Selective Cytotoxic Mechanism of GTP-14564, a Novel Tyrosine Kinase Inhibitor in Leukemia Cells Expressing a Constitutively Active Fms-like Tyrosine Kinase 3 (FLT3)*

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The receptor tyrosine kinase FLT3 is constitutively activated by an internal tandem duplication (ITD) mutation within the juxtamembrane domain in 20–30% of patients with acute myeloid leukemia. In this study, we identified GTP-14564 as a specific kinase inhibitor for ITD-FLT3 and investigated the molecular basis of its specificity. GTP-14564 inhibited the growth of interleukin-3-independent Ba/F3 expressing ITD-FLT3 at 1 μM, whereas a 30-fold higher concentration of GTP-14564 was required to inhibit FLT3 ligand-dependent growth of Ba/F3 expressing wild type FLT3 (wt-FLT3). However, this inhibitor suppressed the kinase activities of wt-FLT3 and ITD-FLT3 equally, suggesting that the signaling pathways for proliferation differ between wt-FLT3 and ITD-FLT3. Analysis of downstream targets of FLT3 using GTP-14564 revealed STAT5 activation to be essential for growth signaling of ITD-FLT3. In contrast, wt-FLT3 appeared to mainly use the MAPK pathway rather than the STAT5 pathway to transmit a proliferative signal. Further analysis demonstrated that the first two tyrosines in an ITD were critical in terms of the proliferation signals of wt-FLT3. These results indicate that an ITD mutation in FLT3 elicits an aberrant STAT5 activation that results in increased sensitivity to GTP-14564. Thus, FLT3-targeted inhibition is an attractive approach, with the potential for selective cytotoxicity, to the treatment of ITD-FLT3-positive acute myeloid leukemia.

Fms-like tyrosine kinase 3 (FLT3), originally isolated as a hematopoietic progenitor cell-specific kinase, belongs to the class of III receptor tyrosine kinase (RTK) family to which c-Fms, c-Kit, and the platelet-derived growth factor (PDGF) receptor also belong (1–3). Normal expression of FLT3 is restricted to hematopoietic progenitors (4, 5), whereas aberrantly expressed FLT3 is observed in acute leukemia cells (6, 7). Recently, an internal tandem duplication (ITD) mutation within the juxtamembrane domain of the FLT3 gene has been found in leukemic cells from 20–30% of patients with acute myeloid leukemia (AML) and is closely associated with a poor prognosis in AML patients (8–11). Also found in 7% of AML patients were activating mutations at aspartic acid 835 (Asp-835) in the kinase domain of FLT3; mutations are also associated with a poor prognosis (12). Both types of FLT3 mutations result in constitutive activation of FLT3 kinase activity and induce autonomous proliferation of factor-dependent hematopoietic cell lines (13, 14).

Targeted inhibition of mutant tyrosine kinase might be an attractive therapeutic strategy for hematological malignancies, just as the BCR-ABL tyrosine kinase inhibitor STI571 has been effectively used for the treatment of chronic myelogenous leukemia harboring gain-of-function mutations in the c-ABL tyrosine kinase (15, 16). Thus, FLT3 is considered to be a rational target for therapeutic intervention aimed at the commonly mutated gene in AML. For this purpose, we screened a small molecular compound library and identified 11 compounds that preferentially inhibited the autonomous growth of Ba/F3 cells expressing ITD-FLT3 (BaF/ITD-FLT3), as compared with Ba/F3 cells expressing wild type FLT3 (Ba/F/wt-FLT3). Among them, GTP-14564 had the most potent and specific inhibitory activity against Ba/F/ITD-FLT3. GTP-14564 inhibited the growth of Ba/F/ITD-FLT3 at 1 μM, whereas a 30-fold higher concentration of GTP-14564 was required for the inhibition of Ba/F/wt-FLT3 growth.

We anticipated that the difference in sensitivity to GTP-14564 between Ba/F/wt-FLT3 and Ba/F/ITD-FLT3 was caused by more potent suppression of the kinase activity of ITD-FLT3 than that of wt-FLT3 by GTP-14564. However, we found that GTP-14564 inhibited the kinase activities of wt-FLT3 and ITD-FLT3 equally. This raises the possibility that the selective cytotoxic potency of GTP-14564 is due to differences in growth signaling mechanisms between Ba/F/wt-FLT3 and Ba/F/ITD-FLT3. To clarify the molecular basis of the selective cytotoxicity of GTP-14564, we analyzed the signaling pathways downstream from wt-FLT3 and ITD-FLT3.
EXPERIMENTAL PROCEDURES

Reagents—Recombinant human FLT3 ligand (FL), PDGF-BB, stem cell factor (SCF), thrombopoietin (TPO), vascular endothelial growth factor (VEGF), and mouse interleukin-3 (mIL-3) were obtained from R&D Systems, Inc. Recombinant human macrophage colony-stimulating factor was a gift of Morinaga Milk Industry Co., Ltd. Polyclonal anti-FLT3, anti-TyK2, anti-extracellular signal-regulated kinase 1 (anti-ERK1), and anti-ERK2 antibodies were purchased from Santa Cruz Biotechnology. Polyclonal anti-STAT5A and anti-STAT5B antibodies were from R&D Systems, Inc. A mitogen-activated protein kinase (MAPK/ERK kinase (MEK) inhibitor, U0126 (17), was from Calbiochem-Novabiochem. The anti-phospho-tyrosine antibody 4G10 and anti-phospho MAPK antibody were purchased from Upstate Biotechnology and Promega, respectively.

Cells—A mouse pro-B cell line, Ba/F3, was maintained in RPMI 1640 containing 10% fetal calf serum (FCS) and 1 ng/ml mIL-3. Ba/F3 cells expressing c-kit or PDGFβR were grown in RPMI 1640 supplemented with 10% FCS, 1 ng/ml mIL-3, and 0.4 mg/ml G418 (Nacalai Tesque, Inc., Tokyo, Japan). Human leukemia cell lines, including Jurkat, K562, HL-60, THP-1, and MOLM13, were cultured in RPMI 1640 with 10% FCS. Murine myeloid leukemia M-NFS-60 cells were maintained in RPMI 1640 with 10% FCS and 100 ng/ml macrophage colony-stimulating factor (18). NIH3T3 cells expressing human kinase insert domain-containing receptor (KDR/Flk-1) were maintained as described (19). An ecotropic retrovirus packaging cell line, Plat-E (20), was maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS, 1 μg/ml puromycin (Sigma), and 10 μg/ml blasticidin S (Funakoshi Co., Tokyo, Japan).

Construction of FLT3 Expression Vectors and the Establishment of Ba/F3 Transfectants Expressing wt-FLT3 or ITD-FLT3—Complementary DNAs encoding human wt-FLT3 and ITD-FLT3 were cloned into the mammalian expression vector pMKTNeo containing a neomycin resistance gene and electroporated into Ba/F3 cells using a Gene Pulser (Bio-Rad). ITD-FLT3 was derived from patients’ leukemic cells harboring a 20-amino acid tandem duplication called M3 (8). These cells were serum starved in the presence of 0.8 ng/ml G418 for 2 weeks to establish BaF/wt-FLT3 and BaF/ITD-FLT3. An Ssp1 site and an AvrII site were introduced upstream and downstream from the ITD domain, respectively, without introducing changes in the amino acid sequence. Using these sites and synthesized oligonucleotides, various combinations of tyrosine to phenylalanine mutations were introduced into wt-FLT3 or ITD-FLT3. Ba/F3 transfectants expressing wt-FLT3 or ITD-FLT3 as well as their mutants were maintained in RPMI 1640 medium containing 10% FCS and 1 ng/ml mL-3 in the presence of 0.4 ng/ml G418 unless otherwise specified, except that Ba/FITD-FLT3 was maintained in the absence of mL-3.

Cell Growth Assay—Cell growth was evaluated by a colorimetric assay using an Alamar Blue solution (21) as described previously (22).

Kinase Assays—GTP-14564 inhibition of substrate phosphorylation by Abl (23), AKT (24), ERK1 (25) and 2 (26), MEK1 (27), epidermal growth factor receptor (EGFR) (28), human epidermal growth factor receptor 2 (HER2) (29), protein kinase A (PKA) (30), protein kinase C (PKC) (31), and Src (32) was assayed as described previously. Cell-based assays were performed by analyzing the effects of GTP-14564 on receptor autophosphorylation induced by ligand stimulation. Briefly, cells were incubated for 2 h in various concentrations of the inhibitor prior to stimulation with macrophage-colony-stimulating factor (100 ng/ml), stem cell factor (100 ng/ml), vascular endothelial growth factor (10 ng/ml), or PDGF-BB (100 ng/ml) for 5 min, respectively. Equal amounts of protein of cell lysates were analyzed by immunoblotting using the anti-phospho-tyrosine antibody.

Immunoprecipitation and Western Blot Analysis—Cells were harvested as explained in each figure legend, and immunoprecipitation and Western blotting were performed as described (22). The filter band antibody was detected using the enhanced chemiluminescence system (Amersham Biosciences).

A Retrovirus Vector Expressing the Dominant Negative STAT5A—The dominant negative STAT5A (dnSTAT5A) missing its C terminus (33) was inserted into the EcoRI and NotI sites of the pMX-internal ribosomal entry site (IRES)-green fluorescent protein (GFP) (34) to generate pMX-dnSTAT5A-IRES-GFP. This vector was transfected into Plat-E (20) to obtain the viruses using FuGENETM 6 transfection reagent (Roche Diagnostics) according to the manufacturer’s recommendations.

RESULTS

Screening of ITD-FLT3-specific Inhibitors—To develop a screening assay for identifying ITD-FLT3-specific inhibitors, we generated indicator cells by introducing wt-FLT3 or ITD-FLT3 into IL-3-dependent Ba/F3 cells. The resulting transfectants, Ba/F/wt-FLT3 and Ba/F/ITD-FLT3, expressed equivalent levels of FLT3 (data not shown). BaF/wt-FLT3 proliferated in the presence of FL, whereas Ba/FITD-FLT3 showed factor-independent growth. Using a colorimetric assay for cell proliferation, we screened a library of small molecular compounds and identified 11 compounds that inhibited the growth of BaF/ITD-FLT3 at concentrations lower than those required for inhibition of FL-driven growth of BaF/wt-FLT3. Among these 11 compounds, GTP-14564 (Fig. 1) had the most potent and specific inhibitory effect on Ba/FITD-FLT3 cells. GTP-14564 is a compound originally synthesized to demonstrate a protocol for synthesizing chemicals (35) and is available from SPECS and BioSPECS BV, Inc. (Rijswijk, Netherlands) under the name 1-phenyl-3-H-8-oxa-2,3-diaza-cyclopenta[a]inden. It has three conjugated rings sharing structural characteristics with the part of staurosporine that inhibits protein kinase C by competing for ATP.

Selective Kinase Inhibition by GTP-14564—The ability of GTP-14564 to inhibit exogenous substrate phosphorylation or receptor autophosphorylation was tested against a wide range of kinases. The concentration of GTP-14564 resulting in a 50% reduction of kinase activity or a 50% reduction in the phosphorylation of receptor tyrosine kinase (IC50) was reported in Table I. GTP-14564 inhibited members of class III RTKs including FLT3, c-Fms, c-KIT, and PDGFβR with IC50 values of 0.3–1.0 μM as assessed in cell-based assays. In contrast, GTP-14564 did not significantly inhibit other protein kinases tested in this
study, including kinase insert domain-containing receptor, epidermal growth factor receptor, human epidermal growth factor receptor 2, and intracellular kinases such as Src, protein kinase C, and ERKs. These studies demonstrated that GTP-14564 is a potent and specific inhibitor of class III RTKs.

GTP-14564 Specifically Inhibits ITD-FLT3-driven Cell Growth—To evaluate the selective cytotoxicity of GTP-14564 against ITD-FLT3, we tested the effects of GTP-14564 on the proliferation of BaF/wt-FLT3, Ba/F/ITD-FLT3, and parental Ba/F3 cells. Also included in this experiment was a Ba/F3 transfectant expressing a kinase-active mutant of FLT3 with a Asp-835 to Val mutation (Ba/F/D835V-FLT3). This mutant also induced autonomous growth in Ba/F3 cells. As shown in Fig. 2A, GTP-14564 inhibited the proliferation of Ba/F/ITD-FLT3 at 1 μM. On the other hand, a 30-fold higher concentration of GTP-14564 was needed to inhibit FL-dependent growth of Ba/F/wt-FLT3. The sensitivity of Ba/F/D835V-FLT3 to GTP-14564 was intermediate, i.e. between those of Ba/F/ITD-FLT3 and Ba/F/wt-FLT3.

We next examined the effects of GTP-14564 inhibition of FLT3 on the cell growth of several human leukemia cell lines. Among the cell lines tested, proliferation of MOLM13 expressing ITD-FLT3 (8) was inhibited by 1 μM GTP-14564. However, other cell lines, including Jurkat, K562 expressing BCR-ABL, THP-1 expressing wt-FLT3, and HL-60, were resistant, requiring at least 300-fold higher concentrations of GTP-14564 (Fig. 2B). IL-3-driven proliferation of Ba/F3 cells was also resistant to GTP-14564 exposure (Fig. 2A). These results revealed GTP-14564 to be a specific inhibitor of ITD-FLT3.

GTP-14564 Inhibits the Kinase Activities of Both wt-FLT3 and ITD-FLT3 with a Similar Efficiency—The above studies demonstrated that ITD-FLT3-expressing cells were more sensitive to GTP-14564 than wt-FLT3-expressing cells. Therefore, we tested whether GTP-14564 suppresses the kinase activity of ITD-FLT3 more efficiently than that of wt-FLT3 by using receptor autophosphorylation as an assay. To our surprise, we found that GTP-14564 suppressed the autophosphorylation of both wt-FLT3 and ITD-FLT3 equally at 0.3 μM (Fig. 3A), indicating that GTP14564 equally suppressed the kinase activities of wt-FLT3 and ITD-FLT3. These results raise the possibility that the selective growth inhibitory potency of GTP-14564 is due to a difference in signaling pathways between wt-FLT3 and ITD-FLT3. Therefore, we investigated the role of two representative signaling pathways, JAK/STAT and MAPK (36, 37), both of which are known to be important for cell proliferation, downstream from the wt-FLT3 and ITD-FLT3 receptors by using GTP-14564.

ITD-FLT3 but Not wt-FLT3 Induces Tyk2 Activation in Ba/F3 Cells, an Event Indirectly Suppressed by GTP-14564—Among members of the Janus kinase family, JAK2, JAK3, and Tyk2, but not JAK1, were expressed in Ba/F3 cells. Phosphorylation of JAK2 and JAK3 was not augmented by either wt-FLT3 or ITD-FLT3 (data not shown). Tyk2 was activated in Ba/F/ITD-FLT3 but not in Ba/F/wt-FLT3 even after FL stimulation. In addition, the activation of Tyk2 in Ba/F/ITD-FLT3 was inhibited by GTP-14564 (Fig. 3B). However, GTP-14564 inhibition of Tyk2 activation is likely to be indirect because of the following result. As we reported, Tyk2 is activated by TPO in Ba/F3 cells expressing TPO receptors (39); however, neither this TPO-induced Tyk2 activation nor TPO-induced cell growth was inhibited by GTP-14564 (Fig. 3B).

ITD-FLT3-induced STAT5 Activation Is More Sensitive to GTP-14564 than wt-FLT3-induced Activation—STAT5A was constitutively phosphorylated in Ba/F/ITD-FLT3 and was only activated in Ba/F/wt-FLT3 cells after factor starvation followed by stimulation with FL (Fig. 3C). The activation of STAT5A was inhibited by much lower concentrations of GTP-14564 in Ba/F/ITD-FLT3 as compared with findings with Ba/F/wt-FLT3 (Fig. 3C). Activation and inhibition of STAT5B were much the same as those of STAT5A (data not shown). It was reported previously that STAT5A, but not STAT5B, was activated by wt-FLT3 (37). However, in our experiments the activation of both STAT5A and STAT5B by wt-FLT3 as well as ITD-FLT3 was detected by several STAT5A- or STAT5B-specific antibodies, including the one used in the previous study (data not shown). A possible explanation for this discrepancy is that Zhang et al. tested STAT5 activation in human embryonic kidney cells (HEK293) but not in hematopoietic cells (37). Phosphorylation of STAT1 and STAT3 was not augmented by either wt-FLT3 or ITD-FLT3 (data not shown).

ERK1 and 2 Play Important Roles in wt-FLT3-driven but Not ITD-FLT3-driven Cell Growth—Activation of ERK1 and 2 was
Essential Roles of STAT5 in Mutant FLT3 Signaling

Actuation of STAT5 Is a Prerequisite for Growth Signals Induced by ITD-FLT3—We and others reported previously that C-terminal truncated mutants of STAT5 worked as dominant negative forms (33, 41). To determine whether activation of STAT5 is critical for growth signals delivered by ITD-FLT3, we expressed dnSTAT5A (33) in BaF/wt-FLT3 and BaF/ITD-FLT3 by using a bicistronic retrovirus vector, pMX-IRES-GFP (34). One day after the transduction of pMX-dnSTAT5A-IRES-GFP, BaF/wt-FLT3 and BaF/ITD-FLT3 cells were cultured in the presence or absence of FL, respectively, and GFP-positive cells (i.e. cells expressing wt-FLT3 or ITD-FLT3) were counted at indicated time intervals to evaluate the growth inhibitory effects of dnSTAT5A (Fig. 5). The cell growth was not affected by the transduction of the blank vector pMX-IRES-GFP, thus serving as a negative control. Factor-independent growth of BaF/ITD-FLT3 was profoundly suppressed by the expression of dnSTAT5A (Fig. 5B), whereas FL-dependent growth of BaF/wt-FLT3 was not significantly affected by the expression of dnSTAT5A (Fig. 5A). These results demonstrate that STAT5 activation is essential for cell growth signals delivered by ITD-FLT3.

Tyrosine Residues of ITD-FLT3 Are Required for STAT5 Activation and Cell Proliferation—To further confirm the importance of STAT5 activation in ITD-FLT3-driven cell growth, we generated a series of ITD-FLT3 mutants harboring phenylalanine instead of tyrosine in various combinations in the ITD domain (ITD-YF mutants) (Fig. 6A). Using these ITD-YF mutants, we attempted to relate phosphorylation of particular tyrosine residues to STAT5 activation and growth induction. When all tyrosines in the ITD region were replaced by phenylalanines, this mutant receptor, FFFFFFFF (designated F8 in Fig. 6), no longer activated STAT5 nor did it induce autonomous growth of Ba/F3 cells (Fig. 6B). To identify the tyrosine residue required for STAT5 activation and growth induction, we further tested the potentials of various ITD-YF mutants to induce STAT5 activation and cell growth. Although the FFFYYYY mutant (F4Y4 in Fig. 6) in which the first four tyrosines were replaced by phenylalanines was not able to activate STAT5, the YYYYYFFFF (Y4F4 in Fig. 6) mutant in which the latter four tyrosines were replaced by phenylalanines did activate STAT5 (Fig. 6B). The YYYYYFFFFF mutant (Y2F6 in Fig. 6), but not the FFYYYYFFFF mutant of ITD-FLT3 (P2Y2F4 in Fig. 6), activated STAT5, thus indicating that the first two tyrosines are critical for STAT5 activation. We also found that one of these tyrosines was adequate to induce STAT5 activation, albeit weakly, in an experiment using the YYYYYFFFFF mutant (YP7 in Fig. 6) or the YYYYYFFFFF mutant (YPY6 in Fig. 6) in which the first or the second tyrosine was retained, respectively. The potential to activate STAT5 correlated well with the potential to induce autonomous cell proliferation in a series of ITD-YF mutants, thereby confirming the importance of STAT5 activation in ITD-FLT3-induced cell growth.

Fig. 3. Signals delivered by ITD-FLT3 and wt-FLT3 and their sensitivity to GTP-14564. A, GTP-14564 inhibited the kinase activities of wt-FLT3 and ITD-FLT3 equally. Five million cells of each BaF/wt-FLT3, which proliferated in the presence of 10 ng/ml FL or BaF/ITD-FLT3, which showed factor-independent growth, were incubated for 2 h in the presence or absence of various concentrations of GTP-14564. The cells were then lysed and immunoprecipitated with polyclonal anti-FLT3 antibodies. The immunoprecipitates were separated through a 7.5% SDS-PAGE and blotted onto a nylon membrane. The membrane was probed with 4G10. The membrane was stripped and reprobed with polyclonal FLT3 antibodies. B, constitutive phosphorylation of Tyk2 in BaF/ITD-FLT3 was inhibited by GTP-14564. The cell lysates of BaF/ITD-FLT3 were prepared as described above. Five million BaF/wt-FLT3 cells and BaF/mpl cells were deprived of serum for 2 h in the presence or absence of 3 μM GTP-14564. The cells were then incubated with or without FL (10 ng/ml) or TPO (10 ng/ml) for 5 min, respectively, and lysed. Tyk2 activation in BaF/mpl cells stimulated by TPO was used as a control for Tyk2 activation and its sensitivity to GTP-14564. The cell lysates were immunoprecipitated with an anti-Tyk2 antibody. The immunoprecipitates were blotted onto a nylon membrane as described above. The membrane was probed with 4G10 and then reprobed with the antibody against Tyk2. C, constitutive activation of STAT5 in BaF/ITD-FLT3 cells was inhibited by GTP-14564. Cell lysates of BaF/wt-FLT3 and BaF/ITD-FLT3 cells were prepared as described above and immunoprecipitated using the anti-STAT5A antibody. The blotted membrane was probed with 4G10 and then reprobed with the anti-STAT5A antibodies. Phosphorylated (top) and non-phosphorylated (bottom) forms of STAT5A appeared in the anti-STAT5A blot.
On the other hand, the FFFF mutant (F4 in Fig. 6) of wt-FLT3 in which all four tyrosines in the juxtamembrane domain of wt-FLT3 were replaced by phenylalanines did not induce STAT5 activation but did induce cell growth in the presence of FL, albeit weakly (Fig. 6B). Activation of ERK1 and 2 by FL was observed in both BaF/wt-FLT3 and Ba/F3 cells expressing the F4 mutant of wt-FLT3 (Fig. 4A, and data not shown). These results suggested that, unlike ITD-FLT3, the MAPK pathway was dispensable and the STAT5 pathway was dispensable for wt-FLT3 growth signals.

**DISCUSSION**

In a search for inhibitors of the oncogenic mutant of FLT3, we developed a screening assay in which two model cell lines were used as indicators. One cell line is BaF/wt-FLT3, which proliferates in the presence of FL, and the other is Ba/F/ITD-FLT3, which shows autonomous cell growth. Using these two lines, we identified a novel kinase inhibitor, GTP-14564, that has both efficacy and selectivity for ITD-FLT3. Although GTP-14564 has a stronger effect on ITD-FLT3 than on its wild type counterpart, the kinase activities of wt-FLT3 and ITD-FLT3 were equally suppressed by GTP-14564, suggesting that the selective cytotoxicity of GTP-14564 depended on the difference in the growth signals delivered by wt-FLT3 and ITD-FLT3. Multiple pathways, including JAK/STAT and RAS-RAF-MEK-ERK, have been implicated in FLT3 signaling (36, 37). To clarify the molecular mechanism underlying the selective inhibition of ITD-FLT3-driven cell growth by GTP-14564, we compared signaling molecules downstream of wt-FLT3 and ITD-FLT3 and their sensitivities to GTP-14564.

Herein we present considerable evidence for the critical roles of STAT5 activation in ITD-FLT3-driven, but not wt-FLT3-driven cell growth. A 30-fold higher concentration of GTP-14564 was required to inhibit STAT5 activation in BaF/wt-FLT3 than in BaF/ITD-FLT3. This difference is consistent with the difference in sensitivities of BaF/ITD-FLT3 and BaF/wt-FLT3 to GTP-14564 in growth suppression. Of greatest importance is the observation that cell proliferation induced by ITD-FLT3, but not wt-FLT3, was dramatically inhibited by dnSTAT5. In addition, the two tyrosine residues at the N-terminus of the intracellular domain of ITD-FLT3 were required for STAT5 activation as well as for the induction of cell growth. On the other hand, the MEK inhibitor U0126 preferentially inhibited wt-FLT3-driven cell proliferation as compared with ITD-FLT3-driven cell proliferation, indicating important roles for the MAPK pathway in growth signaling induced by wt-FLT3. We also found that all of the tyrosine residues in the juxtamembrane domain of wt-FLT3 were dispensable for FL-stimulated cell growth; the FFFF mutant of wt-FLT3 induced FL-dependent growth without detectable activation of STAT5. Moreover, activation of ERK1 and 2 mediated by FL-stimulated wt-FLT3 was not inhibited by GTP-14564 at 3 μM, which suppressed STAT5 activation at much lower concentrations. These findings further support the concept that the proliferative signal through wt-FLT3 mainly depends on the activation of MAPK rather than STAT5. It is obvious that activation of
both pathways is required for full stimulation of FL-dependent cell growth.

Tyk2 was activated by ITD-FLT3 but not wt-FLT3, and this activation was inhibited by GTP-14564. The inhibition of Tyk2 by GTP-14564 was not direct, because GTP-14564 did not inhibit the TPO-induced activation of Tyk2 in the same Ba/F3 cells. Thus, GTP-14564 probably suppressed Tyk2 activation by inhibiting an upstream molecule, most likely ITD-FLT3. It is possible that ITD-FLT3 either activates STAT5 indirectly, through Tyk2 activation, or activates STAT5 directly, independently of Tyk2 activation. It was reported that Tyk2 activation induced cell growth through STAT5 activation (42). To test whether Tyk2 is involved in ITD-FLT3-mediated cell growth through STAT5 activation, it is required to examine whether a dominant negative form of Tyk2 suppresses FLT3-driven STAT5 activation and cell growth. To date, however, an efficient dominant negative form of Tyk2 has not been available. Kelly et al. recently reported that ITD-FLT3 induced myeloproliferative disease in a murine bone marrow transplant model (43). We are currently setting up a mouse leukemia model in which ITD-FLT3 is transduced into bone marrow progenitor cells of either normal mice or tyk2 gene-disrupted mice to determine whether Tyk2 plays critical roles in leukemia development induced by ITD-FLT3. Another receptor, PDGFR, a member of class III RTK family, was reported to induce phosphorylation of JAK and activation of STAT (44). However, PDGFR is not expressed in BaF3 cells and is obviously not involved in the ITD-FLT3 signal shown here. Alternatively, some cytoplasmic kinases such as c-Src may have mediated ITD-FLT3-driven phosphorylation of STAT5. Therefore, it is most likely that GTP-14564 inhibition of STAT5 activation and cell growth is due to the suppression of ITD-FLT3 by GTP-14564, because Tyk2 and c-Src are not the direct targets of GTP-14564.

Several other FLT3 inhibitors, including AG1295, CEP-701, CT5318, and PKC412, have been reported to inhibit the ITD-FLT3-driven proliferation of leukemic cells and transfectants expressing ITD-FLT3 (40–47). CEP-701, CT5318, and PKC412 have proved to be effective for mouse leukemia model induced by ITD-FLT3. However, whether these inhibitors were more effective on ITD-FLT3 than on wt-FLT3 is not known. As shown in this report, GTP-14564 is more effective on ITD and kinase-active mutants of FLT3 than on wt-FLT3. This is an important point when one thinks about the side effect of molec-
ERKs play a crucial role in FL-dependent cell growth, whereas STAT5
activation and that GTP-14564 preferentially inhibits ITD-FLT3-
the FLT3 receptor causes strong and constitutive STAT5 acti-
ivation in ITD-FLT3-driven growth signals, and dnSTAT5 signif-
icaently suppressed cell proliferation mediated by ITD-FLT3.

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