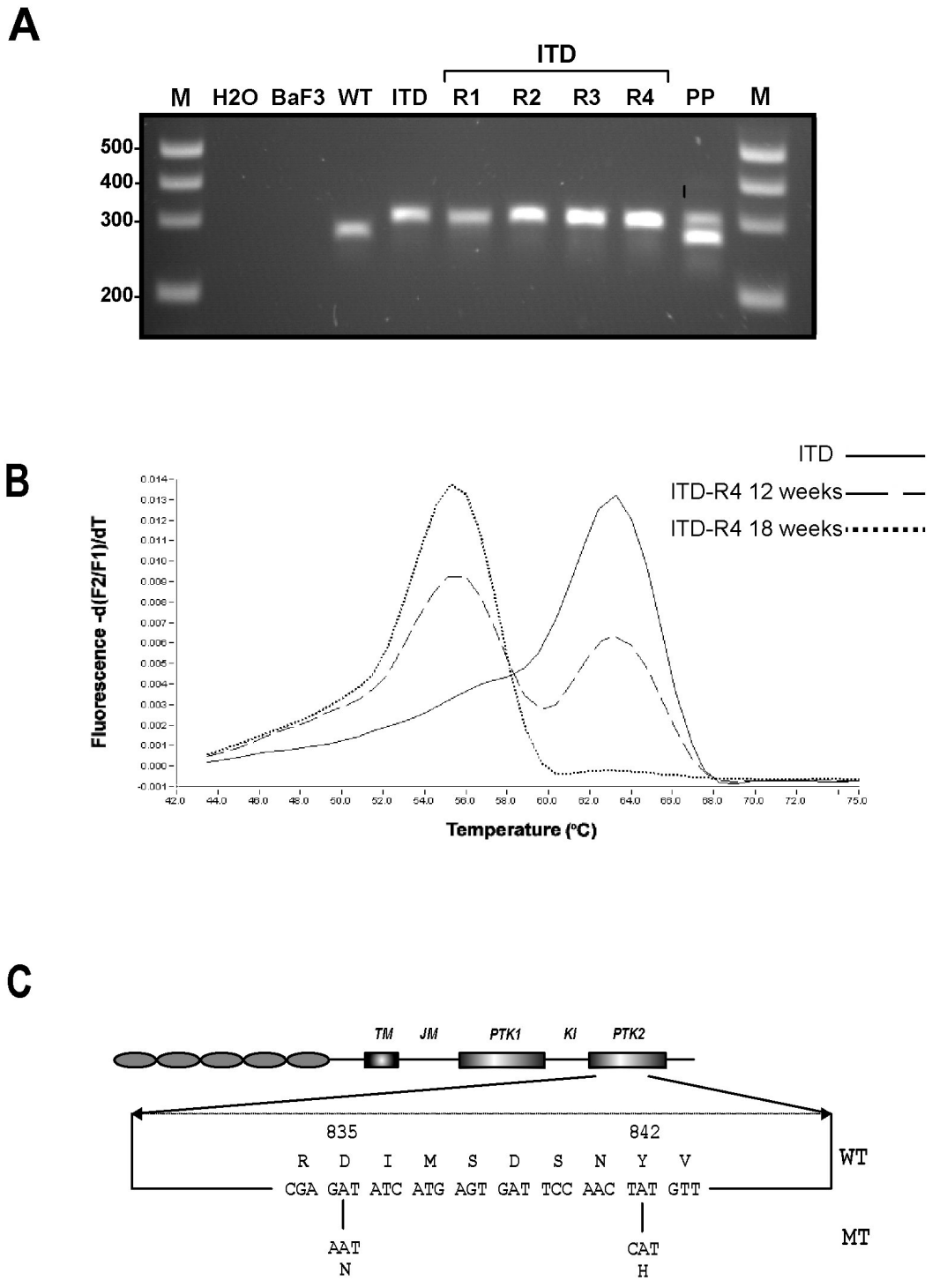


Figure 24: Molecular characterization of SU5614-resistant cells.
A. The juxtamembrane region of FLT3 was amplified by PCR using the published primer pair (11F and 12R) (Kiyoi, Naoe et al. 1997; Schnittger, Schoch et al. 2001)and amplification products were separated by agarose gel electrophoresis and viewed under UV illumination after ethidium bromide staining. M: DNA molecular weight marker, H_2O: water control, Ba/F3: native Ba/F3 cells, WT: Ba/F3 FLT3-WT, ITD: Ba/F3 FLT3-ITD, ITD-R1-4: Ba/F3 FLT3-ITD-R1-4, PP: positive control (AML patient carrying a FLT3-LM). **B.** Detection of FLT3-TKD mutations was performed by melting curve analysis after amplification of a 244 bp fragment by real time PCR as described previously. (Schnittger,

Schoch et al. 2001; Schnittger, Boell et al. 2002)In the presence of the FLT3-TKD wild type sequence or the FLT3 TKD mutant DNA the fluorescence peak is observed at 63°C and 55°C, respectively. **C.** The structural domains of the FLT3 protein with the position of the TKD mutations found in Ba/F3 FLT3-ITD-R1-4 cells (D835N and Y842H) are shown.

After the identification of a D835N mutant in ITD-R3 and R4 cells, we sequenced the entire TKD domain of the ITD-R1 and R2 cell lines to screen for further mutations outside the region that was covered by the hybridization primers used in the light cycler analysis. Direct nucleotide sequencing clearly demonstrated that both the ITD-R1 and R2 cells carried a T→C nucleotide substitution in codon 842 of FLT3 resulting in an Y842H mutation (Figure 24C).

Taken together, these data clearly indicate that distinct mutations in the PTK domain of FLT3 are acquired in Ba/F3 FLT3-ITD cells during selection with the FLT3 PTK inhibitor SU5614.

6.6 “Dual” mutants FLT3 ITD-TKD are resistant to SU5614

6.6.1 The FLT3-ITD/TKD “dual” mutation is responsible for the SU5614 resistant phenotype in FLT3-ITD-R1-4 cells

To confirm that the D835N and Y842H TKD mutations are responsible for the resistance to SU5614 in ITD-R1/2 and ITD-R3/4 cells, we introduced either the D835N or the Y842H mutation in the FLT3-WT and the FLT3-ITD constructs. Then Ba/F3 cell lines expressing the FLT3-D835N, Y842H or the FLT3-ITD mutant alone were generated. In addition, the FLT3 “dual” mutant FLT3-ITD/D835N and FLT3-ITD/Y842H Ba/F3 cell lines were established. All FLT3 mutants conferred IL-3 independent growth to Ba/F3 cell lines (data not shown, summarized in Table 6).

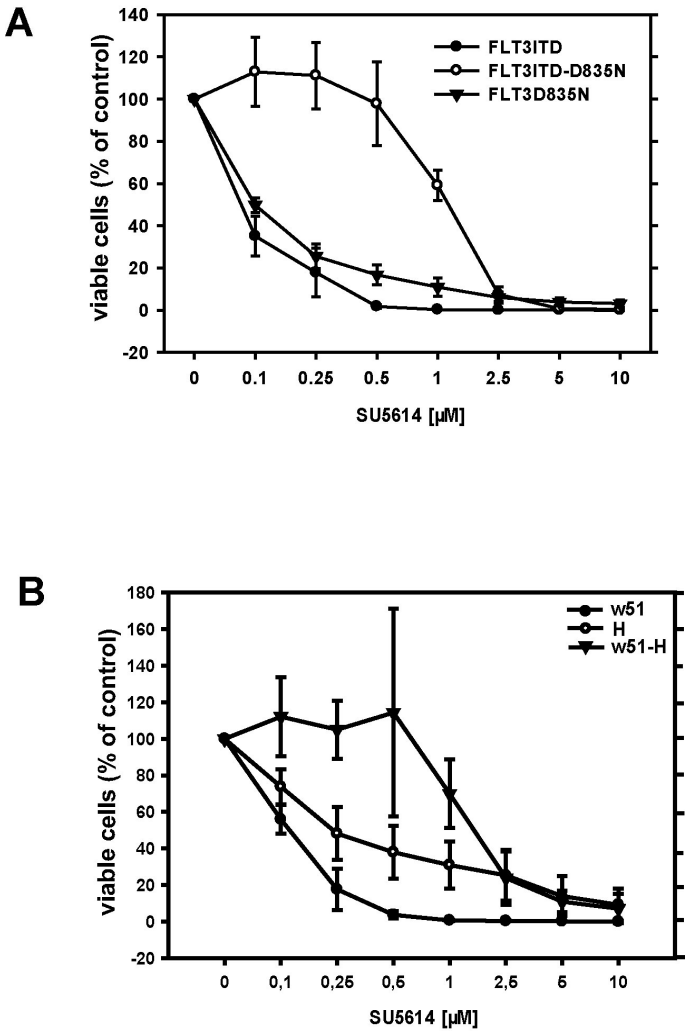
Ba/F3 cell line	IC_{50} SU5614 [μM]	IL-3 independent growth
ITD (W51)	0.1	+
D835N	0.1	+
ITD-D835N	1.0	+
Y842H	0.2	+
ITD-Y842H	1.1	+

Table 6: Characterization of Ba/F3 cells expressing FLT3-ITD/TKD dual mutants.

The IC_{50} of SU5614 in Ba/F3 cell lines expressing either the D835N or Y842H or the FLT3-ITD mutant alone and the FLT3-ITD/D835N, FLT3-ITD/Y842H "dual" mutants was determined as described in Figure 10.

As shown in Figure 25A, the IC_{50} of FLT3-D835N cells to SU5614 did not significantly differ from the IC_{50} of FLT3-ITD cells (IC_{50} =0.1μM and 0.08μM respectively). In contrast, the FLT3-ITD/D835N dual mutant cells were partially resistant to SU5614 at levels comparable to the original ITD-R4 cell line (IC_{50}=1.0 and 1.3μM respectively). Similar results were obtained when FLT3-Y842H and FLT3-ITD/Y842H cell lines were analyzed (Figure 25B, Table 5).

We further analyzed whether the "dual" FLT3 ITD/TKD mutant provides a competitive growth advantage to Ba/F3 cells in the absence or presence of 0.2μM of SU5614 compared to the parental ITD cell line. For this purpose, we mixed MIG FLT3-ITD (GFP+) and MIY FLT3-ITD/D835N (YFP^+) expressing Ba/F3 cells in a ratio of 10:1 and measured the percentage of GFP^+ and YFP^+ cells every 3-4 days for a time period of 2 weeks by FACS analysis (Figure 26A,B).



<u>Figure 25:</u> The FLT3-ITD/D835N and the FLT3-ITD/Y842H dual mutants restore the SU5614-resistant phenotype in Ba/F3 cells.

(**A.**) The Ba/F3 FLT3-ITD, FLT3-D835N and FLT3-ITD/D835N, (**B.**) FLT3-Y842H and FLT3-ITD/Y842H expressing cells were seeded at a density of $4x10^4$ cells/ml in the absence and presence of different concentrations of SU5614 and viable cells were counted after 72 hours by trypan blue exclusion.

We could show that over a time period of 14 days the FLT3-ITD/D835N "dual" mutant cells had a substantial competitive growth advantage in the presence, but not in the absence of SU5614. The percentage of FLT3-ITD-D835N-YFP positive cells in the presence of SU5614 increased from 10% (day 0) to 88% (day 14), whereas the percentage of "dual" mutant YFP positive cells cultured in the absence of SU5614 increased to only 22%.

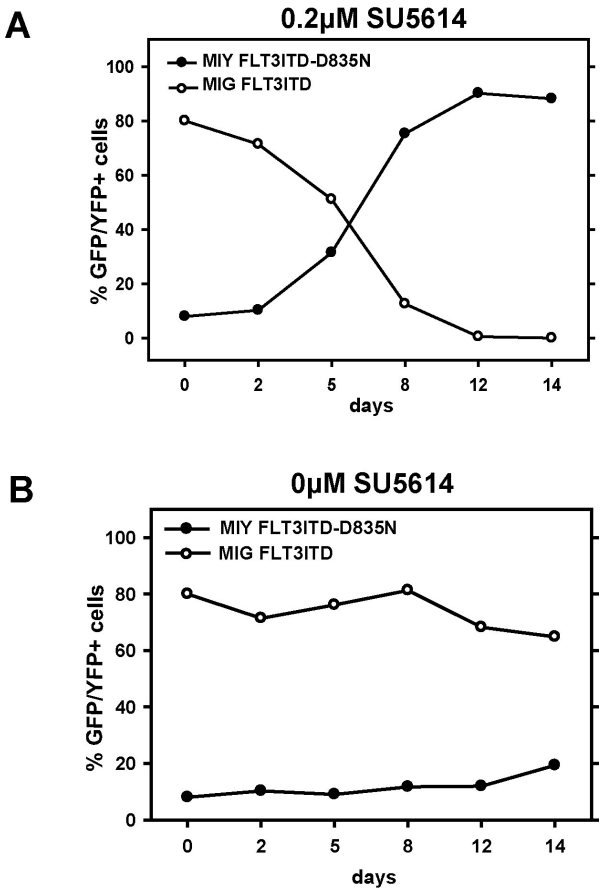


Figure 26: The FLT3-ITD/D835N dual mutant provides a competitive growth advantage to Ba/F3 cells in the presence of SU5614.
A. and B. MIG FLT3-ITD (GFP+) and MIY FLT3-ITD/D835N (YFP^+) expressing Ba/F3 cells were mixed in a ratio of 10:1 and the percentage of GFP^+ and YFP^+ cells was measured every 3-4 days for a time period of 2 weeks by FACS analysis in the presence **(A.)** or absence **(B.)** of 0.2µM of SU5614.

These data clearly show that the FLT3-ITD/TKD dual mutant restores the ITD-R1-4 phenotype. Therefore, the acquisition of specific TKD mutations in the FLT3-ITD gene represents a new mechanism of resistance to FLT3 PTK inhibitors *in vitro*.

6.6.2 "Dual" mutants are resistant to SU5614 independent from kind of mutation in juxtamembrane region of FLT3

We hypothesized, that various length of mutations in JM region can lead to differences in sensitivity to SU5614. For this purpose we generated Ba/F3 cells transformed with FLT3-V592A/D835N and FLT3-NPOS/D835N mutants. The FLT3-NPOS mutant contains a 28-amino acid duplicated sequence inserted

between amino acids 610/611 in contrast to FLT3-W51 mutant, which contains only 6-amino acid duplicated sequence inserted between amino acids 601/602 and FLT3-V592A contains only point mutation.

Our results clearly demonstrate, that all of these dual mutants show partially resistance to SU5614 although at a different degree (IC_{50}=0.3µM for V592A-D835N- only weak resistance and 1µM for NPOS-D835N - more strong) (Figure 27).

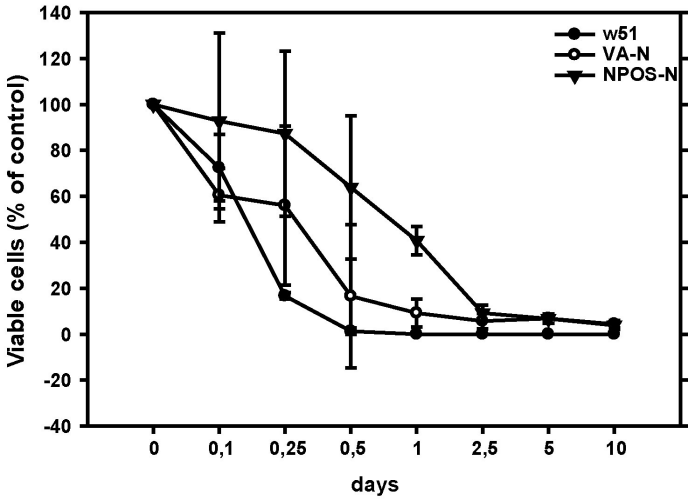


Figure 27: The FLT3-ITD/D835N and the FLT3-ITD/Y842H dual mutants restore the SU5614-resistant phenotype in Ba/F3 cells.
(**A.**) The Ba/F3 FLT3-ITD, FLT3-D835N and FLT3-ITD/D835N, (**B.**) FLT3-Y842H and FLT3-ITD/Y842H expressing cells were seeded at a density of $4x10^4$ cells/ml in the absence and presence of different concentrations of SU5614 and viable cells were counted after 72 hours by trypan blue exclusion.

6.6.3 The presence of FLT3-ITD and FLT3-TKD mutations on the two ‘‘alleles’’ do not confer SU5614 resistance

Furthermore, we analyzed whether the ITD and TKD mutations, which occurred on the different alleles, also might lead to resistance to PTK-inhibitors. For this purpose, we generated Ba/F3 cells transformed with FLT3-ITD//FLT3-D835N and FLT3-ITD//FLT3-WT. As a control we generated Ba/F3 FLT3-ITD cell line. Our data clearly show that occurrence of ITD and TKD mutations on two alleles is not sufficient to induce resistance to PTK-inhibitor (Figure 28).

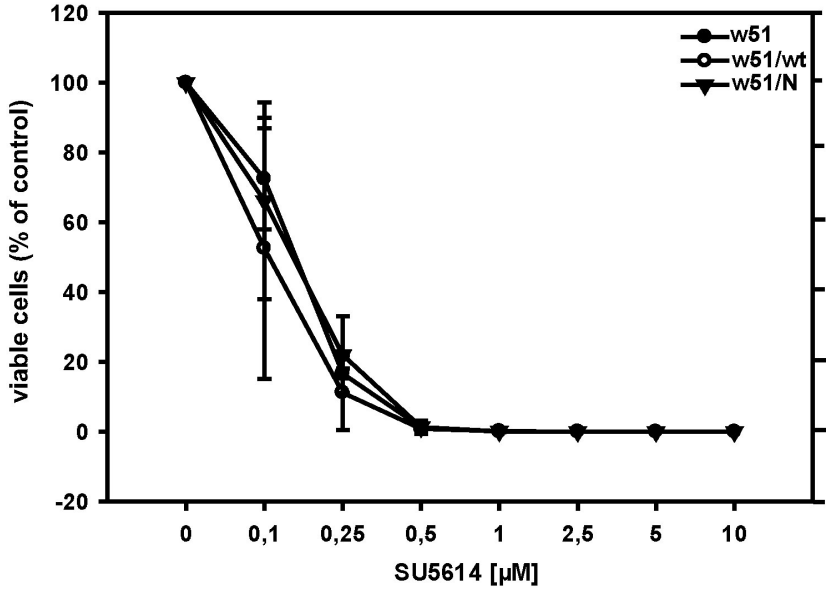


Figure 28: The FLT3-ITD//FLT3-D835N and the FLT3-ITD//FLT3-WT mutants are completely sensitive to SU5614.
The Ba/F3 FLT3-ITD, FLT3-ITD//FLT3-D835N and FLT3-ITD//FLT3-WT expressing cells were seeded at a density of $4x10^4$ cells/ml in the absence and presence of different concentrations of SU5614 and viable cells were counted after 72 hours by trypan blue exclusion.

These data clearly confirm that occurrence of LM and TKD mutations on the same allele, but not on two alleles lead to resistance to the PTK-inhibitor SU 5614.

7. DISCUSSION

7.1 Functional characterization of two “new’’ FLT3 mutations in AML: V592A and 840GS

The data presented here clearly indicate that activating mutations in the FLT3 gene do not only occur within the juxtamembrane domain and in codons D835/836 but also in other positions of the A-loop of the catalytic domain. The A-loop represents a hot spot region for activating mutations in class III RTK which have been described for KIT (D816) and FLT3 (D835/836) (Yamamoto, Kiyoi et al. 2001)These mutations induce a conformational change of the A-loop which results in the opening of the catalytic pocket and a constitutively active kinase activity. Although no structural data on the catalytic domain of FLT3 are available which would allow the detailed structure-function analysis, the close proximity to the AA D835/836 suggests a similar mechanism of kinase activation by the FLT3-840GS mutant.

Activating mutations of the FLT3 gene provide an essential anti-apoptotic and pro-proliferative signal in primary AML cells and cell lines (Hayakawa, Towatari et al. 2000; Mizuki, Fenski et al. 2000; Tse, Mukherjee et al. 2000) Our results clearly indicate that the FLT3840-GS mutant is hyperphosphorylated on tyrosine residues and induces IL-3 independent growth in Ba/F3 cells. These in vitro data underline the pathophysiologic role of this mutant for the leukemic phenotype in patients with AML.

In order to identify downstream targets of FLT3-840GS mutant receptor, we performed Western blot analysis with phosphospecific antibodies against STAT5 and MAPK. Our results confirmed strong MAPK activation by the FLT3-840GS, but only weak STAT5 activation. We hypothesize the weak STAT5 activation translates into a weak transforming activity of this mutation and used IL-3 stimulation to induce maximal proliferation activity. Our results show that IL-3 stimulation leads to an increased growth rate of the Ba/F3 FLT3-840GS cells in

dose-dependent manner. Therefore, we suggest, that the FLT3-840GS mutant requires additional mitogenic signal for full transformation. Possible explanation of these results might be found in recently published article from Y.Minami et al, where the authors show differences between anti-apoptotic pathways of wild-type and mutated FLT3 receptors. The authors showed that STAT5 is the most important downstream target for FLT3-ITD mutants, but the MAPK pathway is indispensable for the FLT3-WT receptor. Based on the data shown here we hypothesize that the FLT3-840GS mutant uses similar pathway compared to the FLT3-WT receptor.

Another mutation that was found and described in this work is the FLT3-V592A. This mutation represents the first point mutation in JM domain of FLT3. The mutation was found in two AML cell lines (MM6 and MM1), but the frequency in patients with AML is unknown. Probably the V592A represents rare mutation, but it represents a good model for future investigations of FLT3 signalling. Recently it was shown by Di Maio et al. that amino acid substitution in JM region of the PDGFR led to activation of the receptor. V592 of FLT3 is also a highly conserved amino acid in class III RTK (Rosnet, Marchetto et al. 1991)and further analysis will be needed to investigate the mechanism of transformation by this mutation.

The data demonstrated here show that FLT3-V592A is an activating mutation, which leads to hyperphosphorylation of the FLT3 receptor and induces IL-3-independent growth in Ba/F3 cells. Our data also demonstrate that the FLT3-V592 mutant receptor activates two known downstream targets of FLT3: STAT5 and MAPK. Furthermore, the specific FLT3 PTK inhibitor SU5614 was able to inhibit IL-3-independent growth of MM6, MM1 (Spiekermann, Dirschinger et al. 2003)and Ba/F3 FLT3-V592A cells.

In order to identify differences in FLT3-TKD and FLT3-V592A signalling, we generated 589/591 Y→F substitution mutants of either FLT3-D835Y or FLT3-V592A. Our data show that the tyrosine residues 589/591 play a crucial role for transformation of Ba/F3 cells by the FLT3-V592A, but not by the FLT3-D835Y mutant. These findings are in good agreement with results from Kyioi et al., which found that these tyrosine residues are important for FLT3-ITD constructs (Kiyoi,

Towatari et al. 1998) Although further studies are required, we suggest, that the FLT3-V592A mutant and FLT3-ITD mutant activate similar signalling pathways.

Although the FLT3-840GS and FLT3-V592A are probably rare mutations, they clearly show that activating mutations other than the FLT3-LM in the JM domain and FLT3D835/836 in the kinase domain exist in patients with AML.

These findings are of significant clinical importance since activating FLT3 mutations could represent selective and specific molecular target structures for therapeutical strategies using PTK inhibitors in AML (Levis, Tse et al. 2001; Tse, Novelli et al. 2001)

7.2 FLT3 as a molecular target for selective therapeutic approaches in AML

Our group and others have previously shown that selective FLT3 PTK inhibitors, like SU5614, AG1295, PKC412 or CEP701 can induce growth arrest and apoptosis in FLT3 transformed cell lines and in primary AML blasts (Levis, Tse et al. 2001; Kelly, Yu et al. 2002; Weisberg, Boulton et al. 2002; Yee, O'Farrell et al. 2002; Spiekermann, Dirschinger et al. 2003) These promising *in vitro* results have stimulated phase I/II clinical studies evaluating the efficacy of FLT3 PTK inhibitors in patients with AML (Foran, Paquette et al. 2002; Smith, Levis et al. 2002; Stone, Klimek et al. 2002)

In contrast to the well studied mechanisms of drug resistance found in patients with BCR-ABL-positive CML and ALL treated with the PTK inhibitor imatinib, little is known about primary and secondary mechanisms of resistance to FLT3 PTK inhibitors. In the present study we could show that certain activating point mutations found in the PTK domain of FLT3 in patients with AML significantly alter the sensitivity to the FLT3 PTK inhibitor SU5614. As a mechanism of secondary resistance we identified that FLT3-ITD transformed cell lines acquire distinct mutations in the TKD domain after prolonged cultivation in the presence of FLT3

PTK inhibitors. Reconstruction of these FLT3 “dual” mutants confirmed that the presence of a certain TKD-mutant on the genetic background of the ITD mutation was responsible for the FLT3-PTK inhibitor resistant phenotype. These data provide genetic evidence that pre-existing and acquired mutations in the TKD domain of FLT3 are sufficient to induce drug resistance to FLT3 PTK inhibitors.

Genetically heterogeneous mutations in the TKD domain of FLT3 have been found in 7-8% of patients with AML and include point mutations at codon D835 and also insertions/deletions in codons 835/836 (Abu-Duhier, Goodeve et al. 2001; Thiede, Steudel et al. 2001; Yamamoto, Kiyoi et al. 2001; Thiede, Steudel et al. 2002) To analyze the sensitivity of these mutants to FLT3 PTK inhibitors, we generated Ba/F3 cell lines expressing the most frequent FLT3-TKD mutants, namely D835Y/V/H. All FLT3-TKD mutants confer IL-3 independent growth to Ba/F3 cells and activate similar signal transduction pathways (STAT5 and MAPK) compared to the FLT3-ITD construct. Importantly, these FLT3-TKD mutants differed significantly in their sensitivity to the growth inhibitory and apoptosis inducing activity of the FLT3 PTK inhibitor SU5614 in the following order: FLT3-ITD = FLT3-D835 Y > V > H.

AML-specific mutations in the TKD-domain of PTKs occur in conserved amino acids at codons 835/836 and 816 in FLT3 and KIT, respectively (Abu-Duhier, Goodeve et al. 2001; Yamamoto, Kiyoi et al. 2001; Ma, Zeng et al. 2002) It has been hypothesized that these mutations change the conformation of the activation loop (A-loop) thereby allowing spontaneous opening of the catalytic pocket and ligand-independent activation of the kinase (Blume-Jensen and Hunter 2001; Wybenga-Groot, Baskin et al. 2001) The conformational change in the A-loop of KIT by the D816V mutations is probably also responsible for the resistance of this mutant to the PTK inhibitor imatinib (Ma, Zeng et al. 2002; Zermati, De Sepulveda et al. 2003) Based on these findings, the observation of decreased SU5614-sensitivity of certain FLT3-TKD mutants, e.g. D835H might also be attributable to the decreased affinity of the inhibitor for the catalytic pocket of FLT3. Recently, Grundler et al. have analyzed the sensitivity of mutants of FLT3 at codon 835/836 to the FLT3 PTK inhibitors SU5614, PKC412 and AG1295. These authors found in

accordance with our results that distinct FLT3-TKD mutations lead to an altered sensitivity to PTK inhibitors (Grundler, Thiede et al. 2003)

These findings have important clinical implications for studies evaluating the efficacy of FLT3 PTK inhibitors in patients with FLT3-TKD mutation positive AML. FLT3-TKD mutations can change the affinity of the kinase to inhibitors and might therefore result in clinical resistance. Treatment of AML patients carrying TKD mutations will therefore require a preclinical sensitivity analysis to select an appropriate inhibitor.

7.3 ‘’Dual’’ mutations in the FLT3 gene as a cause of drug resistance to FLT3 PTK inhibitors

We characterized 4 sublines of the parental FLT3-ITD transformed Ba/F3 cell line after selection in the presence of 0.2-0.8µM SU5614. All cell lines acquired a partial resistance to the FLT3 PTK inhibitor SU5614, but not to other apoptosis inducing agents like Ara-C, and showed an IC_{50} that was 10-25 fold higher compared to that of control cells. Importantly, the ITD-R1-4 lines were completely sensitive to SU5614 at high concentrations of 10µM of inhibitor showing that the proliferation of all cell lines was still dependent on the presence of the FLT3 mutant. Additional experiments using the FLT3 PTK-inhibitor AG1295 clearly showed that the SU5614-resistant phenotype induced a complete cross-resistance with AG1295.

The detailed molecular analysis showed that each of the ITD-R1-4 cell lines acquired a specific TKD mutation resulting either in a D835N or an Y842H FLT3 mutant. In sequential analyses performed in the ITD-R4 cell line during selection with SU5614 the ratio of mutant/wild type DNA increased over time showing a substantial competitive growth advantage of the ITD-D835N dual mutant expressing cell population.

Clinical samples obtained from patients with AML usually carry either the ITD or the TKD mutation (Kottaridis, Gale et al. 2001; Thiede, Steudel et al. 2001; Schnittger, Schoch et al. 2002; Thiede, Steudel et al. 2002) However, in some patients both mutations can be detected and preliminary data suggest that these patients might have an even worse clinical prognosis compared to patients with a single ITD or TKD mutation (Karali, Dimitriadou et al. 2002; Moreno, Martin et al. 2003) In a recent study of 979 patients, Thiede et al. found an additional TKD mutation in 17/200 (8.5%) of AML patients carrying a FLT3-LM. Further analysis revealed that 40% of the LM and TKD mutations occurred on the same allele showing that FLT3-LM/TKD “dual” mutants spontaneously arise *in vivo* (Thiede, Steudel et al. 2002)Although the “dual” FLT3 LM/TKD mutant is infrequently found at diagnosis in patients with AML, our *in vitro* data suggest that these mutants might develop during treatment with FLT3 PTK inhibitors.

7.4 The FLT3-ITD/TKD dual mutation is responsible for the PTK inhibitor resistance.

To prove that the acquisition of the TKD mutation is responsible for the FLT3 PTK inhibitor resistance in the ITD-R1-4 cells we generated Ba/F3 cells expressing either the FLT3-D835N/Y842H mutant alone or on the background of the ITD mutation. Although both TKD mutants alone induced IL-3 independent growth in Ba/F3 cells, these mutants were equally sensitive to SU5614 compared to the FLT3-ITD-construct. In contrast, when introduced on the ITD background, both the D835N and the Y842H mutation restored the SU5614 resistant phenotype in Ba/F3 cells.

Although the FLT3-D835N and the Y842H mutants have transforming activity, these constructs alone did not mediate resistance to SU5614. The explanation for this phenomenon is not obvious, but one has to assume that only the combination of the ITD and the D835N/Y842H mutation changes the conformation of the catalytic domain in a way, which decreases its affinity for the FLT3 inhibitor.

Structural analyses of the Ephrin-receptors have shown that both the activation loop and the JM region directly interact with the PTK domain by the formation of autoinhibitory loops (Blume-Jensen and Hunter 2001; Wybenga-Groot, Baskin et al. 2001) To what extent the exact amino acid sequence and the site of insertion of the FLT3-LM will influence the resistance to a specific FLT3 PTK inhibitor is unknown. The high variability of FLT3-LM found in patients with AML must be taken into account when choosing an appropriate FLT3 PTK inhibitor, as it is likely that resistance or sensitivity to these compounds might also depend on the three dimensional structure of the JM-region.

Although the FLT3-ITD/TKD dual mutant restores the SU5614 resistant phenotype when introduced in FLT3-ITD transformed Ba/F3 cells, we cannot completely rule out that other mechanisms might contribute to the phenotype of ITDR1-4 cells. Such mechanisms of resistance include altered metabolism of the drug, induction of drug efflux, e.g. by ABC transporters or overexpression of the target kinase. Very recently, Weisberg et al. have generated a PKC412 resistant Ba/F3 cell line, that was selected with increasing inhibitor concentrations and showed overexpression of FLT3 as a potential mechanism of drug resistance (Weisberg, Boulton et al. 2002) The detailed analysis of the ITD-R1-4 cell lines described here showed overexpression of FLT3 protein. Although, the FLT3-ITD/TKD dual mutant cells generated by transduction of Ba/F3 cells with the FLT3 cDNA carrying both mutations resembled the Ba/F3 FLT3-ITD-R1-4 phenotype the IC_{50} of SU5614 in ITD-R1-4 cells was still 2-5 times higher (see Table 5 and 6). It is possible that overexpression of the FLT3 found in the ITD-R1-4 cells might contribute to the enhanced SU5614-resistance in these cells compared to the “dual” ITD/TKD mutant Ba/F3 cells. As shown in patients with CML developing clinical resistance during imatinib treatment several mechanisms of resistance can occur in parallel and factors, other than mutations in the gene, e.g. overexpression of the target kinase are likely to contribute to drug resistance *in vivo* (Gambacorti-Passerini, Barni et al. 2000; le Coutre, Tassi et al. 2000; Mahon, Deininger et al. 2000; Weisberg and Griffin 2000).

7.5 Different kind of mutations in the JM region of FLT3 induce FLT3 PTK inhibitor resistance in dual mutant receptors.

To analyze whether different mutations in JM region of FLT3 together with different TKD mutations would also confer resistance to FLT3 PTK inhibitors, Ba/F3 cell lines transformed with different dual FLT3 mutants were generated: Ba/F3 FLT3 W51-D835N, Ba/F3 FLT3 NPOS-D835N, Ba/F3 FLT3 V592A-D835N and Ba/F3 FLT3 W51-D835Y. We hypothesized that a different length of mutation in JM domain could probably lead to different conformation changes of the receptor and in this way to different sensitivity to PTK inhibitor. Our data demonstrated that this hypothesis was only partially right. Though these mutants differ in their sensitivity to SU5614, all of them show partially resistance to SU5614. That means that different kind of mutations in the JM region are able to induce PTK inhibitor resistance in the FLT3 TKD background.

To clarify whether the two mutations have to occur on the same or on different alleles to induce inhibitor resistance, we generated Ba/F3 cell lines transformed with FLT3-ITD and FLT3-WT constructs and FLT3-ITD and FLT3-D835N mutants. In agreement with our theory that ITD-TKD mutation lead to conformation changes, the presence of the ITD and TKD mutations in different constructs was insufficient to confer resistance to SU5614.

Taken together, mutations in the TKD domain of FLT3 can induce primary and secondary resistance to FLT3 PTK inhibitors. These findings have profound impact for clinical studies evaluating FLT3 PTK inhibitors in patients with AML and should be considered when clinical resistance to these compounds occurs.

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9. PUBLICATIONS

Papers:

1. **Bagrintseva K.**, Schwab R., Kohl TM, Schnittger S., Eichenlaub S., Ellwart J.W., Hiddemann W., Spiekermann K. Mutations in the tyrosine kinase domain of FLT3 define a new molecular mechanism of aquired drug resistance to PTK inhibitors in FLT3-ITD transformed hematopoietic cells. Blood, 2003 Nov 6 [Epub ahead of print].

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Abstracts:

1. **Bagrintseva K**., Geisenhof S, Schwab R, Eichenlaub S, Hiddemann W, Spiekermann K. [P48] Dual FLT3-ITD/TKD Mutants Found in AML confer Resistance to FLT3 PTK Inhibitors and Daunorubicin Abstract #48 appears in Annals of Hematology, Suppl1 to Vol 83 (2004)

2. **Bagrintseva K**, Schwab R, Schnittger S, Eichenlaub S, Hiddemann W, Spiekermann K. [P47] Acquisition of Mutations in the PTK Domain of FLT3 represents a New Molecular Mechanism of Drug Resistance to PTK Inhibitors in FLT3-ITD Transformed Cells Abstract #47 appears in Annals of Hematology, Suppl1 to Vol 83 (2004)

3. Schwab R., **Bagrintseva K.**, Wolf U., Eichenlaub S., Hiddemann W., Spiekermann K. [P65] The Transforming Potential of FLT3-TKD Mutants Depends on Tyrosine Residues 589/591 and 597/599. Abstract #65 appears in Annals of Hematology, Suppl1 to Vol 83 (2004)

4. **Bagrintseva K**., Geisenhof S, Schwab R, Eichenlaub S, Hiddemann W, Spiekermann K. [2174] Dual Activating FLT3-ITD/TKD Mutations Found in patients with AML Induce Resistance to FLT3 PTK Inhibitors and Daunorubicin by Upregulation of Bcl-xL. Abstract #2174 appears in Blood, Volume 102, issue 11, November 16, 2003

5. **Bagrintseva K**, Schwab R, Schnittger S, Eichenlaub S, Hiddemann W, Spiekermann K. [2175] Mutations in the Tyrosine Kinase Domain of FLT3 Define a New Molecular Mechanism of Acquired Drug Resistance to PTK Inhibitors in FLT3-ITD Transformed Cells Abstract #2175 appears in Blood, Volume 102, issue 11, November 16, 2003

6. **Bagrintseva K.**, Schwab R., Schnittger S., Eichenlaub S., Hiddemann W., Spiekermann K. Mutations in the tyrosine kinase domain of FLT3 define a new molecular mechanism of aquired drug resistance to PTK inhibitors in FLT3-ITD transformed cells. DGHO (Deutsche Gesellschaft für Hämatologie und Oncologie) 2003

7.Spiekermann K., **Bagrintseva K.**, Schoch C., Haferlach T., Hiddemann W., Schnittger S. (2002): A new and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia. DGHO (Deutsche Gesellschaft für Hämatologie und Oncologie) 2002

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10. ABBREVIATIONS

AML	Acute Myeloid Leukemia
MT	Mutation
FLT3	fms-like tyrosine kinase-3
FCS	Fetal Calf Serum
MAPK	Mitogen-Activated Protein Kinase
STAT	Signal Transducer and Activator of Transcription
MDS	Myelodysplastic syndrom
PBS	Phosphate Buffered Saline
PDGFR	Platelet Derived Growth Factor Receptor
TKD	Tyrosine Kinase Domain
LM	Length Mutation
ITD	Internal Tandem Duplication

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12. CURRICULUM VITAE

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Education

1/04- present **Postdoctoral position**
GSF (National Research Center for Environment and Health),
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7/00- 12/03 **MD, graduate student**
GSF (National Research Center for Environment and Health),
Clinical Cooperative Group ‘‘Leukemia’’, Ludwig-Maximilian University
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Graduate Student; Supervisor: Prof. Dr. W. Hiddemann
Dissertation: The role of the FLT3 mutations in AML. The possible mechanisms of the drug resistance development to specific PTK inhibitors.
Found and characterized a new mutations in FLT3:840GS and V592A.
Generated PTK inhibitor resistant cell lines and characterized the mechanisms of these resistance.

9/93-7/99 **Doctor of Medicine**
Medical Faculty, Russian State Medical University
Moscow, Russia

9/91-7/93 **Diploma Nurse with honor**
Central clinical hospital of the Government of Russian Federation
Moscow, Russia

Experimental Skills

DNA/RNA: recombinant DNA techniques, plasmid DNA isolation, PCR, mutagenesis;
Protein: immunoprecipitation analysis, Western blot analysis;
Cell culture: basic cell culture techniques, transient transfection techniques, retroviral infection, basic FACS analysis, apoptose assay.

Language Skills

Russian (native), English (fluent), German (fluent; Diploma: “Kleines Deutsches Sprachdiplom“, Goethe-Institut, Munich, Germany)

Computer Skills

Microsoft Word, Excel, Power Point, Sigma Plot, Adobe Illustrator, Adobe Photoshop, Winmdi, BioEdit, EndNote.

Activities

2/98-2/99	Russian Red Cross medical help at home to elderly patients in Moscow, Russia
9/98-6/99	Diploma in Therapeutic Massage ; Moscow, Russia
9/85-5/91	School of Music: class piano (Diploma); Moscow, Russia

Participation in scientific meetings:

Posters:

1. **Bagrintseva K**., Geisenhof S, Schwab R, Eichenlaub S, Hiddemann W, Spiekermann K.
[P48] Dual FLT3-ITD/TKD Mutants Found in AML confer Resistance to FLT3 PTK Inhibitors and Daunorubicin
Abstract #48 appears in Annals of Hematology, Suppl1 to Vol 83 (2004)

2. **Bagrintseva K**, Schwab R, Schnittger S, Eichenlaub S, Hiddemann W, Spiekermann K.
[P47] Acquisition of Mutations in the PTK Domain of FLT3 represents a New Molecular Mechanism of Drug Resistance to PTK Inhibitors in FLT3-ITD Transformed Cells
Abstract #47 appears in Annals of Hematology, Suppl1 to Vol 83 (2004)

3. Schwab R., **Bagrintseva K.**, Wolf U., Eichenlaub S., Hiddemann W., Spiekermann K.
[P65] The Transforming Potential of FLT3-TKD Mutants Depends on Tyrosine Residues 589/591 and 597/599.
Abstract #65 appears in Annals of Hematology, Suppl1 to Vol 83 (2004)

4. **Bagrintseva K**., Geisenhof S, Schwab R, Eichenlaub S, Hiddemann W, Spiekermann K.
[2174] Dual Activating FLT3-ITD/TKD Mutations Found in patients with AML Induce Resistance to FLT3 PTK Inhibitors and Daunorubicin by Upregulation of Bcl-xL.
Abstract #2174 appears in Blood, Volume 102, issue 11, November 16, 2003

5. **Bagrintseva K**, Schwab R, Schnittger S, Eichenlaub S, Hiddemann W, Spiekermann K.
[2175] Mutations in the Tyrosine Kinase Domain of FLT3 Define a New Molecular Mechanism of Acquired Drug Resistance to PTK Inhibitors in FLT3-ITD Transformed Cells
Abstract #2175 appears in Blood, Volume 102, issue 11, November 16, 2003

6. **Bagrintseva K.**, Schwab R., Schnittger S., Eichenlaub S., Hiddemann W., Spiekermann K. Mutations in the tyrosine kinase domain of FLT3 define a new molecular mechanism of aquired drug resistance to PTK inhibitors in FLT3-ITD transformed cells. DGHO (Deutsche Gesellschaft für Hämatologie und Oncologie)2003

7.Spiekermann K., **Bagrintseva K.**, Schoch C., Haferlach T., Hiddemann W., Schnittger S. (2002): A new and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia. DGHO (Deutsche Gesellschaft für Hämatologie und Oncologie)2002

8. Spiekermann K, **Bagrintseva K**, Schwab R, Schmieja K, Hiddemann W.
[1280] Overexpression and Constitutive Activation of FLT3 Induces STAT5 Activation in Primary AML Blast Cells. #1280 Blood, Volume 101, issue 10, 2002

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Publications

1. **Bagrintseva K.**, Schwab R., Kohl TM, Schnittger S., Eichenlaub S., Ellwart J.W., Hiddemann W., Spiekermann K. Mutations in the tyrosine kinase domain of FLT3 define a new molecular mechanism of aquired drug resistance to PTK inhibitors in FLT3-ITD transformed hematopoietic cells. Blood, 2003 Nov 6 [in press].

2. Spiekermann K., **Bagrintseva K.**, Schoch C., Haferlach T., Hiddemann W., Schnittger S. (2002): A new and recurrent activating length mutation in exon 20 of the FLT3 gene in acute myeloid leukemia. Blood 100,3423-3425

3. Spiekermann K., Dirschinger R, Schwab R., **Bagrintseva K.**, Faber F., Buske C, Schnittger S., Kelly L. M., Gilliland D. G., Hiddemann W. (2003): The protein tyrosine kinase inhibitor SU5614 inhibits FLT3 and induces growth arrest and apoptosis in AML-derived cell lines expressing constitutively activated FLT3. Blood 101, 1494-1504

4. Spiekermann K., **Bagrintseva K.**, Schwab R., Schmieja K., Hiddemann W.(2003): Overexpression and constitutive activation of FLT3 induces STAT5 activation in primary acute myeloid leukemia blast cells. Clin.Canc.Res. 9, 2140-2150

References

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