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

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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 \textit{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 \textit{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 \textit{EGFR}-activating mutations as well as the \textit{EGFR} T790M resistance mutation both \textit{in vitro} and \textit{in vivo}. Additionally, PF00299804 is a highly effective inhibitor of both the wild-type \textit{ERBB2} and the gefitinib-resistant oncogenic \textit{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 \textit{EGFR} seem to be particularly sensitive to EGFR tyrosine kinase inhibitors both \textit{in vitro} and in NSCLC patients (5–11). However, despite the benefits of gefitinib or erlotinib in \textit{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 \textit{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 \textit{MET} proto-oncogene (16).

A significant portion of patients initially treated with gefitinib or erlotinib do not benefit from these agents (i.e., \textit{de novo} resistance). Somatic mutations in \textit{K-ras} are usually mutually exclusive with \textit{EGFR} mutations and have been associated with \textit{de novo} resistance to gefitinib and erlotinib (17). In addition, patients with cancers harboring \textit{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 \textit{ERBB2} mutations are also resistant to gefitinib and erlotinib (20, 21). Furthermore, \textit{in vitro} studies have shown that \textit{EGFR} exon 20 mutations, which account for 1% to 3% of all \textit{EGFR} mutations, are also resistant to the effects of gefitinib and erlotinib (22). Thus, identification of strategies and/or agents capable of overcoming acquired \textit{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 \textit{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 BBW2992 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...
Table 1. Efficacy comparison of PF00299804, gefitinib, erlotinib, and CI-1033

<table>
<thead>
<tr>
<th>Isolated enzyme assay</th>
<th>EGFR</th>
<th>ERBB2</th>
<th>ERBB4</th>
<th>Cellular EGFR (NIH3T3/EGFR)</th>
<th>Cellular ERBB2 (NIH3T3/ERBB2)</th>
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<tbody>
<tr>
<td></td>
<td>IC_{50}</td>
<td>IC_{50}</td>
<td>IC_{50}</td>
<td>IC_{50}</td>
<td>IC_{50}</td>
</tr>
<tr>
<td>PF-00299804</td>
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<td>45.7</td>
<td>73.7</td>
<td>5.8</td>
<td>41</td>
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<tr>
<td>Gefitinib</td>
<td>3.1</td>
<td>343</td>
<td>476</td>
<td>14.4</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>0.56</td>
<td>512</td>
<td>790</td>
<td>19.3</td>
<td>299</td>
</tr>
<tr>
<td>CI-1033</td>
<td>1.8</td>
<td>11</td>
<td>27</td>
<td>1.6</td>
<td>16</td>
</tr>
</tbody>
</table>

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.
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\times10^6 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: \( \text{length} \times \text{width}^2 \times 0.52 \). 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 quinazoline-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 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\( \mu \)mol/L) and likely reflects off-target effects. In cell lines wild-type for both EGFR and K-ras (H322, H1819, and HCC827 Del E746_A750), gefitinib IC\(_{50}\) was 0.008 \( \mu \)mol/L and PF00299804 IC\(_{50}\) was 0.029 \( \mu \)mol/L. In cell lines wild-type for both EGFR and K-ras (H1975, H820 Del 1747_1751, Ins S/T790M), gefitinib IC\(_{50}\) was 0.42 \( \mu \)mol/L and PF00299804 IC\(_{50}\) was 0.063 \( \mu \)mol/L. In general, PF00299804 was 10-100 fold more effective than gefitinib in these in vitro models.

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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR mutation</th>
<th>ERBB2 mutation</th>
<th>K-ras mutation</th>
<th>Gefitinib IC(_{50}) (( \mu )mol/L)</th>
<th>PF00299804 IC(_{50}) (( \mu )mol/L)</th>
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<tr>
<td>A549</td>
<td>WT</td>
<td>WT</td>
<td>G12S</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>H441</td>
<td>WT</td>
<td>WT</td>
<td>G12V</td>
<td>&gt;10</td>
<td>4</td>
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<tr>
<td>H322</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Calu-3</td>
<td>WT</td>
<td>WT (amplified)</td>
<td>WT</td>
<td>1.4</td>
<td>0.063</td>
</tr>
<tr>
<td>H1819</td>
<td>WT</td>
<td>WT (amplified)</td>
<td>WT</td>
<td>0.42</td>
<td>0.029</td>
</tr>
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<td>H3255</td>
<td>L858R</td>
<td>WT</td>
<td>WT</td>
<td>0.075</td>
<td>0.007</td>
</tr>
<tr>
<td>H3255 GR</td>
<td>L858R/T790M</td>
<td>WT</td>
<td>WT</td>
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<td>0.119</td>
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<tr>
<td>H1975</td>
<td>L858R/T790M</td>
<td>WT</td>
<td>WT</td>
<td>&gt;10</td>
<td>0.44</td>
</tr>
<tr>
<td>H820</td>
<td>Del 1747_1751, Ins S/T790M</td>
<td>WT</td>
<td>WT</td>
<td>&gt;10</td>
<td>0.93</td>
</tr>
<tr>
<td>HCC 827</td>
<td>Del E746_A750</td>
<td>WT</td>
<td>WT</td>
<td>0.008</td>
<td>0.002</td>
</tr>
<tr>
<td>HCC 4006</td>
<td>Del L747_E749</td>
<td>WT</td>
<td>WT</td>
<td>0.050</td>
<td>0.004</td>
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<tr>
<td>PC-9</td>
<td>Del E746_A750</td>
<td>WT</td>
<td>WT</td>
<td>0.023</td>
<td>0.002</td>
</tr>
<tr>
<td>H1781</td>
<td>WT</td>
<td>Ins G776V, C</td>
<td>WT</td>
<td>&gt;10</td>
<td>0.275</td>
</tr>
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</table>

Abbreviation: WT, wild-type.

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

<table>
<thead>
<tr>
<th>EGFR-activating mutations</th>
<th>Gefitinib IC(_{50}) (( \mu )mol/L)</th>
<th>PF00299804 IC(_{50}) (( \mu )mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Del E746_A750</td>
<td>4.8 nmol/L</td>
<td>&lt;1 nmol/L</td>
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<tr>
<td>Del S752_I759</td>
<td>35 nmol/L</td>
<td>2.0 nmol/L</td>
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<td>Del L747_A750InsP</td>
<td>7.4 nmol/L</td>
<td>1.6 nmol/L</td>
</tr>
<tr>
<td>Del L747_P753InsS</td>
<td>4.1 nmol/L</td>
<td>1.9 nmol/L</td>
</tr>
<tr>
<td>Del E746_S752InsV</td>
<td>306 nmol/L</td>
<td>1.4 nmol/L</td>
</tr>
<tr>
<td>L858R</td>
<td>26 nmol/L</td>
<td>2.6 nmol/L</td>
</tr>
<tr>
<td>A767_V769duspASV</td>
<td>3.1 nmol/L</td>
<td>230 nmol/L</td>
</tr>
<tr>
<td>EGFR vIII</td>
<td>2.7 \mu mol/L</td>
<td>1.2 \mu mol/L</td>
</tr>
<tr>
<td>Del E746_A750/T790M</td>
<td>8.3 \mu mol/L</td>
<td>140 \mu mol/L</td>
</tr>
<tr>
<td>Del S752_I759/T790M</td>
<td>&gt;10 \mu mol/L</td>
<td>330 \mu mol/L</td>
</tr>
<tr>
<td>Del L747_A750nsP/T790M</td>
<td>&gt;10 \mu mol/L</td>
<td>240 \mu mol/L</td>
</tr>
<tr>
<td>Del L747_P753InsS/T790M</td>
<td>&gt;10 \mu mol/L</td>
<td>160 \mu mol/L</td>
</tr>
<tr>
<td>Del E746_S752InsV/T790M</td>
<td>&gt;10 \mu mol/L</td>
<td>270 \mu mol/L</td>
</tr>
<tr>
<td>L858R/T790M</td>
<td>&gt;10 \mu mol/L</td>
<td>300 \mu mol/L</td>
</tr>
</tbody>
</table>

Abbreviation: WT, wild-type.
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 EGFR 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. 1A). 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. 1B).

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. 2A, 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 I nmol/L PF299804, whereas 100 nmol/L or greater was required to inhibit EGFR WT/T790M or Del/T790M (Fig. 2A).

**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
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. 2B). This suggests that, at similar concentrations, PF00299804 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. 2C; 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
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. 4A, 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. 4B). Intriguingly, this occurs despite persistent ERBB2 phosphorylation even in the presence of gefitinib (Fig. 4B). 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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR mutation</th>
<th>Expression construct</th>
<th>Gefitinib IC50 (μmol/L)</th>
<th>PF00299804 IC50 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3255 GFP</td>
<td>L858R</td>
<td>GFP</td>
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<td>0.007</td>
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<td>EGFR WT/T790M</td>
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<td>0.137</td>
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<td>EGFR L858R/T790M</td>
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<tr>
<td>H3255 Del/T790M</td>
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<td>EGFR Del L747_S752,P753S/T790M</td>
<td>&gt;10</td>
<td>1.57</td>
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<td>Del E746_A750</td>
<td>GFP</td>
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<td>ERBB2 Ins774YVMA</td>
<td>4.82</td>
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**Table 4.** Comparison of gefitinib and PF00299804 efficacy in EGFR mutant NSCLC cell lines engineered to express different EGFR or ERBB2 constructs

**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).
phosphorylation of ERBB2 Ins774YVMA (Fig. 4A), and treatment of HCC827 ERBB2 Ins774YVMA leads to effective inhibition of EGFR, ERBB3, and Akt (Fig. 4B) 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; IC50 > 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 IC50 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. 4B). 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. 4B). 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. 4B; Table 4). Furthermore, PF00299804 also effectively inhibits the growth of the ERBB2 mutant (Ins G776VC) 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. 4A). 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 RO1CA114665-01 (P.A. Jänne), National Cancer Institute grant K08CA12080-01 (J.A. Engelman), American Cancer Society grant BSG-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).

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

References

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