

Erlotinib Directly Inhibits HER2 Kinase Activation and Downstream Signaling Events in Intact Cells Lacking Epidermal Growth Factor Receptor Expression

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Abstract

Erlotinib (Tarceva), is an orally available, reversible inhibitor of epidermal growth factor receptor (EGFR; HER1) that exhibits inhibitory activity on purified HER2 kinase at much higher concentrations. Despite the minimal activity on purified protein *in vitro*, *in vivo* studies show that erlotinib inhibits the growth of HER2-driven systems effectively. Several hypotheses have been put forward to explain this discrepancy. In particular, it has been suggested that erlotinib might indirectly suppress the activity of HER2 by blocking the ability of EGFR to transactivate it when the two receptors are part of a heterodimer complex. However, an alternative possibility that has not been adequately addressed is whether the direct inhibitory action of erlotinib on the HER2 kinase might account for the observed biological responses. To distinguish between a direct effect of erlotinib on HER2 kinase in intact cells or an indirect effect of erlotinib on HER2 activity that is mediated through EGFR, we generated cell lines that express either EGFR-H2 chimeric receptor or HER2 and HER3 receptors in an EGFR-negative background. We show that dose-dependent inhibition of HER2 was achieved at the receptor level, on downstream signaling molecules, and more importantly was also translated into inhibition of cell growth. Our findings imply that the inhibitory effect of erlotinib in HER2-expressing cells may in part be mediated through direct interaction with HER2 rather than indirectly through a process that requires the presence of EGFR. [Cancer Res 2007;67(3):1228–38]

Introduction

The epidermal growth factor receptor (EGFR) family consists of four members: EGFR/HER1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4. These receptors are essential in modulating cell proliferation, cell differentiation, and cell survival in many tissue types (1). On ligand binding, the receptors form homodimers and heterodimers, and the subsequent activation of the intrinsic kinase then results in receptor autophosphorylation and the activation of downstream signaling mediators (2). Aberrant expression of EGFR and HER2 has been implicated in the development of many types of human cancers, including lung, head and neck, colorectal, pancreatic, breast, and ovarian cancers, and patients with tumors bearing dysregulated EGFR or HER2 receptors have been linked to poor clinical outcome (3, 4). Therefore, EGFR as well as HER2 have been intensely pursued as therapeutic targets.

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Erlotinib (OSI-774, Tarceva) is a selective, orally available low molecular weight inhibitor that binds competitively to the ATP-binding site at the kinase domain of EGFR. The IC₅₀ measured in *in vitro* kinase assays is 2 nmol/L, whereas the potency in intact cells is ~20 nmol/L (5). Preclinical studies with erlotinib have shown potent antitumor activity in a variety of cultured tumor cell lines as well as human tumor xenografts (6, 7), and favorable clinical studies have led to the approval of erlotinib for the use in the treatment of advanced non-small cell lung cancer (NSCLC) and advanced or metastatic pancreatic cancer (8).

Identifying predictors of responsiveness to EGFR tyrosine kinase inhibitors has been a focus of recent research, and a variety of efforts are ongoing to elucidate what confers sensitivity to these inhibitors. In contrast to the HER2 therapeutic antibody trastuzumab that is effective only on HER2-amplified tumors, the role of EGFR expression as a predictor for clinical outcome is less defined (9–11). Consequently, further studies were designed to determine if EGFR gene amplification rather than EGFR overexpression as assessed by immunohistochemistry is a better indicator to identify patients who might benefit from EGFR-directed therapies (12). The recently identified EGFR kinase mutations have proven to be very sensitive to erlotinib (13–15). Yet, tumors with mutated EGFR are not the only tumors that respond to kinase inhibitors (16–18), suggesting that factors other than EGFR mutations contribute to the sensitivity.

The presence or absence of other members of the EGFR family alters the responsiveness of tumor cells to EGFR kinase inhibitors (19) because these receptors modify the signaling properties of EGFR (20). In fact, HER2 is known to form heterodimers with EGFR, and several studies have recently reported that epithelial tumor cell lines and human tumor xenograft models overexpressing HER2 are particularly sensitive to erlotinib and to another EGFR tyrosine kinase inhibitor, gefitinib (21–23). Additionally, a recent clinical study suggests that EGFR-positive NSCLC patients with increased HER2 gene copy numbers respond better to gefitinib than patients that do not overexpress HER2 (24).

Why HER2-overexpressing cells are particularly sensitive to an EGFR inhibitor is still unclear. *In vitro* kinase assays suggest that erlotinib is not effective in inhibiting HER2 kinase directly because the sensitivity of this kinase is 40- to 3,000-fold less when compared with the EGFR kinase (25–27). However, HER2 phosphorylation decreases significantly upon inhibitor treatment (22, 28). Therefore, we wanted to explore whether erlotinib had the capability to directly block HER2 kinase function in intact cells.

HER2 activation arises through either a ligand-dependent or a ligand-independent process. Ligand-independent activation occurs when receptor expression levels are very high, leading to spontaneous receptor homodimerization and kinase activation.

In contrast, ligand-dependent HER2 kinase activation requires the formation of a heterodimeric complex between HER2 and another member of the HER receptor family. In this case, the ligand-occupied partner transactivates HER2.

In the present study, we explored the inhibitory effects of erlotinib on both modes of HER2 activation. We present evidence that erlotinib directly inhibits the HER2 kinase in the absence of EGFR. The inhibition of HER2 kinase at submicromolar levels also translates into the effective inhibition of Akt and mitogen-activated protein kinase (MAPK) signaling pathways. We further show that, in the absence of EGFR, erlotinib effectively inhibits HER2/HER3-driven signaling and cell proliferation. These findings indicate that direct inhibition of HER2 may account for a portion of the overall responsiveness to erlotinib in HER2-driven and HER2/HER3-driven signaling.

Materials and Methods

Materials. NR6 cells were kindly provided by Mark Pegram (University of California at Los Angeles, Los Angeles, CA; ref. 29) and maintained in RPMI 1640 supplemented with 10% calf serum. The NCI-H1666 cell line was purchased from the American Type Culture Collection and maintained in a 50:50 mixture of F12 Ham's medium and DMEM supplemented with 10% fetal bovine serum.

Cetuximab was purchased from St. Mary's pharmacy. The EGF-like domain of HRG $\beta_{177-241}$ was expressed in *Escherichia coli* and purified as described previously (30).

Antibodies. Anti-phosphotyrosine antibody (RC20H) conjugated to horseradish peroxidase was purchased from BD Transduction Laboratories (Lexington, KY). Anti-HER2 (Ab-17) was obtained from Lab Vision Corp. (Fremont, CA). Anti-HER3 (C-17) and anti-EGFR (1005) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against pHER3, pAkt (Ser⁴⁷³), pAkt (Thr³⁰⁸), Akt, pMAPK, and p44/42MAPK were purchased from Cell Signaling.

Expression constructs and infection of NR6 cells. Full-length HER2 cDNA was subcloned into the LNCX retroviral vector, HER3 cDNA was introduced into the LHCX vector, and the EGFR cDNA was subcloned into MSCVneo vector. All retroviral vectors were purchased from BD Biosciences Clontech (Palo Alto, CA). The expression construct for the chimeric EGFR-H2 receptor encoding a molecule containing the extracellular and transmembrane portion of EGFR and the intracellular domain of HER2 was generated by PCR. In detail, amino acids 1 to 680 of EGFR were fused to amino acids 689 to 1,255 of HER2, creating the fusion sequence HIVRKRTLRL/LLQETEL. The entire coding sequence of the chimeric receptor was subcloned into MSCVneo vector, and the accuracy of the construct was verified by DNA sequencing. The retroviral constructs were transfected into the Phoenix amphotropic packaging cell line using LipofectAMINE Plus reagent according to the recommendation of the manufacturer (Invitrogen, Carlsbad, CA). Viral particles were harvested 48 h after transfection and NR6 cells were infected. Stable populations were selected using the appropriate selection markers.

Western blot analysis. For biochemical characterization, cells were plated in six-well plates. Following serum starvation, EGFR and EGFR-H2 chimera-expressing cells were stimulated with the indicated concentrations of transforming growth factor α (TGF α) for 12 min. HER2/HER3-expressing cells were stimulated with the indicated concentrations of heregulin for 8 min. Total cell lysates were run on SDS-PAGE, and Western blots were probed with various antibodies. Western blots were quantitated by densitometry, and the results are shown as percentage of control. H1666 cells were plated in 12-well plates and treated as above.

Cell proliferation assay. Cells were plated in 96-well plates (NR6, 3,000 per well; H1666, 5,000 per well) and incubated overnight at 37°C. The next day, medium was removed and cells were stimulated with indicated concentrations of ligand in 1% (NR6) or 0.1% (H1666) serum-containing growth medium. After 3 to 4 days, Alamar Blue (Trek Diagnostic Systems, Cleveland, OH) was added to the wells and fluorescence was read using a

96-well fluorometer with excitation at 530 nm and emission of 590 nm. The results are expressed in relative fluorescence units (RFU). For growth inhibition assays, erlotinib or cetuximab was administered with ligand, and assay was done as stated above.

Results

Because constitutive, homodimeric activation of HER2 requires very high HER2 levels, we examined the effects of erlotinib on the HER2 kinase using a chimeric receptor composed of the extracellular and transmembrane domains of EGFR fused to the intracellular domain of HER2 (31). This allowed us to activate HER2 using the EGFR ligand TGF α . The chimeric receptor, which we called EGFR-H2, was stably expressed in the NR6 derivative of the NIH3T3 cell line. NR6 cells lack endogenous EGFR and therefore do not respond to EGFR ligands. Although ligand stimulation was required for activation of the EGFR-H2 chimera, the activation did not result from transactivation by a different member of the HER receptor family. Therefore, EGFR-H2 activation was similar to a homodimeric activation that occurs when full-length HER2 is expressed at high levels. An EGFR-expressing NR6 line was generated as a control (Fig. 1A).

Biochemical and *in vitro* characterization of EGFR-H2-expressing cells. To validate the use of our engineered cells for drug studies, we first determined whether the EGFR-H2-expressing line responded appropriately to ligand treatment. Cells were stimulated with TGF α and then lysed, and the levels of receptor autophosphorylation were assessed using anti-phosphotyrosine immunoblots. The Western blots were quantitated by densitometry, and the results were then plotted. As shown in Fig. 1, receptor phosphorylation of EGFR-H2 (Fig. 1B) as well as EGFR (Fig. 1C) was seen in a TGF α dose-dependent manner. The EC₅₀ for EGFR-H2 receptor phosphorylation was 0.49 nmol/L, ~2.5-fold higher than the EC₅₀ value measured for EGFR of 0.19 nmol/L.

The two major downstream signaling pathways that are activated in response to EGFR phosphorylation are the Ras/MAPK and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways. We examined whether the EGFR-H2 chimeric receptor was capable of activating these pathways in NR6 cells. As shown in Fig. 1B, TGF α treatment of cells expressing EGFR-H2 induced phosphorylation of p44/42MAPK in a dose-dependent manner. Similar to the trend for the autophosphorylation of the chimeric receptor, the measured EC₅₀ for the EGFR-H2 cell line was 0.29 nmol/L, whereas the EC₅₀ for activation of p44/42MAPK in the EGFR-expressing cells was 0.17 nmol/L (Fig. 1C). Akt phosphorylation also occurred in EGFR-H2 cells in response to TGF α stimulation. An EC₅₀ of 0.21 nmol/L was observed in the EGFR-H2 cells compared with an EC₅₀ of 0.08 nmol/L in the control EGFR-expressing cells (Fig. 1B and C, respectively).

These data confirmed that the EGFR-H2 kinase can be activated by TGF α and that, once activated, the chimeric receptor transduced a signal similar to that of EGFR. TGF α was ~2.5 times less potent in activating the EGFR-H2 receptor kinase versus wild-type EGFR based on receptor phosphorylation. It was about 1.7 and 2.6 times less potent in activating p44/42MAPK and Akt, respectively, in EGFR-H2 NR6 cells versus the EGFR NR6 control cells.

Results from cell proliferation experiments were consistent with these biochemical data. EGFR-H2, EGFR, and the parental NR6 cells were treated with various concentrations of TGF α for 3 days and their growth rates were measured using Alamar Blue

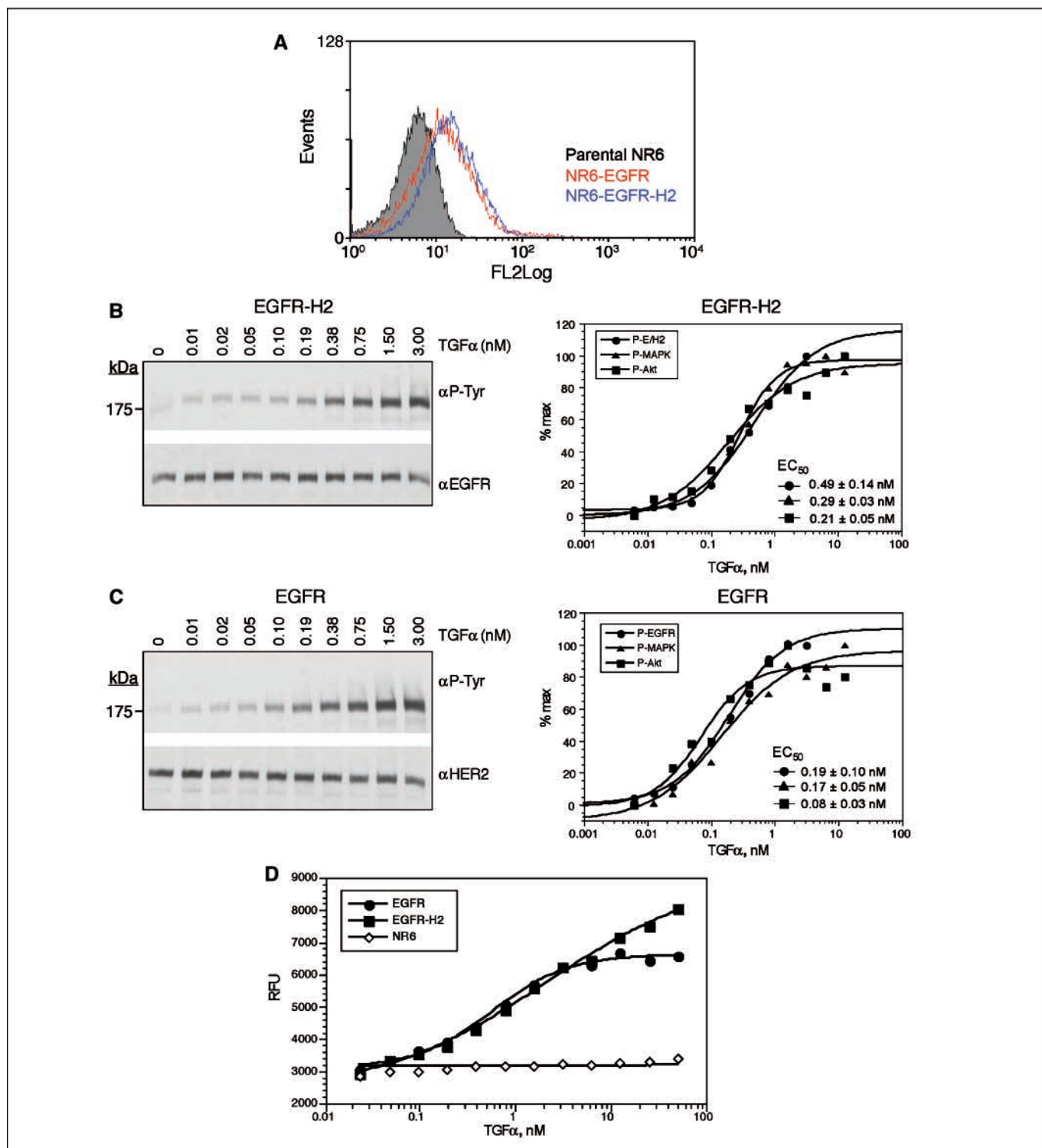


Figure 1. Ligand-dependent signaling in NR6 cells stably expressing EGFR-H2 or EGFR. **A**, surface expression of EGFR and EGFR-H2 as a fluorescence-activated cell sorting histogram. Receptor expression was detected with FITC-labeled anti-EGFR antibody (PharMingen). Receptor levels are similar in EGFR-expressing (red) and EGFR-H2-expressing (blue) cells. EGFR expression was not seen in parental NR6 cells. **B**, EGFR-H2 cells were seeded in 24-well plates and serum starved before TGF α stimulation. Ligand stimulation was done for 12 min with indicated TGF α concentrations. Whole-cell lysates were subjected to SDS-PAGE analysis, and immunoblots were probed with anti-phosphotyrosine or anti-HER2 as loading control. Immunoblots from lysates of EGFR-H2-expressing cells that were probed with anti-phosphotyrosine (α P-Tyr), anti-P-MAPK (P-MAPK), and anti-P-Akt (P-Akt; Ser⁴⁷³ + Thr³⁰⁸) were quantitated by densitometry, and the results are shown as percentage of control. **C**, EGFR-expressing cells were treated as described in (B), and immunoblots were probed with anti-phosphotyrosine or anti-EGFR as loading control. Immunoblots from lysates of EGFR-expressing cells were quantitated as described above. Representative of at least two independent experiments. **D**, EGFR-expressing and EGFR-H2-expressing cells show cell growth upon ligand stimulation. EGFR (●), EGFR-H2 (■), and parental NR6 (◇) cells were seeded in 96-well plates at a density of 3,000 per well. The next day, cells were incubated with indicated concentrations of TGF α in 1% serum-containing medium. After 3 d, Alamar Blue was added and fluorescence was detected using a 96-well fluorometer. The results are expressed in RFUs. Representative of three independent experiments.

reduction. As shown in Fig. 1D, the EGFR-H2 cells as well as EGFR-expressing cells proliferated on ligand stimulation with EC₅₀s of 2.1 and 0.59 nmol/L, respectively. As expected, parental NR6 cells did not grow on addition of TGF α .

Erlotinib inhibits the HER2 kinase directly. The biochemical and biological responses of EGFR-H2 cells to TGF α stimulation were very similar to those of the EGFR line, and we therefore considered it as a good model system for examining the effects of the inhibitor erlotinib on the HER2 kinase. EGFR-H2 and control EGFR-expressing cells were pretreated with various concentrations of erlotinib prior to ligand stimulation, and receptor phosphorylation status was analyzed by immunoblots (Fig. 2A). Interestingly, despite the 40- to 3,000-fold difference in activity in *in vitro* kinase assays (26, 27), erlotinib had a profound effect on the HER2 kinase in our cell-based assays. EGFR-H2 receptor phosphorylation was inhibited by erlotinib with an IC₅₀ of 230 nmol/L. As expected, erlotinib blocked EGFR receptor phosphorylation with an IC₅₀ of 20 nmol/L (Fig. 2B). This measured inhibitory concentration for the EGFR cell line was similar to previously published IC₅₀ data obtained from high EGFR-expressing cancer cell lines (25).

Because erlotinib efficiently blocked receptor phosphorylation in both cell lines, albeit with less potency for the EGFR-H2 kinase, we next examined the effect of erlotinib on the downstream MAPK and Akt signaling pathways. As shown in Fig. 2, erlotinib inhibited phosphorylation of p42/p44 MAPK in EGFR-H2-expressing cells and also in EGFR-expressing cells in a dose-dependent manner. The IC₅₀ values were 290 and 170 nmol/L, respectively. Interestingly, despite the >10-fold difference in IC₅₀ values for receptor phosphorylation for the two cell lines, the IC₅₀ values for p42/p44 MAPK differed by only ~2-fold. Similar experiments done on breast cancer lines have revealed that inhibition of receptor activation and downstream signaling are not necessarily directly proportional (6). We also measured the inhibitory effects of erlotinib on Akt, a major focal point in the cell survival pathway. The measured IC₅₀ value in EGFR-H2-expressing cells was 210 nmol/L, and the IC₅₀ value in the EGFR line was 40 nmol/L.

Inhibition of cell proliferation. After we established that erlotinib prevented signaling by the HER2 kinase, we examined whether this inhibitory effect was also translated into growth inhibition. EGFR and EGFR-H2 cells were treated with various concentrations of erlotinib in the presence of 3 nmol/L TGF α , and cell proliferation was measured after 3 days using Alamar Blue staining. Consistent with what has previously been shown for various cancer cell lines (32), erlotinib strongly inhibited the growth of EGFR-expressing cells with an IC₅₀ of 260 nmol/L. Erlotinib also inhibited the proliferation of the EGFR-H2 line despite the fact that these cells did not express EGFR. As expected, the potency was lower compared with what was observed for the EGFR-expressing line. Cell growth was inhibited with an IC₅₀ of 1.4 μ mol/L in the EGFR-H2 cells (Fig. 2C). In spite of its lower potency against the HER2 kinase, erlotinib effectively blocked proliferation of EGFR-H2 cells.

Generation of HER2/HER3-expressing NR6 cells. As mentioned earlier, the HER2 kinase can also be indirectly activated by ligands through the formation of heterodimeric complexes with other HER receptors that are capable of binding their cognate ligand. We chose to study the effect of erlotinib on the HER2/HER3 heterodimer because HER3 is the preferred partner of HER2 and the resulting complex is more active than any other HER receptor combination in terms of signaling (33) and transforming potential (34, 35).

We engineered NR6 cells to coexpress HER2 and HER3 and confirmed that they would respond biochemically and biologically as expected as we described earlier for the EGFR-H2 cells. In this case, however, the cells were stimulated with the HER3 ligand heregulin. To detect an active receptor complex in the HER2/HER3-expressing line, we stimulated the cells with various concentrations of heregulin for 8 min and then determined HER3 phosphorylation by immunoblots using a HER3 phospho-specific antibody. As shown in Fig. 3A, ligand treatment led to a dose-dependent increase in HER3 phosphorylation. Quantification of the Western blot by densitometry revealed an EC₅₀ of 0.09 nmol/L. Heregulin-induced activation of the receptor complex also resulted in the phosphorylation of the downstream molecules MAPK and Akt. The measured EC₅₀ values were 0.17 and 0.22 nmol/L, respectively (Fig. 3B). The ability of HER2/HER3-expressing cells to proliferate in response to heregulin was also assessed. The cells were treated with various concentrations of heregulin for 3 days, and their growth response was determined by Alamar Blue staining. HER2/HER3-expressing cells responded to heregulin with an EC₅₀ of 0.38 nmol/L, whereas the parental cells did not grow under these conditions (Fig. 3C).

Erlotinib inhibits heregulin-stimulated HER2 kinase activity. HER3 lacks intrinsic kinase activity and cannot transduce a signal by itself (33). Instead, it requires the presence of a partner receptor, such as HER2, which is catalytically active and can be transactivated. For this reason, any inhibitory effects of erlotinib on the HER2/HER3 cell line result from the effects of erlotinib on the HER2 kinase. We first explored whether erlotinib could block ligand-induced receptor phosphorylation in the HER2/HER3-expressing cells. Cells were pretreated with various concentrations of erlotinib prior to ligand stimulation, and HER2/HER3 phosphorylation status was analyzed by immunoblots (Fig. 4A). Erlotinib inhibited the HER2 kinase in the HER2/HER3-expressing cells with an IC₅₀ of 150 nmol/L (Fig. 4B). Erlotinib also inhibited the phosphorylation of p42/p44 MAPK in HER2/HER3-expressing cells in a dose-dependent manner with an IC₅₀ value of 160 nmol/L. Moreover, it also inhibited Akt phosphorylation in HER2/HER3 cells with a measured IC₅₀ value of 720 nmol/L (Fig. 4B).

Inhibition of heregulin-stimulated cell proliferation. We next investigated if the growth of HER2/HER3-expressing cells was also inhibited by erlotinib. Cells were stimulated with 1 nmol/L heregulin in the presence of various concentrations of erlotinib. The extent of cell proliferation was measured after 3 days using Alamar Blue staining. As with the EGFR-H2 line, erlotinib inhibited the proliferation of the HER2/HER3-expressing line with an IC₅₀ value of 690 nmol/L despite the fact that these cells did not express EGFR (Fig. 4C).

Effect of erlotinib on the NSCLC line H1666. After we established that erlotinib was capable of inhibiting the HER2 kinase in the engineered cell lines, we asked whether the inhibitory effect could also be seen in a nonengineered cancer cell line. H1666 cells were chosen because this NSCLC line expresses HER2 and HER3 and responds well to heregulin stimulation (Fig. 5A, lane 2). Like most other lung cancer lines, H1666 cells also express EGFR and respond to EGFR ligands (Fig. 5A, lane 11). To examine the effects of erlotinib on HER2 in the absence of EGFR signaling, we blocked the ligand binding and dimerization capability of EGFR with the EGFR ectodomain-specific antibody cetuximab. Cetuximab is a monoclonal antibody that sterically hinders the EGFR ectodomain and prevents it from adopting the extended conformation necessary for ligand binding and dimerization (36).

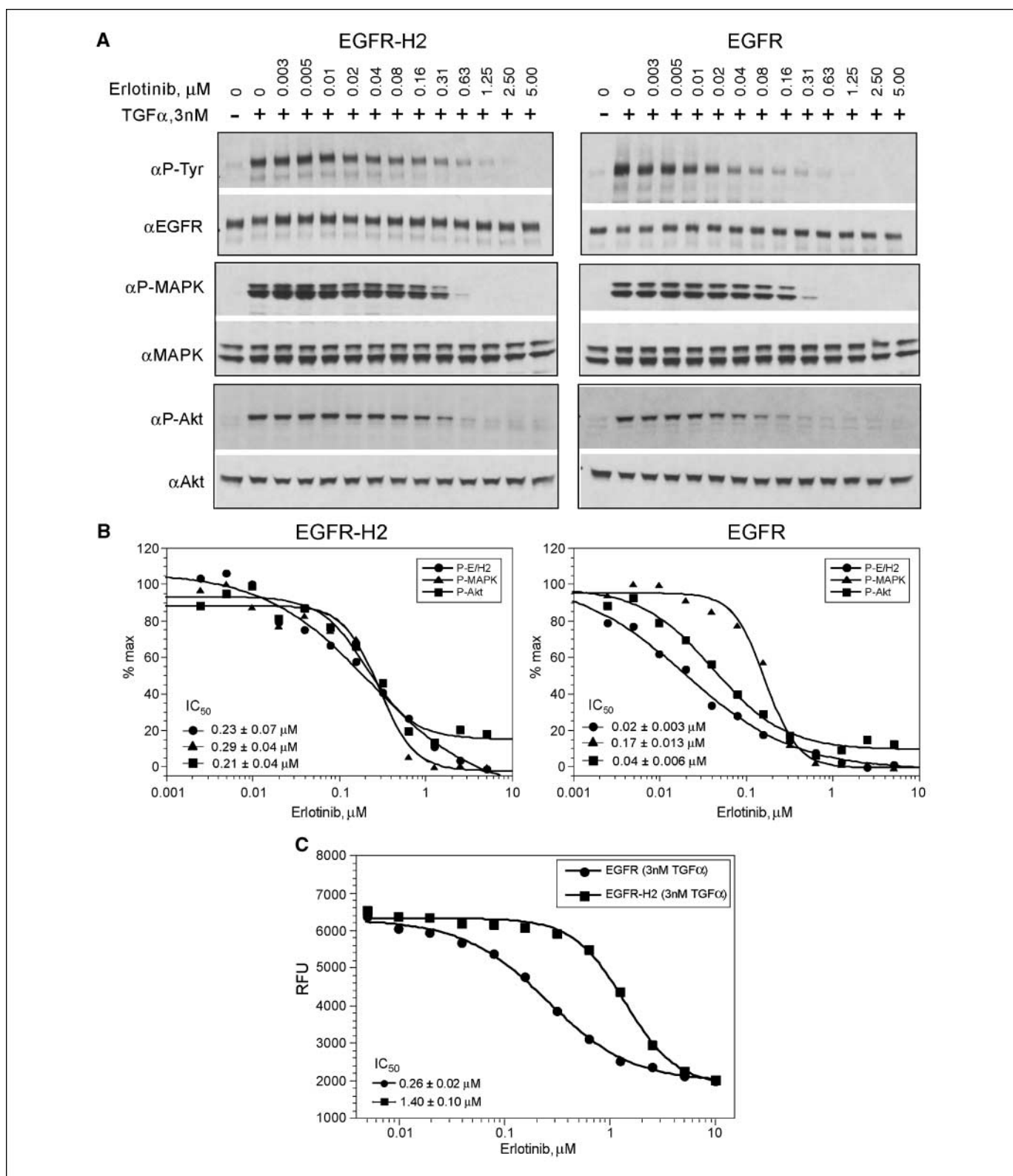


Figure 2. Erlotinib inhibits receptor phosphorylation as well as MAPK and Akt activation in a dose-dependent manner. *A*, following serum starvation for 3 to 5 h, cells were treated with indicated concentrations of erlotinib for 1 h and then stimulated with 3 nmol/L TGF α for 12 min. Western blot analysis was done as described in Fig. 1. *Top*, receptor phosphorylation and total receptor levels; *middle*, P-MAPK and total MAPK levels; *bottom*, P-Akt and total Akt levels. *B*, immunoblots were quantitated by densitometry using ImageJ software and the results are percentage of control. Representative of three independent experiments. *C*, erlotinib inhibits TGF α -induced cell proliferation. EGFR-H2-expressing (\blacksquare) and EGFR-expressing (\bullet) cells were seeded in 96-well plates at a density of 3,000 per well. The following day, cells were treated simultaneously with indicated concentrations of erlotinib and 3 nmol/L TGF α in 1% serum-containing medium. After 3 d, Alamar Blue was added and fluorescence was detected using a 96-well fluorometer. The results are expressed in RFUs. Representative of three independent experiments.

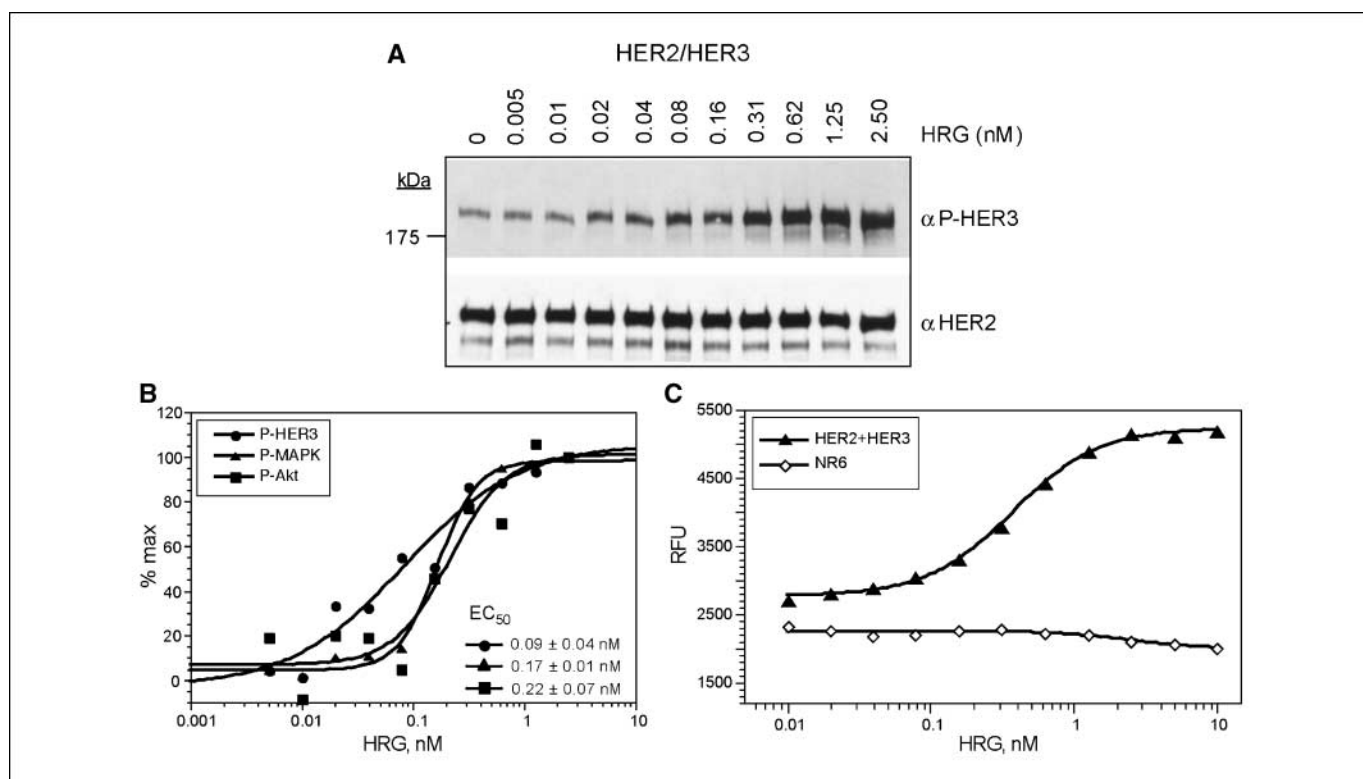


Figure 3. Heregulin (HRG) induces receptor phosphorylation, MAPK, and Akt activation and induces cell growth in HER2/HER3-expressing cells. *A*, cells were seeded in 24-well plates and serum starved for 3 h before ligand stimulation. Heregulin stimulation was done for 8 min with indicated concentrations. Whole-cell lysates were subjected to SDS-PAGE analysis, and immunoblots were probed with anti-P-HER3 (α P-HER3) or anti-HER2 (α HER2) as loading control. *B*, immunoblots were also probed with P-MAPK, total MAPK, P-Akt (Ser⁴⁷³ + Thr³⁰⁸), and total Akt antibodies. The blots were quantitated by densitometry, and the results are percentage of control. *C*, HER2/HER3-expressing cells and parental NR6 cells were stimulated with indicated concentrations of heregulin, and proliferation assay was done as described in Fig. 1. Representative of three independent experiments.

Treating H1666 cells with saturating amounts of cetuximab eliminated EGFR signaling, although the receptor was still physically present on the cells (Fig. 5A, lane 15).

Erlotinib inhibits heregulin-dependent activation of H1666 cells in the presence or absence of cetuximab. Heregulin treatment induced receptor phosphorylation in H1666 cells regardless of whether cetuximab was present or not. As shown in Fig. 5A, addition of 2 nmol/L heregulin resulted in a strong increase of HER3 phosphorylation (Fig. 5A, lanes 2 and 3) in both the absence and the presence of 20 μ g/mL cetuximab. In contrast, the TGF α -induced EGFR receptor phosphorylation was fully inhibited by the presence of 20 μ g/mL cetuximab (Fig. 5A, lane 15). These results confirmed that heregulin-mediated signaling, in contrast to TGF α -mediated signaling, does not depend on EGFR.

We next incubated the cells with various concentrations of erlotinib prior to ligand stimulation both in the presence and in the absence of cetuximab. HER3 receptor phosphorylation was completely abrogated with 5 μ mol/L erlotinib (Fig. 5A, lane 6), whereas 1 μ mol/L erlotinib decreased receptor phosphorylation by 76% (Fig. 5A, lane 5). As anticipated and shown previously, erlotinib inhibited TGF α -induced receptor phosphorylation more potently. Treatment with 0.1 μ mol/L erlotinib decreased EGFR phosphorylation by 65% (Fig. 5A, lane 12), and treatment with 1 μ mol/L erlotinib abolished receptor phosphorylation completely (Fig. 5A, lane 13). The presence of saturating concentrations of cetuximab in heregulin-treated cells did not change the activity of erlotinib (Fig. 5A, lanes 7–9), again indicating that EGFR is not participating

in this aspect of the inhibitory activity of erlotinib on the HER2/HER3 complex.

The inhibitory effect of erlotinib was also assessed on the downstream target MAPK using phospho-specific antibodies. MAPK was inhibited by erlotinib in the absence (Fig. 5A, lanes 4–6) or presence (Fig. 5A, lanes 7–9) of cetuximab, indicating that EGFR signaling is not required for drug sensitivity in heregulin-stimulated cells. Cetuximab alone, however, completely inhibited MAPK activation in TGF α -stimulated cells (Fig. 5A, lane 15).

Erlotinib abrogates heregulin-induced growth in H1666 cells in the presence of cetuximab. Ultimately, we wanted to determine whether erlotinib could inhibit heregulin-driven growth in H1666 cells. H1666 cells were plated in 96-well plates in 0.1% serum-containing medium. The cells underwent different treatments, and cell growth was measured after 5 days using Alamar Blue staining.

As shown in Fig. 5B, heregulin treatment stimulated the growth of H1666 cells, and cetuximab had essentially no effect on this stimulation. TGF α also induced proliferation of the H1666 cells but, as anticipated, the effect was abrogated by the presence of cetuximab. To obtain IC₅₀ values, we treated H1666 cells with various concentrations of erlotinib and measured ligand-dependent cell growth in the presence or absence of cetuximab (Fig. 5C). Heregulin-driven proliferation was inhibited by erlotinib with an IC₅₀ of 700 nmol/L regardless of whether cetuximab was present or not. TGF α -stimulated growth was repressed by erlotinib with an

IC₅₀ of 300 nmol/L. As expected, significant cell growth did not occur when TGF α -stimulated cells were treated additionally with cetuximab. The heregulin-stimulated proliferation of H1666 cells in the presence of saturating concentrations of cetuximab indicated that HER2/HER3 heterodimer complexes play an important role in the growth and survival of these cells. The fact that erlotinib suppressed the proliferation in a dose-dependent manner showed that this inhibitor effectively blocked the HER2 kinase, albeit with a lower potency in comparison with its effect on the EGFR kinase.

The engineered EGFR-H2 and HER2/HER3 cell lines enabled us to study the effect of erlotinib on the HER2 kinase in the absence of endogenous EGFR. With this system, we were able to clearly distinguish a direct effect of erlotinib on HER2 from possible indirect effects that might be facilitated through EGFR/HER2 cooperation. Using the EGFR-H2 chimera, we showed that erlotinib

can directly inhibit the HER2 kinase when it is activated through the formation of a homodimeric complex. We also showed that erlotinib effectively inhibits the most prominent heterodimeric complex in the HER receptor family—the HER2/HER3 heterodimer. The dose-dependent inhibition was seen at the receptor level, on downstream signaling molecules, and was also translated into inhibition of cell growth.

Discussion

EGFR tyrosine kinase inhibitor treatment in HER2-overexpressing cells results in decreased basal receptor phosphorylation of EGFR and HER2 (21, 28). Thus far, it is unclear whether this decreased phosphorylation is due to direct inhibition of both HER2 and EGFR receptors or to direct inhibition of EGFR and the subsequent loss of HER2 transactivation.

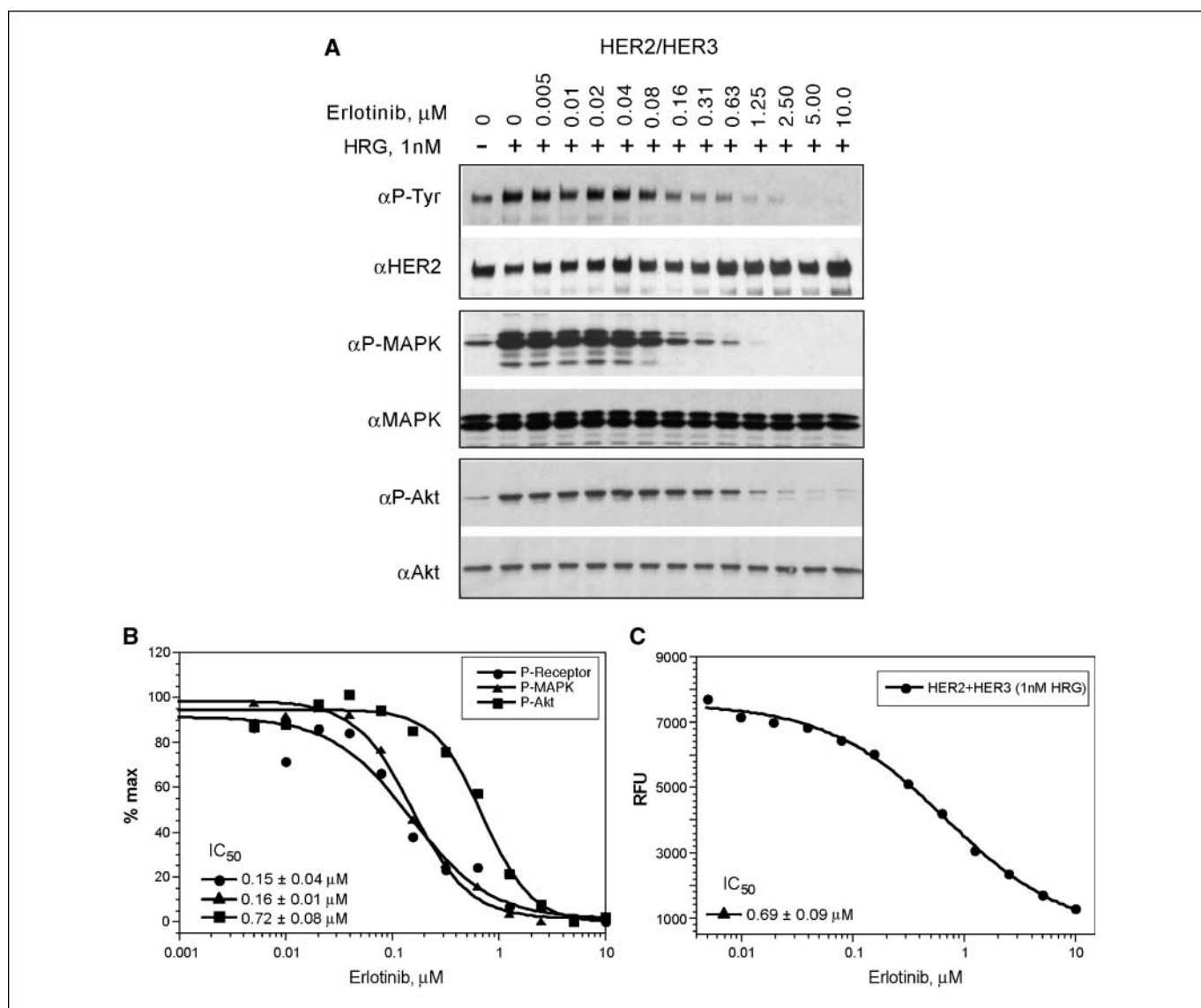


Figure 4. Erlotinib inhibits heregulin-induced signaling in HER2/HER3-expressing cells. **A**, following serum starvation, cells were treated with indicated concentrations of erlotinib for 1 h and then stimulated with 1 nmol/L heregulin for 8 min. Western blot analysis was done as described in Fig. 1. *Top*, HER2/HER3 phosphorylation status and total HER2 receptor levels; *middle*, P-MAPK and total MAPK levels; *bottom*, P-Akt and total Akt levels. **B**, quantitative analysis by densitometry was done on the immunoblots, and results are percentage of control. **C**, inhibition of cell growth of heregulin-stimulated HER2/HER3-expressing cells was done as described in Fig. 2.

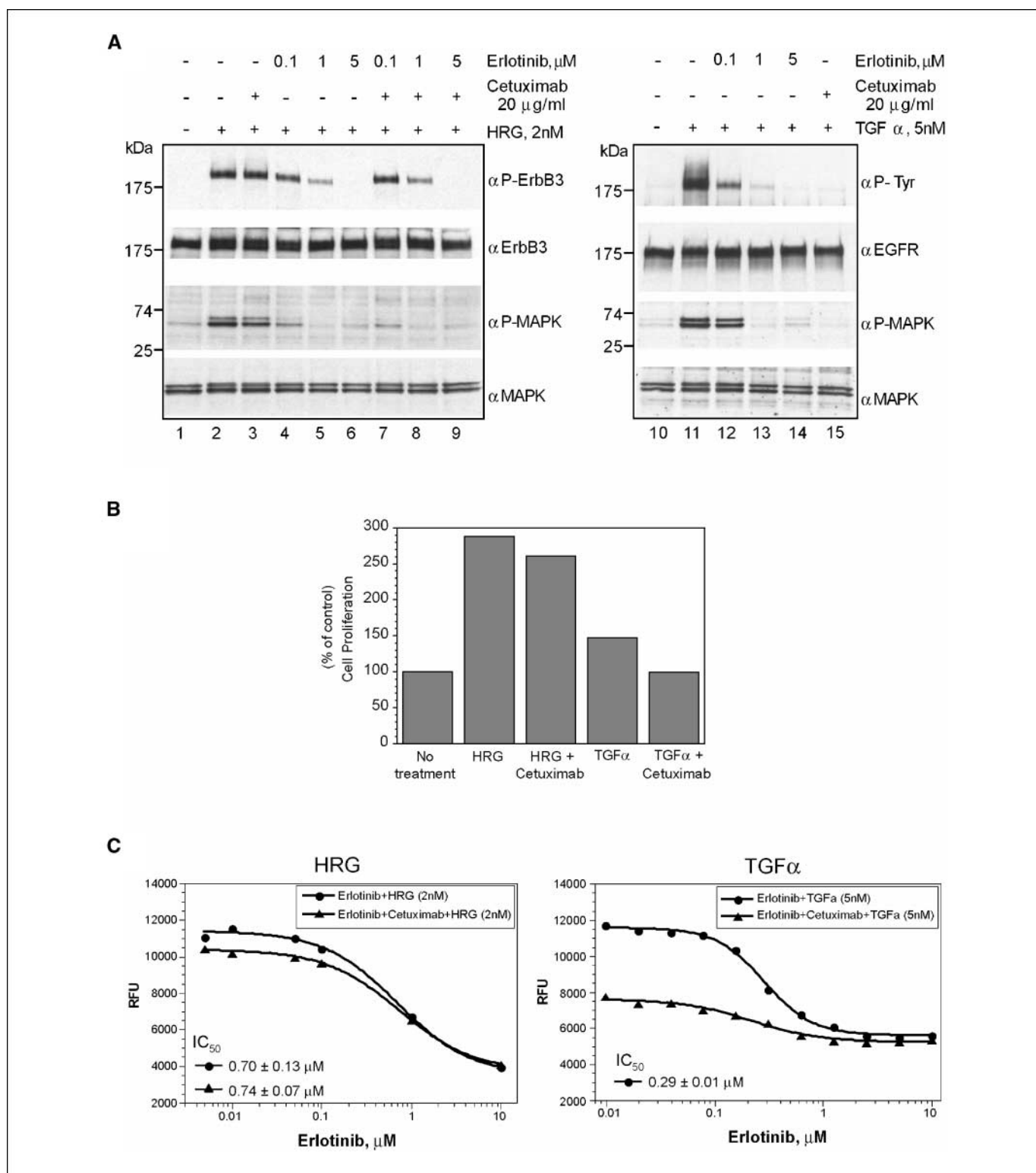


Figure 5. Erlotinib inhibits heregulin-induced signaling and proliferation in H1666 cells in the presence or absence of cetuximab. *A*, H1666 cells were serum starved for 4 h and stimulated with 2 nmol/L heregulin (*lanes 1–9*) for 8 min or as a control with 5 nmol/L TGF α (*lanes 10–15*) for 12 min. *Lanes 4 to 9 and 12 to 14*, prior to ligand stimulation, cells were treated for 1 h with erlotinib; *lanes 7 to 9*, simultaneously treated with 20 $\mu\text{g/ml}$ cetuximab; *lanes 3 and 15*, incubated with cetuximab before ligand stimulation. Whole-cell lysate were subjected to immunoblot analysis. *Top*, HER3 phosphorylation, total HER3 (*lanes 1–9*), and total EGFR and EGFR phosphorylation using an anti-phosphotyrosine antibody (*lanes 10–15*); *bottom*, P-MAPK status and total MAPK. *B*, ligand-induced cell proliferation was measured using Alamar Blue staining. H1666 cells were plated in 96-well plates at a density of 5,000 per well. The next day, cells were stimulated with heregulin (2 nmol/L) or TGF α (5 nmol/L) in the presence or absence of cetuximab (20 $\mu\text{g/ml}$) in 0.1% serum-containing medium. Four days later, Alamar Blue was added and fluorescence was detected using a 96-well fluorometer. Results are percentage of control cells. Erlotinib sensitivity was determined in the presence or absence of 20 $\mu\text{g/ml}$ cetuximab in a dose-dependent manner. Cell growth was stimulated with either 2 nmol/L heregulin or 5 nmol/L TGF α , and assay was done as described above.

In this study, we set out to determine whether some portion of the antiproliferative effects of erlotinib can be attributed to direct inhibition of the HER2 kinase. We first showed that, in intact cells, erlotinib was capable of inhibiting an EGFR-H2 chimeric receptor at submicromolar levels following activation by an EGFR ligand. The inhibition at the receptor level translated into the potent inhibition of downstream signaling molecules and resulted in a marked decrease in cell proliferation. The EGFR-H2 chimeric receptor enabled us to recapitulate homodimeric activation of the HER2 kinase; the mode of activation that is predominantly seen in HER2-overexpressing cells. Because NR6 cells lack EGFR expression, we showed that erlotinib blocked the HER2 kinase directly. We found that erlotinib was only ~12-fold less active against HER2 kinase versus the EGFR kinase in intact cells. These results are in strong contrast to the 40- to 3,000-fold difference in potency that has been seen in purified enzymatic assays. One potential explanation for the substantial difference may be that erlotinib is a far better inhibitor of the HER2 kinase in intact cells because the *in vitro* kinase format may not recapitulate how HER2 is presented in the cell membrane. Hence, higher IC_{50} values are obtained in *in vitro* kinase assays.

The potent inhibition of HER2 kinase by EGFR tyrosine kinase inhibitors has been reported previously (21–23). However, since the *in vitro* kinase assays suggested that erlotinib or gefitinib was incapable of inhibiting the HER2 kinase at submicromolar levels, an alternative mechanism of action was proposed. In the presence of EGFR, HER2 forms functional heterodimers with EGFR upon ligand stimulation that in turn result in the transphosphorylation of HER2. It was suggested that EGFR inhibitors efficiently hinder the transphosphorylation of HER2 but do not block HER2 function directly. However, based on our data, the activity of EGFR kinase inhibitors on HER2-overexpressing cells can be attributed to the direct inhibition of HER2 kinase. In accordance with our findings, a study by Wels et al. (37) shows that an EGFR-neutralizing antibody is unable to inhibit cell proliferation of HER2-overexpressing

SKBR3 cells, yet SKBR3 cells are very sensitive to EGFR tyrosine kinase inhibitors. Because SKBR3 cells express HER2 in large excess over EGFR, it is unlikely that the potent down-regulation of HER2 in SKBR3 cells is solely due to the inhibition of cross-talk between EGFR and HER2. It is more likely that the direct inhibition of HER2, in conjunction with some disruption of EGFR/HER2 cross-talk, leads to the profound antiproliferative effect of EGFR tyrosine kinase inhibitors on HER2-overexpressing cells.

Although HER2 overexpression is observed in 20% to 30% of all breast cancers, the frequency of HER2 overexpression in most other tumor types is low (38). When HER2 levels are low, HER2 signaling is mediated by a second receptor in a heterodimeric complex. Because HER3 is the preferred heterodimeric partner of HER2 and is the most common mediator of ligand-stimulated HER2 activation (2, 33), we examined the inhibitory effects of erlotinib on the heregulin-dependent activation of HER2/HER3 heterodimers in cells that lack EGFR. Inhibition of receptor phosphorylation as well as Akt and MAPK activation was achieved using submicromolar concentrations of erlotinib. Heregulin-induced cell growth was also effectively inhibited in these cell lines. Because HER3 is catalytically inactive and cannot signal by itself, these results indicated that erlotinib directly blocked HER2 kinase and, consequently, prevented HER3 transphosphorylation.

Our findings agree with a previously published report that gefitinib potently inhibits ligand-driven HER2/HER3 signaling in various breast cancer cells (28). Contrary to our interpretation, Anido et al. postulate that inhibition of HER2 occurs through the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers and not through direct inhibition of HER2. These inactive heterodimers sequester HER2 and prevent the formation of active HER2/HER3 heterodimers. This model, however, implies that only cells that express significant amounts of EGFR in relation to HER2 will respond to EGFR tyrosine kinase inhibitors. Our findings and a recent report describing the

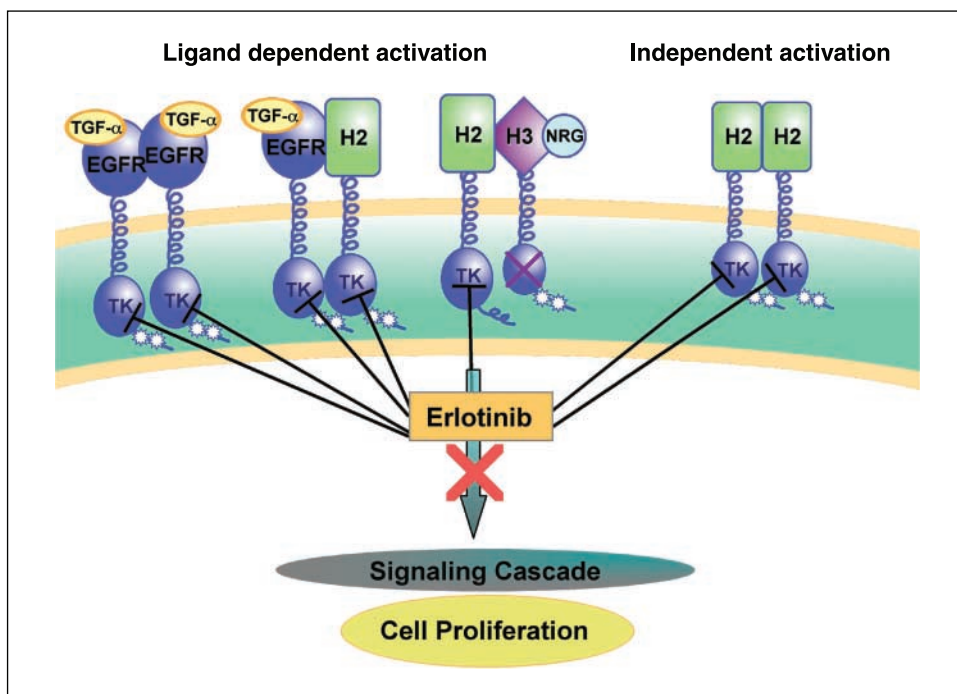


Figure 6. Schematic diagram of the inhibitory action of erlotinib. Erlotinib effectively inhibits EGFR (blue) kinase and signaling when it is activated by either homodimeric or heterodimeric receptor-receptor interactions. These interactions are driven by ligand binding as indicated in the first two representations on the left. HER2 (green) also forms activated homodimeric and heterodimeric complexes, but HER2 homodimers (right) can form in the absence of ligand stimulation. Erlotinib is capable of blocking the signaling activity of HER2 in homodimeric and heterodimeric complexes even when EGFR is not present, albeit with lower potency in comparison with its effect on the EGFR kinase.

potent effect of gefitinib on the inhibition of HER2/HER3 signaling in NSCLC cells that have no detectable EGFR (39) do not support this model.

As mentioned previously, EGFR, HER2, and HER3 activate the PI3K/Akt signaling pathway. The activation of this pathway is most efficiently mediated through HER3 because only HER3 has multiple binding sites for the p85 regulatory subunit of PI3K (40, 41). In contrast, EGFR predominantly activates PI3K via the adaptor protein Grb2-associated binder 1 (42, 43). Additionally, in the presence of EGFR and HER3, PI3K can be stimulated via ligand-induced EGFR/HER3 heterodimers (44). Gefitinib sensitivity in NSCLC lines has been strongly linked to the down-regulation of the PI3K/Akt pathway, and loss of HER3 activation seems to be an important prerequisite for the sensitivity (45, 46). The authors propose the direct inhibition of EGFR/HER3 as the mechanism of action. Interestingly, all of the sensitive lines also express HER2. Given our data, one can imagine that the potent down-regulation of the survival pathway in HER2-overexpressing cells may also occur via the inhibition of HER2/HER3 heterodimers especially in cases where EGFR expression is low.

In conclusion, we have shown that erlotinib directly blocks HER2 activity regardless of whether HER2 was activated by either homodimeric association or heterodimeric transactivation with HER3. These modes of inhibition are summarized schematically in Fig. 6. In both cases, erlotinib also inhibited the activation of

downstream signaling intermediates and suppressed cell proliferation in the absence of EGFR.

Based on our study, we speculate that erlotinib may have a much wider therapeutic potential and that, in addition to EGFR-expressing tumors, it may also be useful against HER2-overexpressing tumors. Pharmacokinetic studies determined that a daily oral dose of 150 mg erlotinib, which is the maximal tolerated dose, translates into a mean serum concentration of 3 to 10 $\mu\text{mol/L}$ (47, 48). Because $\sim 90\%$ of erlotinib is bound to serum protein, the free drug concentration is approximately 0.3 to 1 $\mu\text{mol/L}$. Therefore, it is unlikely that erlotinib effectively inhibits HER2 activity in a wide range of patients. Yet, some individuals might benefit from this therapy because plasma concentrations of erlotinib vary substantially among individuals (49). If higher drug exposure could be achieved potentially through pulse dosing or i.v. administration, erlotinib may very well efficiently block HER2 function in a substantial number of patients bearing tumors that are driven by either EGFR or HER2 activation.

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References

- Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2000;103:211–5.
- Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127–37.
- Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene* 2000;19:6550–65.
- Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 2005; 5:341–54.
- Kim TE, Murren JR. Erlotinib OSI/Roche/Genentech. *Curr Opin Investig Drugs* 2002;3:1385–95.
- Akita RW, Sliwkowski MX. Preclinical studies with erlotinib (Tarceva). *Semin Oncol* 2003;30:15–24.
- Grunwald V, Hidalgo M. Development of the epidermal growth factor receptor inhibitor Tarceva (OSI-774). *Adv Exp Med Biol* 2003;532:235–46.
- Johnson JR, Cohen M, Sridhara R, et al. Approval summary for erlotinib for treatment of patients with locally advanced or metastatic non-small cell lung cancer after failure of at least one prior chemotherapy regimen. *Clin Cancer Res* 2005;11:6414–21.
- Arteaga CL. Epidermal growth factor receptor dependence in human tumors: more than just expression? *Oncologist* 2002;7 Suppl 4:31–9.
- Perez-Soler R. HER1/EGFR targeting: refining the strategy. *Oncologist* 2004;9:58–67.
- Parra HS, Cavina R, Latteri F, et al. Analysis of epidermal growth factor receptor expression as a predictive factor for response to gefitinib (Iressa, ZD1839) in non-small-cell lung cancer. *Br J Cancer* 2004;91:208–12.
- Hirsch FR, Bunn PA, Jr. Epidermal growth factor receptor inhibitors in lung cancer: smaller or larger molecules, selected or unselected populations? *J Clin Oncol* 2005;23:9044–7.
- Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004;304:1497–500.
- Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2004;350:2129–39.
- Johnson BE, Janne PA. Epidermal growth factor receptor mutations in patients with non-small cell lung cancer. *Cancer Res* 2005;65:7525–9.
- Shigematsu H, Gazdar AF. Somatic mutations of epidermal growth factor receptor signaling pathway in lung cancers. *Int J Cancer* 2006;118:257–62.
- Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer—molecular and clinical predictors of outcome. *N Engl J Med* 2005;353:133–44.
- Calvo E, Baselga J. Ethnic differences in response to epidermal growth factor receptor tyrosine kinase inhibitors. *J Clin Oncol* 2006;24:2158–63.
- Fujimoto N, Wislez M, Zhang J, et al. High expression of ErbB family members and their ligands in lung adenocarcinomas that are sensitive to inhibition of epidermal growth factor receptor. *Cancer Res* 2005;65: 11478–85.
- Olayioye MA, Graus-Porta D, Beerli RR, Rohrer J, Gay B, Hynes NE. ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol Cell Biol* 1998;18:5042–51.
- Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* 2001;61: 7184–8.
- Moulder SL, Yakes FM, Muthuswamy SK, Bianco R, Simpson JF, Arteaga CL. Epidermal growth factor receptor (HER1) tyrosine kinase inhibitor ZD1839 (Iressa) inhibits HER2/neu (erbB2)-overexpressing breast cancer cells *in vitro* and *in vivo*. *Cancer Res* 2001;61:8887–95.
- Anderson NG, Ahmad T, Chan K, Dobson R, Bundred NJ. ZD1839 (Iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potentially inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression. *Int J Cancer* 2001; 94:774–82.
- Cappuzzo F, Varella-Garcia M, Shigematsu H, et al. Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small-cell lung cancer patients. *J Clin Oncol* 2005;23:5007–18.
- Moyer JD, Barbacci EG, Iwata KK, et al. Induction of apoptosis and cell cycle arrest by CP-358774, an inhibitor of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 1997;57:4838–48.
- Rusnak DW, Affleck K, Cockerill SG, et al. The characterization of novel, dual ErbB-2/EGFR, tyrosine kinase inhibitors: potential therapy for cancer. *Cancer Res* 2001;61:7196–203.
- Fabian MA, Biggs WH III, Treiber DK, et al. A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol* 2005;23:329–36.
- Anido J, Matar P, Albanell J, et al. ZD1839, a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, induces the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers and prevents heregulin signaling in HER2-overexpressing breast cancer cells. *Clin Cancer Res* 2003;9:1274–83.
- Pruss RM, Herschman HR, Klement V. 3T3 variants lacking receptors for epidermal growth factor are susceptible to transformation by Kirsten sarcoma virus. *Nature* 1978;274:272–4.
- Holmes WE, Sliwkowski MX, Akita RW, et al. Identification of heregulin, a specific activator of p185erbB2. *Science* 1992;256:1205–10.
- Sorkin A, Di Fiore PP, Carpenter G. The carboxyl terminus of epidermal growth factor receptor/erbB-2 chimera is internalization impaired. *Oncogene* 1993;8: 3021–8.
- Yauch RL, Januario T, Eberhard DA, et al. Epithelial versus mesenchymal phenotype determines *in vitro* sensitivity and predicts clinical activity of erlotinib in lung cancer patients. *Clin Cancer Res* 2005;11:8686–98.
- Pinkas-Kramarski R, Soussan L, Waterman H, et al. Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J* 1996;15:2452–67.
- Alimandi M, Romano A, Curia MC, et al. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene* 1995;10:1813–21.
- Wallasch C, Weiss FU, Niederfellner G, Jallat B, Issing W, Ullrich A. Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. *EMBO J* 1995;14:4267–75.

36. Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM. Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* 2005;7:301-11.
37. Wels W, Beerli R, Hellmann P, et al. EGF receptor and p185erbB-2-specific single-chain antibody toxins differ in their cell-killing activity on tumor cells expressing both receptor proteins. *Int J Cancer* 1995;60:137-44.
38. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989;244:707-12.
39. Hirata A, Hosoi F, Miyagawa M, et al. HER2 over-expression increases sensitivity to gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, through inhibition of HER2/HER3 heterodimer formation in lung cancer cells. *Cancer Res* 2005;65:4253-60.
40. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF III, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A* 2003;100:8933-8.
41. Fedi P, Pierce JH, di Fiore PP, Kraus MH. Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase C γ or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. *Mol Cell Biol* 1994;14:492-500.
42. Mattoon DR, Lamothe B, Lax I, Schlessinger J. The docking protein Gab1 is the primary mediator of EGF-stimulated activation of the PI-3K/Akt cell survival pathway. *BMC Biol* 2004;2:24.
43. Sithanandam G, Smith GT, Fields JR, Fornwald LW, Anderson LM. Alternate paths from epidermal growth factor receptor to Akt in malignant versus nontransformed lung epithelial cells: ErbB3 versus Gab1. *Am J Respir Cell Mol Biol* 2005;33:490-9.
44. Soltoff SP, Carraway KL III, Prigent SA, Gullick WG, Cantley LC. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. *Mol Cell Biol* 1994;14:3550-8.
45. Engelman JA, Janne PA, Mermel C, et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. *Proc Natl Acad Sci U S A* 2005;102:3788-93.
46. Amann J, Kalyankrishna S, Massion PP, et al. Aberrant epidermal growth factor receptor signaling and enhanced sensitivity to EGFR inhibitors in lung cancer. *Cancer Res* 2005;65:226-35.
47. Frohna P, Lu J, Eppler S, et al. Evaluation of the absolute oral bioavailability and bioequivalence of erlotinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in a randomized, crossover study in healthy subjects. *J Clin Pharmacol* 2006;46:282-90.
48. Zhang W, Siu LL, Moore MJ, Chen EX. Simultaneous determination of OSI-774 and its major metabolite OSI-420 in human plasma by using HPLC with UV detection. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005;814:143-7.
49. Lu JF, Eppler SM, Wolf J, et al. Clinical pharmacokinetics of erlotinib in patients with solid tumors and exposure-safety relationship in patients with non-small cell lung cancer. *Clin Pharmacol Ther* 2006;80:136-45.