Treatment of HER-2/neu Overexpressing Breast Cancer Xenograft Models with Trastuzumab (Herceptin) and Gefitinib (ZD1839): Drug Combination Effects on Tumor Growth, HER-2/neu and Epidermal Growth Factor Receptor Expression, and Viable Hypoxic Cell Fraction

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ABSTRACT

Purpose: The purpose of this research was to assess the effects of single agent and combination treatment with trastuzumab and gefitinib on tumor growth and tumor microenvironment in two HER-2/neu overexpressing breast xenograft models, MDA-MB-435/LCC6HER-2 (LCC6HER-2; estrogen receptor negative) and MCF-7HER-2 (estrogen receptor positive).

Experimental Design: LCC6HER-2 and MCF-7HER-2 cells, both in tissue culture and xenografts grown in SCID-Rag 2M mice, were treated with trastuzumab and gefitinib, alone or in combination. The rate of tumor growth was determined in addition, tumor HER-2/neu and epidermal growth factor receptor expression, cell viability, cell cycle distribution, and proportion of viable hypoxic cells were determined by flow cytometric analyses of single tumor cell suspensions.

Results: Both tumor models were very sensitive to trastuzumab and moderately sensitive to gefitinib in vivo. The combination resulted in therapeutic effects, as judged by inhibition of tumor growth, which was greater (albeit not statistically significant) than that observed with trastuzumab administered as a single agent. Trastuzumab was effective in down-regulating HER-2/neu, and gefitinib mediated a reduction in epidermal growth factor receptor expression on tumor cells. In LCC6HER-2 tumors, trastuzumab significantly reduced tumor cell viability, which was not improved by the addition of gefitinib. Gefitinib dramatically reduced the proportion of viable hypoxic cells in LCC6HER-2 and MCF-7HER-2 tumors. This effect was abrogated by the addition of trastuzumab.

Conclusions: Although in vivo efficacy studies in two HER-2/neu overexpressing breast xenograft models showed that the combination of trastuzumab and gefitinib was effective, analyses of various cellular parameters failed to reveal beneficial effects and argue that this drug combination may not be favorable.

INTRODUCTION

Approximately 25–30% of human breast cancers overexpress HER-2/neu, most commonly due to amplification of the c-erbB-2 proto-oncogene (1–4). In breast and ovarian carcinoma, overexpression of this receptor tyrosine kinase is associated with younger patient age, earlier disease recurrence, lymph node involvement, and increased level of metastases, resistance to endocrine therapy and poor survival (2–8). High levels of HER-2/neu have also been detected in a range of additional malignancies, including prostate, lung, uterine serous papillary, gastramias, and thyroid carcinomas (9–15). HER-2/neu belongs to a family of four type I receptor tyrosine kinases with an overall sequence homology of 40–50% (16–18). High affinity binding of a peptide ligand to the extracellular domains of family members epidermal growth factor receptor (EGFR), HER-3, and HER-4, but not HER-2/neu, initiates receptor homo- or heterodimerization, followed by auto- or cross-phosphorylation of specific tyrosine residues within the COOH terminus of the dimerization partners (19). Selective recruitment of cytoplasmic adapter proteins to these phosphorylated tyrosines activates downstream signaling cascades, most prominently the mitogen-activated protein kinase pathway and the phosphatidylinositol 3-kinase/Akt pathway. The diverse signals prompt the transcriptional machinery of the cell to modulate apoptosis, survival, and mitogenesis (10, 20–27).

Low levels of expression in normal adult human tissue (28, 29) in conjunction with a relatively reliable expression profile over time and in distant metastases compared with the primary tumor (4, 30, 31) have made HER-2/neu an attractive target for therapy. Effective targeted therapy against HER-2/neu overexpressing breast cancers has been in place since trastuzumab, a humanized monoclonal antibody directed against the juxtamembrane domain of HER-2/neu (32), was approved for use in the...
clinic in 1998. Several investigators have demonstrated that trastuzumab exerts additive or synergistic effects on tumor cell growth inhibition in combination with a range of cytotoxic cancer drugs (33–35). These results have been confirmed in the clinic. Women suffering from metastatic breast cancer expressing high levels of HER-2/neu have benefited from treatment with trastuzumab, both in combination with classic cytotoxic drug regimens as well as single agent and first line treatment (36–38). This is reflected by statistically significant increases in overall response rate and survival time, and a reported improvement in quality of life (37, 39). The effects of monoclonal antibodies against HER-2/neu include down-regulation and a decrease in phosphorylation of HER-2/neu (40, 41), inhibition of cell cycle progression via up-regulation of the cell cycle inhibitor p27KIP1 (41, 42), and impairment of signaling through other HER family members (43). Furthermore, trastuzumab is capable of mediating antibody dependent cellular cytotoxicity in the presence of human peripheral blood leukocytes (14, 32, 44–46) as well as complement dependent cytotoxicity (14). Importantly, trastuzumab acts exclusively against tumor cells expressing high levels of HER-2/neu (23, 37).

HER-2/neu overexpressing tumor cells have been shown recently to be remarkably sensitive to gefitinib, a selective and reversible EGFR tyrosine kinase inhibitor of the 4-anilinoquinazoline-class (47–50). Gefitinib treatment contributed to regression in EGFR overexpressing xenografts in combination with cytotoxic drugs (51–53) and with radiotherapy (54). In addition, gefitinib has effectuated growth inhibition of tumor cells expressing only low levels of EGFR (48, 55, 56). Most importantly, patients with disease refractory to hormone therapy, radiotherapy, and/or several different chemotherapy regimens have benefited from single agent gefitinib in the form of partial responses, disease stabilization, and symptom improvement (57).

HER-2/neu, although unable to bind to any known ligand with high affinity, has been shown to act as a favored coreceptor in partnership with other HER family members (58, 59), where, for example, epidermal growth factor-like factor ligands stimulate the creation of EGFR-HER-2/neu heterodimers (21). Importantly, ligand-independent activation of HER-2/neu and of EGFR in HER-2/neu overexpressing tumor cells has been reported by Worthylake et al. (60). HER-2/neu enhances the signaling potency of its dimerization partner on multiple levels: heteroassociation with HER-2/neu leads to decreased ligand-dissociation from the early endosome to the cell surface, rather than shuttled to the lysosome for degradation as would be the fate of activated EGFR-EGFR homodimers (27, 61). As a result, increased numbers of activated receptors are available on the surface of HER-2/neu overexpressing cells, causing prolonged signaling (60, 61).

Treatment of HER-2/neu overexpressing breast tumors with a combination of both trastuzumab and gefitinib appears promising due to their distinct chemistries and sites of attack, precluding stereochemical interference and precipitation of chemistry-related side effects. Moreover, their biochemical effects, such as dephosphorylation of receptors, down-regulation of activated signaling molecules (47–49, 62), and cell cycle arrest through the up-regulation of p27KIP1 (47, 63), seem to converge. More specifically, previous studies by Normanno et al. (49) used in vitro cell-based screening assays to demonstrate that combinations of gefitinib and trastuzumab resulted in synergistic interactions as determined by the median effect principle developed by Chou and Talalay (64). Moulder et al. (47) augmented these results with in vitro studies demonstrating that gefitinib treatment could inhibit HER-2/neu phosphorylation and in vivo studies, which suggested that gefitinib enhanced the activity of trastuzumab when the combination was used to treat mice bearing established BT-474 (EGFR-positive, HER-2/neu-overexpressing) tumors. In the present study we investigated the effects of trastuzumab, gefitinib, and their combination on two mouse xenograft models for HER-2/neu overexpressing aggressive breast cancer: the MDA-MB-435/LCC6 HER-2/LCC6HER-2 model is characterized by negative estrogen receptor (ER) status, whereas the MCF-7HER-2 model is ER+ (65). Similar to previous studies, it is demonstrated that both xenograft models are highly sensitive to trastuzumab and moderately sensitive to gefitinib. The combination of drugs achieved improved effects as judged by inhibition of tumor growth. Because we have demonstrated recently that HER-2/neu overexpression is associated with a higher percentage of viable hypoxic tumor cells in the LCC6 model (66), flow cytometric analyses of cells obtained from tumors were completed to assess treatment effects on viable hypoxic cell fraction, as well as tumor cell expression of molecular targets. The results indicated that gefitinib treatment effected a dramatic reduction in the proportion of viable hypoxic tumor cells, a novel observation that is consistent with reports suggesting that gefitinib can act as a radiosensitizer (67). When combined with trastuzumab, this effect of gefitinib is lost.

MATERIALS AND METHODS

Cell Lines and Cell Culture. MDA-MB-435/LCC6 (LCC6) breast carcinoma cells were generously provided by Dr. Robert Clarke, Georgetown University (Washington, DC; Ref. 68). LCC6HER-2 cells were generated by electroporation of LCC6 cells (230 V, 500 μF) with the human expression plasmid pREP9 (Invitrogen, Burlington, Ontario, Canada) containing the full-length human c-erbB-2 cDNA. The human c-erbB-2 cDNA was a kind gift from Dr. Moulay Alaoui-Jamali (McGill University, Montreal, Quebec, Canada; Ref. 69). All of the cells were grown in DMEM (Stem Cell Technologies, Vancouver, Canada; 10% fetal bovine serum (FBS); Hyclone, Logan, UT) and 500 μg/ml Geneticin (Invitrogen), followed by three consecutive cycles of flow cytometry sorting of the top 5% expressors. MCF-7HER-2 and MCF-7NEO cells were a kind gift from Dr. Moulay Alaoui Jamali (McGill University, Montreal, Quebec, Canada; Ref. 69). All of the cells were grown in DMEM (Stem Cell Technologies, Vancouver, British Columbia, Canada), supplemented with 2 mM l-glutamine (Stem Cell Technologies), 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 500 μg/ml Geneticin (LCC6HER-2) or 100 μg/ml Geneticin (MCF-7HER-2), respectively. Cells were maintained at 37°C and 5% CO2 in a humidified atmosphere. For in vitro as well as animal studies, all of the cells were cultured without Geneticin for 1 week before the experiment. In vitro studies were performed in either normal serum conditions (10% FBS) or reduced serum conditions (0.1% FBS) in DMEM. HER-2/neu-transfected cell lines were used in these studies for three reasons: (a) many of the human cells lines...
that overexpress HER-2/neu due to gene amplification (e.g., SK-BR-3 cells) grow poorly or not at all in immune compromised mice; (b) the parental cell lines selected do not exhibit sufficient HER-2/neu levels to measure expression by standard immunohistochemical methods or flow based assay; and (c) the parental cell lines were not sensitive to Herceptin in vitro or in vivo.

In Vitro Drug Sensitivity Assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). To determine sensitivity of the LCC6HER-2 and MCF-7HER-2 breast cancer cell lines to both trastuzumab and gefitinib, cells were cultured under normal serum conditions (10% FBS) or serum-starved conditions (0.1% FBS). Drug was added at increasing doses of a fixed ratio of 0.1 μM gefitinib to 1.0 μg/ml trastuzumab, and after 96 h cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. LCC6HER-2 cells (1,000 and 4,000, normal or reduced serum, respectively) and 5,000 and 20,000 MCF-7HER-2 cells (normal or reduced serum conditions, respectively) were grown in triplicate in tissue culture-treated flat-bottomed 96-well plates (Falcon; BD, Franklin Lakes, NJ). Cells were allowed to adhere, and after 24 h serial dilutions of trastuzumab, gefitinib, or combination were added and incubated for 96 h. Trastuzumab (Hoffman-La Roche, Mississauga, Ontario, Canada; purchased from the British Columbia Cancer Agency Pharmacy) was reconstituted in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 2 mM KH2PO4) at a stock concentration of 21 mg/ml, and gefitinib (a kind gift from EF5 was a generous gift from Dr. Cameron Koch, University of Pennsylvania (Philadelphia, PA). Harvested tumors were rinsed in HBSS (Stem Cell Technologies) with 0.4% BSA (Fraction V; Sigma). The tumor was scalped into small pieces and incubated for 2 h, rotating at 37°C in HBSS, 1% BSA fraction V, and 250 units/ml collagenase type 2 and 4 (Worthington Biochemical Corporation, Lakewood, NJ). After incubation, tumor tissue pieces were disaggregated in three 1-min cycles in 50 μm Medicones in the Medimachine (both by BD Biosciences, San Jose, CA), with washes of ice cold PBS with 0.1% BSA fraction V (PBSB) between each run. Combined cells were pelleted and incubated with 1 ml 0.25 mM EDTA for 5 min at 37°C. Tumor cells were washed and resuspended in cold PBSB and kept on ice for additional processing.

Tumor Cell Viability. The flow cytometric analysis of propidium iodide (PI) stained cellular DNA content is the most commonly used assay to determine the fraction of apoptotic/necrotic cells after drug treatment in vitro. These in vitro assays are short term and focused on early apoptotic events taking place after exposure to drugs, where cellular fragmentation is not very extensive. Using untreated tumors, the trypan-blue exclusion assay correlated well with the assessment of the apoptotic/necrotic cell fraction (sub-G1/G0) by the PI staining method (R2 = 0.779; described below in “Flow Cytometric Analyses”). Treated tumors were harvested within 7–12 days after the last treatment; therefore, a significant cellular fragmentation of cells that have died as a result of drug treatment was anticipated. For this reason the PI method used to calculate the percentage of live/dead cells may overestimate the proportion of dead cells. By using the trypan-blue exclusion assay, the quality of the tumor cell suspension can be visually evaluated during the counting process, and cellular debris (identified as cellular membranes/paris without blue-stained nuclear material) as well as small cells of host origin (cells with 2 × host erythrocyte volume or smaller) can be easily excluded from viability counts.

Flow Cytometric Analyses. The EPICS ELITE ESP flow cytometer (Beckman-Coulter, Miami, FL), with the Innovex Enterprise 621 laser (Coherent, Santa Clara, CA), was used for all of the flow cytometric analyses. The Neu 24.7-FITC antibody (BD Biosciences, San Jose, CA) was used as an isotype control. EGFR cell surface expression was detected with NCL-R1-FITC (Novo Castra, Newcastle, United Kingdom; 1:1 v/v), and amplified with goat antianimal phycoerythrin (Tago, Inc., Burlingame, CA; 1:80 v/v); Goat-anti-human-FITC (Caltag Laboratories, Burlingame, CA) was used as isotype control. EGFR cell surface expression was detected with NCL-R1-FITC (Novo Castra, Newcastle, United Kingdom; 1:1 v/v), and amplified with goat antianimal phycoerythrin (Tago, Inc., Burlingame, CA; 1:80 v/v). Goat-anti-human-FITC (1:160, v/v; Caltag Laboratories) was used to detect trastuzumab bound to LCC6HER-2 tumor cells. Five × 10⁶ cells were blocked in 20% human serum in PBS with 1% BSA (Sigma; PBSB) for 10 min on ice and subsequently incubated with the appropriate antibody for 30 min. After three washes, cells were resuspended in PBSB containing 0.3 μg/ml PI (Sigma), used to discriminate between live and dead cells. Quantification of surface marker expression was performed as described previously (70).

Flow cytometric cell cycle and DNA analyses were performed on tumor cells fixed in 70% ethanol. One × 10⁶ cells were stained in PI buffer [50 μg/ml PI, 1 mg/ml RNase A (Sigma), and 0.1% Triton X-100 (Bio-Rad, Richmond, CA) in PBS] and incubated at 37°C for 15 min. Cells were then chilled on ice for 1 h and filtered through a 40-μm filter.
EF5 adducts bound to macromolecules in hypoxic cells were detected using the Cy3-conjugated ELK3–51 antibody, a gift from Dr. Cameron Koch. Tumor cells were fixed in 2% formaldehyde in PBSB and permeabilized in 1% Tween 20 (Sigma) followed by overnight blocking at 4°C. Cells (10⁶) were rotated for 3 h at room temperature with ELK3–51-Cy3 antibody, washed three times in PBSB with 0.5% Tween 20, and resuspended in 0.2 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma) in PBSB. On the basis of DAPI staining (DNA content) and Time of Flight parameters, cells with <2n DNA content, representative of host cells, necrotic/apoptotic cells, and debris, as well as cell aggregates, were gated out. The cells, which passed the above gating criteria, were considered viable. After gating, the percentage of viable hypoxic cells in tumor cell suspensions were calculated using a threshold set on fluorescence intensity of LCC6HER-2 control cells exposed to hypoxic (99.995% N₂ and 0.005% O₂) conditions for 3 h with 100 µM EF5.

**Definition of Controls.** An important factor in the study was the choice of appropriate untreated LCC6HER-2 and MCF-7HER-2 control tumors against which to compare cell surface marker expression, viability, and percentage of viable hypoxic tumor cells. Control tumors were harvested at different time points (maturity controls), as well as at different volume end points (volume controls), but grown for the same number of days. We found that the proportion of viable versus necrotic cells and percentage of viable hypoxic cells in cells recovered from tumors obtained from untreated LCC6HER-2 control mice was independent of tumor volume and tumor age at the time of harvest (R² < 0.129 in all cases), but that surface expression of HER-2/neu declined with advancing tumor age (R² = 0.794; results not shown). In untreated MCF-7HER-2 tumors, however, we observed a moderate to strong positive correlation between tumor age at time of harvest, and HER-2/neu expression (R² = 0.781) and EGFR-expression (R² = 0.759), as well as a strong negative correlation between tumor age and the percentage of viable tumor cells (R² = 0.952). The percentage of viable hypoxic cells was correlated with tumor age (R² = 0.33). These parameters bore no correlation to the volume (250–1000 mm³) of untreated control MCF-7HER-2 tumors harvested on the same day (R² < 0.16). For these reasons all of the cellular parameters of tumors obtained from treated mice were compared with control tumors of comparable age.

**Statistical Analyses.** All of the statistical analyses were performed using the STATISTICA software program. In vivo data were analyzed by performing a Scheffé test. This one-way ANOVA analysis can be used to compare unequal sample sizes and is often used to make unplanned comparisons. As such it tends to be viewed as a very conservative procedure that is less likely to show statistical significance. Our data, within the small group size, was shown to be normally distributed, and the variances were not different as determined by the Levene test. Multiple comparisons of drug-mediated tumor growth inhibition were performed on the final measurements obtained from all of the treatment groups. Combination index analyses were performed with the CalcuSyn program, based on the multiple drug effect equation by Chou and Talalay (Biosoft, Cambridge, United Kingdom; Ref. 64).

**RESULTS**

**Analysis of HER-2/neu and EGFR on the Surface of LCC6HER-2 and MCF-7HER-2 Cells**

Fig. 1. A and B, show HER-2/neu and EGFR expression patterns for LCC6HER-2 and MCF-7HER-2 cells, respectively. Using antibody binding capacity to estimate the number of molecules expressed on the cell surface, the LCC6HER-2 cells exhibited approximately 2.2–4.5 × 10⁵ HER-2/neu molecules and <5000 molecules of EGFR. In MCF-7HER-2 cells the estimated number of HER-2/neu molecules per cell was 1.6 × 10⁶, and the number of EGFR molecules was 1.3 × 10⁵. Moderate to strong HER-2/neu expression on the cell membrane in >10% of tumor cells is clinically required for eligibility for treatment with trastuzumab (71).

**In Vitro Treatment of LCC6HER-2 and MCF-7HER-2 Cell Lines with Trastuzumab, Gefitinib, or a Combination of Both**

Under normal serum conditions (10% FBS) the LCC6HER-2 breast cancer cell line was resistant to trastuzumab as well as to gefitinib treatment; incubation with 1.0 µM gefitinib and 10.0 µg/ml trastuzumab, respectively, left LCC6HER-2 cells unaffected by single agent as well as combination treatment (Fig. 2A). Treatment with 10 µg/ml trastuzumab in 10% FBS had no effect on MCF-7HER-2 cells, and 1 µM gefitinib achieved a growth inhibition of 13% (± 9.5%) versus control (Fig. 2B). Combination of both drugs resulted in a significant (26.1% ± 9.9%) growth inhibition of MCF-7HER-2 cells compared with untreated controls (P = 0.0153; Fig. 2B). Because cell culture medium supplemented with 10% FBS could provide the cells with an excess of growth factors overriding possible effects of trastuzumab (72), we repeated the experiment under reduced serum conditions (0.1% FBS). Under those conditions LCC6HER-2 cells were marginally affected by trastuzumab (up to 10% growth inhibition), whereas 1 µM gefitinib alone caused a 65.7% (± 7.1%) inhibition in cell proliferation compared with...
untreated controls \((P < 0.001; \text{Fig. 2C})\). Addition of 10 \(\mu\)g/ml trastuzumab to 1 \(\mu\)M gefitinib did not significantly increase cytotoxicity (Fig. 2C).

For MCF-\(7^\text{HER-2}\) cells, a 70.2\% (\pm 10.9\%) growth inhibition was observed when the cells were incubated with 1.0 \(\mu\)M gefitinib under serum-starved conditions, and single agent trastuzumab, at 10.0 \(\mu\)g/ml, resulted in 48.3\% (\pm 5.6\%) growth inhibition \((P = 0.0001 \text{ and } P = 0.0035, \text{respectively; Fig. 2D})\). The combination of trastuzumab and gefitinib achieved a growth inhibition of 83.2\% (\pm 6.7\%) versus control, which is not significantly different from gefitinib alone \((P = 0.638; \text{Fig. 2D})\). No additional benefit was observed at concentrations above 1.0 \(\mu\)M gefitinib and 10 \(\mu\)g/ml trastuzumab (data not shown).

Combination index analyses performed with the CalcuSyn program indicated that trastuzumab did not potentiate the effect of gefitinib on LCC6\(^\text{HER-2}\) tumor cells cultured in 0.1\% FBS. However, the combination effect of lower doses of trastuzumab and gefitinib on MCF-\(7^\text{HER-2}\) cells appeared to be synergistic under serum-starved conditions. Combination index (CI) values for the combinations of 2.5 -10 \(\mu\)g/ml trastuzumab with 0.25–1.0 \(\mu\)M gefitinib were between 0.442 and 0.61; however, at a combination of 25 \(\mu\)g/ml trastuzumab and 2.5 \(\mu\)M gefitinib the CI value rose to 1.21, a value that is considered to be slightly antagonistic (data not shown). Due to the lack of activity of trastuzumab in 10\% serum conditions, combination index analyses were not performed on cells cultured in 10\% FBS.

**In Vivo Response of the LCC6\(^\text{HER-2}\) and MCF-\(7^\text{HER-2}\) Xenograft Models to Trastuzumab and Gefitinib**

Because we did not observe any strong interactions between trastuzumab and gefitinib in vitro (Fig. 2), we analyzed the effects of trastuzumab and gefitinib, alone and in combination, on the growth of established solid LCC6\(^\text{HER-2}\) and MCF-\(7^\text{HER-2}\) breast cancer xenografts. Dose titration studies revealed that growth inhibition of established LCC6\(^\text{HER-2}\) tumor xenografts after treatment with trastuzumab was dose-dependent and that a 54.4\% (\pm 17.1\%) growth inhibition versus vehicle-treated control could be achieved with trastuzumab doses as low as 0.25 mg/kg (Fig. 3A). A dose of 1.0 mg/kg trastuzumab yielded tumor growth inhibition from 52.5\% (\pm 12.9\%; \(P = 0.000063\)) to 68.4\% (\pm 8.2\%; \(P = 0.00163\); Fig. 3, A and C, respectively). LCC6\(^\text{HER-2}\) tumor-bearing animals treated with 1 mg/kg trastuzumab continued to exhibit smaller tumors 40 days after the last administration of trastuzumab (Fig. 3A). Gefitinib mediated growth delays of LCC6\(^\text{HER-2}\) tumors ranging from 30.4\% (\pm 4.4\%; \(P = 0.033\); Fig. 3C) to 62.3\% (\pm 3.6\%; \(P = 0.0011\); Fig. 3B) versus control, with tumors resuming the growth characteristics of controls soon after cessation of treatment (Fig. 3B). The dose-response curve suggested that activity was not significantly altered with increasing doses >50 mg/kg (Fig. 3B); however, toxicities at 200 mg/kg included loose stool, dry eyes, and weight loss up to 15\% in 10\% of the...
mice. These results show that the LCC6HER-2 breast cancer xenograft model was highly sensitive to low concentrations of trastuzumab, whereas gefitinib was required at higher doses to reduce tumor growth. Similar dose titration data were obtained for mice bearing MCF-7HER-2 tumors (data not shown) and from these data a ratio of gefitinib to trastuzumab was defined for the combination studies (shown in Fig. 3D). In brief, the maximum dose that could be administered for the gefitinib formulation used was 200 mg/kg (daily p.o.). The lowest dose of trastuzumab used to achieve optimal efficacy was then used to define the ratio for the combination studies. In the LCC6HER-2 model and the MCF-7HER-2 model the lowest trastuzumab dose used to provide significant therapeutic effects was 1 mg/kg and 10 mg/kg, respectively. Thus, the dose used to assess combination effects was set at 1 mg/kg trastuzumab: 200 mg/kg gefitinib for the LCC6HER-2 model and 10 mg/kg trastuzumab: 200 mg/kg gefitinib for MCF-7HER-2.

In LCC6HER-2 xenografts the combination of trastuzumab and gefitinib yielded 60.5% (±14.9%) growth inhibition compared with controls (P = 0.000005), which was not significantly greater than single agent trastuzumab (P = 0.856; Fig. 3C). Treatment of MCF-7HER-2 xenografts with 10 mg/kg trastuzumab effected an 80.2% (±23.8%) inhibition of tumor growth (P = 0.000005). Treatment with gefitinib (200 mg/kg) achieved a 56.2% (±22.2%) inhibition of tumor growth (P = 0.00156). The combination of trastuzumab and gefitinib resulted in an 89.1% (±14.3%) inhibition of tumor growth (P = 0.000001; Fig. 3D).

We conclude that both the ER⁻ LCC6HER-2 model and the ER⁺ MCF-7HER-2 model are sensitive to trastuzumab and moderately sensitive to gefitinib in vivo. The effect of combination of these two agents, however, was not significantly greater than the more potent agent, trastuzumab, alone (P = 0.856 for LCC6HER-2 and P = 0.92 for MCF-7HER-2).

Effects of Trastuzumab, Gefitinib, and the Combination on Tumor Parameters

Cell Surface Marker Expression in LCC6HER-2 and MCF-7HER-2 Tumors. To better understand the biological effects of trastuzumab and gefitinib, we investigated consequences of in vivo treatment on cells isolated from tumors excised from both treated and control mice. We used a tissue disaggregation method established in our laboratory, which yields single cell suspensions representative of entire tumor cell populations. The method allows for high cell recovery as well as excellent retention of cell surface markers, and the results ob-

Fig. 3 In vivo efficacy curves for LCC6HER-2 and MCF-7HER-2 tumors established in SCID Rag 2M mice, treated with trastuzumab, gefitinib, or combination. A and B, LCC6HER-2 xenografts treated for 5 weeks with increasing concentrations of trastuzumab (A) and gefitinib (B; n = 4 mice per group); C, LCC6HER-2 tumors treated for 5 weeks with 1 mg/kg trastuzumab, 200 mg/kg gefitinib, or the combination (n = 4 mice per group); D, MCF-7HER-2 tumors treated for 3.5 weeks with 10 mg/kg trastuzumab, 200 mg/kg gefitinib, or combination (n = 8-10 mice per group); bars, ±SE.
Combination Treatment with Trastuzumab and Gefitinib

levels of cell surface HER-2/\textit{neu} expression. The mean fluorescence intensity of HER-2/\textit{neu}-FITC on tumor cells was reduced by 54.6\% (±2.0\%) shortly after treatment \((P = 0.000001)\); however, 40 days after the last trastuzumab injection, mean fluorescence intensity of HER-2/\textit{neu}-FITC on tumor cells had recovered to levels that were only 35.1\% (±13.4\%) lower than that observed for control cells \((P = 0.0283\) versus HER-2/\textit{neu} expression immediately after treatment; Fig. 4A). Gefitinib treatment did not significantly affect HER-2/\textit{neu} cell surface expression of \textit{LCCG\textit{\textit{HER}}-2} cells derived from disaggregated xenografts after treatment \((P = 0.74\); Fig. 4A). Furthermore, the combination of trastuzumab and gefitinib was less effective in down-regulating cell surface HER-2/\textit{neu} expression than single agent trastuzumab \((P = 0.0671\) combination versus trastuzumab alone); treatment with the combination of trastuzumab and gefitinib reduced the mean fluorescence intensity of the HER-2/\textit{neu}-FITC-stained \textit{LCCG\textit{\textit{HER}}-2} tumors by only 39.4\% (±10.8\%) versus controls \((P = 0.0000043\); Fig. 4A). These data suggest that effective down-regulation of cell surface HER-2/\textit{neu} could be achieved with trastuzumab, and that this down-regulation was gradually reversible. It is important to note that binding of the Neu 24.7-FITC antibody to the cell surface of trastuzumab treated \textit{LCCG\textit{\textit{HER}}-2} tumor cells was not inhibited by bound trastuzumab molecules.\footnote{W. H. Dragowska and C. Warburton, unpublished observations.}

All of the treatment regimens achieved significant reduction of surface HER-2/\textit{neu} in cells derived from disaggregated MCF-\textit{\textit{HER}}-2 \footnote{W. H. Dragowska and C. Warburton, unpublished observations.} xenografts (Fig. 4B). Trastuzumab treatment mediated down-regulation of cell surface HER-2/\textit{neu} by 59.6\% (±5.5\%) compared with cells derived from maturity matched untreated control tumors \((P = 0.000001\); Fig. 4B). Gefitinib caused a 39.7\% (±8.9\%) reduction in cell surface HER-2/\textit{neu} expression \((P = 0.0185\), and the combination of trastuzumab and gefitinib yielded a reduction in HER-2/\textit{neu} expression on MCF-\textit{\textit{HER}}-2 tumor cells by 57.6\% (±6.9\%); \(P = 0.0102\); Fig. 4B). These results suggest that the most effective reduction in cell surface HER-2/\textit{neu} expression was achieved with trastuzumab treatment. The combination of trastuzumab and gefitinib did not augment this effect in MCF-\textit{\textit{HER}}-2 \footnote{W. H. Dragowska and C. Warburton, unpublished observations.} tumors (Fig. 4B).

Because MCF-\textit{\textit{HER}}-2 tumor cells expressed surface EGFR, we also determined the effects of both trastuzumab and gefitinib on EGFR cell surface expression (Fig. 4C). Both trastuzumab and gefitinib mediated significant reduction of EGFR cell surface expression compared with controls of equal maturity. A 45.9\% (±11.3\%: \(P = 0.00603\)) and 71.7\% (±0.9\%: \(P = 0.00109\)) reduction was noted after treatment with trastuzumab and gefitinib, respectively (Fig. 4C). Adding trastuzumab to the more potent agent, gefitinib, led to slight inhibition of the gefitinib-mediated reduction in EGFR cell surface expression, as the combination achieved only 53.7\% (±6.3\%: \(P = 0.0034\)) down-regulation compared with controls (Fig. 4C). Taken together, these data show that both trastuzumab alone and gefitinib alone mediated significant down-regulation of EGFR expression on the cell surface of MCF-\textit{\textit{HER}}-2 tumors, and the combination of gefitinib and trastuzumab was less effective than gefitinib alone. These findings are concordant with our obser-

tained correlate well with observations derived from immuno-
histochemistry stained tumor sections \(66\). As illustrated in Fig. 4A, trastuzumab-treated \textit{LCCG\textit{\textit{HER}}-2} xenografts yielded cell suspensions with significantly reduced

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Selective down-regulation of HER-2/\textit{neu} and epidermal growth factor receptor as a consequence of targeted treatment. \textit{LCCG\textit{\textit{HER}}-2} (A) and MCF-\textit{\textit{HER}}-2 (B–C) tumors. A, expression of HER-2/\textit{neu} on viable cells derived from disaggregated \textit{LCCG\textit{\textit{HER}}-2} tumors treated with trastuzumab, gefitinib, or the combination, relative to untreated control tumor cells (\#). Reduced HER-2/\textit{neu} surface expression on \textit{LCCG\textit{\textit{HER}}-2} tumor cells mediated by trastuzumab, as observed 1 week after treatment (B) is reversible over a period of 5.5 weeks after treatment (C). B, HER-2/\textit{neu} surface expression on viable cells derived from disaggregated MCF-\textit{\textit{HER}}-2 tumors treated with trastuzumab, gefitinib, or the combination relative to untreated control tumors. C, expression of epidermal growth factor receptor on viable cells derived from disaggregated MCF-\textit{\textit{HER}}-2 tumors treated with trastuzumab, gefitinib, or the combination relative to untreated control tumors; bars, ±SD. Statistically significant \((P < 0.05)\) results are indicated by *.
\end{figure}
Tumor Viability. Tumor cell viability was determined by trypan blue exclusion in single cell suspensions obtained from LCC6HER-2 and MCF-7HER-2 tumor xenografts harvested after cessation of drug administration. Previous studies using tumor cells derived from disaggregated control LCC6HER-2 and MCF-7HER-2 tumors had shown that the percentage of trypan blue excluding cells correlated well with the percentage of clonogenic cells in disaggregated tumor cell suspensions (data not shown). Therefore, trypan blue excluding cells were considered viable, because they retained their proliferative capacity after the tumor disaggregation procedure.

Treatment with 1 mg/kg trastuzumab significantly reduced the percentage of viable LCC6HER-2 tumor cells from 25.8% (±6.0%) observed in the control group to 11.5% (±5.6%) in the treated group (P = 0.0272; Fig. 5A). Single agent gefitinib had no significant effect on LCC6HER-2 tumor cell viability (P = 0.478). Tumors isolated from animals treated with both drugs exhibited 11.9% (±3.7%) viable tumor cells. These data suggest that trastuzumab had a significant cytotoxic effect on LCC6HER-2 xenografts and that this effect was not augmented by the addition of gefitinib to the treatment regimen. In contrast to effects seen with LCC6HER-2 tumor cells, trastuzumab had no effect on the percentage of live, trypan blue excluding MCF-7HER-2 tumor cells (P = 0.953; Fig. 5B). The proportion of viable cells in gefitinib-treated tumors was reduced to 33.4% (±16.5%); however, this was not significantly different from controls (47.1 ± 13.6% viable cells; P = 0.164; Fig. 5B). Cell suspensions from tumors obtained from mice treated with both drugs exhibited 35.5% ± 10.6% cell viability, but this was not statistically different from the control (P = 0.43) or gefitinib alone groups (P = 0.99). As might be expected from the viability data, large portions of the isolated tumors (control or treated) were necrotic as judged by histology (data not shown).

Effects on Cell Cycle Distribution. Previous studies (47) suggested that treatment with trastuzumab and gefitinib, alone and in combination, engendered a reduction in tumor cells in S phase. For this reason PI staining was performed to study cell cycle distribution after treatment. Thus, unlike previous studies, which determined drug induced changes in cell cycle during treatment, the analyses summarized here were completed at a time point well after treatment was stopped. DNA content of fixed, PI-stained cells obtained from control and trastuzumab/ gefitinib-treated LCC6HER-2 and MCF-7HER-2 tumor xenografts harvested after cessation of drug treatment was analyzed by flow cytometry, and cell cycle distribution patterns were calculated from the resulting histograms. Cell cycle distribution of LCC6HER-2 xenografts was not affected by either treatment regimen (Fig. 6A). Gefitinib-treated MCF-7HER-2 tumor cells experienced a small but significant arrest in G1/G0 (P = 0.0336 versus control), which was slightly augmented by the addition of trastuzumab (P = 0.0068 versus control; Fig. 6B). Treatment with trastuzumab did not alter cell cycle distribution of MCF-7HER-2 tumor cells, as compared with untreated controls (Fig. 6B).

Proportion of Viable Hypoxic Cells in LCC6HER-2 and MCF-7HER-2 Tumors. EF5-labeled LCC6HER-2 control tumors after disaggregation produced a cell suspension that contained 18.7% (±7.6%) viable hypoxic cells, whereas after treatment with gefitinib tumor cell suspensions contained 11.0% (±3.8%) hypoxic cells (P = 0.022; Fig. 7A). Trastuzumab treatment exerted no effects on the percentage of viable hypoxic cells within LCC6HER-2 tumors (P = 0.616; Fig. 7A). Cells isolated from tumors treated with the combination of trastuzumab and gefitinib appeared to have levels of viable hypoxic cells slightly greater than controls (22.0% ± 3.4%; P = 0.800; Fig. 7A).

Similarly, when MCF-7HER-2 tumor-bearing mice were treated with gefitinib alone, we observed a dramatic reduction in the proportion of viable highly hypoxic cells in disaggregated tumor cell suspensions (2.1% ± 1.0% for gefitinib treated as opposed to 12.2% ± 5.9% for controls; P = 0.0000023; Fig. 7B). Treatment with trastuzumab, as well as the combination of gefitinib and trastuzumab, had no effect on the proportion of viable hypoxic cells isolated from MCF-7HER-2 tumors (Fig. 7B). Taken together, these data suggest that the reduction in the population of viable hypoxic tumor cells observed after treatment with gefitinib can be lost if trastuzumab is coadministered.
DISCUSSION

Several human breast cancer xenograft models are currently being used to investigate effects of novel treatments on HER-2/neu overexpressing breast cancer, and these models are often used interchangeably. However, the naturally occurring diversity among breast tumors can only in part be mimicked by xenograft models. For example, the parental cell line for the LCC6HER-2 model, MDA-MB-435, and the MCF-7 cell line show differences in ER/H11002, p53/H11002, and Bcl-2/Mcl-1 status (65).

The MCF-7 HER-2 tumor xenograft model has been shown to be sensitive to the humanized anti-HER-2/neu monoclonal antibody trastuzumab and is subject to additive and synergistic growth inhibition by trastuzumab in combination with various cytotoxic drugs (35, 73). Furthermore, several groups have recently demonstrated sensitivity to the selective EGFR-tyrosine kinase inhibitor gefitinib in breast cancer cell lines, which overexpress HER-2/neu (47–49). The data summarized in this report have confirmed these previous observations using two HER-2/neu overexpressing solid breast cancer xenograft models, LCC6HER-2 (ER-) and MCF-7HER-2 (ER+). Importantly, on the basis of preclinical data Moulder and Arteaga (74) have already initiated Phase I/II clinical trials assessing the therapeutic potential of trastuzumab and gefitinib in patients with HER-2/neu-positive metastatic breast cancer. Our studies have assessed the effects of trastuzumab and gefitinib, alone and in combination, in the context of tumor cell expression of the defined molecular targets as well as the potential of these agents alone and in combination to influence tumor microenvironment.

This contrasts efforts directed toward understanding the impact of this drug combination on cellular biochemistry and intracellular pathway function, topics that have been addressed by many previous investigations (47–49).

Our in vitro results show that the LCC6HER-2 and MCF-7HER-2 cell lines were relatively resistant to treatment with trastuzumab and gefitinib, as well as their combination, when cultured in the presence of serum. Only under serum-starved conditions were LCC6HER-2 and MCF-7HER-2 cells more susceptible to gefitinib and combination treatment, whereas trastuzumab remained ineffective in the killing of LCC6HER-2 cells and reduced MCF-7HER-2 cell viability by only 40% at the highest doses tested. These results were in concordance with findings by O’Donovan et al. (75) in BT474 breast cancer cells, and by Lu et al. (72), who demonstrated that trastuzumab did not inhibit growth of MCF-7HER-2 cells grown in 10% FBS, but was able to reduce cell proliferation by 42% under reduced serum conditions. Furthermore, Lu et al. (72) established that...
resistance to trastuzumab in 10% FBS was due to enhanced activation of the insulin-like growth factor 1 receptor signaling pathway. Woodburn et al. (56) found no correlation between EGFR expression and antitumor activity of tyrosine kinase inhibitors, and a recent study by Bishop et al. (76) found 33 of 43 EGFR-positive cell lines to be relatively insensitive to small molecule tyrosine kinase inhibitors. The fact that LCC6HER-2 cells, which express only few EGFR molecules, show some sensitivity to gefitinib, may be due in part to the absence of the ER. Endocrine-insensitive cells have been shown to be highly sensitive to gefitinib (77).

To be able to characterize effects of combination treatment, trastuzumab, gefitinib, and the combination were evaluated in vivo in established solid s.c. LCC6HER-2 and MCF-7HER-2 tumors in SCID mice. Trastuzumab and gefitinib alone were able to significantly delay tumor growth in both xenograft models, but in combination their activity, albeit improved, was not statistically better than that observed with trastuzumab treatment alone. These in vivo data obtained with LCC6HER-2 and MCF-7HER-2 tumors were comparable with results obtained by Moulder et al. (47) who reported on the therapeutic benefit of treating estrogen-supplemented mice bearing established BT-474 xenografts with 10 mg/kg trastuzumab (i.p. twice per week for 4 weeks) and gefitinib (oral gavage, 200 mg/kg/day). These data clearly suggest that the drug combination can provide effective therapy in vivo. Importantly, neither our study nor the study of Moulder et al. (47) was completed in a manner that can define whether the in vivo effects were a result of additive or synergistic interactions. It is important that conclusions about drug interactions (whether additive, antagonistic, or synergistic) must be derived from studies assessing effects over a broad range of effective doses. Similar to the methods developed by Chou and Talalay (64) these interactions should also be assessed at different drug ratios. More specifically, drug interactions defining synergy, antagonism, or additivity cannot be made on the basis of data obtained at a single dose. It should be noted that in our study trastuzumab effected sustained growth inhibition for 5 weeks after the end of treatment. We detected trastuzumab bound specifically to 26.1% of live LCC6HER-2 tumor cells treated with 1 mg/kg trastuzumab 40 days after the final injection into the mice. This is in concordance with a half-life in humans of >3 weeks (78). These observations could suggest that extended binding of trastuzumab to the surface of HER-2/neu-positive tumor cells may play a potential role in its sustained growth inhibitory effect (79–81).

To obtain a more comprehensive understanding of how trastuzumab/gefitinib influence tumor biology after treatment we sought to examine surrogate markers in cells from disaggregated tumors such as the expression of target receptors, viability of tumor cells, cell cycle distribution, and proportion of viable hypoxic cells. Reversible HER-2/neu down-regulation by trastuzumab, as observed in the LCC6HER-2 model (Fig. 4A), was first described by Drehin et al. (40) in transfected NIH 3T3 cells. In fact, target receptor down-regulation may constitute a (passive) evasion mechanism that allows a proportion of cells to survive exposure to trastuzumab, only to replenish surface receptor numbers after cessation of treatment. Viable tumor cells obtained from MCF-7HER-2 xenografts treated with single agent gefitinib reproducibly displayed significantly lower HER-2/neu and EGFR levels than cells recovered from untreated tumor xenografts. Our findings appear to be in contrast to studies suggesting that gefitinib inhibits activation of EGFR and HER-2/neu, but does not alter the total expression levels (47, 53, 82); however, these discrepancies could be explained by different times of harvesting tumors for analysis (i.e., right after last treatment in case of studies cited in Refs. 47, 53, 82 versus several days after the last treatment in our studies). Also, it is important to note that the work summarized in our article was not designed to evaluate HER-2/neu and EGFR activation or signaling. The effects of gefitinib and trastuzumab, alone and in combination, on receptor activation has been well characterized by other investigators (47–49). These previous studies indicate that during drug exposure there may not be a significant effect on EGFR and HER-2/neu expression levels, but the drugs will interfere with signaling. In the studies reported here we wanted to determine how treatment impacted on tumor cell population dynamics and tumor microenvironment, thus providing an assessment of the tumor cell population after treatment was stopped. Regardless, it should be noted that treatment-induced reductions in target molecule expression may be tumor model-specific. It could be argued from our studies, and those of others, that correlative studies assessing the impact of these agents alone and in combination should consider treatment-induced effects on expression levels as well as receptor tyrosine kinase signaling. It is interesting to note that in our studies, gefitinib slightly impaired trastuzumab-mediated down-regulation of HER-2/neu in the LCC6HER-2 model. Likewise, trastuzumab treatment inhibited the ability of gefitinib to decrease EGFR levels in MCF-7HER-2 tumors. Nearly all of the treatment regimens affected a decrease in the percentage of viable tumor cells obtained from LCC6HER-2 and MCF-7HER-2 tumors. The extent of the decrease in tumor cell viability, however, closely reflected: (a) trastuzumab-mediated HER-2/neu down-regulation in LCC6HER-2; or (b) gefitinib-induced EGFR down-regulation in MCF-7HER-2.

Inhibition of cell cycle progression via up-regulation of cyclin inhibitors of the p27KIP1 family, and subsequent arrest of breast cancer cells in the G1/G0 phase, has been attributed to both trastuzumab and EGFR-tyrosine kinase inhibitors (41, 42, 47, 63). In our in vivo studies, however, substantial cell cycle arrest of live cells recovered from LCC6HER-2 and MCF-7HER-2 xenografts was not achieved by either treatment regimen (Fig. 6). Others demonstrated that in cell culture, peak arrest of cancer cells in G1/G0 has been noted 30 h after treatment with trastuzumab (42) and 24–72 h after exposure to gefitinib (83). Gefitinib has a relatively short half life of 28 h in humans (84); it is therefore possible that transient cell cycle arrest arising during treatment with both trastuzumab and gefitinib was missed, and/or that the high dose of gefitinib applied (200 mg/kg) led to cell death rather than cell cycle arrest (53).

We have demonstrated previously that in the LCC6 tumor model, HER-2/neu overexpressing xenografts (LCC6HER-2) showed significantly higher levels of viable hypoxic cells than untransfected LCC6 wild-type or LCC6VECTOR tumors (66). The majority of viable hypoxic cells within a tumor are non-cycling and are known to be resistant to radiation and chemotherapy treatment (85). For this reason we determined whether trastuzumab, gefitinib, and their combination affected the pro-
portion of viable hypoxic cells in the HER-2/neu overexpressing LCC6HER-2 and the MCF-7HER-2 xenograft models. In both cases we found gefitinib to be able to significantly reduce the percentage of viable hypoxic cells in the tumor populations, whereas trastuzumab was ineffective and the combination abrogated this potentially beneficial effect of gefitinib. Gefitinib-mediated reduction of the proportion of viable hypoxic cells within the tumor could contribute to the radiosensitization effect attributed to agents, which inhibit EGFR signaling (67). Because gefitinib is a small molecule, it is possible that it could reach hypoxic tumor areas remote from vessels, causing apopotic death of hypoxic cells. In addition, hypoxic cells switch to a glycolytic metabolism (86), hence an inhibitor that additionally competes for ATP binding might be anticipated to confer enhanced cytotoxic effects in an ATP-deprived environment. Others had shown that trastuzumab can mediate toxicity on tumor vasculature (79, 81), so it is possible that new hypoxic areas continuously emerge in treated tumors, thereby diminishing the reduction in the viable hypoxic cell fraction achieved with gefitinib alone.

Trastuzumab has shown good efficacy and limited toxicity in the clinic (36–38) as well as excellent tumor control properties in several HER-2/neu overexpressing xenograft models in preclinical studies (47). It appears as if addition of gefitinib to a trastuzumab-based therapy may enhance tumor growth inhibition, but our studies would suggest that the impact of the drug combination on tumor cells may not be optimal. It can be argued, for example, that combination chemotherapy regimes must include strategies designed to affect unique cell populations within the tumor. It is known that viable repopulating cells, which are in a unique microenvironment (e.g., hypoxic environment), may escape agents that are selective against proliferating cell populations. Thus, the combination of gefitinib and trastuzumab may be effective against a defined cell population, but secondary effects induced by the drugs on other cell populations within the tumor may not be favorable and, perhaps more importantly, these effects on cell populations may suggest that the selected combination is actually not beneficial. Our data support the importance of carefully selecting correlative end points in treatment studies, where treatment-induced effects need to be characterized at a level beyond monitoring changes in tumor growth. Our results suggest that a possible combination with a selective hypoxia-activated topoisomerase II inhibitor such as tirapazamine, which causes DNA strand breaks under hypoxic conditions, could target distinct viable hypoxic cell populations, even in micrometastases of 1 mm in diameter (85), thereby limiting the regrowth of tumor cells after trastuzumab treatment. Conversely, the substantial reduction of the percentage of viable hypoxic tumor cells by gefitinib might enhance the effect of DNA damage inflicted by antibiotic drugs like bleomycin.

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Treatment of HER-2/neu Overexpressing Breast Cancer Xenograft Models with Trastuzumab (Herceptin) and Gefitinib (ZD1839): Drug Combination Effects on Tumor Growth, HER-2/neu and Epidermal Growth Factor Receptor Expression, and Viable Hypoxic Cell Fraction


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