Response to trastuzumab, erlotinib, and bevacizumab, alone and in combination, is correlated with the level of human epidermal growth factor receptor-2 expression in human breast cancer cell lines

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Abstract

Human epidermal growth factor receptor-2 (HER2) and epidermal growth factor receptor (EGFR) heterodimerize to activate mitogenic signaling pathways. We have shown previously, using MCF7 subcloned cell lines with graded levels of HER2 expression, that responsiveness to trastuzumab and AG1478 (an anti-EGFR agent), varied directly with levels of HER2 expression. HER2 and EGFR up-regulate vascular endothelial growth factor (VEGF), a growth factor that promotes angiogenesis and participates in autocrine growth-stimulatory pathways that might be active in vitro. Here, we show that trastuzumab, erlotinib, and bevacizumab, individually and in combination, inhibit cell proliferation in a panel of unrelated human breast cancer cell lines, in proportion to their levels of HER2 expression. The combination of all three drugs provided a greater suppression of growth than any single drug or two-drug combination in the high HER2–expressing cell lines (P < 0.001). Combination index analysis suggested that the effects of these drugs in combination were additive. The pretreatment net level of VEGF production in each cell line was correlated with the level of HER2 expression (r = 0.883, P = 0.016). Trastuzumab and erlotinib each reduced total net VEGF production in all cell lines. Multiparameter flow cytometry studies indicated that erlotinib alone and the triple drug combination produced a prolonged but reversible blockade of cells in G1, but did not increase apoptosis substantially. These studies suggest that the effects of two and three-drug combinations of trastuzumab, erlotinib, and bevacizumab might offer potential therapeutic advantages in HER2-overexpressing breast cancers, although these effects are of low magnitude, and are likely to be transient. [Mol Cancer Ther 2007;6(10):2664 – 74]

Introduction

Human epidermal growth factor receptor-2 (HER2), a member of the epidermal growth factor receptor (EGFR) family of tyrosine kinases, is overexpressed in 25% to 30% of human breast cancers (1). It has been implicated in cancer progression (2, 3), and has been identified as a prognostic and predictive marker for breast cancer outcome (4). Clinically, chemotherapeutic regimens that include trastuzumab (Herceptin), a humanized monoclonal antibody directed against the extracellular domain of HER2, prolong the time to progression and increase overall survival in patients with advanced disease whose tumors amplify/overexpress HER2 (5, 6). In the adjuvant setting, trastuzumab increases disease-free survival and overall survival in patients whose tumors amplify/overexpress HER2 (7, 8).

EGFR is overexpressed in 15% to 35% of breast cancers, and EGFR overexpression is associated with a poor prognosis (9, 10). Agents that target EGFR, including erlotinib (Tarceva/OSI-774), are currently approved for the management of non–small cell lung cancer, colon cancer, pancreatic, and head and neck cancers (11, 12). Responses are seen in 8% to 20% of patients with non–small cell lung cancer, with higher response rates in a subset of patients whose tumors contain mutations involving the kinase domain of EGFR (13, 14). Although preclinical cell culture and xenograft studies suggest that EGFR-targeted therapies may be active in breast cancer, clinical trials have shown responses in <10% of patients (15).

HER2 and EGFR heterodimerize with one another to drive mitogenic signaling pathways, and this interaction is likely to play a key role in cancer progression in breast cancer cells that overexpress HER2 and express EGFR. In preclinical studies, combinations of EGFR and HER2 targeting agents were found to be more effective than single targeted therapy agents. Combinations of EGFR-targeted agents erlotinib or gefitinib (Iressa/ZD1839) with the HER2-targeted agents trastuzumab or pertuzumab (Omnitarg/2C4) have shown additive or synergistic effects on growth inhibition of cancer cells in vitro and/or in vivo (16–19). Clinical results with a combination of erlotinib and
trastuzumab suggests that this combination is well tolerated, and the addition of erlotinib may be able to overcome resistance to trastuzumab, but the in vitro activity of the combination has not yet been borne out in the clinic (20).

Breast cancer cell lines and primary breast cancers can express vascular endothelial growth factor (VEGF), or its receptors (21–25), and increased VEGF expression in breast cancers is associated with tumor progression and increased risk of recurrence (26). Although the antiangiogenic effects of agents targeted against VEGF have been emphasized in cancer (27–29), tumors that express both VEGF and its receptors can participate in autocrine/paracrine feedback loops (30–32), and the specific effects of anti-VEGF agents on these autocrine loops can be studied in vitro, where angiogenesis is not at issue.

HER2 can regulate VEGF production in human breast cancer cell lines (33, 34), and VEGF levels are correlated with HER2 expression in primary breast cancers (35, 36). EGFR can also up-regulate the expression of VEGF in cancer cell lines (37–39), and inhibition of EGFR activity reduces VEGF production and angiogenesis in vitro (38, 40, 41).

Given that HER2 and EGFR can heterodimerize to initiate signal transduction, and that each can regulate VEGF production, it would seem reasonable to compare the effects of inhibition of EGFR, HER2, and VEGF, individually and in combination, in breast cancer cell lines. We have shown previously that responses to AG1478 (a small molecule EGFR inhibitor) alone, or in combination with trastuzumab, were correlated with levels of expression of HER2 in a series of MCF7 breast cancer cell line subclones that expressed graded levels of HER2 (42).

In this study, we examine this relationship in genetically unrelated human breast cancer cell lines using clinically relevant concentrations of the EGFR inhibitor erlotinib, and we have extended our studies to include the VEGF-inhibiting antibody bevacizumab (Avastin). We show that the triple combination of erlotinib, trastuzumab, and bevacizumab provided a greater suppression of growth than any of these drugs alone or any possible two-drug combination. The pretreatment net level of VEGF production in each cell line was correlated with the level of HER2 expression, and trastuzumab and erlotinib each reduced net VEGF production in all cell lines. Multiparameter flow cytometry studies indicated that all three drugs produced a prolonged, reversible blockade of cells in G1 but did not increase apoptosis substantially at clinically relevant concentrations.

Materials and Methods

Cell Culture

The HER2 stable transfectant cell lines NH29, NH47, and NH27 (derivatives of the MCF7 breast cancer cell line), were generated as described previously (42), and were maintained in RPMI 1640 containing 100 units/mL of penicillin and streptomycin, 0.25 μg/mL of amphotericin B, and 10% fetal bovine serum supplemented with 500 μg/mL of geneticin. MCF7, MDA-231, and SKBR3 breast cancer cell lines (American Type Culture Collection) were cultured in the same media without geneticin. The BT474 cell line (American Type Culture Collection) was maintained in DMEM containing 100 units/mL of penicillin and streptomycin, 0.25 μg/mL of amphotericin B, and 10% fetal bovine serum. The MDA-361 cell line (American Type Culture Collection) was maintained in Leibovitz's L-15 Media containing 100 units/mL of penicillin and streptomycin, 0.25 μg/mL of amphotericin B, and 10% fetal bovine serum in a CO2-free incubator.

Immunoblot Analysis

Cell lysate (50 μg) was subjected to SDS-PAGE on 4% to 20% tris/glycine gels, transferred to nitrocellulose filters, and blocked for 30 min in TTBS [100 mmol/L Tris (pH 7.5), 0.9% NaCl, 0.1% Tween 20] with 5% nonfat dry milk. A monoclonal antibody against HER2 (CB11; Novacastra Labs.) was used at a 1:1,000 dilution. The secondary antibody was goat anti-mouse horseradish peroxidase–conjugated antibody (Santa Cruz Biotechnology), and was used at a 1:1,000 dilution. For the supplemental data, a polyclonal antibody against the insulin-like growth factor-IR (Santa Cruz Biotechnology) was used at a 1:200 dilution, and the secondary antibody was goat anti-rabbit horseradish peroxidase–conjugated antibody (Santa Cruz Biotechnology), used at a 1:1,000 dilution. Antibody signal was visualized using the SuperSignal West Fico Chemiluminescent Substrate (Fierce) exposed to Kodak BioMax XAR film. Densitometry was done using a Personal Densitometer SI (Molecular Dynamics), and the data were analyzed using Scion Image for Windows (Scion Corporation).

Growth Inhibition Assays

Trastuzumab and bevacizumab were provided by Genentech, erlotinib was provided by OSI Pharmaceuticals, and the anti–insulin-like growth factor-IR antibody IMC-A12 was provided by ImClone Systems. Trastuzumab, bevacizumab, and IMC-A12 were stored at 4°C. Erlotinib, VEGF receptor (VEGFR) inhibitor (Calbiochem), and etoposide (Calbiochem) were stored as 10 mmol/L stocks in DMSO at −20°C. Cell stocks were grown to 80% confluence, trypsinized, and plated in duplicate into 24-well tissue culture plates at 1 to 2 × 104 cells/well. The following day, duplicate wells (for day 0 counts) were trypsinized and each well was counted with a hemocytometer. The remaining wells were fed on days 0 and 4 with regular cell culture media supplemented with the inhibitors, DMSO, or human IgG as vehicle controls. On day 6, the cells were trypsinized and counted with a hemocytometer, and the data were graphed in Excel. The data presented are from at least three separate experiments.

VEGF Analysis by ELISA

Endogenous VEGF production and secretion from cell lines was assayed using the Quantikine human VEGF immunoassay (R&D Systems). Cells were plated and grown as for the growth inhibition assays above (no drug, or 1 μg/mL trastuzumab, or 2 μmol/L erlotinib), and prior to cell counting, the medium from each well was removed, spun down to remove particulate matter, and used for the immunoassay according to the manufacturer’s instructions.
using fresh medium as a negative control, and a concentration standard provided with the immunoassay. The assay was analyzed using an ELx800 microplate reader (BioTek). The amount of VEGF (pg/mL) in each cell line was derived using the provided standard, then divided by the number of cells to obtain the value (pg/mL/cell), and the data were graphed in Excel. The data presented are from at least three separate experiments.

Flow Cytometry Studies of Cell Cycle and Apoptosis

SKBR3 cells were plated into T75 flasks at 1.65 \times 10^6 cells/flask. The following day, the cells were treated with 1 \mu g/mL of trastuzumab, 2 \mu m or 10 \mu mol/L of erlotinib, 1 \mu g/mL of bevacizumab, or a combination of all three, with or without 500 nmol/L of etoposide for 0, 12, 24, 36, 48 or 72 h. After drug treatment, the media were collected, adherent cells were trypsinized and pooled with their respective media, spun down, and washed once with PBS. Samples were divided into two aliquots, and one aliquot was fixed in 1.0 mL of cold PBS and 3.0 mL of cold 90% methanol for DNA analysis and the other aliquot was fixed in 3.0 mL of 0.5% paraformaldehyde at room temperature for 15 min, washed once in PBS, and resuspended in 1.0 mL of cold PBS and 3.0 mL of cold 90% methanol. Samples were stored at \(-20^\circ C\) until the time of staining and analysis.

For DNA histogram analysis, cells fixed with 90% methanol were stained with 50 \mu g/mL of propidium iodide and 1 mg/mL of RNase (Sigma) for 1 h at 4°C, and analyzed by flow cytometry. For multiparameter flow cytometry apoptosis studies, paraformaldehyde/methanol-fixed samples were first subjected to the terminal transferase dUTP nick end labeling method to detect apoptosis using the APO-DIRECT KIT (BD Biosciences), according to the manufacturer’s instructions. The samples were washed in PBS and stained with 4',6-diamidino-2-phenylindole (Sigma) for 1 h at 4°C to measure cell DNA content.

Measurements were made on each of several thousand cells using an EPICS ELITE flow cytometer (Beckman-
Coulter) using a 15-mW air-cooled argon laser emitting at 488 nm with a 550 nm dichroic long-pass filter and a 525 nm band pass filter for FITC, and a water-cooled 5 W laser with UV capabilities emitting at 325 nm with a 381 nm band pass filter for 4,6-diamidino-2-phenylindole. All measurements were collected and stored in listmode. DNA measurements were collected in 1,024 channels and later converted to a 256-channel format, and the FITC measurements were collected in 256 channels. The data presented are from at least three separate experiments.

**Data Analysis**

Statistical analyses were done using SPSS version 12.0 for Windows. HER2 expression, cell growth, and drug treatment data were analyzed by one-way ANOVA followed by a two-sided Dunnett’s test post hoc for the determination of differences between groups. Pearson correlation coefficients of linear regression were calculated for the $-\log$ of surviving cell fraction versus relative HER2 expression and net VEGF production versus relative HER2 expression.

The combination index (CI) for the triple drug effect in each cell line was calculated from the experimentally determined surviving cell fractions of cells treated with each drug alone, SF$_A$, SF$_B$, and SF$_C$, and the surviving cell fraction for the triple drug combination, SF$_{ABC}$. The CI was calculated as the observed surviving fraction/expected

![Figure 2](image-url)

**Figure 2.** Effects of drugs and drug combinations on cell proliferation, and correlation between growth inhibition and HER2 expression level. A, cells were plated and treated in duplicate with DMSO or human IgG (controls), 2 $\mu$mol/L of erlotinib alone (red columns), 1 $\mu$g/mL of trastuzumab alone (yellow columns), 1 $\mu$g/mL bevacizumab alone (light blue columns), erlotinib + trastuzumab (burgundy columns), erlotinib + bevacizumab (orange columns), trastuzumab + bevacizumab (dark blue columns), or a combination of all three drugs (black columns) for 8 days. Final cell numbers from the drug-treated samples were normalized to those of control cells treated with vehicle only. Columns, means; bars, SE. B, drug-induced growth inhibition, expressed as $-\log$ surviving cell fraction, from trastuzumab (1 $\mu$g/mL), erlotinib (2 $\mu$mol/L), bevacizumab (1 $\mu$g/mL), IMC-A12 (1 $\mu$g/mL), and the triple drug combination (trastuzumab at 1 $\mu$g/mL, erlotinib at 2 $\mu$mol/L, bevacizumab at 1 $\mu$g/mL) was plotted against relative HER2 expression levels of each cell line to determine the correlation between drug effects and HER2 expression level. Drug effect, as $-\log$ of surviving cell fraction, is plotted on the ordinate, and mean HER2 expression is plotted on the abscissa. Top, the correlation coefficient ($r$) and $P$ value ($P$) for each correlation. Bars, confidence intervals.
Table 1. Comparisons of effects of drug combinations on surviving cell fraction by HER2 expression level

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<th>HER2 expression level</th>
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<td>Low</td>
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<td>0.89 ± 0.01</td>
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<td>Intermediate</td>
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<td>P = 0.011</td>
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NOTE: Paired t tests were done on the data from Fig. 2A to compare the results of treatment regimens by HER2 expression level group. The means (± SE), and P value (P) of single-drug treatments are compared with those of the two-drug regimens (One-drug to two-drug regimen), and to the three-drug regimen (One drug to three-drug regimen); also, the results of the two-drug regimens are compared with the three-drug regimen (Two-drug to three-drug regimen), and the data are grouped according to HER2 expression level (Low, Intermediate, High). Comparisons that are statistically significant (P < 0.05) are in boldface. Abbreviations: E, erlotinib; T, trastuzumab; B, bevacizumab; ET, erlotinib + trastuzumab; EB, erlotinib + bevacizumab; TB, trastuzumab + bevacizumab; ETB, erlotinib + trastuzumab + bevacizumab.

Dose Response Effects of Targeted Agents

Dose response curves were obtained in different cell lines for trastuzumab, erlotinib, bevacizumab, and the anti-insulin-like growth factor-IR antibody IMC-A12 (Fig. 1C). For trastuzumab, the lowest drug concentration that produced the maximum or near-maximum drug effect was 1 μg/mL (Fig. 1C1). For erlotinib, in the most sensitive cell lines studied (SKBR3 and NH27), the IC50 was close to 1 μmol/L (Fig. 1C2). For bevacizumab, SKBR3 was the most sensitive cell line, with an IC50 of close to 1 μg/mL (Fig. 1C3), with no further increase in drug effect at higher concentrations. In contrast to all other drugs, IMC-A12 was most sensitive in the low HER2-expressing cell lines MCF7 and MDA-231, where the IC50 concentration was 1 μg/mL (Fig. 1C4). In subsequent studies, trastuzumab, bevacizumab, and IMC-A12 were each used at 1 μg/mL. Erlotinib was used at 2 μmol/L, in keeping with previously reported clinical steady state plasma concentrations in the range of 2 to 4 μmol/L (44, 45). These studies suggest that high HER2-expressing breast cancer cell lines exhibit greater growth inhibition in response to trastuzumab, erlotinib, or bevacizumab, than the low HER2-expressing cell lines over a range of concentrations. On the other hand, the low HER2-expressing breast cancer cell lines exhibit the greatest growth suppression with IMC-A12. The effects of IMC-A12 are shown here solely for contrast purposes, to demonstrate that not all ligand or receptor-targeted drugs suppress growth in proportion to HER2 expression level. However, although we suggest here that the combinations of drugs,
the effects of which positively correlate with HER2 expression, should be considered for combination therapy, the effects of IMC-A12 suggest that one should not rule out investigations of other combinations. IMC-A12 shows a negative correlation with HER2 expression (Fig. 2), but can provide additional benefits in high HER2–expressing breast cancer cell lines in combination with trastuzumab (Supplemental Data),1 in keeping with the suggestion that the insulin-like growth factor-IR can contribute to resistance to trastuzumab (46).

**Growth-Inhibitory Effects of Targeted Agent Combinations and Correlations of Drug Effects with Levels of HER2 Expression**

We compared the growth-inhibitory effects of individual erlotinib, trastuzumab, and bevacizumab with the effects of all possible two-drug combinations and the three-drug combination in a larger panel of human breast cancer cell lines (Fig. 2A). In the low HER2–expressing cell lines, the single agents and the two-drug combinations produced growth inhibition that was in the range of 30% or less, and the three-drug combination provided no advantage over the best single drug or two-drug combination. In cell lines with intermediate and high levels of HER2 overexpression, trastuzumab, erlotinib, and bevacizumab were progressively more effective in inhibiting tumor cell growth, especially when used in combination. In the intermediate HER2–expressing cell line group, although the three-drug combination and many two-drug combinations provided greater inhibition of proliferation over the single agents, not all of the possible one-drug to two-drug comparisons were statistically significant (Table 1). In the high HER2–expressing group, the three-drug combination produced a statistically significantly greater inhibition of cell proliferation over any single drug or two-drug combination (Table 1), and whereas the overall magnitude of growth suppression was relatively minor, produced the greatest growth inhibition overall in these experiments (Fig. 2A).

Correlation of growth-inhibitory effect of individual drugs to HER2 expression level showed statistically significant ($P = 0.05$) and strong correlations in the different cell lines in response to trastuzumab, erlotinib, and bevacizumab, and an inverse correlation between the degree of growth inhibition and level of HER2 expression in response to trastuzumab, erlotinib, and bevacizumab, and an inverse correlation between the degree of growth inhibition and level of HER2 expression (Fig. 2B). However, the strongest correlation was achieved with the three-drug combination ($r = 0.974, P = 0.0001$; Fig. 2B). CI were done for the three-drug combination (ref. 43; Table 2). Overall, the CIs suggest that the combined drug effects were additive, but in the low and intermediate HER2–expressing groups, in which there were no statistically significant differences in drug effect between the single agents and the combinations, the CI results are difficult to interpret.

**Evidence for VEGF Autocrine Loops in Breast Cancer Cell Lines**

Although the role of bevacizumab as an antiangiogenic agent has been emphasized in studies *in vitro* (27–29), other mechanisms must be invoked to account for its effects *in vivo*. Autocrine loops involving VEGF have been identified in human tumor cell lines and in various cancers (30–32). To investigate the role of such autocrine loops in our studies, we did dose response studies on these cell lines using a VEGFR inhibitor that has specificity for the VEGFRs Flt1 and KDR (Fig. 3A). This VEGFR inhibitor, like bevacizumab, suppressed cell growth to a greater degree in high HER2–expressing cell lines (SKBR3, MCF7, BT474), than in low HER2–expressing cell lines (MDA-231, MCF7). Figure 3B shows the net production of VEGF (pg/mL/cell), secreted into the culture medium over 6 days of growth by

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1 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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the cell lines, which suggests that the cells expressing high levels of HER2 produced the highest levels of VEGF per cell. As shown in Fig. 3C, there is a statistically significant correlation between VEGF production and the level of HER2 expression per cell (r = 0.883, P = 0.016). Trastuzumab and erlotinib each reduced net VEGF production to varying degrees in comparison with untreated controls (Fig. 3D), but there was no significant correlation between the degree of reduction of VEGF and HER2 expression. The VEGF-reducing effect of bevacizumab could not be determined by ELISA because the drug itself interferes with the assay.

Inhibition of Cell Cycle Progression and Apoptosis

The inhibition of tumor cell growth may be due to the inhibition of cell cycle progression, due to apoptosis, or both. We compared the effects of the three-drug combination to the effects of erlotinib alone on these processes in the high HER2–expressing cell line SKBR3 (Fig. 4). Serial DNA histograms obtained over 72 h from the initiation of continuous drug exposure are shown for untreated control cells (Fig. 4A1), erlotinib alone at a concentration of 2 μmol/L (Fig. 4A2), and the three-drug combination with erlotinib at 2 μmol/L (Fig. 4A3). The effects of erlotinib alone on the G1 cell fraction over time seem relatively small (compare Fig. 4A2 with Fig. 4A1), but they persist in replicate studies (see Fig. 4B1). To better appreciate the contribution of erlotinib to G1 cell cycle blockade, we used etoposide, a topoisomerase II inhibitor, which blocks cells in the G2 phase of the cell cycle. This agent prevents cells from proceeding through mitosis and reentering G1, where they might obscure the G1 blocking effects of the targeted agents. When the effects of erlotinib plus etoposide are compared with those of etoposide alone, the G1 blocking effect of erlotinib is shown more clearly by 72 h of treatment (see arrows at 72 h in Fig. 4A4 and A5). When comparing the G1 blocking effects of the three-drug combination to erlotinib alone, adding etoposide also reveals a greater effect of the triple drug combination over erlotinib alone (arrows, Fig. 4A5 and A6).

Quantitative comparisons of the cell cycle effects are shown in Fig. 4B. The triple drug combination with erlotinib at 2 μmol/L produced a modest increase of up to 1.2-fold in the G1 cell fraction over the 72 h treatment period (Fig. 4B1), and an ~0.7-fold decrease in the fraction of cells in S phase (Fig. 4B2), but there were no clear changes in the fraction of cells in G2-M with either erlotinib alone or in the triple drug combination (Fig. 4B3). Because erlotinib has been used at conservative doses in the clinic (44, 45), we increased the concentration of erlotinib to 10 μmol/L and examined its effects alone and in the three-drug combination. G1 blockade (Fig. 4B4), and the decrease in the fraction of cells in S phase (Fig. 4B5) are more pronounced during the 72-h observation period, and a late decrease in the fraction of cells in G2-M can be observed (Fig. 4B6).

Figure 4C shows the normalized cell concentration and the corresponding apoptotic fractions at the end of 72 h in control cells and triple drug–treated cells with erlotinib at 2 and 10 μmol/L. There was no substantial net decrease in total cell concentration, even with the high erlotinib concentration (Fig. 4C1), suggesting that much of the growth-inhibitory effects observed in our studies might be due to transient cell cycle arrest rather than to apoptosis. The time course of the apoptotic cell fraction measured by the terminal transferase dUTP nick end labeling assay (see Materials and Methods), showed that considerable apoptosis was observed after only 24 h with exposure to the drug combination with the 10 μmol/L erlotinib concentration (Fig. 4C2).

Discussion

In a previous study using a panel of clonally related breast cancer cell lines (42) we showed that a combination of trastuzumab and AG1478 (an EGFR inhibitor), produced

Table 2. Mean survival fractions for the individual drugs and the three-drug combination, and CI for the three-drug combination treatment

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<tr>
<th></th>
<th>Mean survival fraction with erlotinib</th>
<th>Mean survival fraction with trastuzumab</th>
<th>Mean survival fraction with bevacizumab</th>
<th>Mean expected survival fraction (for additivity)</th>
<th>Mean observed survival fraction with the erlotinib + trastuzumab + bevacizumab combination</th>
<th>Mean CI</th>
<th>P value (CI)</th>
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<td>MDA-231</td>
<td>0.852 ± 0.052</td>
<td>0.971 ± 0.053</td>
<td>0.870 ± 0.024</td>
<td>0.718 ± 0.024</td>
<td>0.703 ± 0.108</td>
<td>0.978 ± 0.140</td>
<td>0.778</td>
</tr>
<tr>
<td>NH29</td>
<td>0.749 ± 0.043</td>
<td>0.805 ± 0.021</td>
<td>0.833 ± 0.036</td>
<td>0.502 ± 0.022</td>
<td>0.572 ± 0.092</td>
<td>1.134 ± 0.141</td>
<td>0.153</td>
</tr>
<tr>
<td>NH47</td>
<td>0.773 ± 0.014</td>
<td>0.768 ± 0.045</td>
<td>0.736 ± 0.031</td>
<td>0.438 ± 0.047</td>
<td>0.592 ± 0.028</td>
<td>1.370 ± 0.206</td>
<td>0.091</td>
</tr>
<tr>
<td>MDA-361</td>
<td>0.656 ± 0.083</td>
<td>0.654 ± 0.063</td>
<td>0.712 ± 0.051</td>
<td>0.309 ± 0.083</td>
<td>0.566 ± 0.135</td>
<td>1.860 ± 0.422</td>
<td>0.072</td>
</tr>
<tr>
<td>NH27</td>
<td>0.428 ± 0.066</td>
<td>0.639 ± 0.035</td>
<td>0.611 ± 0.078</td>
<td>0.170 ± 0.051</td>
<td>0.195 ± 0.081</td>
<td>1.126 ± 0.306</td>
<td>0.550</td>
</tr>
<tr>
<td>SKBR3</td>
<td>0.485 ± 0.059</td>
<td>0.643 ± 0.055</td>
<td>0.490 ± 0.095</td>
<td>0.155 ± 0.046</td>
<td>0.252 ± 0.091</td>
<td>1.710 ± 0.730</td>
<td>0.095</td>
</tr>
<tr>
<td>BT474</td>
<td>0.583 ± 0.094</td>
<td>0.410 ± 0.029</td>
<td>0.794 ± 0.065</td>
<td>0.188 ± 0.026</td>
<td>0.157 ± 0.069</td>
<td>0.807 ± 0.256</td>
<td>0.230</td>
</tr>
</tbody>
</table>

NOTE: Mean survival fraction values for the individual drug treatments and the three-drug treatment, and CI values for the three-drug treatment were obtained for each cell line from triplicate studies shown Fig. 2A. The P values for each cell line indicate the level of statistical significance of the CI compared with a CI of 1.0. Mean CI values significantly greater than 1.0 (P < 0.05), indicate antagonism, values not significantly different from 1.0 (P > 0.05) indicate additivity, and values less than 1.0 (P < 0.05) indicate synergy (±SE).
greater growth inhibition than the effects of either drug individually, and that the magnitudes of the effect of these agents correlated with the levels of HER2 expression. In this article, we have examined the effects of trastuzumab, erlotinib (a clinically relevant EGFR inhibitor), and the VEGF-inhibiting antibody bevacizumab on cell proliferation, cell cycle arrest, and apoptosis in relation to levels of HER2 expression in a panel of unrelated human breast cancer cell lines. We have shown that erlotinib (like AG1478), produces growth inhibition that varies directly with the level of HER2 expression (Fig. 2A and B). This finding additionally supports the possibility that HER2 expression level might be useful as a clinical marker for the combination of anti-HER2 and anti-EGFR therapies in breast cancer.

Clinical trials investigating the effects of various two-drug combinations of trastuzumab, erlotinib, and bevacizumab are currently under way in several different cancer types, with promising results (20). An early trial of the combination of erlotinib and trastuzumab in patients with trastuzumab-resistant breast cancer suggested that the two-drug combination might overcome trastuzumab resistance (20). Lapatinib, a drug that inhibits both EGFR and HER2, has shown response rates as a first line agent (20), and has increased the time to progression in combination with capecitabine in patients with breast cancer refractory to trastuzumab.

The rationale for pursuing bevacizumab in combination with other agents has been based largely on its known antiangiogenic effects (27–29). In addition, there are active autocrine growth loops involving VEGF in various tumor cell types including breast cancer cells (30–32). Because the antiangiogenic effects of bevacizumab are not applicable in vitro, the growth-inhibitory effects of this agent in our studies (Figs. 1 and 2) might well be due to interference with autocrine VEGF signaling loops. Our findings that the effects of an agent targeted against VEGFR were similar to those of bevacizumab support this premise (Fig. 3A). That is, they suggest that VEGFRs are present and active in our cell lines, and that interference with their function produced effects that were comparable to those produced by interfering with VEGF itself. The finding that both agents were more effective in cell lines with high HER2 overexpression suggests the possibility that VEGF autocrine loops might be more active in tumors with high HER2 expression.
The activation of HER2 and/or EGFR signaling pathways is known to lead to increased VEGF production (33, 34, 37, 39). This is supported by our findings that trastuzumab and erlotinib each reduced net VEGF production in the tumor cell lines (Fig. 3D). This raises the possibility that the growth-inhibitory effects of trastuzumab and erlotinib in breast cancer might be due in part to the disruption of VEGF autocrine loops. However, neither trastuzumab nor erlotinib reduced net VEGF production in a manner that directly correlated with HER2 expression level, suggesting that the effects of these drugs on VEGF production are not the sole mechanism for their growth suppression.

We have shown that the three-drug combination of trastuzumab, erlotinib, and bevacizumab produced a statistically significant decrease in growth over any of the two-drug combinations or single agents in the cell lines with the highest levels of HER2 expression (Fig. 2A; Table 1). CI analysis indicates that the effect of these agents in the three-drug combination is neither antagonistic nor synergistic (Table 2), suggesting that the effects are additive. Although the overall magnitude of effects of all individual drugs and drug combinations are minor, the three-drug combination provided the best suppression of growth.

These findings support the pursuit of breast cancer clinical trials using the three-drug combination, or the use of small molecule inhibitors that have shown activity against HER2, EGFR and VEGFR, such as ZD6474 (Zactima) or AEE788 (20, 47). Based on our studies, one might anticipate that these agents would be even more effective against tumors that express very high levels of

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**Figure 4.** Effects of drug treatment on the cell cycle and apoptosis. A, DNA histograms of drug effects on cell DNA content and cell cycle perturbation at 0, 12, 24, 36, 48, and 72 h of drug treatment. A1, no drug; A2, 2 μmol/L of erlotinib; A3, three-drug combination with 2 μmol/L of erlotinib; A4, etoposide alone; A5, etoposide + 2 μmol/L of erlotinib; A6, etoposide + the three-drug combination with 2 μmol/L of erlotinib. Arrows, G1 peak. B, normalized drug-induced changes in the fractions of cells in the G1, S, and G2-M phases of the cell cycle. B1–3, changes in the fractions with cells treated with no drug, erlotinib alone at 2 μmol/L, or the three drug combination with erlotinib at 2 μmol/L. B4–6, changes in the fractions with cells treated with no drug, erlotinib alone at 10 μmol/L, or the three drug combination with erlotinib at 10 μmol/L. C, effects of the concentration of erlotinib in the triple drug combination on net cell proliferation (T) and on total apoptosis as assayed by terminal transferase dUTP nick end labeling (2).
HER2 than trastuzumab alone. However, we would emphasize that our in vitro studies do not take into account the additional antiangiogenic effects of these agents, and the potential effects of antibody-dependent cell-mediated cytotoxicity associated with trastuzumab and other antibodies (48), which apply only in vivo, and which may act independently of HER2 expression levels.

The observed effects of therapeutic agents in the growth inhibition assay may be due to general cytostasis, due to the transient blockade of cells in one or more phases of the cell cycle, or due to cell death, commonly through apoptosis. In the absence of actual cell loss, one would not expect to observe an increase in objective tumor response rates. This basic principle is shown in the present study, in which the three-drug combination is shown to produce greater and more prolonged G1 blockade than erlotinib alone at clinically relevant drug concentrations (Fig. 4A and B), but without increasing the baseline apoptotic rate by terminal transferase dUTP nick end labeling assay throughout the 72-h course of these experiments (Fig. 4C2). This, in turn, was associated with a net increase in total cell number from time zero to the end of the growth inhibition study in Fig. 4C1. The transient sequestration of the majority of the drug-treated cells in G1 phase, and their delayed progression through S phase over a 48-h period (Fig. 4A and B) may be sufficient to account for most, if not all, of the differences between final control and drug-treated cell concentrations in the growth inhibition assay (Fig. 4C1). If these pharmacodynamic effects apply in vivo, then one might anticipate the need to rely on a clinical strategy that combines these targeted agents with chemotherapy.

We have shown here that the rate of apoptosis can be increased by increasing the concentration of erlotinib (Fig. 4C2) to levels that can be estimated to be ~3-fold higher than those commonly achieved with current clinical regimens. This would suggest that dose escalation of targeted agents might have the potential to improve clinical results, provided that clinical toxicity remains tolerable. In this regard, dose escalation of small molecule–targeted agents like erlotinib may be easier to manage clinically in comparison with targeted antibodies, largely because of the relatively short biological half-lives of the former.

In summary, we have shown here that in a panel of human breast cancer cell lines in vitro, the growth-inhibitory effects of trastuzumab, erlotinib, and bevacizumab, alone and in combination, are strongly correlated with HER2 expression, and that the combination of all three drugs provides a statistically significantly greater inhibition of cell proliferation than any single drug or two-drug combination in high HER2–expressing cell lines. These effects are independent of antiangiogenic effects and antibody-dependent cell-mediated cytotoxicity, which occur only in vivo. They seem to be due, at least in part, to interference with VEGF-mediated autocrine loops. These agents produce acute pharmacodynamic changes that consist of transient blockade of cells in G1 phase, in the absence of significant apoptosis, when used at clinically relevant drug concentrations. Apoptosis was shown to increase with erlotinib dose escalation, suggesting that dose response may be an issue for targeted agents that might deserve attention in the clinical setting.

Acknowledgments

We thank Genentech for providing the trastuzumab and bevacizumab, OSI Pharmaceuticals for providing the erlotinib, and ImClone Systems for providing IMC-A12.

References

Response to trastuzumab, erlotinib, and bevacizumab, alone and in combination, is correlated with the level of human epidermal growth factor receptor-2 expression in human breast cancer cell lines

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