PF00299804, an Irreversible Pan-ERBB Inhibitor, Is Effective in Lung Cancer Models with *EGFR* and *ERBB2* Mutations that Are Resistant to Gefitinib

Jeffrey A. Engelman,[1,2,3] Kreshnik Zejnullahu,[4,5] Christopher-Michael Gale,[3] Eugene Lifshits,[6] Andrea J. Gonzales,[9] Takeshi Shimamura,[4,5,7] Feng Zhao,[4,5] Patrick W. Vincent,[10] George N. Naumov,[6] James E. Bradner,[5] Irene W. Althaus,[9] Leena Gandhi,[4,5,7] Geoffrey I. Shapiro,[4,5,7] James M. Nelson,[9] John V. Heymach,[11] Matthew Meyerson,[4,5,8,12] Kwok-Kin Wong,[4,5,7] and Pasi A. Jänne[4,5,7]

[1]Massachusetts General Hospital Cancer Center; [2]Department of Systems Biology, Harvard Medical School; [3]Department of Signal Transduction, Beth Israel Deaconess Medical Center; [4]Lowe Center for Thoracic Oncology and [5]Department of Medical Oncology, Dana-Farber Cancer Institute; [6]Department of Surgery and Vascular Biology Program, Children's Hospital; [7]Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School; [8]Department of Pathology, Harvard Medical School, Boston, Massachusetts; [9]Pfizer Global Research and Development, Cancer Drug Discovery, Ann Arbor, Michigan; [10]Pfizer Global Research and Development, Groton, Connecticut; [11]Department of Thoracic/Head and Neck Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; and [12]The Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts

Abstract

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors gefitinib and erlotinib are effective treatments for a subset of non–small cell lung cancers. In particular, cancers with specific *EGFR*-activating mutations seem to be the most sensitive to these agents. However, despite their initial response, such cancers almost invariably develop resistance. In 50% of such cancers, a secondary *EGFR* mutation, T790M, has been identified that renders gefitinib and erlotinib ineffective inhibitors of EGFR kinase activity. Thus, there is a clinical need to develop novel EGFR inhibitors that can effectively inactivate T790M-containing EGFR proteins. In this study, we evaluate the effectiveness of a novel compound, PF00299804, an irreversible pan-ERBB inhibitor. The results from these studies show that PF00299804 is a potent inhibitor of *EGFR*-activating mutations as well as the *EGFR* T790M resistance mutation both *in vitro* and *in vivo*. Additionally, PF00299804 is a highly effective inhibitor of both the wild-type *ERBB2* and the gefitinib-resistant oncogenic *ERBB2* mutation identified in lung cancers. These preclinical evaluations support further clinical development of PF00299804 for cancers with mutations and/or amplifications of ERBB family members. [Cancer Res 2007;67(24):11924–32]

Introduction

The small-molecule epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors gefitinib (Iressa, Astra-Zeneca) and erlotinib (Tarceva, OSI Pharmaceuticals) have been evaluated in clinical trials for patients with non–small cell lung cancer (NSCLC). Both agents cause partial responses in 10% to 20% of all NSCLC patients (1–4). Tumors that possess activating mutations in *EGFR* seem to be particularly sensitive to EGFR tyrosine kinase inhibitors both *in vitro* and in NSCLC patients (5–11). However, despite the benefits of gefitinib or erlotinib in *EGFR* mutant NSCLC, most, if not all, patients ultimately develop acquired resistance to these agents. In ~50% of these individuals, acquired resistance is associated with the emergence of a secondary mutation in *EGFR* (a methionine for threonine substitution at amino acid 790; T790M), which blocks gefitinib or erlotinib from being able to inhibit the activation of EGFR (12–15). Initial studies suggest that ~20% of patients become resistant to gefitinib due to an acquired amplification of the *MET* proto-oncogene (16).

A significant portion of patients initially treated with gefitinib or erlotinib do not benefit from these agents (i.e., *de novo* resistance). Somatic mutations in *K-ras* are usually mutually exclusive with *EGFR* mutations and have been associated with *de novo* resistance to gefitinib and erlotinib (17). In addition, patients with cancers harboring *ERBB2* mutations, which are observed in 2% to 3% of lung cancers, do not clinically respond to gefitinib and erlotinib (18, 19). Additionally, lung cancer cell lines with insertion *ERBB2* mutations are also resistant to gefitinib and erlotinib (20, 21). Furthermore, *in vitro* studies have shown that *EGFR* exon 20 mutations, which account for 1% to 3% of all EGFR mutations, are also resistant to the effects of gefitinib and erlotinib (22). Thus, identification of strategies and/or agents capable of overcoming acquired and *de novo* resistance mechanisms are needed.

Gefitinib and erlotinib are both quinazalone-based inhibitors of EGFR. Both act as ATP mimetics and are reversible inhibitors of the tyrosine kinase domain. In contrast, irreversible EGFR inhibitors not only act as ATP mimetics but also covalently bind Cys-797 of EGFR. This enables them to inhibit EGFR kinase activity even in the presence of *EGFR* T790M. Irreversible EGFR inhibitors CL387,785, EKB-569, and HKI-272 have all been shown to inhibit the phosphorylation of EGFR in the presence of T790M and also the growth of NSCLC cell lines harboring T790M mutations (23, 24). HKI-272 and BIBW2992 are currently being evaluated in clinical studies in patients with NSCLC.

PF00299804 is an irreversible pan-ERBB inhibitor presently under clinical development. In comparison with a first-generation irreversible pan-ERBB inhibitor, CI-1033, PF00299804 has more attractive pharmacokinetic properties including greater bioavailability, longer

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

J.A. Engelman and K. Zejnullahu contributed equally to this work.

Requests for reprints: Pasi A. Jänne, Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, D820A, 44 Binney Street, Boston, MA 02115. Phone: 617-632-6076; Fax: 617-582-7683; E-mail: pjanne@partners.org.

doi:10.1158/0008-5472.CAN-07-1885

Table 1. Efficacy comparison of PF00299804, gefitinib, erlotinib, and CI-1033

	Isolated enzyme assay				
	EGFR	ERBB2	ERBB4	Cellular EGFR (NIH3T3/EGFR)	Cellular ERBB2 (NIH3T3/ERBB2)
	IC_{50}	IC_{50}	IC_{50}	IC_{50}	IC_{50}
PF-00299804	6.0	45.7	73.7	5.8	41
Gefitinib	3.1	343	476	14.4	>500
Erlotinib	0.56	512	790	19.3	299
CI-1033	1.8	11	27	1.6	16

NOTE: Shown are the IC_{50} values of each drug using an *in vitro* kinase assay against wild-type ERBB receptors (left) and the IC_{50} values against wild-type EGFR and ERBB2 expressed in NIH-3T3 cells (right). All values are shown in nmol/L.

half-life, larger volume of distribution, and lower clearance. PF00299804 is presently under evaluation in phase I clinical studies (25). Based on its potential efficacy as an irreversible pan-ERBB inhibitor, we examined the efficacy of PF00299804 in cancer models with *EGFR* and *ERBB2* mutations that are resistant to gefitinib.

Materials and Methods

Cell culture and reagents. The *EGFR* mutant NSCLC cell lines H3255 (L858R), HCC827 (del E746_A750) and H1975 (L858R/T790M), HCC820 (del 747_L751, Ins S/T790M), HCC4006 (Del L747_E749), and PC-9 (Del E746_A750) were used in this study and have been extensively characterized (5, 13, 26–30). H3255 GR were selected *in vitro* for their resistance to gefitinib, contain an *EGFR* T790M in cis with L858R in a limited number of alleles, and have been previously characterized (23). *EGFR* wild-type cell lines (A549, H441, H322, Calu-3, and H1819) and the *ERBB-2* mutant cell line (H1781) were obtained from American Type Culture Collection. NIH3T3 cells transfected to express the full-length EGFR or a chimeric receptor containing the extracellular domain of EGFR and the intracellular domain of ERBB2 were a gift from Dr. Bruce Cohen (Pfizer Global Research and Development, Groton, CT; ref. 31).

Gefitinib was obtained from commercial sources and was purified through an ethyl acetate extraction. The resulting product was verified by liquid chromatography and mass spectrometry. PF00299804 was a kind gift from Pfizer. Stock solutions (10 mmol/L) of both drugs were prepared in DMSO and stored at −20°C.

Anti–phospho-Akt (Ser-473), anti-total Akt, anti-EGFR, anti–phospho-ERBB-3 (Tyr-1289), anti–phospho-extracellular signal-regulated kinase (ERK) 1/2 (pT185/pY187), and anti-total ERK1/2 antibodies were obtained from Cell Signaling Technology. Anti–ERBB-3 antibody was obtained from Santa Cruz Biotechnology. The phosphospecific EGFR (pY1068) antibody was purchased from Biosource International, Inc.

Cell proliferation and growth assays. Growth and inhibition of growth was assessed by 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay. This assay, a colorimetric method for determining the number of viable cells, is based on the bioreduction of MTS by cells to a formazan product that is soluble in cell culture medium, can be detected spectrophotometrically, and was performed according to previously established methods (5, 27, 29). The cells were exposed to treatment for 72 h, and the number of cells used per experiment were determined empirically and has been previously established (27, 29). All experimental points were set up in 6 to 12 wells, and all experiments were repeated at least thrice. The data were graphically displayed using GraphPad Prism version 3.00 for Windows (GraphPad Software).[13] The curves were fitted using a nonlinear regression model with a sigmoidal dose response.

Immunoblotting. Cells grown under the previously specified conditions were lysed in the following lysis buffer composition: 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP40, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium PPi, 50 mmol/L NaF, 10 nmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 0.5 mmol/L DTT, 4 μg/mL leupeptin, 4 μg/mL pepstatin, 4 μg/mL apoprotein, and 1 mmol/L phenylmethylsulfonyl fluoride. After cell lysis, lysates were centrifuged at 16,000 × *g* for 5 min at 4°C. The supernatant was used for subsequent procedures. Western blot analyses were conducted after separation by SDS-PAGE and transfer to nitrocellulose membranes. Immunoblotting was performed according to the antibody manufacturers' recommendations. Antibody binding was detected using an enhanced chemiluminescence system (New England Nuclear Life Science Products, Inc.).

***In vitro* kinase assays.** The catalytic domains of ERBB1, ERBB2, and ERBB4 tagged with glutathione *S*-transferase were expressed in insect cells and purified according to ref. (32). ELISA-based enzyme assays and IC_{50} determinations for ERBB1, ERBB2, and ERBB4 were performed as described in ref. (32). Enzyme assays and IC_{50} determinations for all other kinases used in this study were performed as previously described (33–35).

EGFR and ERBB2 mutant constructs and retroviral infection. The human *EGFR* and *ERBB2* cDNA coding regions were cloned into pDNR-Dual (BD Biosciences). The *EGFR* mutants, L858R and del L747_S752, P753S, and *ERBB2* Ins 774YVMA, were constructed with Quick Change Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions and have been previously published (29). The T790M mutation was introduced into the *EGFR* L858R or L747_S752del, P753S constructs using site-directed mutagenesis. Similarly, the *ERBB2* insertion (Ins774YVMA) was introduced using site-directed mutagenesis into the *ERBB2* cDNA. The oligonucleotide sequences are available upon request. All constructs were confirmed by DNA sequencing. As a control, a retroviral construct expressing green fluorescent protein (GFP) was used and has been previously described (36). All constructs (including GFP) were shuttled into the retroviral vector JP1520 using the BD Creator System (BD Biosciences). NSCLC or NIH-3T3 cells were infected with retrovirus according to standard protocols, as described previously (36, 37). Stable populations were obtained by selection in median containing 2 μg/mL puromycin (Sigma-Aldrich).

Ba/F3 cells. *EGFR* constructs were introduced into Ba/F3 cells by retroviral infection using previously described methods (21, 22, 38). The Ba/F3 cells were cultured as previously described (39). Polyclonal cell lines stably expressing different *EGFR* mutant constructs were established by puromycin selection and subsequently cultured in the absences of interleukin-3 (IL-3). Pooled stable cells that were transformed to IL-3 independence were used for drug-sensitivity testing by the method previously described (18).

[13] http://www.graphpad.com

Table 2. Comparison of gefitinib and PF00299804 efficacy in *EGFR, ERBB2*, and *K-ras* mutant cell lines

Cell line	*EGFR* mutation	*ERBB2* mutation	*K-ras* mutation	Gefitinib IC_{50} (μmol/L)	PF00299804 IC_{50} (μmol/L)
A549	WT	WT	G12S	>10	>10
H441	WT	WT	G12V	>10	4
H322	WT	WT	WT	>10	>10
Calu-3	WT	WT (amplified)	WT	1.4	0.063
H1819	WT	WT (amplified)	WT	0.42	0.029
H3255	L858R	WT	WT	0.075	0.007
H3255 GR	L858R/T790M	WT	WT	>10	0.119
H1975	L858R/T790M	WT	WT	>10	0.44
H820	Del 747_L751, Ins S/T790M	WT	WT	>10	0.93
HCC 827	Del E746_A750	WT	WT	0.008	0.002
HCC 4006	Del L747_E749	WT	WT	0.050	0.004
PC-9	Del E746_A750	WT	WT	0.023	0.002
H1781	WT	Ins G776V, C	WT	>10	0.275

Abbreviation: WT, wild-type.

Xenograft studies. Nude mice (*nu/nu*; 6–8 weeks old; Massachusetts General Hospital) were used for *in vivo* studies and were cared for in accordance with the standards of the Institutional Animal Care and Use Committee under a protocol approved by the Animal Care and Use Committee of the Children's Hospital Boston. Mice were anesthetized using a 2% isoflurane (Baxter) inhalation oxygen mixture. A suspension of 5×10^6 HCC827-GFP or HCC827-Del/T790M lung cancer cells (in 0.2 mL of PBS) were inoculated s.c. into the lower-right quadrant of the flank of each mouse. Five mice were inoculated with either HCC827-GFP or HCC827-Del/T790M cells in the gefitinib treatment group. Tumors were measured twice weekly using calipers, and volume was calculated using the following formula: length $\times$ width^2 $\times$ 0.52. Mice were monitored daily for body weight and general condition. Mice were randomized to treatment when the mean tumor volume was 400 to 500 mm^3. Gefitinib was administered at 150 mg/kg/d by daily oral gavage as previously described (40). PF00299804 was administered at 10 mg/kg/d by daily oral gavage. The experiment was terminated when the mean size of the control tumors reached 2000 mm^3.

Results

PF00299804 is a specific inhibitor of the ERBB family of kinases. PF00299804 is a quinazalone-based irreversible pan-ERBB inhibitor structurally related to CI-1033. We examined the specificity of PF00299804 by performing *in vitro* kinase assays against purified ERBB family kinases (Table 1) and against 38 other protein kinases (Supplementary Table S1). PF00299804 effectively inhibited the *in vitro* kinase activity of wild-type *EGFR* with similar efficacy as gefitinib, erlotinib, and CI-1033. In contrast to gefitinib and erlotinib, PF00299804 also effectively inhibited wild-type ERBB2. LCK and SRC were the only other kinases inhibited by PF00299804 although with >10 fold higher IC_{50} than against *EGFR* (Supplementary Table S1).

PF00299804 is effective in gefitinib-sensitive and gefitinib-resistant NSCLC cell lines. The *in vitro* sensitivity of NSCLC cell lines to gefitinib is most closely associated with the presence of activating mutations in *EGFR*. However, some *EGFR* mutations, such as T790M or exon 20 insertion mutations, are associated with gefitinib resistance *in vitro* and *in vivo* (12, 13, 18, 19, 22). In addition, both K-ras and ERBB2 mutations are associated with resistance to gefitinib in NSCLC patients and *in vitro*, respectively (17, 41). Thus, we compared the efficacy of PF00299804 to gefitinib in NSCLC *in vitro* models. We examined NSCLC cell lines with known *EGFR*, K-ras, or ERBB2 mutations (Table 2). K-ras mutant NSCLC cell lines (A549 and H441) were resistant to both gefitinib and PF00299804 consistent with prior observations (5, 27). In H441, an IC_{50} was reached with PF00299804 but only at a very high concentration (4 μmol/L) and likely reflects off-target effects. In cell lines wild-type for both *EGFR* and K-ras (H322, H1819, and

Table 3. Comparison of gefitinib and PF00299804 efficacy in Ba/F3 cells engineered to express different *EGFR* mutations

EGFR-activating mutations			EGFR-activating mutations in *cis* with T790M		
EGFR mutation	Gefitinib IC_{50}	PF00299804 IC_{50}	*EGFR* mutation	Gefitinib IC_{50}	PF00299804 IC_{50}
Del E746_A750	4.8 nmol/L	<1 nmol/L	Del E746_A750/T790M	8.3 μmol/L	140 nmol/L
Del S752_I759	35 nmol/L	2.0 nmol/L	Del S752_I759/T790M	>10 μmol/L	330 nmol/L
Del L747_A750InsP	7.4 nmol/L	1.6 nmol/L	Del L747_A750InsP/T790M	>10 μmol/L	240 nmol/L
Del L747_P753InsS	4.1 nmol/L	1.9 nmol/L	Del L747_P753InsS/T790M	>10 μmol/L	160 nmol/L
Del E746_S752InsV	306 nmol/L	1.4 nmol/L	Del E746_S752InsV/T790M	>10 μmol/L	270 nmol/L
L858R	26 nmol/L	2.6 nmol/L	L858R/T790M	>10 μmol/L	300 nmol/L
A767_V769duspASV	3.1 μmol/L	230 nmol/L			
EGFR vIII	2.7 μmol/L	1.2 nmol/L			

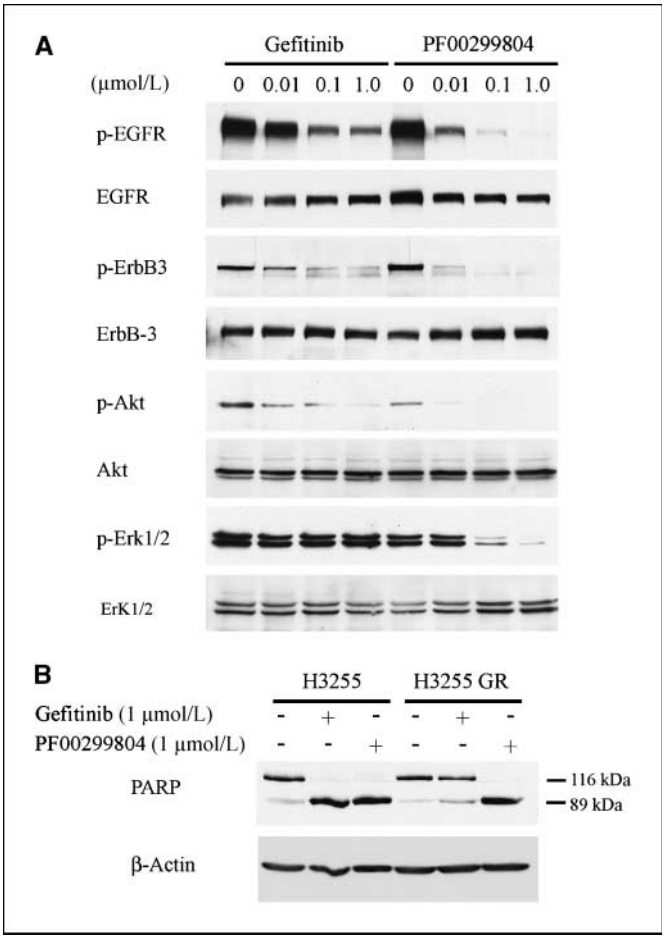

Figure 1. PF00299804 inhibits EGFR signaling and induces apoptosis in the *EGFR* T790M–containing H3255 GR cell line. *A,* H3255 GR cells were exposed to indicated concentrations of gefitinib or PF00299804 for 24 h. Cells were lysed and the proteins were separated by Western blotting, transferred to a nitrocellulose membrane, and probed with the indicated antibodies. The H3255 GR cells maintain activation of EGFR/ErbB-3/Akt signaling in the presence of gefitinib. In contrast, PF299804 effectively abrogates EGFR, ERBB3, and Akt phosphorylation. *B,* Western blot analysis for PARP with H3255 and H3255 GR after treatment with either gefitinib (1 μmol/L) or PF00299804 (1 μmol/L) for 72 h. As can be seen, there is a marked increase in the 89 kDa cleaved product in H3255 after both gefitinib and PF00299804 treatment. However, only PF00299804 treatment induces substantial PARP cleavage in H3255 GR.

Calu-3), gefitinib and PF00299804 both effectively inhibited growth of H1819 and Calu-3 cells but not of H322 cells. Both H1819 and Calu-3 contain amplifications of the *ERBB2* locus (42, 43). Increased ERBB2 copy number has been associated with increased efficacy of gefitinib in NSCLC cell lines and patients treated with gefitinib (44). In both cell lines, PF00299804, which is a much more potent ERBB2 inhibitor than gefitinib, was significantly more effective and yielded IC_{50} values at 10-fold lower concentrations (Table 2). The NSCLC cell lines (H3255, HCC4006, HCC827, and PC-9) containing the two common *EGFR*-activating mutations (L858R and exon 19 deletions), although all sensitive to gefitinib, also had lower IC_{50} values with PF00299804. Similarly, in the Ba/F3 cell models, PF00299804 was more effective than gefitinib at inhibiting the growth of cells containing the common *EGFR*-activating mutations (Table 3). Whereas we observed a 70-fold range (4.1–306 nmol/L) in the IC_{50} for gefitinib against the five most common exon 19 deletions, the IC_{50} values were between 1 and 2 nmol/L for PF00299804 for all five mutations. Consistent with the greater activity of PF00299804 in H3255 cells (L858R; Table 2), the IC_{50} for Ba/F3 cells expressing *EGFR* L858R was 10-fold lower with PF00299804 compared with gefitinib (Table 3). Furthermore, the IC_{50} for PF00299804 was substantially lower against two other *EGFR* mutations (A767_V769duspASV and *EGFRvIII*) than for gefitinib.

We next examined whether PF00299804 can inhibit the growth of NSCLC or Ba/F3 cell lines, which contain the *EFGR* T790M resistance mutation. H1975 and H3255 GR are NSCLC cell lines that contain both *EGFR* L858R and T790M mutations, whereas H820 contains an EGFR exon 19 deletion (Del 747_L751, Ins S) and a T790M mutation (13, 23, 45). All three are resistant to gefitinib *in vitro*, but PF00299804 is able to effectively inhibit the growth of these cell lines (IC_{50} <1 μmol/L for all three cell lines; Table 2). Similarly, and also in contrast to gefitinib, PF00299804 potently inhibits the growth of Ba/F3 cells engineered to express *EGFR* T790M *cis* to any of the five different exon 19 deletions or *cis* to the L858R mutation (Table 3).

To determine if PF00299804 inhibited the growth of *EGFR* T790M–containing cells via on-target effects on EGFR T790M, we examined the phosphorylation state of EGFR and downstream signaling molecules. Our previous studies showed that down-regulation of ERBB3/phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is necessary for gefitinib to inhibit the growth of *EGFR* mutant NSCLC cell lines (16, 23, 36). The gefitinib-resistant H3255 GR cell line contains a limited number of T790M alleles within the amplified *EGFR* locus and maintains ERBB3 and Akt phosphorylation in the presence of gefitinib (23). In H3255 GR, PF00299804, but not gefitinib, leads to complete inhibition of EGFR, ERBB3, and Akt phosphorylation (Fig. 1*A*). Although both gefitinib and PF00299804 are able to induce apoptosis in H3255, as indicated by cleaved poly(ADP)ribose polymerase (PARP), only PF00299804 treatment leads to substantial apoptosis in the H3255 GR cell line (Fig. 1*B*).

Because PF00299804 is a pan-ERBB inhibitor and most *EGFR* mutant cell lines express multiple ERBB family members, the effects on EGFR phosphorylation could potentially be indirect (11, 28, 36). Thus, we determined whether PF00299804 is able to specifically inhibit *EGFR* T790M. We expressed *EGFR* T790M alone (WT/T790M) or in *cis* with L858R (L858R/T790M) or an exon 19 deletion (L747_S752del,P753S; referred to as Del/T790M) in NIH3T3 cells that do not contain significant quantities of endogenous ERBB family members. As can be seen in Fig. 2*A*, PF00299804 inhibited EGFR phosphorylation in all of the different EGFR T790M proteins whereas gefitinib was ineffective even at 10 μmol/L. These results are consistent with the greater effectiveness of PF00299804 on the growth of NSCLC and Ba/F3 cells harboring the *EGFR* T790M mutation (Tables 2 and 3). In the NIH3T3 cells, phosphorylation of EGFR L858R/T790M was completely inhibited by 1 nmol/L PF299804, whereas 100 nmol/L or greater was required to inhibit EGFR WT/T790M or Del/T790M (Fig. 2*A*).

PF00299804 inhibits the growth of H3255 and HCC827 cells engineered to express EGFR T790M. We have previously shown that expression of T790M in *cis* to an *EGFR*-activating mutation in H3255 or HCC827 renders these cell lines resistant to gefitinib (23). However, these cell lines are sensitive to the effects of PF00299804 (Table 4). Thus, cancer cell lines that have acquired resistance via acquisition of an *EGFR* T790M remain sensitive to PF00299804 at submicromolar concentrations. We compared the effects of gefitinib and PF00299804 on the phosphorylation of EGFR and

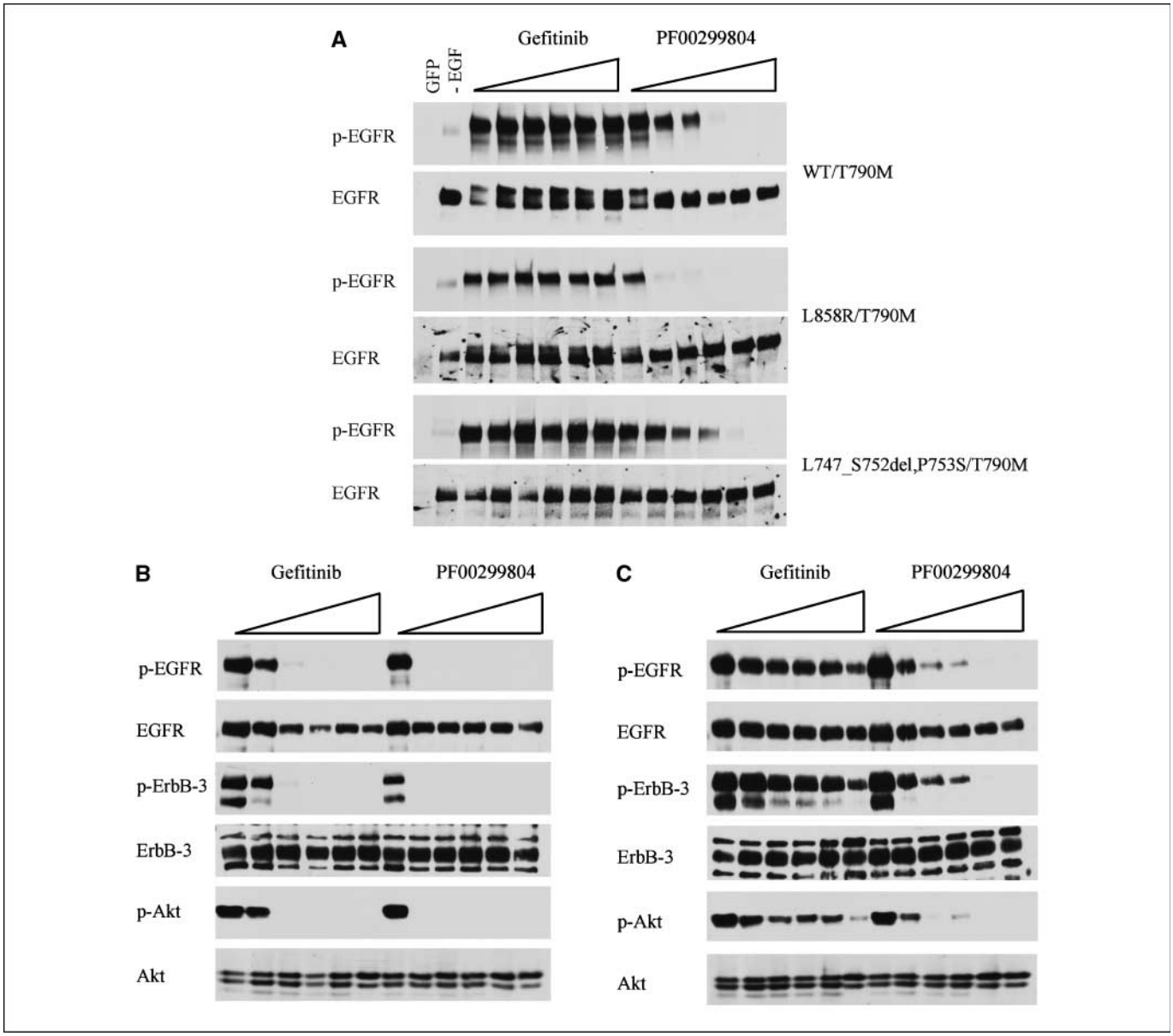

Figure 2. PF00299804 inhibits EGFR phosphorylation in the presence of the T790M mutation. *A,* NIH-3T3 cells were stably infected with either GFP or *EGFR* T790M in the background of wild-type (*WT*) *EGFR* or in *cis* with L858R or an exon 19 deletion (L747_S752del,P753S). Cells treated with either gefitinib or PF00299804 (concentrations, 0, 1 nmol/L, 10 nmol/L, 100 nmol/L, and 1 and 10 μmol/L for both drugs) for 6 h followed by EGF stimulation (10 ng/mL) for 15 min. Cells were lysed and the proteins were separated by Western blotting, transferred to a nitrocellulose membrane, and probed with indicated antibodies. Gefitinib does not inhibit EGFR phosphorylation in any of the cell lines. PF00299804 effectively inhibits EGFR phosphorylation in all *EGFR* T790M–containing constructs. *B,* HCC827 cells expressing either GFP control or Del/T790M (*C*) were treated with increasing concentrations of gefitinib or PF00299804 (0, 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 and 10 μmol/L for both drugs) for 12 h and lysed as described Materials and Methods. The resulting extracts were probed with the indicated antibodies. Both gefitinib and PF00299804 effectively inhibit EGFR, ERBB3, and Akt phosphorylation in HCC827 GFP cells, whereas in the HCC827 Del/T790M cells only PF00299804 is effective.

downstream signaling molecules in HCC827 cells expressing either GFP or Del/T790M. The effects of gefitinib and PF00299804 are similar in the control GFP-infected cell lines although complete inhibition of EGFR, ERBB3, and Akt phosphorylation is observed with only 1 nmol/L of PF00299804 compared with 10 nmol/L of gefitinib (Fig. 2*B*). This suggests that, at similar concentrations, PF00298804 is a more effective inhibitor of mutant *EGFR* than gefitinib. This observation is also consistent with the lower IC_{50} values obtained with PF00299804 compared with gefitinib in all of the *EGFR* mutant (both L858R and exon 19 deletion) NSCLC cell lines (Table 2). However, in HCC827 Del/T790M, gefitinib is unable to inhibit EGFR, ERBB3, and Akt phosphorylation (except minimally at clinically unachievable doses; i.e., 10 μmol/L; ref. 46). In contrast, PF00299804 inhibits the phosphorylation of these proteins starting at 10 nmol/L, consistent with its dramatic effects on growth of this cell line (Fig. 2*C*; Table 4).

To evaluate the *in vivo* efficacy of PF00299804, we generated xenografts in *nu/nu* mice using HCC827 GFP and HCC827 Del/T790M cells and treated the mice with gefitinib or PF00299804. As can be seen in Fig. 3, both agents effectively inhibited the growth of HCC827 GFP xenografts. In contrast, HCC827 Del/T790M xenografts were resistant to gefitinib, whereas PF00299804

Table 4. Comparison of gefitinib and PF00299804 efficacy in *EGFR* mutant NSCLC cell lines engineered to express different *EGFR* or *ERBB2* constructs

Cell line	*EGFR* mutation	Expression construct	Gefitinib IC_{50} (μmol/L)	PF00299804 IC_{50} (μmol/L)
H3255 GFP	L858R	GFP	0.071	0.007
H3255 WT/T790M	L858R	EGFR WT/T790M	>10	0.137
H3255 L858R/T790M	L858R	EGFR L858R/T790M	>10	1.62
H3255 Del/T790M	L858R	EGFR Del L747_S752,P753S/T790M	>10	1.57
HCC 827 GFP	Del E746_A750	GFP	0.004	0.002
HCC 827 WT/T790M	Del E746_A750	EGFR WT/T790M	0.008	0.003
HCC 827 L858R/T790M	Del E746_A750	EGFR L858R/T790M	0.927	0.014
HCC 827 Del/T790M	Del E746_A750	EGFR Del L747_S752,P753S/T790M	1.93	0.061
HCC 827 ERBB2	Del E746_A750	ERBB2	0.026	0.002
HCC 827 InsERBB2	Del E746_A750	ERBB2 Ins 774YVMA	4.82	0.083

treatment was substantially more effective at inhibiting growth of this xenograft model. Thus, these preclinical models suggest that PF00299804 may be quite effective against lung cancers that become resistant to gefitinib or erlotinib via acquisition of a T790M mutation in *EGFR*..

PF00299804 inhibits both wild-type and mutant ERBB2. Recently, *ERBB2* mutations have been identified in lung cancer, and preclinical studies suggest that these oncogenic mutations are resistant to erlotinib and gefitinib (20, 41). We examined the effects of PF00299804 in NSCLC cell lines with and without mutations in *ERBB2*. H1781 contains a homozygous mutation in *ERBB2* and is wild-type at the *EGFR* locus. This cell line is resistant to gefitinib but is highly sensitive to PF00299804 (Table 2). To determine whether PF00299804 can specifically inhibit the insertion *ERBB2* mutant, we expressed wild-type *ERBB2* or the common *ERBB2* mutant found in NSCLC (Ins774YVMA) in NIH-3T3 cells and compared the efficacy of gefitinib and PF00299804 on autophosphorylation of ERBB2. As can be seen in Fig. 4*A*, gefitinib has virtually no effect on ERBB2 phosphorylation of either the wild-type or insertion mutant except at 10 μmol/L. In contrast, PF00299804 effectively inhibits ERBB2 phosphorylation of both the wild-type and Ins774YVMA mutant at concentrations of 100 nmol/L and greater.

We subsequently introduced either wild-type *ERBB2* or the insertion *ERBB2* mutant (Ins774YVMA) into HCC827 cells (referred to herein as HCC827 ERBB2 or HCC827 ERBB2 Ins774YVMA). Expression of wild-type *ERBB2* did not render the HCC827 cells resistant to gefitinib (Table 4). In contrast, the insertion *ERBB2* mutant rendered HCC827 cells resistant to gefitinib but not to PF00299804 (Table 4). In HCC827 ERBB2, both gefitinib and PF00299804 effectively inhibited phosphorylation of EGFR, ERBB3, and Akt (Fig. 4*B*). Intriguingly, this occurs despite persistent ERBB2 phosphorylation even in the presence of gefitinib (Fig. 4*B*). Thus, maintenance of wild-type ERBB2 activation is insufficient to maintain ERBB3 phosphorylation (and subsequently PI3K activation) in HCC827 cells. However, in HCC827 ERBB2 Ins774YVMA, gefitinib is unable to inhibit phosphorylation of EGFR, ERBB3, or Akt except at 10 μmol/L. Thus, unlike wild-type ERBB2, the ERBB2 Ins774YVMA is able to activate ERBB3/PI3K/Akt despite EGFR inhibition. However, PF00299804 retains the ability to inhibit

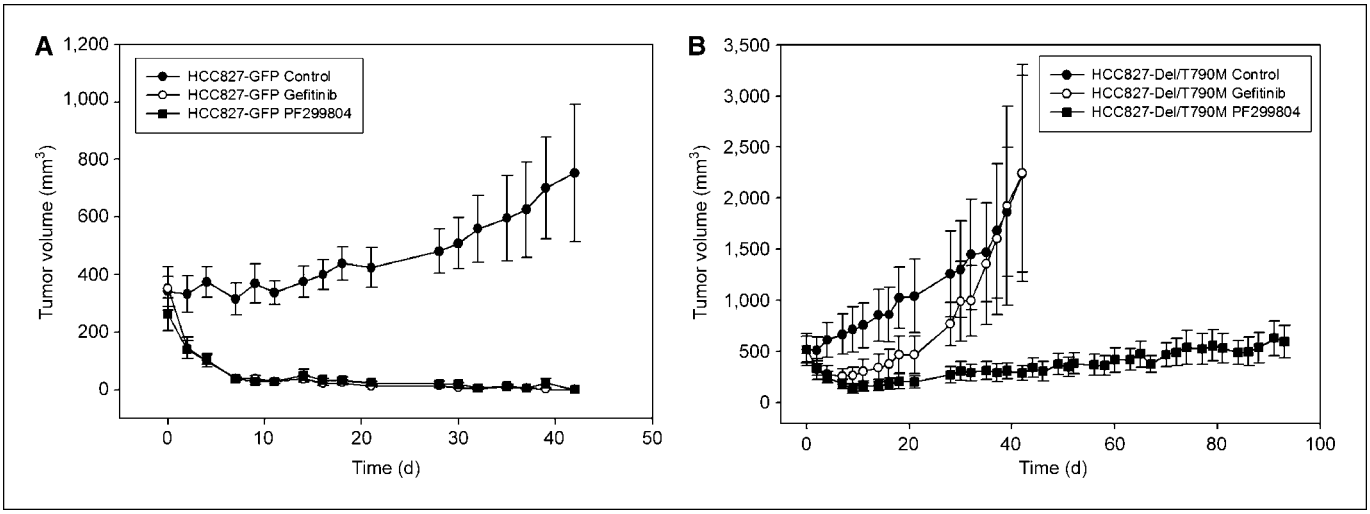

Figure 3. PF00299804 inhibits growth of HCC827 Del/T790M *in vivo*. Xenografts in *nu/nu* mice were generated as described in Materials and Methods. Gefitinib and PF00299804 treatments were administered by oral gavage and tumors were measured thrice weekly. Both gefitinib and PF00299804 led to complete disappearance of tumors in HCC827 GFP xenografts (*A*). The HCC827 Del/T790M xenografts grew while treated with gefitinib but were effectively inhibited by PF00299804 (*B*). PF00299804 treatment was continued for 95 d. The growth curves for control and gefitinib-treated animals have been previously published but were performed at the same time as the studies with PF00299804 (23).

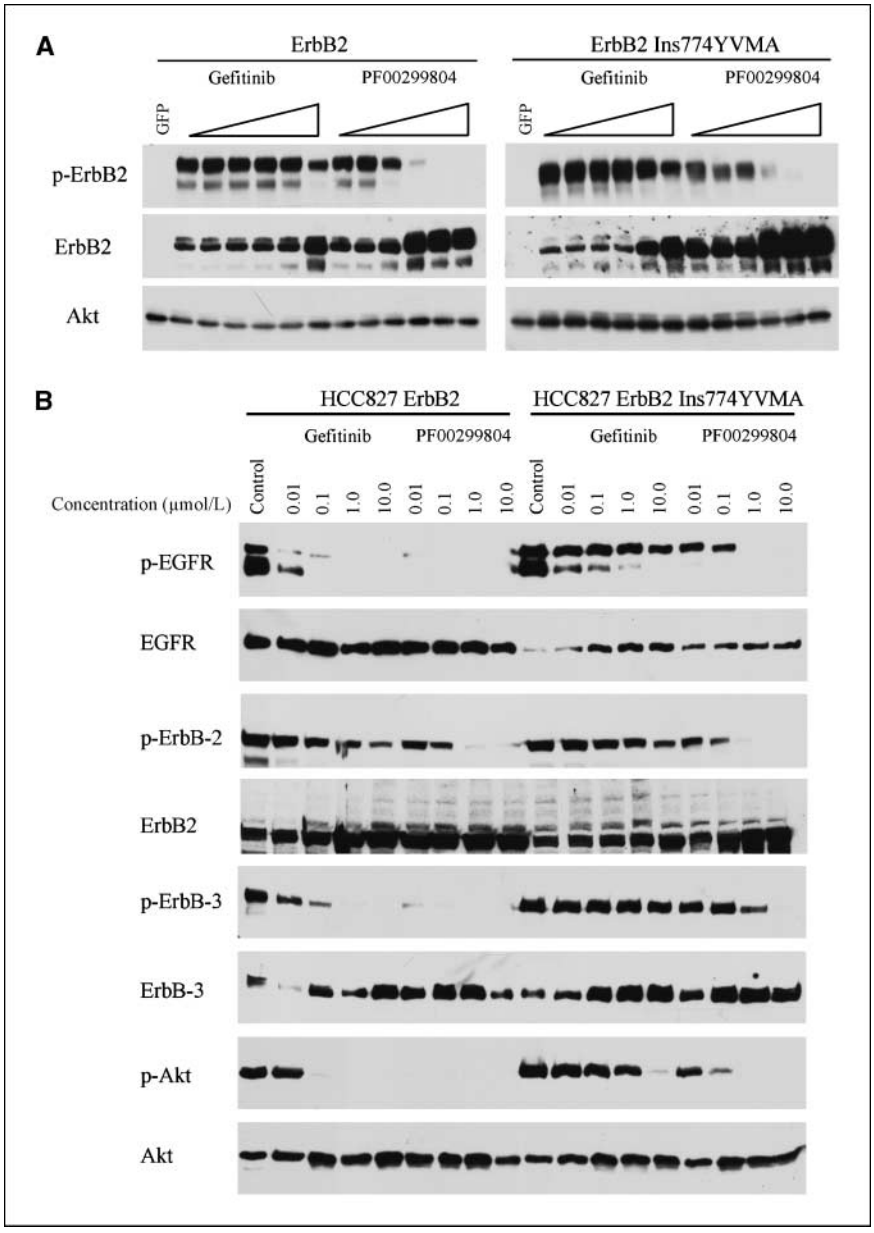

Figure 4. PF00299804 inhibits wild-type and Ins774YVMA *ERBB2*. *A,* NIH-3T3 cells were retrovirally infected with either *ERBB2* or *ERBB2* Ins774YVMA. Cells were treated with either gefitinib or PF00299804 (0, 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 and 10 μmol/L for both drugs) for 6 h and lysed as described in Materials and Methods. The resulting extracts were probed with the indicated antibodies. PF00299804 effectively inhibits both wild-type and Ins774YVMA *ERBB2* at concentrations >100 nmol/L, whereas gefitinib is ineffective. The blots were stripped and probed with total Akt as a loading control. *B,* the same retroviral constructs were used to stably infect HCC827 cells, and the resulting cell lines were treated with either gefitinib or PF00299804 as in *A*. In HCC827 ERBB2 cells, both gefitinib and PF00299804 effectively inhibit EGFR, ERBB3, and Akt phosphorylation, whereas in HCC827 ERBB2 Ins774YVMA only PF00299804 effectively inhibits their phosphorylation.

phosphorylation of ERBB2 Ins774YVMA (Fig. 4*A*), and treatment of HCC827 ERBB2 Ins774YVMA leads to effective inhibition of EGFR, ERBB3, and Akt (Fig. 4*B*) consistent with its capacity to effectively reduce cell viability at submicromolar concentrations.

Discussion

In the present study, we examine the efficacy of PF00299804, an irreversible pan-ERBB inhibitor, using *in vitro* and *in vivo* models of gefitinib sensitivity and resistance. The reversible EGFR tyrosine kinase inhibitors, gefitinib and erlotinib, have emerged as effective treatments for NSCLC patients whose tumors harbor activating mutations in *EGFR* (47, 48). However, clinical and preclinical studies suggest that these agents are not effective against all *EGFR* mutations, and in particular, the T790M resistance mutation. Thus, the development of additional agents targeting EGFR is warranted (12, 13, 22). Because many such agents are presently in clinical development, preclinical studies are necessary to determine which agents to potentially evaluate in clinical trials.

This study shows that PF00299804 is an effective agent *in vitro* and *in vivo* in tumors with *EGFR* T790M acquired resistance mutation, which has been detected in 50% of patients who clinically develop acquired resistance to gefitinib or erlotinib (12–15). This was evaluated using NSCLC cell lines harboring endogenous T790M mutations, and NSCLC cell lines and Ba/F3 cells engineered to express *EGFR* T790M in *cis* to an activating mutation. In addition, PF00299804 is highly effective in a xenograft model of T790M-mediated acquired resistance to gefitinib (Fig. 3). Our studies also suggest that PF00299804 is effective against a T790M mutation that is *cis* to either an L858R or an exon 19 deletion activating mutation (Table 3). Although not a direct comparison, PF00299804 seems more effective in H1975 and H3255 GR cells than CL-387,785 in our prior studies (23). Based on these findings, PF00299804 warrants evaluation in NSCLC patients who have

developed T790M-mediated acquired resistance to gefitinib or erlotinib.

We recently identified *MET* amplification as a second mechanism of acquired resistance to gefitinib in *EGFR* mutant NSCLC (16). In such gefitinib-resistant tumors, MET phosphorylates ERBB3 and activates PI3K/Akt signaling in an EGFR-independent manner. Thus, irreversible EGFR inhibitors alone would not be expected to effectively inhibit the growth of gefitinib-resistant *EGFR* mutant tumors that also contain a *MET* amplification. Consistent with this notion, the HCC827 gefitinib-resistant cell line with a *MET* amplification (HCC827 GR; *EGFR* del E746_A750/*MET* amplification) is significantly more resistant to PF00299804 than the HCC827 gefitinib-resistant cell line with a T790M mutation [HCC827 Del/T790M; IC_{50} > 3 μmol/L (data not shown) versus 0.06 μmol/L (Table 4), respectively]. Thus, it is possible that PF00299804 will be clinically less effective against resistant tumors that also contain a *MET* amplification although they may contain a concurrent *EGFR* T790M.

The *EGFR* mutations in exon 19 (deletion mutations) and the L858R point mutation account for 85% of all known mutations (49). In patients treated with gefitinib or erlotinib, the median time to progression and overall survival is significantly longer for patients whose tumors contain exon 19 deletions compared with those with L858R (50, 51). This may be due to a pharmacologic effect as analyses using purified intracellular EGFR kinase domain constructs show that at high ATP concentrations, erlotinib is 20-fold less effective at inhibiting L858R compared with the common exon 19 deletion (del E746_A750) mutation (52). Thus, more potent L858R inhibitors may improve overall survival and median time to progression in patients with this *EGFR* mutation. In support of this notion, PF00299804, which is more potent against the L858R mutation, has 10-fold lower IC_{50} values in a NSCLC cell line with an L858R mutation (H3255) as well as Ba/F3 cells expressing L858R (Tables 2 and 3). Thus, PF00299804 may also be clinically more effective than gefitinib in cancers harboring *EGFR* L858R mutations.

Somatic mutations in *ERBB2* have been associated with resistance to gefitinib *in vitro* and in NSCLC patients (18, 41). Consistent with those findings, this study shows that the *ERBB2* mutant, when expressed in the highly sensitive *EGFR* mutant HCC827 cell line, confers resistance to gefitinib by maintaining phosphorylation of EGFR, ERBB3, and Akt despite gefitinib treatment (Table 4; Fig. 4*B*). Of note, in the HCC827 ERBB2 cell line, the wild-type ERBB2 remains phosphorylated even in the presence of gefitinib; however, it fails to maintain ERBB3 phosphorylation in the presence of gefitinib (Fig. 4*B*). Prior studies in NSCLC cell lines have shown that down-regulation of ErbB3/PI3K/Akt signaling is necessary for gefitinib to lead to growth inhibition and apoptosis (23, 36, 53). The findings with the HCC827 *ERBB2* cell line suggest that the growth-inhibitory effects of gefitinib do not necessarily require inhibition of ERBB2. However, because many *EGFR* mutant NSCLC cell lines and tumors concurrently express ERBB2 and/or contain increased *ERBB2* copy number, ERBB2 activity may promote the tumorigenic potency of the *EGFR* mutants. In contrast to gefitinib, PF00299804 effectively inhibits growth and signaling in the HCC827 ERBB2 Ins774YVMA cell line (Fig. 4*B*; Table 4). Furthermore, PF00299804 effectively inhibits the growth of the *ERBB2* mutant (Ins G776V,C) H1781 cell line. PF00299804 is also a more potent inhibitor of wild-type *ERBB2* than gefitinib, and it more effectively inhibits the growth of NSCLC cell lines (Calu-3 and H1819) that harbor amplifications of wild-type *ERBB2* (Table 2; Fig. 4*A*). Thus, PF00299804 may also be clinically efficacious against NSCLCs harboring either *ERBB2* mutations or an amplification of wild-type *ERBB2*.

Our findings suggest that PF00299804 may be clinically effective against NSCLCs with *EGFR* or *ERBB2* mutations as well as those harboring the *EGFR* T790M mutation. As there are already therapies for the majority of *EGFR* mutant tumors, the greatest clinical need at present is to identify effective treatments for patients that develop acquired resistance to gefitinib or erlotinib. Our preclinical studies suggest that PF00299804 should be studied in clinical trials for this patient population.

Acknowledgments

Received 5/22/2007; revised 10/15/2007; accepted 10/22/2007.

Grant support: NIH grant 1RO1CA114465-01 (P.A. Jänne), National Cancer Institute grant K08CA120060-01 (J.A. Engelman), American Cancer Society grant RSG-06-102-01-CCE (P.A. Jänne and J.A. Engelman), American Society of Clinical Oncology (J.A. Engelman), AACR (J.A. Engelman), and the International Association for the Study of Lung Cancer (J.A. Engelman).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

P.A. Jänne and M. Meyerson are part of a pending patent application on *EGFR* mutations.

References

1. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353:123–32.
2. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 2003;21:2237–46.
3. Kris MG, Natale RB, Herbst RS, et al. Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA 2003;290:2149–58.
4. Perez-Soler R, Chachoua A, Hammond LA, et al. Determinants of tumor response and survival with erlotinib in patients with non-small-cell lung cancer. J Clin Oncol 2004;22:3238–47.
5. 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.
6. 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.
7. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306–11.
8. Mitsudomi T, Kosaka T, Endoh H, et al. Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small-cell lung cancer with postoperative recurrence. J Clin Oncol 2005;23:2513–20.
9. Han SW, Kim TY, Hwang PG, et al. Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small-cell lung cancer patients treated with gefitinib. J Clin Oncol 2005;23:2493–501.
10. Takano T, Ohe Y, Sakamoto H, et al. Epidermal growth factor receptor gene mutations and increased copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer. J Clin Oncol 2005;23:6829–37.
11. Cappuzzo F, Hirsch FR, Rossi E, et al. Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small-cell lung cancer. J Natl Cancer Inst 2005;97:643–55.
12. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
13. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:1–11.
14. Balak MN, Gong Y, Riely GJ, et al. Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. Clin Cancer Res 2006;12:6494–501.
15. Kosaka T, Yatabe Y, Endoh H, et al. Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and

acquired resistance to gefitinib. Clin Cancer Res 2006;12:5764–9.
16. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
17. Pao W, Wang TY, Riely GJ, et al. KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med 2005;2:e17.
18. Cappuzzo F, Ligorio C, Janne PA, et al. Prospective study of gefitinib in epidermal growth factor receptor fluorescence *in situ* hybridization-positive/phospho-Akt-positive or never smoker patients with advanced non-small-cell lung cancer: the ONCOBELL trial. J Clin Oncol 2007;25:2248–55.
19. Sasaki H, Endo K, Takada M, et al. EGFR exon 20 insertion mutation in Japanese lung cancer. Lung Cancer. Epub 2007 Aug 6.
20. Stephens P, Hunter C, Bignell G, et al. Lung cancer: intragenic ERBB2 kinase mutations in tumours. Nature 2004;431:525–6.
21. Shimamura T, Ji H, Minami Y, et al. Non-small-cell lung cancer and Ba/F3 transformed cells harboring the ERBB2 G776insV_G/C mutation are sensitive to the dual-specific epidermal growth factor receptor and ERBB2 inhibitor HKI-272. Cancer Res 2006;66:6487–91.
22. Greulich H, Chen TH, Feng W, et al. Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants. PLoS Med 2005;2:e313.
23. Engelman JA, Mukohara T, Zejnullahu K, et al. Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. J Clin Invest 2006;116:2695–706.
24. Kwak EL, Sordella R, Bell DW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci U S A 2005;102:7665–70.
25. Schellens JH, Britten CD, camidge DR, et al. First-in-human study of the safety, tolerability, pharmacokinetics (PK), and pharmacodynamics (PD) of PF-00299804, a small molecule irreversible pan-HER inhibitor in patients with advanced cancer. ASCO Annual Meeting Proceedings 2007. J Clin Oncol 2007;19S:Abstract 3599.
26. Fujishita T, Loda M, Turner RE, et al. Sensitivity of non-small-cell lung cancer cell lines established from patients treated with prolonged infusions of Paclitaxel. Oncology 2003;64:399–406.
27. Tracy S, Mukohara T, Hansen M, et al. Gefitinib induces apoptosis in the EGFRL858R non-small-cell lung cancer cell line H3255. Cancer Res 2004;64:7241–4.
28. 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.
29. Mukohara T, Engelman JA, Hanna NH, et al. Differential effects of gefitinib and cetuximab on non-small-cell lung cancers bearing epidermal growth factor receptor mutations. J Natl Cancer Inst 2005;97:1185–94.
30. Helfrich BA, Raben D, Varella-Garcia M, et al. Antitumor activity of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefitinib (ZD1839, Iressa) in non-small cell lung cancer cell lines correlates with gene copy number and EGFR mutations but not EGFR protein levels. Clin Cancer Res 2006;12:7117–25.
31. Cohen BD, Goldstein DJ, Rutledge L, et al. Transformation-specific interaction of the bovine papillomavirus E5 oncoprotein with the platelet-derived growth factor receptor transmembrane domain and the epidermal growth factor receptor cytoplasmic domain. J Virol 1993;67:5303–11.
32. Klutchko SR, Zhou H, Winters RT, et al. Tyrosine kinase inhibitors. 19. 6-Alkynamides of 4-anilinoquinazolines and 4-anilinopyrido[3,4-*d*]pyrimidines as irreversible inhibitors of the erbB family of tyrosine kinase receptors. J Med Chem 2006;49:1475–85.
33. Changelian PS, Flanagan ME, Ball DJ, et al. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. Science 2003;302:875–8.
34. Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. Biochem J 2003;371:199–204.
35. Fry DW, Harvey PJ, Keller PR, et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. Mol Cancer Ther 2004;3:1427–38.
36. 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.
37. Zhao JJ, Gjoerup OV, Subramanian RR, et al. Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. Cancer Cell 2003;3:483–95.
38. Yuza Y, Glatt KA, Jiang J, et al. Allele-dependent variation in the relative cellular potency of distinct egfr inhibitors. Cancer Biol Ther 2007;6.
39. Jiang J, Greulich H, Janne PA, et al. Epidermal growth factor-independent transformation of Ba/F3 cells with cancer-derived epidermal growth factor receptor mutants induces gefitinib-sensitive cell cycle progression. Cancer Res 2005;65:8968–74.
40. Sirotnak FM, Zakowski MF, Miller VA, Scher HI, Kris MG. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. Clin Cancer Res 2000;6:4885–92.
41. Wang SE, Narasanna A, Perez-Torres M, et al. HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. Cancer Cell 2006;10:25–38.
42. Zhao X, Weir BA, LaFramboise T, et al. Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. Cancer Res 2005;65:5561–70.
43. Kasprzyk PG, Sullivan TL, Hunt JD, et al. Activity of anti-erbB-2 recombinant toxin OLX-209 on lung cancer cell lines in the absence of erbB-2 gene amplification. Clin Cancer Res 1996;2:75–80.
44. 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.
45. Li D, Shimamura T, Ji H, et al. Bronchial and peripheral murine lung carcinomas induced by T790M-L858R mutant EGFR respond to HKI-272 and rapamycin combination therapy. Cancer Cell 2007;12:81–93.
46. Albanell J, Rojo F, Averbuch S, et al. Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. J Clin Oncol 2002;20:110–24.
47. Inoue A, Suzuki T, Fukuhara T, et al. Prospective phase II study of gefitinib for chemotherapy-naive patients with advanced non-small-cell lung cancer with epidermal growth factor receptor gene mutations. J Clin Oncol 2006;24:3340–6.
48. Paz-Ares L, Sanchez JM, García-Velasco A, et al. A prospective phase II trial of erlotinib in advanced non-small cell lung cancer (NSCLC) patients (p) with mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR). J Clin Oncol 2006;24:Abstract 7020.
49. Janne PA, Engelman JA, Johnson BE. Epidermal growth factor receptor mutations in non-small-cell lung cancer: implications for treatment and tumor biology. J Clin Oncol 2005;23:3227–34.
50. Jackman DM, Yeap BY, Sequist LV, et al. Exon 19 deletion mutations of epidermal growth factor receptor are associated with prolonged survival in non-small cell lung cancer patients treated with gefitinib or erlotinib. Clin Cancer Res 2006;12:3908–14.
51. Riely GJ, Pao W, Pham D, et al. Clinical course of patients with non-small cell lung cancer and epidermal growth factor receptor exon 19 and exon 21 mutations treated with gefitinib or erlotinib. Clin Cancer Res 2006;12:839–44.
52. Carey KD, Garton AJ, Romero MS, et al. Kinetic analysis of epidermal growth factor receptor somatic mutant proteins shows increased sensitivity to the epidermal growth factor receptor tyrosine kinase inhibitor, erlotinib. Cancer Res 2006;66:8163–71.
53. Bianco R, Shin I, Ritter CA, et al. Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. Oncogene 2003;22:2812–22.