Impact of Common Epidermal Growth Factor Receptor and HER2 Variants on Receptor Activity and Inhibition by Lapatinib

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Abstract

The goal of this study was to characterize the effects of non–small cell lung carcinoma (NSCLC)-associated mutations in epidermal growth factor receptor (EGFR/ErbB1) and HER2 (ErbB2) on interactions with the dual tyrosine kinase inhibitor lapatinib. Biochemical studies show that commonly observed variants of EGFR [G719C, G719S, L858R, L861Q, and Δ746–750 (del15)] are enzyme activating, increasing the tyrosine kinase activity. Mutations G719C and L861Q had minor effects on lapatinib potency. [Ca 2+ ] is an enzyme activating, increasing the tyrosine kinase activity. These data suggest that structural flexibility and possible stabilization of the active-like conformation could interfere with lapatinib binding, particularly to the EGFR deletion mutants. Furthermore, EGFR deletion mutants were relatively resistant to lapatinib in recombinant cells expressing the variants, whereas EGFR potentiated the ability of epidermal growth factor (EGF) to activate the receptor, and that the activated mutant EGFR is more susceptible to drug-dependent inhibition (11, 17, 18). This is consistent with the fact that the activating mutations are located in the tyrosine kinase domain and cluster in or near the ATP binding pocket of EGFR. Cell-based studies suggest that the sensitizing mutations in EGFR potentiate the ability of epidermal growth factor (EGF) to activate the receptor, and that the activated mutant EGFR is more susceptible to drug-dependent inhibition (11, 17, 18). This is consistent with the fact that the activating mutations are located in the tyrosine kinase domain and cluster in or near the ATP binding pocket of EGFR. Cell-based studies typically measure the steady-state level of receptor phosphorylation on tyrosine, so it is currently unclear if the mutations result in enhanced kinase activity, enhanced activation by ligand, or reduced down-regulation. Biochemical studies with purified receptors and structural studies, including cocystals with inhibitors, are needed to understand the mechanism by which these mutations alter clinical outcome for different drugs and different cancers.

Lapatinib, erlotinib, and gefitinib are members of the 4-anilinoquinazoline group of tyrosine kinase inhibitors. Although erlotinib and gefitinib are specific inhibitors of EGFR, lapatinib is a (1–12). It is reasonable to propose that such variation in efficacy reflects genetic heterogeneity in the tumors of treated patients, and recent evidence strongly supports this possibility. For example, the ability to respond to selective kinase inhibitors, such as gefitinib, erlotinib, and imatinib, has been correlated with mutations in the target receptor (13–16). Detailed studies with gefitinib-responsive (non–small cell lung carcinoma) NSCLC patients showed that gefitinib efficacy is linked to activating mutations in the epidermal growth factor receptor (EGFR) that sensitize the tumor to the drug, possibly by stimulating drug-induced apoptosis (17). A causal relationship between EGFR genotype and drug susceptibility is also supported by the fact that some drug-resistant alleles carry second-site mutations that seem to act as suppressors of EGF-activating, drug-sensitizing mutations (18, 19). Similar observations have also been made for chronic myelogenous leukemia and gastrointestinal stromal tumors–derived cells that acquire resistance to imatinib (20).

A number of EGFR mutations have been described that are enriched in NSCLC adenocarcinomas and in female never smokers of Asian ethnicity (21). The EGFR mutations include in-frame deletions affecting residues 746 to 753, multiple isolates of EGFR-L858R, and other point mutations in exons 18 to 21 (11). EGFR-T790M is a second-site mutation that has been reported in several NSCLC tumors that is strongly associated with resistance to gefitinib (19). Similar mutational patterns have been reported in HER2, and Shigematsu and coworkers (22) recently reported that 11 of 671 (1.6%) of patients' tumors contained HER2 mutant alleles in a study of NSCLC. As observed for EGF, the mutations included primarily in-frame mutations in exon 20 and were highly enriched in adenocarcinomas in female never smokers of Asian ethnicity.

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p.o. potent dual inhibitor of EGFR (ErbB1) and HER2 (ErbB2; refs. 23, 24) that strongly down-regulates the antiapoptotic Akt pathway in responsive cells (25). Biochemical studies show that the EGFR-lapatinib complex has a long half-life and is characterized by a much slower dissociation rate than the erlotinib- or gefitinib-receptor complexes (24). This mode of binding may potentiate drug efficacy by prolonging drug-induced down-regulation of receptor-mediated tyrosine kinase activity. Indeed, lapatinib has shown clinical benefit for the treatment of metastatic breast cancers that overexpress HER2 when given alone or in combination with chemotherapy (26, 27) and was recently approved by the Food and Drug Administration for the treatment of patients with advanced or metastatic breast cancer whose tumors over-express HER2.

The goal of this study was to characterize the interactions of lapatinib with wild-type EGFR and several of the common mutant variants of EGFR observed in NSCLC. For comparison, receptor variants were also challenged with gefitinib. In addition, the effect of lapatinib on four variants of HER2 was also examined. The results presented here reveal distinct similarities and differences in the interaction between lapatinib or gefitinib and EGFR/HER2.

Materials and Methods

Cell culture and transfections. Chinese hamster ovary (CHO)-K1 cells were from American Type Culture Collection (ATCC) and cultured in Ham’s F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone). CHO-K1 cells were seeded at 2.42 × 10^6 per 100-mm dish 24 h before transfection. Cells were then incubated at 37°C and 10% CO_2 in an incubator in complete medium containing 0.18 mL Lipofectamine 2000 (Invitrogen) and 12 μg plasmid DNA per dish for 24 h. EGFR and HER2 expression was confirmed by immunoprecipitation/Western analyses. Human NSCLC cell lines NCI H1975, NCI H1650, NCI H1734, NCI H358, NCI H322, NCI H1781S, Calu-3, and A549 were obtained from the ATCC. All NSCLC lines were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS.

Lapatinib and gefitinib treatment. Lapatinib and gefitinib were synthesized as described (28, 29). Transfected CHO-K1 cells were exposed to lapatinib or gefitinib at increasing concentrations (0, 0.14, 0.41, 0.12, 0.37, 1.1, and 3.3 μmol/L). Compounds were prepared in DMSO and diluted in complete medium before dosing. Cells transfected with EGFR constructs were exposed to drug for 4 h and stimulated with 10 ng/mL EGF for 10 min before cell lysis. Cells transfected with HER2 constructs were exposed to drug for 4 h and then immediately harvested for cell lysate.

Immunoprecipitation and immunoblotting. EGF was from Sigma. The anti-EGFR antibody for Western detection (αEGFR monoclonal antibody-12) and the anti-EGFR antibody for immunoprecipitation (αEGFR EGFR Ab-13) were from Lab Vision. The antiphosphotyrosine antibody αPT-66 was from Sigma. The peroxidase-conjugated anti-mouse IgG secondary antibody was from Jackson Immunoresearch Laboratories. The anti-HER2 antibody for Western detection (αHER2 C-18) was from Santa Cruz. The anti-HER2 antibody for immunoprecipitation (αHER2 Ab-4 Clone N12) was from Lab Vision. The Alexa Fluor antibody for secondary detection of HER2 was from Invitrogen. Cell lysis, immunoprecipitation, and immunoblotting were performed as described previously (25).

Cellular growth inhibition studies. The NSCLC cell lines were plated in 100 μL of growth medium in 96-well tissue culture plates at the following densities: H1975, 5,000 cells per well; H1650, 10,000 cells per well; H1734, 10,000 cells per well; H358, 5,000 cells per well; H322, 5,000 cells per well; H1781S, 10,000 cells per well; A549, 4,000 cells per well and Calu-3, 10,000 cells per well and the NSCLC cell lines were placed in a humidified 37°C incubator containing 5% CO_2 and 95% air, overnight. Stock solutions and compound dilutions as well as the cell proliferation studies were performed as described in the Supplementary Text. Values for the IC_{50} were determined by the method of Levenberg and Marquardt (30) with Eq. A:

\[ y = V_{\text{max}} - \left(1 - \left(\frac{x^\alpha}{K^\alpha + x^\alpha}\right)\right) \]  

\[ \text{IC}_{50} = K^\alpha \left(\frac{V_{\text{max}}}{\left(1 - \left(\frac{x^\alpha}{K^\alpha + x^\alpha}\right)\right)}\right) \]

PCR amplification, sequencing, and cloning. Amplification of the EGFR gene was performed using genomic DNA from NSCLC cell lines H358, H1734, H1975, H1650, H322, and A549 (ATCC). PCR primers were designed within flanking intronic regions to amplify coding region (exons 2–28) in the EGFR gene (NM_005228.3). Sequence data was not obtained for exon 1 in any cell line due to high guanine-cytosine content. Primer pairs used for EGFR amplification are listed in Supplementary Table S1. For direct sequencing of amplicons, all sequence-specific forward primers were tagged with the universal M13 forward primer sequence (5’-TGTTAAAAGACGGCCAGTGTAG-3’), and all sequence-specific reverse primers were tagged with the universal M13 reverse primer sequence (5’-CAGGAAAAACACGTACGAC-3’).

Sequence analysis of the HER2 (NM_004482.2) gene tyrosine kinase domain (exons 18–24) was accomplished using genomic DNA from NSCLC cell lines H1781S and Calu-3 (ATCC). Intron-based primers used for PCR amplification of the HER2 tyrosine kinase domain were reported by Shigematsu et al. (22) and are listed in Supplementary Table S3. See Supplementary Text for detailed PCR and sequencing protocols and for description of cloning and expression of EGFR and HER2 mutants.

Structural modeling. Due to the different EGFR conformations observed in the erlotinib and lapatinib crystal structures, mutations were considered in the context of both the active and inactive-like conformations. Models were constructed in both conformations for each mutant and examined for interactions that would stabilize or destabilize inhibitor binding. Coordinates from PDB1m17 and PDB1xkk were used as the starting models for the active and inactive conformations of EGFR. It was assumed that gefitinib would bind to the same conformation of the protein as erlotinib.

Enzyme kinetic analysis: determination of catalytic rates \( V_{\text{max}} \) and \( K_{\text{m(app)}} \) for ATP-Mg. \( V_{\text{max}} \) and \( K_{\text{m(app)}} \) for ATP-Mg were determined at a fixed concentration of peptide substrate and variable concentrations of ATP-Mg. The constructs of the enzyme were based on the intracellular domain of EGFR (amino acids 671–1210). Enzyme purification and kinase reaction conditions were as described by Brignola et al. (31). Briefly, phosphorylated product was detected using 1 μCi [γ-32P]ATP per reaction and the phosphocellulose product detection method. Reaction buffers contained 50 mM/L 3-(N-morpholino) propane sulfonic acid (pH 7.5), 10 mM/L MgCl₂, 0.01% Tween 20, 1 mM/L DTT, and 100 mM/L enzyme. Incubations were at 23°C. The peptide substrate, bovine-(amino-hexanoic acid)-(EEEEEELVAKKK-CONH₂), was used at a fixed concentration of 100 μmol/L. Eleven concentrations of ATP-Mg were used ranging from 4 to 300 μmol/L. For each substrate concentration, initial rates were determined from the linear portion of a 30-min time course sampled at 0, 10, 20, and 30 min. In all cases, product formation was <10% of the initial ATP substrate and <20% of the peptide substrate. \( V_{\text{max}} \) and \( K_{\text{m(app)}} \) values were estimated from these experiments by fitting the data to Eq. B:

\[ v = \frac{V_{\text{max}}(\text{ATP})}{K_{\text{m(app)}} + (\text{ATP})}. \]
Estimation of $K_i$ for lapatinib and gefitinib. $K_i$ values for lapatinib and gefitinib were estimated for wild-type and mutant EGFR using second-order assumptions because these enzyme-inhibitor complexes exhibit tight-binding behavior. Reactions were carried out with variable inhibitor concentration at several fixed enzyme concentrations. The lapatinib-EGFR interaction also exhibits significant time-dependent (or slow-binding) behavior. However, a 30-min preincubation period was sufficient to allow EGFR-lapatinib binding to reach equilibrium under the conditions of these experiments. For the EGFR-gefitinib complex, a 5-min preincubation was sufficient because binding reaches equilibrium more quickly than for the EGFR-lapatinib complex.

The concentration range for each EGFR-inhibitor pair used in this analysis was independently selected to optimize the accuracy of the $K_i$ estimate. For each experiment, six fixed concentrations of enzyme were used that ranged up to six times the initial concentration. The initial nominal concentration used for each enzyme was as follows: wild-type, 8 nmol/L; L861Q, 8 nmol/L; G719C, 10 nmol/L; Δ746-750, 40 nmol/L; and L858R, 7 nmol/L. Thus, for wild-type EGFR, the six nominal concentrations were used as 8, 16, 24, 32, 40, and 48 nmol/L. A very similar concentration range was used for all EGFR variants except Δ746-750, which was used at a significantly higher nominal concentration because a relatively small fraction of this enzyme variant was capable of binding the inhibitor (see Results). Eleven concentrations of inhibitor were used evenly, spanning concentrations from 10-fold lower than the apparent $IC_{50}$ to 10-fold higher than the apparent $IC_{50}$.

The rate of product formation was determined using the phosphocellulose filter–binding method (31). The concentration of ATP-Mg was 20 mmol/L, the concentration of peptide, biotin-(aminohexanoic acid)-EEEEEYFEL-VAKKK-CONH2, was 100 mmol/L, and the time of incubation was 20 min. Each experimental condition was performed in quadruplicate, and the average rate of product formation was determined. Reaction rates were expressed as a percentage of the no-inhibitor control reaction for each concentration of enzyme used. $K_i^{(app)}$ estimates were obtained according to the general method described by Morrison (32) by globally fitting the data to Eq. C:

$$v_i = v_o \times \frac{[E] + [I] + K_i^{(app)}}{[E] + [I] + K_i^{(app)} - 4a[E]/[I]} + B$$

where the experimental variable $a$ is the factor from 1 to 6 representing the incremental increase in the volume of enzyme used and $[I]$ is the concentration of inhibitor. For experimental results, $v_i$ is the initial rate of product formation normalized to the control value in the absence of inhibitor. For curve fit variables, $v_o$ is the uninhibited rate of the reaction (maximum asymptote of the inhibition curve), $B$ is the residual rate of catalysis at saturating concentrations of inhibitor (minimum asymptote of the inhibition curve), $E$ is the concentration of enzyme at $a = 1$, and $K_i^{(app)}$ is the apparent $K_i$ estimated from the experiment.

$K_i^{(app)}$ values can be converted to $K_i$ values because the inhibitors are competitive with ATP. The equation that explains this relationship is as follows (33):

$$K_i^{(app)} = K_i \left(1 + \frac{[ATP]}{K_{m(app)}}\right)$$

## Results

Truncated forms of EGFR, HER2, HER3, and HER4 have been crystallized as apo proteins or as binary complexes. The binary complex structures include inhibitors that target the intracellular kinase domain or antibodies that bind the extracellular domain (24–34). From these structures, it is apparent that EGFR is capable of relatively large conformational changes upon inhibitor or ligand binding. Recent cocrystal structures of EGFR-erlotinib or EGFR-lapatinib are very different from one another. EGFR-erlotinib has a structure very similar to apo-EGFR. We assume that gefitinib binds in a similar fashion. In contrast, lapatinib binding to EGFR involves several structural changes (24, 41). The EGFR-lapatinib structure suggested that lapatinib binding may be affected by some of the common NSCLC mutations. This report is focused on the following seven EGFR variants, which are found in human NSCLC tumors: Δ746-750 (del15), Δ746-753S (del18), G719C, G719S, L858R, L861Q, and T790M. Four HER2 mutants, YVMA, G776VinsC, H878Y, and V659E, were also characterized in this study.

### Kinetic analysis of tyrosine kinase activity of wild-type and mutant EGFR

To understand the effect of specific EGFR mutations on EGFR tyrosine kinase activity, the kinetic variables of EGFR variants were determined using an in vitro kinase assay. For these experiments, the intracellular domains of wild-type EGFR and the mutants were expressed in insect cells using a baculovirus expression system, and mutant and wild-type proteins were purified to ~80% homogeneity. The apparent $K_m$ and $V_{max}$ values for ATP-Mg were determined for wild-type and mutant EGFR at a fixed concentration of peptide substrate (Table 1). Wild-type EGFR had a $V_{max}$ of 0.52 mol/min/mol. All EGFR mutants in this study had higher intrinsic catalytic activity than wild-type EGFR. L861Q and L858R were the most active (23- and 17-fold higher than that for wild-type EGFR, respectively). The $V_{max}$ for G719C and del15 was 4- and 29-fold higher than that for wild-type EGFR, respectively. At present, the mechanism for this activation is not known; however, all the EGFR mutants characterized here have a higher $K_m$ for ATP-Mg than wild-type EGFR.

### Table 1. Biochemical properties of EGFR mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Catalytic activity</th>
<th>Lapatinib inhibition</th>
<th>Gefitinib inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max}$ (mol/min/mol)</td>
<td>$K_m^{(app)}$ (μmol/L)</td>
<td>$K_i$ (nmol/L)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.52 ± 0.01</td>
<td>9 ± 0.9</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>del15</td>
<td>15 ± 1</td>
<td>118 ± 20</td>
<td>15.01 ± 0.13</td>
</tr>
<tr>
<td>L861Q</td>
<td>12.4 ± 0.6</td>
<td>55 ± 6</td>
<td>0.42 ± 0.18</td>
</tr>
<tr>
<td>G719C</td>
<td>2.2 ± 0.2</td>
<td>97 ± 16</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td>L858R</td>
<td>8.6 ± 0.2</td>
<td>55 ± 3</td>
<td>3.05 ± 0.75</td>
</tr>
</tbody>
</table>

NOTE: Values are the average of at least three independent experiments ± SD. Abbreviation: nd, not determined.
Estimation of mutant and wild-type \( K_i \) for lapatinib and gefitinib. The estimation of \( K_i \) for lapatinib and gefitinib is complicated because EGFR exhibits slow-binding and tight-binding characteristics with these inhibitors. The EGFR-lapatinib interaction is defined as slow-binding because it takes a relatively long time for the protein and inhibitor to reach equilibrium at the concentrations used in the studies (24). The EGFR interaction with either inhibitor is defined as tight binding because the \( K_i \) values are below the concentration of the enzyme needed to catalyze the formation of measurable amounts of product. Under these conditions, the enzyme-inhibitor interaction is no longer first order, and \( K_i \) must be determined using relatively complicated experiments and data analysis methods. To account for this kinetic behavior, we determined \( K_i \) using the general analytic method described by Morrison (32), incorporating an enzyme-inhibitor preincubation to allow the system to reach equilibrium before initiating the reaction with substrate (31).

The \( K_i \) for the lapatinib interaction with wild-type and EGFR del15 was determined (Fig. 1A and B). With wild-type EGFR, increasing the concentration of enzyme used in the assay results in a shift in the apparent IC\(_{50}\). This occurs because the \( K_{i,app} \) for the inhibitor is below the concentration of enzyme, and the apparent IC\(_{50}\) is increased arithmetically by a factor of [Enzyme]/2 (33). With EGFR del15, there is no shift in the apparent IC\(_{50}\) with increasing concentrations of enzyme. This occurs because the \( K_{i,app} \) value is above the highest concentration of enzyme used in the experiment. A global fit of these data to Eq. C gives accurate estimates of both the \( K_{i,app} \) value and the concentration of enzyme competent for inhibitor binding. Lapatinib and gefitinib have been shown to be competitive with ATP (24). Therefore, the \( K_i \) values can be converted to \( K_i \) using Eq. D. The results showed that the \( K_i \) of lapatinib for wild-type EGFR is 0.10 nmol/L; in contrast, the \( K_i \) of lapatinib for EGFR del15 is 15 nmol/L, indicating a 150-fold decrease in the affinity of lapatinib for EGFR. The \( K_i \) of EGFR L858R is 30-fold higher than wild-type EGFR, whereas the \( K_i \) values of G719C and L861Q are slightly higher than wild-type EGFR. Lapatinib inhibition of EGFR and ErbB2 is time-dependent due to a very slow off rate compared with other quinazoline inhibitors such as gefitinib (24). The off rate of lapatinib for the mutant EGFR proteins was determined (Fig. 1C and D). We found that the increase in \( K_i \) observed with del15 and L858R corresponded to a significant decrease in the EGFR-lapatinib half-life (Table 1). The effects of these mutations on the \( K_i \) values for gefitinib are less pronounced (Table 1): the \( K_i \) values of EGFR del15 and G719C are slightly lower than for wild-type EGFR, whereas the other mutations have little or no effect on the \( K_i \) for the drug.

**Figure 1.** Lapatinib \( K_{i,app} \) and off rate for wild-type and mutant EGFR. A, lapatinib \( K_{i,app} \) for wild-type EGFR. Inhibition curve was determined at the following nominal concentrations of EGFR: ●, 8; ○, 16; ▼, 24; △, 32; ■, 40; and □, 48 nmol/L. \( K_{i,app} \) was estimated as described in Materials and Methods. Lines, a global fit of the data to Eq. A. B, lapatinib \( K_{i,app} \) for EGFR del15. Experiment and analysis as in A using 40 nmol/L (●), 80 (○), 120 (▼), 160 (△), 200 (■), and 240 (□) nmol/L nominal enzyme concentration. C, recovery of enzyme activity after dilution of a preformed EGFR-lapatinib complex. ●, residual inhibition control. The EGFR-lapatinib complex was not preformed. Lapatinib was added to the reaction at the final diluted concentration of 0.5 nmol/L. ○, lapatinib and EGFR (0.5 μmol/L of each) were preincubated to form a complex. The reaction was initiated by diluting this complex 1,000-fold into substrate (final concentration, 0.5 nmol/L each). ▼, full inhibition control. Lapatinib and EGFR (0.5 μmol/L of each) were preincubated to form a complex. This mixture was diluted 1,000-fold into substrate mix containing 0.5 μmol/L lapatinib. Off rates were estimated from these results by fitting the data to Eq. B. D, off rate for lapatinib and EGFR del15. Experiment and analysis as in C.
In this study, we have estimated lapatinib $K_i$ for wild-type EGFR to be 0.1 nmol/L. In a previous publication (24), we used a different method to estimate the $K_i$ that generated a value of 3 nmol/L. In the previous study, we did not include an enzyme inhibitor preincubation. Thus, the estimate was higher because the system had not reached equilibrium during the course of the experiment. Because of the significant time dependence of lapatinib binding, we believe that the method presented herein better estimates the affinity of the drug.

**Structural modeling of mutant EGFR-inhibitor interactions.** EGFR del15 and del18 were unique among the mutants studied here, in that they showed significant differential sensitivity to gefitinib and lapatinib. These mutants have deletions in the flexible loop (residues 748–754) that connects the C helix with strand 3 of the β-sheet in the NH$_2$-terminal lobe (Fig. 2A). In the EGFR-lapatinib structure, the NH$_2$ terminus of the C helix is shifted ~9 Å relative to its position in the apo or erlotinib structures, and this shift accommodates the larger 3-fluorobenzyl-oxy substituent in lapatinib (Fig. 2B). It is possible that this shift at the NH$_2$ terminus of the C helix is accommodated by the flexible loop, and that the shift cannot occur in EGFR mutants with a deletion in this loop. Thus, the active conformation of the C helix might be stabilized in EGFR del15 and del18, such that gefitinib binding would be favorable over lapatinib binding due to steric hindrance of the bulky head group on lapatinib.

EGFR L858R also showed a slight differential sensitivity to lapatinib and gefitinib. L858 is located on the EGFR activation loop representation of the kinase domain of EGFR. Mutations are highlighted in yellow. The activation loop and C helix are shown in white and purple, respectively. The figure was prepared using PYMOL. B, difference in the C helix positions of EGFR in the lapatinib and erlotinib cocrystal structures. The EGFR-lapatinib structure is shown in green, whereas the EGFR-erlotinib structure is shown in magenta. A loop connecting the C helix to the preceding β-strand is partially disordered in the EGFR-lapatinib crystal structure and, therefore, is shown as a dashed line. The figure was prepared using PYMOL. C, position of the T790M mutation in the ATP binding site of EGFR. A methionine residue (magenta) was modeled into the active site of EGFR (green) based on the crystal structure of EGFR complexed with lapatinib (yellow). (PDB 1xkk). The figure was prepared using PYMOL.
In the crystal structures of apoEGFR and EGFR-erlotinib, the hydrophobic side chain of L858 points toward a charged and polar region of the substrate-binding cleft. Mutation of this residue to arginine did not lead to any structural perturbations in the active conformation of EGFR L858R complexed with gefitinib (42). In the crystal structure of EGFR-lapatinib, the side chain of L858 forms part of the back pocket that binds the 3-fluorobenzyl-oxy substituent. Mutation of L858 to R could influence the shape and size of the back pocket. However, because the side chain of L858 is solvent exposed in the EGFR-lapatinib structure, it seems from the cell and enzyme data that the arginine side chain can be accommodated. Structural studies with this mutant and related quinazoline inhibitors indicates that the arginine side chain can rotate and point out into solution (data not shown).

T790 is located at the back of the ATP binding site (Fig. 2C) and is often called the gatekeeper residue (43). This mutation is analogous to the T315I mutation in bcr-abl that leads to imatinib resistance (44). In the EGFR-erlotinib structure, the side chain of T790 makes a water-mediated hydrogen bond to N3 of the quinazoline. In the EGFR-lapatinib structure, the threonine side chain is rotated relative to its position in the erlotinib structure and makes a number of direct and water-mediated hydrogen bonds to the protein. Mutation of this residue to methionine is predicted to sterically block inhibitor binding because the methionine side chain would occupy part of the same space as the quinazoline ring for both inhibitors (45).

The last three EGFR mutations examined in this study, G719C, G719S, and L861Q, have moderate effects on the $K_i$ for lapatinib or gefitinib. Thus, it is unlikely that G719 or L861 play significant roles

**Figure 3.** Inhibition of receptor autophosphorylation by lapatinib and gefitinib. A, CHO-K1 cells were transiently transfected with wild-type (wt) or mutant EGFR or HER2 constructs as indicated. Cells transfected with EGFR constructs were incubated with the indicated concentrations of lapatinib or gefitinib for 4 h, stimulated with 10 ng/mL EGF for 10 min, and lysed in radioimmunoprecipitation assay buffer buffer and immunoprecipitated with anti-EGFR antibody. Cells transfected with HER2 constructs were exposed to drug for 4 h and immunoprecipitated with anti-HER2 antibody. Western blot analysis and quantification was carried out using antiphosphotyrosine antibody PT-66 as described in Materials and Methods. Western quantification values are shown in Table 2 and represent at least two independent experiments.
inhibitor binding. Structural modeling suggested that G719C might sterically interact with the ribose ring of ATP, perhaps explaining the observation that the $K_m$ for ATP is ~10-fold higher for EGFR719C than for wild-type EGFR.

**Inhibition of wild-type and mutant receptor autophosphorylation by lapatinib in CHO cells.** The effects of EGFR mutations on EGFR activity in cells were tested by overexpressing each EGFR variant in CHO cells in the presence or absence of lapatinib or gefitinib. Cultures were exposed to EGF to activate kinase activity, EGFR was immunoprecipitated from cell extracts, and EGFR autophosphorylation was measured by quantitative Western blot analyses with an antiphosphotyrosine antibody. The results show that the sensitivity to lapatinib and gefitinib is similar for wild-type EGFR and EGFR point mutants G719C, G719S, and L861Q (Fig. 3 and Table 2). The only point mutant that is differentially sensitive to either drug is L858R, which seems to be slightly more sensitive to gefitinib than wild-type EGFR and has about the same sensitivity to lapatinib as wild-type EGFR. In contrast, the two EGFR deletion mutants, del15 and del18, are much less sensitive to lapatinib inhibition than wild-type EGFR. Gefitinib inhibits these deletion mutants with better potency than wild-type EGFR. Interestingly, T790M is resistant to inhibition by both drugs. These results are in general agreement with the biochemical results described above.

Recent studies identified several HER2 mutants expressed in tumor cells from female never-smoker Asian NSCLC patients (22). The most common variants observed were HER2 YVMA and HER2 G776VinsC. The latter variant is also expressed in human NCI cell line H1781. These two variants were overexpressed in CHO cells, and receptor autophosphorylation was measured in the presence and absence of lapatinib and gefitinib (Fig. 3B and Table 2). Wild-type HER2 is very sensitive to lapatinib inhibition, but not to gefitinib inhibition, as expected. However, HER2 YVMA and HER2 G776VinsC are less susceptible to inhibition by either lapatinib or gefitinib. In contrast, lapatinib was a potent inhibitor (25–50 nmol/L) of receptor autophosphorylation of the point mutation HER2 H878Y, which has been described in hepatomas and the activating point mutation HER2 V659E when expressed in CHO cells (Table 2).

**Inhibition of wild-type and mutant cell growth by lapatinib.** The ability of lapatinib and gefitinib to inhibit proliferation of human NSCLC cell lines expressing wild-type or mutant EGFR was also examined. The lapatinib $IC_{50}$ for cell growth varied up to 10-fold in four cell lines expressing wild-type EGFR (Table 3), and the reason for this variation is not understood. However, $IC_{50}$ for cell growth was similar for lapatinib and gefitinib in each of these four cell lines. Two of these wild-type–expressing cell lines were tested for inhibition of phosphorylated EGFR (pEGFR), and both compounds were effective (Supplementary Table S1). This is consistent with the fact that lapatinib and gefitinib have similar potency for wild-type-EGFR inhibition. NSCLC cell lines expressing mutant variants of EGFR were also examined (Table 3). H1650 cells, expressing EGFR del15 and H1975 cells, which are heterogeneous for EGFR L858R and T790M, were relatively resistant to lapatinib and gefitinib. These data are consistent with recently published reports examining the effect of gefitinib on the growth of NSCLC cell lines (46) and the effects of gefitinib and lapatinib on the growth of H1975 (47). H1781S cells, expressing mutant HER2 G776VinsC, were relatively insensitive to growth inhibition by both lapatinib and gefitinib, although HER2 receptor autophosphorylation is susceptible to inhibition by lapatinib (Supplementary Table S1). CalLu-3 cells, which overexpress wild-type HER2, were highly sensitive to growth inhibition by lapatinib, with an $IC_{50} <100$ nmol/L. Gefitinib was 4-fold less effective on that cell line (Table 3).

**Table 2. EGFR and HER2 mutant–dependent inhibition of autophosphorylation in CHO cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$IC_{50}$ (nmol/L) immunoblot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lapatinib</td>
</tr>
<tr>
<td>EGFR wild-type</td>
<td>22 ± 13</td>
</tr>
<tr>
<td>EGFR G719C</td>
<td>&lt;14</td>
</tr>
<tr>
<td>EGFR G719S</td>
<td>&lt;14</td>
</tr>
<tr>
<td>EGFR L858R</td>
<td>24 ± 14</td>
</tr>
<tr>
<td>EGFR L861Q</td>
<td>&lt;14</td>
</tr>
<tr>
<td>EGFR del15</td>
<td>361 ± 119</td>
</tr>
<tr>
<td>EGFR del18</td>
<td>155 ± 25</td>
</tr>
<tr>
<td>EGFR T790M</td>
<td>&gt;3,300</td>
</tr>
<tr>
<td>HER2 wild-type</td>
<td>87 ± 107</td>
</tr>
<tr>
<td>HER2 YVMA</td>
<td>20, 78, and 80% inhibition at 3,300*</td>
</tr>
<tr>
<td>HER2 G776VinsC</td>
<td>20, 69, and 77% inhibition at 3,300*</td>
</tr>
<tr>
<td>HER2 H878Y</td>
<td>26 ± 10</td>
</tr>
<tr>
<td>HER2 V659E</td>
<td>47 ± 18</td>
</tr>
</tbody>
</table>

NOTE: Values are the average of at least two independent experiments ± SD.

*Values are the maximum inhibition at the highest concentration tested for two or three independent experiments.

**Discussion**

Several variables contribute to the effects of ErbB family receptor mutations on receptor oncogenicity and kinase inhibitor efficacy. Listed in order of increasing complexity, these variables include the effect of the mutation on (a) the affinity of the drug for the receptor, (b) the kinetic properties of the enzyme, (c) receptor signaling and regulation, and (d) dependence of the tumor cell on the signal from the receptor.

At the simplest level, the mutations may result in changes in tyrosine kinase activity and inhibitor binding. The effect of mutations on the biochemical properties of the receptor was obtained by purifying EGFR catalytic domains and determining enzyme kinetic variables with an *in vitro* tyrosine kinase assay. The results indicate that EGFR mutations that are common in NSCLC are highly activating (Table 1). For example, the $V_{max}$ values of EGFR L858R and del15 were 17- and 30-fold higher than for wild-type EGFR, respectively. These activating mutations may be advantageous to tumor cell growth, which may explain why they are frequently isolated in NSCLC. The mutations also affect drug affinity for EGFR. The lapatinib $K_i$ was 150-fold higher for del15 and 30-fold higher for L858R than for wild-type EGFR. Modest effects on lapatinib $K_i$ were observed for other mutants and on the affinity of del15 for gefitinib. The effects on inhibitor $K_i$ can be explained by structural models derived from EGFR-inhibitor co-crystals (24, 41). Lapatinib binding seems to require a conformational change in the apo-EGFR structure, which allows the bulky anilinoquinazoline headgroup to protrude into the back pocket of the enzyme. The models suggest that EGFR deletions affecting residues 748 to 754 (del15 and del18) reduce the
and L858R EGFR (L858R. In spite of this structural similarity, they found that EGFR binding to wild-type EGFR is identical to gefitinib binding to EGFR. The gefitinib complex is in the active-like conformation, and gefitinib enzyme. Yun et al. (42) also found that the structure of the EGFR-wild-type variants.

Recently, other studies comparing the biochemical properties of wild-type EGFR to EGFR harboring mutations have been published (42, 48, 49). The kinetic properties that we have measured are in general agreement with these reports. For example, all of these studies report that L858R or del15 result in an increase in $K_{\text{cat, ATP}}$ and significantly increase the catalytic activity of the enzyme. Yun et al. (42) determined the structure of EGFR L858R in complex with the ATP analogue AMP-PNP or the inhibitor, gefitinib. They found that the L858R mutation seems to stabilize the active conformation of EGFR by preventing the activation loop from adopting the inactive-like helical conformation. Presumably, these findings explain the increase in measured catalytic rate ($V_{\text{max}}$) of this enzyme. Yun et al. (42) also found that the structure of the EGFR-gefitinib complex is in the active-like conformation, and gefitinib binding to wild-type EGFR is identical to gefitinib binding to EGFR L858R. In spite of this structural similarity, they found that EGFR L858R binds gefitinib tighter than wild-type EGFR ($K_{i} = 2.6$ versus $53.5$ nmol/L, respectively). They propose that this increase in affinity for gefitinib results from the stabilization of the active-like conformation of EGFR by the mutation. In contrast, we have obtained essentially identical $K_{i}$ values for gefitinib with wild-type and L858R EGFR ($K_{i} = 0.2$nmol/L for both). Our $K_{i}$ determinations were obtained by measuring substrate phosphorylation in an in vitro kinase assay and are similar to other such measurements of gefitinib affinity to wild-type EGFR (24, 50). Yun et al. (42) determined the inhibitor $K_{d}$ by measuring changes in the intrinsic fluorescence of the EGFR protein induced by inhibitor addition. One possible explanation for these discrepancies is that fluorescent changes are induced by compound binding to the total population of EGFR molecules in solution, whereas substrate phosphorylation assays are only sensitive to inhibitor binding to the fraction of active EGFR molecules.

Because gefitinib and lapatinib compete with ATP for receptor binding, their potency in cells depends upon several factors including the receptor $K_{d}$ for ATP, the concentration of ATP in the cell, and inhibitor $K_{i}$. The relationship between these variables is described by Eq. D (33).

From Eq. D it is apparent that a higher $K_{d, \text{ATP}}$ will result in a lower IC$_{50}$ for a kinase inhibitor in cells even if the inhibitor $K_{i}$ does not change. Using these relationships, one may predict the expected IC$_{50}$ for inhibition of receptor autophosphorylation in cells. For lapatinib, the predicted IC$_{50}$ for wild-type and del15 EGFR are 22 and 270 nmol/L, respectively. These numbers are in very good agreement with the measured values, 22 and 361 nmol/L, respectively.

The susceptibility of human tumor cell lines expressing EGFR mutations to lapatinib-mediated growth inhibition was also examined in this study. Interestingly, lapatinib-mediated inhibition of the growth of these cell lines did not correlate directly with biochemical and autophosphorylation data. For example, the lapatinib IC$_{50}$ for receptor autophosphorylation was reduced to ≤40 nmol/L in cells expressing wild-type EGFR (H1734) and expressing the HER2 mutation G776VinsC (H1781S), but the lapatinib IC$_{50}$ for cell growth was above 2 μmol/L. In addition, Wang and coworkers (51) recently reported a decrease in colony formation of H1781 tumor cells with lapatinib but at a concentration of 5 μmol/L. Furthermore, the lapatinib and gefitinib IC$_{50}$ for cell growth both varied up to 10-fold in four NSCLC cell lines expressing wild-type EGFR. The resistance to lapatinib shown for the H1650 cell line is not surprising because the del15 mutation reduces the affinity of lapatinib binding and pEGFR is not inhibited in these cells. The reason for the low potency of gefitinib on cell proliferation, however, is not clear, and pEGFR is effectively reduced with gefitinib treatment in these cells. This may indicate that the cell lines are not dependent on EGFR or HER2 for growth, and additional factors contribute to the susceptibility of these cells to growth inhibition by lapatinib and gefitinib.

In summary, this is the first study that we are aware of to report biochemical, structural, and biological data on the inhibition of common NSCLC EGFR and HER2 variants by a dual tyrosine kinase inhibitor. We have fully characterized the inhibition of intrinsic catalytic activity of receptor variants by lapatinib through structure-, enzyme-, and cell-based assays. Structural models of EGFR-lapatinib interactions show that the effect of EGFR mutations also depends on the magnitude of the effect on conformation and structural flexibility. In addition, cell line heterogeneity modulates the sensitivity of lapatinib in EGFR/HER2 regulation of cell growth and survival. In this study, we show that the HER2-overexpressing NSCLC cell line was most responsive to growth inhibition by lapatinib and is consistent with results from clinical trials of lapatinib in patients with HER2 overexpressing breast cancer (26, 27). The biochemical and biological data on EGFR and HER2 presented here for NSCLC highlights the complexity of receptor sensitivity and targeted therapies and shows how understanding the potency of specific tyrosine kinase inhibitors toward specific receptor variants can contribute significantly to the design, execution, and interpretation of clinical trials for these agents.

### Acknowledgments

Received 6/26/2007; revised 10/19/2007; accepted 11/15/2007.

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Conflict of interest: All authors were employees of GlaxoSmithKline at the time the work was performed.

We thank David McKee and Earnest Horne for their contributions to protein expression and purification; Hong Shi, Li Liu, and Yawei Li for technical help with HER2 mutant assays; and Miriam Sander and Francine Carrick for editorial assistance.

**Table 3. Lapatinib and gefitinib-mediated inhibition of cell growth and receptor status**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Receptor status</th>
<th>IC$_{50}$ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lapatinib</td>
<td>Gefitinib</td>
</tr>
<tr>
<td>H1975</td>
<td>EGFR L858R/T790M</td>
<td>10.1 ± 1.3</td>
</tr>
<tr>
<td>H1650</td>
<td>EGFR del15</td>
<td>6.9 ± 2.4</td>
</tr>
<tr>
<td>H1734</td>
<td>EGFR wild-type</td>
<td>4.0 ± 1.4</td>
</tr>
<tr>
<td>H338</td>
<td>EGFR wild-type</td>
<td>0.64 ± 0.09</td>
</tr>
<tr>
<td>A549</td>
<td>EGFR wild-type</td>
<td>5.0 ± 0.49</td>
</tr>
<tr>
<td>H322</td>
<td>EGFR wild-type</td>
<td>0.92 ± 0.02</td>
</tr>
<tr>
<td>H1781S</td>
<td>HER2 G776VinsC</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>CaLu-3</td>
<td>HER2 overexpressed wild-type</td>
<td>0.057 ± 0.006</td>
</tr>
</tbody>
</table>

NOTE: Values represent the average of two independent experiments ± SD.
References
Impact of Common Epidermal Growth Factor Receptor and HER2 Variants on Receptor Activity and Inhibition by Lapatinib

Tona M. Gilmer, Louann Cable, Krystal Alligood, et al.


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