Differential Effects of Tyrosine Kinase Inhibitors on Normal and Oncogenic EGFR Signaling and Downstream Effectors

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Abstract

Constitutive activation of EGFR due to overexpression or mutation in tumor cells leads to dysregulated downstream cellular signaling pathways. Therefore, EGFR as well as its downstream effectors have been identified as important therapeutic targets. The FDA-approved small-molecule inhibitors of EGFR, gefitinib (Iressa) and erlotinib (Tarceva), are clinically effective in a subset of patients with non–small cell lung cancer (NSCLC) whose tumors harbor activating mutations within the kinase domain of EGFR. The current study examined effects of these drugs in 32D cells expressing native (WT) or oncogenic (L858R) EGFR as well as in cancer cell lines A431 and H3255. Distinct patterns for gefitinib and erlotinib inhibition of EGFR autophosphorylation at individual tyrosines were revealed for wild-type (WT) and L858R EGFR. Phosphorylation of Y845 has been shown to be important in cancer cells and Y1045 phosphorylation is linked to Cbl-mediated ubiquitination and degradation. Dramatic differences were observed by greater potency of these drugs for inhibiting downstream effectors for L858R EGFR including Cbl and STAT5. Selective targeting of Cbl may play a role in oncogene addiction and effects on STAT5 identify features of signaling circuitry for L858R EGFR that contribute to drug sensitivity and clinical efficacy. These data provide new understanding of the EGFR signaling environment and suggest useful paradigms for predicting patient response to EGFR-targeted therapy as well as combination treatments.

Implications: This study offers fundamental insights for understanding molecular mechanisms of drug sensitivity on oncogenic forms of EGFR and downstream signaling components as well as considerations for further drug optimization and design of combination therapy. Mol Cancer Res; 13(4): 765–74. ©2015 AACR.

Introduction

Cancer is the leading cause of death worldwide, and there are limited options for treatment, which typically includes surgery, irradiation, and/or chemotherapy. Since the successful development of the first kinase-targeted drug, imatinib (Gleevec), which inhibits the Abelson tyrosine kinase that promotes chronic myelogenous leukemia (1), many drugs have been subsequently developed that target specific proteins that are mutationally activated or overexpressed in various forms of cancers. Because EGFR is overexpressed or constitutively active due to mutations in various cancers, it is an important target for drug development. Erlotinib and gefitinib are 2 of the first FDA-approved drugs specifically targeting EGFR in non–small cell lung cancer (NSCLC). These compounds are small ATP mimetics that inhibit autophosphorylation of EGFR at its cytoplasmic tyrosine residues by binding to the catalytic site. Notably, these 2 drugs are more effective in patients with activating mutations in the kinase domain of EGFR. The most common activating EGFR mutations are an in-frame deletion affecting exon 19 (delE746-A750) and a point mutation at residue 858 in exon 21 (L858R; L858R is also known as L834R; the difference in numbering is based upon the 24 amino acid nuclear localization sequence that is absent in the mature form of EGFR; ref. 2). The underlying mechanisms for enhanced potency are unclear. Previous structural studies show similar inhibitor binding in the kinase domain for native (wild-type, WT) and L858R-mutant forms of EGFR (3), and subsequent work has suggested altered ATP affinity, dimerization propensity, and conformational enzyme dynamics as possible factors governing increased drug sensitivity (4–6).

Phosphorylated tyrosine residues in EGFR serve as specific recruitment sites for downstream signaling molecules that are involved in various cellular processes such as proliferation, migration, and apoptosis (7). Although studies have shown that different signaling molecules in pathways downstream of EGFR are activated in many cancers, few global studies address linking autophosphorylation of a tyrosine residue in EGFR to the activation of specific downstream signaling molecules.

Studies by Wu and colleagues show that many tyrosine residues in EGFR are differentially phosphorylated in lung cancer compared with normal lung tissue and provide a phosphosignature that is strongly correlated with the lung cancer phenotype (8). Similarly, Klammer and colleagues looked at the phosphoproteome profile from NSCLC cell lines identifying predictive
phosphosignatures that differ between sensitive and resistant cell lines (9). In another study, Chen and colleagues report distinctive activation patterns in constitutively active and gatekeeper EGFR mutants compared with WT EGFR (10). It was observed that gefitinib has variable growth-inhibitory effects in cells expressing different EGFR mutants. These studies show that various cell lines with distinct phosphorylated EGFR due to mutations have specific phosphosignatures that respond differently to the drugs. Therefore, correlating specific phosphosignatures to drug responsiveness may be a useful strategy for individualized therapy.

Previously, our biochemical studies examined the temporal resolution of autophosphorylation for WT and oncogenic L858R EGFR using the recombinant cytoplasmic domain of EGFR expressed in Sf9 cells (5, 11). Differences in autophosphorylation kinetics and the unique signature patterns of drug sensitivity were observed between WT and L858R EGFR. With these biochemical studies as a foundation, we extended our studies at a cellular level using 32D cells, a myeloid cell line lacking endogenous EGFR. Isogenic 32D cells overexpressing either native (WT) or oncogenic L858R-mutant forms of EGFR allowed the study of normal and aberrant EGFR signaling and drug responsiveness without concern for cell line heterogeneity. Additional studies examined WT and L858R-mutant forms of EGFR in the setting of cancer cells. A431 is a human epidermoid carcinoma cell line overexpressing EGFR and H3255 is a human lung cancer cell line expressing L858R EGFR. These cell lines were included as part of an earlier study to understand the effects of the EGFR antibody cetuximab in lung cancer cells and xenografts expressing oncogenic forms of EGFR (12). The current study was designed to address the following mechanistic questions related to the clinical efficacy of gefitinib and erlotinib: (i) Are differences in drug responsiveness observed in EGFR autophosphorylation patterns for individual tyrosines in 32D cells expressing WT and L858R forms of EGFR? (ii) Are some downstream pathways more significant than others when comparing normal and oncogenic EGFR signaling?; and (iii) Can we identify key tyrosines in EGFR or downstream signaling molecules that may play prominent roles in determining drug sensitivity in the context of oncogenic EGFR signaling?

The current study establishes that gefitinib and erlotinib have differential effects at a cellular level as assessed by examining autophosphorylation of individual tyrosines in 32D cells expressing WT or L858R-mutant forms of EGFR, consistent with our previous biochemical studies. In addition, it was observed that there were marked differences in drug sensitivity with respect to inhibition of downstream signaling proteins. By examining normal and oncogenic EGFR signaling in 32D cells, it was found that both drugs significantly impacted the activation of the Y845 residue in L858R EGFR compared with WT EGFR. Among downstream signaling proteins, STAT5 activation was substantially diminished by erlotinib (288-fold) and Cbl activation was most affected by gefitinib (267-fold) in L858R EGFR signaling relative to WT EGFR signaling. Our results suggest that L858R EGFR signaling may be mediated through activation of EGFR by autophosphorylation or Src phosphorylation of Y845 followed by STAT5 activation. Inhibition of this pathway for L858R EGFR may be linked to the effectiveness of gefitinib. Likewise, the potent inhibition of Cbl activation in L858R signaling by erlotinib relative to WT EGFR may circumvent receptor degradation and contribute to an oncogene-addicted cellular phenotype. This in-depth analysis of receptor activation, downstream signaling, and differential effects of clinically important drugs aids in understanding mechanistic differences in normal and oncogenic EGFR signaling. These major findings in 32D cell lines were well-translated to cancer cell lines. These results provide insights into the complexity of the EGFR signaling network in human tumors and pinpoint key features of downstream signaling that may be essential for designing even more selective therapies.

Materials and Methods

Cell lines and culture conditions

The cell lines used in this study (32D, A431, and H3255 cells) were kindly provided by Dr. Carlos Arteaga at Vanderbilt University School of Medicine (Nashville, TN; refs. 12, 13). The 32D and A431 cells were originally obtained from ATCC. The NCI-H3255 cells have been previously characterized (14–16); they are heterozygous for the L858R missense mutation in exon 21 of the EGFR gene. The IL3-dependent hematopoietic 32D cell lines stably transfected with EGFR-WT and EGFR-L858R were maintained in RPMI-1640 containing 10% FBS and 10% WEHI conditioned medium (as a source of IL3) and supplemented with antibiotics (penicillin/streptomycin, 100 μg/mL). The human lung cancer cell line NCI-H3255 was grown in RPMI-1640 supplemented with 10% FBS plus antibiotics (penicillin/streptomycin, 100 μg/mL; gentamicin, 50 μg/mL). The human epidermoid carcinoma cell line A431 was maintained in Improved MEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and antibiotics (penicillin/streptomycin, 100 μg/mL). All cells were grown at 37°C in a humidified atmosphere with 5% CO₂.

Reagents and antibodies

EGF was purchased from R&D Systems. We used the following antibodies: anti-phospho-EGFR (to phosphotyrosines 1045, 1065, 1188, and 1173), total EGFR, Akt (#4691), phospho-Akt (Thr#38, #9272), MAPK (#4695), phospho-MAPK (Thr#38, #4370), PI3K (#11889), phospho-PI3K (Tyr#458, #4228), PLCγ1 (#5690), phospho-PLCγ1 (Thr783, #2821), Src (#2123), phospho-Src (Thr416, #2101), Stat3 (#4304), phospho-Stat3 (Tyr705, #9145), phospho-Stat5 (Tyr694, #4322), Cbl (#7477), and horseradish peroxidase (HRP)-linked rabbit IgG secondary antibody from Cell Signaling Technology; anti-phospho-EGFR (phosphotyrosines 845 and 992) from Millipore; and Stat5 (sc-835), phospho-Cbl (Tyr700, sc-377571), and goat anti-mouse IgM-HRP (sc-2064) from Santa Cruz Biotechnology.

Rapid cell stimulation with EGF

Before EGF stimulation, the 32D nonadherent cells grown to a density of approximately 2 × 10⁶ cells/mL were starved for 3 hours in serum-free RPMI-1640. After starvation, equal volumes of the cell suspension and EGF ligand in RPMI-1640 medium (200 ng/mL) were mixed using a KinTek RQF-3 rapid-quench instrument. The reactions were quenched at different time points with lysis buffer [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, 25 mmol/L NaF, protease inhibitors (Complete tablets, Roche Diagnostics), and 1 mmol/L sodium vanadate]. The zero time point was collected using RPMI-1640 media alone in the absence of EGF ligand and represented the unstimulated state. Lysates were incubated on ice for 30 minutes, cleared by centrifugation at 13,000 × g for 10 minutes and the supernatants were collected for further analysis or stored at –80°C.
Inhibition of EGFR phosphorylation

32D-EGFR WT and 32D-EGFR L858R stable cell lines were serum-starved as described previously (17). A431 and H3255 cells grown to 80% confluence were starved overnight in serum-free medium. Before stimulation, cells were preincubated with a range of concentrations of gefitinib or erlotinib (0–10 μmol/L for WT and 0–0.1 μmol/L for L858R EGFR) for 30 minutes and then stimulated with 100 ng/mL EGF for 2 minutes. 32D cells were lysed directly with lysis buffer, whereas A431 and H3255 cells were washed with ice-cold PBS and scrapped immediately after adding lysis buffer. All lysates were cleared by centrifugation and supernatants were collected. Receptor phosphorylation was detected by Western blotting with specific phospho-EGFR antibodies.

Western blot analyses

Equal amounts of protein extracts (10 ng) were resolved by SDS-PAGE (BioRad, 7.5% Tris-HCl gels) and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was first incubated in blocking buffer (3% BSA in TBST-Tris-buffered saline with 0.05% Tween-20), then incubated with a primary antibody (dilution of antibodies were determined as described previously; ref. 5), washed, and blotted with secondary antibody conjugated with HRP (1:2,000). Blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech) and exposed to X-ray films. The level of receptor phosphorylation was evaluated from the Western blotting using a Molecular Imager FX. Standard curves for each antibody were generated to determine the dilution factor necessary to obtain a signal that is linear in response to the amount of protein loaded. Adjusted antibody dilution was as follows: 1:2,000 for 845, 1:5,000 for 992, 1:200 for 1045, 1:1,000 for 1068, 1:250 for 1148, 1:5,000 for 1173, and 1:2,000 for EGFR. To estimate the rate of EGFR phosphorylation, the intensity of bands was plotted against increasing times and the data were fitted to equation 1. To determine the concentration of kinase inhibitor (gefitinib or erlotinib) that resulted in 50% EGFR activity (IC50), the data were fitted to the equation B.

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\text{Intensity} = \frac{\text{Intensity}_{\text{max}} \times (1 - \exp(-\text{time} \times \text{rate of autophosphorylation})) + \text{constant}}{\text{activity}_{\text{max}} \times \text{IC}_{50} + \text{Ik}}
\]

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\text{Percent activity} = \frac{\text{activity}_{\text{max}} \times \text{IC}_{50} + \text{Ik}}{\text{activity}_{\text{max}} \times \text{IC}_{50} + \text{Ik}}
\]

Results

Differential effect of gefitinib and erlotinib on autophosphorylation of EGFR

Previously, we showed that the pattern of gefitinib sensitivity toward tyrosine residues determined at a biochemical level is unique in WT and L858R EGFR (5, 11). Using recombinant EGFR expressed in Sf9 insect cells, we observed that gefitinib was more potent for L858R EGFR than WT and the ranges of drug sensitivity required to inhibit individual tyrosine residues were very different. To further examine drug sensitivity in a cell culture context, we performed similar experiments using 32D cell lines. 32D cells are an IL3-dependent myeloid cell line and EGFR-expressing 32D cells become EGF-dependent in the absence of IL3. Because there

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**Figure 1.**

A and B, effect of gefitinib on WT (A) and L858R EGFR (B) autophosphorylation in 32D cells. C, bar graph representation of IC50 values of gefitinib for WT and L858R EGFR in 32D cells. Parentheses indicate the ratio of IC50 values for gefitinib for WT relative to those of L858R EGFR. After serum starvation, cells were preincubated with the indicated concentrations of gefitinib for 30 minutes and then stimulated with 100 ng/mL EGF for 2 minutes. Cell lysates were run on SDS-PAGE and receptor phosphorylation at different tyrosine residues was detected by Western blotting with specific phospho-EGFR antibodies.

is no endogenous expression of EGFR. 32D cell lines with stable expression of EGFR are widely used as an isogenic model system for studying EGFR signaling. To obtain IC50 values of gefitinib and erlotinib for EGFR inhibition, serum-starved 32D cells expressing WT or L858R EGFR were incubated with increasing concentrations of inhibitors for 30 minutes and then stimulated with EGF for 2 minutes as described in Materials and Methods. EGF stimulation of 32D cell lines expressing WT and L858R-mutant forms of EGFR and kinetic analysis of EGFR phosphorylation established that a maximum level of EGFR activation was achieved after 2 minutes (data not shown). Levels of phosphorylation of individual tyrosine residues for WT and L858R-mutant EGFR in the presence of gefitinib or erlotinib were followed by Western blot analysis using phospho-specific EGFR antibodies (Figs. 1 and 2). Similar to the biochemical data obtained previously, IC50 values for drugs inhibiting the various tyrosine residues of EGFR varied widely at a cellular level (Supplementary Table S1). For example, IC50 values for gefitinib for WT EGFR ranged from 0.019 to 0.083 μmol/L and those for erlotinib ranged from 0.015 to 0.079 μmol/L. L858R EGFR was more sensitive to both gefitinib and erlotinib than WT. In addition to the overall differences in IC50 values, with erlotinib being more potent than gefitinib, there were differential effects on the inhibition of tyrosine autophosphorylation in EGFR. For WT EGFR, gefitinib was most selective...
for Y1045 and Y992 (≈0.02 μmol/L), followed by Y1173 (0.037 μmol/L), and Y1148, Y1068, and Y845 (0.07–0.083 μmol/L).

Erlotinib inhibited phosphorylation of tyrosine residues in a similar order of potency as gefitinib. For L858R EGFR, the pattern in drug sensitivity was altered relative to the WT. All tyrosine residues except Y1068 were similarly sensitive to gefitinib with IC₅₀ of 0.008 to 0.018 μmol/L versus 0.054 μmol/L for Y1068. As with WT EGFR, erlotinib showed a similar pattern of sensitivity, but with greater potency. Interestingly, gefitinib and erlotinib were most potent for inhibition of Y1045 in both WT and L858R EGFR. This is consistent with our observations at a biochemical level (5). Y1045 of EGFR is a docking site for Cbl proteins, which are E3 ligases responsible for ubiquitination and degradation of EGFR (18, 19). Therefore, these results suggest that drugs inhibit phosphorylation at Y1045 and prevent EGFR from ubiquitination.

To quantitatively assess differential drug effects on tyrosine residues of WT and L858R EGFR, the ratios of IC₅₀ values (IC₅₀ values of WT EGFR/IC₅₀ values of L858R EGFR) were compared.

These values are represented as fold differences in parentheses in Figs. 1C and 2C. Even though gefitinib and erlotinib were more potent toward L858R EGFR than WT EGFR in general (up to 6-fold for gefitinib and up to 30-fold for erlotinib), the most striking difference observed was in potency of the 2 drugs on Y845 phosphorylation. As shown in Figs. 1C and 2C, the IC₅₀ of gefitinib in inhibiting Y845 phosphorylation in L858R EGFR was about 6-fold lower than that seen for WT EGFR and erlotinib had about 30-fold lower IC₅₀ in inhibiting Y845 of L858R EGFR than WT. Y845 is located in the activation loop and has been suggested to be phosphorylated by the Src kinase, which is required for EGFR-induced tumorigenesis (20–22). The Y845 residue could also be autophosphorylated, but this is not required for EGFR activation, unlike for many other receptor tyrosine kinases (22). These results suggest that the 2 EGFR inhibitors are more effective toward L858R EGFR by specifically inhibiting phosphorylation of the Y845 residue, and that this effect is greater with erlotinib than with gefitinib.

Effect of gefitinib and erlotinib on phosphorylation of downstream signaling proteins

Autophosphorylation of EGFR leads to activation of downstream signaling (23). Gefitinib and erlotinib have been shown to target EGFR by inhibiting autophosphorylation of EGFR tyrosine residues; however, further effects of these drugs on downstream signaling proteins have not been previously studied in isogenic cells in a comprehensive manner. Here, we examined the effect of gefitinib and erlotinib on inhibition of signaling proteins downstream of WT and L858R EGFR by measuring phosphorylation levels using Western blot analysis. Brieﬂy, serum-starved 32D cells expressing WT or L858R EGFR were incubated with increasing concentrations of drugs for 30 minutes and stimulated with EGF for 2 minutes. Phosphorylation levels of downstream signaling proteins including Src, PI3K, Akt, STAT3, STAT5, ERK, PLCγ, and Cbl were followed by Western blot analysis using total and phosphospeciﬁc antibodies (Supplementary Fig. S1). These particular signaling proteins were selected because their overexpression and activation have been linked to EGFR activation and are associated with cancer (24–27). Supplementary Table S2 summarizes IC₅₀ values of gefitinib and erlotinib with respect to 8 downstream signaling proteins. These IC₅₀ values do not represent direct inhibition of signaling proteins by drugs, rather they examine how the upstream inhibition of EGFR phosphorylation by these drugs is manifested downstream and whether inhibition of phosphorylation is amplified or dampened in the context of WT and L858R EGFR signaling.

As shown in Supplementary Table S2A, in 32D cells expressing WT EGFR, the IC₅₀ values of gefitinib for downstream EGFR signaling proteins indicate that STAT3 and STAT5 are the most sensitive (0.017–0.068 μmol/L), followed by PLCγ and PI3K (0.13–0.16 μmol/L), then Akt and ERK (0.92–1.08 μmol/L), and finally, Cbl and Src (3.74–4.12 μmol/L). Also in Supplementary Table S2A are results for the 32D cells expressing L858R EGFR, showing that gefitinib was much more potent in exerting effects on downstream EGFR signaling proteins. Distinct drug sensitivity patterns were observed. The IC₅₀ values of gefitinib revealed that the downstream proteins most sensitive were STAT5 and PLCγ (0.0038–0.008 μmol/L), followed by ERK, Cbl, STAT3, and Akt (0.012–0.028 μmol/L), whereas Src and PI3K were not inhibited by as much as the highest concentration of gefitinib tested
(0.5 μmol/L). A comparison of fold differences for WT/L858R cells is shown in the right hand column.

Similar to gefitinib, erlotinib was more potent toward L858R EGFR than WT EGFR, with the exception of Src and PI3K phosphorylation, as shown in Supplementary Table S2B. However, the pattern of erlotinib inhibition of downstream signaling proteins was not the same as for gefitinib. In this case, the IC50 values for erlotinib toward WT EGFR signaling proteins showed that STAT3 was the most sensitive (0.0061 μmol/L), followed by Src and Cbl (0.042 and 0.05 μmol/L, respectively), and PI3K, Akt, STAT5, PLCγ, and ERK (0.46–0.84 μmol/L). For the L858R-mutant EGFR signaling, the IC50 values of erlotinib for inhibition of downstream phosphorylation of signaling proteins showed that STAT3 was the most sensitive (0.00087 μmol/L), followed by Cbl, STAT5, and PLCγ (0.0015–0.0048 μmol/L), and ERK and Akt (0.019 and 0.023 μmol/L, respectively). Src and PI3K phosphorylation was not inhibited by as much as 0.5 μmol/L of erlotinib, as was seen with gefitinib.

A summary of the ratio of IC50 values for each drug in the context of signaling proteins in WT and L858R EGFR-expressing cells is shown as bar graphs in Fig. 3A (gefitinib) and B (erlotinib). The data reveal that these drugs exhibit distinct patterns of inhibition for downstream signaling pathways in WT and L858R EGFR-expressing cells. Those values having >5-fold difference are shown. One of the most striking results was the effect of gefitinib on Cbl phosphorylation, where the drug was 288-fold more potent on L858R EGFR relative to WT (Fig. 3A). Cbl is an ubiquitin ligase and is responsible for ubiquitination and ultimate targeting of EGFR for receptor degradation. On the other hand, erlotinib showed the greatest fold difference in IC50 values...
between WT and L858R EGFR-expressing cells in the context of inhibition of downstream signaling protein STAT which in this case was 267-fold (Fig. 3B). These results suggest that the downstream pathway effects of gefitinib and erlotinib are achieved in unique ways despite their similar chemical properties and direct inhibition on EGFR.

To examine whether the effect of drugs on phosphorylation of downstream EGFR signaling proteins seen in 32D cells is also observed in cancer cell lines, these studies were extended to evaluate A431 and H3255 cell lines. The A431 cancer cells overexpress WT EGFR, whereas the H3255 cells overexpress the missense oncogenic L858R EGFR. The data for the effects of gefitinib and erlotinib on A431 and H3255 are found in Supplementary Table S3A and S3B and Supplementary Fig. S2A and S2B, respectively. Figure 4 summarizes in bar graphs the fold difference in the ratio of IC_{50} values for WT (A431) and L858R (H3255) inhibition of downstream EGFR proteins for gefitinib (Fig. 4A) and erlotinib (Fig. 4B). Those values showing >5-fold inhibition are shown. Consistent with the results in 32D cells, in cancer cells expressing WT or L858R EGFR, the largest effect on downstream EGFR signaling observed for gefitinib was on Cbl (100-fold) as shown in Fig. 4A. Also in accord with 32D cells as illustrated in Fig. 4B, for erlotinib, the most notable effect on EGFR-mediated downstream signaling partners for WT versus L858R was STAT5 (404-fold).

**Discussion**

Cellular signaling via EGFR is initiated by ligand-stimulated autophosphorylation of key tyrosine residues of EGFR that have been shown to recruit a host of downstream signaling proteins leading to activation of various cellular pathways responsible for controlling growth and survival (28). Our previous study with FGFR1 (fibroblast growth factor receptor 1) have shown that the
order of autophosphorylation is important in providing both temporal and spatial resolutions to receptor signaling and this order is perturbed in an oncogenic form of EGFR (29, 30). Therefore, the pattern and timing of phosphorylation events on EGFR may be important for proper orchestration of downstream partner recruitment and activation of relevant pathways in a defined manner for appropriate regulation of cell growth. Identifying specific phosphosignatures of global EGFR signaling may offer insights that may be useful for predicting patient response to EGFR-targeted therapy as well as combination treatments. Our previous biochemical studies using a recombinant form of the cytoplasmic domain for EGFR expressed in S9P cells have shown that there is a unique signature pattern of drug sensitivity between WT and L858R EGFR, indicating that the activation of specific tyrosine residues determines drug sensitivity (5). The current study was designed to extend those findings in a cellular context to determine how inhibition of EGFR autophosphorylation by gefitinib and erlotinib may differentially affect specific downstream signaling proteins. EGFR autophosphorylation patterns may emerge that predict drug sensitivity and explain the effectiveness of gefitinib and erlotinib in cells expressing L858R oncogenic form of EGFR relative to WT. Downstream signaling partners and pathways may be identified that are also incongruent influenced in oncogenic signaling and define essential features of drug efficacy.

Similar to our findings at the biochemical level, tyrosine 1045 within the C-terminal tail of EGFR was identified as being one of the most sensitive to drug inhibition (Supplementary Table S1 and Figs. S1 and S2; ref. 5). Phosphorylation of Y1045 has been established as a mechanism to recruit Cbl, an ubiquitin ligase, for EGFR ubiquitination followed by degradation in the lysosome (31, 32). In another study by Gu and colleagues, hypophosphorylation at Y1045 was shown to cause impaired degradation and enhanced recycling of EGFR (33). Our results with gefitinib suggest that this drug may prevent the oncogenic L858R form of EGFR from degradation by inhibiting phosphorylation at Y1045. If this mutant form of EGFR is less susceptible to degradation, this in turn might render cell growth more dependent on EGFR activation, thereby enhancing oncogene "addiction" (34). Accordingly, the mode of action for gefitinib in cells expressing L858R EGFR may, in part, be linked to inhibition of Y1045 and subsequent prevention of Cbl activation, as Cbl is downstream of Y1045. However, relative to L858R-expressing cells, gefitinib is a less potent inhibitor of Cbl phosphorylation in cells expressing WT EGFR (Figs. 3A and 4A). In this case, Cbl might be activated through a different pathway. These observations may explain the molecular features of inhibiting key tyrosines on EGFR and downstream effectors responsible for the clinical effectiveness of gefitinib in treating patients having EGFR mutations. Recent studies have suggested that Cbl inhibitors may be effective in some cancers (35).

Our findings with erlotinib imply that additional mechanisms may be operative in patients with tumors harboring EGFR mutations such as L858R. The most significant difference in terms of EGFR activation in comparing cells expressing WT and oncogenic EGFR was found to be erlotinib sensitivity toward inhibition of phosphorylation of Y845 in EGFR, with an observed 30-fold difference in IC_{50} WT/IC_{50} L858R as shown in Supplementary Table S1B. Further examination of downstream signaling in the presence of erlotinib revealed differences in the ratios for IC_{50} WT/IC_{50} L858R for STAT5 and PLC\gamma phosphorylation, with the largest difference being STAT5 (Fig. 3B). This large difference implies that effectiveness of erlotinib in patients with EGFR mutation may be largely driven by inactivation of the STAT5 pathway perhaps in concert with PLC\gamma1. Similar results were obtained with cancer cell lines in which a 404-fold difference in erlotinib sensitivity was noted for STAT5 (Fig. 4B).

The differential erlotinib activity against Y845 suggests that L858R EGFR might also be more dependent on Y845 activation relative to WT EGFR. Therefore, inhibiting Y845 phosphorylation by erlotinib might provide a further explanation for the effectiveness in the context of EGFR L858R mutation. Phosphorylation of Y845 has shown to be ligand-independent and associated with STAT-dependent gene expression (36). Upon phosphorylation, the Y845 residue interacts with Src through an SH2 domain that in turn activates STAT5 signaling. Y845 phosphorylation could be indirectly inhibited because of Src inhibition by drugs. However, as shown in Supplementary Tables S2 and S3, Src activity is not substantially changed by the 2 drugs. Therefore, it seems plausible that Y845 phosphorylation is directly inhibited by drugs rather than via Src inhibition. The lack of Src inhibition by gefitinib suggests that L858R activation is Src-independent. Interestingly, studies done by Fu and colleagues showed that phosphorylation of Y845 is important for constitutive activation of L858R EGFR. They also found that L858R EGFR is more sensitive to PP2, a Src inhibitor, compared with WT EGFR (36). This implies that L858R EGFR uses Y845 phosphorylation as an activation mechanism and that Src inhibition by PP2 would preclude Y845 phosphorylation from exhibiting enhanced sensitivity toward PP2. Other work by Yang and colleagues suggests that mutation in the kinase domain of EGFR alone is sufficient for phosphorylation of Y845 and that Src kinase activity is dispensable (36). An additional study by Chung and colleagues showed that NSCLC-associated mutant forms of EGFR displayed enhanced association with Src (37). Activation of Y845 in L858R EGFR was also observed by Sordella and colleagues (38).

Whether or not Y845 phosphorylation is Src-dependent, L858R EGFR signaling seems to involve Y845 activation. The results described in the current study of gefitinib and erlotinib are in accord with a similar mechanism in which L858R EGFR becomes constitutively active due to the phosphorylation of the Y845 residue. Therefore, the inhibitory drugs are more effective toward L858R EGFR by specifically targeting Y845.

Another important consequence of Src-mediated phosphorylation of Y845 in EGFR signaling is the activation of STAT5 (39, 40). Phosphorylation of Y699 within STAT5b is responsible for mediating EGF-induced DNA synthesis. Interestingly, a connection in EGFR signaling between EGFR Y845 and STAT5 is represented in Figs. 3B and 4B and Supplementary Tables S2B and S3B. In the case of erlotinib, the level of phosphorylation for STAT5 was much lower in the presence of drug suggesting a possible link between Y845 phosphorylation and STAT5 activation. Src phosphorylation of EGFR on Y845 has a major role in regulation of cellular functions, as described in reports by Sato and colleagues (41). Src is negatively regulated by phosphorylation and its signal-dependent transient activation contributes to cellular responses such as proliferation and differentiation. Although Y845 is dispensable for cellular functions regulated by EGFR, transphosphorylation of Y845 by Src plays an important role in several aspects of cellular functions. Thus, it was reported by Gotoh and colleagues (42) that the Y845F mutant of EGFR was able to transform NIH3T3 cells. STAT is a possible mediator of the...
Y845 phosphorylation–dependent synergy between EGFR and Src. In breast cancer cells, STAT5b was identified as a major tyrosine-phosphorylated protein and expression of the Y845F mutant of EGFR had a negative effect on activation of STAT5b. Moreover, activated EGFR–STAT3 signaling promotes tumor survival in vivo in NSCLC (43).

Our study suggests that the efficacy of erlotinib in L858R EGFR-expressing 32D cells is a result of drug effects on Y845 and STAT5 phosphorylation. Sordella and colleagues reported that activating mutations in EGFR selectively activate Akt and STAT pathways that are important in lung cancer cell survival by activating an anti-apoptotic pathway and gefitinib inhibits this survival pathway in EGFR-dependent cells (38). Other studies support a role for Src family kinases to link signals from growth factors to downstream effector pathways (44, 45). An understanding of these phosphorylation events in signal propagation enables prediction of drug sensitivity to small-molecule inhibitors such as gefitinib and erlotinib. Song and colleagues showed that a Src kinase inhibitor, dasatinib, selectively induces apoptosis in lung cancer cells dependent on EGFR signaling for survival (46) suggesting that Src is in fact the main player in EGFR signaling. Our studies further validate the importance of Src in linking EGFR signaling to activation of the STAT pathway via phosphorylation of tyrosine 845 in EGFR and suggest that drugs such as gefitinib and erlotinib are much more effective in the context of EGFR-activating mutations due to their specific inhibition of phosphorylation of tyrosine residue 845, leading to apoptosis. Activation of Src and STAT5 has also been reported in squamous cell carcinoma of the head and neck (SCCHN; refs. 47, 48). Koppikar and colleagues showed that combined treatment with Src inhibitor AZD0530 and gefitinib led to greater inhibition of SCCHN compared with single-inhibitor treatment (49). In another study, Koppikar and colleagues presented that STAT5 activation contributes to the resistance of SCCHN cells to the EGFR tyrosine kinase receptor, erlotinib (50). Taken together with our studies, combination therapy using inhibitors of both STAT5 and EGFR would be more effective treatment in patients with lung cancer. Many inhibitors targeting STAT pathway in various cancers have been developed (51).

In summary, we have examined the effect of 2 FDA-approved tyrosine kinase inhibitors, gefitinib and erlotinib on inhibition of EGFR and its downstream signaling proteins. The IC50 values for gefitinib and erlotinib inhibition with respect to key tyrosine residues in EGFR and essential downstream signaling proteins were determined for normal and oncogenic EGFR signaling. The most significant difference in terms of EGFR activation was found to be erlotinib activity toward tyrosine 845 in L858R EGFR signaling, which exhibited about 30-fold lower IC50 value than normal WT signaling. For downstream signaling proteins, a 288-fold difference in IC50 WT/IC50 L858R was observed for Cbl inhibition following gefitinib in 32D cells. Erlotinib showed a 267-fold difference in IC50 WT/IC50 L858R for STAT5 phosphorylation in 32D cells. Similarly, in comparing A431 cells and H3255, there was 100-fold difference in IC50 WT/IC50 L858R for Cbl inhibition following gefitinib and 404-fold difference in IC50 WT/IC50 L858R for STAT5 inhibition following erlotinib. Figure 5 provides a diagrammatic representation of gefitinib and erlotinib effects on EGFR downstream signaling pathways. These studies highlight the importance of Y1045 in EGFR and downstream signaling protein Cbl for the efficacy of gefitinib on L858R EGFR. In addition, they suggest that constitutive activation of L858R EGFR is mediated through phosphorylation of Y845 followed by activation of the STAT5 downstream pathway, and erlotinib seems to specifically target these pathways in the context of oncogenic mutant forms of EGFR. This information provides a more detailed understanding of the effectiveness of gefitinib and erlotinib. Moreover, this in-depth analysis may point to new avenues to explore for designing better drugs and combination therapy targeting not only EGFR but also its downstream signaling proteins when treating various forms of cancer.

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No potential conflicts of interest were disclosed.

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Effect of Gefitinib and Erlotinib on Normal and Oncogenic EGFR Signaling

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