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# Constitutive Activation of c-*kit* by the Juxtamembrane but Not the Catalytic Domain Mutations Is Inhibited Selectively by Tyrosine Kinase Inhibitors STI571 and AG1296

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**Abstract**

The c-*kit* receptor tyrosine kinase (KIT) is constitutively activated by 2 types of naturally occurring mutations, the Val559→Gly (G559) mutation in the juxtamembrane domain and the Asp814→Val (V814) mutation in the catalytic domain. We evaluated the effects of the tyrosine kinase inhibitors STI571 and AG1296 on BaF3 cells expressing wild-type KIT ($KIT^{WT}$) or activating mutants of KIT ($KIT^{G559}$ and $KIT^{V814}$) in the presence or absence of the KIT ligand, stem cell factor (SCF). Both STI571 and AG1296 inhibited SCF-dependent activation of $KIT^{WT}$ and SCF-independent activation of $KIT^{G559}$ more efficiently, whereas SCF-independent activation of $KIT^{V814}$ was scarcely affected. Furthermore, both inhibitors inhibited SCF-dependent growth of BaF3-$KIT^{WT}$ cells and, with higher potencies, SCF-independent growth of BaF3-$KIT^{G559}$ cells through the induction of apoptosis. In contrast, the inhibitors had little or no effect on SCF-independent growth of BaF3-$KIT^{V814}$ cells or on IL-3–dependent growth of BaF3-Mock cells. These results suggested that both inhibitors may be effective therapeutic agents for oncogenic KIT with the juxtamembrane domain mutation, but not with the catalytic domain mutation, and that the activation mechanism of the catalytic domain mutant KIT is complex and entirely different from that of the wild-type KIT or the juxtamembrane domain mutant KIT. *Int J Hematol.* 2002;76:427-435.


*Key words:* KIT; Activating mutation; Juxtamembrane domain; Catalytic domain; Tyrosine kinase inhibitor

## 1. Introduction

c-kit is a receptor tyrosine kinase (RTK) that, with the receptors for platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF-1), and flt-3 ligand [1,2], belongs to a type III RTK subfamily. The type III RTKs are characterized by an extracellular region with 5 immunoglobulin-like domains and a cytoplasmic region consisting of a kinase domain interrupted by a kinase insert. c-*kit* receptor (KIT) and its ligand, stem cell factor (SCF), play a crucial role in the proliferation, differentiation, migration, and survival of hematopoietic stem cells, mast cells, melanocytes, primordial germ cells, and interstitial cells of Cajal (ICCs) [3-7]. In addition, the c-*kit* gene product has been associated with various forms of neoplasms. Activating mutations of c-*kit* have been identified as the cause of transformation of hematopoietic stem cells, mast cells, and gastrointestinal stromal cells [8-10]. We have found that human mast cell

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leukemia cell line HMC-1 carried 2 types of constitutively activating mutations of KIT: the $Val^{560}$-to-Gly mutation ($Val^{559}$ in mouse) in the juxtamembrane domain and the $Asp^{816}$-to-Val mutation ($Asp^{814}$ in mouse) in the catalytic domain [11]. Most of the constitutive active mutations of KIT cluster on these 2 domains. The mutations in the corresponding Asp residue of the catalytic domain were also detected in tumor cells of rodents and, importantly, in peripheral blood mononuclear cells from patients with myelodysplastic disorders accompanying mastocytosis, in mast cells from patients with urticaria pigmentosa and mastocytosis, and in leukemia cells from patients with acute myelogenous leukemia [11-20]. These data indicated that the mutation of the Asp residue in the catalytic domain of KIT plays an important role in a part of hematopoietic malignancy, especially in mast cell neoplasms. In contrast to the low frequency of mutations in the juxtamembrane domain of KIT in hematopoietic malignancies [11,21], the activating mutations in the juxtamembrane domain have frequently been detected in gastrointestinal stromal tumors (GISTs) [22-25]. Confirming the pathogenetic role of the juxtamembrane domain mutation in GISTs, we and others have also found germ line mutations of the juxtamembrane domain in familial GIST patients [26-28].

The tyrosine kinase inhibitor STI571, a 2-phenylaminopyrimidine, was developed as an adenosine triphosphate (ATP) competitive inhibitor of the Abl protein kinase and was found to inhibit BCR-ABL kinase activity and the growth of chronic myelogenous leukemia (CML) cells [29-31]. The use of STI571 has achieved excellent success in the treatment of CML patients and has realized a novel therapy targeted for oncogenic signal transduction. Because STI571 inhibits not only the kinase activity of Abl but also that of PDGF receptor and KIT [32], we evaluate in this article the possibility of targeted inhibition of KIT mutants by STI571. In addition to STI571, we examined the effects of another tyrosine kinase inhibitor, AG1296, which belongs to the class of tyrphostins and blocks the kinase activities of the PDGF receptor and KIT [33]. We have found that both tyrosine kinase inhibitors are more effective against the juxtamembrane domain mutant than against the wild type and that, in sharp contrast, the catalytic domain mutant completely resisted these inhibitors. These results suggest that STI571 and AG1296 may work as a specific targeting therapy for the juxtamembrane domain mutant of KIT and that the activation mechanisms are completely different for the juxtamembrane domain mutant and the catalytic domain mutant.

## 2. Materials and Methods

### *2.1. Reagents and Antibodies*

Highly purified recombinant murine (rm) SCF and rmIL-3 were kindly provided by Kirin Brewery Co Ltd (Tokyo, Japan). STI571 was generously provided by Novartis Pharma (Basel, Switzerland). AG1296 and the anti-Abl antibody were purchased from Calbiochem (San Diego, CA, USA). The anti-KIT antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the antiphosphotyrosine antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). G418 sulfate (Geneticin) was purchased from Gibco BRL (Grand Island, NY, USA).

### *2.2. Construction of Expression Plasmids*

The mammalian expression vector pEF-BOS was donated by Dr. S. Nagata (Osaka University, Osaka, Japan) [34]. The gene encoding murine c-*kit* wild-type cDNA was cloned into an *Eco*RV site of Bluescript I KS (–) (Stratagene, La Jolla, CA, USA). The Blunted *Hind*III-*Eco*RI fragment of Bluescript I KS (–) containing c-*kit* wild-type cDNA was introduced into the blunted *Xba*I site of pEF-BOS. The mutated murine c-*kit* genes encoding $KIT^{G559}$ and $KIT^{V814}$ were constructed by site-directed mutagenesis and cloned into the pEF-BOS vector as described previously [11]. The $KIT^{V814}$ gene was also cloned into the *Kpn*I and *Xho*I sites of pMAM/BSD, the blasticidin S–resistant expression vector (Kaken Pharmaceutical, Tokyo, Japan). The expression vector of p210 form BCR-ABL was kindly gifted from Dr. R.A. Van Etten, Harvard Medical School.

### *2.3. Cells and Transfection*

The IL-3–dependent murine pro–B-cell line, BaF3, was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1 ng/mL rmIL-3. We transfected the pEF-BOS vector carrying various types of c-*kit* cDNA and pSV2neo carrying the neomycin resistance gene into BaF3 cells by electroporation. Following gene transfer, cells were cultured in IL-3–containing medium for 24 hours and then selected in G418 at a concentration of 1 mg/mL. Multiple clones were expanded for further analysis. As for the transfection of pMAM/BSD containing the $KIT^{V814}$ mutant, cells were selected with 30 μg/mL blasticidin S (Invitrogen, Carlsbad, CA, USA) after electroporation. To detect KIT proteins on the cell surface, cells were treated for 30 minutes at 4°C with biotin-conjugated rat anti-murine KIT antibody (Immunotech, Marseilles, France). After washing, cells were incubated for 30 minutes at 4°C with a 1:40 dilution of avidin–fluorescein isothiocyanate (avidin-FITC) (Becton Dickinson, San Jose, CA, USA). Samples were analyzed with a FACSCalibur (Becton Dickinson).

### *2.4. Cell Proliferation Assay*

To investigate the effect of tyrosine kinase inhibitors on cell proliferation, we used an MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl tetrazolium bromide] (Sigma Chemical Company, St. Louis, MO, USA) rapid colorimetric assay as previously described [11]. Briefly, $1 \times 10^4$ BaF3 cells transfected with c-*kit* constructs were suspended in 100 μL medium supplemented with 10% FCS and cultured in 96-well microtiter plates for 72 hours at 37°C with various concentrations of tyrosine kinase inhibitors. MTT was incubated in each well for the final 4 hours of culture. After thoroughly dissolving the dark crystals formed by MTT with acid isopropanol, the optical density (OD) was measured on a microELISA plate reader (Corona Electric Co, Ibaragi, Japan) with a test wavelength of 540 nm and a reference wavelength of 620 nm.

### 2.5. Detection of Cells in Apoptosis

Cell viability and apoptosis in STI571-treated cells were assayed with the Annexin V–FITC staining kit (Beckman Coulter, Marseilles, France). Briefly, $1 \times 10^5$ cells cultured in the presence or absence of STI571 for 24 or 48 hours were washed once with phosphate-buffered saline (PBS) and resuspended in 100 μL diluted binding buffer containing 1 μL Annexin V–FITC solution and 5 μL propidium iodide solution. Cells were incubated for 10 minutes on ice in the dark, diluted in 400 μL of ice-cold diluted binding buffer, and analyzed by FACSCalibur. Morphologic changes in cells were examined under light microscopy after cytospin preparation and May-Grünwald staining. DNA fragmentation was examined by agarose gel electrophoresis.

### 2.6. Immunoprecipitation and Western Blot Analysis

BaF3 cells transfected with c-*kit* constructs were starved of IL-3 and serum for 12 hours in RPMI 1640 medium supplemented with 0.1% FCS. Cells were subsequently resuspended in 1 mL medium for 10 minutes at 37°C with or without 100 ng/mL SCF, washed twice with ice-cold PBS, and lysed with buffer containing 50mM HEPES (pH 7.4), 10% glycerol, 150mM sodium chloride, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 50μM zinc chloride, 25mM sodium fluoride, proteinase inhibitors (Complete; Boehringer-Mannheim, Mannheim, Germany), 1μM pepstatin, and 1mM sodium orthovanadate. Cell lysates were clarified by centrifugation at 20,000*g* for 20 minutes. For immunoprecipitation, cell lysates were incubated with rabbit polyclonal antibody to murine KIT and with Protein A/G-Plus-Sepharose (Santa Cruz Biotechnology). The immunoprecipitates were washed 3 times with lysis buffer. Immunoprecipitates and total lysates were resuspended in sodium dodecyl sulfate (SDS) sample buffer, heated, and separated by SDS polyacrylamide gel electrophoresis. Gels were blotted on Immobilon P membrane (Millipore, Bedford, MA, USA) and stained with the indicated antibody. Antibody binding was detected by incubation with a horseradish peroxidase–labeled secondary antibody followed by chemiluminescence detection (ECL-Plus, Amersham Pharmacia Biotech, Buckinghamshire, UK).

## 3. Results

### 3.1. Effects of STI571 and AG1296 on KIT Autophosphorylation in BaF3 Cells Expressing Wild-Type KIT or Constitutive Active Mutants of KIT

STI571 is a known inhibitor of the c-abl, v-abl, and bcr-abl tyrosine kinases. Additionally, STI571 has been found to potently inhibit the kinase activities of the α- and β-PDGF receptors and KIT. To determine whether STI571 inhibits the tyrosine kinase activity of constitutive active mutants of KIT other than wild-type KIT, we examined the receptor autophosphorylation of BaF3 cells transfected with wild-type KIT (BaF3/WT) or constitutive active mutants, $KIT^{G559}$ or $KIT^{V814}$ (BaF3/G559, BaF3/V814) (Figure 1A). In BaF3/WT cells, SCF-dependent KIT autophosphorylation was inhibited completely at a dose of 1.0μM STI571. In BaF3/G559 cells, the receptor was phosphorylated constitutively, and this phosphorylation was inhibited at a dose of 0.1μM STI571, both in the absence and presence of SCF, indicating the higher sensitivity of the $KIT^{G559}$ mutant to STI571 inhibition compared with wild-type KIT. On the contrary, the autophosphorylation of $KIT^{V814}$ could not be inhibited by STI571, even at the quite high concentration of 10μM. We next examined the inhibitory effect of another tyrosine kinase inhibitor, AG1296, which belongs to the tyrphostin class and has been reported to inhibit the kinase activity of the PDGF receptor and KIT. Although AG1296 inhibited the SCF-induced autophosphorylation of wild-type KIT completely at a concentration of 10μM, it inhibited the constitutive autophosphorylation of $KIT^{G559}$ completely at 1μM, suggesting the higher sensitivity of the $KIT^{G559}$ mutant to AG1296 inhibition (Figure 1B). In the presence of SCF, however, 10μM AG1296 was able to inhibit the phosphorylation of the immature form of $KIT^{G559}$ but not the phosphorylation of the mature form. On the other hand, similar to the reaction seen with STI571, autophosphorylation of $KIT^{V814}$ completely resisted inhibition by AG1296, even at 10μM and irrespective of the presence of SCF.

### 3.2. Effects of STI571 and AG1296 on Cellular Proliferation in BaF3 Cells Expressing Wild-Type KIT or Constitutive Active Mutants of KIT

The proliferation of BaF3 cell lines transfected with KIT constructs was measured by the MTT assay at various concentrations of STI571 (Figure 2A). In BaF3/WT cells, STI571 inhibited SCF-induced proliferation in a dose-dependent manner with a 50% inhibitory concentration ($IC_{50}$) value of 0.5μM. In BaF3/G559 cells, factor-independent growth was completely inhibited at a concentration of 0.03μM ($IC_{50}$, 0.015μM). In the presence of SCF, the proliferation of BaF3/G559 cells was completely inhibited at a concentration of 0.1μM ($IC_{50}$, 0.05μM), a concentration slightly higher than without SCF. By contrast, both in the absence and presence of SCF, the proliferation of BaF3/V814 cells was not affected by the addition of STI571, even at a dose of 10μM. We next examined the inhibitory effect of AG1296 (Figure 2A). In BaF3/WT cells, AG1296 inhibited SCF-induced proliferation in a dose-dependent manner with an $IC_{50}$ value of 5μM. In BaF3/G559 cells, factor-independent growth was completely inhibited by AG1296 at a concentration of 1μM ($IC_{50}$, 0.36μM), indicating that AG1296 was also a specific inhibitor of the $KIT^{G559}$ mutant. However, different from STI571, the addition of SCF attenuated the proliferation-inhibitory effects of AG1296 on BaF3/G559 to a level similar to that of wild type ($IC_{50}$, 8.4μM). Both in the absence and presence of SCF, AG1296 did not affect the proliferation of BaF3/V814 cells, even at a dose of 10μM. The inhibitory effects of STI571 and AG1296 in BaF3/WT and BaF3/G559 cells were completely rescued by the addition of 1 ng/mL IL-3 (data not shown). The inhibitory effects of STI571 on cell proliferation were also examined by the measurement of viable cell counts. BaF3/WT, BaF3/G559, and BaF3/V814 cells were cultured at 0.1μM and 1μM STI571, and the total number of viable cells were counted (Figure 2B). At 0.1μM STI571, the

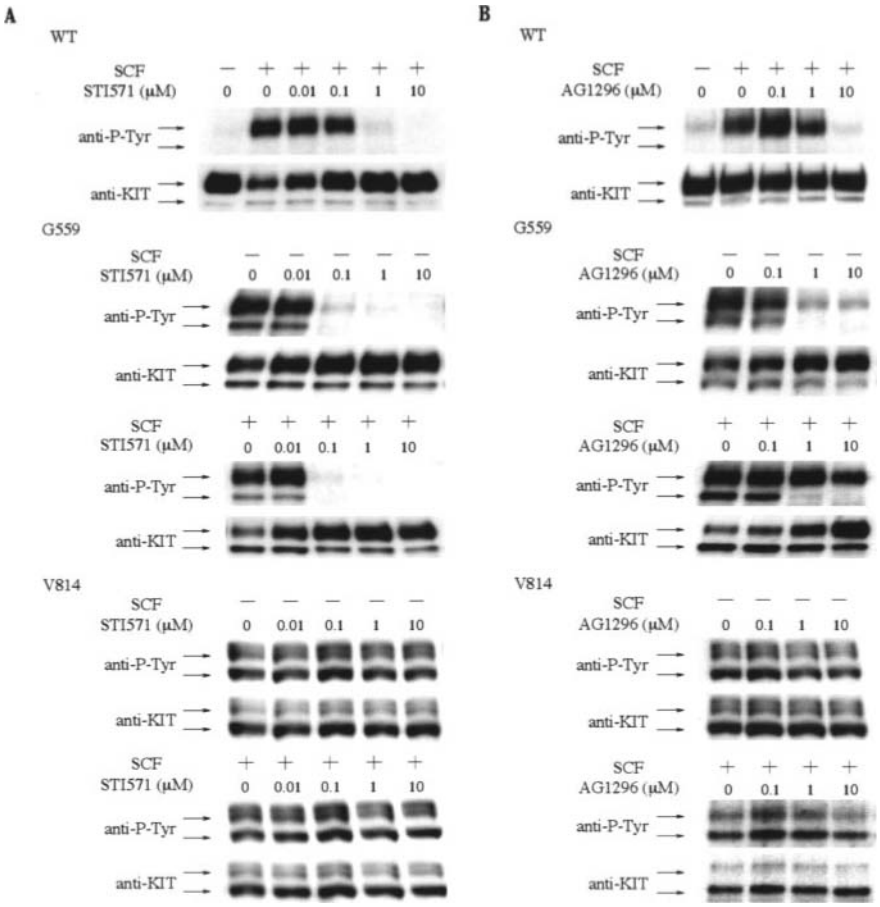


**Figure 1.** Effects of STI571 or AG1296 tyrosine kinase inhibitors on tyrosine phosphorylation of wild-type (WT) c-*kit* receptor (KIT) and constitutively active mutants of KIT in the juxtamembrane domain (G559) or the catalytic domain (V814). BaF3/WT, BaF3/G559, and BaF3/V814 cells were starved of IL-3 for 12 hours. After incubation with various concentrations of STI571 (A) or AG1296 (B) for 90 minutes, cells were then stimulated with 100 ng/mL stem cell factor (SCF) for 10 minutes and lysed. Cell lysates were immunoprecipitated with anti-KIT antibody, separated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate, and immunoblotted with antiphosphotyrosine antibody (4G10). The membranes were stripped and reblotted with anti-KIT antibody. The mobilities of the mature (145 kD) and immature (125 kD) forms of KIT are indicated at left. Three independent experiments showed comparable results.

cell proliferation of BaF3/G559 cells was completely abolished, whereas the proliferation of BaF3/WT and BaF3/V814 cells was not affected. On the contrary, at the 1μM concentration of STI571 at which BaF3/WT and BaF3/G559 cells had completely ceased to proliferate, the proliferation of BaF3/V814 cells was not inhibited. Taking these results together, we conclude that only the activating mutation in the juxtamembrane domain of KIT showed high sensitivity to the 2 different tyrosine kinase inhibitors, STI571 and AG1296.

### *3.3. Low-Dose STI571 Selectively Induces Apoptosis in BaF3 Cells Expressing the Activating Mutation in the Juxtamembrane Domain of KIT*

For further definition of the inhibitory effects of STI571 on cell survival, Annexin V–propidium iodide staining was performed on BaF3/WT, BaF3/G559, and BaF3/V814 cells treated with 0.1μM STI571 (Figure 3). Although 0.1μM STI571 did not induce apoptosis in BaF3/WT and BaF3/V814 cells 24 hours after the initiation of treatment, it significantly increased the early and late apoptotic fractions in BaF3/G559 cells as early as 24 hours after treatment initiation (percent apoptosis at 24 hours, 31.2%; at 48 hours, 58.8%). The apoptosis effects on BaF3/G559 cells by STI571 were also proven by the morphologic change and the DNA fragmentation assay (data not shown). These results indicated that low-dose STI571 could selectively induce apoptosis in cells expressing the juxtamembrane domain mutant of KIT.

### *3.4. Combined Effect of STI571 and AG1296 on the Growth of BaF3 Cells Expressing Wild-Type KIT and Constitutive Active Mutants of KIT*

Because STI571 and AG1296 belong to different classes of tyrosine kinase inhibitor and because the mode of inhibition in the presence of SCF is different for these 2 drugs, we

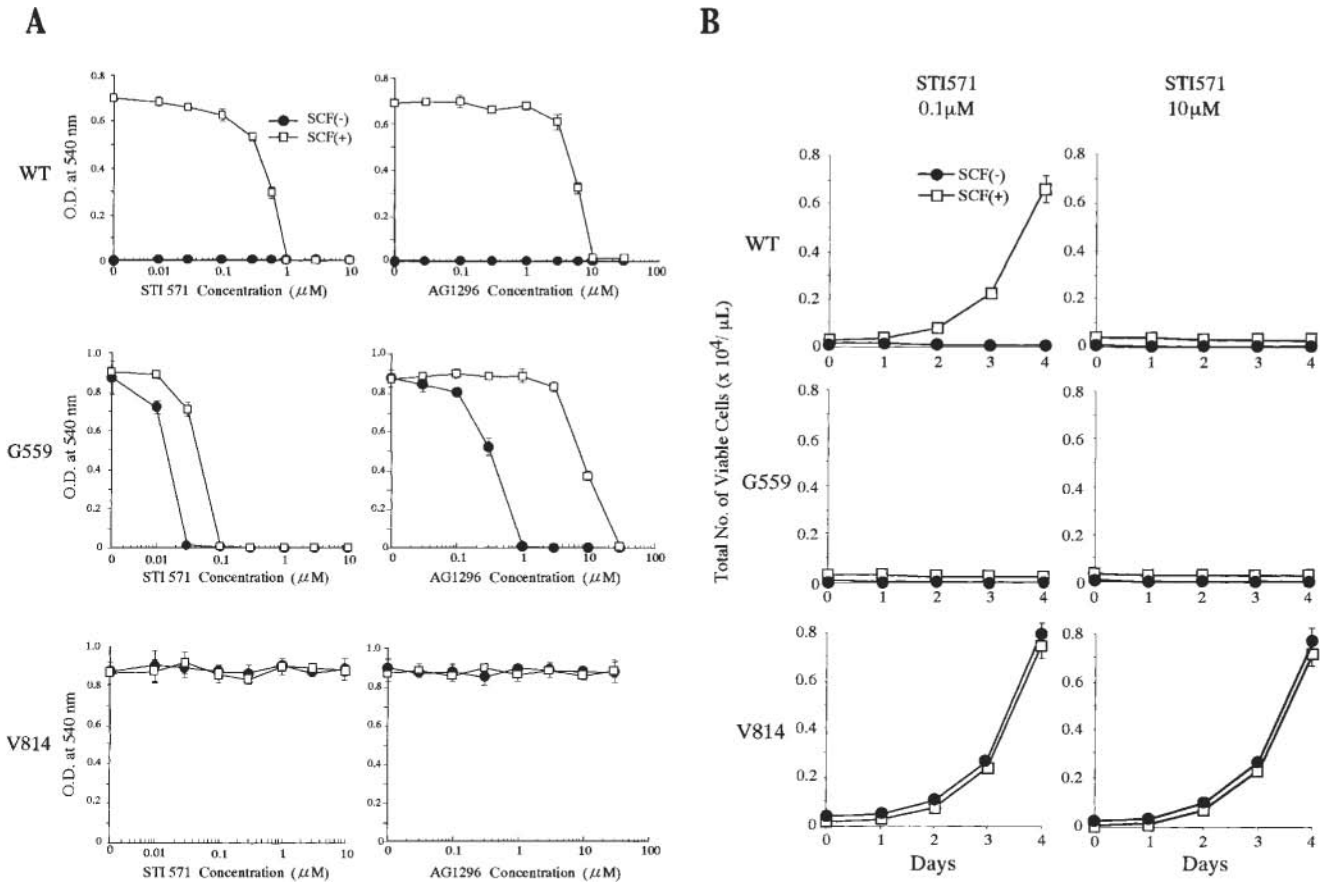


**Figure 2.** The effects of STI571 and AG1296 tyrosine kinase inhibitors on the proliferation of BaF3 cells expressing wild-type (WT) c-*kit* receptor (KIT) and the constitutive active mutants of KIT (G559 and V814). A, BaF3/WT, BaF3/G559, and BaF3/V814 cells were incubated with various concentrations of STI571 or AG1296 for 72 hours in the presence or absence of 100 ng/mL stem cell factor (SCF), and cell proliferation was measured using an MTT colorimetric assay. The results are shown as means ± SD of triplicate cultures. B, BaF3/WT, BaF3/G559, and BaF3/V814 cells were seeded at a cell density of 100/μL and cultured in the presence of STI571 at the indicated concentration. The total number of viable cells was counted by the trypan blue dye exclusion method. The results are shown as the means ± SD of triplicate cultures. Both results represent 3 independent experiments.

evaluated the combined effect of STI571 and AG1296. Figure 4A shows that although either 0.01μM STI571 or 0.2μM AG1296 had little inhibitory effect on the factor-independent growth of BaF3/G559 cells, the growth of BaF3/G559 cells was reduced to about 40% of normal levels when both compounds were used in combination. This combination did not suppress the factor-dependent growth of BaF/WT cells. BaF3/V814 cells were entirely resistant to either STI571 or AG1296, as was shown by the results that neither 10μM STI1571 nor 10μM AG1296 inhibited the proliferation of BaF3/V814 cells. Even when both of these compounds were used in combination, the proliferation of BaF3/V814 cells was not affected (Figure 4B).

### *3.5. Resistance of KIT$^{V814}$ to STI571*

We considered 2 possible mechanisms to explain the resistance of BaF3/V814 cells to STI571 and AG1296. One was that the catalytic domain mutation of KIT itself resisted these inhibitors, possibly through conformation change in the receptor. The other possible mechanism was that the intracellular concentration of the inhibitor decreased in BaF3/V814 cells because of altered drug metabolism or an accelerated efflux of the drugs by the multidrug-resistant protein family. To exclude the latter possibility, we prepared BaF3 cells expressing both the p210 form of BCR-ABL and KIT$^{V814}$ proteins and assessed the effect of STI571 on the tyrosine phosphorylation and growth-supporting activities of BCR-ABL and KIT$^{V814}$. We also transfected the BCR-ABL expression vector into BaF3 cells and obtained BCR-ABL–expressing clones after selection with G418. BaF3 cells expressing BCR-ABL (BaF3/BCR-ABL) were able to proliferate in the absence of IL-3. The clones were designed as BaF3/BCR-ABL/KIT$^{V814}$ or BaF3/BCR-ABL/Mock. As shown in Figure 5A, the proliferation of BaF3/BCR-ABL/V814 cells was entirely resistant to STI571 inhibition, whereas the growth of BaF3/BCR-ABL/Mock cells was completely inhibited at concentrations of about 1μM to 10μM. In both BaF3/BCR-ABL cells and BaF3/BCR-ABL/V814 cells, the autophosphorylation of BCR-ABL was

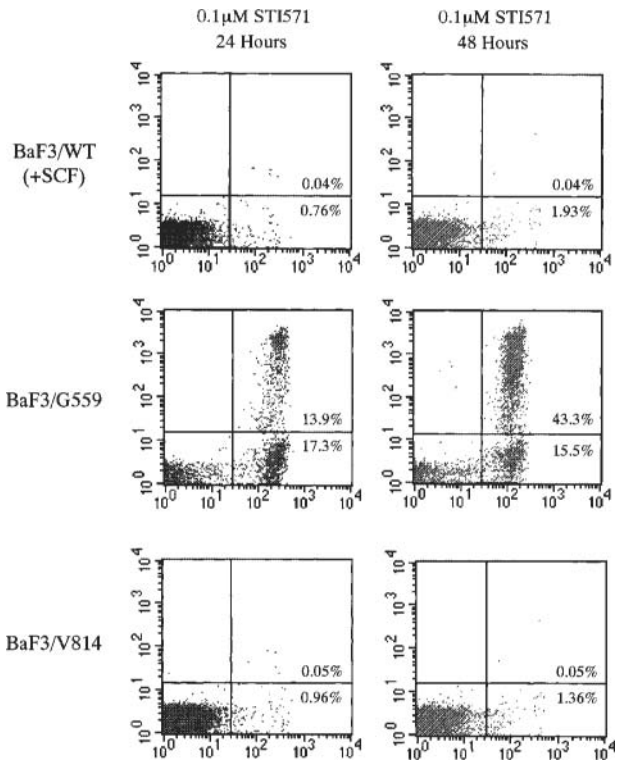


**Figure 3.** Low-dose STI571 selectively induces apoptosis in BaF3 cells expressing the activating mutation in the juxtamembrane domain of c-*kit* receptor (KIT). The viabilities of BaF3/WT, BaF3/G559, and BaF3/V814 cells were assayed by staining with Annexin V and propidium iodide (PI) after culture with 0.1μM STI571 for 24 and 48 hours. The lower right Annexin V(+) PI(–) population indicates early apoptotic cells, and the upper right Annexin V(+) PI(+) population indicates late apoptotic or necrotic cells. Similar results were obtained from 3 independent experiments.

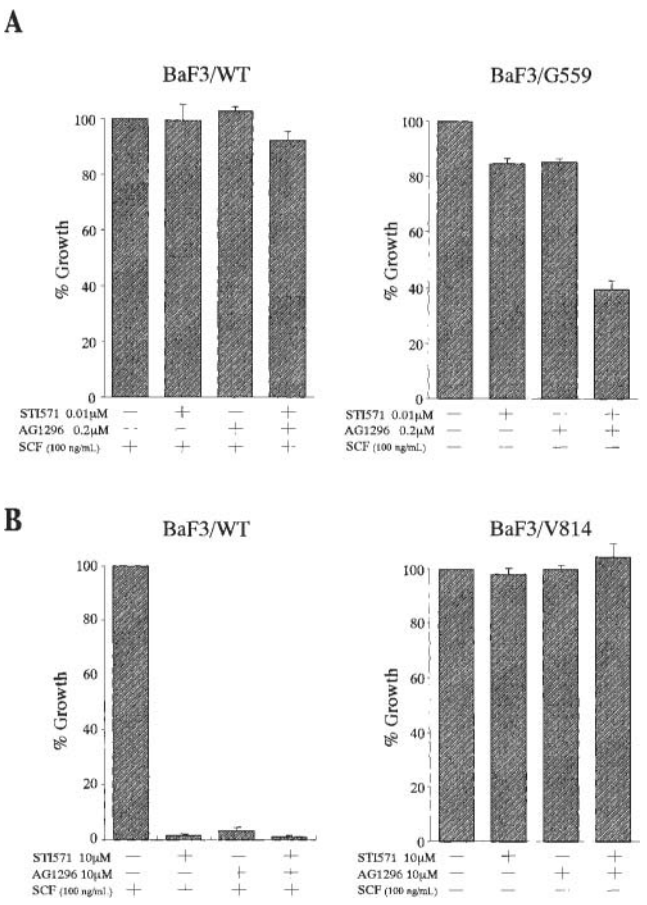


**Figure 4.** Combined effects of STI571 and AG1296 tyrosine kinase inhibitors on the growth of BaF3 cells expressing wild-type (WT) c-*kit* receptor (KIT) and constitutive active mutants of KIT (G559 and V814). BaF3/WT, BaF3/G559, and BaF3/V814 cells were incubated in the presence or absence of STI571 and/or AG1296 at the indicated concentration for 72 hours. Cell proliferation was measured with an MTT colorimetric assay. Data are shown as percentages of the control without inhibitor. Similar results were obtained from 3 independent experiments. SCF indicates stem cell factor.

nearly completely inhibited at a dose of 1.0μM STI571 (Figure 5B). This result indicated that the intracellular concentration of STI571 was similar in both BaF3/BCR-ABL/Mock cells and BaF3/BCR-ABL/V814 cells. Nevertheless, the autophosphorylation of $KIT^{V814}$ was not inhibited by STI571 in BaF3/BCR-ABL/V814 cells. These findings suggest that the catalytic domain mutation of KIT itself is entirely resistant to STI571 inhibition.

## 4. Discussion

It is known that STI571 inhibits KIT receptor tyrosine kinase activity over a range of 0.1μM to 1μM. Nevertheless, little is known about whether STI571 is effective against constitutive active mutants of KIT in comparison with wild type. Ma et al, using the 2 mastocytoma lines HMC1.1 and HMC1.2, recently reported that the activating KIT mutation in the juxtamembrane domain is sensitive to STI571 but that the activating KIT mutation in the catalytic domain is resistant to STI571 [35]. In our study, we demonstrated that the activating mutation in the juxtamembrane domain of KIT conferred a higher sensitivity to the tyrosine kinase inhibitor STI571, compared with wild-type KIT. A low dose of STI571 selectively inhibited cell proliferation and induced apoptosis in BaF3/G559 cells, but not in BaF3/WT cells. These results indicated that STI571 might be a highly selective therapeutic agent for gastrointestinal stromal tumors (GISTs), in many of which juxtamembrane domain mutations of KIT have been detected [8,23,25]. Recently, it has been reported that STI571 shows an excellent clinical effect in patients with gastrointestinal stromal tumors [36-38].

On the other hand, the activating $Asp^{814}$-to-Val mutation in the catalytic domain of KIT lacked sensitivity to STI571. This result suggests that STI571 has little or no effect on most hematologic malignancies induced by the KIT mutant, especially mast cell neoplasms that possess the $Asp^{816}$ mutation. These results also indicate that the molecular mechanisms regulating constitutive activation appear to be different for

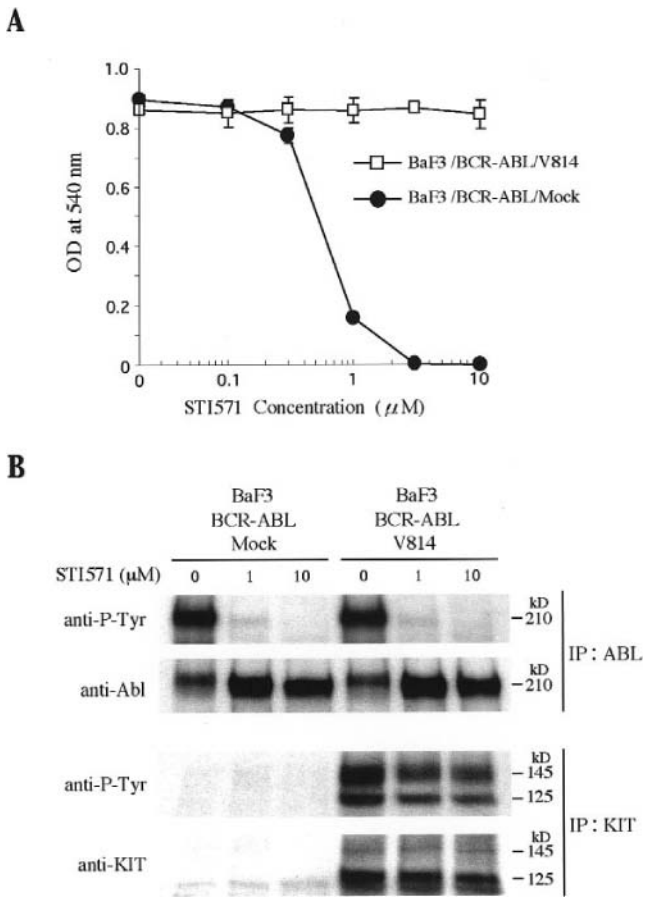


**Figure 5.** Tyrosine kinase inhibitor STI571 inhibits kinase and growth-supporting activities of BCR-ABL but not of $KIT^{V814}$ in BaF3 cells expressing both oncoproteins. A, BaF3/BCR-ABL/Mock and BaF3/BCR-ABL/V814 cells were incubated with various concentrations of STI571 for 72 hours in the absence of cytokines, and cell proliferation was measured with an MTT colorimetric assay. The results are shown as means ± SD of triplicate cultures. B, BaF3/BCR-ABL/Mock and BaF3/BCR-ABL/V814 cells were pretreated with indicated concentrations of STI571 for 90 minutes. Cells were lysed, and cell lysates were immunoprecipitated with anti-Abl or anti-KIT antibody, separated by polyacrylamide gel electrophoresis with sodium dodecyl sulfate, and immunoblotted with antiphosphotyrosine antibody (4G10). The membranes were stripped and reblotted with the indicated antibody.

the juxtamembrane domain mutant and the catalytic domain mutant. In fact, we have previously shown that although the juxtamembrane domain mutant of KIT led to constitutive dimerization of KIT at the extracellular domain, the catalytic domain mutant did not lead to such a dimerization but instead caused receptor self-association in the cytoplasmic domain, possibly via a conformation change [9,39]. On the basis of the known structure of the ATP-binding site of protein kinases, STI571 was designed and selected for its specificity for the ABL tyrosine kinase. This compound is considered to act as a competitive inhibitor of ATP binding to the kinase domain. Recently, a point mutation in the ATP-binding pocket of the ABL kinase domain was found to confer STI571 resistance in some CML patients resistant to STI571 [40]. On the other hand, Weisberg and Griffin suggested that the resistance of BCR-ABL to STI571 could be mediated by an increased expression of BCR-ABL or a decreased intracellular concentration due to an altered drug metabolism or transport [41]. In our study, the expression level of $KIT^{V814}$ protein was not significantly different from the expression of $KIT^{WT}$ or $KIT^{G559}$. In addition, the comparable effect of STI571 on BCR-ABL protein in both BaF3/BCR-ABL/V814 cells and BaF3/BCR-ABL/Mock cells clearly excluded the possibility of a decreased intracellular STI571 concentration in $KIT^{V814}$-expressing cells. These results suggest that the $Asp^{814}$-to-Val mutation in the catalytic domain of KIT may cause a conformation change that impedes the binding of STI571. Consistent with our findings, von Bubnoff et al has reported that a mutation similar to $KIT^{V814}$ in the activation loop of ABL kinase found in STI571-refractory Ph+ leukemia caused the resistance to STI571 [42].

In this study, we also examined whether another tyrosine kinase inhibitor of the tyrphostin class, AG1296, inhibits KIT activation. AG1296 is known as a selective tyrosine kinase inhibitor for PDGF and KIT receptor kinase. AG1296 inhibited SCF-induced wild-type KIT autophosphorylation in a dose-dependent manner, with complete inhibition observed at 10μM. AG1296 completely inhibited SCF-induced cell proliferation of BaF3/WT cells at 10μM. Similar to STI571, AG1296 inhibited factor-independent growth of BaF3/G559 cells more effectively than it did BaF3/WT cells and did not block the proliferation of BaF3/V814 cells, suggesting again the higher susceptibility of the juxtamembrane domain mutant to the inhibitor. It was of great interest to note that, unlike STI571, the growth inhibition by AG1296 in BaF3/G559 cells could be rescued by the addition of SCF. The addition of SCF also attenuated the inhibitory effects of AG1296 on the autophosphorylation of the mature form of the $KIT^{G559}$ receptor without affecting the inhibitory effects on the autophosphorylation of the immature form of the receptor. Because the ligand can stimulate only the mature form that is expressed on the cell membrane, this result indicated that ligand stimulation altered the sensitivity of the G559 mature-form receptor to AG1296. Kovalenko et al have reported that for the nonactivated PDGF receptor, AG1296 exerted pure competitive inhibition toward ATP and mixed competitive inhibition toward the peptide, whereas it showed mixed competitive inhibition toward both ATP and the peptide substrate for the ligand-activated PDGF receptor [43]. Thus, the type of AG1296-mediated inhibition changes upon ligand stimulation according to the possible conformational changes at the ATP-binding site. Although the G559 receptor exhibits constitutive dimerization involving the extracellular domain, the addition of ligand may further stabilize the dimerization and change the conformation of the receptor, decreasing the contact between AG1296 and the ATP-binding site and resulting in less inhibition. In contrast, STI571 inhibited the receptor activation of G559 to a comparable extent, both in the presence and absence of SCF, suggesting that STI571 and AG1296 may inhibit KIT activation at different points. In fact, STI571 and AG1296 were found to synergistically inhibit the factor-independent growth of BaF3/G559 cells.

These data raise the possibility that the combination of STI571 and other tyrosine kinase inhibitors such as AG1296 might become a more effective therapy for diseases, including GISTs, that possess an activating mutation in the juxtamembrane domain of KIT. BaF3/V814 cells were still resistant to the combination of STI571 and AG1296. These data further strengthened the possibility that the activation mechanism of the $Asp^{814}$ mutation in the catalytic domain of KIT is complex and entirely different from that of wild-type KIT. Further studies will be necessary to identify novel specific inhibitors against the oncogenic activation of catalytic domain mutations of KIT for developing highly targeted therapies for diseases such as mast cell neoplasms.

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