Quantitative Chemical Proteomics Profiling Differentiates Erlotinib from Gefitinib in EGFR Wild-Type Non–Small Cell Lung Carcinoma Cell Lines

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Abstract

Although both erlotinib and gefitinib target the EGF receptor (EGFR), erlotinib is effective in patients with EGFR wild-type or mutated tumors, whereas gefitinib is only beneficial for patients with activating mutations. To determine whether these differences in clinical outcomes can be attributed to their respective protein interaction profiles, a label-free, quantitative chemical proteomics study was conducted. Using this method, 24 proteins were highlighted in the binding profiles of erlotinib and gefitinib. Unlike gefitinib, erlotinib displaced the ternary complex formed by integrin-linked kinase (ILK), α-parvin, and PINCH (IPP). The docking of erlotinib in the three-dimensional structure of ILK showed that erlotinib has the ability to bind to the ATP-binding site, whereas gefitinib is unlikely to bind with high affinity. As the IPP complex has been shown to be involved in epithelial-to-mesenchymal transition (EMT) and erlotinib sensitivity has been correlated with EMT status, we used a cellular model of inducible transition and observed that erlotinib prevented EMT in a more efficient way than gefitinib by acting on E-cadherin expression as well as on IPP levels. A retrospective analysis of the MERIT trial indicated that, besides a high level of E-cadherin, a low level of ILK could be linked to clinical benefit with erlotinib. In conclusion, we propose that, in an EGFR wild-type context, erlotinib may have a complementary mode of action by inhibiting IPP complex activities, resulting in the slowing down of the metastatic process of epithelial tumors. Mol Cancer Ther; 12(4); 520–9. ©2013 AACR.
transition (EMT). EMT is a known mechanism through which epithelial cells lose adhesion molecules such as E-cadherin and acquire a mesenchymal phenotype, which allows them to migrate into the stroma and become invasive (11). The correlation between erlotinib sensitivity and EMT has been shown previously in vitro and in vivo (12, 13) and suggests that patients with epithelial-type NSCLC tumors, that is, which had not undergone the transition, could have a better outcome with an erlotinib-containing regimen (12). The complex formed by integrin-linked kinase (ILK), α-parvin, and PINCH is bound to erlotinib but not gefitinib in the chemical proteomics profiling of several EGFR wild-type NSCLC cell lines. This complex, also known as ILK–PINCH–Parvin complex (IPP) complex, forms a structural and signaling entity in the integrin pathway (14) and has been shown to influence EMT. ILK and PINCH overexpression are able to trigger EMT, by inducing loss of E-cadherin (15, 16). TGF-β–induced EMT in mammary epithelial cells required functional ILK (17). The next steps were to investigate a potential structural explanation for this binding discrepancy between the 2 compounds, as well as using an EMT-inducible cellular model to assess their respective functional effect on EMT. These findings were further correlated with gene expression data from the MERIT open-label, multicenter, phase II clinical trial, which aimed to identify genes with potential as biomarkers for clinical benefit from erlotinib therapy (18).

Materials and Methods

Reagents and cell lines

Anti-ILK, E-cadherin, and α-parvin were obtained from Cell Signaling Technology, anti-PINCH1 and glyceraldehyde-3-phosphate dehydrogenase–horse radish peroxidase (GAPDH-HRP) from Santa Cruz Biotechnology, and anti-vimentin from BD Biosciences. All cell lines were obtained from the American Type Culture Collection (ATCC) without further authentication. Cell cultures H292, H226, H322, H358, H441, H460, and H1299 were maintained in RPMI-1640, and Calu6 in EMEM; both supplemented with 10% FBS.

Chemical proteomics profiling in H292 cells

Erlotinib and gefitinib were modified with a 6-(3-aminopropoxy) side chain for covalent linking to epoxy-activated Sepharose 6B beads (GE Healthcare) to generate affinity matrices, as described by Brehmer and colleagues (19). Starting material 4-(3-ethylphenyl)aminol-7-(2-methoxyethoxy)-6-quinazolinol was prepared as described in WO9630347A1 (20). Coupling of the derivatives to the epoxy-activated beads was done in 50% dimethylformamide (DMF)/0.1 mol/L Na2CO3, pH 11 in the presence of 10 mmol/L NaOH, overnight at room temperature in the dark. After washing with 50% DMF/0.1 mol/L Na2CO3, any remaining reactive groups were blocked with 1 mol/L ethanolamine. Subsequent washing steps were conducted according to the manufacturer’s instructions. Binding efficiency was estimated by comparing the optical densities of the compound solutions before and after coupling at the maximal absorbance wavelengths 247 and 330 nm. Cells were lysed in 50 mmol/L HEPES pH 7.3 buffer containing 150 mmol/L NaCl, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 1% NP-40, protease inhibitors (Complete EDTA free; Roche Applied Sciences), and 1 mmol/L orthovanadate; samples were cleared by centrifugation at 12,000 × g for 15 minutes at 4°C. Cell lysates were preincubated with increasing concentrations (0, 0.0856, 0.2862, 0.957, 3.2, 10.7, 35.78, 119.6, and 400 mmol/L final concentration) of unmodified erlotinib or gefitinib for 1 hour at 4°C under rotation. The pretreated samples were loaded onto Sepharose beads immobilized with the respective compound (erlotinib or gefitinib) for 3 hours at 4°C under rotation. Beads were washed 3 times with lysis buffer, treated with SDS-PAGE buffer for 5 minutes at 85°C, and eluted proteins separated on 4% to 20% Tris–glycine SDS-PAGE. After protein fixation in 40% ethanol and 10% acetic acid, gels were stained overnight with Coomassie Brilliant blue. For protein in-gel digestion, an adapted protocol of Shevchenko and colleagues (21) was used. In brief, each gel lane was cut into 5 slices between the 20 and 150 kDa region. Proteins were reduced with 50 mmol/L dithioerythritol for 45 minutes at 56°C, alkylated with 55 mmol/L iodoacetamide for 1 hour at room temperature in the dark, and digested with trypsin (Promega) overnight at room temperature. Peptides were extracted twice with 1:2 (v/v) acetonitrile/25 mmol/L ammonium bicarbonate, and 1:2 (v/v) acetonitrile/5% formic acid, respectively, for 15 minutes at 37°C, dried down using a speedvac, and stored at −20°C before analysis. Samples were reconstituted in 2% acetonitrile/5% formic acid and run in triplicate by LC/MS. Nanoflow liquid chromatography/tandem mass spectrometry (LC/MS-MS) was carried out by coupling an Easy-nLC system (Proxeon) to an LTQ Orbitrap Velos (Thermo Fisher Scientific) equipped with a Proxeon nanoelectrospray and an active background ion reduction device (ABIRD; ESI Source Solutions). Peptides were loaded on a 10-mm Aqua C18 trapping (packed in-house, 100 μm inner diameter) and separated on a 200-mm Reprosil-Pur C18-AQ analytic (Dr. Maisch GmbH) column (75 μm inner diameter, 3 μm particle size, 120 Å). Individual raw data files were processed with SEQUEST (version 27.0, revision 12, Thermo Fisher Scientific) and searches were conducted against a concatenated forward/reverse human UniProt/SwissProt database (June 2009 release, 34,275 entries, containing splice-variants) allowing for a spectrum false-discovery rate of 2.5%. A mass tolerance of 5 ppm for precursor and 1.0 Da for fragment ions was set. Oxidized methionines (+15.9949 Da) and carbamidomethylated cysteines (+57.0215 Da) were considered as differential and fixed modifications, respectively. Only fully tryptic peptides with not more than one miscleavage were considered for data analysis. Detailed LC/MS and data analysis procedure can be found in the Supplementary Data.
Chemical proteomics profiling of erlotinib and gefitinib in other NSCLC cell lines

H358, H322, H226, and Calu6 cells were lysed in 50 mmol/L HEPES pH 7.3 buffer containing 150 mmol/L NaCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 1% NP-40, protease inhibitors (Complete EDTA free, Roche Applied Sciences), 1 mmol/L orthovanadate, and samples were cleared by centrifugation at 12,000 × g for 15 minutes at 4°C. Cell lysates were loaded on erlotinib-Sepharose and gefitinib-Sepharose, using the same experimental conditions as in the profiling experiment described earlier but without competing with the unmodified erlotinib or gefitinib. The elution fractions were analyzed by LC/MS. Spectral counts corresponding to EGFR, GAK, LOK (Stk10), Ab12, SLK, MEKK1 (M3K1), ILK, α-parvin (PARVA), and PINCH1 (LIMS1) were extracted and normalized toward the sum of all spectral counts corresponding to all proteins identified in the respective fractions.

Alignment of EGFR and ILK crystal protein structures

Publicly available crystal structure protein data bank (pdb) files of EGFR-bound erlotinib or gefitinib and the crystal structure of ILK complexed with ATP were downloaded from the crystallographic database [ref. 22; pdb codes 1M17, 2ITY, 3KMW; (23–25)]. The 3 kinase structures were aligned using the coordinates from CCA atoms of a subset of the residues forming the ATP-binding site [residue numbers based on EGFR numbering 691–693, 701–703, 719–722 (21) 1, 751, 766–772, 820–823, 831–832]. Alignment and distance measurements were conducted using the PyMOL visualization package (25).

Testing EMT on H358 NSCLC cell line

H358 cells were treated with recombinant 10 ng/mL TGF-β3 (Peprotech) and 5 μmol/L erlotinib or gefitinib for 3, 6, 10, and 13 days. Cell culture medium was renewed every 3 days. Cell lysis was conducted in PBS containing 1% NP-40 and protease inhibitors. After clearing by centrifugation at 12,000 × g for 15 minutes at 4°C, proteins were separated by SDS-PAGE and processed for Western blot analysis. Images were acquired using a compact digital camera (Luminescent Image Analyzer; Fujifilm) and quantified with ImageQuant TL (GE Healthcare). Statistical analysis was conducted using JMP 8.0.2 (SAS Institute Inc.). To determine the influence of erlotinib and gefitinib on ILK and PINCH after TGF-β3 stimulation, the following model was applied: ExpressionD13 ~ Treatment + ExpressionD32. For analysis of IPP expression in NSCLC cell lines, simple f tests were conducted.

Analysis of MERIT data

Gene expression profiles were measured in tumor biopsy samples from 102 patients with stage IIIb/IV NSCLC, being treated with erlotinib (150 mg/day). Tumor biopsies were analyzed using gene expression profiling with Affymetrix GeneChip Human Genome U133A Array (Affymetrix). All gene expression results described here (fold changes and P values) are derived from the statistical analysis already reported (18). We specifically looked at differences between gene expression profiles of patients with and without clinical benefit from erlotinib. Clinical benefit was defined as an objective response (complete responses or partial responses were confirmed by repeated assessments >4 weeks apart at any time during the treatment period) or maintenance of stable disease for ≥12 weeks after study entry.

The following 21 proteins, potentially involved in transcriptional regulation of EMT or in IPP complex, were mapped to the corresponding genes and microarray probe sets: Twist, Snail (Sma1), Zeb (TCF8), Lef-1, E12/E47 basic loop helix (bHLH), Slug (Sma2), E-cadherin (CDH1), N-cadherin (CDH2), Myc, vimentin, catenin-β, catenin-δ, fibronectin, epimorphin, ILK, α-parvin, β-parvin, PINCH 1, PINCH 2, metastasis-associated protein 3 (MTA3), and Y box–binding protein 1 (YB-1).

Results

Interaction profile of erlotinib and gefitinib in H292 NSCLC cell line

A quantitative chemical proteomics method was applied to erlotinib and gefitinib (Fig. 1A) using the same wild-type EGFR-expressing NSCLC cell line H292 as a biological target to identify the interaction profiles of the compounds. Cell lysates were preincubated with increasing concentrations of unmodified erlotinib or gefitinib before fractionation on the respective affinity matrix, captured proteins were eluted, separated by SDS-PAGE and identified by LC/MS and protein database mining (Supplementary Fig. S1). A total of 1,285 and 1,303 protein groups were identified in the bound fractions to erlotinib and gefitinib, respectively (Supplementary Table S1). A linear model (27, 28) was applied on mass spectrometry signals to derive concentration-dependent signal decrease of specific binders. Comparison of the binding profiles of the 2 compounds was carried out using a robust, simple, statistical procedure adapted from Bretz and Hothorn (29) on peptide signals and extended to multiple testing of corresponding protein groups. Nine proteins were found with an adjusted P value below 5% in the samples displaced with erlotinib (Fig. 1B, Regions A, B and x-axis; Supplementary Table S2), and 19 in those displaced with gefitinib (Fig. 1B; regions A, C, and y-axis; Supplementary Table S2), including EGFR, which was specifically displaced by both compounds, confirming the validity of the approach. The presence of 2 other proteins, GAK and Stk10, was also significant in both compound profiles (Fig. 1B; region A; Supplementary Table S2), whereas NUD12, K0564, SN1L1, KC1E, and EPHA1 were only identified in the gefitinib-bound fraction (Fig. 1B, y-axis; Supplementary Table S2); M3K1 and LIMS1 only in the erlotinib-bound fraction (Fig. 1B, x-axis; Supplementary Table S2).

While the majority of proteins displaced by either erlotinib or gefitinib were protein kinases (7 for erlotinib, i.e., EGFR, GAK, STK10, ILK, SLK, ABL2, and M3K1; and...
of the other cell lines. In addition, we observed that the spectral counts of Abl2, Slk, MEKK1 (M3K1), ILK, α-parvin (PARVA), and PINCH1 (LIMS1) in the erlotinib-bound fraction were higher than those in the gefitinib-bound fraction. This was particularly noticeable for the IPP, which was enriched on the erlotinib but not the gefitinib column from these EGFR wild-type NSCLC cell lysates.

Interaction profile of erlotinib and gefitinib in the ILK DVK loop region

Erlotinib and gefitinib have a high degree of similarity in their chemical structure (Fig. 1A). Knowing the resolved crystal structures of EGFR and ILK (24, 25), we examined whether the binding of erlotinib to ILK was different from gefitinib, which might explain the discrepancies in their binding profiles toward the IPP complex. As expected, when erlotinib or gefitinib were in a complex with EGFR, they displayed the same mode of binding to the ATP-binding sites. The overall protein conformation of EGFR-erlotinib, EGFR-gefitinib, and the ATP-binding pocket was very similar, resulting in an exact overlay of the 2 structures after aligning (data not shown).

The pseudokinase ILK shows some crucial differences in fold and conformation to other kinases, including EGFR (25). However, when ignoring sequence differences, the buried part of the ILK ATP-binding site is very similar to the ATP-binding site of EGFR and allows a reliable structural alignment of ILK with EGFR when in a complex with either erlotinib or gefitinib (Fig. 3). A crucial difference in the ATP-binding site is the conformation of the DFG loop and Ala338, the residue upstream of the DVK loop in ILK (residues 339–341, which form the typical DFG). This residue is part of a turn that is not observed in EGFR and is close to the space occupied by the aniline portions of erlotinib and gefitinib.

The different substitutions in the erlotinib and gefitinib aniline rings (the 3 position is substituted with chlorine in gefitinib but with acetylene in erlotinib and the 4 position is unsubstituted in erlotinib but substituted with fluorine in gefitinib) and the resulting differences in atom proximities suggest that erlotinib is more likely than gefitinib to bind favorably to the ATP-binding site of ILK.

Potential influence of erlotinib on the integrin signaling pathway, connecting to EMT

The IPP complex functions as a hub in the integrin signaling pathway (14). It plays a structural role by connecting integrins to the cytoskeleton and also transduces signals from the extracellular matrix to various intracellular pathways involved in cell proliferation, migration, and survival. In addition, overexpression of both ILK and PINCH seems not only to follow the occurrence of EMT (15, 16), but ILK activity is also required for TGF-β1-mediated EMT in mammary epithelial cells (17). On the basis of the fact that erlotinib binds to the IPP complex, as shown in our chemical proteomics approach, we hypothesized that in NSCLC cell lines that have not undergone
EMT, erlotinib could potentially slow down the transition, by inhibiting the IPP-mediated E-cadherin pathway.

To test this hypothesis, we used a model NSCLC cell line, H358, which had been previously described as switching from epithelial-to-mesenchymal state upon TGF-β3 treatment (30). The results show a clear decrease of E-cadherin after 13 days of TGF-β3 treatment, indicating that the cells are transitioning (Fig. 4A). Treatment with erlotinib prevents this, whereas gefitinib fails to inhibit the TGF-β3–mediated decrease of E-cadherin levels.

We also examined the effects of the transition on ILK and PINCH expression (α-parvin could not be tested because of low detection by Western blot analysis). Figure 4B and C shows the average quantified Western blot analysis signals from both proteins, for 3 and 13 days of treatment. The expression of both ILK and PINCH was increased by the transition. Both proteins gave a lower signal when cells were treated with TGF-β3 and erlotinib.

Thus, this EMT model indicates that erlotinib reduces the extent of EMT by inhibiting the loss of E-cadherin and
also seems to prevent a TGF-β3–mediated increase of both members of the IPP complex, ILK and PINCH. The levels of expression of E-cadherin (A), ILK, and PINCH were analyzed by Western blot analysis. For ILK (B) and PINCH (C), Western blot analysis signals were quantified and averaged from 2 biological replicates. The effect of 13 days of treatment with erlotinib and gefitinib was estimated on TGF-β3-stimulated cells after adjustment to the level at 3 days of respective treatment. Compared with TGF-β3-stimulated untreated cells, a downregulation (in the sense of a "non-upregulation") by gefitinib on ILK (one-sided \( P = 0.085 \)) and PINCH-1 (one-sided \( P = 0.12 \)) did not reach significance. In contrast, there was a significant downregulation by erlotinib on ILK (one-sided \( P = 0.03 \)) and PINCH-1 (one-sided \( P = 0.02 \)). Note: all cell lines were obtained from ATCC without further authentication.

**Figure 4.** Erlotinib slows down the transition of H358 by stabilizing E-cadherin. H358 cells were treated for 3, 6, 13, and 20 days with TGF-β3 alone or together with erlotinib and gefitinib. The levels of expression of E-cadherin (A), ILK, and PINCH were analyzed by Western blot analysis. For ILK (B) and PINCH (C), Western blot analysis signals were quantified and averaged from 2 biological replicates. The effect of 13 days of treatment with erlotinib and gefitinib was estimated on TGF-β3-stimulated cells after adjustment to the level at 3 days of respective treatment. Compared with TGF-β3-stimulated untreated cells, a downregulation (in the sense of a "non-upregulation") by gefitinib on ILK (one-sided \( P = 0.085 \)) and PINCH-1 (one-sided \( P = 0.12 \)) did not reach significance. In contrast, there was a significant downregulation by erlotinib on ILK (one-sided \( P = 0.03 \)) and PINCH-1 (one-sided \( P = 0.02 \)). Note: all cell lines were obtained from ATCC without further authentication.

**Expression of IPP complex in NSCLC cell lines**

ILK and PINCH overexpression has been correlated with tumor progression and/or aggressiveness in several human malignancies (14). At present, the 3 components of the complex are studied mainly on an individual basis, not addressing the possibility of the expression of the entire complex being correlated with EMT status. Eight EGFR wild-type NSCLC cell lines were selected on the basis of this status (13, 30), which was assessed by the expression of E-cadherin as an epithelial marker and vimentin as a mesenchymal marker. All the IPP proteins were detected, with varying intensities, in all 8 cell lines except for α-parvin in H358 (Fig. 5A). After quantification and normalization, the overall sum of IPP complex intensities was almost 2 times higher in mesenchymal versus epithelial cells (Fig. 5B).

**ILK expression in erlotinib-treated patients—data from the MERIT trial**

Taking into account the data from NSCLC cell lines, and considering that the EMT status of biopsies from patients with NSCLC has been previously correlated with clinical benefit from erlotinib treatment (12), we evaluated the
Among the 31 probe sets (corresponding to 16 unique genes), only 2 were differentially expressed in tumor cell lines were obtained from ATCC without further authentication. (one-sided $P = 0.011$) from epithelial-to-mesenchymal cell lines. Note: all substitutions of the aniline moieties of the compounds gefitinib, are aligned with the ILK structure, the different crystal structures, complexed either with erlotinib or docking of erlotinib in the 3-dimensional structure of ILK shows that erlotinib is able to bind to the ATP-binding site among human protein kinases, the focus had been placed on potential binding activity with other protein kinases. Indeed, Abl2, Slk, and STK10 (LOK) have been confirmed to bind to erlotinib in vitro with at least 25-fold higher affinity than gefitinib (33). However, these assays revealed also at least 30-fold lower affinity of erlotinib for Abl2, Slk, and STK10 by comparison with EGFR. A recent study using native kinase binding profiling in HL60 and PC3 cell lines also reported inhibitory activities of erlotinib on STK10 (LOK), MAP3K1 (M3K1), Slk, and ILK, which correlates well with our findings (34). Further experiments would be required to confirm the effect of erlotinib on these kinases in the appropriate in vivo background. Our data show that substantial information can also be deduced from the nonkinase proteins that form complexes with the direct binders. Unlike gefitinib, erlotinib interacted with the ternary complex formed by ILK, $\alpha$-parvin, and PINCH in our assay. We propose a structural explanation for the difference in binding to IPP between erlotinib and gefitinib. The docking of erlotinib in the 3-dimensional structure of ILK shows that erlotinib is able to bind to the ATP-binding site of the pseudokinase, as opposed to gefitinib, which is less likely to bind with high affinity. Indeed, when the EGFR crystal structures, complexed either with erlotinib or gefitinib, are aligned with the ILK structure, the different substitutions of the aniline moieties of the compounds seem to have crucial implications for their potential relationship between IPP, a subset of genes involved in EMT, and response to erlotinib. Specifically, we looked at the differences between their expression profiles in patients with and without clinical benefit from erlotinib.

In total, 16 of 21 preselected genes were identified with detected probe sets and statistically analyzed [Twist, Snail (Snai1), Zeb (TCF8), Lef-1, E12/E47 basic loop helix (bHLH), Slug (Sna2), E-cadherin, Muc, vimentin, catenin-$\beta$, catenin-$\delta$, fibronectin, epimorphin, ILK, $\alpha$-parvin, and $\beta$-parvin]. Data for the following 5 genes: PINCH 1, PINCH 2, metastasis-associated protein 3 (MTA3), Y box-binding protein 1 (YB-1), and N-cadherin were not available. Among the 31 probe sets (corresponding to 16 unique genes), only 2 were differentially expressed in tumor samples from patients with and without clinical benefit: ILK and CDH1 (E-cadherin), which were downregulated (0.93-fold; $P = 0.011$) and upregulated (1.38-fold; $P = 0.033$), respectively, in patients with clinical benefit. It should be noted that these genes exhibited only small trends of regulation that were not significant when a procedure of $P$ value adjustment for multiple testing was applied, as in the initial work from Tan and colleagues (ref. 18; Fig. 6).

**Discussion**

In tumors dependent on an EGFR-mutated receptor, the mutation triggers a decreased affinity of the kinase for ATP but its affinity to erlotinib is kept intact. This, taken together, leads to an increased sensitivity to the TKI. The difference in efficacy between erlotinib and gefitinib has been at least partially attributed to a difference in pharmacokinetic properties, that is, erlotinib at 150 mg has an area under the curve 7 times higher than gefitinib at 250 mg. In this study, we hypothesize that an additional activity besides the inhibition of EGFR could explain the difference in clinical benefit seen between erlotinib and gefitinib in EGFR wild-type tumors, for example, inhibition of ILK. Indeed, the chemical proteomics profiling of erlotinib and gefitinib has highlighted differences in the binding profiles of the 2 compounds in at least 5 different EGFR wild-type NSCLC cell lines. Many kinase inhibitors, which have been approved for clinical use, have been profiled with this method leading to the discovery of new activities, for example, on DDR1 for imatinib (31) or CAMK2G for bosutinib (32). Given the conservation of the ATP-binding site among human protein kinases, the potential relationship between IPP, a subset of genes involved in EMT, and response to erlotinib. Specifically, we looked at the differences between their expression profiles in patients with and without clinical benefit from erlotinib.

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In tumors dependent on an EGFR-mutated receptor, the mutation triggers a decreased affinity of the kinase for ATP but its affinity to erlotinib is kept intact. This, taken together, leads to an increased sensitivity to the TKI. The difference in efficacy between erlotinib and gefitinib has been at least partially attributed to a difference in pharmacokinetic properties, that is, erlotinib at 150 mg has an area under the curve 7 times higher than gefitinib at 250 mg. In this study, we hypothesize that an additional activity besides the inhibition of EGFR could explain the difference in clinical benefit seen between erlotinib and gefitinib in EGFR wild-type tumors, for example, inhibition of ILK. Indeed, the chemical proteomics profiling of erlotinib and gefitinib has highlighted differences in the binding profiles of the 2 compounds in at least 5 different EGFR wild-type NSCLC cell lines. Many kinase inhibitors, which have been approved for clinical use, have been profiled with this method leading to the discovery of new activities, for example, on DDR1 for imatinib (31) or CAMK2G for bosutinib (32). Given the conservation of the ATP-binding site among human protein kinases, the focus had been placed on potential binding activity with other protein kinases. Indeed, Abl2, Slk, and STK10 (LOK) have been confirmed to bind to erlotinib in vitro with at least 25-fold higher affinity than gefitinib (33). However, these assays revealed also at least 30-fold lower affinity of erlotinib for Abl2, Slk, and STK10 by comparison with EGFR. A recent study using native kinase binding profiling in HL60 and PC3 cell lines also reported inhibitory activities of erlotinib on STK10 (LOK), MAP3K1 (M3K1), Slk, and ILK, which correlates well with our findings (34). Further experiments would be required to confirm the effect of erlotinib on these kinases in the appropriate in vivo background. Our data show that substantial information can also be deduced from the nonkinase proteins that form complexes with the direct binders. Unlike gefitinib, erlotinib interacted with the ternary complex formed by ILK, $\alpha$-parvin, and PINCH in our assay. We propose a structural explanation for the difference in binding to IPP between erlotinib and gefitinib. The docking of erlotinib in the 3-dimensional structure of ILK shows that erlotinib is able to bind to the ATP-binding site of the pseudokinase, as opposed to gefitinib, which is less likely to bind with high affinity. Indeed, when the EGFR crystal structures, complexed either with erlotinib or gefitinib, are aligned with the ILK structure, the different substitutions of the aniline moieties of the compounds seem to have crucial implications for their potential
binding to ILK. Acknowledging that the distance measured between overlaid structures depends on the alignment method used and may vary slightly, a few observations can be made from our structural alignment. Gefitinib has 2 substitutions on the aniline ring, whereas erlotinib has only 1, which points inside the ATP-binding site, close to the DVK loop. The 4-position is not substituted in erlotinib, whereas in gefitinib it features fluorine. Compared with hydrogen, the van der Waal’s radius of the fluorine atom is larger and the carbon–fluorine bond is significantly longer than the carbon–hydrogen bond (35). This hydrogen-to-fluorine substitution seems to be important, as the fluorine sits in an environment where it clashes and causes steric repulsions with the Cβ-methyl and the carbonyl oxygen of Ala338 (~1.5 and 2 Å, respectively; Fig. 5). Furthermore, the fluorine also creates an unfavorable electrostatic interaction with the carbonyl oxygen of Ala338. In contrast, erlotinib projects a slightly polarized hydrogen atom from the aniline 4-position that can establish an attractive interaction with the carbonyl oxygen of Ala338. The repulsion from the fluorine atom in gefitinib could either prevent it from binding to ILK with reasonable affinity or it could trigger a rearrangement of the loop containing the DVK motif upon binding. Because this loop is connected to the activation loop of ILK that is crucial for binding α-parvin (25), such a rearrangement is likely to have an influence on the ability of ILK to form a complex with α-parvin. More sophisticated methods such as molecular dynamics simulations could be applied to further elucidate the favorable and unfavorable interactions between ligands and proteins. Measurements of in vitro binding affinity of the compounds to the purified complex will be required to experimentally validate the results of this in silico approach.

Our study shows that erlotinib prevents EMT induced by TGF-β3 in a more efficient way than gefitinib in the H358 cellular model. As expected, this does not influence cellular proliferation in our in vitro assays, where the different cell lines used in this study were growth inhibited to a varying extent but equally by both compounds as previously reported (12, 36). EMT will be more relevant for invasive tumor growth in vivo, and clinical studies have already shown the link between E-cadherin (CDH1) levels and response to erlotinib (12). The retrospective analysis presented here on data from the MERIT trial confirms that patients with higher expression of E-cadherin (CDH1) have an increased clinical benefit from treatment with erlotinib, as previously published (18). A similar analysis has been conducted on gefitinib by Kook and colleagues and did not show any correlation of E-cadherin expression with response rate or with progression-free survival and overall survival in patients with inoperable stage IIIIB/IV NSCLC treated with gefitinib (37). Interestingly, a recent study has shown a causative relation between loss of E-cadherin and overexpression of ILK in squamous cell carcinoma and adenocarcinoma of the lung (38).

In addition, in our cellular model, erlotinib showed a tendency to prevent the overexpression of ILK and PINCH induced by TGF-β3. It should be noted that neither...
erlotinib nor gefitinib have been shown to bind the TGF-β receptors in vitro (33), excluding the possibility of a direct effect in our model on TGF-β receptor signaling. Overexpression of both ILK and PINCH has been shown to induce EMT by lowering E-cadherin levels (16, 39, 40). Interestingly, ILK is captured in our chemical proteomics experiments to the same extent in all the cell lines, PINCH is most enriched in H226 and Calu6 fractions (Fig. 2), which are mesenchymal cell lines (30). Overall, these observations indicate that erlotinib may exert a long-term effect on the expression of ILK and PINCH, which could explain the observed consequences on the EMT. Because the expression of both proteins is regulated, at least in part, by proteasomal degradation (41), we could hypothesize that erlotinib would accelerate this degradation and therefore counteract the EMT process.

The respective overexpression of ILK and PINCH has been associated with poor prognosis in NSCLC (42–44). Although PINCH could not be monitored in the MERIT trial, low expression of ILK seems to indicate a better benefit with erlotinib. This strengthens our theory that in patients with a low expression of IPP, the remaining free erlotinib can still act on the complex, whereas in cases of overexpression, this effect would not be significant.

In conclusion, we propose that, in a wild-type EGFR context, unlike gefitinib, erlotinib targets both EGFR and the IPP complex activities, resulting in the slowing down of the metastatic process of epithelial tumors. Further investigations on additional models will be necessary to confirm this observation and explore the early effects of erlotinib on the activities of the IPP complex, for example in deciphering the details of the signaling pathway leading to hindrance of EMT.

Disclosure of Potential Conflicts of Interest
All authors were employees of F. Hoffmann La Roche Ltd. at the date of the study.

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Chemical Proteomics Differentiates Erlotinib from Gefitinib


26. The PyMOL Molecular Graphics System V, Schrödinger, LLC.


Molecular Cancer Therapeutics

Quantitative Chemical Proteomics Profiling Differentiates Erlotinib from Gefitinib in EGFR Wild-Type Non-Small Cell Lung Carcinoma Cell Lines

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