Stromal niche cells protect early leukemic FLT3-ITD⁺ progenitor cells against first generation FLT3 tyrosine kinase inhibitors

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Abstract
Targeting constitutively activated FLT3 (FLT3-ITD) by tyrosine kinase inhibition (TKI) in acute myeloid leukemia (AML) leads to clearance of blasts in the periphery but not in the bone marrow, suggesting a protective effect of the marrow niche on leukemic stem cells. In this study, we examined the effect of stromal niche cells on CD34+ progenitors from patients with FLT3-ITD+ or wild-type FLT3 (FLT3-WT) AML treated with the tyrosine kinase inhibitors SU5614 or sorafenib. TKI effectively and specifically inhibited FLT3, and increased the fraction of undivided progenitors in both FLT3-ITD+ and FLT3-WT samples. Treatment with SU5614 and sorafenib also reduced the number of mature leukemic progenitors, while contact with stroma protected against this cell loss. In contrast, primitive long-term progenitors from both FLT3-ITD+ and FLT3-WT AML were resistant to TKI. Additional contact with niche cells significantly expanded long-term FLT3-ITD+ but not FLT3-WT progenitors in the presence of SU5614 but not sorafenib. Thus, TKI with first generation inhibitors fails to eradicate early leukemic stem/progenitor cells in FLT3-ITD+ AML. Further, we defined a specific interaction between FLT3-ITD+ progenitors and niche cells that enables the maintenance of leukemic progenitors in the presence of TKI. Collectively, our findings suggest that molecular therapy may have unpredicted effects on leukemic progenitors, underscoring the necessity of developing strategies to selectively eliminate the malignant stem cell clone.
Introduction

Acute myeloid leukemia (AML) is organized as a hierarchy resembling normal hematopoiesis with leukemic stem cells (LSC) responsible for producing the bulk of leukemic blasts as well as sustaining the disease by their ability to self-renew (1, 2). Conventional chemotherapy induces high rates of remission but cures only a small percentage of patients with AML (3). The persistence of LSC in the bone marrow after chemotherapy is thought to be responsible for the high rate of relapse (4, 5). Therefore, new strategies to eradicate residual LSC are urgently needed. Targeting key signaling pathways with small molecule inhibitors is one therapeutic approach currently being evaluated.

FMS-like tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase over-expressed on leukemic blasts in almost all cases of AML (6). Activating mutations in the FLT3 gene in form of internal tandem duplications (FLT3-ITD) can be identified in one-third of AML patients and are associated with poor prognosis and increased relapse rates (7, 8). These mutations induce constitutive tyrosine kinase activity in the absence of FLT3 ligand (FL) and confer growth factor independence, proliferation and survival to myeloid cells in mouse models. Introduction of FLT3-ITD into murine bone marrow induces myeloproliferative disease, indicating the importance of FLT3 mutations in malignant transformation (9-11). FLT3-ITD mutations were shown to be present in primitive human CD34+CD38- cells, demonstrating that the mutation can occur within the hematopoietic stem cell compartment (12). Moreover, detection of FLT3-ITD in the CD34+CD33- stem/progenitor cell fraction in children with FLT3-ITD+ AML was associated with a particularly poor prognosis (13). Thus, targeting FLT3-ITD may improve prognosis by enabling eradication of leukemic CD34+ stem/progenitor cells. Indeed, inhibition of constitutively active FLT3 has been shown to prolong survival in a mouse model of FLT3-ITD+ leukemia (14, 15) and several tyrosine kinase inhibitors (TKI) have entered clinical trials (16-18). However, although inhibition of mutant FLT3 leads to clearance of leukemic blasts in the periphery, the
bone marrow often remains unchanged and remissions are usually short-lived (17, 19), raising the question whether a protective effect of the marrow niche on leukemic stem cells exists.

The stem cell niche of the bone marrow provides a supportive microenvironment for normal hematopoietic stem cells (20, 21) and regulates the balance between self-renewal and differentiation in the stem cell pool (20). Newer data suggests that leukemic stem cells are also protected by the stem cell niche and may even manipulate the microenvironment to their advantage (21, 22). If so, the interaction between leukemic FLT3-ITD+ stem/progenitors and the niche may influence the efficacy of TKI on these cells. We addressed this question by investigating the effects of the tyrosine kinase inhibitors SU5614 and sorafenib on leukemic CD34+ stem/progenitor cells from AML patients with wild-type (FLT3-WT) or mutated (FLT3-ITD) FLT3 receptor in the presence or absence of niche cells. The murine embryonic stromal cell line EL08-1D2 was used as an in vitro model for the stem cell niche (23).
Materials and Methods

Bone marrow samples

Bone marrow samples were obtained from patients recruited into the German AMLSG trials between 2002 and 2010 (Table 1). Written informed consent in accordance with the Declaration of Helsinki was obtained from all patients prior to bone marrow aspiration according to a protocol approved by the local Ethics Committee. All patients were newly diagnosed and untreated. Bone marrow samples underwent standardized processing including cytogenetics, fluorescence in situ hybridization and molecular genetics. All samples were screened for the presence of FLT3-ITD mutation as recently described(24) as well as for mutations in the tyrosine kinase domain of FLT3 (FLT3-TKD), partial tandem duplications of the MLL gene (MLL-PTD) and mutations of the nucleophosmin gene (NPM).

Cell isolation, enrichment, and CFSE staining

Mononuclear cells were enriched for CD34+ cells using magnetic selection as described by the manufacturers (Miltenyi Biotech). Enriched cells were analyzed for expression of CD34 and in some cases CD38 and FLT3 (CD135). Purity of CD34+ enriched cells ranged from 87-98%. Further lineage depletion of CD34+ cells was not performed due to primary sample size constraints. In some experiments, CD34-enriched cells were labeled for 10 min at 37°C with 2 µM of the fluorescent dye 5-(and 6-)carboxy-fluorescein succinimidyl ester (CFSE, Molecular Probes, Leiden the Netherlands) in IMDM (Gibco, Invitrogen, Karlsruhe Germany), 1% FCS and 10 mM Hepes (Gibco) as described (25). To determine the location of undivided cells for cell division tracking, a control culture was set up in the same manner but additionally supplemented with colcemid (Karyomax, Gibco) as described (31). Stromal cell lines FBMD-1 and EL08-1D2 were cultured as described (23). The RS4;11 and MV4-11 human leukemia cell lines were obtained from and propagated as suggested by the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig,
Germany) and were authenticated by DSMZ using DNA-typing and PCR analysis as well as cytogenetic testing. Cells used for all experiments were passaged for fewer than 6 months after receipt.

Culture of CD34\(^+\) progenitor cells from primary AML samples

CD34\(^+\) progenitors from AML bone marrow samples were cultured in serum-free medium supplemented with five growth factors (5GF): kit-ligand (KL), FLT3-ligand (FL), thrombopoietin (TPO), interleukin-3 (IL-3) and Hyper-IL-6, a designer cytokine consisting of IL-6 and soluble IL-6 receptor (H-IL-6, kind gift from S. Rose-John, Kiel, Germany)(26). Cells were cultured in suspension or on confluent EL08-1D2 stromal cells and treated with SU5614, sorafenib or DMSO as indicated. Cells were maintained at 37°C in a humidified atmosphere with 5% CO\(_2\). After 4 days, cells were harvested and assayed for short and long-term hematopoietic activity.

Analysis of apoptosis in CD34\(^+\) cells

CD34\(^+\) bone marrow cells were cultured in serum-free medium with 5GF and DMSO or SU5614. After the indicated time, cells were harvested and stained for Annexin V and propidium iodide (PI) as described by the manufacturers (ApoTest, Becton Dickinson, Heidelberg). Cells were washed in PBS supplemented with 1% BSA, resuspended in PBS buffer containing RNase (100 µg/ml) and propidium iodide (50 µg/ml), followed by flow cytometry performed on an Epics XL Cytometer (Beckman Coulter, Krefeld, Germany). Acquired data was analyzed using FlowJo Software Version 8.7.3 for Macintosh.

Hematopoietic progenitor cell clonogenic assays

Mature hematopoietic progenitors were assessed by colony formation before and after 4 days of 5 GF supplemented serum-free culture. 1000 to 5000 input cell equivalents were plated in growth factor-supplemented methylcellulose (H4435,
Stem Cell Technologies) and incubated at 37°C in a humidified atmosphere with 5% CO₂. After 14 days, colony-forming units (CFU) were scored using standard criteria. Immature hematopoietic stem/progenitors were determined after long-term culture on the FBMD-1 stromal cell line in the presence of TPO and FL as described (25). After 6 weeks of culture, the entire culture was harvested by trypsin detachment and assayed for the presence of long-term culture-derived colony-forming cells (LTC-CFC) in methylcellulose.

Polymerase chain reaction for FLT3

AML bone marrow samples were validated for the presence of FLT3-ITD by polymerase chain reaction (PCR) after isolation of mononuclear cells as well as after CD34-enrichment and after culture in methylcellulose. For PCR from hematopoietic colonies, single hematopoietic colonies were picked from methylcellulose. Total genomic DNA was isolated using the QiaAmp Micro kit (Qiagen) and eluted into 30 µl TE buffer. Eight microliters of single colony-derived genomic DNA were amplified by FLT3-ITD PCR. Products were resolved on 2% agarose gels and visualized under ultraviolet light after ethidium bromide staining.

Western blot analysis

MV4-11 or RS4;11 cells or primary CD34⁺ cells were starved overnight in suspension culture or on the stromal cell line EL08-1D2 at 37°C in 5% CO₂. Cells were incubated with SU5614 (5 µM), sorafenib (100 nM) or DMSO at 37°C and 5% CO₂ for indicated time periods. Cells were placed in pre-chilled tubes containing ice-cold PBS with Na₂VO₄ (1 mM, Sigma) and washed once in cold PBS with Na₂VO₄. Cell lysis, SDS-PAGE and immunoblotting were done as described (25). Antibodies to FLT3 (S-18, sc-480 Santa Cruz) and pFLT3 (3461, Cell Signalling Technologies), AKT1/pS473 and AKT1/pT308 AKT (all from Cell Signaling), ERK1 (K-23, Santa Cruz), pY204-ERK1 (E4, Santa Cruz), β-actin (Sigma), pY694STAT5 and STAT5
(Cell Signalling Technologies), phosphotyrosine (4G10 and PY20, Transduction Laboratories) were used as described in manufacturer’s instructions. Signals were visualized on Kodak film using polyclonal secondary horse-radish peroxidase-labeled antibodies (Pierce) and enhanced chemoluminescence (Pierce). Band intensities were determined using the ImageJ software package (NIH, USA).

**ELISA for FLT3 Ligand**

Levels of murine and human FL in stroma co-culture were measured using species-specific Quantikine ELISA kits for murine (MFK00) and human FLT3 ligand (DFK00) following the manufacturer’s instructions (R & D Systems, Minneapolis, MN, USA).

**Statistics**

For analysis of functional assays of patient samples, we used non-parametric tests for both unpaired (between WT and ITD) and paired (within either WT or ITD) comparisons. For unpaired comparisons we used the Mann-Whitney U test, for paired samples the Wilcoxon matched pairs signed-rank test. To test for possible differences between other groups in other experiments we used the paired $t$ test. Statistical testing was performed using InStat software (Graphpad Software, San Diego, CA, USA).
Results

Characteristics of AML patient samples

The characteristics of AML bone marrow samples used in this study are summarized in Table 1. A total of 21 AML samples harboring the FLT3-ITD mutation were analyzed and compared to 13 AML samples without the mutation (FLT3-WT). The majority of samples (23/34) had normal cytogenetics. With the exception of sample 2, which additionally harbored an MLL-PTD mutation and samples 10-12 as well as 20, 21, 27, which were NPM mutated, none of the other molecular abnormalities routinely screened for were detected in the remaining 27 AML samples. Primary FLT3-ITD+ AML samples showed variable expression of CD34 (Table 1), CD38 and FLT3 receptor (CD135), as shown in Figure 1. Enrichment for CD34 by magnetic bead isolation was successful in all samples.

Cell death induction in primary leukemic FLT3-ITD+CD34+ cells by SU5614

The effect of TKI on growth factor stimulated leukemic CD34+ cells was first studied using the tyrosine kinase inhibitor SU5614, which has been shown to effectively inhibit FLT3-ITD as well as activated FLT3-WT kinase (27). We confirmed the selective inhibitory effect of SU5614 in human leukemia cell lines containing either the wild-type FLT3 receptor (RS4;11) or FLT3-ITD mutation (MV4-11) (Figure 2A). Proliferation of MV4-11 cells was inhibited by SU5614 whereas growth of RS4;11 cells was not affected. Ligand stimulation did not increase susceptibility of RS4;11 cells to SU5614 while inhibition of MV4-11 cells by SU5614 was partially overcome by stimulation with FL. Constitutively active FLT3-ITD in MV4-11 cells was completely inhibited by treatment with SU5614 (Figure 2B). Direct contact of leukemic cells with EL08-1D2 stromal cells did not significantly influence FLT3 activation or its inhibition by SU5614.

In primary CD34+FLT3-ITD+ cells, induction of apoptosis by SU5614 was moderate (Figure 2C), while apoptosis induction in CD34+FLT3-WT cells was not statistically
significant (Figure 2C). Culture of primary CD34+ leukemic cells on EL08-1D2 stroma prevented SU5614 induced apoptosis. This was not observed for MV4-11 cells (Figure 2C).

FLT3-ITD specific downstream signaling is uncoupled from FLT3-ITD in CD34+ leukemic cells

Phosphorylation of FLT3 in CD34+FLT3-ITD+ AML cells was suppressed by treatment with SU5614 (Figure 3A), confirming inhibition of the target mutation in primary leukemic cells. Co-culture on EL08-1D2 stroma partially overcame inhibition of phosphorylation through SU5614 (Figure 3A and B). We therefore looked downstream from FLT3 to investigate whether signaling differences occurred during co-culture on stroma. Of particular interest was STAT5, whose phosphorylation and subsequent activation is a hallmark of the oncogenic FLT3-ITD pathway (28, 29). In MV4-11 cells, SU5614 inhibited phosphorylation of STAT5 in suspension and when co-cultured with EL08-1D2 (Figure 3C and 3D). Phosphorylation of both T308 and S473 AKT and ERK were also inhibited by SU5614 (Figure 3D). In EL08-1D2 stromal cells analyzed as a control, both AKT phosphorylation sites were found to be constitutively phosphorylated and were not inhibited by SU5614 (Figure 3D).

In untreated AML patient samples, activated STAT5 was more readily detectable in CD34+FLT3-ITD+ cells (7/7 samples) than FLT3-WT cells (4/7 samples) and was not inhibited by SU5614 (Figure 3C and 3E). In CD34+FLT3-ITD+ cells cultured in suspension, SU5614 inhibited phosphorylation of AKT at T308 as well as phosphorylation of ERK, while phosphorylation of AKT at S473 was not affected (Supplementary Figure S3). Co-culture on EL08-1D2 cells did not influence signaling pathways downstream of FLT3 either in CD34+FLT3-ITD+ or FLT3-WT cells (Figure 3E and S3). Thus, while SU5614 inhibits FLT3 as well as T308AKT and ERK pathways, STAT5 and S473AKT phosphorylation appear to be uncoupled from FLT3-ITD activation status in primary leukemic CD34+ cells.
SU5614 increases the fraction of undivided CD34+ leukemic cells

Analysis of primary CD34+ AML cells revealed the majority to be in G0/G1 phase of the cell cycle after four days of growth factor stimulation (data not shown), precluding detection of an inhibitory effect of SU5614 on cell cycle. Therefore, although one mechanism of action for TKI is cell cycle arrest, this effect is not readily detectable in primary CD34+ cells. We reasoned that an analysis of cell division might provide a better discrimination of TKI effect. Cell division was indeed detectable by CFSE staining, with the majority of samples dividing once during the 4-day period. FLT3-ITD+ AML samples contained a significantly higher proportion of cells that did not divide during the 4-day culture compared to FLT3-WT samples (Supplementary Figure S1). Treatment with SU5614 significantly increased the fraction of non-dividing cells in both FLT3-ITD+ and FLT3-WT samples. This effect was almost completely reversed by co-culture with EL08-1D2 stroma for CD34+FLT3-WT cells but not for CD34+FLT3-ITD+ cells (Supplementary Figure S1C).

TKI eradicates short-term committed leukemic progenitor cells but stromal contact counteracts this effect

Next, we investigated hematopoietic activity of primary CD34+ AML bone marrow cells cultured for 4 days in the presence or absence of SU5614 with or without EL08-1D2 cells. The 4-day incubation was chosen as this is the time period within which progenitors undergo at least one division but do not lose CD34 expression, as we have shown for normal hematopoiesis (30) and have confirmed by our CFSE experiments for leukemic progenitors. All of the five FLT3-WT AML samples studied yielded colony growth at day 0 (i.e. input) as well as after the 4-day culture. However, 3 of the 12 FLT3-ITD+ AML samples (samples 3, 6 and 7) used for progenitor cell assays did not form colonies either at day 0 or after 4 days of in vitro culture (Table 1 and supplementary data, Table S1). Overall, untreated FLT3-ITD+CD34+ progenitors produced significantly fewer colonies than CD34+ cells from
FLT3-WT AML samples (Figure 4A and Table S1), in accordance with published observations (31). Treatment with SU5614 reduced committed progenitors in suspension culture by 77% for FLT3-ITD+ and 44% for FLT3-WT samples, demonstrating that SU5614 targets committed leukemic progenitors (Figure 4A and Table S1). Direct contact with EL08-1D2 did not significantly enhance expansion of committed CFU in either FLT3-ITD+ or FLT3-WT AML samples. However, culture with EL08-1D2 stromal cells completely abrogated the inhibitory effect of SU5614 on leukemic progenitor cell growth (Figure 4A and Table S1). Production of FL by either EL08-1D2 stromal cells or autocrine secretion of FL by AML cells was not responsible for the protective effect of stroma, since neither murine nor human FL were secreted at detectable levels in co-culture (Supplementary Figure S2).

Inhibition of activated FLT3 by SU5614 does not eradicate primitive leukemic progenitors in FLT3-ITD+ or FLT3-WT AML

To address the question whether inhibition of activated FLT3 can effectively target the most primitive leukemic progenitor cell population, we performed long-term in vitro culture experiments, resulting in a functional read-out for the early progenitor cell fraction (30). As shown in Figure 4B and Table S2, the number of more primitive LTC-CFC was very heterogeneous within untreated FLT3-ITD+ and FLT3-WT samples and not statistically different between the two groups. Exposure to SU5614 over four days in suspension culture did not eliminate primitive leukemic progenitors, either in FLT3-ITD+ or FLT3-WT samples (Figure 4B and C, Table S2).

Early FLT3-ITD+ stem/progenitor cells protected by stromal niche cells are amplified by treatment with SU5614

To determine the contribution of stromal support on maintenance of leukemic LTC-CFC, we assessed the effect of SU5614 on primitive CD34+ progenitors cultured on EL08-1D2 stroma. We have previously shown that this murine embryonic stromal
cell line supports long-term production of both mature and immature human hematopoietic progenitors and can therefore mimic the stem cell niche in vitro (23, 32). Culture on EL08-1D2 effectively prevented loss of FLT3-ITD^+ LTC-CFC during the 4-day in vitro culture (Figure 4B). In contrast, there was no significant difference in LTC-CFC number between suspension cultures and stromal supported cultures for FLT3-WT samples (Figure 4B). Absolute colony numbers from all bone marrow samples are summarized in supplementary Table S2.

An unexpected finding was the expansion of LTC-CFC in the context of stromal support and treatment with SU5614 in FLT3-ITD^+ AML samples. As depicted in Figure 4B, LTC-CFC were expanded 3.5-fold compared to day 0 in the presence of EL08-1D2 and SU5614. Compared to progenitors cultured on stroma without TKI, expansion of LTC-CFC was 2.6-fold in the presence of SU5614. The increase in LTC-CFC on stroma in the presence of SU5614 was observed for 8/9 FLT3-ITD^+ patient samples, ruling out the possibility of singular outliers distorting the overall results. To better assess the contribution of EL08-1D2 stroma to stimulation of leukemic progenitors, a ratio between colony numbers for SU5614-treated vs. DMSO-treated cultures was formed. As shown in Figure 4C, stromal support led to significant stimulation of colony growth in FLT3-ITD^+ progenitors while this was not the case for FLT3-WT progenitor cells, indicating that stromal support is necessary for expansion for FLT3-ITD^+ progenitors.

Expanded LTC-CFC are of leukemic origin

Since primitive AML progenitors are thought to have a growth advantage over their normal counterparts (33), we sought to ascertain whether expanded LTC-CFC from FLT3-ITD^+ samples were of leukemic origin or if stromal contact conferred a survival advantage to healthy progenitor cells. PCR for FLT3-ITD in individual hematopoietic colonies from methylcellulose confirmed presence of the FLT3
mutation in LTC-CFC from all patient samples (Supplementary data, Figure S4),
indicating persistence of early leukemic progenitors.

Confirmatory Studies with Sorafenib

To confirm the general applicability of our findings, we repeated key experiments using a second TKI, sorafenib. This compound is a more selective and potent inhibitor of FLT3 than SU5614 and is currently widely used in the clinic (34-36). As shown in Figure 5A, sorafenib effectively inhibited activated FLT3 in WT as well as FLT3-ITD+ AML. Inhibition of FLT3 phosphorylation by sorafenib was not influenced by stromal contact. As with SU5614, STAT5 signaling downstream of FLT3-ITD was not inhibited by sorafenib (Figure 5B), confirming uncoupling of FLT3-ITD and STAT5 in primary CD34+FLT3-ITD+ AML cells.

Colony-forming assays revealed that like with SU5614, treatment with sorafenib also did not eliminate short-tem or long-term leukemic progenitors (Figure 5C). Co-culture with EL08-1D8 stromal cells again abolished the inhibitory effect of sorafenib, although expansion of progenitor cells was not statistically significant. Analysis of colonies by PCR again confirmed that a substantial number of colonies recovered from short- as well as long-term cultures contained the FLT3-ITD mutation despite treatment with sorafenib (Supplementary Figure S4E).
Discussion

Despite entry of FLT3 inhibitors into clinical trials for FLT3-ITD+ AML, it has so far not been established whether inhibition of aberrant FLT3 signaling can actually eradicate the earliest stem/progenitor cells responsible for propagating the disease. Using the FLT3 inhibitors SU5614 and sorafenib as proof of concept, we show here in primary bone marrow samples from patients with newly diagnosed FLT3-ITD+ AML that treatment with TKI does not eliminate early CD34+FLT3-ITD+ leukemic stem/progenitor cells. In addition, we demonstrate a protective effect of the stromal microenvironment on these cells, conferring a growth advantage to FLT3-ITD+ leukemic progenitors over normal ones in the presence of TKI.

We show that primary early leukemic CD34+FLT3-ITD+ progenitors are insensitive to the cytotoxic effects of TKI. The majority of CD34+FLT3-ITD+ cells divided upon cytokine stimulation in vitro, with 50% of cells undergoing at least one cell division within 4 days. Treatment with SU5614 significantly increased the fraction of undivided cells, suggesting the predominant effect of the inhibitor on this population is a decrease in cell division, not induction of cell death.

The inherent unresponsiveness of early FLT3-ITD+ leukemic progenitors to TKI may be due to the fact that they are not dependent on mutant FLT3 signaling for survival. In contrast to chronic myeloid leukemia (CML), more than one genetic alteration is necessary to cause AML. Therefore, inhibiting activated FLT3-kinase may not be sufficient to eliminate the stem cell fraction, or FLT3-ITD may not be the relevant target in these early cells. For CML, it has been elegantly shown that primitive CD34+ stem/progenitors are insensitive to first and second generation Bcr-Abl inhibitors and are instead induced into quiescence by treatment with TKI (37, 38). Our results suggest that this principle may be true for primitive FLT3-ITD+ progenitors from AML as well. This counterproductive effect on leukemic stem/progenitor cells has also been reported for other novel treatment strategies in AML, such as the histone deacetylase inhibitor valproate (39).
An alternative explanation may be that current TKI are not efficient enough to completely prevent constitutive activation of FLT3. Since it has been suggested that complete and enduring inhibition of FLT3 phosphorylation is critical for achieving clinical efficacy (40, 41), failure to completely inhibit FLT3 may contribute to persistence of leukemic progenitors after treatment. However, to our knowledge it has not yet been definitively demonstrated that complete inhibition of FLT3 actually translates into improved clinical outcome in FLT3-ITD+ AML. Increased allelic ratios of FLT3-ITD to WT receptor have also been linked to poor prognosis (42). In our small cohort, we could not observe a correlation between allelic ratio and outcome of progenitor assays.

Additionally, continued activation of signaling pathways downstream of FLT3 through other mechanisms may contribute to TKI resistance in CD34+FLT3-ITD+ progenitors. Our results demonstrate uncoupling of FLT3-ITD from STAT5 signaling in primary CD34+FLT3-ITD+ cells in the presence of TKI. Activation of STAT5 by FLT3 has been shown to be dependent on intracellular localization of the receptor (29), thus restricted to mutated FLT3 and not found in FLT3-WT signaling. In addition, FLT3-ITD itself has recently been demonstrated to be differentially phosphorylated on different tyrosine residues in a compartment-dependent manner (29). In this context, our results suggest that inhibition of mutated FLT3 by TKI in primary patient samples may take place mainly at the plasma membrane, allowing intracellular activation of STAT5 to persist.

Our data extend findings previously made using leukemic cell lines with acquired resistance to TKI demonstrating continued STAT5 activation in resistant cells (43, 44). Prolonged exposure of leukemic cell lines or AML blasts to TKI selects for cells that are FLT3 independent, leading to pharmacological resistance (35, 43, 44). However, our results suggest that at least primitive CD34+FLT3-ITD+ progenitors are insensitive to TKI from the onset of treatment. The inability of TKI to sustain suppression of leukemic blasts may therefore be due not only to outgrowth of
resistant blasts but also to the fact that the more primitive leukemic stem/progenitors
maintaining the disease persist despite treatment with TKI. Thus, our data offer an
additional explanation to the transient clinical responses seen so far with TKI in
FLT3-ITD⁺ AML. However, these results should be interpreted with caution as third-
generation FLT3 inhibitors with improved pharmacokinetic properties now entering
clinical trials (45) may yield different results.

Finally, we show that interaction of CD34⁺FLT3-ITD⁺ progenitors with stromal
niche cells mimicking the bone marrow environment protects these cells from the
effects of TKI. Since FL was not significantly produced by either stromal cells or AML
cells during co-culture, this protective effect is not dependent on FL in our in-vitro
system, as has been observed in vivo in response to chemotherapy(46). Although
similar protective effects of stroma have been reported for normal progenitors, our
observation that the combination of niche cells and concomitant TKI actually may in
some instances even lead to expansion of malignant progenitors is unexpected. This
effect was specific for FLT3-ITD⁺ progenitors and not observed for FLT3-WT cells,
pointing to a differential response of FLT3-ITD⁺ cells to the niche. However, we did
not observe this expansion when leukemic progenitors were treated with sorafenib.
Since SU5614 and sorafenib show differential inhibitory effects on FLT3-WT and
FLT3-ITD, the difference in observed effects on long-term CFC-producing cultures
may be due to the weaker efficiency of SU5614 in inhibiting FLT3 or to an altered
balance between inhibition of WT and mutant FLT3. The mechanistic basis for our
finding is still unclear but suggests that the niche generates specific self-renewal or
survival signals, perhaps in reaction to leukemic cells themselves, to which FLT3-
ITD⁺ progenitors are able to uniquely respond.

Taken together, these data highlight the fact that molecular therapy may have
unpredicted effects on leukemic stem/progenitor cells and underscores the
importance of developing strategies to selectively eliminate the malignant stem cell
cloned. Our data point to an altered interaction between FLT3-ITD⁺ stem/progenitors
and the stem cell niche. To efficiently target FLT3-ITD+ stem/progenitor cells in AML, future investigations should focus on how the bone marrow microenvironment regulates these cells. Our results suggest that combining inhibitors to additionally block downstream pathways (e.g. STAT5) or adding agents that disrupt the interaction between leukemic stem cell and niche (e.g. CXCR4 antagonists) may be necessary to overcome the unresponsiveness of FLT3-ITD+ leukemic stem/progenitor cells to TKI (44, 47). Finally, our findings have potentially important clinical implications for the use of TKI to treat FLT3-ITD+ AML as they raise the possibility of unwittingly amplifying leukemic stem/progenitor cells.

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References


Table 1. AML sample characteristics

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* Other molecular mutations screened for were FLT3-TKD, MLL-PTD and NPM1. n/a, not available
Figure Legends

Figure 1. Expression of CD34, CD38 and FLT3 receptor before and after CD34 enrichment in FLT3-ITD+ primary AML samples.

Bone marrow mononuclear cells from patients with FLT3-ITD+ AML were stained for expression of CD34, CD38 and FLT3 (CD135) before and after enrichment for CD34 by magnetic bead isolation. Bars on top of each plot show number of CD34+ cells (as % of total cells), boxes represent either CD34+CD38- cells or CD34+FLT3+ cells. Results from four representative AML samples (13, 18, 19, 21, Table 1) are shown.

Figure 2. SU5614 induces death in MV4-11 cells and CD34+FLT3-ITD+ cells but contact with niche cells confers protection to leukemic progenitors.

(A) Dose response for MV4-11 and RS4;11 cells treated with SU5614 for 96 hours ± FL (50 ng/ml). Proliferation was determined by MTT assay in triplicate. Results shown are mean ± standard error of the mean (SEM) of 3 independent experiments. * p ≤ 0.05, ** p ≤ 0.01.

(B) MV4-11 and RS4;11 cells were treated with SU5614 for 30 min. ± FL (50 ng/ml) and EL08-1D2 stroma. FLT3 was immunoprecipitated from cell lysate and resolved by SDS-PAGE. Blots were probed with anti-phospho-FLT3 antibody (591Y) and membrane stripped and reprobed for total FLT3 to confirm equal loading.

(C) MV4-11 cells (grey bars), CD34+FLT3-WT (open bars) or CD34+FLT3-ITD+ cells (black bars) were treated in serum-free medium with 5 GF with SU5614 (5µM) or DMSO for 96 hours with or without co-culture on EL08-1D2 stroma. Percentage of live cells (defined as AnnexinV and PI negative) was determined by flow cytometry. Results are representative of 3 (MV4-11), 3 (FLT3-WT, samples 26-28) or 5 (FLT3-ITD+ samples 10-14) independent experiments. Error bars indicate SEM.
Figure 3. SU5614 inhibits phosphorylation of FLT3, AKT and ERK but not STAT5 in CD34+FLT3-ITD+ cells.

(A) FLT3 phosphorylation in primary CD34+ cells following treatment with SU5614. CD34+ cells were incubated in serum-free medium with 5GF overnight in suspension or co-cultured with EL08-1D2 stromal cells as indicated. Cells were incubated with DMSO or SU5614 (5µM) for 30 min. FLT3 was immunoprecipitated with FLT3 antibody (S-18). PVDF membranes were probed with anti-phosphotyrosine antibodies (4G10 and YP20), stripped and reprobed for FLT3 to confirm equal loading. Results of 2 representative samples (21 and 34) are shown.

(B) Quantitation of FLT3 inhibition by SU5614 in primary CD34+ AML cells treated as in A. Western blots were quantitated using ImageJ. Results shown are SEM from 3 individual blots each for FLT3-WT (samples 28, 31, 34) and FLT3-ITD (samples 12, 13, 21).

(C) Quantitation of STAT5 phosphorylation in MV4-11 cells and primary CD34+ cells from FLT3-ITD+ or FLT3-WT AML following treatment with SU5614. Cells were treated as in (A). Lysed samples were subjected to immunoblotting with anti-phosphoSTAT5. Quantitation of western blots was performed with ImageJ. Results are representative of 7 experiments with MV4-11, 7 FLT3-ITD+ AML samples (12-17, 21) and 7 FLT3-WT AML samples (28-34, only those samples with detectable phosphorylation of STAT5 were used for quantitation: 29, 30, 31, 34). Error bars indicate SEM. *** p=0.005; * p=0.04.

(D) Analysis of signaling pathways downstream of FLT3. MV4-11 cells were treated as in (A). Lysed samples were subjected to immunoblotting with antibodies as indicated. EL08-1D2 cells were starved over night in serum-free medium, incubated with DMSO or SU5614 (5µM) for 30 min and subjected to immunoblotting. PVDF membranes were probed with anti-phosphotyrosine antibodies, stripped and reprobed for total protein.
(E) Analysis of signaling pathways downstream of FLT3 in primary AML cells. CD34+FLT3-ITD+ and CD34+FLT3-WT cells were treated as in (A) and subjected to immunoblotting as in (D). Shown are blots prepared from one representative patient sample each for CD34+ FLT3-WT (sample 28) and FLT3-ITD+ (sample 13) AML.

Figure 4. Effect of stromal niche cells on progenitor cell activity of CD34+ AML cells treated with SU5614.

(A) Short-term colony forming assay. CD34+ FLT3-WT or FLT3-ITD+ AML cells were incubated for 4 days in serum-free medium with 5 GF and DMSO or SU5614 (5µM) in suspension or co-cultured with EL08-1D2 stroma as indicated. After 4 days, cells were harvested and plated in methylcellulose to determine colony-forming units (CFU). Colonies were scored after 14 days using standard criteria. 'Input' represents CFU assay of untreated CD34+ cells on day 0. Results are shown as colony number per input cell number at day 0. Left panel shows mean number of CFU from 5 individual samples for FLT3-WT AML (open bars, samples 22-26) and 9 individual samples for FLT3-ITD+ AML (black bars, samples 1, 2, 4, 5, 8-12). Error bars indicate SEM. Right panel shows individual results for FLT3-ITD+ AML samples. Each icon represents a separate patient sample.

(B) Long-term colony forming assay. CD34+ FLT3-WT or FLT3-ITD+ AML cells treated as in (A). After 4 days, cells were harvested and subjected to long-term culture on FBMD-1 stromal cells in the presence of TPO and FL. After 6 weeks, cells were harvested and plated in methylcellulose to determine number of long-term colony forming cells (LTC-CFC). Left panel shows mean number of LTC-CFC from same samples as in (A). Error bars indicate SEM. Right panel shows the distribution of individual FLT3-ITD+ AML samples. Each icon represents a separate patient sample.

(C) To determine the contribution of stroma support to leukemic colony growth, a ratio of SU5614/DMSO colony numbers was formed. Values below the shaded area
indicate inhibition, values above the shaded area stimulation of colony growth for the conditions indicated. Each icon represents an individual patient sample.

**Figure 5. Confirmatory studies with sorafenib.**

(A) FLT3 phosphorylation in primary CD34^+FLT3-ITD^+ cells following treatment with sorafenib. CD34^+ cells were incubated in serum-free medium with 5GF overnight in suspension or co-cultured with EL08-1D2 stroma as indicated. Cells were incubated with DMSO or sorafenib (100 nM) for 30 min. FLT3 was immunoprecipitated with FLT3 antibody (S-18). PVDF membranes were probed with anti-phosphotyrosine antibodies (PY20 and 4G10), stripped and reprobed for FLT3 to confirm equal loading. Results of 2 representative samples (21, 28) are shown.

(B) Quantitation of STAT5 phosphorylation in primary CD34^+FLT3-ITD^+ cells following treatment with sorafenib. Cells were treated as in (A). Quantitation of western blots was performed with ImageJ. Results shown are representative of 3 individual samples (13, 16, 21). Error bars indicate SEM.

(C) Short- and long-term colony forming assays. CD34^+FLT3-ITD^+ cells were incubated for 4 days in serum-free medium with 5 GF and DMSO or sorafenib (100 nM) in suspension or co-cultured with the adherent stromal cell line EL08-1D2 as indicated. After 4 days, cells were harvested and assayed as in Figure 4. Left panel shows mean number of CFU from 5 individual samples (13, 16, 17, 18, 21), right panel shows mean number of LTC-CFC from same samples. Error bars indicate SEM.
FIGURE 2

A

\[ \text{OD}_{490} \]

- RS4;11
- RS4;11 + FL
- MV4-11
- MV4-11 + FL

SU5614 (uM)

B

- RS4;11
- MV4-11

EL08-1D2

pY591 FLT3

FLT3

C

\[ \text{Annexin V: PI} \ (\%) \]

- MV4-11
- CD34*FLT3-WT AML
- CD34*FLT3-ITD+ AML

5uM SU5614

EL08-1D2

p=0.01

p=0.03

p=n.s.

p=0.02

p=0.04

p=0.01

p=0.03

p=0.03
**FIGURE 3**

A

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B

![Bar chart showing the ratio of pTyr-FLT3 between FLT3-WT and FLT3-ITD+ cells with treatment with SU5614 and EL08-1D2.](chart)

C

![Graph showing pY694 STAT5 levels in MV4-11, FLT3-WT, and FLT3-ITD+ cells with treatment with SU5614 and EL08-1D2.](chart)

D

![Western blot analysis of MV4-11 and EL08-1D2 cells with and without treatment with EL08-1D2 and SU5614 showing the following proteins: pY694 STAT5, STAT5, pT308 AKT, AKT, pS473 AKT, AKT, pT202/4 ERK1/2, ERK 1/2, and beta-actin.](blot)

E

![Western blot analysis of CD34+ AML FLT3-WT and FLT3-ITD+ cells with and without treatment with EL08-1D2 and SU5614 showing the following proteins: pY694 STAT5, STAT5, pT308 AKT, AKT, pS473 AKT, AKT, pT202/4 ERK1/2, ERK 1/2, and beta-actin.](blot)
Stromal niche cells protect early leukemic FLT3-ITD+ progenitor cells against first generation FLT3 tyrosine kinase inhibitors

Amanda Parmar, Stefanie Marz, Sally Rushton, et al.

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