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# STI 571 inhibition effect on KIT$^{Asn822Lys}$-mediated signal transduction cascade

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***Objective*. Alterations in growth factor signaling pathways may be a frequent collaborating event in AML1-ETO-mediated leukemogenesis. Gain-of-function KIT receptor mutations have been reported in adult AML patients, especially those with core binding factor leukemia (CBFL).**

**We have previously reported a new gain-of-function KIT$^{Asn822Lys}$ mutation that is constitutively expressed in the Kasumi-1 CBFL cell line, and has recently been described in two childhood AML patients. To explore the molecular basis of the effects of this mutation in the appropriate context of hemopoietic dysregulation, we investigated KIT downstream signaling in the Kasumi-1 cell line by means of STI 571 (Imatinib, Gleevec) pharmacological inhibition.**

***Materials and Methods*. We investigated KIT$^{Asn822Lys}$ mutant-initiated signaling in Kasumi-1 cell line, and characterized the inhibitory effect of the STI 571 protein tyrosine kinase inhibitor on downstream signaling.**

***Results*. The use of STI 571-mediated inhibition impaired the tyrosine phosphorylation of KIT$^{Asn822Lys}$ and its association with the p85 subunit of phosphatidylinositol 3′-kinase (p85$^{PI3K}$). The downstream constitutive phosphorylation of JNK1/2 and STAT3 was also significantly inhibited, but STI 571 had no effect on the constitutive activation of Akt, thus suggesting that it is due to other signaling in Kasumi-1 cells. STI 571 inhibited the KIT-mediated proliferation of Kasumi-1 cells in a dose-dependent manner.**

***Conclusions*. These findings show the role of PI3K in KIT$^{Asn822Lys}$-mediated constitutive activation through the Akt-independent downstream signaling pathway of JNK, and also demonstrate the mutant's susceptibility to STI 571, which may therefore have therapeutic potential in CBFL patients with susceptible KIT mutations.** 

## Introduction

Tyrosine kinase receptor (RTK) signaling involves the formation of a symmetric ligand-bound dimer of the receptor extracellular domain, the activation of intrinsic receptor kinase, the self-phosphorylation of substrates, and the association of the activated receptor with intracellular signaling molecules [1]. Ligand binding promotes receptor dimerization and phosphorylation at specific tyrosine residues, which can act as docking sites for downstream signal transduction molecules containing Src homology 2 (SH2) domains. The associating proteins have catalytic activity themselves, or serve as adaptor molecules for other catalyzing proteins, which then generate small second messenger molecules or modify other proteins and thus initiate signaling cascades for the different biological functions of the receptor [2,3].

Tyrosine-phosphorylated KIT can bind a unique array of intracellular signaling molecules, including phosphatidylinositol 3′-kinase (PI3K)/Akt, the members of the Src family, the effectors of the Janus family kinase/signal transducers, and the activators of transcription (JAK-STAT) pathways and the Ras-Raf-MAP (mitogen-activated protein) kinase cascade [4]. Constitutively activating KIT mutations in the second tyrosine kinase domain cause receptor self-association beyond the ligand-induced dimerization site, thus leading to the activation of the downstream effectors required for factor-independent growth and tumorigenicity [5,6].

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A number of studies have suggested some differences in the molecular mechanisms, enzymatic specificity, and transduction signals between kinase mutations and the KL-stimulated wild-type KIT receptor [6–9]. In human KIT, the substitution of aspartic acid 816 by valine or tyrosine leads to constitutive increases in kinase activity and alterations in the receptor's substrate preference. Recent work has provided some insights into the signaling pathways mediating the transformation of hematopoietic cells by $KIT^{Asp816}$ mutants, and it has been found that $Asp^{816}$ mutants induce the constitutive activation of STAT3, but not to an extent that is sufficient by itself to give M-07e cells stem cell factor–independent survival [9]. KIT mutant activation of other signaling components in the PI3K pathway has been demonstrated, and may also be needed for mutant KIT-mediated biological effects [8]. However, Akt is not the only effector of PI3K, whose full range of action may also require other proteins such as Rac1 and JNK [10].

Gain-of-function mutations in the catalytic domain of KIT are found in a substantial fraction of patients with core binding factor acute myelogenous leukemia (CBFL) [11–13] and the majority of patients with mastocytosis and mast cell leukemia [14,15], as well as in germ-cell tumors [16]. We identified a novel Asn822Lys ligand-independent KIT activating mutation [17] in the Kasumi-1 CBFL cell line with t(8;21) [18], which has also been reported in childhood AML [19].

We here describe the functional impact of the Asn822Lys mutation on the signaling intermediates regulated by KIT (MAPK Erk1 and Erk2, STAT3, PI3K/AKT and JNK), and the different inhibitory effects of STI 571 on these KIT mutant-activated pathways.

STI 571 (Imatinib, Gleevec) is a potent inhibitor of KIT juxtamembrane mutants in vitro [20,21], which has been found to have no effect in the KIT mutations in the kinase domain that interfere with its ATP binding. Only the prevalent Asp816 mutation has been studied so far, and so it is worth investigating its effects on other exon 17 mutations that may be differentially sensitive to inhibitors [22].

As increasingly effective targeted therapies have been developed to treat adult cancer patients, it is critically important to devise appropriate in vitro experimental models in order to study their use [23,24]. The availability of the Kasumi-1 cell line provides a cell context operating in vivo in which the distinct consequences of abnormal KIT signaling pathway activation can be studied and, by means of biochemical, signal transduction, and proliferation assays, we found that the activation of the PI3K/JNK pathway is a consequence of the $KIT^{Asn822Lys}$ mutation in Kasumi-1 cells. We also observed that STI 571 has a dose-dependent inhibitory effect on the growth of Kasumi-1 cells and inhibits the ligand-independent activation of the $KIT^{Asn822Lys}$ mutant and its downstream PI3K/JNK and STAT3 pathways, but a negligible effect on the constitutive activation of Akt.

## Materials and methods

*Cell cultures*

The Kasumi-1 human AML cell line (AML M2, AML1-ETO+), was maintained in RPMI 1640 (PBI International, Milan, Italy) with 20% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA).

The proliferation of the M07e human myeloid leukemia cell line depends on the growth factors interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), or stem cell factor (SCF). The M07e cells were cultured in RPMI 1640 (PBI International) supplemented with 10% fetal calf serum, and supplied with 10 ng/mL GM-CSF.

*Phosphorylation analysis of mutant KIT transduction pathways*

The M07e and Kasumi-1 cells ($2 \times 10^7$) were starved overnight at 37°C in serum-free RPMI 1640, and then incubated with or without 100 ng/mL of hu-SCF (KIT ligand) (Cell Signaling Technology, Inc., Beverly, MA, USA) for 6 or 12 minutes at 37°C. Subsequently, the cell pellets were lysed with 1 mL of protein lysis buffer (50 mM/L Tris, 150 mM/L sodium chloride, 1% NP-40, 0.25% deoxycholate with the addition of the inhibitors aprotinin, leupeptin, pepstatin (Roche, Mannheim, Germany), sodium orthovanadate, and phenylmethyl sulfonyl fluoride).

The lysates were precleared by incubation with 50 μL of protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) for 10 minutes at 4°C, centrifuged at 12000*g* for 20 minutes, and subsequently immunoprecipitated with mouse anti-KIT, rabbit anti-JNK, mouse anti-STAT3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit anti-PI3K antibody (Upstate Biotechnology, Dundee, Scotland, UK) at a dilution of 1:50, and with 20 μL of protein A-Sepharose.

The precipitated samples were resuspended in reduced loading buffer, boiled for 3 minutes, and separated by SDS-PAGE. The gels were electroblotted to polyvinylidene difluoride (PVDF) membranes (Roche), which were incubated with rabbit anti-phospho-KIT, rabbit anti-phospho-PI3K (Cell Signaling Technology, Inc.), mouse anti-phospho-JNK, and mouse anti-phosphotyrosine antibodies (Santa Cruz Biotechnology, Inc.). They were incubated in stripping buffer (1.5 mM Tris-HCl, 2% SDS, pH 6.8, and 50 mM mercaptoethanol) for 15 minutes at 70°C, and then reprobed with anti-KIT, anti-JNK, anti-STAT3, and anti-PI3K. The signals were developed using the Super Signaling West Pico chemiluninescent substrate (Pierce Biotechnology, Erembodegem, Belgium).

For the Western blot analysis of whole-cell lysates, aliquots of protein were electrophoresed, transferred to PVDF membranes, and incubated with anti-MAPK and anti-phospho-MAPK (Cell Signaling Technology, Inc.), or with anti-Akt and anti-phospho-Akt (Cell Signaling Technology, Inc.). The signals were developed using the Super Signaling West Pico chemiluminescent substrate (Pierce). The data are expressed as mean values (± SD) of three independent experiments.

*STI 571 assay*

The M07e and Kasumi-1 cells ($2 \times 10^7$) were starved overnight at 37°C in serum-free RPMI 1640, and then incubated for 90 minutes in the presence of various concentrations of STI 571 (0.1, 1, 10, and 20 μM/L). The M07e cells were incubated with or without 100 ng/mL of hu-SCF (KIT ligand) at 37°C. Subsequently, the cell pellets were lysed with 1 mL of protein lysis buffer. KIT, PI3K, STAT3, JNK, and Akt were analyzed by means of Western

immunoblotting as described above. The data are expressed as mean values (± SD) of three independent experiments.

*In vitro proliferation assay*

The Kasumi-1 cells treated with STI 571 underwent 5-bromo-2′-deoxy-uridine (BrdU) immunofluorescence assay.

Briefly, the cells were plated in a 24-well plate at a concentration of $1 \times 10^6$ cells/well, cultured in RPMI 1640 supplemented with 20% FBS in the presence of STI 571 at concentrations of 0.1, 1, 10, or 20 μM for 24 and 48 hours, and then further cultured in the presence of BrdU labeling medium (10 μM) in a volume of 0.5 mL. Immunofluorescence was measured using a cytospin preparation in accordance with the manufacturer's instructions (Roche). The data were expressed as the mean values (± SD) of five wells.

## Results

*Signaling pathways activated in Kasumi-1 cells constitutively expressing the KIT$^{Asn822Lys}$ mutant*

As reported previously, the growth factor–independent Kasumi-1 cell line [18] expresses the KIT$^{Asn822Lys}$ mutant constitutively phosphorylated on tyrosine residues [17].

The Ras-Raf-MAP kinase pathway, which is crucial to neoplastic transformation, was the first shown to be activated by wild-type KIT [25,26]. In order to evaluate whether p44 and p42 MAP kinases (Erk1 and Erk2) are constitutively activated by KIT$^{Asn822Lys}$, we compared their phosphorylation in Kasumi-1 cells with that observed following the huSCF-induced activation of M-07e cells. HuSCF induced the tyrosine phosphorylation of Erk1 and Erk2 in both the M-07e cells expressing wild-type KIT and the Kasumi-1 cells expressing KIT$^{Asn822Lys}$ (Fig. 1). The Kasumi-1 did not show any constitutive Erk phosphorylation, thus suggesting that the catalytic domain mutant does not affect the activity of the MAPK pathway.

An immediate consequence of KIT self-phosphorylation is the recruitment of PI3K to the receptor complex [8]. The association of KIT with p85$^{PI3K}$ (and therefore PI3-kinase) activity is necessary for a full mitogenic and adhesive response [10,26,27]. In order to evaluate whether p85$^{PI3K}$ was constitutively associated with KIT$^{Asn822Lys}$, the KIT immunoprecipitates were analyzed by SDS-PAGE and blotted with anti-p85$^{PI3K}$ antibody, and it was found that those from KIT$^{Asn822Lys}$-expressing Kasumi-1 cells contained p85$^{PI3K}$ (Fig. 2B).

We then addressed the question as to whether the constitutive activation of PI3K by KIT$^{Asn822Lys}$ led to the constitutive activation of Akt as a result of the increase in serine phosphorylation. Kasumi-1 cell lysates were immunoblotted with antibodies specific for phosphorylated or total Akt, and it was found that Akt results constitutively phosphorylated in Kasumi-1 cells expressing the KIT$^{Asn822Lys}$ mutation (Fig. 3).

PI3K activates multiple signaling pathways and, when dysregulated, many of them have biological properties that are consistent with oncogenesis [28]. We investigated the JNK family of signaling components activated by SCF downstream of the PI3K pathway [10] in order to determine whether they are constitutively activated in Kasumi-1 cells. As shown in Figure 2C, whole-cell lysates immunoblotted with antibodies specific for either phosphorylated or total JNK (both specific for JNK1 and 2) showed constitutive JNK phosphorylation.

Previous studies have shown that STAT1, 3, and 5 are activated in primary AML blasts and hematopoietic cell lines upon the stimulation of KIT receptor by SCF [29–32]. In order to determine whether the activation of STAT plays a role in mutant KIT$^{Asn822Lys}$-mediated effects, as has been observed in the case of STAT3 in the KIT$^{Asp816}$ mutant [9], we examined the status of STAT3 activation. Western blot analysis demonstrated the constitutive tyrosine phosphorylation of STAT3 in the Kasumi-1 cell line (Fig. 4).

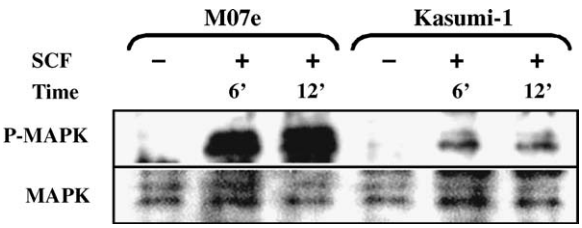


**Figure 1.** Activation status of p44 and p42 MAP kinase (Erk1 and Erk2) in Kasumi-1 cells. The M-07e and Kasumi-1 cells were treated with huSCF or vehicle. Whole-cell lysate was prepared and immunoprecipitated with the rabbit polyclonal antibody anti-p44/42, and then immunoblotted with a rabbit polyclonal antibody specific for the phosphorylated form of p44/42 Map kinase (Thr202/Tyr204). The membrane was stripped and reprobed with an antibody for total p44/42 Map kinase. Representative results from one of three independent experiments.

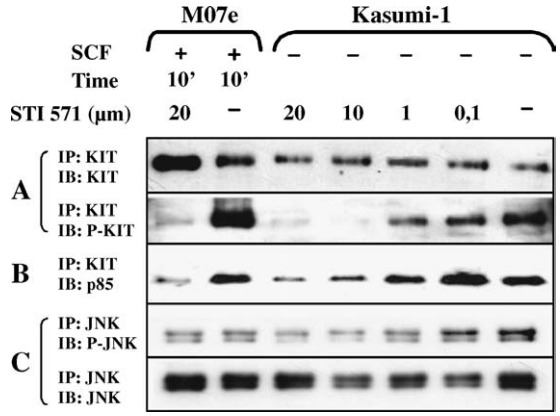


**Figure 2.** Inhibitory effect of STI 571 on the KIT$^{Asn822Lys}$-mediated activation of p85$^{PI3K}$ and JNK. After pretreatment with STI 571 or vehicle, the M-07e cells were treated with huSCF. **(A)** Whole cell lysate was immunoprecipitated with mouse monoclonal antibody (clone Ab81), and then immunoblotted with anti-phosphoKIT (P-KIT-Tyr719) polyclonal antibody. The membrane was stripped and reprobed with **(A)** anti-KIT polyclonal antibody, and **(B)** anti-p85. **(C)** Whole-cell lysate was prepared and immunoblotted with a polyclonal antibody specific for the doubly phosphorylated forms of JNK1 and 2. The membrane was stripped and reprobed with an antibody for total JNK1 and 2. Representative results from one of three independent experiments.

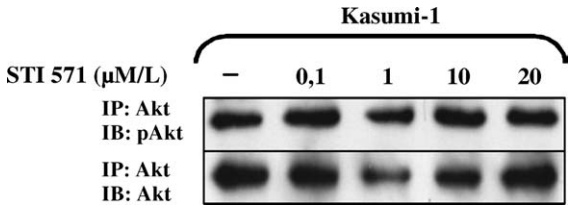


**Figure 3.** Spontaneous phosphorylation of Akt is resistant to STI 571. The Kasumi-1 cells were pretreated with various concentrations of STI 571 for 90 minutes. Whole-cell lysate was prepared and immunoprecipitated with mouse monoclonal antibody anti-Akt (clone B-1), and then immunoblotted with a rabbit polyclonal antibody specific for the phosphorylated form of Akt (Ser473). The membrane was stripped and reprobed with an antibody for total Akt. Representative results from one of three independent experiments.

*Effect of STI 571 (Gleevec) on KIT$^{Asn822Lys}$-activated downstream signaling pathways*

Western blot analysis was used to determine the signaling effect of STI 571 on the activation of KIT and its downstream signaling pathways by measuring self-phosphorylation in cells preincubated with the indicated concentrations of STI 571. In the control M07e cells, the tyrosine residues of wild-type KIT were strongly phosphorylated only after stimulation by huSCF.

In order to determine the effect of STI 571 on the self-phosphorylation of KIT$^{Asn822Lys}$, we performed immunoprecipitation experiments using anti-KIT antibody and Western blots for either KIT or phosphotyrosine (Fig. 2A). The quantitation of phosphotyrosine levels revealed that, at an STI 571 dose of 0.1 μM/L, KIT phosphorylation in Kasumi-1 cells was approximately 60% that observed in untreated cells.

Unlike in the M-07e cell line, in which the association of p85$^{PI3K}$ is a result of the SCF-dependent activation of the KIT receptor, Kasumi-1 cells bearing the KIT$^{Asn822Lys}$ mutation show a constitutive association (Fig. 2B). In order to assess the effect of STI 571 on this pathway, we probed KIT immunoprecipitates from M-07e cells treated with huSCF (with and without STI 571) and from Kasumi-1 cells with an antibody specific for p85$^{PI3K}$. At doses ranging from 0.1 to 20 μM/L, STI 571 markedly inhibited the p85$^{PI3K}$-mediated association of PI3K with KIT$^{Asn822Lys}$ in the Kasumi-1 cells and the huSCF-mediated association in the M-07e cells (Fig. 2B).

We then determined whether STI 571 (which inhibits the p85$^{PI3K}$-mediated association of PI3K with KIT$^{Asn822Lys}$) reduces the constitutive activation of JNKs, and found partial dose-dependent inhibition (Fig. 2C) of the KIT$^{Asn822Lys}$-dependent activation of JNKs in Kasumi-1 cells, but no inhibition of the phosphorylation of JNK in huSCF-stimulated M-07e cells (Fig. 2C).

In order to test whether STI 571 could also inhibit mutant KIT$^{Asn822Lys}$ receptor activation of the signal transduction pathways leading to Akt, we undertook similar studies using an antibody specific for Akt and one recognizing a serine phosphorylated form. At the concentrations indicated in Figure 3, STI 571 had no effect on Akt activation.

The partial inhibitory effects of the same concentrations of STI 571 on the phosphorylation of STAT3 were similar to those on JNK (Fig. 4).

Figure 5 shows the overall quantitation of the dose-dependent inhibitory effect of STI 571 on the activation status of KIT and downstream effectors. As can be seen, the constitutive activation of PI3K, JNK, and STAT3 in Kasumi-1 cells was partially inhibited by STI 571 as a result of its efficient inhibition of the KIT$^{Asn822Lys}$ mutant receptor. However, the Kasumi-1 cells contained high levels of phosphorylated Akt, which was not inhibited by STI 571, thus suggesting

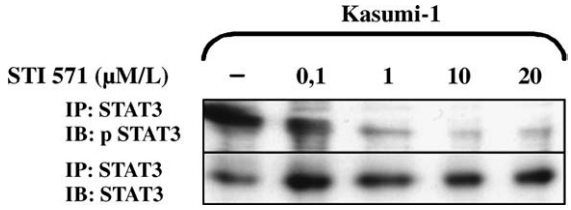


**Figure 4.** Inhibitory effect of STI 571 on phosphorylation of STAT3. The Kasumi-1 cells were pretreated with various concentrations of STI 571 for 90 minutes. Whole-cell lysate was prepared and immunoprecipitated with rabbit polyclonal antibody anti-STAT3 (clone C-20), and then immunoblotted with a mouse monoclonal antibody specific for the phosphorylated form of STAT3 (Tyr705). The membrane was stripped and reprobed with an antibody for total STAT3. Representative results from one of three independent experiments.

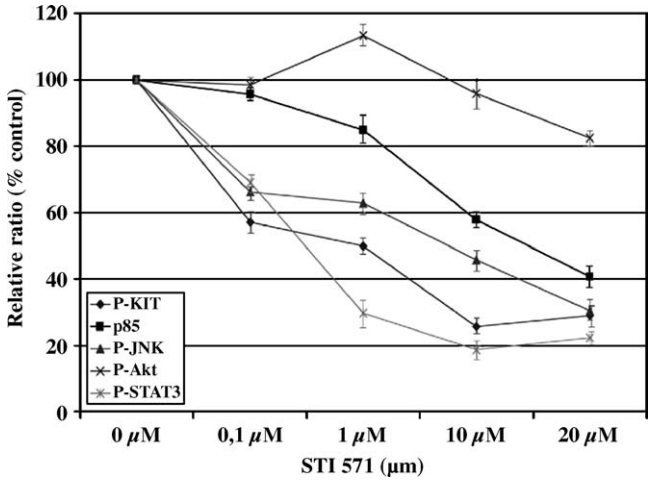


**Figure 5.** Effect of STI 571 on the phosphorylation of KIT, JNK, Akt, and STAT3, and on the association of p85$^{PI3K}$ with KIT. The results are expressed as the percentage of maximum phosphorylation and represent the ratio of phosphorylated:total protein. The p85$^{PI3K}$ results are expressed as the percentage of maximum protein associated with the KIT receptor. STI 571 inhibited the phosphorylation of KIT, JNK, and STAT3, and p85$^{PI3K}$ recruitment to the KIT receptor at a concentration of 10 μM; it had no dose-dependent inhibitory effect on the phosphorylation of Akt. The phosphorylation was quantified using a phosphoimage analyzer, with the activity in nontreated cells being assumed to be 100%. The data represent the mean values and standard deviations of three independent experiments.

that Akt phosphorylation in the Kasumi-1 cell line is independent of KIT$^{Asn822Lys}$ activation.

*STI 571 (Gleevec) inhibits the growth of Kasumi-1 cells*

Proliferation assays were used to evaluate the effect of STI 571 on the SCF-independent KIT$^{Asn822Lys}$-mediated proliferation of Kasumi-1 cells.

In order to test the biological effect of inhibiting the kinase activity of the KIT$^{Asn822Lys}$ mutant receptor, we cultured Kasumi-1 cells for 48 hours in the presence of various concentrations of STI 571. At concentrations of 0.1 to 20 μM/L, proliferation was 80 to 95% less than that observed in the cells propagated in an STI 571-free medium. Complete inhibition was obtained at a concentration of 10 μM/L (Fig. 6).

These results showed that STI 571 had a dose-dependent inhibitory effect on the autonomous growth of Kasumi-1 cells in the same dose-response range as that observed for the inhibition of KIT receptor self-phosphorylation, PI3K activation and recruitment to the receptor, and STAT3 and JNK phosphorylation.

## Discussion

The role of KIT mutations has been evaluated because of their involvement in myeloid leukemogenesis, and the need to determine their significance has been made more pressing as a result of the development of targeted therapies aimed at counteracting their effects. Activating KIT mutations have been found in childhood AML and nearly half of the adult CBFL cases, including various kinds of gain-of-function mutations [12,13]. Patients with KIT activating mutations have a higher diagnostic white blood cells (WBC) count than patients without [33], and this adversely affects their relapse rate [12].

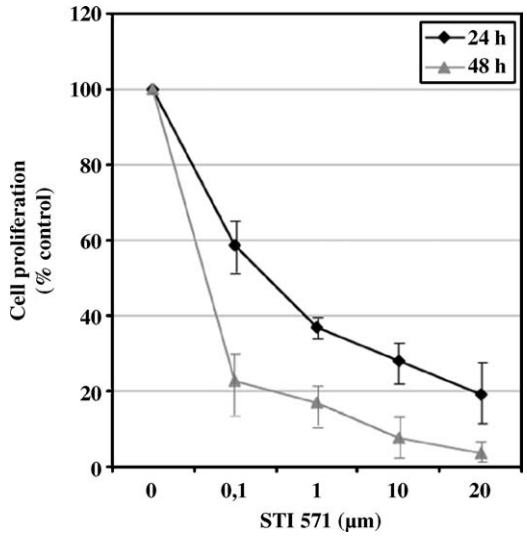


**Figure 6.** STI 571 inhibits the proliferation of Kasumi-1 cells. After pretreatment with various concentrations of STI 571, the proliferation of Kasumi-1 cells was measured using a 5-bromo-2′-deoxy-uridine (BrdU) immunofluorescence assay. Three independent assays were performed with comparable results after 24 and 48 hours. Each point represents the mean values and standard deviations of three independent experiments.

The kinase domain mutant at codon 816 is resistant to STI 571 (Gleevec), but the fact that juxtamembrane mutants are more sensitive [21] suggests that the effective dose for treating malignancies in which KIT is the main target depends on the presence and type of inherent mutations. We therefore examined the effects of STI 571 on KIT$^{Asn822Lys}$ phosphorylation and downstream signaling.

Receptor phosphorylation in the presence of STI 571 was analyzed in the Kasumi-1 cell line that constitutively expresses the KIT$^{Asn822Lys}$ mutant by means of immunoprecipitation and Western blotting. The results were in keeping with previous reports showing that sensitivity to Imatinib depends on the type of mutation [21,34] insofar as the phosphorylation of KIT$^{Asn822Lys}$ was not completely inhibited by an STI 571 concentration of 1 μM/L.

However, the self-phosphorylation of KIT$^{Asn822Lys}$ was inhibited by concentrations of greater than 1 μM/L, which supports the hypothesis that kinase mutants are less sensitive to STI 571 (a higher $IC_{50}$) than juxtamembrane mutants. As the KIT$^{Asn822Lys}$ allele is about five times amplified compared to the normal allele [17], this may at least partially explain the STI 571 sensitivity of the mutant in Kasumi-1 cells.

It has been previously demonstrated that there are differences in signaling between kinase domain mutants and wild-type KIT [8,35], and we found that, although p44/42 MAP kinase (Erk1/2) phosphorylation was strongly activated by wild-type KIT stimulated with SCF, it was not constitutively activated by KIT$^{Asn822Lys}$. It has been shown that the signal transduction pathway involving the PI3K or STAT pathway is constitutively activated as a consequence of an intrinsic activating mutation at KIT receptor level [9,35], and that the signal generated by the activated KIT receptor is transduced via both pathways, and thus constitutively activates both systems. Although STAT3 is not activated by wild-type KIT, it is constitutively activated by the KIT$^{Asn822Lys}$ mutant, as has been observed in the case of Asp816 mutants [9]. The activation of PI3K is essential for KIT-mediated survival and proliferation, and it has been demonstrated that KIT$^{Asp816Val}$ associates with activated PI3K [8,36]. In Kasumi-1 cells, activated PI3K is constitutively associated with the mutant receptor, and our evidence that the PI3K signaling pathway converges to activate JNK suggests that these molecules play an important role in the KIT$^{Asn822Lys}$-mediated mitogenic response.

Significant JNK activation has also been observed in the case of a number of growth factors, including EGF, NGF, IL-3, and G-CSF [37–39]. The JNK activation mediated by growth factors is blocked by PI3K inhibitors, thus suggesting that JNK is in a downstream effector pathway of PI3K [40]. We found that Akt, which acts downstream of PI3K,

is constitutively activated in Kasumi-1 cells expressing KIT$^{Asn822Lys}$ but, although STI 571 partially inhibited the activation of PI3K, JNK, and STAT3 in these cells, it did not modify Akt activation, thus suggesting that signaling other than by KIT$^{Asn822Lys}$ may be responsible for the constitutive activation of Akt in Kasumi-1 cells. Recent studies have shown that AML cells produce and secrete VEGF, and coexpress one or both VEGF receptors [41]. An autocrine loop between VEGF and VEGFR1 has been described in Kasumi-1 cells and might be responsible for constitutive Akt activation [32]. In line with this view, recent observations indicate that VEGFR-1 activation stimulates the rapid and sustained phosphorylation of Akt, and generates a clonogenic response in leukemia cells that is mediated by PI3K-dependent Akt activation [42].

Activating PDGFRα mutations have been rarely reported in CBFL [43], which may explain why we did not detect any constitutive PDGFRα and β phosphorylation in our Kasumi-1 cells (data not shown).

Furthermore, a BrdU assay showed that the in vitro ligand-independent proliferation of KIT$^{Asn822Lys}$-expressing Kasumi-1 cells was markedly inhibited by an STI 571 concentration of 1 μM/L, a finding that has also been reported by Spiekermann et al. [44]. These results also suggest that the activating mechanism of the KIT$^{Asn822Lys}$ mutant is different from that of KIT$^{Asp816}$ mutants, whose proliferation rate is unaffected by STI 571 even at a concentration of 10 μM/L [34].

The inhibition of KIT$^{Asn822Lys}$ induced by STI 571 indicates its potential use in the treatment of CBFL patients carrying KIT mutations other than KIT$^{Asp816}$. Early reports showed that extra-membrane (exon 8 insertion/deletion) and juxtamembrane KIT mutations are relatively common in CBFL, thus suggesting that these more STI 571-sensitive mutations may be useful targets in clinical trials.

In conclusion, our results indicate that the kinase mutant KIT$^{Asn822Lys}$ and, consequently, its downstream effector pathways are inhibited by STI 571 in the constitutive KIT mutant-expressing Kasumi-1 cell line. Targeting the downstream signaling elements (such as PI3K/JNK) that integrate signals from various kinds of cell-surface receptors may circumvent certain resistance mechanisms that may otherwise limit the efficacy of KIT receptor blockade. Although laboratory research involves many molecular signals in the AML neoplastic process, the Kasumi-1 cell line is a rare example of a model system which, by recapitulating the correct cell context, can be used to screen for small molecules that specifically inhibit the aberrant activation of KIT receptor in leukemia cells.

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## References

1. Blume-Jensen P, Hunter T. Oncogenic kinase signaling. Nature. 2001;411:355–365.
2. Hubbard SR, Mohammadi M, Schlessinger J. Autoregulatory mechanisms in protein-tyrosine kinases. J Biol Chem. 1998;273:11987–11990.
3. Ashman LK. The biology of stem cell factor and its receptor c-KIT. Int J Biochem Cell Biol. 1999;31:1037–1051.
4. Linnekin D. Early signaling pathways activated by c-KIT in hematopoietic cells. Int J Biochem Cell Biol. 1999;31:1053–1074.
5. Tsujimura T, Hashimoto K, Kitayama H, et al. Activating mutation in the catalytic domain of c-KIT elicits hematopoietic transformation by receptor self-association not at the ligand-induced dimerization site. Blood. 1999;93:1319–1329.
6. Hashimoto K, Matsumura I, Tsujimura T, et al. Necessity of tyrosine 719 and phosphatidylinositol 3′-kinase–mediated signal pathway in constitutive activation and oncogenic potential of c-KIT receptor tyrosine kinase with the Asp814Val mutation. Blood. 2003;101:1094–1102.
7. Moriyama Y, Tsujimura T, Hashimoto K, et al. Role of aspartic acid 814 in the function and expression of c-KIT receptor tyrosine kinase. J Biol Chem. 1996;271:3347–3350.
8. Chian R, Young S, Danilkovitch-Miagkova A, et al. Phosphatidylinositol 3 kinase contributes to the transformation of hematopoietic cells by the D816V c-KIT mutant. Blood. 2001;98:1365–1373.
9. Ning ZQ, Li J, Arceci RJ. Signal transducer and activator of transcription 3 activation is required for Asp(816) mutant c-KIT-mediated cytokine-independent survival and proliferation in human leukemia cells. Blood. 2001;97:3559–3567.
10. Timokhina I, Kissel H, Stella G, Besmer P. KIT signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. EMBO J. 1998;17:6250–6262.
11. Beghini A, Peterlongo P, Ripamonti CB, et al. C-KIT mutations in core binding factor leukemias. Blood. 2000;95:726–727.
12. Care RS, Valk PJM, Goodeve AC, et al. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukemias. Br J Haematol. 2003;121:775–777.
13. Beghini A, Ripamonti CB, Cairoli R, et al. KIT activating mutations: incidence in adult and pediatric AML, and identification of an internal tandem duplication. Haematologica. 2004;89:920–925.
14. Nagata H, Worobec AS, Oh CK, et al. Identification of a point mutation in the catalytic domain of the protooncogene c-KIT in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder. Proc Natl Acad Sci U S A. 1995;92:10560–10564.
15. Longley BJ, Tyrrell L, Lu SZ, et al. Somatic c-KIT activating mutation in urticaria pigmentosa and aggressive mastocytosis: establishment of clonality in a human mast cell neoplasm. Nat Genet. 1996;12:312–314.
16. Tian Q, Frierson HF Jr, Krystal GW, Moskaluk CA. Activating c-KIT gene mutations in human germ cell tumors. Am J Pathol. 1999;154:1643–1647.
17. Beghini A, Magnani I, Ripamonti CB, Larizza L. Amplification of a novel c-KIT activating mutation Asn(822)-Lys in the Kasumi-1 cell line: a t(8;21)-KIT mutant model for acute myeloid leukemia. Hematol J. 2002;3:157–163.
18. Asou H, Tashiro S, Hamamoto K, Otsuji A, Kita K, Kamada N. Establishment of a human acute myeloid leukemia cell line (Kasumi-1) with 8;21 chromosome translocation. Blood. 1991;77:2031–2036.
19. Zwaan CM, Miller M, Goemans BF, et al. Frequency and clinical significance of c-KIT exon 17 mutations in childhood acute myeloid leukemia [abstract]. Blood. 2002;100(11):746a. Abstract 2952.

20. Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler AJ. Inhibition of c-KIT receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. Blood. 2000;96:925–932.
21. Frost MJ, Ferrao PT, Hughes TP, Ashman LK. Juxtamembrane mutant V560GKIT is more sensitive to Imatinib (STI571) compared with wild-type c-KIT whereas the kinase domain mutant D816VKIT is resistant. Mol Cancer Ther. 2002;12:1115–1124.
22. Ma Y, Zeng S, Metcalfe DD, et al. The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors; kinases with enzymatic site mutation show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory-type mutations. Blood. 2002;99:1741–1744.
23. Debiec-Rychter M, Cools J, Dumez H, et al. Mechanisms of resistance to imatinib mesylate in gastrointestinal stromal tumors and activity of the PKC412 inhibitor against imatinib-resistant mutants. Gastroenterology. 2005;128:270–279.
24. Rahmani M, Reese E, Dai Y, et al. Co-treatment with SAHA and 17-AAG synergistically induces apoptosis in Bcr-Abl$^{+}$ cells sensitive and resistant to STI-571 in association with down-regulation of Bcr-Abl, abrogation of STAT5 activity, and Bax conformational change. Mol Pharmacol. 2005;67:1166–1176.
25. Okuda K, Sanghera JS, Pelech SL, et al. Granulocyte-macrophage colony-stimulating factor, interleukin-3, and steel factor induce rapid tyrosine phosphorylation of p42 and p44 MAP kinase. Blood. 1992;79: 2880–2887.
26. Serve H, Yee NS, Stella G, Sepp-Lorenzino L, Tan JC, Besmer P. Differential roles of PI3-kinase and KIT tyrosine 821 in KIT receptor-mediated proliferation, survival and cell adhesion in mast cells. EMBO J. 1995;14:473–483.
27. Shivakrupa R, Bernstein A, Watring N, Linnekin D. Phosphatidylinositol 3′-kinase is required for growth of mast cells expressing the KIT catalytic domain mutant. Cancer Res. 2003;63:4412–4419.
28. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer. 2002;2:489–501.
29. Gotoh A, Takahira H, Mantel C, Litz-Jackson S, Boswell HS, Broxmeyer HE. Steel factor induces serine phosphorylation of Stat3 in human growth factor–dependent myeloid cell lines. Blood. 1996;88: 138–145.
30. Deberry C, Mou S, Linnekin D. Stat1 associates with c-KIT and is activated in response to stem cell factor. Biochem J. 1997;327:73–80.
31. Ryan JJ, Huang H, McReynolds LJ, et al. Stem cell factor activates STAT-5 DNA binding in IL-3-derived bone marrow mast cells. Exp Hematol. 1997;25:357–362.
32. Spiekermann K, Pau M, Schwab R, Schmieja K, Franzrahe S, Hiddemann W. Constitutive activation of STAT3 and STAT5 is induced by leukemic fusion proteins with protein tyrosine kinase activity and is sufficient for transformation of hematopoietic precursor cells. Exp Hematol. 2002;30:262–271.
33. Cairoli R, Grillo G, Beghini A, et al. c-KIT point mutations in core binding factor leukemias: correlation with white blood cell count and the white blood cell index. Leukemia. 2003;17:471–472.
34. Chen H, Isozaki K, Kinoshita K, et al. Imatinib inhibits various types of activating mutant KIT found in gastrointestinal stromal tumors. Int J Cancer. 2003;105:130–135.
35. Piao X, Paulson R, van der Geer P, Pawson T, Bernstein A. Oncogenic mutation in the KIT receptor tyrosine kinase alters substrate specificity and induces degradation of the protein tyrosine phosphatase SHP-1. Proc Natl Acad Sci U S A. 1996;93:14665–14669.
36. Serve H, Yee NS, Stella G, Sepp-Lorenzino L, Tan JC, Besmer P. Differential roles of PI3-kinase and KIT tyrosine 821 in KIT receptor-mediated proliferation, survival and cell adhesion in mast cells. EMBO J. 1995;14:473–483.
37. Minden A, Lin A, Claret FX, Abo A, Karin M. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell. 1995;81:1147–1157.
38. Nagata Y, Nishida E, Todokoro K. Activation of JNK signaling pathway by erythropoietin, thrombopoietin and interleukin 3. Blood. 1997;89: 2664–2669.
39. Rausch O, Marshall CJ. Tyrosine 763 of the murine granulocyte colony-stimulating factor receptor mediates Ras-dependent activation of the JNK/SAPK mitogen-activated protein kinase pathway. Mol Cell Biol. 1997;17:1170–1179.
40. Logan SK, Falasca M, Hu P, Schlessinger J. Phosphatidylinositol 3-kinase mediates epidermal growth factor–induced activation of the c-Jun N-terminal kinase signaling pathway. Mol Cell Biol. 1997;17: 5784–5790.
41. Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor (VEGF) and its receptors in hematopoietic malignancies. Cancer Res. 1999;59:728–733.
42. List AF, Glinsmann-Gibson B, Stadheim C, Meuillet EJ, Bellamy W, Powis G. Vascular endothelial growth factor receptor-1 and receptor-2 initiate a phosphatidylinositide 3-kinase–dependent clonogenic response in acute myeloid leukemia cells. Exp Hematol. 2004;32:526–535.
43. Johan MF, Goodeve AC, Reilly JT. Activating loop mutations in the PDGFRa and b genes are rare in core binding factor acute myeloid leukaemia. Br J Haematol. 2004;127:123–124.
44. Spiekermann K, Faber F, Voswinckel R, Hiddemann W. The protein tyrosine kinase inhibitor SU5614 inhibits VEGF-induced endothelial cell sprouting and induces growth arrest and apoptosis by inhibition of c-kit in AML cells. Exp Hematol. 2002;30:767–773.