Terminal myeloid differentiation in vivo is induced by FLT3 inhibition in FLT3/ITD AML

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Abstract

A hallmark of cancer is the disruption of differentiation within tumor cells. Internal tandem duplication mutations of the FLT3 kinase (FLT3/ITD) occur commonly in acute myeloid leukemia (AML) and are associated with poor survival, leading to efforts to develop FLT3 kinase inhibitors. However, FLT3 inhibitors have thus far met with limited success, inducing only a clearance of peripheral blasts with minimal marrow responses. Quizartinib is a novel potent and selective FLT3 inhibitor currently being studied in clinical trials. In 13 out of 14 FLT3/ITD AML patients with normal karyotype treated with quizartinib, we observed terminal myeloid differentiation of marrow blasts in association with a clinical differentiation syndrome. The single patient whose blasts failed to differentiate had a pre-existing C/EBPα mutation, while another developed a C/EBPα mutation at disease progression, suggesting a mechanism of resistance to FLT3 inhibition. In vitro, in primary blasts co-cultured with human bone marrow stroma, FLT3 inhibition with quizartinib induced cell cycle arrest and differentiation rather than apoptosis. This is the first description of terminal differentiation of cancer cells in patients treated with a tyrosine kinase inhibitor. These data highlight the importance of the differentiation block in the pathogenesis of AML.

(NCI clinical trials # NCT00989261)
Introduction

A characteristic of cancer cells is their ability to avoid terminal differentiation,\(^1\) and acute myeloid leukemia (AML) has been recognized as representing a paradigm for this phenomenon.\(^2\) Disruption of a differentiation-inducing transcription factor can be identified in over one third of newly diagnosed cases of AML.\(^3-6\) While carcinogenesis is generally believed to be a process requiring multiple mutations, some have proposed that AML develops out of a central core of 2 classes of mutations – one blocking differentiation and the other promoting proliferation via effects on growth factor pathways.\(^7\) Internal tandem duplication mutations of the FLT3 receptor tyrosine kinase (FLT3/ITD), one of the most common mutations in AML,\(^8\) constitutively activate this cytokine receptor and are members of the “growth factor” class in this 2-step model.

Because FLT3/ITD mutations confer a negative prognostic effect on the clinical outcome of AML, FLT3 TKIs have been investigated as a potential targeted therapy for this disease. Clinical responses to FLT3 inhibitors were initially confined to clearance of peripheral blasts with minimal effects on the bone marrow.\(^9-13\) However, the use of more potent FLT3 inhibitors such as sorafenib and quizartinib has resulted in a higher rate of marrow blast reduction in FLT3/ITD AML patients.\(^14\)

In an exploratory group of relapsed/refractory FLT3/ITD AML patients treated with quizartinib (as part of a phase 2 trial), a remarkably high clinical response rate of 71% was recently reported.\(^15\) The bone marrow and peripheral blood specimens from these patients, particularly those with normal cytogenetics, displayed an unexpected finding. Instead of the typical rapid loss of cellularity seen following conventional cytotoxic chemotherapy, the marrows remained hypercellular, continued to express the FLT3/ITD mutation, and displayed progressive myeloid differentiation over time. This is similar to the response of acute
promyelocytic leukemia (APL) to all-trans retinoic acid (ATRA), in which a cellular marrow and persistence of the leukemia-specific mutation PML-RAR\(\alpha\) is observed as the ATRA promotes differentiation from immature leukemic promyelocytes to mature neutrophils.\(^{16}\)

To study this phenomenon further, we developed an in vitro model of FLT3 inhibitor-induced differentiation using co-culture of leukemia cells with human bone marrow stroma. We conclude that in FLT3/ITD AML, aberrant signaling from the mutant FLT3 receptor is responsible for the block in differentiation that is so characteristic of this disease. Previous in vitro data suggested a potential role of FLT3/ITD in myeloid differentiation.\(^{17,18}\) Here we provide the first evidence that TKI therapy induces terminal differentiation of human cancer cells in vivo. Our data provides new insight into the role of tyrosine kinases in the pathogenesis of cancer, and help define the clinical and biological consequences of FLT3 inhibition for FLT3/ITD AML.
Methods

Patients

This is a laboratory and clinical correlative study using bone marrow and blast samples from a subset of patients enrolled on protocol AC220-002 (NCI clinical trials # NCT00989261). AC200-002 is a phase 2 multi-center, international study of quizartinib administered as monotherapy to patients with relapsed or refractory FLT3/ITD AML (as well as a smaller cohort of patients with wild type FLT3) that accrued 333 patients. Although interim results from a safety subset of patients from this trial have been presented, final analysis of the clinical data is not yet available. This correlative study is based on 28 patients with FLT3/ITD mutations meeting eligibility requirements who were accrued to the trial at two institutions. Additionally, patient blasts were collected and banked separately as part of the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins Tumor and Cell Procurement Bank, supported by the Regional Oncology Research Center Grant # 2 P30 CA 006973-44. Whole blood and bone marrow aspirates from healthy donors were also collected under this procurement protocol. All patients gave informed consent according to the Declaration of Helsinki.

Clinical specimens

Bone marrow aspirates were collected on days 0, 15, and 29. Slides were fixed in methanol and stained with Wright-Giemsa stain (Harleco/VGD Inc., Lawrence, KS). A 100-cell manual differential was performed using standard clinical protocols. Unstimulated cultures of bone marrow were performed overnight and slides were prepared. G-banding was carried out according to standard clinical protocols. Twenty metaphases were analyzed for each case. No additional growth factors were used for conventional cytogenetic analysis. For analysis of cell
morphology, cytospins were prepared using 1x10^5 cells, which were centrifuged onto a glass slide, and then fixed and stained with Modified Wright-Giemsa stain (Sigma, St. Louis, MO). Cells were analyzed by light microscopy using an Olympus BX41 clinical microscope. Photographs were taken using an attached Olympus DP72 digital camera (Opelco, Sterling, VA) with cellSens Standard software (Olympus, Center Valley, PA). For isolation of neutrophils, whole blood was centrifuged over a layer of Ficoll-paque PLUS (GE Healthcare, Fairfield, CT), and the monolayer was extracted and then overlayed onto Histopaque (Sigma, St. Louis, MO) and centrifuged a second time. The monolayer was then isolated, washed, and cytospins were prepared. Light microscopy was used to confirm the isolated cells were more than 95% neutrophils.

**Cell culture and reagents**

All cell lines and primary blast samples were cultured as described. Molm14 cells were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Sorafenib and quizartinib were dissolved in dimethyl sulfoxide (DMSO) at stock concentrations of 10 mM. Quizartinib was supplied by Ambit Biosciences, Inc. (La Jolla, CA). Sorafenib was obtained from LC Laboratories, Inc. (Woburn, MA).

**Bone marrow stroma co-culture**

Leftover bone marrow from healthy donor harvests was collected, re-suspended in RPMI (Invitrogen, Carlsbad, CA), and cells were centrifuged over a layer of Ficoll-paque PLUS (GE Healthcare, Fairfield, CT). Mononuclear cells were collected, washed twice with RPMI, and counted using a Coulter Counter (Beckman Coulter, Indianapolis, IN). Cells were re-suspended in stroma medium, consisting of RPMI/10% fetal bovine serum/1% L-Glutamine/1% Penicillin-
Streptomycin/1μM Hydrocortisone (L-Glutamine and Penicillin/Streptomycin: Invitrogen, Carlsbad, CA; Hydrocortisone: Sigma, St. Louis, MO). Cells were then plated into a 75 cm² tissue culture flask with an average number of 50 x 10⁶ cells/flask and were grown at 33°C in 5% CO₂. After 24-48 hours, medium containing non-adherent cells was removed and replaced with 20ml of fresh stroma medium. Cells were cultured until 95-100% confluency was reached. Cultures were passaged every 4-8 weeks after harvesting with 0.25% Trypsin-EDTA (Sigma, St. Louis, MO). For bone marrow stroma co-culture experiments, stromal cells were plated into 6-well plates (Becton Dickinson, Franklin Lakes, NJ) and allowed to grow until 95-100% confluency was reached. Adherent cells were then washed with PBS 3 times and medium was replaced with culture medium. Molm14 cells or primary patient blasts were then added to the culture medium, and plates were incubated at 37°C in 5% CO₂.

DNA isolation and sequencing

DNA was isolated from primary patient blasts using the DNeasy Mini Kit (Qiagen, Valencia, CA). DNA sequencing, PCR for FLT3, and estimation of FLT3 mutant allelic burden were performed as described.¹⁹ Direct sequencing of the PCR products covering the entire C/EBPα coding sequence was done using primers described recently.²⁰,²¹

Flow cytometry

Flow cytometric immunophenotyping was performed as described²² on primary patient blasts grown on stromal co-culture in the presence or absence of quizartinib. For cell cycle experiments, cells were seeded in 6-well plates containing a confluent stromal cell monolayer at a density of 2 x10⁶ suspension cells/ml and incubated with or without quizartinib. After 24 hours
(Molm14 cells) or 48 hours (primary patient blasts), cells were harvested and analyzed for propidium iodide staining as described.23

**Immunoblotting**

Electrophoresis and immunoblotting for FLT3 and ERK were performed as described.19
Antibodies to MMP-9 and to lactoferrin were obtained from Cell Signaling (Bedford, MA). Anti-C/EBPα and anti-phospho-C/EBPα antibodies were obtained from Santa Cruz (Santa Cruz, CA) and Cell Signaling (Bedford, MA), respectively.

**Nitroblue Tetrazolium (NBT) Reduction**

For NBT reduction assays, reagents were obtained from Sigma-Aldrich (St. Louis, MO). Whole blood taken from quizartinib-treated patients and healthy controls was collected in heparinized tubes and incubated with NBT Solution, with or without Stimulant Solution (bacterial extract) per the manufacturer’s instructions. Blood smears were prepared using the manual wedge method and were stained according to the manufacturer’s instructions. Cells treated with quizartinib in vitro were also incubated as described above, cytopsins were prepared as described under “Clinical Specimens,” and staining was performed per the manufacturer’s instructions.

**C/EBPα knockdown**

For C/EBPα knockdown experiments, Silencer Select C/EBP-alpha siRNA and the Amaxa Nucleofector V kit were obtained from Invitrogen (Carlsbad, CA). For each sample, 2 million cells were incubated with 320 pM siRNA (C/EBP-alpha or scrambled control) and electroporated using program T-019. Cells were lysed after 24 and 48 hours for analysis of C/EBPα protein levels by western blotting.
Results

Peripheral blood and bone marrow responses to quizartinib

AC220-002 is a multi-center, international phase 2 study of quizartinib administered as a single agent to adult patients with relapsed or refractory AML. While the trial has completed accrual, final clinical results are not yet available, although interim clinical results from a safety subset of patients have been presented in abstract form. The primary outcome of the trial was composite complete response rate (complete remission + complete remission with incomplete platelet recovery + complete remission with incomplete count recovery), with a secondary outcome of survival. Results regarding the primary outcomes (overall remission rates, survival, etc…) will be presented elsewhere. The data presented in this report are derived from a laboratory correlative study of patients enrolled at 2 centers.

AML is a molecularly heterogeneous disease. Patients are classified for prognostic purposes according to both cytogenetic and molecular risk groups, and FLT3/ITD AML with a diploid karyotype represents a unique category that has been well characterized from a prognostic standpoint. Of the 28 patients enrolled at the 2 centers, 17 had normal diploid cytogenetics, 7 had intermediate risk karyotype, and 4 had a poor-risk/complex karyotype, according to conventional classification. Because we noted the differentiation effect occurring predominantly in those patients with diploid karyotype, and because FLT3/ITD mutations are most closely associated with a normal karyotype (in non-M3 AML), we restricted our evaluation to include only these patients. We had genomic DNA samples, bone marrow aspirates, and bone marrow core biopsies from pre-treatment, day 15, and day 29 time points available from 14 out of the 17 patients. These 14 patients (with FLT3/ITD mutation, diploid
karyotype, and samples available for correlative studies) represent the study set for this laboratory correlative evaluation. The treatment regimens received by the 14 patients are summarized in Supplementary Table 1.

Patients treated with quizartinib displayed rapid clearance of peripheral blasts consistent with responses observed with other FLT3 inhibitors.\textsuperscript{9,10,12,13} However, while neutrophil levels tended to be very low in these patients at the start of treatment, we noted a surge of neutrophils in the peripheral blood after several weeks of therapy (Figure 1A) in 13 out of the 14 study subjects. This rise in the absolute neutrophil count (ANC) typically peaked around day 40, and then fell again (Table 1), remaining below 1000 per cubic millimeter for the duration of the trial. Although formal outcome results from this trial are not yet available for publication, the patients experiencing this surge of neutrophils typically achieved a complete remission with incomplete count recovery (CRi), as reported in the interim results.\textsuperscript{15} In general, the peripheral blood neutrophils circulating in these patients during this period appeared morphologically normal (Figure 1B). In parallel with this neutrophil surge, several patients developed fever and inflammatory infiltrates in the lungs, soft tissue, or skin. No infectious etiologies were identified, and treatment with steroids led to their rapid resolution. Biopsy of the skin lesions revealed neutrophilic lobular panniculitis, consistent with Sweet’s Syndrome. This finding can occasionally be observed in patients with APL treated with ATRA.\textsuperscript{28} In general, the fevers and steroid-responsive lung and skin nodules that accompanied the neutrophil surge were reminiscent of retinoic acid differentiation syndrome.\textsuperscript{29}

Based on these observations, we hypothesized that quizartinib was inducing differentiation of marrow blasts. Bone marrow core biopsies and aspirates were obtained within 2 weeks of treatment initiation and on days 15 and 29 of the first cycle of quizartinib. Most
patients began therapy with hypercellular marrows (> 70% cellularity). Table 2 compares cellularity between pre-treatment and day 29 specimens for the 14 patients. In the majority, there was little or no difference in overall cellularity of the marrows among the pre-treatment, day 15 (not shown), and day 29 marrow samples, indicating that no significant cell killing had occurred throughout the first 4 weeks of quizartinib therapy. However, in 13 out of 14 patients, there were striking changes in the cellular morphology. Examination of day 15 aspirates revealed the majority of the marrow cells were myelocytes, and by day 29 most were mature-appearing neutrophils. An example is shown in Figure 1C (patient 2). For the 13 patients that exhibited differentiation, the mean marrow cellularity was 75% prior to treatment and 68% on day 29, while blasts decreased from 77% to 6% over the same time period, and maturing myeloid cells (myelocytes, metamyelocytes, and neutrophils) increased from 9% to 57% (Table 2). Interestingly, the FLT3/ITD mutant allelic ratios changed minimally between the pre-treatment and day 29 samples. For example, in patients 2, 7, 8, 9, 10, and 11, whose marrows on day 29 displayed predominantly mature neutrophils, very little wild type FLT3 allele was detectable by PCR on either pre-treatment or day 29, indicating that the maturing myeloid elements were derived directly from leukemic precursors, with the cells retaining their ITD allele. Samples with low allelic ratios, which likely harbored heterozygous ITD mutations, likewise showed minimal change in ratio over the treatment period. Shown in Figure 1D are flow cytometric analyses of the clinical marrow samples from patient 7, who demonstrated the typical myeloid differentiation we observed. During treatment, the cells within the marrow displayed increased side scatter, decreased expression of the immature surface markers CD34 and CD117, and increased expression of the maturation marker CD15.
Neutrophils are derived from the FLT3/ITD blasts

As patients accrued to this clinical protocol, it became apparent that quizartinib might be inducing the blasts to undergo differentiation. We therefore isolated and characterized the neutrophils produced during a surge. We prepared purified peripheral blood neutrophils from a patient at the peak of the surge (Figure 2A). We then prepared genomic DNA from the isolated neutrophils and from blasts collected from this same patient prior to beginning quizartinib therapy. PCR of the FLT3 juxtamembrane domain from both samples yielded fragments that migrated with an identical pattern on ethidium-stained agarose (Figure 2B). DNA sequencing of the mutant bands (not shown) confirmed that these were identical 75 bp ITD mutations, establishing that the neutrophils were derived from leukemic progenitors. We next analyzed these same purified neutrophils for expression of FLT3, lactoferrin, and MMP-9 (gelatinase) by immunoblotting. On normal mature neutrophils, FLT3 is not expressed, while lactoferrin and MMP-9 are found within the granules. Figure 2C shows the expression of constitutively activated FLT3 in the pre-treatment blasts of this patient but not in the isolated neutrophils, while lactoferrin and MMP-9 are appropriately expressed in both the patient-derived and control neutrophils. The leukemia-derived neutrophils exhibited normal oxidative burst activity in a nitroblue tetrazolium (NBT) assay (Figure 2D).

These data indicate that in 13 out of these 14 FLT3/ITD AML patients, treatment with quizartinib resulted in terminal granulocytic differentiation of bone marrow blasts. In one patient (Patient 14; Table 2), however, there was no evidence of differentiation at any time during treatment. While this patient experienced complete clearance of peripheral blasts, no neutrophil surge occurred (Table 1), and there was no clinically significant reduction in marrow blasts on day 15 or day 29. The patient had a normal karyotype, so the ongoing block in differentiation
could not be explained by translocation of a transcription factor such as core binding factor. However, mutation analysis of the CEPBα coding sequence revealed a b-ZIP domain mutation (insertion of 4 base pairs – ACCG – at position 1449 of the reference sequence U34070, leading to a frameshift at R287), expected to result in loss of C/EBPα–mediated cell cycle control. CEPBα is a well-described transcription factor central to the induction of granulocyte development, and is associated with transcriptional activation of genes such as lactoferrin. Mutations in CEPBα occur in approximately 10-15% of normal karyotype AML, and are known to act in a dominant negative manner to disrupt granulocytic differentiation. The presence of this CEPBα mutation offered a potential explanation for this patient’s lack of differentiation in response to FLT3 inhibition. In a second patient (Patient 2; Table 2), a trans-activation domain (TAD) CEPBα mutation (deletion of 17 base pairs – CCGCCCCGAGCGGCTG – at position 731 of the reference sequence U34070, leading to a frameshift at P46) was detected at disease progression four months after beginning quizartinib treatment.

**Differentiation of Molm14 cells in vitro**

FLT3 inhibition has been reported to induce granulocytic differentiation in FLT3/ITD AML cells. These studies postulated that FLT3/ITD signaling leads to phosphorylation and potential inactivation of C/EBPα on residue ser21 via P-ERK1/2, resulting in a differentiation block that could be overcome with FLT3 inhibition. However, these observations were made using in vitro models with FLT3/ITD-expressing cell lines in suspension culture. The differentiation we observed in our patients occurred within the bone marrow. Therefore, we wished to develop an in vitro model of FLT3/ITD AML differentiation which more closely mimicked the conditions of the bone marrow microenvironment, with direct interactions between...
the leukemia and bone marrow stromal cells. Monolayers of stromal cells from healthy bone marrow donors were co-cultured with Molm14 cells, a FLT3/ITD-expressing AML line previously used to study differentiation in response to FLT3 inhibition.\textsuperscript{18,35} This type of model has been previously used to study hematopoiesis, as well as chemoresistance conferred by the marrow microenvironment.\textsuperscript{36,37}

To determine if differentiation in response to FLT3 inhibition represents a class effect of FLT3 TKIs, we cultured Molm14 cells on stromal layers with increasing doses of two different FLT3 TKIs, sorafenib and quizartinib.\textsuperscript{19,38-40} In suspension culture, Molm14 cells undergo apoptosis on exposure to sustained, potent FLT3 inhibition.\textsuperscript{13} In contrast, while the cells co-cultured on stroma arrested in G1 in response to the inhibitors, the degree of apoptosis induced by either drug was diminished (Figures 3A, 3B). After 24 hours of treatment, morphologic differentiation of the cells was evident in the presence of either quizartinib (Figure 3C) or sorafenib (\textit{not shown}) at an equipotent FLT3-inhibitory concentration,\textsuperscript{19} and respiratory burst activity by NBT assay was present (Figure 3D). This difference in response to FLT3 inhibition in suspension culture as compared with stromal co-culture parallels the responses observed clinically, in which peripheral blasts are rapidly cleared while marrow blasts remain viable but undergo differentiation. It should be noted that while quizartinib inhibits both FLT3 and c-KIT, the IC\textsubscript{50} for inhibition of c-KIT is 35 nM (\textit{data not shown}), whereas the IC\textsubscript{50} for FLT3, is approximately 1 nM.\textsuperscript{40} Accordingly, the differentiation effects from this drug are most likely due to FLT3 inhibition, and although FLT3 autophosphorylation was fully inhibited under the conditions of this experiment (Figure 3E), P-ERK was only partially inhibited, in contrast to what is routinely observed with FLT3/ITD cell lines in suspension culture.\textsuperscript{18,41} C/EBP\textalpha{} was readily detectable and phosphorylated despite FLT3 inhibition (Figure 3E). Because a previous
study in murine 32D cells expressing a FLT3/ITD receptor indicated that C/EBPα levels increased in response to FLT3 inhibition, we examined C/EBPα levels over 24 hours of quizartinib exposure (Figure 3F). C/EBPα (and P-C/EBPα) was maximally expressed during the first 4-8 hours of stromal contact, and then decreased as the cells differentiated.

**C/EBPα knockdown impedes FLT3 inhibitor-induced differentiation**

Because of the central role of C/EBPα in myeloid differentiation, and because the single patient in our series who failed to display differentiation harbored a C/EBPα mutation, we predicted that the loss of C/EBPα in FLT3/ITD cells would prevent FLT3 inhibitor-induced differentiation. Molm14 cells co-cultured with stroma differentiated in a dose-responsive fashion to quizartinib (Figure 4A). We then used siRNA treatment of the Molm14 cells to knock down C/EBPα levels (Figure 4B), which resulted in a significant decrease in differentiation induced by quizartinib (Figure 4C). This experiment demonstrates the importance of C/EBPα in mediating differentiation induced by FLT3 inhibition.

**Differentiation of primary FLT3/ITD AML patient blasts in vitro**

We had sufficient viable blasts from patient 7 (Tables 1 and 2, and Figure 1D), who exhibited the typical differentiation response while on treatment, to study in our stromal co-culture system. When treated in suspension culture, these blasts displayed a cytotoxic response to quizartinib (*not shown*), resembling the typical response we and others have described previously with numerous FLT3 inhibitors including quizartinib. The blasts were plated onto stroma with or without 200 nM quizartinib, a concentration of drug chosen to approximate
the steady state levels (accounting for plasma protein binding) achieved in patients treated with 135 mg quizartinib per day. The cells were examined daily by light microscopy to monitor viability and morphology. Viable cell number, as assessed by trypan blue staining, did not decrease over the duration of the experiment in either treated or untreated samples (not shown). However, the proliferative fraction decreased significantly during the first 48 hours of quizartinib treatment, indicative of a cell cycle arrest (Figure 5A). Differentiation was evident in the quizartinib-treated samples, but the time course was very different as compared to Molm14 cells. Not until day 14 of co-culture with stroma and quizartinib did the cells display morphologic changes consistent with differentiation (Figures 5B, and quantified in Figure 5C), although functional evidence (NBT) was present by day 5 (not shown). This time course is consistent with that observed in the clinical specimens from patients, in which the maturation occurred over 2-4 weeks (Figure 1C). We confirmed that the ITD mutation present in the blasts at the start of the experiment was also detectable in the differentiated cells at an identical ratio (not shown).

Flow cytometric analysis of the cells from patient 7 after 14 days of drug exposure on stroma revealed loss of CD34 and CD117 and gain of CD15 in the blasts treated with quizartinib (Figure 5D). The blasts co-cultured on stroma without quizartinib also showed some flow cytometric evidence of differentiation, but less than the quizartinib-treated cells, and there was little evidence of morphologic differentiation (Figure 5B, D). As with the Molm14 cells, the primary cells displayed only partial inhibition of P-ERK despite complete inhibition of FLT3 autophosphorylation (Figure 5E). Likewise, C/EBPα was expressed at the protein level (increased in cells on stroma compared with suspension cells) and remained phosphorylated despite quizartinib treatment (Figure 5E).
We repeated the experiment using a second FLT3/ITD AML sample (with a mutant to wild type allelic ratio of 19) and again achieved morphologic differentiation of the blasts, but this time in 9 days (Figure 5F).
Discussion

This is the first report of in vivo terminal differentiation of leukemia cells occurring as a result of therapeutic inhibition of an oncogenic tyrosine kinase. Two previous studies have used in vitro models to predict that FLT3 inhibition would relieve the differentiation block that characterizes AML cells. While clinically apparent differentiation has not been seen with other FLT3 inhibitors, quizartinib is 10-50 times more potent in vivo than previously studied drugs, and the reported interim response rate is significantly higher than that seen with other inhibitors. The formal trial results for all patients, including the exploratory group, are not yet available.

The release of the differentiation block appears to be an on-target effect of FLT3 inhibitors, as we induced differentiation in vitro with sorafenib as well as quizartinib, and two prior studies used lestaurtinib and tandutinib. In general, therefore, the clinical response to potent sustained FLT3 inhibition appears to consist of clearance of peripheral blasts over a few days and terminal differentiation of marrow blasts over several weeks. This difference in the response of blasts in the peripheral blood compared with the marrow highlights the influence of the bone marrow microenvironment on the pathogenesis of leukemia.

In previous studies, FLT3/ITD-expressing cells in suspension culture, both cell lines and primary patient blasts, responded to FLT3 inhibition in vitro with rapid apoptosis, which parallels the clinical peripheral blood response. However, in our stromal co-culture system, the response mimicked the differentiation observed in the bone marrows of the quizartinib-treated patients. The protective effect that bone marrow stroma confers on leukemia cells is well-described, so it is not surprising that apoptosis was not induced with FLT3 inhibition in cells on
stroma. Our in vitro model was successful in reproducing the differentiation observed in vivo, and so provided the opportunity to investigate some of the mechanisms regulating this process.

Differentiation is tightly coupled with cell cycle arrest, and in normal myelopoiesis, C/EBPa is generally considered to play a central role in both processes. The function of C/EBPa is frequently perturbed in AML through a variety of mechanisms, and FLT3/ITD mutations have been reported to inhibit C/EBPa function through P-ERK-mediated phosphorylation. In the present study, while the level of C/EBPa protein was increased in the blasts on stroma as compared to the cells in suspension, it remained phosphorylated, possibly due to the persistent activation of ERK. ERK, in turn, remained active presumably due to stromal-derived factors acting outside of the FLT3 signaling pathway. This failure of FLT3 inhibition to inhibit ERK in blasts on stroma has been reported by others, and stands in distinct contrast to the consistent findings in suspension cells. In blasts on stroma, FLT3 inhibition results in a cell cycle arrest, which appears to be sufficient to trigger differentiation. Given that the single patient in our series who failed to differentiate with quizartinib harbored a C/EBPa b-ZIP mutation, and that clinical resistance to quizartinib in another patient was associated with the emergence of a C/EBPa TAD mutation, it appears that FLT3 inhibitor-induced differentiation requires the presence of a functional C/EBPa.

ATRA is effective in treating APL by induction of terminal differentiation but fails to permanently clear the leukemic clone (as can be achieved by combining ATRA with anthracyclines or arsenic). Only through these combination therapies is the eradication of the leukemia achieved. With quizartinib, it would seem that truly potent in vivo FLT3 inhibition can finally be accomplished, offering the hope that this drug, combined with chemotherapy, may result in significant cure rates for FLT3/ITD AML.
The detection of leukemia-derived neutrophils and of a clinical differentiation syndrome represent striking parallels between FLT3/ITD AML and APL treated with molecularly targeted therapy. There are important differences, however. The rise in the white blood cell count that commonly occurs during the treatment of APL is presumably due to a much larger fraction of the leukemic burden undergoing differentiation. In FLT3/ITD AML, the majority of the malignant cells undergo apoptosis relatively rapidly, followed by gradual differentiation of the residual marrow blasts. The “surge” of the ANC following this differentiation is therefore much more modest than that seen in APL.

Taken together, our findings highlight an important role for differentiation therapy but suggest an incomplete understanding of relevant targets in most cases of AML, including those with FLT3/ITD. In summary, FLT3 inhibition by quizartinib induces terminal differentiation of FLT3/ITD AML blasts and demonstrates a largely unrecognized role of tyrosine kinase mutations in generating the maturation arrest so characteristic of AML.
Authorship

Contribution: A.S. and M.L. designed the study, performed experiments, enrolled patients, analyzed the data, and wrote the manuscript. A.P. contributed to study design, analyzed data, enrolled patients, and helped edit the manuscript. C.T. analyzed samples for C/EBPα mutations. X.Y. and T.R. performed experiments. M.B. contributed to study design and helped analyze the flow cytometry data. C.G. and G.E.N. helped analyze the data. M.F., B.D.S., K.P., and J.K. enrolled patients.

Conflict-of-interest disclosure: M.L., A.P., and C.T. are on the clinical advisory board for Ambit Biosciences, Inc., whose product was investigated in this study.

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Table 1. Clinical characteristics, and peripheral blood neutrophil and blast counts for patients treated with quizartinib. ANC = absolute neutrophil count. PB = peripheral blood. RL = relapsed. RF = refractory to most recent therapy. PR = primary refractory (i.e., patient was refractory to induction therapy at diagnosis). All cell counts are in cells/mm³. “ANC baseline” refers to the lowest ANC during the first week of therapy. “ANC peak” refers to the highest ANC observed after the first week of therapy. “PB blasts baseline” refers to the absolute peripheral blood blast count at the start of therapy. Mean values are shown for patients 1-13.

* For patient 14, the ANC decreased during therapy with quizartinib, but peripheral blasts were cleared after 1 week.
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<th>Myelocytes, Neutrophils D.29 (%)</th>
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**Table 2. Bone marrow cellularity, differentials, and FLT3 and C/EBPα mutations for patients treated with quizartinib.** Bone marrow core biopsy samples (decalcified, slide mounted, and stained with hematoxylin and eosin) were examined by light microscopy to visually estimate cellularity within 2 weeks prior (“Pre”) and after 4 weeks of continuous therapy (D.29) with quizartinib. Bone marrow aspirates (obtained from the same procedure as the core biopsies) were stained with Wright-Giemsa stain and examined by light microscopy. For each specimen, 100 cells were counted and scored by morphology as blasts, myelocytes, metamyelocytes, bands, or neutrophils, and expressed as a percentage (%) of the total. FLT3 allelic ratios were derived from the aspirate specimens. C/EBPα mutation status was determined as described in Methods. Mean values are shown for patients 1-13.

*The TAD mutation was detectable only at disease progression, after 4 months of quizartinib therapy.
Figure Legends

**Figure 1. In vivo terminal myeloid differentiation in patients treated with quizartinib.** A. Graph of peripheral total white blood cell count (WBC), absolute neutrophil count (ANC), and absolute blast count in a representative patient (Table 1; patient 2) during the first 2 months of treatment with quizartinib. B. Photomicrograph of a peripheral blood neutrophil from a whole blood sample collected from the patient shown in A on day 35 of treatment. C. Bone marrow aspirates collected from a representative patient (Table 1; patient 7) one week prior to, and on days 15 and 29 of treatment with quizartinib. D. Flow cytometry analysis of bone marrow aspirate specimens collected pre-treatment, Day 15, and Day 29 from patient 7. The analysis was performed on the day of collection, and the dot plots are overlayed, organized by color.

**Figure 2. Neutrophils are derived from the leukemic blasts.** A. Graph of peripheral blood ANC from patient 4 during treatment with quizartinib. Neutrophils from Day 60 were isolated to > 95% homogeneity as described in “Methods” which was confirmed by cytospin (inset). B. Genomic DNA samples isolated from pre-treatment blasts, the day 60 neutrophils, and neutrophils from a healthy donor were analyzed by PCR for the FLT3/ITD mutation as described in “Methods.” Shown is an ethidium bromide-stained agarose gel. C. Whole cell lysates were prepared from Molm14 cells, pre-treatment blasts from patient 4, day 60 neutrophils from patient 4, and neutrophils from a healthy donor. Lysates were analyzed for total FLT3, phosphorylated FLT3, lactoferrin, and MMP9 as described in “Methods.” D. Nitroblue tetrazolium reduction assay of peripheral blood collected from a healthy donor (“Control”) compared with a quizartinib-treated patient during the neutrophil surge. Cells “with stimulation” were exposed to bacterial extract to induce respiratory burst activity.
Figure 3. Treatment of Molm14 cells with FLT3 inhibitors. A and B. Molm14 cells were exposed to quizartinib (10 nM) or sorafenib (100 nM) in suspension culture or co-culture with bone marrow stroma and analyzed for Annexin V binding (A) and propidium iodide staining (B) by flow cytometry. C. and D. Molm14 cells were co-cultured with stroma in the presence and absence of 10 nM quizartinib for 24 hours, and then cells were collected and analyzed for morphology (C) and nitroblue tetrazolium reduction activity (D). E. Molm14 cells were co-cultured on stroma in the presence and absence of 10 nM quizartinib. After one hour, cells were collected and lysates were analyzed by immunoblotting as described in “Methods.” F. Molm14 cells were co-cultured with stroma and quizartinib at the indicated concentrations. Cells were collected after 0, 4, 8, 12, and 24 hours of drug exposure and analyzed by immunoblotting for phosphorylated and total C/EBPα.

Figure 4. Knockdown of C/EBPa blocks the differentiation induced by FLT3 inhibition.

A. Molm14 cells were co-cultured with stroma in the presence of increasing concentrations of quizartinib for 24 hours, and then cells were collected and analyzed for morphology. For each condition, 100 viable cells were counted and categorized as undifferentiated or differentiated. B. Molm14 cells (2 million per sample) were incubated with siRNA for C/EBP-alpha (or scrambled control) and electroporated, then co-cultured with stroma. After 24 hours, the cells were harvested, lysed, and analyzed for C/EBPα protein levels by western blotting. Incubation for 48 hours led to identical results (not shown). C. Molm14 cells were subject to siRNA C/EBPa knockdown as in (B), co-cultured with stroma for 24 hours, then treated with 5 nM quizartinib. After 24 hours, the cells were harvested and examined and scored for morphologic changes as in (A).
Figure 5. Treatment of primary patient blasts with FLT3 inhibitors. A. Blasts from patient 7 were co-cultured with stroma or grown in suspension culture overnight, and then 200 nM quizartinib was added to both cultures. Cells were incubated with drug for 48 hours, and were stained with propidium iodide and analyzed by flow cytometry. Blasts from patient 7 were co-cultured with stroma in the presence and absence of 200 nM quizartinib for 14 days, and cells were collected and analyzed by light microscopy (B) and scored for differentiation (C). D. Cells from (B) were also analyzed for the presence of differentiation markers by flow cytometry as described in “Methods.” E. Blasts from patient 7 were co-cultured with stroma overnight, and then 200 nM quizartinib was added. After one hour, cells were collected, and lysates were analyzed by immunoblotting as described in “Methods.” F. Blasts from a 52 year old man with relapsed, refractory FLT3/ITD AML were co-cultured on stroma as in Figure 5B. Shown are cells harvested after 9 days of culture with or without quizartinib.
Figure 1D
Figure 2A
Figure 2C
Figure 2D
Figure 3A
Propidium Iodide Count

Off stroma
No drug
54.5%
5.39%
35.6%

Off stroma
+ quizartinib
38.7%
39.1%
18.1%

On stroma
No drug
51.2%
3.72%
40.8%

On stroma
+ quizartinib
54.9%
27.3%
15.5%

Figure 3B
Figure 3C

Control

Untreated

Quizartinib
Figure 3D
Figure 3E

Graph showing the effect of Quizartinib on various proteins.

X-axis: Quizartinib concentration in nM (0, 10, 50, 100)

Y-axis: Protein levels

Proteins analyzed:
- P-FLT3
- FLT3
- P-ERK
- ERK
- P-CEBPα
- CEBPα
- Actin
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Figure 3F
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Figure 4B
Figure 4C
Figure 5A
Figure 5B
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Figure 5C
Figure 5D
Figure 5E

- P-FLT3
- FLT3
- P-ERK
- ERK
- P-CEBPα
- CEBPα
- Actin

On stroma

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Figure 5F
Terminal myeloid differentiation in vivo is induced by FLT3 inhibition in FLT3/ITD AML

Amy Sexauer, Alexander Perl, Xiaochuan Yang, Michael Borowitz, Christopher Gocke, Trivikram Rajkhowa, Christian Thiede, Mark Frattini, Grant E. Nybakken, Keith Pratz, Judith Karp, B. Douglas Smith and Mark Levis