Activated Phosphoinositide 3-Kinase/AKT Signaling Confers Resistance to Trastuzumab but not Lapatinib

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Abstract
Trastuzumab and lapatinib provide clinical benefit to women with human epidermal growth factor receptor 2 (HER2)-positive breast cancer. However, not all patients whose tumors contain the HER2 alteration respond. Consequently, there is an urgent need to identify new predictive factors for these agents. The aim of this study was to investigate the role of receptor tyrosine kinase signaling and phosphoinositide 3-kinase (PI3K)/AKT pathway activation in conferring resistance to trastuzumab and lapatinib. To address this question, we evaluated response to trastuzumab and lapatinib in a panel of 18 HER2-amplified cell lines, using both two- and three-dimensional culture. The SUM-225, HCC-1419, HCC-1954, UACC-893, HCC-1569, UACC-732, JIMT-1, and MDA-453 cell lines were found to be innately resistant to trastuzumab, whereas the MDA-361, MDA-453, HCC-1569, UACC-732, JIMT-1, HCC-202, and UACC-893 cells are innately lapatinib resistant. Lapatinib was active in de novo (SUM-225, HCC-1419, and HCC-1954) and in a BT-474 cell line with acquired resistance to trastuzumab. In these cells, trastuzumab had little effect on AKT phosphorylation, whereas lapatinib retained activity through the dephosphorylation of AKT. Increased phosphorylation of HER2, epidermal growth factor receptor, HER3, and insulin-like growth factor I receptor correlated with response to lapatinib but not trastuzumab. Loss of PTEN or the presence of activating mutations in PI3K marked resistance to trastuzumab, but lapatinib response was independent of these factors. Thus, increased activation of the PI3K/AKT pathway correlates with resistance to trastuzumab, which can be overcome by lapatinib. In conclusion, pharmacologic targeting of the PI3K/AKT pathway may provide benefit to HER2-positive breast cancer patients who are resistant to trastuzumab therapy. Mol Cancer Ther; 9(6); OF1–14. ©2010 AACR.

Introduction
The human epidermal growth factor receptor 2 (HER2; ErbB2/neu) oncogene encodes a receptor tyrosine kinase (RTK) that is amplified and overexpressed in ∼25% of human breast cancers (1,2). Women whose malignancies contain this alteration have an aggressive form of the disease with significantly shortened disease-free and overall survival (1,2). As a result of this observation, the HER2 alteration was evaluated and shown to play a role in the pathogenesis of these cancers (3,4). Subsequent to that, HER2 has been successfully targeted by trastuzumab (Herceptin, Genentech), a monoclonal antibody directed against the extracellular domain of HER2, and by lapatinib (Tykerb, GlaxoSmithKline), a dual kinase inhibitor that targets the intracellular ATP binding domain of HER2 and its dimerization partner epidermal growth factor receptor (EGFR), rendering it kinase inactive (5–8). Despite the proven clinical benefits of HER2-targeted therapies, resistance to both agents exists. Less than 35% of patients with metastatic disease carrying the HER2 alteration respond to trastuzumab monotherapy, although this number increases to over 50% when given in combination with chemotherapy, and several of those who initially respond will acquire resistance within 24 to 36 months (8–10). Preclinical and clinical studies have also shown evidence for both primary and acquired resistance to lapatinib (11–13). Understanding the molecular mechanisms responsible for intrinsic (de novo) and acquired resistance to HER2-targeted therapy is critical to improving the survival rates of patients with this subtype of breast cancer.

Currently, there are no clinically validated markers of resistance to HER2-targeted therapy, although several potential mechanisms have been proposed. Trastuzumab binds to the extracellular domain IV of HER2 and induces G1-S cell cycle arrest (14), by as yet poorly defined mechanisms. However, it has been shown that the binding...
of trastuzumab to HER2 does not prevent ligand-induced HER2 heterodimerization with ErbB1/EGFR and ErbB3/HER3 (15, 16). It is possible that increased activity of ligands and receptors of the HER signaling network may be responsible for continued proliferation in the presence of trastuzumab. Increased levels of phosphorylated EGFR and EGFR-activating ligands such as transforming growth factor-α and heparin-binding EGF have been detected in HER2-positive breast cancer cell lines with reduced response to trastuzumab (17, 18). There is also evidence to suggest a role for HER3 in trastuzumab resistance. HER2-HER3 heterodimers are potent activators of the downstream RAS-MAPK and phosphoinositide 3-kinase (PI3K)/AKT proliferative survival pathways (19, 20). Trastuzumab has been shown to have activity against ligand-independent HER2-HER3 heterodimerization but not against ligand-dependent heterodimerisation (21). Studies have shown that the ligand stimulation of HER3 can confer resistance to trastuzumab-sensitive cells, and the inhibition of HER3 activation arrests proliferation in HER2-positive breast cancer cells (20, 22). Insulin-like growth factor IR (IGF-IR) signaling also activates the RAS-MAPK and PI3K/AKT pathways, and this has been implicated in trastuzumab resistance. Cell lines that coexpress IGF-IR and HER2 are less responsive to trastuzumab (23), and inhibition of IGF-IR resensitizes resistant cells to trastuzumab (24). Enzymatic cleavage of the HER2 extracellular domain results in a 95-kDa kinase-active COOH-terminal fragment of HER2 that lacks a binding site for trastuzumab. A small-scale patient study (n = 46) showed that HER2-positive breast cancer patients expressing p95HER2 were less likely to respond to trastuzumab than those expressing the full-length receptor (25).

Activity of the PI3K/AKT pathway is frequently altered in breast cancer. Expression of the tumor suppressor PTEN, which negatively regulates PI3K signaling, is lost in ~50% of breast cancers (26) and has been associated with poor prognosis and resistance to trastuzumab (27, 28). A preclinical study also suggested that PTEN loss is associated with lapatinib resistance (29). Specific mutations in the catalytic (E454K) and kinase domains (H1047R) of the p110α subunit of PI3K have been shown to activate PI3K/AKT signaling (30) and play a role in resistance to both trastuzumab and lapatinib (27, 29, 31). A recent study reported that measuring both PTEN loss and activating mutations of PI3K in combination rather than either alone is a superior marker of trastuzumab response (27).

Despite these advances, there is a lack of consensus within the literature as to which factors (if any) are most consistently altered in resistance to HER2-targeted therapies. A major limitation of most previous studies investigating trastuzumab response has been that they were carried out using only a limited (one to three) number of cell line models of resistance, and that they classified drug response based on only a single assay platform or restricted the studies to short term assays. To address this issue, we have evaluated the trastuzumab and lapatinib responses of a panel of 18 HER2-positive breast cancer cell lines, including a trastuzumab-conditioned cell line, by both two- and three-dimensional drug response assays. We also examined the relationship between HER2-targeted drug response and both RTK and PI3K/AKT signaling.

Materials and Methods

Cell lines, cell culture, and reagents

The effects of trastuzumab and lapatinib on cell growth were studied on a panel of 18 HER2-amplified breast cancer cell lines, including a trastuzumab-conditioned HER2-amplified cell line selected for long-term outgrowth in a trastuzumab-containing medium. The BT-474, SKBR3, MDA-MB-361, HCC-2218, HCC-202, HCC-1954, HCC-1419, HCC-1569, UACC-893, UACC-812, UACC-732, MDA-MB-453, and ZR-75-30 cell lines were obtained from the American Type Culture Collection. The EFM-192A and JIMT-1 cells were obtained from the German Tissue Repository DSMZ, and the SUM190 and SUM225 cells were obtained from the University of Michigan. BT-474, EFM-192A, and MDA-MB-453 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, and 1% penicillin G-streptomycin-fungizone solution (PSF, Irvine Scientific). SKBR3 and JIMT-1 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, and 1% PSF. UACC-812 and MDA-MB-361 cells were grown in L15 medium supplemented with 15% heat-inactivated FBS, 2 mmol/L glutamine, and 1% PSF. UACC-893 were grown in the same L15 media but supplemented with 20 ng/mL EGF (Invitrogen Life Technologies), 10 μg/mL insulin, and 10 μg/mL gluthathione. UACC-732 cells were maintained in MEM supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, 1% PSF, 1% MEM nonessential amino acids, and 1 μmol/L sodium pyruvate. SUM190 and SUM225 cells were grown in DMEM-F12 medium supplemented with 10% heat-inactivated FBS, 1% PSF, 10 μg/mL insulin, and 1 μg/mL hydrocortisone. The remaining cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, 1% PSF, 4.5 g/L D-glucose, and 1 μmol/L sodium pyruvate.

Cells were routinely assessed for Mycoplasma contamination using a multiplex PCR method (32). Mitochondrial DNA from the cells was sequenced to confirm their correct identity (33). PCR products were sequenced using a 3730 DNA Analyzer (Applied Biosystems). The trastuzumab-conditioned BT-TR cell line (34) was established by culturing the BT-474 cell line in the appropriate media supplemented with 105 μg/mL of recombinant humanized monoclonal HER2 antibody, trastuzumab (Herceptin, Genentech, Inc.). Trastuzumab was removed from the culture medium 7 days before assay. Lapatinib was provided by GlaxoSmithKline as a 10-mmol/L concentrated stock solution in DMSO.
Two-dimensional proliferation assays

Cells were seeded in triplicate in 12-well plates at a density of 8,000 to 12,000 cells per well and allowed to adhere and enter growth phase before treating with or without 15 μg/mL trastuzumab for 0, 3, 5, and 7 days in the appropriate culture media. Cells were then harvested by trypsinization and counted using a Coulter Z2 particle counter (Beckman Coulter, Inc.). Response to trastuzumab was quantified by calculating the change in the growth rate of the cells in the presence and absence of the drug. The mean cell doubling time of each cell line was calculated using the equation \( \log (N_t) = (\log_2 / DT) \times T + \log (N_0) \), in which \( N_0 \) is the cell count at the first time point, \( N_t \) is the cell count at the second time point, \( T \) is time between points, and \( DT \) is the doubling time (35). Fold change in growth rate was ascertained from the ratio of doubling time in the presence of drug divided by the doubling time in the absence of drug for each cell line. The distribution of the response data was used to determine a cutoff for sensitivity and resistance of 20%. Thus, for classification purposes, cell lines with a fold decrease in growth rate of ≥1.2 or a decrease in colony number of ≥20% in response to trastuzumab were considered sensitive.

Lapatinib response assays were done in triplicate in 24-well plates at 6,000 to 8,000 cells per well. Cells were allowed to adhere and enter growth phase before treating with increasing concentrations of lapatinib (0.01–10 μmol/L). Cells were harvested by trypsinization on day 5 and counted using the Coulter Z2 particle counter. Growth inhibition was calculated as a percentage of the nontreated controls. The log of the fractional growth inhibition was then plotted against the log of the drug concentration, and the IC_{50} values were interpolated from the linear regression curve fit as previously described (34). IC_{50} values of <1 μmol/L were considered sensitive (34, 36).

Three-dimensional proliferation assays

Cells were seeded in quadruplicate at a density of 7,000 cells per well in 12-well plates containing a 5:1 (v/v) mixture of media to 3% agarose on top of a solidified layer of 0.5% agarose in culture medium. Media/agarose mixes were prepared with or without 15 μg/mL trastuzumab or lapatinib (IC_{50} concentration). Before seeding, cells were also preincubated for 30 minutes with and without trastuzumab and lapatinib. Cells were allowed to form colonies for 3 weeks before staining with neutral red, and viable colonies were counted using a light microscope. Fresh drug was added to the cells each week. Cell lines with a ≥20% decrease in colony number in treated versus control were considered sensitive.

Western blots and protein quantification

Protein lysates were obtained from two-dimensional cultures as follows: Cells were washed twice in ice-cold PBS and lysed for 15 minutes at 4°C using a mild lysis buffer (Cell Signaling Technology) containing a mixture of protease inhibitors (Calbiochem) and 1 mmol/L phenylmethylsulfonylfluoride. Insoluble material was removed by centrifugation at 10,000 x g for 10 minutes. Protein was quantified by BCA (Pierce), resolved by SDS-PAGE, and transferred to nitrocellulose membranes (iBlot, Invitrogen Life Technologies). Total (p185 and p95) and phosphorylated (Ty-r/1221,1222) HER2 protein were detected by the monoclonal anti-HER2 (Ab-3, Calbiochem) and polyclonal anti-pHER2 (Cell Signaling Technology) antibodies. Monoclonal anti-PTEN (A2B1, Santa Cruz Biotechnology) and anti-α-tubulin (DMA1, Calbiochem) antibodies, and polyclonal anti-HER3 (C-17, Santa Cruz Biotechnology), anti-AKT, anti-pHER3 (Tyr-r1289), and anti-pAKT (Ser-r473; Cell Signaling Technology) antibodies were used for detection. All blots were repeated in triplicate and relative expression of individual proteins was quantitated using the ECL Plus chemiluminescent reagent (Amersham Biosciences) and the Typhoon 9400 (Amersham Biosciences) system. Densitometry was performed using the ImageQuant software (Amersham Biosciences). Densitometry results were normalized using α-tubulin as a control.

Analysis of the effects of trastuzumab and lapatinib on AKT phosphorylation

Cells were seeded in six-well plates at a density of 50,000 cells per well and allowed to adhere and enter growth phase before treating with or without 15 μg/mL trastuzumab or 1 μg/mL lapatinib for 24 hours in the appropriate culture media. The HCC-2218, UACC-732, and UACC-812 had low baseline levels of pAKT and were thus excluded from this analysis. Assays were repeated in triplicate. Western blotting and protein quantitation was done as described above. Percentage loss of pAKT was calculated as a ratio of the decrease in AKT phosphorylation in treated versus nontreated control.

Enzyme-linked immunosorbant assays

As levels of EGFR and IGF-IR were low in some of the HER2-positive cell lines, total and phosphorylated EGFR and IGF-IR were measured using commercially available quantitative ELISAs (R&D Systems) according to the manufacturer’s instructions. Lysates were prepared as described above. Total EGFR and IGF-IR levels are presented as nanogram per milligram of total protein, and phosphorylated protein levels are presented as a ratio of the sample with the highest level of phosphorylation.

Determination of PIK3CA mutation status

The PIK3CA mutation status of the majority of cell lines in the panel were publicly available from the Catalogue of Somatic Mutations in Cancer database from the Sanger Institute (37). The remaining three cell lines (JIMT-1, UACC-732, and MDA-MB-361) were sequenced using known primers for exon 9 and exon 20 regions of the PIK3CA gene (38). PCR products were sequenced using the 3730 DNA Analyzer.
Statistical analysis

The results of the two-dimensional and three-dimensional drug response assays were expressed as means (±SD) of at least three independent experiments. Relationships between continuous variables such as protein expression and drug response were measured using the Spearman-Rank correlation. All correlations were assessed between drug response and protein levels in two-dimensional culture. Differences in growth rate and protein expression between cell lines were assessed using the Student’s t-test.

All statistics were calculated using StatView for Windows version 5.0.1 (SAS Institute, Inc.).

Results

Trastuzumab and lapatinib activity in a panel of HER2-positive breast cancer cell lines

The antiproliferative effects of trastuzumab and lapatinib were measured on a panel of 17 HER2-positive breast cancer cell lines using two-dimensional and three-dimensional drug response assays (Table 1). Response to trastuzumab ranged from a >7-fold decrease in growth rate (ZR75-30) to a <1.2-fold decrease (SUM-225) by two-dimensional monolayer assay over 7 days of treatment. Assessment of trastuzumab responses by three-dimensional assay were similar to that observed in two-dimensional culture with the exception of the SUM-190 and HCC-202 cell lines, which were resistant in the two-dimensional assay yet showed significant sensitivity by soft agar assay (Table 1).

We used a combination of data from both the two-dimensional and three-dimensional assays to identify cells that are either sensitive or de novo resistant to trastuzumab. Using a cutoff of <1.2-fold decrease in proliferation by two-dimensional assay and >20% decrease in colony number by three-dimensional assay; cell lines with distinct sensitivities are those resistant by two-dimensional assay yet sensitive by dimensional assay.

Lapatinib sensitivity was defined as an IC50 of <1 μmol/L.

Table 1. Trastuzumab and lapatinib responses in a panel of HER2-amplified breast cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Trastuzumab (15 μg/mL, ± SD)</th>
<th>Lapatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Two-dimensional growth rate</td>
<td>Three-dimensional colony number</td>
</tr>
<tr>
<td></td>
<td>(fold change)</td>
<td>(% decrease)</td>
</tr>
<tr>
<td>ZR75-30</td>
<td>7.11 ± 0.32</td>
<td>56.5 ± 12.9</td>
</tr>
<tr>
<td>BT-474</td>
<td>5.00 ± 1.05</td>
<td>37.2 ± 8.1</td>
</tr>
<tr>
<td>BT-TR‡</td>
<td>1.16 ± 0.21</td>
<td>1 12.1 ± 3.6</td>
</tr>
<tr>
<td>HCC-2218</td>
<td>2.60 ± 0.60</td>
<td>34.1 ± 9.7</td>
</tr>
<tr>
<td>EFM192A</td>
<td>1.86 ± 0.54</td>
<td>69.8 ± 4.3</td>
</tr>
<tr>
<td>UACC-812</td>
<td>1.49 ± 0.27</td>
<td>50.2 ± 8.7</td>
</tr>
<tr>
<td>SKBR3</td>
<td>1.45 ± 0.09</td>
<td>65.3 ± 3.1</td>
</tr>
<tr>
<td>MDA-361</td>
<td>1.21 ± 0.22</td>
<td>—</td>
</tr>
<tr>
<td>UACC-732</td>
<td>1.17 ± 0.01</td>
<td>1.8 ± 3.1</td>
</tr>
<tr>
<td>HCC-1419</td>
<td>1.18 ± 0.02</td>
<td>1.0 ± 12.6</td>
</tr>
<tr>
<td>JIMT-1</td>
<td>1.15 ± 0.10</td>
<td>19 ± 1.2</td>
</tr>
<tr>
<td>HCC-202</td>
<td>1.13 ± 0.07</td>
<td>47.8 ± 6.4</td>
</tr>
<tr>
<td>UACC-893</td>
<td>1.12 ± 0.20</td>
<td>3.4 ± 1.1</td>
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<tr>
<td>HCC-1954</td>
<td>1.08 ± 0.10</td>
<td>9.3 ± 14.9</td>
</tr>
<tr>
<td>HCC-1569</td>
<td>1.04 ± 0.06</td>
<td>17.3 ± 1.7</td>
</tr>
<tr>
<td>MDA-453</td>
<td>1.04 ± 0.04</td>
<td>—</td>
</tr>
<tr>
<td>SUM-190</td>
<td>1.02 ± 0.10</td>
<td>55.4 ± 9.0</td>
</tr>
<tr>
<td>SUM-225</td>
<td>1.01 ± 0.12</td>
<td>13.3 ± 7.8</td>
</tr>
</tbody>
</table>

NOTE: MDA-453 and MDA-361 did not form colonies in soft agar.
Abbreviations: % increase in colony number; S, sensitive; R, resistant; D, distinct, resistant by two-dimensional assay yet sensitive by three-dimensional assay.
*For trastuzumab, sensitive was defined as a response of >1.2-fold decrease in growth rate by two-dimensional assay and >20% decrease in colony number by three-dimensional assay; cell lines with distinct sensitivities are those resistant by two-dimensional assay yet sensitive by dimensional assay.
†Lapatinib sensitivity was defined as an IC50 of <1 μmol/L.
‡TR, trastuzumab-conditioned BT-474 cells.
responsive to lapatinib when grown in soft agar in the presence of the IC\textsubscript{50} concentration of lapatinib as determined by two-dimensional assay (Supplementary Table S1). Using a cutoff IC\textsubscript{50} of $>1$ μmol/L, the MDA-361, MDA-453, HCC-1569, UACC-732, JIMT-1, HCC-202, and UACC-893 cell lines were considered lapatinib resistant (Table 1). Interestingly, the SUM-225, HCC-1419, and HCC-1954 cells were lapatinib sensitive despite being identified as trastuzumab resistant in two-dimensional and three-dimensional culture, whereas the SUM-190 cells are lapatinib sensitive in two-dimensional culture despite showing trastuzumab resistance in two-dimensional culture. The MDA-361 and HCC-202 cell lines also have opposing sensitivities as they are lapatinib resistant yet trastuzumab sensitive.

The relative levels of HER2 protein were quantified across the panel of HER2-amplified cell lines by immunoblotting, and a range of HER2 protein levels was observed (Fig. 1). Response to trastuzumab did not correlate with the levels of HER2 protein (Fig. 1B). However, lapatinib response correlated significantly with the levels of total HER2 protein ($P = 0.011$; Fig. 1C). Based on these findings, we evaluated if autophosphorylation of HER2 or levels of the kinase-active 95-kDa cleaved form of HER2, p95HER2, were markers of response to HER2-targeted therapy. Trastuzumab response was not significantly associated with either HER2 phosphorylation or p95HER2 levels, whereas lapatinib response correlated significantly with both increased levels of phosphorylated HER2 ($P = 0.043$) and levels of p95 HER2 ($P = 0.019$; Figure 1).

![Figure 1. HER2 and response to HER2-targeted therapy.](https://example.com/figure1.png)

Figure 1. HER2 and response to HER2-targeted therapy. A, HER2, p95, and HER3 levels were quantified by Western blot as described in Materials and Methods. HER2 phosphorylation levels were determined using an anti-pHER2 (Tyr\textsuperscript{1221/1222}) antibody. T/L, cell lines that are either sensitive to both trastuzumab and lapatinib (S), resistant to one but sensitive to the other (O), or resistant to both agents (R). B and C, correlations between total, p95, and phosphorylated HER2 (pHER2), and between full-length HER2 and p95 HER2, and between full-length HER2 and phosphorylated HER2 in the cell line panel. For all scatters, white points represent cell lines sensitive to both agents, black are resistant to both, and gray represent cell lines that are sensitive to one but resistant to the other, i.e., opposing (O). Quantitation of Western blots was done by densitometry using the ImageQuant software. *, $P$ values according to the Spearman Rank correlation.
Both phosphorylated and p95HER2 levels also correlated significantly ($P=0.011$ and $P<0.001$, respectively) with total HER2 levels, suggesting that these factors may not be independent markers of response to lapatinib (Fig. 1D).

**Acquired trastuzumab resistance and response to lapatinib**

A model of acquired trastuzumab resistance (BT-TR) was established by culturing the trastuzumab-sensitive BT-474 breast cancer cell line in 105 μg/mL trastuzumab for 9 months. Trastuzumab inhibited the proliferation of the parental BT-474 cells by 5-fold compared with 1.16-fold for the BT-TR cells by two-dimensional assay (Table 1; Fig. 2). The three-dimensional growth of the parental BT-474s was inhibited by 37.2% in contrast to the BT-TR cells, which showed an average increase of 12.1% in colony number in the presence of trastuzumab (Fig. 2B). The significant difference in the responses of the BT-474 and BT-TR cells by both two-dimensional ($P=0.003$) and three-dimensional assay ($P=0.018$) confirms that the BT-TR cells have acquired trastuzumab resistance and fit the criteria of less than 1.2-fold decrease in growth rate and less than 20% decrease in colony number that we used to classify de novo resistance. Full-length and p95HER2 protein levels were unchanged in the BT-TR cells relative to their isogenic controls; however, significantly increased levels of phosphorylated HER2 ($P=0.005$) were detected in the BT-TR cells (Fig. 2D). Despite the acquired resistance to trastuzumab and increased levels of activated HER2, the BT-TR cells remained sensitive (IC$_{50} = 77$ nmol/L) to lapatinib (Table 1).

**RTK activation and response to HER2-targeted therapy**

To investigate the possible mechanisms of resistance to HER2-targeted therapies, we measured the levels of other RTKs known to be involved in HER2 signaling. No correlation was observed between trastuzumab response by two-dimensional assay and levels of total or phosphorylated HER3, EGFR, or IGF-IR levels (Fig. 3). In contrast, lapatinib response correlated significantly with increased phosphorylation of HER3 ($P=0.006$), EGFR ($P=0.008$), and IGF-IR ($P=0.009$; Fig. 3B). No correlation was observed between lapatinib response
and levels of total HER3, EGFR, or IGF-IR (Supplementary Table S2).

We also investigated the role of RTK activation in acquired trastuzumab resistance. No significant difference in total or phosphorylated HER3 or IGF-IR was detected in the BT-TR cells relative to parental cells. However, phosphorylation of EGFR ($P < 0.001$) was significantly increased in the trastuzumab-conditioned cells (Fig. 3C). Significantly increased levels of pAkt were also detected in the BT-TR cells ($P = 0.008$; Fig. 4).

**PI3K/AKT pathway signaling and response to HER2-targeted therapy**

We evaluated the role of PTEN protein levels, AKT phosphorylation, and PI3K mutation status in response to trastuzumab and lapatinib. The level of AKT phosphorylation in the cell line panel did not correlate with HER2 phosphorylation ($P = 0.724$), indicating that AKT activation can occur independently of HER2. High levels of PTEN protein correlated significantly with trastuzumab sensitivity in our panel of cell lines ($P = 0.008$; Fig. 4).

PTEN protein levels were inversely correlated with pAKT levels ($P = 0.024$). A significant association between pAKT levels and resistance to trastuzumab was also observed ($P = 0.034$). In contrast, lapatinib response was independent of both PTEN and pAKT levels (Fig. 4).

The cell lines were classified as either PI3K mutant (MUT), if they contained either the E454K or H1047R mutations, or wild-type (WT; Table 2; Fig. 5). Cell lines were also classified as PTEN high or low based on whether the PTEN levels were above or below median. Finally, we divided the cell lines into either PI3K “pathway activated” or not activated (normal) based on the criteria previously described by Berns et al. (27), i.e., cells with either low PTEN or a mutation in PI3K were considered PI3K pathway activated. Significantly, increased levels of pAKT were detected in the PI3K mutant cell lines ($P = 0.017$; Fig. 5A). Cell lines with low PTEN had significantly higher pAKT than cells with high PTEN ($P = 0.033$), and cell lines designated as PI3K pathway activated had a marginally significant association with increased pAKT levels ($P = 0.043$; Fig. 5A).

**Figure 3.** RTK activation and response to HER2-targeted therapy. A, total and phosphorylated (Tyr1289) HER3 was measured in each cell line by Western blot. Total and phosphorylated EGFR and IGF-IR levels were determined by ELISA as described in Materials and Methods. T/L, cell lines that are either sensitive to both trastuzumab and lapatinib (S), resistant to one but sensitive to the other (O), or resistant to both agents (R). B, correlations between HER3, EGFR, and IGF-IR phosphorylation, and response to trastuzumab and lapatinib in the cell line panel; ΔGR, change in growth rate. *, $P$ values according to the Spearman Rank correlation. C, quantification of phosphorylated HER3, EGFR, and IGF-IR in the BT-TR cells relative to their isogenic control BT-474 cells. Quantitation of triplicate Western blots was done by densitometry using ImageQuant software. $\$, $P$ values according to the Student’s $t$ test. Columns, mean; bars, SD.
Although response to trastuzumab was lower in PI3K mutant cell lines, this did not reach statistical significance. PTEN status ($P = 0.048$) showed a weak association with trastuzumab resistance, whereas the combination of both factors, i.e., PI3K pathway activation status ($P = 0.009$), correlated significantly with resistance to trastuzumab (Fig. 5B). The BT-TR cells were assessed for PI3K mutation status and were found to be wild-type. Interestingly, lapatinib response was independent of PI3K mutation status, PTEN status, and PI3K pathway activation status (Fig. 5C).

**Activity of trastuzumab and lapatinib on AKT phosphorylation**

To further investigate the role of PI3K/AKT signaling in resistance to HER2-targeted therapy, we evaluated the effects of trastuzumab and lapatinib on AKT phosphorylation in each of the response subsets, i.e., (a) sensitive to both trastuzumab and lapatinib, (b) resistant to both, and (c) resistant to trastuzumab yet sensitive to lapatinib. Three of the four cell lines sensitive to both trastuzumab and lapatinib displayed a greater than 60% reduction in AKT phosphorylation in response to treatment by either agent (Fig. 6). Of the five cell lines resistant to trastuzumab and lapatinib, four showed a <60% reduction in pAKT in response to lapatinib, whereas all five had less than 50% reduction in pAKT in response to trastuzumab. In contrast to the other cell lines, AKT phosphorylation was completely ablated in the HCC-202 cells in response to lapatinib, despite these cells being lapatinib resistant. In cell lines resistant to trastuzumab yet sensitive to lapatinib, only one (HCC-1419) had a >60% reduction in
pAKT in response to trastuzumab, whereas four of the five cell lines had a dramatic reduction (>80%) in pAKT in response to lapatinib (Fig. 6). In summary, the activity of trastuzumab on AKT phosphorylation correlated highly significantly with response to trastuzumab across the cell line panel ($P = 0.001$), whereas the activity of lapatinib on AKT phosphorylation showed a marginally significant association with response ($P = 0.048$; Fig. 6C). We also observed that in contrast to trastuzumab, lapatinib has activity on AKT phosphorylation independent of PI3K mutations status (UACC-893, MDA-453, HCC-202, and SUM-190; Fig. 6A).

**Discussion**

To investigate mechanisms of resistance to HER2-targeted agents, we screened a total of 18 HER2-positive breast cancer cell lines for response to trastuzumab and lapatinib by both two-dimensional and by three-dimensional drug response assay and identified subgroups of cells that were either sensitive or resistant to these agents. Cell lines were considered to be resistant or at least have a reduced response to trastuzumab if we observed a <1.2-fold change in growth rate by two-dimensional assay and a <20% response with three-dimensional assays. This approach enabled us to differentiate between cell lines that are truly resistant to trastuzumab from those that display resistance specific to their cell culture conditions.

There is an increasing volume of literature suggesting that three-dimensional drug response assays are superior to traditional two-dimensional assays as they may better reflect the microenvironmental conditions of tumor cells growing in vivo (39, 40). However, the challenge three-dimensional response assays present is that they are not “high throughput” and not all cell lines will grow in these conditions, limiting the spectrum of models that can be studied. In the present study, 2 of the 18 cell lines tested had differing responses by two-dimensional and three-dimensional assay. For example, the growth rate of the SUM-190 cells was unchanged in the presence of trastuzumab by monolayer cell count assay, indicating resistance, consistent with previous results (41). However, there was a 55% reduction in the number of colonies when these cells were grown in anchorage-independent conditions in the same concentration of trastuzumab. It has been reported that signaling downstream of HER2 can switch between the PI3K/AKT and RAS-MAPK pathways depending on cell culture conditions (40). This may explain why we see differing trastuzumab sensitivities for the SUM-190 cells between two-dimensional and three-dimensional culture. For the purposes of this study, we have measured the expression of signaling proteins in two-dimensional culture and related expression to drug response by two-dimensional culture. However, it will be interesting to determine if further analysis of protein expression in three-dimensional culture identifies alternative pathways associated with resistance.

The combination of data from both the two-dimensional and three-dimensional assays showed that 8 of 17 HER2-amplified cell lines are resistant to trastuzumab by both assays (SUM-225, HCC-1419, HCC-1954, UACC-893, HCC-1569, UACC-732, JIMT-1, and MDA-453). The

### Table 2. HER2-amplified cell lines with increased PI3K/AKT signaling

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exon 9</th>
<th>Exon 20</th>
<th>PTEN</th>
<th>PI3K/AKT signaling</th>
<th>T/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR75-30</td>
<td>WT</td>
<td>WT</td>
<td>High</td>
<td>Normal</td>
<td>S/S</td>
</tr>
<tr>
<td>BT-474</td>
<td>WT</td>
<td>WT</td>
<td>High</td>
<td>Normal</td>
<td>S/S</td>
</tr>
<tr>
<td>HCC-2218</td>
<td>WT</td>
<td>WT</td>
<td>High</td>
<td>Normal</td>
<td>S/S</td>
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<tr>
<td>EFM192A</td>
<td>WT</td>
<td>WT</td>
<td>High</td>
<td>Normal</td>
<td>S/S</td>
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<tr>
<td>UACC-812</td>
<td>WT</td>
<td>WT</td>
<td>High</td>
<td>Normal</td>
<td>S/S</td>
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<tr>
<td>SKBR3</td>
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<td>WT</td>
<td>Low</td>
<td>Activated</td>
<td>S/S</td>
</tr>
<tr>
<td>UACC-732</td>
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<td>High</td>
<td>Normal</td>
<td>R/R</td>
</tr>
<tr>
<td>JIMT-1</td>
<td>WT</td>
<td>WT</td>
<td>Low</td>
<td>Activated</td>
<td>R/R</td>
</tr>
<tr>
<td>UACC-893</td>
<td>WT</td>
<td>H1047R</td>
<td>Low</td>
<td>Activated</td>
<td>R/R</td>
</tr>
<tr>
<td>HCC-1569</td>
<td>WT</td>
<td>WT</td>
<td>Low</td>
<td>Activated</td>
<td>R/R</td>
</tr>
<tr>
<td>MDA-453</td>
<td>WT</td>
<td>H1047R</td>
<td>Low</td>
<td>Activated</td>
<td>R/R</td>
</tr>
<tr>
<td>MDA-361</td>
<td>E545K</td>
<td>WT</td>
<td>High</td>
<td>Activated</td>
<td>S/R</td>
</tr>
<tr>
<td>HCC-1419</td>
<td>WT</td>
<td>WT</td>
<td>Low</td>
<td>Activated</td>
<td>R/S</td>
</tr>
<tr>
<td>HCC-202</td>
<td>E545K</td>
<td>WT</td>
<td>High</td>
<td>Activated</td>
<td>D/R</td>
</tr>
<tr>
<td>HCC-1954</td>
<td>WT</td>
<td>H1047R</td>
<td>Low</td>
<td>Activated</td>
<td>R/S</td>
</tr>
<tr>
<td>SUM-190</td>
<td>WT</td>
<td>H1047R</td>
<td>Low</td>
<td>Activated</td>
<td>D/S</td>
</tr>
<tr>
<td>SUM-225</td>
<td>WT</td>
<td>WT</td>
<td>Low</td>
<td>Activated</td>
<td>R/S</td>
</tr>
</tbody>
</table>

**NOTE:** Median expression level used for high/low cutoff.
Abbreviation: T/L, response to either trastuzumab or lapatinib, respectively.
SUM-225, HCC-1419, and HCC-1954 cell lines were lapatinib sensitive by both two-dimensional (IC50 < 1 μmol/L) and three-dimensional assay despite being trastuzumab resistant. The SUM-190 cells were trastuzumab resistant by two-dimensional culture yet lapatinib sensitive in two-dimensional culture. Lapatinib was active in the BT-TR cells that were conditioned to acquire trastuzumab resistance, suggesting that lapatinib has activity in de novo and acquired trastuzumab resistance. Although, it has previously been reported that HER2-positive cells selected for resistance to trastuzumab remain sensitive to lapatinib (17, 34, 42), the present study is the first to show that lapatinib has activity in cell lines with primary trastuzumab resistance. Furthermore, clinical data have proven that lapatinib has efficacy in patients who have progressed on trastuzumab (43). Seven of the HER2-positive cell lines display primary lapatinib resistance, four of which are also trastuzumab resistant (MDA-453, HCC-1569, JIMT-1, and UACC-893) and three (MDA-361, UACC-732, and HCC-202) of which are trastuzumab sensitive. Collectively, these data suggest that trastuzumab and lapatinib can have nonoverlapping mechanisms of resistance and that this combination continues to be a rational therapeutic intervention for HER2-positive breast cancers as we initially showed (34).

The level of expression and activation HER2 protein has been proposed as a marker of response/resistance to HER2-targeted therapy (34, 41). However, we found no association between these factors and response to trastuzumab. Previous studies included both HER2-amplified and nonamplified cell lines in their analysis (34, 41), showing that amplification of HER2 is associated

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**Figure 5.** PI3K/AKT activation and response to trastuzumab and lapatinib. A, markers of PI3K/AKT pathway activation. Cell lines with below median PTEN protein levels were defined as PTEN low. Samples were classified as PI3K mutant if they contained either of the E454K- or H1047R-activating mutations. Cell lines were classified as PI3K/AKT activated if they were low PTEN (below median) or PI3K mutant. AKT phosphorylation measured by triplicate Western blot. B and C, relationship between PI3K/AKT activation, and trastuzumab and lapatinib response. ΔGR: change in growth rate. *, P values according to the Student's t test. White points, cell lines sensitive to both agents; black points, resistant to both; gray points, cell lines that are sensitive to one but resistant to the other. Dashed line, median response or expression level.
with response to trastuzumab. However, within a panel of HER2-amplified cell lines, we have shown that the level of HER2 overexpression or activation do not predict response to trastuzumab. In contrast, lapatinib sensitivity was significantly associated with increased levels of total and phosphorylated HER2. Expression of p95HER2 has been shown, in a small patient cohort (n = 46), to be a marker of resistance to trastuzumab, and cell lines transfected with p95HER2 are sensitive to lapatinib but not trastuzumab (25). We found no association between p95HER2 levels and trastuzumab response but did observe a positive correlation with lapatinib response. It is important to note however that in this study, p95HER2 levels directly correlate with total and phosphorylated HER2 within the cell line panel, indicating that p95HER2 may not be an independent marker of response.

Our data agree with a study (17) that detected increased levels of pEGFR and pHER2 in acquired trastuzumab resistance, yet we found no association between increased pEGFR levels and de novo trastuzumab resistance. Thus, it is possible that increased signaling through EGFR-HER2 dimers is responsible for the elevated levels of pAKT we observe in these cells and is a mechanism specific to acquired resistance brought about by long-term exposure to trastuzumab. This may also explain why the dual EGFR-HER2 tyrosine kinase inhibitor, lapatinib, retains activity in these cells. Although previous studies have suggested an association between both

| Figure 6. Activity of trastuzumab and lapatinib on AKT phosphorylation. A, all cell lines were treated with 1 μmol/L lapatinib (L) or 15 μg/mL trastuzumab (T) for 24 h before lysis. Western blotting for total and phosphorylated AKT was done as described in Materials and Methods. Cell lines were grouped by their response to trastuzumab and lapatinib, i.e., cell lines sensitive to both trastuzumab and lapatinib, cell lines resistant to both trastuzumab and lapatinib, and cell lines resistant to trastuzumab yet sensitive to lapatinib. B, the levels of dephosphorylation of AKT by trastuzumab and lapatinib within the subgroups of cell lines. Percentage loss of pAKT is the percent decrease in phosphorylation of AKT in the treated samples relative to the nontreated controls. C, correlations between two-dimensional response to trastuzumab and lapatinib and the level of dephosphorylation of AKT by each agent. ΔGR, change in growth rate. White points, cell lines sensitive to both agents; black points, resistant to both; gray lines, cell lines that are sensitive to one but resistant to the other, i.e., opposing sensitivities. *P value according to the Spearman Rank correlation. H1047R and E545K: PI3K mutation status, others wild-type. |
HER3 (20, 22) and IGF-IR (24, 44) and trastuzumab resistance, we found no association between the expression or activation of these RTKs and response to trastuzumab in our cell line panel. Trastuzumab has been shown to be active against ligand-independent HER2-HER3 heterodimers but not against ligand-induced dimers (21). Further analysis of our cell line panel in the absence of ligand stimulation will determine if ligand-independent HER2-HER3 heterodimerization plays a role in resistance to trastuzumab. Another study found no association between either HER3 or IGF-IR expression and response to trastuzumab in HER2-positive breast tumors, although IGF-IR expression combined with the phosphorylation of S6 ribosomal protein was predictive of resistance in that patient cohort \( n = 68 \); ref. 45).

In contrast to trastuzumab, response to lapatinib correlated with the increased activation of each of the type I RTKs measured in this study: HER2, EGFR, and HER3. This suggests that lapatinib not only has activity on HER2-EGFR signaling but also on HER2-HER3–activated signaling. Increased phosphorylation of the type II RTK, IGF-IR, also correlates with lapatinib response in the cell lines, indicating that lapatinib may have activity on activated IGF-IR signaling. These data agree with another study that showed lapatinib treatment reduced pIGF-IR levels in trastuzumab-conditioned SKBR3 cells (42).

Resistance to HER2-targeted therapy can occur through altered signaling downstream of RTK activation. Several independent studies have shown that the loss of the protein phosphatase PTEN, which results in activated PI3K/AKT signaling, correlated with trastuzumab resistance (28, 46). As with acquired trastuzumab resistance, we detected significantly higher levels of pAKT in the innately resistant cells; however, it seems that in innate resistance, this activation of AKT is brought about by an alternative mechanism, most likely due to a loss of PTEN or the presence of activating mutations of PI3K. In this investigation, PTEN protein levels in the cell line panel were inversely related to phosphorylation of AKT, confirming that decreased PTEN leads to the activation of AKT. Lower levels of PTEN correlated significantly with primary resistance to trastuzumab in the panel.

In addition, we observed higher pAKT levels in the PIK3CA mutant lines, although the presence of the mutations alone was not significantly associated with resistance to trastuzumab. Because PTEN loss and PIK3CA activating mutations can have similar downstream effects on the PI3K/AKT pathway and are events that rarely occur in the same breast tumor (27, 47), measuring these factors in combination may be a more accurate marker of resistance to therapy. Consistent with the findings of a previous study (27), we found that the evaluation of these two factors in combination is a stronger predictor of trastuzumab resistance than either factor alone. We also report that dephosphorylation of AKT by trastuzumab correlates significantly with response, indicating that cell lines maintaining high levels of AKT phosphorylation in the presence of trastuzumab, possibly either through PTEN loss or PI3K mutation, have a reduced response to trastuzumab.

Activation of the PI3K/AKT pathway has also been implicated in lapatinib resistance. Knockdown of PTEN conferred resistance to lapatinib, as did transfection with mutant PIK3CA (29). However, we found no association between PTEN protein levels and resistance to lapatinib across our panel of 17 HER2-amplified cell lines. This is consistent with the findings of two studies that used multiple cell lines or patient tissues to show no association between PTEN and lapatinib response (48, 49). We found no association between PI3K mutation status, either alone or in combination with PTEN status, and response to lapatinib. Similar to trastuzumab, response to lapatinib correlated significantly with the dephosphorylation of AKT. However, as previously discussed, lapatinib has activity in a different subset of cell lines than trastuzumab. By two-dimensional assay, the HCC-1954, SUM-190, SUM-225, and BT-TR cells were all resistant to trastuzumab, yet remain sensitive to lapatinib. In these cells, trastuzumab has a reduced effect on AKT phosphorylation, whereas lapatinib greatly reduces the levels of AKT phosphorylation. Interestingly, two of these cell lines, HCC-1954 and SUM-190, harbor the H1047R PI3K mutation, suggesting that lapatinib, contrary to what has previously been published (29), can have activity in the presence of PI3K mutations. However, both cell lines (MDA-361 and HCC-202) harboring E545K mutations of PI3K are resistant to lapatinib. These results indicate that the two activating mutations may have differing downstream effects. Analysis of a larger sample set will be required to address this question. Nevertheless, these data show that unlike trastuzumab, lapatinib remains active in the presence of increased PI3K/AKT signaling and illustrate the importance of using multiple cell line models to accurately determine molecular markers of drug response. None of the markers measured in this study associated with resistance to lapatinib. It is possible that aberrant signaling through pathways activated by EGFR, also a target of lapatinib, play a role in lapatinib resistance. Our laboratory is currently developing cell line models of acquired lapatinib resistance; these cell lines in combination with the de novo resistant cells identified in the present study will be used to study the signaling alterations associated with lapatinib resistance.

Taken together, these data indicate that HER2-amplified breast cancer cells fall into one of three response categories with respect to the two approved HER2 inhibitors. Some are sensitive to both trastuzumab and lapatinib as individual agents. Some are sensitive to one agent but resistant the other, and some are resistant to both HER2-targeting agents. The molecular data indicate that overlapping as well as nonoverlapping mechanisms of resistance to these agents are likely to occur in HER2-positive breast cancers. Increased RTK activity may be a marker of sensitivity to lapatinib, whereas increased PI3K/AKT signaling may be associated with resistance to trastuzumab. Lastly, dephosphorylation of AKT may be a biomarker of response.
to both agents. These markers of response and resistance could facilitate the development of strategies to overcome resistance, such as pharmacologic targeting of additional RTK pathways or inhibition of the PI3K/AKT pathway to overcome trastuzumab resistance. These data also provide additional rationale for combined therapy with both HER2-targeting agents used together in HER2-positive breast cancers. The initial preclinical studies in HER2-positive breast cancer cells indicated that these two agents do act synergistically and could provide superior efficacy when compared with using either alone (34). The early clinical data from small studies indicate that this may be the case (50, 51), and this hypothesis is now being formally evaluated in the Adjuvant Lapatinib and/or Trastuzumab Optimization (ALTO) study.

Disclosure of Potential Conflicts of Interest

D.J. Slamon: GlaxoSmithKline, honoraria from Speakers Bureau. No other potential conflicts of interest were disclosed.

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