Dacomitinib (PF-00299804), an Irreversible Pan-HER Inhibitor, Inhibits Proliferation of HER2-Amplified Breast Cancer Cell Lines Resistant to Trastuzumab and Lapatinib

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Abstract
The human EGF (HER) family of receptors has been pursued as therapeutic targets in breast cancer and other malignancies. Trastuzumab and lapatinib are standard treatments for HER2-amplified breast cancer, but a significant number of patients do not respond or develop resistance to these drugs. Here we evaluate the in vitro activity of dacomitinib (PF-00299804), an irreversible small molecule pan-HER inhibitor, in a large panel of human breast cancer cell lines with variable expression of the HER family receptors and ligands, and with variable sensitivity to trastuzumab and lapatinib. Forty-seven human breast cancer and immortalized breast epithelial lines representing the known molecular subgroups of breast cancer were treated with dacomitinib to determine IC50 values. HER2-amplified lines were far more likely to respond to dacomitinib than nonamplified lines (RR, 3.39; P < 0.0001). Furthermore, HER2 mRNA and protein expression were quantitatively associated with response. Dacomitinib reduced the phosphorylation of HER2, EGFR, HER4, AKT, and ERK in the majority of sensitive lines. Dacomitinib exerted its antiproliferative effect through a combined G0–G1 arrest and an induction of apoptosis. Dacomitinib inhibited growth in several HER2-amplified lines with de novo and acquired resistance to trastuzumab. Dacomitinib maintained a high activity in lines with acquired resistance to lapatinib. This study identifies HER2-amplified breast cancer lines as most sensitive to the antiproliferative effect of dacomitinib and provides a strong rationale for its clinical testing in HER2-amplified breast cancers resistant to trastuzumab and lapatinib. Mol Cancer Ther; 11(9); 1978–87. ©2012 AACR.

Introduction
The HER (ErbB) receptor family consists of 4 type I receptor tyrosine kinases, which include the EGF receptor (EGFR, HER1, and ErbB-1), HER2 (HER2/neu, ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4), and their associated ligands, including EGF, neuregulins (NRG1-4), TGF-α, amphiregulin, betacellulin, heparin-binding EGF-like growth factor and epiregulin. Ligand binding to the extracellular domain leads to homodimerization or heterodimerization of the receptors and autophosphorylation within the intracellular domain. This results in the activation of signaling cascades involved in mediating cell growth and differentiation. Unlike other family members, HER2 has no known ligand and, under normal conditions, relies on forming heterodimers with other family members that have ligand interactions. In cancer, this tight regulation of HER family signaling is disrupted and contributes to transformation (1).

HER2 amplification occurs in 20% to 25% of patients with breast cancer. It is associated with a poor prognosis and is a validated target for therapy (2, 3). Trastuzumab (Herceptin; Genentech), a humanized monoclonal antibody that binds to the extracellular domain of HER2, has been shown to improve survival in the metastatic and adjuvant settings (4–11), and lapatinib (Tykerb; GlaxoSmithKline), a small-molecule selective inhibitor of the HER2 and EGFR tyrosine kinases, is approved for HER2-positive advanced breast cancer that progressed after trastuzumab-based therapy (12).

Pan-HER inhibitors were developed with the goal of improving therapeutic response and overcoming drug resistance that is seen with trastuzumab and lapatinib. Unlike lapatinib, which is a reversible inhibitor that competes with ATP at the binding sites (13), most pan-HER inhibitors bind to the kinases in a covalent and irreversible form (14, 15). It is hypothesized that the prolonged suppression of multiple targets within the HER receptor

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family may overcome the problems with adaptability and redundancy in signaling pathways.

Dacomitinib (PF-00299804; chemical structure in Fig. 1) is a second-generation irreversible pan-HER receptor tyrosine kinase inhibitor (selectively inhibiting EGFR, HER2, and HER4) under clinical development (16, 17). Dacomitinib showed marked activity in several xenograft models with variable levels of HER family receptors, including lung tumors resistant to EGFR small molecule tyrosine kinase inhibitors (16, 18). The primary aim of this study was to describe the in vitro activity of dacomitinib in a panel of 47 human breast cancer and immortalized breast cell lines, to identify potential biomarkers of response and/or resistance to guide clinical development.

Materials and Methods

Cell lines, cell culture, and reagents

The cell line panel included 44 breast cancer lines and 3 immortalized breast epithelial cell lines representing the known molecular subgroups of breast cancer and has been described in detail previously (19). The panel included MDA-MB-415, MDA-MB-134, HCC-1500, ZR-75-30, HCC-202, HCC-1419, HCC-38, HCC-70, HCC-1187, HCC-1806, HCC-1937, HCC-1954, MDA-MB-436, HCC-1569, Hs578T, HCC-1143, MDA-MB-175, BT-474, SK-BR-3, MDA-MB-361, UACC-893, UACC-812, UACC-732, T-47D, MDA-MB-453, MDA-MB-468, CAMA-1, MDA-MB-157, MCF-7, MDA-MB-435, ZR-75-1, BT-20, MDA-MB-231, BT-549, DU4475, HCC-1395, HCC-2218, 184A1, 184B5, and MCF-10A that were purchased from American Type Culture Collection. The cell lines EFM-192A, KPL-1, COLO-824, and CAL-51 were obtained from the German Tissue Repository DSMZ. Both cell line banks were authenticated and checked for contamination using a multiplex PCR method (20), and mitochondrial DNA from the cells was sequenced to detect Mycoplasma contamination as described above.

The cell line panel included 44 breast cancer lines and 3 immortalized breast epithelial cell lines representing the known molecular subgroups of breast cancer and has been described in detail previously (19). The panel included MDA-MB-415, MDA-MB-134, HCC-1500, ZR-75-30, HCC-202, HCC-1419, HCC-38, HCC-70, HCC-1187, HCC-1806, HCC-1937, HCC-1954, MDA-MB-436, HCC-1569, Hs578T, HCC-1143, MDA-MB-175, BT-474, SK-BR-3, MDA-MB-361, UACC-893, UACC-812, UACC-732, T-47D, MDA-MB-453, MDA-MB-468, CAMA-1, MDA-MB-157, MCF-7, MDA-MB-435, ZR-75-1, BT-20, MDA-MB-231, BT-549, DU4475, HCC-1395, HCC-2218, 184A1, 184B5, and MCF-10A that were purchased from American Type Culture Collection. The cell lines EFM-192A, KPL-1, COLO-824, and CAL-51 were obtained from the German Tissue Repository DSMZ. Both cell line banks carried out the cell lines authentication by short tandem repeat analysis. The cell lines SUM-190 and SUM-225 were obtained from the University of Michigan (Ann Arbor, MI). Upon receipt, all cell lines were assessed for Mycoplasma contamination using a multiplex PCR method (20), and mitochondrial DNA from the cells was sequenced to confirm their correct identity (21). Cell lines were then expanded and these procedures were repeated for all cell lines before cryopreservation. All cell lines were passaged for less than 6 months before use in this study. Trastuzumab-resistant BT-474 (BT-474-TR) and SK-BR3 (SK-BR-3-TR) cell lines (pools of resistant cells) were established after serial passage in the presence of gradually increasing concentrations of lapatinib (0.1–7 μmol/L). These lines were established in our laboratories, authenticated, and checked for Mycoplasma contamination as described above.

HER2 amplification was defined as greater than 2 HER2/neu FISH signals per chromosome 17 centromere FISH signal (22). A SpectrumOrange labeled HER2/neu probe (ABBOTT Molecular) and SpectrumGreen labeled chromosome 17 alpha-satellite centromere probe were used (ABBOTT Molecular).

Proliferation assays

Cells were seeded in duplicate at 5 × 10³ to 5 × 10⁴ cells per well in 24-well plates, and growth inhibition data was calculated as described previously (19). Briefly, day after plating, dacomitinib was added at 10 μmol/L and 2-fold dilutions over 12 concentrations were carried out to generate a dose–response curve. Control wells without the drug were also seeded. The cells were counted on day 1 when the drug was added, as well as after 6 days when the experiment ended. After the trypsinization cells were placed in an Isotone solution and immediately counted using a Coulter Z1 particle counter (Beckman Coulter, Inc.). The suspension cultures were counted using a Coulter Vi-Cell counter (Beckman Coulter, Inc.).

Microarray analysis of cell lines

Agilent microarray analyses were developed for each cell line as described previously (23, 24). These data are available with GEO accession number GSE18496.

Western blots and protein quantification

To determine the effect of dacomitinib on the expression of analyzed proteins, cells in log-phase growth were treated with 0.1 or 1 μmol/L dacomitinib and lysates were taken as described (19). Details about antibodies and immunoprecipitation techniques can be found in Supplementary Material.

Cell-cycle analysis and apoptosis studies

The effects of dacomitinib on the cell cycle were assessed using Nim-DAPI (4′, 6-diamidino-2-phenylindole) staining (NPE Systems). The cells were plated...
evenly in control and experimental wells, allowed to grow to log phase, and then treated with 100 nmol/L or 1 μmol/L dacomitinib for 48 hours or 5 days and analyzed as described previously (19).

For apoptosis, cells were plated evenly in control and experimental wells, allowed to grow to log phase, and then treated with 100 nmol/L or 1 μmol/L dacomitinib for 48 hours or 5 days and analyzed using Annexin-V–fluorescein isothiocyanate (FITC) as described (19).

Statistical analysis
Cochran–Mantel–Haenszel (CMH) χ² analysis was carried out using the PROC FREQ function in SAS for Windows version 9.2 (SAS Institute, Inc.). Simple linear regression was also carried out in SAS using PROC REG function.

Pearson correlation coefficients and their corresponding P values were calculated using the PROC CORR function in SAS. Graphs were created in Microsoft Excel.

Results
Dacomitinib preferentially inhibits growth of HER-2-amplified breast cancer cell lines in vitro
A panel of 44 human breast cancer cell lines, representing luminal, nonluminal subtypes, and 3 immortalized breast epithelial lines (19), was used to test the antiproliferative effect of dacomitinib (PF-00299804). The calculated IC₅₀ values for each cell line and its molecular classification, as well as HER2 amplification status (by FISH) and estrogen receptor (ER) status, were determined (Table 1 and Fig. 2). Sensitivity was defined as IC₅₀ < 1 μmol/L (the rationale for selecting this cut-off is discussed below).

As a group, the HER2-amplified cell lines were most sensitive to growth inhibition by dacomitinib (IC₅₀ < 1 μmol/L in 14 of 16 lines; 87.5%) as compared with 5 of 28 (17.9%) of HER2-nonamplified lines (excluding immortalized lines). MDA-MB-453 and UACC-732 were the only HER2-amplified lines resistant to the antiproliferative effect of the compound (IC₅₀ > 1 μmol/L). The nonluminal lines were the most resistant to this compound. χ² (CMH) analysis was carried out to compare the response classification (response defined as IC₅₀ < 1 μmol/L) in the HER2-amplified versus nonamplified groups of cell lines. HER2-amplified lines were far more likely to be classified as responders to dacomitinib [RR, 3.39; 95% confidence interval (CI), 1.82–6.33; P < 0.0001].

ER status correlated with response when treated as a separate, independent variable (P = 0.015). However, ER status was highly cross-correlated with HER2 status. After controlling for HER2 status by stratified analysis, ER status was no longer a statistically significant predictor of response (P = 0.17).

Quantitative HER2 expression associates with response to dacomitinib
We also aimed to explore the quantitative effect of HER2 expression on response to dacomitinib, in comparison with the qualitative amplified/nonamplified approach discussed above. Specifically, we wanted to determine whether a higher relative expression of HER2 mRNA is associated with a stronger response to dacomitinib, even within the HER2-amplified and nonamplified groups. For this purpose, we ran a linear regression analysis using log(IC₅₀) as the response variable and log-ratio of expression by microarray (compared with mixed breast cancer RNA reference pool) as the predictor variable. This analysis was carried out first on the entire panel of lines, then separately on each group of HER2-amplified and nonamplified lines. The association was significant in all 3 analyses. (Full panel: β = -1.07, SE = 0.15, P < 0.0001, r = -0.73; HER2 amplified: β = -1.6, SE = 0.57, P = 0.014, r = -0.6; HER2-nonamplified: β = -1.57, SE = 0.6, P = 0.014, r = -0.44; Supplementary Fig. S1).

However, the significance of this relationship in cell lines with higher IC₅₀ values (resistant) is unknown. In a separate analysis for HER2-amplified lines, we found that the total HER2 protein levels (quantified by Western blot) correlated with HER2 mRNA levels by microarray (r = 0.67, P = 0.004). Finally, we found an association between the HER2 protein levels and response to dacomitinib in HER2-amplified lines (β = -1.99, SE = 0.35, P = 0.003, r = -0.69; Supplementary Fig. S2).

All 4 HER family receptors were analyzed for their relationship between mRNA expression and IC₅₀. The association between HER2 expression and response to dacomitinib was by far the strongest. In addition, there was a marginal inverse correlation between HER3 mRNA levels and log(IC₅₀) values (r = -0.25, P = 0.09).

No correlation was observed between the response to dacomitinib and EGFR mRNA (r = -0.07, P = 0.62) and HER4 mRNA (r = 0.05, P = 0.75) levels, respectively (Supplementary Fig. S3).

Biochemical effects of dacomitinib on signal transduction
The effects of dacomitinib on HER2, EGFR, HER4, AKT, and ERK activation were determined using Western blot analysis of a subset of lines with variable levels of HER receptors and sensitivities to dacomitinib.

After a 10-minute exposure to 100 nmol/L or 1 μmol/L of dacomitinib, we observed no effect on total HER2, AKT, and ERK in a majority of lines and a minor decrease in total EGFR in several lines. Most of the selected lines expressed very low baseline levels of total HER4 that mostly did not change after the treatment with dacomitinib. Dacomitinib caused an inhibition of HER2 phosphorylation in all lines with detectable baseline phosphorylation (Fig. 3, densitometry in Supplementary Table S1). Dacomitinib caused an inhibition of EGFR phosphorylation in sensitive lines, but only 2 resistant lines (MDA-MB-453 and MDA-MB-231). Dacomitinib decreased the phosphorylation of HER4 in sensitive lines with detectable baseline phosphorylation. Dacomitinib inhibited phosphorylation of AKT in all tested sensitive lines, but only 2 resistant lines (HCC-70 and MDA-MB-453). Similarly, dacomitinib phosphorylation.
Table 1. The calculated IC$_{50}$ for each cell line and its molecular classification

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC$_{50}$ value ($\mu$mol/L)</th>
<th>IC$_{50}$ SE</th>
<th>Breast cancer subtype</th>
<th>HER2 status</th>
<th>ER status</th>
</tr>
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<tr>
<td>HCC-202</td>
<td>&lt;0.005</td>
<td>n/a</td>
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<td>MDA-MB-175</td>
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<td>HCC-2218</td>
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<td>SUM-225</td>
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<td>HCC-1419</td>
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<td>0.002</td>
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<td>18A1</td>
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<td>0.0003</td>
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<td>Negative</td>
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<tr>
<td>MCF-10A</td>
<td>0.008</td>
<td>0.001</td>
<td>Immortalized</td>
<td>n/a</td>
<td>Negative</td>
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<td>SK-BR-3</td>
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<td>EFM-192A</td>
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<td>UACC-893</td>
<td>0.017</td>
<td>0.006</td>
<td>Luminal</td>
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<td>Positive</td>
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<td>BT-474</td>
<td>0.018</td>
<td>0.011</td>
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<td>MDA-MB-361</td>
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<td>0.01</td>
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<td>UACC-812</td>
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<td>0.00</td>
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<td>Positive</td>
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<td>HCC-1954</td>
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<td>0.01</td>
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<tr>
<td>184B5</td>
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<td>0.00</td>
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<td>SUM-190</td>
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<td>ZR-75-1</td>
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<td>0.30</td>
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<td>0.07</td>
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<td>0.05</td>
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<td>HCC-1569</td>
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<td>0.17</td>
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<tr>
<td>EFM-19</td>
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<td>COLO-824</td>
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<td>MDA-MB-157</td>
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<td>T-47D</td>
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<td>0.69</td>
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<td>MDA-MB-468</td>
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<td>0.03</td>
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<td>HCC-70</td>
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<td>HCC-1187</td>
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<tr>
<td>UACC-732</td>
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<td>0.09</td>
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<td>MDA-MB-134</td>
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<td>0.17</td>
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<tr>
<td>HCC-1395</td>
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<td>0.64</td>
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</tr>
<tr>
<td>MDA-MB-453</td>
<td>2.00</td>
<td>0.11</td>
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<td>HCC-1937</td>
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<td>MDA-MB-436</td>
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<td>BT-20</td>
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<td>CAMA-1</td>
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<td>CAL-51</td>
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<td>MDA-MB-435</td>
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<td>Hs578T</td>
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<td>MCF-7</td>
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<td>0.35</td>
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<td>MDA-MB-231</td>
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<td>KPL-1</td>
<td>4.88</td>
<td>0.57</td>
<td>Luminal</td>
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</table>

NOTE: Included are molecular subtype and HER2 and ER status. HER2-amplified cell lines were most sensitive to the growth inhibition effects of dacomitinib. Post-EMT, cell lines classified as representing breast cancers that had undergone an epithelial-to-mesenchymal transition.

Abbreviation: n/a, not applicable.
caused a decrease in phosphorylation of ERK in all lines, except for resistant MDA-MB-231.

Effects of dacomitinib on cell cycle and apoptosis

The effects of dacomitinib on the cell cycle were analyzed in a subset of both sensitive and resistant cell lines. Cells were exposed to dacomitinib at 1 μmol/L for 48 hours, and then flow cytometry was carried out using Nim-DAPI staining. We observed a significant G0–G1 cell-cycle arrest in the very sensitive HER2-amplified BT-474 and SK-BR-3 lines and less pronounced yet statistically significant G0–G1 cell-cycle arrest in the resistant HER2-normal MCF-7 line. Dacomitinib caused no changes in cell cycle in the less sensitive ZR-75-1 and HCC-1143 lines and in the resistant MDA-MB-231 line (Fig. 4).

Similarly, the effect of dacomitinib on apoptosis was determined in the same subset of lines. For this assay, cells were exposed to 1 μmol/L of dacomitinib for 5 days and then analyzed with a dual stain flow cytometry protocol using Annexin-V–FITC and propidium iodide. A significant increase in apoptosis was seen in the very sensitive HER2-amplified cell lines with IC50 values of 0.031 and 0.039 μmol/L, respectively.

Together, these data suggested that the antiproliferative effect of dacomitinib is mediated by both inhibition of the cell cycle and the induction of apoptosis.

Dacomitinib overcomes acquired resistance to trastuzumab and lapatinib in vitro

BT-474-trastuzumab–resistant (BT-474-TR) and SK-BR-3-trastuzumab–resistant (SK-BR-3-TR) cell lines were established in our laboratory after prolonged exposure of the parental cell lines in medium with 105 μg/mL trastuzumab over several months (25). Despite resistance to trastuzumab, dacomitinib inhibited the proliferation of both these cell lines with a similar potency as their parental cell lines (Table 2). Although the exact mechanism of trastuzumab resistance in these lines is unknown, they clearly maintain dependence on HER signaling.

BT-474-lapatinib–resistant (BT-474-LR) and SK-BR-3-lapatinib–resistant (SK-BR-3-LR) cell lines were developed in our laboratory after prolonged exposure of the parental cell lines in medium with 105 μg/mL lapatinib over several months. Unlike the trastuzumab-resistant cell lines, the sensitivity of these lines to dacomitinib was less than their parental counterparts. For the BT-474-LR, there was a 1.7-fold increase in IC50, and for the SK-BR-3-LR, there was a 23-fold increase in IC50 (Table 2). However, the decrease in sensitivity to dacomitinib in the lapatinib-resistant lines compared with their parental lines was much less pronounced than their decrease in sensitivity to lapatinib. Specifically, there is an 89-fold and 119-fold increase in lapatinib IC50 for BT-474-LR and SK-BR-3-LR compared with respective parental cell lines, and both lapatinib-resistant cell lines would still be considered sensitive to dacomitinib with low IC50 values of 0.031 and 0.339 μmol/L, respectively.

These data indicated that, in the HER2-amplified cell lines, dacomitinib overcomes the acquired resistance to...
The effects of dacomitinib on apoptosis in trastuzumab-resistant lines was stronger than in the parental lines. In lapatinib-resistant lines, dacomitinib at 100 nmol/L did not induce apoptosis, and the effect at 1 μmol/L was less pronounced than in the parental lines (Supplementary Fig. S6).

In addition, the effect of dacomitinib on the phosphorylation of HER2, EGFR, HER4, AKT, and ERK in the acquired resistant lines was analyzed by Western blots (Supplementary Fig. S7, densitometry in Supplementary Table S2). Dacomitinib decreased the phosphorylation of HER2 in all sensitive lines. Dacomitinib caused a decrease in phosphorylation of ERK in all lines, except for resistant MDA-MB-231. Densitometry data are available in Supplementary Table S1.

Figure 3. The effects of dacomitinib on total and phosphorylated HER2, EGFR, HER4, AKT, and ERK. The effects of dacomitinib on total and phosphorylated HER2, EGFR, HER4, AKT, and ERK were measured by Western blot as described in Materials and Methods. All cell lines were treated with 100 nmol/L or 1 μmol/L dacomitinib for 10 minutes. Cell lines are arranged from most sensitive (low IC50) to least sensitive (high IC50) [left to right]. Dacomitinib had no effect on total HER2, AKT, and ERK in a majority of lines and caused a minor decrease in total EGFR in several lines. Most of the selected lines expressed low levels of total HER4, which remained mostly unchanged posttreatment. Dacomitinib inhibited HER2 phosphorylation in all lines with detectable baseline phosphorylation. Dacomitinib inhibited EGFR phosphorylation in sensitive lines, but only 2 resistant lines (MDA-MB-453 and MDA-MB-231). Dacomitinib decreased HER4 phosphorylation in sensitive lines with detectable baseline levels. Dacomitinib inhibited phosphorylation of AKT in all sensitive lines, but only 2 resistant lines (HCC-70 and MDA-MB-453). Dacomitinib caused a decrease in phosphorylation of ERK in all lines, except for resistant MDA-MB-231.

Discussion

Using a broad panel of 44 human breast cancer cell lines and 3 immortalized breast epithelial lines, we have shown...
that HER2 amplification status was a strong predictor of response to dacomitinib, with IC$_{50}$ values below 1 µmol/L in the vast majority of HER2-amplified lines. MDA-MB-453 and UACC-732 were the only HER2-amplified lines that were resistant to dacomitinib. Of note, these 2 lines have some of the lowest levels of HER2 DNA copy number by comparative genomic hybridization (data not shown) and HER2 mRNA among the HER2-amplified lines. In addition, these 2 lines express low baseline levels of total and phosphorylated HER2 (25) and total and phosphorylated EGFR (data not shown). These 2 cell lines were also previously shown to be resistant to trastuzumab (25) and lapatinib (25, 26). These findings may have potential clinical implications for selecting the patients for clinical trials with dacomitinib.

Dacomitinib showed an antiproliferative activity superior to trastuzumab in vitro. The HER2-amplified lines SUM-225, HCC-1419, HCC-1954, UACC-893, and HCC-1569 were resistant to trastuzumab (25) but sensitive to dacomitinib (IC$_{50}$ < 1 µmol/L). These data suggest that dacomitinib can potentially overcome de novo trastuzumab resistance. Lapatinib and dacomitinib generated comparable IC$_{50}$ values in HER2-amplified lines; only HCC-1569 was resistant to lapatinib (IC$_{50}$ > 1 µmol/L) but sensitive to dacomitinib (25). Similarly to trastuzumab and lapatinib, the nonluminal, HER2-nonamplified cell lines were the most resistant to dacomitinib.

We have confirmed that dacomitinib acts by blocking the phosphorylation of HER2, EGFR, and HER4 in breast cancer cell lines. The inhibition of HER2 phosphorylation was dose dependent and specific to HER2-amplified lines. The blockage of EGFR phosphorylation was more pronounced in HER2-amplified lines but was also present in some resistant lines. The inhibition of HER4 phosphorylation was observed in several sensitive HER2-amplified lines. These effects lead to the inhibition of the PI3K/AKT signaling pathway in sensitive lines and 2 resistant lines, as evidenced by a reduction of phosphorylated AKT. We have also observed a loss of phosphorylated ERK in most lines, suggesting that dacomitinib also acts by inhibiting the RAS/MAPK pathway. These effects of dacomitinib on HER receptors and downstream signaling pathways ultimately resulted in cell-cycle inhibition and apoptosis.

In this study, 1 µmol/L was chosen based on the distribution of IC$_{50}$ values in our dataset, which has biologic significance (i.e., there seemed to be a natural increase in the IC$_{50}$ distribution around the 1 µmol/L mark; cell lines below this cutoff point were mostly HER2 amplified; dacomitinib induced changes in cell cycle and apoptosis in cell lines with IC$_{50}$ values below 1 µmol/L but not above). In addition, phase I data was recently reported (17). In general, the maximum plasma levels of dacomitinib in the phase I study were between 200 to 300 nmol/L and are within the range of the 1,000 nmol/L cut-off used here.

Trastuzumab and lapatinib have an important role in the current clinical therapy of HER2-amplified breast cancer. Unfortunately, less than half of patients respond to monotherapy, and though the response rates increase when combined with chemotherapy, the responses are often only temporary in a significant number of patients (4–11, 27–30). For these reasons, it is important to keep searching for new therapies for this subset of patients.
Our findings suggest different, largely nonoverlapping mechanisms of resistance to dacomitinib, trastuzumab, and lapatinib. We found that 2 HER2-amplified cell lines that we had conditioned for acquired resistance to trastuzumab (BT-474-TR and SK-BR-3-TR) were very sensitive to the antiproliferative effect of dacomitinib. This sensitivity to dacomitinib was comparable with the sensitivity observed in their parental cell lines. To examine the effect of dacomitinib in the models of acquired resistance to lapatinib, we have generated 2 lines with acquired lapatinib resistance (BT-474-LR and SK-BR-3-LR). We found these 2 lines to be sensitive to dacomitinib, though their sensitivity was less pronounced than in the parental lines.

Several mechanisms of resistance to trastuzumab have been proposed; however, their significance is not yet clearly defined. Several studies have implicated an increased activation of EGFR, HER3, and other receptor tyrosine kinases (e.g., IGF-1R) and their ligands in resistance to trastuzumab (25, 31–35). Alterations in the PI3K/AKT pathway through PIK3CA mutation (36, 37) and/or a loss of expression of the PTEN tumor suppressor (38, 39) have also been associated with resistance to trastuzumab. We have shown previously that, contrary to dacomitinib, the response to trastuzumab was not significantly associated with total HER2 protein levels (vs. amplification; ref. 25). A recent publication exploring mechanisms of trastuzumab resistance (40) also supports the use of a pan-HER inhibitor in overcoming resistance to trastuzumab.

The mechanisms of lapatinib resistance are not well understood either. Among the proposed mechanisms of

![Figure 5](image-url). Effects of dacomitinib on apoptosis. A, sensitive cell lines BT-474 and SK-BR-3 show significant increase in the percentage of Annexin-V-positive cells. Less sensitive cell lines ZR-75-1 and HCC-1143 show less pronounced increase in Annexin-V-positive cells (statistically significant in HCC-1143). B, resistant cell lines MCF-7 and MDA-MB-231 show no changes. Cells were incubated with 1 µmol/L dacomitinib for 5 days. Solid bars, control samples; striped bars, treated samples. Error bars represent SE for 2 separate experiments. *: P < 0.05 compared with control.
lapatinib resistance are HER2 mutations (41), activation of prosurvival pathways through ER signaling (42), and overexpression of membrane-bound receptor tyrosine kinase AXL (43). There are several possible explanations for the effect of dacomitinib in overcoming the acquired resistance to lapatinib. Unlike lapatinib, dacomitinib binds to the active site of kinases in a covalent and irreversible form, permanently blocking the kinase activity. In addition to blocking HER2 and EGFR, dacomitinib also inhibits HER4 kinase activity; however, the clinical significance of HER4 inhibition remains to be determined. We confirmed that dacomitinib blocks the phosphorylation of HER4 in several sensitive lines. However, we did not find a correlation between the HER4 mRNA levels and the response to dacomitinib.

In summary, this study shows that dacomitinib has a strong antiproliferative activity in HER2-amplified breast cancer cell lines and maintains this activity in HER2-amplified cell lines with de novo and acquired resistance to trastuzumab and acquired resistance to lapatinib. Given the importance of finding new therapies for drug-resistant breast cancer, these findings are a strong rationale for clinical development of this compound.

Table 2. Comparison of dacomitinib and lapatinib responses (IC50) in parental and trastuzumab- and lapatinib-resistant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dacomitinib IC50 (μmol/L)</th>
<th>Lapatinib IC50 (μmol/L)</th>
<th>Dacomitinib IC50 fold change compared with parental line</th>
<th>Lapatinib IC50 fold change compared with parental line</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT-474</td>
<td>0.018 ± 0.011</td>
<td>0.016 ± 0.011</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BT-474-TR</td>
<td>0.010a</td>
<td>0.077 ± 0.039</td>
<td>0.56a</td>
<td>4.81</td>
</tr>
<tr>
<td>BT-474-LR</td>
<td>0.031 ± 0.020</td>
<td>1.424 ± 0.120</td>
<td>1.72</td>
<td>89</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>0.015 ± 0.003</td>
<td>0.054 ± 0.008</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SK-BR-3-TR</td>
<td>0.005a</td>
<td>0.039 ± 0.010</td>
<td>0.33a</td>
<td>0.72</td>
</tr>
<tr>
<td>SK-BR-3-LR</td>
<td>0.339 ± 0.137</td>
<td>6.400 ± 1.119</td>
<td>22.60</td>
<td>118.52</td>
</tr>
</tbody>
</table>

NOTE: Growth rate decrease (fold change) in the presence or absence of 15 μg/mL of trastuzumab: BT-474 (5.00 ± 1.05), BT-474-TR (1.16 ± 0.21), SK-BR-3 (1.45 ± 0.09), SK-BR-3-TR (1.19 ± 0.05); both resistant lines fit the criteria for trastuzumab resistance (<1.2-fold decrease in growth rate; data in part published by O’Brien and colleagues; ref. 25). Abbreviations: TR, trastuzumab-resistant cell lines; LR, lapatinib-resistant cell lines. *Approximate average value from a minimum of 2 experiments.

References


Disclosure of Potential Conflicts of Interest

C.D. Briten received a commercial research grant from Pfizer Inc. (major) and was paid for travel to a scientific meeting (minor, Pfizer). I. Taylor is an employee of Pfizer Inc. as Senior Director, has received compensation (major) and has ownership interest in Pfizer Inc. (major). D.J. Slamon has received honoraria from Speakers Bureau of Genentech (minor), Sanofi-Aventis (minor), and GlaxoSmithKline (minor), has ownership interest in Amgen (major), and is a consultant and an advisory board member of Novartis Pharmaceuticals (minor). No potential conflicts of interest were disclosed by the other authors.

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