Gefitinib (Iressa) induces myeloid differentiation of acute myeloid leukemia

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Abstract

Cure rates for patients with acute myeloid leukemia (AML) remain low despite ever-increasing dose-intensity of cytotoxic therapy. In an effort to identify novel approaches to AML therapy, we recently reported a new method of chemical screening based on the modulation of a gene expression signature of interest. We applied this approach to the discovery of AML differentiation-promoting compounds. Among the compounds inducing neutrophilic differentiation was 4,5-dianilinophthalimide (DAPH1), previously reported to inhibit epidermal growth factor receptor (EGFR) kinase activity. Here, we report that the FDA-approved EGFR inhibitor gefitinib (Iressa) similarly promotes the differentiation of AML cell lines and primary patient-derived AML blasts *in vitro*. Gefitinib induced differentiation based on morphological assessment, nitro-blue tetrazolium reduction, cell surface markers, genome-wide patterns of gene expression, and inhibition of proliferation at clinically achievable doses. Importantly, EGFR expression was not detected in AML cells, indicating that gefitinib functions through a previously unrecognized, EGFR-independent mechanism. These studies indicate that clinical trials testing the efficacy of gefitinib in patients with AML are warranted.
Introduction

Despite an improved understanding of the pathogenesis of acute myeloblastic leukemia (AML), long-term survival remains poor. While the current approach to AML therapy is based on cytotoxic agents, increasing evidence points to the potential of therapy aimed at overcoming a block in differentiation that is characteristic of AML.\(^1\)\(^2\) This approach is particularly striking in acute promyelocytic leukemia (APL), where treatment of patients with all-trans retinoic acid (ATRA) results in the induction of myeloid differentiation of the leukemic blasts, and long-term survival has improved dramatically.\(^3\)\(^-\)\(^5\) Other AML subtypes similarly have defects in differentiation caused in part by mutations in differentiation-promoting transcription factors (e.g. C/EBPalpha, PU.1, GATA-1).\(^6\)\(^-\)\(^9\) The potential of differentiation therapy in non-APL AML has yet to be realized, however, in large measure because chemical inducers capable of triggering differentiation have yet to be described.

The challenges in identifying AML differentiation-inducing agents are two-fold. First, in many cases the mechanism by which differentiation is abrogated is unknown, thereby precluding a traditional biochemical screen for compounds that activate the differentiation program. Second, even for those cases in which differentiation-blocking mutations are known, such mutations have been primarily in transcription factors, generally considered to be “undruggable.” We recently addressed these issues by developing a gene expression-based screening method (Gene Expression-based High-throughput Screening (GE-HTS)), whereby a small molecule library was screened for compounds that induced the gene expression signature of myeloid differentiation.\(^10\) Among the chemicals confirmed to induce neutrophilic differentiation was 4,5-dianilinophthalimide (DAPH1). DAPH1 induced morphological, biochemical and functional changes indicative of myeloid maturation, consistent with its induction of a differentiation gene expression program.

DAPH1 was previously identified as an epidermal growth factor receptor (EGFR) kinase inhibitor, but the compound has not been developed clinically, thus precluding its evaluation as differentiation therapy for patients with AML.\(^11\)\(^12\) In the present report, we describe the preclinical efficacy of the FDA-approved EGFR inhibitor gefitinib (Iressa). We find that gefitinib induces myeloid differentiation in AML cell lines and primary patient-derived AML blasts at concentrations that are achievable in humans. These results indicate that gefitinib warrants evaluation as potential differentiation therapy for patients with AML.

Materials and Methods

Cells and gefitinib treatment

HL-60, Kasumi-1, and U937 cells were maintained in culture in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO\(_2\). Primary patient AML blasts were collected from peripheral blood or bone marrow aspirate after obtaining patient informed consent under a Dana-Farber Cancer Institute Internal Review Board approved protocol. They were isolated using Ficoll-Paque Plus (Amersham Biosciences,
Uppsala, Sweden) separation and maintained in culture in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C with 5% CO2. Cells were treated with gefitinib (Astra-Zeneca, London, UK and WuXi PharmaTech Co., Ltd., Shanghai, China) resuspended in dimethyl sulfoxide (DMSO) at concentrations ranging from 10 µM down to 0.078 µM. DMSO was used as a vehicle control in differentiation and viability experiments. HL-60 cells were also treated with cetuximab (Erbitux) (Bristol-Myers Squibb Company, Princeton, NJ) at 10-20 µg/ml and trastuzumab (Herceptin) (Genentech Inc., San Francisco, CA) at 0.1-1000 µg/ml.

Viability assays
Viability experiments were performed in 96-well format in replicates of four using the Promega Cell-Titer Glo (Madison, WI) ATP-based assay per the manufacturer’s instructions. Cells were evaluated at 0, 3 days, and 6 days with gefitinib in a 2-fold dilution from 10 µM down to 0.078 µM versus DMSO control treated cells. The concentration at which cell viability was reduced to 50% of DMSO treated controls (EC50) was determined. Values for EC50 were calculated by interpolating a polynomial fit to the measured viability data. Curve fitting was performed in MATLAB (Mathworks, Natick, MA) using the least-squares curve-fitting function (polyfit). Model order for the polynomial was set to n=3 except in the cases where the estimated EC50 point falls outside the range of the measured values where it was set to n=1 (one of the normal controls). The EC50 value was found by interpolating the curve for the value of 50 with the MATLAB one-dimensional interpolation function (interp1).

Differentiation assays
Differentiation induction with gefitinib was confirmed by morphology, Nitro-Blue Tetrazolium (NBT) reduction, cell surface marker expression, and whole genome changes in expression. For morphological assessment, cytospin preparations of treated AML blasts stained with May-Grunwald Giemsa were evaluated with light microscopy. For NBT reduction assays, experiments were performed in triplicate. Gefitinib treated cells were compared to DMSO treated controls after five days of treatment. Cells were incubated at 37°C for 1 hour in a mixture containing total medium, 0.1% NBT (Sigma, St. Louis, MO), and 1µg/ml 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma, St. Louis, MO). The percentage of blue cells was counted by light microscopy for at least 200 cells per sample. Gefitinib treated cells were compared to DMSO treated cells with a one-tailed t-test analysis assuming two samples with unequal variance. Analysis for myeloid maturation with cell surface markers was performed by fluorescence-activated cell sorting (FACS) with FITC-labeled antibodies for CD11b and CD14 (Becton Dickinson, San Jose, CA). Live cells were gated based upon forward and side scatter patterns. Fluorescence was analyzed by FACS with a Becton Dickinson FACScan and CELLQuest analytical software.

Expression profiling
HL-60 and Kasumi-1 cells were treated in replicate of three with 10 µM gefitinib or DMSO vehicle control for 6 hours and 24 hours. Primary patient APL cells (Patient 1) were treated with 5 µM gefitinib or DMSO in duplicate or triplicate for 3 days. Primary patient M5-AML cells (Patient 2) were treated in duplicate with 5µM gefitinib or DMSO.
in duplicate for 6 hours. Primary patient M4-AML cells (Patient 7) were treated in triplicate with 2.5 µM gefitinib or DMSO for 6 hours, 24 hours, and 3 days and with 5 µM gefitinib for 6 hours and 24 hours. RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) as per the manufacturer’s guidelines and 10 µg used to create target for hybridization to Affymetrix U133A DNA microarrays (Affymetrix, Santa Clara, CA) as previously described. GeneChip MAS5 Software (Affymetrix, Santa Clara, CA) was used for preprocessing of the raw data, and all scans within an experiment were scaled to the array with the median overall microarray intensity, as previously described. Raw microarray data are available at http://www.broad.mit.edu/cancer/pub/AML_gefitinib.

The Mantel test was used to assess whether gefitinib induced changes on a whole genome level consistent with differentiation. A Mantel test is a non-parametric, randomization-based procedure that estimates the correlation between two distance matrices. We compared previously reported sets of primary AML versus normal mature neutrophils to sets of undifferentiated versus gefitinib treated AML cells according to their level of expression to see if genes across the whole genome were being up-regulated and down-regulated similarly. Specifically, we compared the expression patterns (measured on Affymetrix’s U133A microarray) observed in data sets composed of nine primary AML versus three normal, mature neutrophil samples to those expression patterns observed in data sets composed of DMSO treated samples versus gefitinib treated samples. Next, we compared previously reported sets of primary AML versus normal mature monocytes to sets of gefitinib treated AML cells according to level of expression to see if genes across the whole genome were being up-regulated and down-regulated similarly. Specifically, we compared the expression patterns observed in the data sets composed of nine primary AML versus three normal, mature monocyte samples to those expression patterns observed in data sets composed of DMSO treated samples versus gefitinib treated samples. For a given gene expression data set \( X \) and its corresponding class labels, the distance of each feature from the class labels was calculated using the signal-to-noise statistic. The signal-to-noise statistic is calculated as follows:

\[
X_i = \frac{\mu_{i1} - \mu_{i2}}{\sigma_{i1} + \sigma_{i2}}
\]

where \( \mu_{i1} \) represents the mean expression of samples from class 1 for feature \( i \) and \( \sigma_{i1} \) represents the standard deviation of class 1 for feature \( i \). Similarly, the signal-to-noise statistic is calculated for the second set of samples \( Y \). The elements of vector \( X \) and vector \( Y \) correspond to the same set of objects (U133A probe sets). The Pearson correlation was computed between the corresponding elements of the two vectors to produce the Mantel correlation \( R_m \). The Pearson correlation was calculated as follows:

\[
R_m = \frac{\sum_{i=1}^{n} x_i y_i - \left( \frac{\sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{n} \right)}{\sqrt{\left( \sum_{i=1}^{n} x_i^2 - \left( \frac{\sum_{i=1}^{n} x_i}{n} \right)^2 \right) \left( \sum_{i=1}^{n} y_i^2 - \left( \frac{\sum_{i=1}^{n} y_i}{n} \right)^2 \right)}}
\]
where $X_i$ is the signal-to-noise statistic for feature $i$ of sample set $X$ and $Y_i$ is the signal-to-noise statistic for feature $i$ of sample set $Y$. The Mantel correlation $R_m$ was used as the reference value in the Mantel test. To calculate the significance level, the elements of one of the vectors were randomly permuted to produce a permuted vector $X^*$. As before, the Mantel statistic $R_{m*}$ was computed between $X^*$ and $Y$. The permutation-computation steps were repeated 2500 times and the resulting distribution was used to estimate the $P$-value by examining the proportion of $R_{m*}$ values that are greater than $R_m$.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from HL-60 and Kasumi-1 cells using TRIZOL Reagent (Invitrogen, Carlsbad, CA). Universal Human Reference RNA (Stratagene, La Jolla, CA) was used as a positive control. cDNA was synthesized from 1 µg of total RNA from each sample using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and oligo d(T)$_{16}$ primers in a 20 µl reaction system. Two microliters of cDNA was amplified using HotStarTaq DNA Polymerase (Qiagen, Valencia, CA) in the DNA Engine (PTC-200) Peltier Thermal Cycler (MJ Research, Waltham, MA) in a 20 µl reaction system.

PCR was performed at 94°C for 9 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The primer pairs were as follows: GAPD: 5’ AGCCACATCGCTCAGACAC 3’; 5’ CTCATGTGTGAAGACGCTAC 3’; EGFR: 5’ CCGGACATAGTCAGTGA 3’; 5’ ACTGTTGTGGCAGCAGTC 3’; ERBB2: 5’ GTTTGAGTGCCATGCCC 3’; 5’ GTAACYGGTCTCAGCT 3’.

**Immunoprecipitation and Western blot analysis**

A-431 whole cell lysate (20 ug) (sc-2201, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a positive control for EGFR detection. HL-60 and Kasumi-1 cells were collected by centrifugation at 1500 rpm for five minutes. Cells were lysed in RIPA buffer (10 mM Tris-Cl pH 7.6, 100 mM NaCl, 1mM EDTA [ethylenediaminetetraacetic acid], 1% Triton X, 0.5% sodium deoxycholate, and 0.1% SDS [sodium dodecyl sulfate]) with protease inhibitor (Complete Mini EDTA free protease inhibitor tablets, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor (1 mM sodium vanadate) and incubated on ice for 30 minutes. The supernatant was retained and the protein concentration was determined using the Bio-Rad Protein Assay reagent per the manufacturer’s instructions (BioRad Laboratories, Hercules, CA). For total EGFR determination, 50 µg of whole cell lysate was denatured by boiling in SDS sample buffer, separated on a 10% Tris-HCl pre-cast Ready Gel (BioRad Laboratories, Hercules, CA), and transferred to Millipore Immobilon P-PVDF membranes (Millipore, Billerica, MA). The membrane was blocked in non-fat dry milk (5% in TBST) for one hour at room temperature and blotted with anti-EGFR antibody (sc-03, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membrane was washed three times in Tris buffered saline Tween-20 (TBST) and incubated with anti-rabbit secondary horseradish peroxidase-linked antibody (Amersham Biosciences, Buckinghamshire, UK) for 45 minutes at room temperature. It was then washed three times in TBST. Antibody binding was detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc., Boston, MA) and exposed to Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK) film. Membranes were stripped and re-probed using...
a pan-actin antibody (ACTN05, NeoMarkers, Fremont, CA) to assure consistent sample loading.

Immunoprecipitation was performed as follows. Cells were washed with ice cold phosphate buffered saline and then incubated with 0.5 ml of 1X ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA) containing protease inhibitor (Complete Mini EDTA free protease inhibitor tablets) for 30 minutes. 500 µg of each lysate (HL-60 and SKBR3) was incubated overnight at 4°C with anti-ERBB2 antibody (neu Ab-11, Neomarkers, Fremont, CA) or Mouse IgG control antibody (Jackson Labs, West Grove, PA) and then incubated with Ultralink Protein G (Pierce, Rockford, IL) at room temperature for two hours. The immunoprecipitants were then washed five times with 1X cell lysis buffer (Cell Signaling Technology, Beverly, MA) with protease inhibitor. They were resuspended in SDS sample buffer, heated, and then separated by electrophoresis with a 5% Tris-HCl pre-cast Ready Gel (BioRad Laboratories, Hercules, CA) and transferred to Millipore Immobilon P-PVDF membranes (Millipore, Billerica, MA). They were analyzed as above with anti-phosphotyrosine antibodies 4G10 (Upstate, Waltham, MA) and pY100 (Cell Signaling Technology, Beverly, MA) and anti-ERBB2 (neu Ab-17, Neomarkers, Fremont, CA).

**Results**

**Gefitinib induces differentiation in AML cell lines**

Using a new chemical genomic screening method, we previously screened a small molecule library for agents inducing myeloid maturation in the AML cell line HL-60. One of the chemicals confirmed to induce HL-60 differentiation based upon multiple phenotypic and functional assays of differentiation was DAPH1, a 4,5-dianilinophthalimide class member, initially developed as an EGFR inhibitor. We therefore hypothesized that inhibition of EGFR or a related kinase may be an important mechanism of myeloid differentiation and as such may have clinical implications for AML therapy.

Because DAPH1 is not an FDA-approved drug, we extended testing to the FDA-approved EGFR inhibitor, gefitinib. HL-60 cells treated with 10 µM gefitinib for four days underwent striking evidence of differentiation with condensation and lobulation of the nucleus consistent with neutrophil maturation (Figure 1A and B) and the t(8;21) containing AML cell line, Kasumi-1, similarly demonstrated morphological evidence of gefitinib-induced neutrophilic differentiation (Figure 1C and D). The monocytic AML cell line, U937, underwent evidence of macrophage differentiation (Figure 1E and F) consistent with its proclivity toward monocyte/macrophage differentiation. Thus, gefitinib does not appear to only induce neutrophilic differentiation but rather myeloid differentiation depending on the cellular context.

We next performed an NBT reduction assay to assess for functional evidence of myeloid maturation. One feature of myeloid maturation is the production of superoxide anion, utilized by the mature myeloid cell to kill ingested microorganisms. Superoxide anion production can be measured by the reduction of nitro-blue tetrazolium (NBT). After five
days of treatment with gefitinib at 10 µM, over 80% of the HL-60 cells were positive for NBT reduction ($P < 0.001$). At concentrations of gefitinib as low as 1.25 µM at five days, there was enhanced induction of NBT reduction compared to DMSO controls ($P = 0.001$) (Figure 1G). Gefitinib treatment of U937 cells with 7.5 to 10 µM for five days increased NBT reduction compared to DMSO treated controls from 1% positive to 13% ($P = 0.04$) and 34% ($P = 0.03$) respectively (data not shown).

We then evaluated for the cell surface markers associated with myeloid maturation, CD14 and CD11b. HL-60 cells and Kasumi-1 showed a striking increase in expression of both CD14 and CD11b by flow cytometric analysis while the U937 cells showed only a subtle increase in CD11b and CD14 (Figure 2). Although CD14 expression is generally associated with a more monocytic phenotype, it is also expressed on mature neutrophils and CD11b is expressed on neutrophils and monocytes. Thus, a neutrophil versus monocyte differentiation distinction cannot be made solely based on limited cell surface marker expression.

**Gefitinib induces gene expression program of myeloid differentiation**

To obtain a more global, non-biased view of gefitinib effects on AML cells, we evaluated whether gefitinib induces genome-wide gene expression changes consistent with neutrophil or monocyte maturation. HL-60 and Kasumi-1 cells were treated in replicates of three with gefitinib at 10 µM or DMSO, and RNA was prepared at 6 and 24 hours for hybridization to Affymetrix U133A microarrays. In order to evaluate whether gefitinib induced a whole genome program of differentiation, we applied the Mantel test, a statistical, global measurement of similarity. We compared the genes that distinguish primary patient-derived AML blasts from normal human neutrophils or monocytes to the genes that distinguish DMSO treated HL-60 cells from gefitinib treated HL-60 cells. As early as 6 hours, gefitinib treatment of HL-60 cells recapitulated the gene expression pattern of *bona fide* neutrophil maturation ($P < 0.001$), and the differentiation program was similarly evident following 24 hours of gefitinib treatment ($P < 0.001$) (Figure 3). By 24 hours, gefitinib treatment of Kasumi-1 cells recapitulated the gene expression pattern of neutrophil differentiation ($P = 0.006$), whereas a gene expression pattern of monocyte differentiation was not significantly induced in either cell line ($P > 0.1$). This result indicates that gefitinib induced global changes in gene expression consistent with neutrophil differentiation in the HL-60 and Kasumi-1 cell lines. Detailed results are available at [http://www.broad.mit.edu/cancer/pub/AML_gefitinib](http://www.broad.mit.edu/cancer/pub/AML_gefitinib).

**Gefitinib-induced differentiation is EGFR-independent**

Gefitinib is well characterized as an inhibitor of *in vitro* and *in vivo* EGFR tyrosine kinase activity. In order to assess whether gefitinib is inducing AML differentiation via EGFR inhibition, we first evaluated whether EGFR transcript or protein is expressed. EGFR transcript was undetectable in HL-60 and Kasumi-1 cells (Figure 4A). Accordingly, no protein expression was detected by Western immunoblotting with anti-EGFR antibody, whereas EGFR-expressing A431 epidermoid carcinoma cells were strongly positive (Figure 4B). Additionally, stimulation of cells with 100 ng/ml EGF failed to induce expression of EGFR protein, and the monoclonal anti-EGFR antibody, cetuximab (Erbitux), failed to induce evidence of myeloid differentiation by morphology.
and NBT reduction (data not shown). These findings indicate that EGFR, the principle target of gefitinib, is not expressed in AML cells, consistent with prior reports, and therefore is unlikely to be the target of gefitinib in AML.19,20

Gefitinib and DAPH1 have both been reported to inhibit the activity of the EGFR-related receptor tyrosine kinase ERBB2 (HER-2/neu).12,17,21 While ERBB2 transcript was detected (Figure 4A), ERBB2 protein was undetectable either by total lysate Western blotting or by anti-ERBB2 immunoprecipitation followed by anti-phosphotyrosine or anti-ERBB2 Western blotting (Figure 4C), consistent with recent observations of lack of detectable ERBB2 protein in ERBB2 transcript-expressing AML cells.22 To further exclude ERBB2 as the target of gefitinib’s differentiation-inducing activity, HL-60 cells were treated with the anti-ERBB2 monoclonal antibody trastuzumab (Herceptin), and this failed to induce any evidence of myeloid maturation (data not shown). These data, taken together, suggest that gefitinib induces myeloid differentiation via a non-EGFR, non-ERBB2 mechanism, thereby implicating a new mechanism of action for gefitinib.

**Gefitinib inhibits viability and induces differentiation in primary AML blasts**
The effect of small molecules in cell lines does not always parallel that in primary patient material. We therefore extended the evaluation of gefitinib-induced differentiation to primary patient AML blasts obtained from patients with de novo or relapsed AML. We first examined the effects of gefitinib on cellular viability in a gefitinib dose-response series. Six of eight patients responded with an EC50 less than 5 μM, regardless of FAB subtype (mean EC50 2.0 μM amongst responders, range 1.0-8.1 μM; see Figure 5 and Table 1). In contrast normal donor peripheral blood mononuclear cells from five independent donors showed decreased viability in response to gefitinib only at high dose (mean EC50 = 9.15, SD = 2.6). Consistent with this lack of a gefitinib effect on normal hematopoietic cell viability, no hematologic toxicity has been observed in clinical trials evaluating gefitinib in patients with lung cancer and other solid tumors.23-25

For patients from whom sufficient numbers of leukemic cells were available, we explored the effects of gefitinib on differentiation by evaluating morphological and genome-wide gene expression changes. For example, in a patient with M3-AML with the t(15;17) and trisomy 8 (Patient 1) and in a patient with M4-AML with normal cytogenetics (Patient 7), gefitinib induced morphological changes consistent with neutrophil differentiation (Figure 6 A-D). Additionally, in a patient with M1-AML with monocytic features and trisomy 8 (Patient 8), gefitinib induced morphological evidence of macrophage differentiation (Figure 6 E-F). Furthermore, microarray-based expression profiling indicated that 3-day exposure to 5 μM gefitinib induced a gene expression program consistent with neutrophil differentiation as assessed by the Mantel test (P = 0.001) for Patient 1 and with a 3-day exposure to 2.5 μM gefitinib for Patient 7 (P < 0.001). Inadequate RNA was available from Patient 8. Similarly, a patient with M5-AML with a FLT-3 mutation and monosomy 7 (Patient 2) induced the gene expression program of myeloid differentiation in response to gefitinib (P < 0.001). These experiments indicate that clinically achievable doses of gefitinib induce bona fide myeloid differentiation of primary AML blasts in vitro.
Discussion

The road to developing new therapeutic agents for AML is a long and expensive one. The fact that AML is a relatively rare disease further adds to a market disincentive to develop new drugs for the disease. Accordingly, very few new agents have been FDA-approved for patients with AML over the past decade. The problem is further compounded by the fact that for the majority of cases of AML, validated therapeutic targets are unknown and therefore conventional biochemical screens cannot be initiated.

As an alternate approach to the problem, we undertook a chemical screen based not on an isolated target, but rather on a gene expression signature of the differentiated state, reasoning that any compound capable of inducing the gene expression program of neutrophilic differentiation would be of potential biological and clinical interest. As with any chemical screening approach, the output of the primary screen rarely yields a compound that can be tested directly in humans. We therefore deliberately screened a library of compounds about which much was known with respect to biochemical properties and mechanism of action. The chemical identified in that screen, 4,5 dianilinophthalimide (DAPH1) was one such compound. It is known to have EGFR kinase inhibitory activity, but its development as a drug ceased, and it is no longer commercially available. Nevertheless, this functional knowledge of DAPH1 biochemical activity allowed us to move directly to the characterization of other EGFR family kinase inhibitors including gefitinib, which, given its FDA-approval status, might be rapidly translated into the clinic.

Indeed we observed that gefitinib induces striking morphological, biochemical and gene expression evidence of myeloid differentiation both in AML cell lines and primary patient-derived AML blasts. As expected with terminal differentiating cells, gefitinib also inhibited the proliferation of AML blasts in vitro. The potential for gefitinib or other EGFR inhibitors as anti-AML agents has not been previously explored, likely in large measure because EGFR is not thought to be expressed in AML blasts. Our experiments confirm lack of expression of EGFR at the RNA or protein level, indicating that EGFR is not a target of gefitinib in these cells. Our observation that low micromolar concentrations of gefitinib are required to induce the differentiation effect is consistent with a non-EGFR target, given the well-documented IC50 of EGFR inhibition in the < 50 nM range based on in vitro kinase profiling. The ability of gefitinib to inhibit the ERBB2 kinase has been well recognized. ERBB2 protein was undetectable in gefitinib-responsive AML cells, and the anti-ERBB2 antibody trastuzumab failed to recapitulate the differentiation effect.

These experiments strongly indicate that the differentiation-promoting effects of gefitinib occur through a non-EGFR, non-ERBB2 target, thereby implicating an as yet uncharacterized target of gefitinib. In that regard, a recent report described the systematic identification of protein targets of a panel of kinase inhibitors in clinical development. That report indicates, quite surprisingly, that most kinase inhibitors, including gefitinib, hit a large number of kinase targets, the majority of which were
previously unknown. Of the 119 protein kinases evaluated, gefitinib, in particular, was shown to bind to at least 18 kinases at less than 10 µM. Each of these, either alone, or in combination, should now be evaluated as the critical target through which the differentiative effects of gefitinib are exerted.

In parallel to such laboratory investigations of gefitinib mechanism of action in AML cells, we believe that the striking biological effects of gefitinib on AML cells warrant immediate clinical translation. Gefitinib has been used to treat over 100,000 patients with non-small cell lung cancer and has been FDA-approved as third line therapy for patients with locally advanced or metastatic disease. It is orally administered and has an excellent safety profile with no reported deleterious effects on normal hematopoiesis. Dose limiting toxicity was most commonly diarrhea and rash.27 At the maximum tolerated dose (MTD; 700-1000 mg/day), the reported geometric mean Cmax was approximately 2.5-5 µM,23,24,28 in the range required to induce myeloid differentiation \textit{in vitro} in our studies (mean responder EC50 2 µM). We therefore expect that treatment of patients at the MTD will be well tolerated and has the potential to induce AML differentiation \textit{in vivo}. Given its outstanding safety profile, ease of administration, and compelling pre-clinical data, clinical trials testing gefitinib in relapsed or refractory patients with AML are therefore warranted.

\textbf{Acknowledgments}

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Table 1. Patient sample characteristics and *in vitro* gefitinib EC50.

<table>
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<th>Diagnosis</th>
<th>EC50 (µM)</th>
<th>Cytogenetic Findings</th>
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<tr>
<td>1</td>
<td>M3-AML</td>
<td>1.84</td>
<td>47, XX, +8, t(15;17) (q22;q21)</td>
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<tr>
<td>2</td>
<td>M5-AML</td>
<td>1</td>
<td>45, XY, -7</td>
</tr>
<tr>
<td>4</td>
<td>M1-AML</td>
<td>8.11</td>
<td>46, XY, der(6) ins(6;?) (q23;?) t(6;7) (q25;p13-14), der(7) add(7) (p13-14) del(7) 9 (q22q34), del(11) (q2?3), del(12) (q13q24) .ish der(6) (MLL+), del(11) (MLL-)</td>
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<tr>
<td>6</td>
<td>M5-AML</td>
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Primary patient leukemia blasts were collected by ficoll separation from bone marrow aspirate or peripheral blood. FAB subclass and cytogenetic findings are reported above. The gefitinib concentration at which cell viability is 50% of DMSO treated control cells (EC50) was determined at 6 days by an ATP-based assay performed in four replicates.
Figure Legends

Figure 1. Morphological and functional changes induced by gefitinib in AML cell lines. May-Grunwald Giemsa staining of HL-60 cells treated with (A) 0.02% DMSO and (B) 10 µM gefitinib for 4 days, Kasumi-1 cells treated with (C) 0.01% DMSO and (D) 5 µM gefitinib for 3 days, and U937 cells treated with (E) 0.02% DMSO and (F) 8 µM gefitinib for 3 days. Doses were chosen at which optimal differentiation occurs. Images were acquired with an Olympus BH-2 microscope, 100X/1.25 magnification under oil, an Olympus Q-Color 5 digital camera, and Adobe Photoshop CS version 8.0 software. (G) HL-60 cells were treated in triplicate for 5 days with gefitinib in a 2-fold dose response series from 10 µM to 1.25 µM and the percentage of NBT positive cells compared to DMSO treated controls with a one-tailed $t$-test analysis.

Figure 2. Gefitinib induces maturation associated myeloid cell surface markers. Fluorescence-activated cell sorting analysis was performed with FITC labeled antibodies for CD14 and CD11b. AML cell lines were incubated for 5 days before analysis with DMSO vehicle or with gefitinib at a dose optimized for morphological changes: HL-60 (10 µM), Kasumi-1 (5 µM), and U937 (7.5 µM). Viable cells were gated based on forward and side scatter patterns and fluorescence determined. Shaded in grey are the DMSO treated controls and outlined in black the gefitinib treated cells.

Figure 3. Gefitinib induces whole-genome expression modulation consistent with neutrophil maturation. Gene expression profiling was done in triplicate 6 hours and 24 hours after treatment with either 10 µM gefitinib or 0.02% DMSO in HL-60 cells. These patterns were compared with expression profiles distinguishing primary patient AML cells from normal human neutrophils. Using the signal-to-noise (SNR) metric, the genes distinguishing the nine primary AML samples from the three normal neutrophil samples were identified and then reordered according to their degree of regulation by gefitinib in HL-60 cells. The top 25 genes in each direction are shown.

Figure 4. Gefitinib induced differentiation is EGFR-independent. (A) RT-PCR (40 cycles) was performed to evaluate for EGFR and ERBB2 transcript presence in HL-60 and Kasumi-1 cells. GAPD was amplified as a control to demonstrate intact cDNA. Negative water controls and a positive cDNA control (uRNA) synthesized from universal Stratagene RNA were also included. (B) Western immunoblotting was performed to evaluate for the presence of EGFR protein in HL-60 and Kasumi-1 whole cell lysates (50µg) with anti-EGFR antibody. An EGFR expressing positive control, A431epidermoid carcinoma cells, was included. (C) Immunoprecipitation was performed with anti-ERBB2 and a mouse IgG control antibody to evaluate for the presence of ERBB2 protein in HL-60 cells with and without 10 µM gefitinib stimulation. Total lysate from SKBR3, a breast cancer cell line known to overexpress ERBB2, was used as a positive control. Western
immunoblotting was performed with ERBB2 or a cocktail of 4G10 and pY100 antiphosphotyrosine antibodies.

**Figure 5. Gefitinib inhibits cell viability in the majority of primary patient AML cells.** Primary patient AML blasts were isolated from bone marrow aspirate or peripheral blood samples by ficoll separation and treated in a dose-response series with gefitinib. Cell viability was evaluated at 6 days with an ATP-based assay and plotted as a percentage of control cells. Samples were evaluated in replicates of four. Blasts from peripheral blood mononuclear cells from normal human donors show markedly decreased response to gefitinib compared to AML samples. One illustrative example is included. FAB class and EC50 evaluation are reported in Table 1.

**Figure 6. Morphological changes induced by gefitinib in primary patient AML blasts.** May-Grunwald Giemsa staining of primary patient M3-AML blasts (Patient 1) containing the t(15;17) and trisomy 8 treated *in vitro* for 3 days with (A) 0.01% DMSO and (B) 5 μM gefitinib, primary patient M4-AML blasts (Patient 7) treated *in vitro* for 4 days with (C) 0.01% DMSO and (D) 2.5 μM gefitinib, and primary patient M1-AML blasts (Patient 8) containing trisomy 8 treated *in vitro* for 7 days with (E) 0.01% DMSO and (F) 10 μM gefitinib. Images were acquired with an Olympus BH-2 microscope, 100X/1.25 magnification under oil, an Olympus Q-Color 5 digital camera, and Adobe Photoshop CS version 8.0 software.

**References**


21. Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839


Figure 2

HL-60  Kasumi-1  U937

CD14

CD11b
Figure 3
Figure 4

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Gefitinib (10 μM)

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IP: ERBB2
Western: ERBB2

IP: ERBB2
Western: PTyr
Figure 5

![Graph showing the percentage of control of different patients' CD8+ T cells treated with gefitinib at various concentrations.](figure5.png)

Legend:
- **Patient 1**
- **Patient 2**
- **Patient 3**
- **Patient 4**
- **Patient 5**
- **Patient 6**
- **Patient 7**
- **Patient 8**
- **PBMC**

X-axis: Gefitinib concentration (μM)
Y-axis: % of Control
Figure 6
Gefitinib (Iressa) induces myeloid differentiation of acute myeloid leukemia

Kimberly Stegmaier, Steven M Corsello, Kenneth N Ross, Jenny S Wong, Daniel J DeAngelo and Todd R Golub