Treatment of Human Epidermal Growth Factor Receptor 2–Overexpressing Breast Cancer Xenografts With Multiagent HER-Targeted Therapy

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Background Human epidermal growth factor receptor 2 (HER2) is a member of the HER signaling pathway. HER inhibitors partially block HER signaling and tumor growth in preclinical breast cancer models. We investigated whether blockade of all HER homo- and heterodimer pairs by combined treatment with several inhibitors could more effectively inhibit tumor growth in such models.

Methods Mice carrying xenograft tumors of HER2-overexpressing MCF7/HER2-18 (HER2-transfected) or BT474 (HER2-amplified) cells were treated with estrogen supplementation or estrogen withdrawal, alone or combined with tamoxifen. One to three HER inhibitors (pertuzumab, trastuzumab, or gefitinib) could also be added ($n \geq 8$ mice per group). Tumor volumes, HER signaling, and tumor cell proliferation and apoptosis were assessed. Results were analyzed with the *t* test or Wilcoxon rank sum test and survival analysis methods. All statistical tests were two-sided.

Results Median time to tumor progression was 21 days for mice receiving estrogen and 28 days for mice receiving estrogen and pertuzumab (difference = 7 days; $P = .001$; hazard ratio [HR] of progression in mice receiving estrogen and pertuzumab versus mice receiving estrogen = 0.27, 95% confidence interval [CI] = 0.09 to 0.77). Addition of gefitinib and trastuzumab to estrogen and pertuzumab increased this time to 49 days (difference = 21 days; $P = .004$; HR of progression = 0.28, 95% CI = 0.10 to 0.76). MCF7/HER2-18 tumors disappeared completely and did not progress (for ≥189 days) after combination treatment with pertuzumab, trastuzumab, and gefitinib plus tamoxifen (19 of 20 mice) or plus estrogen withdrawal (14 of 15 mice). Both combination treatments induced apoptosis and blocked HER signaling and proliferation in tumor cells better than any single agent or dual combination. All BT474 tumors treated with pertuzumab, trastuzumab, and gefitinib disappeared rapidly, regardless of endocrine therapy, and no tumor progression was observed for 232 days.

Conclusion Combined treatment with gefitinib, trastuzumab, and pertuzumab to block signals from all HER homo- and heterodimers inhibited growth of HER2-overexpressing xenografts statistically significantly better than single agents and dual combinations.

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For patients with estrogen receptor (ER)–expressing breast cancer, tamoxifen, which inhibits ER functions, is the most commonly prescribed drug. Its role has expanded from treatment of breast cancer to chemoprevention for patients at high risk of developing the disease (1,2). However, not all patients with ER-positive breast cancer respond to tamoxifen (i.e., tumors with de novo resistance), and those who do frequently develop resistance to tamoxifen over time (i.e., tumors that have acquired resistance). Aromatase inhibitors inhibit the synthesis of estrogen and thus inhibit tumor growth by depriving ER of its ligand, estrogen. Treatment with aromatase inhibitors is proving superior to treatment with tamoxifen at least in some patients (3–5). However, de novo and acquired resistance to aromatase inhibitors also occur. Signaling from growth factor receptors has been implicated as a cause of this resistance (6).

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Human epidermal growth factor receptor 2 (HER2) belongs to the HER family of tyrosine kinase receptors, which also includes HER1, HER3, and HER4. The binding of specific ligands (such as epidermal growth factor, transforming growth factor α, or heregulin) induces formation of HER homodimers or heterodimers that initiate a phosphorylation signaling cascade that results in enhanced cell proliferation and survival (7–13). The HER2 gene (also known as c-ErbB2 or HER2/neu) is amplified in 10%–15% of ER-positive breast cancers (14). The HER2 protein does not have a ligand, but it is activated by heterodimerization with other HER family members or by homodimerization when it is highly expressed (15).

Clinical and laboratory evidence support an important role for cross talk between the ER and HER2 signaling pathways in resistance to hormone therapies (5,16–22). In tumors with hyperactive HER2 signaling, both estrogen and tamoxifen can activate the HER signaling pathway and stimulate tumor growth through nongenomic (i.e., non-nuclear or membrane) ER activity (20). We previously reported (23) that, in a xenograft model using MCF7 human breast cancer cells stably transfected with HER2 (MCF7/HER2-18), tamoxifen stimulated tumor growth, leading to de novo tamoxifen resistance. The selective HER1 tyrosine kinase inhibitor gefitinib temporarily reversed tamoxifen stimulation of tumor growth, but complete tumor regression was not observed (20). In this system, estrogen-deprivation therapy also inhibited tumor growth, but again inhibition was temporary, with tumors progressing as HER signaling increased and acquired resistance developed (24). This endocrine resistance was temporarily reversed by gefitinib (24).

Several drugs that are in clinical use or in clinical trials inhibit HER family receptor activation. Gefitinib blocks signals from HER1 homo- and heterodimers (25) but not from HER2–HER3 dimers. Pertuzumab and trastuzumab are directed against distinct epitopes of the extracellular domain of HER2. Trastuzumab, which is currently used to treat patients with HER2-overexpressing breast cancer (26–28), has various cellular effects (29,30), including inhibiting the interaction between HER2 and Src (31), but trastuzumab does not block HER2-HER3 heterodimer signaling effectively, and de novo and acquired resistance to it is common (32). Pertuzumab, which is currently in phase I and II clinical trials (33), binds to the heterodimerization domain of HER2 and inhibits its heterodimerization with other HER family members (32), including HER3 (10,32,34–38). Given the differences in the mechanisms of action of these drugs, we investigated whether combinations of these agents would more completely inhibit the HER pathway than single-agent treatment and thus overcome the rapidly acquired resistance observed with single agents in the MCF7/HER2-18 xenograft model system.

CONTEXT AND CAVEATS

Prior knowledge

Human epidermal growth factor receptor (HER) inhibitors partially block HER signaling in breast cancer model systems.

Study design

Human xenograft HER2-overexpressing tumors in mouse model systems.

Contribution

The combination of three HER inhibitors (gefitinib, trastuzumab, and pertuzumab) inhibited the growth of HER2-overexpressing xenograft tumors better than single-agent or dual-agent combinations.

Implications

Further investigation into the mechanisms of action and the mechanisms of resistance to the three-drug combination therapy is warranted.

Limitations

The superiority of the three-inhibitor combination (gefitinib, trastuzumab, and pertuzumab) was tested in only two cell lines in an experimental mouse model system and cannot necessarily be extrapolated to other HER2-positive cell lines or to patients with HER2-amplified breast cancer.

Materials and Methods

Reagents, Hormones, and Antibodies

17β-Estradiol pellets (0.36-mg 60-day release) were from Innovative Research (Sarasota, FL). Tamoxifen citrate was from Sigma (St Louis, MO). Gefitinib (Iressa) was from AstraZeneca (Macclesfield, U.K.). Trastuzumab (Herceptin) and pertuzumab (Omnitarg, 2C4) were from Genentech (South San Francisco, CA). Antibodies against total HER2, phosphorylated HER2 (at Tyr^{887} and Tyr^{1248}), total Akt, phosphorylated Akt (at Ser^{437}), total p42/44 mitogen-activated protein kinase (MAPK, total MAPK), and phosphorylated p42/44 MAPK (at Thr^{202} or Tyr^{204}) were from Cell Signaling Technology (Beverly, MA). β-Actin antibody was from Chemicon (Temecula, CA).

Cells and Cell Culture Conditions

Two breast cancer cell lines were used to grow xenografts in mice: the MCF/HER2-18 cell line, a derivative clone of the ER-positive MCF7 cells that was stably transfected with HER2 overexpression vector (23), and the BT474 cell line (obtained from AstraZeneca), which expresses ER and is naturally gene amplified for HER2. Cells were maintained in high-glucose Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin (100 IU/mL), and streptomycin (100 μg/mL), under a humidified atmosphere of 5% CO_2/95% air and at 37 °C, as described previously (23,20). Medium for the MCF/HER2-18 cell line also contained Geneticin (G418; Gibco) at 400 μg/mL.

Immunohistochemistry for Cell Proliferation, Apoptosis, and Human Epidermal Growth Factor Signaling

Tumor tissue was fixed in 4% neutral-buffered formalin overnight before processing and paraffin embedding. One 3-μm section of tissue was examined by hematoxylin–eosin staining to verify that adequate tumor tissue was present, and blocks were then randomly arrayed in a 4-mm tissue array (more than five tumors per study group and two sections per marker). For immunohistochemical staining, 4-μm sections from these arrays were used. Briefly, after deparaffinization of the sections, endogenous peroxidase was blocked by 3% H_2O_2, and endogenous avidin and biotin were blocked as described by the AB blocking kit (Vector, Burlingame, CA).

Bromodeoxyuridine (BrdU) labeling of tumor cell nuclei was performed by injecting each mouse intraperitoneally with 0.2 mL of BrdU solution (product RPN201, Cell Proliferation Labeling Reagent; GE Healthcare Life Sciences, Piscataway, NJ) 2 hours before harvesting the tumors. Mice were killed, tumors were excised, and tumor-incorporated BrdU was stained by using the BrdU In-Situ Detection Kit (product 551321; BD Pharmingen, San Diego, CA), according to the manufacturer's instruction manual. Briefly, the deparaffininized slides were pretreated with 10 mM sodium citrate (pH = 6.0) in a pressure cooker at full pressure for 10 minutes and then washed. After washing, slides were quickly immersed in 3% H_2O_2 for 5 minutes and then a biotinylated anti-BrdU antibody (1:10 dilution) was applied for 1 hour in a humidified chamber. Slides were rinsed in 1× TBS-20 (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, and 0.05% Tween 20), three to four drops of ready-to-use streptavidin from the detection kit above was added to each slide, and the slide was incubated for 30 minutes at room temperature. Slides were washed again, and liquid diaminobenzidine substrate chromogen (Dakocytomation, Dako, Glostrup, Denmark) was added for 15 minutes, until the desired color intensity developed. BrdU was visualized by light microscopy after staining with anti-BrdU antibody (BD Pharmingen) followed by a 5-minute incubation with Sparkle DAB Enhancer (Biocare, Concord, CA). To assess apoptosis, additional sections were stained for apoptotic cells with an antibody against cleaved caspase 3 that also crossreacts with cleaved caspase 7 (product 9661, Cell Signaling Technology). Briefly, the protocol above was followed, but the antigen retrieval was done by use of 100 mM Tris–HCl (pH 9.0), and anti–cleaved caspase 3 rabbit antibody in dilution of 1:50 was used and followed by three to four drops Envision-labeled polymer-horseradish peroxidase–labeled anti-rabbit secondary antibodies (Dakocytomation) for 30 minutes at room temperature. To assess HER signaling in other sections, cells containing activated MAPK were identified with anti–phosphorylated MAPK rabbit antibody in a 1:80 dilution (product 9101, Cell Signaling Technology; 2-hour incubation), followed by antigen retrieval with a buffer containing 10 mM Tris and 1 mM EDTA (pH 8.0), as previously described (39).

Tumors were scored by the percentage of cells positive for BrdU (as a measure of cell proliferation) and for cleaved caspase 3 or 7 (as a measure of apoptosis) and by an Allred score (40) that estimates the proportion of positively stained tumor cells on a scale of 0–5 and the intensity of staining on a scale of 1–3 (in which 0 = none, 1 = weak, 2 = intermediate, and 3 = strong) for activated MAPK staining. Scoring criteria were based on the estimated fraction of positively staining cells as follows: 0 = none, 1 = fewer than one in 100 cells, 2 = one in 100 cells to one in 10 cells, 3 = one in 10 cells to one in three cells, 4 = one in three cells to two in three cells, and 5 = more than two in three cells (24,40). Tumor markers were visualized and studied by light microscopy (original magnification = ×10, ×20, and ×40).

Tumor Extracts and Immunoblots to Assess Human Epidermal Growth Factor Signaling

Frozen tumors from the different treatment groups were manually homogenized in lysis buffer (Cell Signaling Technology) supplemented with 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN). Tumor lysates were collected, sonicated (for five 5-second periods on ice), and microcentrifuged at 15 300*g* for 20 minutes at 2 °C. Cell supernatants were separated into aliquots and stored at −70 °C. Protein concentration was measured by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's directions. Equivalent amounts of protein (25 μg) from each sample were separated, under denaturing conditions, by electrophoresis on 8%–10% polyacrylamide gels containing sodium dodecyl sulfate, and protein bands were transferred by electroblotting to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The blots were first stained with Ponceau S to confirm uniform loading and transfer of proteins and then immunoblotted with the specific antibodies according to the manufacturer's directions. Total HER2, total Akt, total MAPK, and phosphorylated HER2 (at Tyr^{887} and Tyr^{1248}), as well as the HER2 downstream signaling proteins—phosphorylated Akt (at Ser^{437}) and phosphorylated p42/44 MAPK (at Thr^{202} or Tyr^{204})—were assessed by immunoblot analysis. Briefly, blots were blocked with blocking buffer (5% [wt/vol] nonfat dry milk in phosphate-buffered saline [PBS] containing 0.3% Trition X-100 at pH 7.5], 0.9% NaCl, and 0.1% Tween 20 [PBST]) for 1 hour and then incubated with primary antibodies at dilutions recommended by the manufacturer. For all antibodies to phosphorylated proteins, the reaction mixture contained 5% bovine serum albumin (Sigma) in PBST, and the reaction was incubated overnight at 4 °C or otherwise for 2 hours at room temperature. Blots were then washed three times in PBST and incubated for 1 hour at room temperature with horseradish peroxidase–labeled anti-rabbit antibody as secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) in 5% nonfat dry milk in PBST. Blots were then washed in PBST, and the labeled protein was visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and exposure of the membranes on x-ray film (Kodak, Rochester, NY). Immunoblots were reproduced at least two times, each with similar results. Protein expression and phosphorylation levels were quantified by densitometry with the Alpha Imager Detection System (Alpha Innotech Corporation, San Leandro, CA). In each tumor sample, protein levels were normalized to that of β-actin as a loading control (i.e., protein levels/actin levels × 100). Median of normalized expression and its interquartile range (IQR) are presented for each treatment group as well as representative blots.

Xenograft Studies

MCF7/HER2-18 cells (which were transfected with HER2) and BT474 cells (in which HER2 is naturally amplified) were maintained as previously described (23,41). Animal care was in accordance with institutional guidelines. MCF7/HER2-18 and BT474 xenografts were established by injecting 5×10^6 cells subcutaneously into ovariectomized 5- to 6-week–old athymic female mice (Harlan Sprague Dawley, Madison, WI) that had been implanted with 0.36-mg 60-day–release estrogen pellets (Innovative Research), as described (42). Before injection, BT474 cells were suspended in 300 μL of growth factor–reduced Matrigel (BD Biosciences, Bedford MA), and MCF7/HER2-18 cells were suspended in their regular medium. When tumors reached a size of 200–250 mm^3 (2–4 weeks), mice bearing MCF7/HER2-18 xenografts were randomly allocated to continued

estrogen treatment or to estrogen withdrawal alone (by removal of the estrogen pellets) or to estrogen withdrawal plus tamoxifen treatment (500 μg of tamoxifen citrate, administered subcutaneously in peanut oil for 5 days/wk) (42). Mice treated with estrogen withdrawal plus tamoxifen were then randomly assigned to treatment with gefitinib (100 mg/kg of body weight, administered by gavage in 1% Tween 80 for 5 days/wk), pertuzumab (12 mg/kg of body weight intraperitoneally for the first week and then 6 mg/kg intraperitoneally in 1% sterile PBS weekly), or trastuzumab (10 mg/kg, administered intraperitoneally in sterile H_2O for 2 days/wk) alone; to a double dose of trastuzumab; or to a drug combination (trastuzumab + gefitinib or pertuzumab, trastuzumab or pertuzumab + gefitinib, pertuzumab + trastuzumab + gefitinib). Mice treated with estrogen supplementation and mice treated by estrogen withdrawal were randomly allocated to treatment with vehicle alone, single-agent pertuzumab, or a combination of all three HER inhibitors (i.e., three-drug combination treatment). Each treatment group contained a minimum of 15 mice.

In a second experiment with MCF7/HER2-18 xenografts, mice were randomly allocated to continued estrogen treatment, estrogen withdrawal plus tamoxifen treatment (500 μg of tamoxifen citrate, administered subcutaneously in peanut oil for 5 days/wk), or treatment with estrogen and tamoxifen. Mice treated with estrogen plus tamoxifen were then further randomly assigned to receive the three-drug combination or vehicle alone (peanut oil). Each treatment group contained a minimum of eight mice.

To determine whether tumor cells were completely eradicated by combined treatment with tamoxifen and the three-drug regimen or estrogen withdrawal and the three-drug combination, in another experiment, in the group of mice (n = 9) receiving tamoxifen and the three-drug regimen and in the group of mice (n = 10) treated with estrogen withdrawal and the three-drug combination therapy, all therapy was stopped at day 112 or day 189, respectively. For both groups, 0.36-mg 60-day release estrogen pellets were reintroduced when the combined therapy was discontinued, and mice were followed for an additional 60 days for tumor regrowth.

Mice bearing BT474 xenografts were randomly allocated to treatment with continued estrogen, estrogen withdrawal alone (by removal of the estrogen pellets), or estrogen withdrawal plus tamoxifen treatment. Mice in these groups were then randomly assigned to treatment with vehicle alone or the three-drug combination. Mice treated with tamoxifen and mice treated with estrogen were also randomly assigned to receive pertuzumab as single agent. Each treatment group contained a minimum of eight mice.

Tumor size was assessed weekly by measuring the two largest tumor diameters with a caliper, and tumor volumes were calculated from these diameters as described previously (42). Mice were killed 3–5 days after treatment or when tumors reached a volume of 1000 mm^3 by cervical dislocation while under general surgical anesthesia, and tumors were harvested for immunoblot and immunohistochemical analyses. Each mouse carried a single tumor; tumor tissue was removed and maintained at −190 °C for later analyses.

Statistical Analysis

We have had considerable experience with this xenograft model and have computed previously (data not shown) that 12 or more animals generally provide greater than 80% power (alpha = 5%) to detect twofold differences in time to tumor progression. These experiments were sized accordingly. We have developed a standard approach to similar xenograft animal models used in these studies in which time to progression curves are modeled as a log-normal or an empirically estimated distribution and are then used in Monte Carlo simulation to estimate the effect sizes that will be detectable with various sample sizes and follow-up times.

Tumor growth curves were constructed by use of the mean tumor volume at each measurement point along with 95% confidence intervals (95% CIs). On the basis of our previous studies in similar xenograft models (24), time to tumor progression was defined a priori as the time when tumor volume had increased 2.5 times from the baseline value for each mouse. The Kaplan–Meier method was used to determine the median time to tumor progression. All *P* values for the xenograft studies were based on comparisons of time to tumor progression among groups by use of the log-rank test. Estimates of hazard ratios (HRs) and 95% confidence intervals were derived from the Cox regression model. The assumption of proportionality of hazards between treatment groups was verified by performing hypothesis tests of treatment groups as time-dependent variables in the Cox model. Complete tumor regression was defined as complete tumor disappearance (no measurable disease or no palpable nodule) for at least 150 days of follow-up. Complete tumor regression rates and 95% exact binomial confidence intervals were calculated for each group of animals. The two-sample *t* test or Wilcoxon rank sum test was used for two-group comparisons of tumor proliferation and survival pathways by use of immunoblot and immunohistochemical assessments. All statistical tests were two-sided.

Results

Human Epidermal Growth Factor Pathway Inhibition and Estrogen-Stimulated Growth of MCF7/HER2-18 Tumors

To determine whether treatment with additional HER inhibitors, singly or in combination, could more completely inhibit tumor growth in MCF7/HER2-18 xenografts than treatment with gefitinib alone, we first used treatment with pertuzumab, which should inhibit signaling from HER2 homo- and heterodimers. Combined treatment with pertuzumab and estrogen slowed xenograft tumor growth minimally (Fig. 1, A). Median time to tumor progression, as defined by tumors reaching 2.5 times baseline size, was 21 days for mice receiving estrogen and 28 days for mice receiving estrogen and pertuzumab (difference = 7 days; *P* = .001; HR for tumor progression with estrogen and pertuzumab compared with estrogen alone = 0.27, 95% CI = 0.09 to 0.77). Similar results were observed with combined treatment with trastuzumab and estrogen (data not shown). Addition of gefitinib and trastuzumab to treatment with pertuzumab (i.e., the three-drug combination) further delayed median time to tumor progression to 49 days (difference = 21 days; *P* = .004; HR = 0.28, 95% CI = 0.10 to 0.76) (Fig. 1, A). However, the effect of the three-drug combination was only transient, as

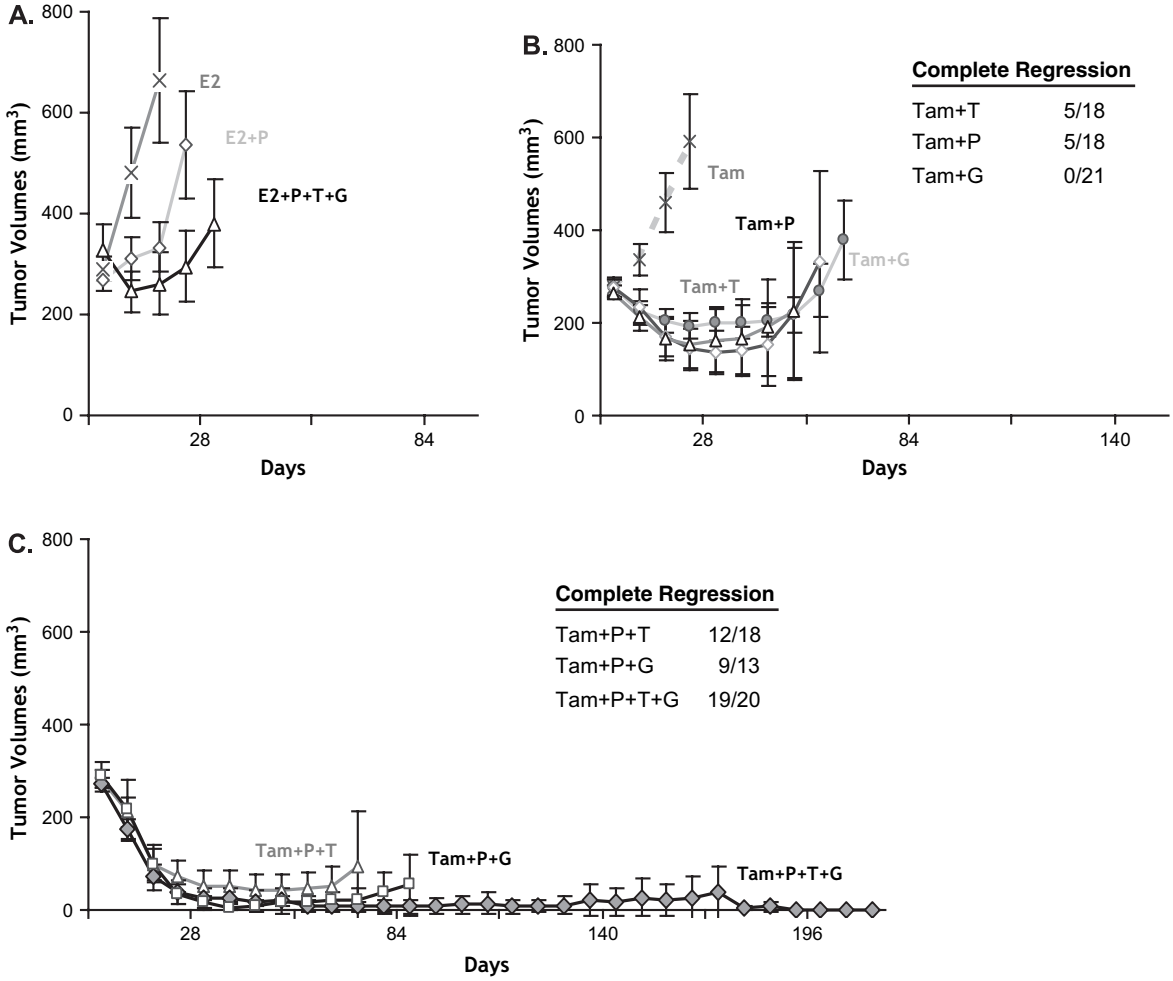

Fig. 1. Growth of MCF7/HER2-18 xenograft tumors in athymic female mice treated with human epidermal growth factor receptor (HER) family inhibitors—pertuzumab (P), trastuzumab (T), or gefitinib (G)—in the presence of estrogen (E2) or tamoxifen (Tam). The maximum length for each treatment was 189 days. **A**) E2 treatment alone or with pertuzumab or with the combination of pertuzumab, trastuzumab, and gefitinib. E2 = **times symbol**, E2 + P = **diamonds**, and E2 + P + T + G = **triangles**. **B**) Tamoxifen treatment alone or with pertuzumab, trastuzumab, or gefitinib. Tam = ×, Tam + T = **triangles**, Tam + P = **diamonds**, and Tam + G = **circles**. **C**) Tamoxifen treatment with various combinations of pertuzumab, trastuzumab, and gefitinib. Tam + P + T = **triangles**, Tam + P + G = **squares**, Tam + P + T + G = **diamonds**. Results are presented as the mean tumor volume ($n \geq 15$ mice, **error bars** are 95% confidence intervals). In panels **B** and **C**, for each group, the number of mice with complete tumor regression and the total number of mice are shown. Complete regression was defined as complete tumor disappearance. In panel **C**, all treatments were stopped on day 189, and mice were followed for another 30 days.

shown by tumor regrowth after 49 days. Thus, pertuzumab used alone or in combination with gefitinib and trastuzumab was not an effective therapy when estrogen was present to activate ER.

Human Epidermal Growth Factor Pathway Inhibition and Tamoxifen-Stimulated Growth of MCF7/HER2-18 Tumors

We next investigated the effect of HER inhibition on tumor growth in tamoxifen-treated mice. Tamoxifen, like estrogen, stimulated the growth of MCF7/HER2-18 tumors (Fig. 1, B), as reported previously (20,23,43). However, in contrast to the growth of estrogen-stimulated tumors, growth of tumors in mice treated with tamoxifen and trastuzumab, pertuzumab, or gefitinib was substantially inhibited. Complete tumor regression was observed in five (28%, 95% CI = 10% to 53%) of 18 mice treated with tamoxifen and trastuzumab or pertuzumab but not in any of the 21 mice treated with tamoxifen and gefitinib. Tumor growth inhibition, however, lasted only 2 months before resistance to tamoxifen and the HER2 inhibitors evolved, and tumor growth resumed. Median time to tumor progression was statistically significantly longer after treatment with tamoxifen and gefitinib (94 days; difference = 59 days; HR for tumor progression in mice receiving combined treatment versus tamoxifen alone = 0.25, 95% CI = 0.13 to 0.48), tamoxifen and trastuzumab (105 days; difference = 70 days; HR = 0.43, 95% CI = 0.28 to 0.66), or tamoxifen and pertuzumab (84 days; difference = 49 days; HR = 0.16, 95% CI = 0.06 to 0.42) than after treatment with tamoxifen alone (35 days) (for all, $P<.001$).

We then investigated whether the development of resistance to tamoxifen and to each of the three HER inhibitors individually was due to incomplete blockade of the HER pathway. To do so, we used treatment with tamoxifen and various two-drug combinations or the three-drug combination. Treatment with tamoxifen, trastuzumab, and gefitinib was slightly more effective than that with any drug alone, but resistance to the combined treatment still developed quickly. The most successful combinations to delay the development of resistance are shown in Fig. 1, C. Treatment

with tamoxifen, pertuzumab, and trastuzumab or with tamoxifen, pertuzumab, and gefitinib was more effective than that with tamoxifen and pertuzumab alone during the 189-day follow-up period (compare Fig. 1, C versus B); visible tumors completely disappeared in 12 (67%, 95% CI = 41% to 87%) of 18 mice and in nine (69%, 95% CI = 38% to 91%) of 13 mice, respectively, compared with five (28%, 95% CI = 10% to 53%) of 18 mice after pertuzumab alone. However, after 2 months, seven (39%, 95% CI = 17% to 64%) of the 18 mice treated with tamoxifen, pertuzumab, and trastuzumab and five (38%, 95% CI = 14% to 68%) of the 18 mice treated with tamoxifen, pertuzumab, and gefitinib had developed resistance to their treatment. The greater effectiveness in delaying tumor progression by these combined anti-HER inhibitor regimens in comparison to treatments with a single anti-HER inhibitor was not a dose effect. Time to progression was statistically significantly delayed in mice treated with trastuzumab and pertuzumab compared with mice treated with a double dose of trastuzumab ($P = .008$, data not shown), confirming that increasing the dosage of a single anti-HER agent is not as effective as using a combination regimen with inhibitors with different mechanism of action.

As shown by previous molecular studies, neither pertuzumab nor trastuzumab blocks signals from HER1 homodimers or HER1–HER3 heterodimers (31–38). We therefore hypothesized that adding gefitinib to this combination could further improve therapeutic efficacy. Even though HER1 is expressed at low levels in these cells, tumors disappeared completely in 19 (95%, 95% CI = 75% to 100%) of 20 mice mostly after 2 or 3 weeks of treatment (Fig. 1, C). The single mouse whose tumor did not disappear completely had a small residual mass that did not progress during the 189-day follow-up period.

To confirm that the estrogen antagonist properties of tamoxifen were restored to tumors with de novo tamoxifen resistance after HER blockade, we examined the effects of tamoxifen treatment in the presence of estrogen from implanted estrogen pellets. In such mice, treatment with tamoxifen alone minimally inhibited estrogen-stimulated tumor growth after 2 weeks, and no tumor regression was observed at a follow-up of 150 days (Fig. 2). However, when mice were treated with the three-drug combination of HER family inhibitors plus tamoxifen, estrogen-stimulated tumor growth was statistically significantly inhibited by tamoxifen, and complete tumor regression was observed in eight of 15 mice (53%, 95% CI = 26% to 79%; $P<.001$). The remaining seven mice in this treatment group had small residual masses, and tumor progression was observed in only one mouse, at a follow-up of 150 days. Thus, HER blockade restored the ability of tamoxifen to antagonize estrogen in these tumors.

Human Epidermal Growth Factor Pathway Inhibition and Estrogen Deprivation Therapy in MCF7/HER2-18 Tumors

Estrogen deprivation, which mimics aromatase inhibitor therapy in postmenopausal patients, is another method to block ER signaling, and so we investigated this therapy in combination with complete HER blockade. Treatment with pertuzumab and estrogen withdrawal caused complete tumor regression in nine of 15 mice (60%, 95% CI = 32% to 84%), whereas no cases of complete tumor regression were observed in 16 mice treated with estrogen withdrawal alone (Fig. 3). Median time to tumor progression was statistically significantly longer after treatment with estrogen withdrawal and pertuzumab (126 days) than after treatment with estrogen withdrawal alone (73.5 days) (difference = 52.5 days; $P<.001$; HR = 0.25, 95% CI = 0.10 to 0.62) (Fig. 3). Treatment with gefitinib, trastuzumab, and pertuzumab was the most effective therapy, with complete tumor regression in 14 of the 15 mice (93%, 95% CI = 68% to 100%), and no tumor progression or regrowth was observed for as long as 189 days.

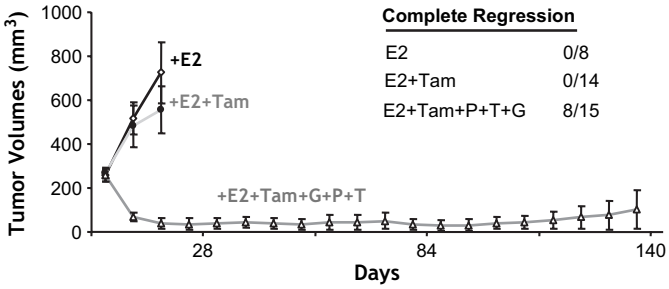

Fig. 2. Growth of MCF7/HER2-18 xenograft tumors in athymic mice treated with estrogen (E2) alone or with tamoxifen (Tam), with or without the three-drug combination of human epidermal growth factor receptor (HER) family inhibitors—pertuzumab (P), trastuzumab (T), and gefitinib (G). The maximum length for each treatment was 150 days. E2 = **diamonds**, E2 + Tam = **circles**, and E2 + Tam + P + T + G = **triangles**. Results are presented as the mean tumor volumes ($n \geq 8$ mice, **error bars** are 95% confidence intervals). Complete regression was defined as complete tumor disappearance. For each group, the number of mice with complete tumor regression and the total number of mice in that group are shown.

MCF7/HER2-18 Tumors and Results of Long-Term Combination Treatment

To determine whether tumor cells were completely eradicated by combined treatment with tamoxifen and the three-drug regimen, all treatments were stopped on day 189, and mice were followed for another 30 days (Fig. 1, C). No tumors regrew during the 30-day observation period, and no microscopic evidence of residual tumor was found in any tumor specimen. To exclude the possibility of microscopic residual disease that was not detected by histologic evaluation, all therapy was stopped in another group of nine mice receiving this combination treatment at 112 days, estrogen pellets were reintroduced, and mice were followed for an additional 60 days. Tumors reappeared in five of nine mice (55%, 95% CI = 21% to 86%), indicating that tumor cell eradication had been achieved in four of the nine mice.

To determine whether tumors had disappeared in mice treated with estrogen withdrawal and the three-drug combination, treatment was stopped at day 189, and estrogen pellets were reintroduced. At a follow-up of 60 days, tumor regrowth was observed in only three of the 10 mice (30%, 95% CI = 7% to 65%) in this group, indicating that, under this therapeutic regimen, tumor cells appear to have been completely eradicated in a substantial proportion of these mice.

Human Epidermal Growth Factor Pathway Inhibition and BT474 Tumors

Although estrogen treatment is required to establish BT474 xenograft tumors in mice (41), in our experiments established tumors

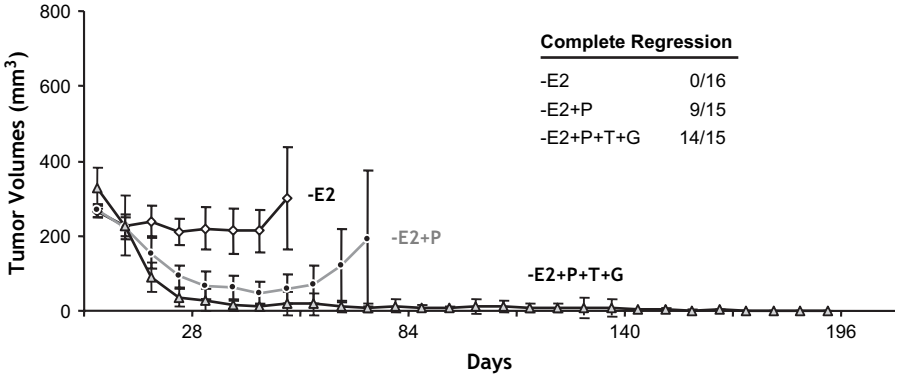

Fig. 3. Growth of MCF7/HER2-18 xenograft tumors in athymic mice treated with estrogen withdrawal (–E2) and various combinations of human epidermal growth factor receptor (HER) family inhibitors—pertuzumab (P), trastuzumab (T), and/or gefitinib (G). The maximum length for each treatment was 189 days. –E2 = **diamonds**, –E2 + P = **circles**, –E2 + P + T + G = **triangles**. Results are mean tumor volumes; **error bars** are 95% confidence intervals. Complete regression was defined as complete tumor disappearance. For each group, the number of mice with complete tumor regression and the total number of mice in that group are shown.

became completely estrogen independent, exhibiting the same pattern of progressive growth in mice treated with estrogen, with estrogen withdrawal, or with estrogen withdrawal and tamoxifen (i.e., tamoxifen therapy), even though the tumors continued to express ER (Fig. 4). The median time to tumor progression was 21 days for all groups.

Adding pertuzumab to tamoxifen therapy slightly inhibited the growth of BT474 tumors. Median time to tumor progression after treatment with pertuzumab and tamoxifen was not reached, but time to tumor progression was statistically significantly longer in these mice than in mice treated with tamoxifen alone (HR compared with tamoxifen alone = 0.37, 95% CI = 0.18 to 0.76), estrogen alone (HR = 0.03, 95% CI = 0.003 to 0.29), or estrogen withdrawal alone (HR = 0.04, 95% CI = 0.005 to 0.34) (all $P<.001$). No difference was observed in time to tumor progression in mice treated with pertuzumab and tamoxifen therapy compared with mice treated with pertuzumab and estrogen ($P = .58$; HR = 1.14, 95% CI = 0.70 to 1.84, data not shown). Complete tumor regression was observed in only two (22%, 95% CI = 3% to 60%) of nine mice treated with pertuzumab and tamoxifen, and tumor regrowth was evident at 49 days after treatment (Fig. 4). In mice treated with gefitinib, trastuzumab, and pertuzumab (i.e., the three-drug combination), all tumors rapidly disappeared—regardless of endocrine therapy—and no tumor progressed during the 2-month treatment period, even when mice were also treated with estrogen. When therapy was discontinued on day 112 in mice treated with estrogen withdrawal and the three-drug combination, no tumor regrowth was detected in the next 60 days. Estrogen treatment was then reintroduced to these mice at day 172, and no tumor regrowth was detected, even after an additional 60 days of follow-up, for a total follow-up of 232 days.

Estrogen Receptor and Human Epidermal Growth Factor Receptor 2 Blockade, Tumor Proliferation, and Human Epidermal Growth Factor Receptor Signaling

To better understand the antitumor activity of the three-drug combination, we examined HER downstream signaling molecules, cell proliferation, and apoptosis in tumors of mice treated with this regimen. To investigate HER signaling, we assessed levels of HER2 phosphorylation, Akt phosphorylation, and MAPK phosphorylation. After 3 days of treatment, phosphorylation at the HER2 Tyr^{1248} autophosphorylation site was lower after treatment with the three-drug combination (normalized median protein level = 0.08, IQR = 0.06–0.26) than after treatment with tamoxifen alone (normalized median protein level = 3.6, IQR = 1.49–4.93) or with pertuzumab (normalized median protein level = 1.22, IQR = 0.54–1.92), although the difference was not statistically significant ($P = .06$ for both comparisons) (Fig. 5, A). HER2 phosphorylation at Tyr^{877}, a Src-dependent phosphorylation site, was statistically significantly lower after treatment with the three-drug combination (normalized median protein level = 15.4, IQR = 14.7–16.0) than after treatment with tamoxifen alone (normalized median protein level = 29.6, IQR = 25.4–38.0) or with pertuzumab (normalized median protein level = 19.1, IQR = 17.0–23.0) ($P = .03$, for both comparisons) (Fig. 5, A). The slight reduction in total HER2 after treatment compared with baseline was not statistically significant (Fig. 5, A).

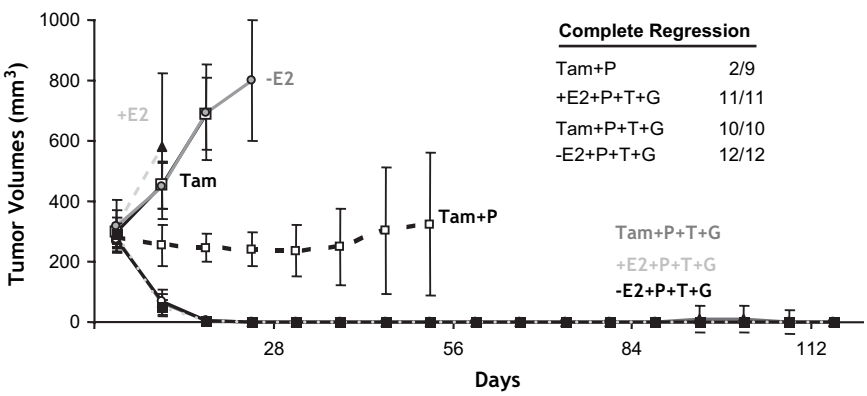

Fig. 4. Growth of BT474 xenograft tumors in mice treated with estrogen supplementation (+E2), tamoxifen (Tam), or estrogen withdrawal (–E2) alone or with various combinations of human epidermal growth factor receptor family inhibitors—pertuzumab (P), trastuzumab (T), and/or gefitinib (G). The maximum length for each treatment was 120 days. +E2 = **triangles**, –E2 = **circles**, Tam = **open squares** and **solid lines**, Tam + P = **open squares** and **dashed lines**, Tam + P + T + G = **open circles**, +E2 + P + T + G = **dotted lines** and **open triangles**, and –E2 + P + T + G = **solid black line** and **solid squares**. Results are mean tumor volumes; **error bars** are 95% confidence intervals. Complete regression was defined as complete tumor disappearance. For each group, the number of mice with complete tumor regression and the total number of mice are shown.

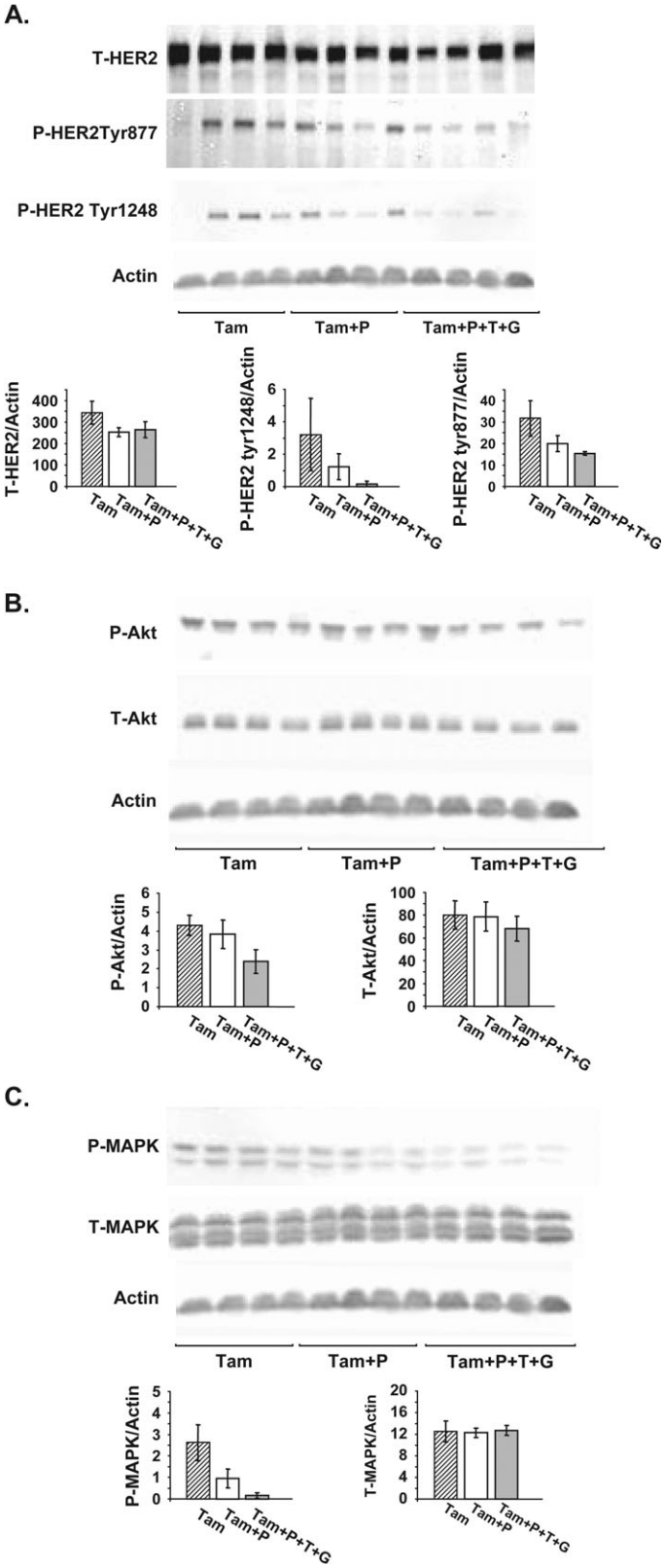

Fig. 5. Human epidermal growth factor receptor (HER) downstream signaling in MCF7/HER2-18 tumors from mice treated with tamoxifen (Tam) and pertuzumab (P) as a single anti-HER agent or the three-drug combination of HER inhibitors—pertuzumab, trastuzumab (T), and gefitinib (G). For all panels, the maximum length for each treatment was 3 days. Proteins from tumor lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to immunoblot analysis with antibodies specific for total HER2, total Akt, total MAPK, and phosphorylated HER2 (at Tyr^{887} and Tyr^{1248}); phosphorylated Akt (at Ser^{437}) and phosphorylated p42/44 MAPK (at Thr^{202} or Tyr^{204}); or β-actin. Protein expression and phosphorylation levels were quantified by densitometry with an Alpha Imager Detection System. In each tumor sample, protein levels were normalized to the level of β-actin (the loading control; by the formula, protein level/actin level × 100). The means of corrected expression levels (n = 4) are presented for each treatment group; **error bars** are 95% confidence intervals. **A**) Levels of total HER2 and phosphorylated HER2 (on Tyr^{887} and Tyr^{1248}) in the indicated tumor extracts. **B**) Levels of total Akt (T-Akt) and phosphorylated Akt (on Ser^{437}) (P-AKT) in the indicated tumor extracts. **C**) Levels of total p42/44 mitogen-activated protein kinase (MAPK; total MAPK [T-MAPK]) and phosphorylated p42/44 MAPK (P-MAPK; on Thr^{202} and Tyr^{204}) in tumor extracts as indicated.

Activated Akt was also reduced by the treatment. Specifically, with the three-drug combination, the level of phosphorylated Akt was statistically significantly lower (normalized median protein level = 2.6, IQR = 2.1–2.7) than after treatment with tamoxifen alone (normalized median protein level = 4.1, IQR = 3.9–4.7) or with tamoxifen and pertuzumab (normalized median protein level = 3.9, IQR = 3.2–4.4) (P = .03 for both comparisons) (Fig. 5, B). Tumor levels of phosphorylated Akt were similar after treatment with pertuzumab and tamoxifen or with tamoxifen alone (P = .47, Fig. 5, B).

Levels of phosphorylated MAPK were also statistically significantly lower after treatment with the three-drug combination (normalized median protein level = 0.09, IQR = 0.07–0.22) than after treatment with tamoxifen alone (normalized median protein level = 2.5, IQR = 1.9–3.3) or with tamoxifen and pertuzumab (normalized median protein level = 0.89, IQR = 0.61–1.31) (P = .03 for both comparisons) (Fig. 5, C). When the level of phosphorylated MAPK was assessed by immunohistochemistry, we found substantially lower expression after treatment with the three-drug combination (median Allred score = 3.5, IQR = 2.5–5.0) (Fig. 6, A) than after tamoxifen (median Allred score = 7.2, IQR = 7–8) (P = .02) (Fig. 6, B) or tamoxifen and pertuzumab (median Allred score = 5.5, IQR = 5–7) (P = .04) (Fig. 6, C). Furthermore, levels of phosphorylated MAPK were lower after treatment with tamoxifen, pertuzumab, and gefitinib than after treatment with tamoxifen and pertuzumab, although statistical significance was not achieved. No substantial inhibition was observed after treatment with tamoxifen, pertuzumab, and trastuzumab. The most extensive inhibition was observed after treatment with tamoxifen and the three-drug combination (data not shown).

We investigated cell proliferation by assessing the uptake of BrdU by tumor after 5 days of therapy. The fraction of actively proliferating cells was two- to threefold lower after treatment with the three-drug combination (median percentage of cells positive for BrdU = 10%, IQR = 9%–11%) than after treatment with tamoxifen (median percentage of cells positive for BrdU = 29%, IQR = 24%–34%) or with tamoxifen and pertuzumab (median percentage of cells positive for BrdU = 29%, IQR = 21.5%–35.5%), in agreement with findings for phosphorylated MAPK (Fig. 7, A).

Apoptosis at day 3 of treatment was assessed by immunohistochemistry with an antibody against cleaved caspases 3 and 7 (Fig. 7, B). The level of apoptosis induced by treatment with tamoxifen and any of the three HER inhibitors used as single agents was not statistically significantly different from that induced by tamoxifen

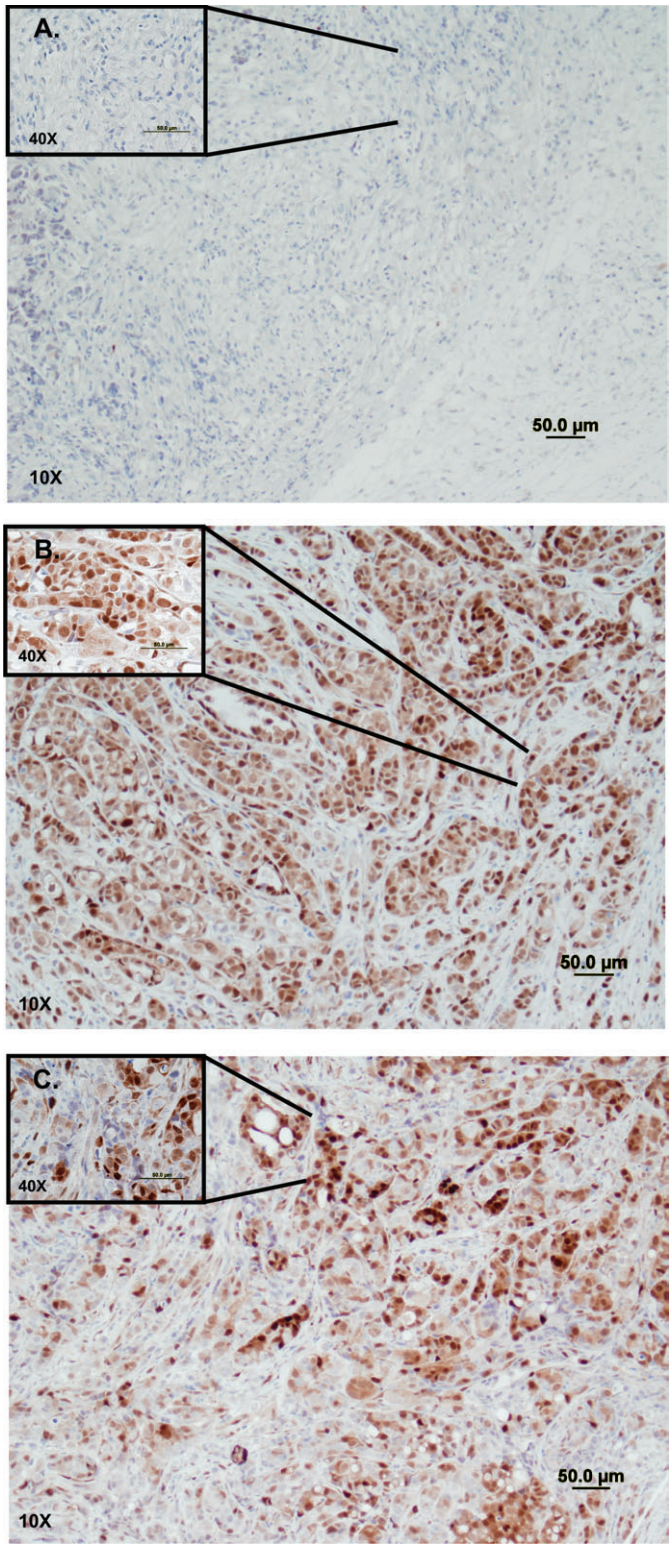

Fig. 6. Expression of phosphorylated p42/44 mitogen-activated protein kinase (MAPK, phosphorylation on Thr^{202} and/or Tyr^{204}) in tamoxifen-treated tumors. For all panels, the maximum length for each treatment was 3–5 days. Phosphorylated MAPK was assessed by immunohistochemical staining (original magnification = ×10, **insets** = ×40). **A**) Tamoxifen-treated tumor treated with the three-drug combination. **B**) Tumor treated with tamoxifen alone. **C**) Tamoxifen-treated tumor treated with single-agent pertuzumab.

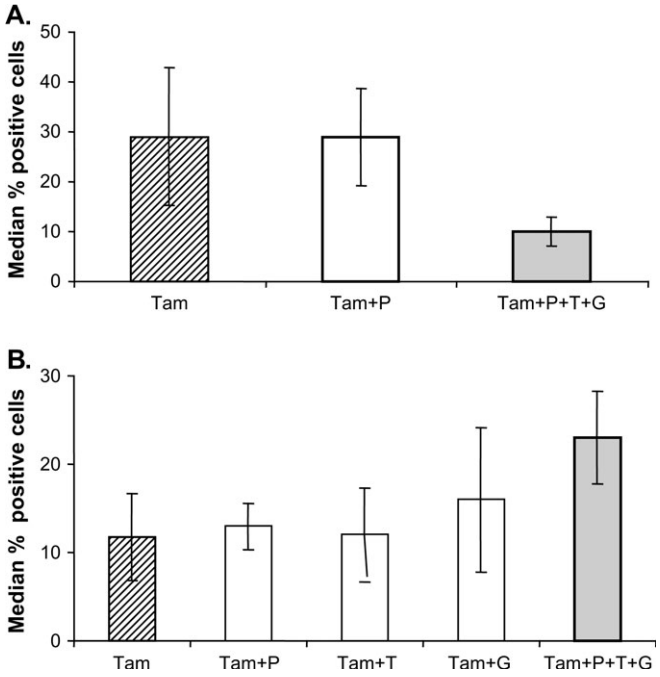

Fig. 7. Treatment of MCF7/HER2-18 tumors with tamoxifen (Tam) and single-agent pertuzumab (P) or the three-drug combination—pertuzumab, trastuzumab (T), and gefitinib (G)—and cell proliferation and apoptosis. **A**) Cell proliferation as measured by bromodeoxyuridine staining. Proliferation was measured in tumors, two from each treatment group, after 5 days of treatment. Results are the percentage of cells positive for bromodeoxyuridine; **error bars** are 95% confidence intervals. **B**) Apoptosis as assessed by immunohistochemical analysis of levels of cleaved caspase 3 or 7 after 3 days of treatment. Apoptosis was measured in more than 10 tumors from each treatment group. Results are the percentage of cells positive for cleaved caspase 3 or 7; **error bars** are 95% confidence intervals.

alone. In contrast, apoptosis was strongly induced by treatment with the three-drug combination and tamoxifen. The median percentage of cells positive for cleaved caspase 3 or 7 was statistically significantly higher after treatment with the three-drug combination and tamoxifen (median percentage of cells positive for cleaved caspases 3 and 7 = 25%, IQR = 19%–31.6%) than after treatment with tamoxifen (median percentage of cells positive for cleaved caspases 3 and 7 = 11.7%, IQR = 9%–13.6%) ($P = .008$), with tamoxifen and pertuzumab (median percentage of cells positive for cleaved caspases 3 and 7 = 13%, IQR = 12.4%–13.5%) ($P = .002$), or with tamoxifen and trastuzumab (median percentage of cells positive for cleaved caspases 3 and 7 = 12%, IQR = 10.5%–18%) ($P = .011$). Although apoptosis on day 3 after treatment with the three-drug combination was higher than after treatment with tamoxifen and gefitinib, the increase did not reach statistical significance. In a subsequent limited analysis that compared two-drug combinations with the three-drug combination, higher apoptotic rates were observed with the three-drug combination, although, again, statistical significance was not achieved (data not shown).

Discussion

Our data from two different HER2-overexpressing breast cancer xenograft models indicate that the three-drug combination designed to block signaling from various HER dimer pairs is more effective

than the inhibitors used alone or in dual combinations. This regimen even eradicated tumors in a substantial proportion of mice. These results support the hypothesis that acquired resistance to the individual agents is the result of incomplete blockade of this complex network at the receptor level and not activation of a different survival pathway. Although both cell lines also express ER, only MCF7/HER2-18 cells required simultaneous treatment with tamoxifen or estrogen deprivation to block ER stimulation of tumor growth. Additional study is needed to clarify whether both ER and HER2 need to be blocked in patients with tumors expressing both proteins.

Several in vitro and in vivo studies investigating possible synergistic mechanisms of different HER family inhibitors have been published (30,44–48). The robust cooperative efficacy of trastuzumab and pertuzumab has been attributed to enhanced endocytosis of HER2 (49) and/or to a more complete blockade of the various HER dimers (32). In our study, even in the presence of all three HER inhibitors, we did not detect statistically significantly decreased HER2 protein levels, although both HER2 activation and HER2 downstream signaling were reduced. It is possible that treatment longer than 3–5 days may be needed in vivo to detect loss of HER2. However, treatment with the three-drug combination for 3–5 days robustly inhibited the activation of MAPK and AKT, reduced proliferation, and increased apoptosis. Thus, it may not be necessary to decrease HER2 expression substantially to achieve potent antitumor effects. Several weeks of trastuzumab treatment did not decrease the expression of HER2 in patient samples, but it did induce apoptosis, indicating that receptor loss was not linked to trastuzumab activity (50).

Trastuzumab treatment is currently used alone or with chemotherapy in patients. However, only 30% of patients with metastatic disease respond to trastuzumab treatment despite having tumors with amplified HER2. Trastuzumab is more effective in the adjuvant setting than as a single agent, but tumors also recur in many of these patients (51). Combination treatment with trastuzumab and gefitinib was tested recently in a clinical trial but was not effective when compared with previous data from treatment with trastuzumab alone (52). One possible explanation for this clinical result is that this combination failed to block signaling from HER2–HER3 heterodimers, so that signaling was forced down an antiapoptotic pathway, increasing the resistance of tumors to cell death signals. Similarly, combination treatment with only two of the three anti-HER inhibitors was not effective in our xenograft models, and complete and durable tumor regression in nearly all mice was achieved only with combination treatment with all three anti-HER inhibitors. Our finding that, in the presence of the two HER2 antibodies, gefitinib was still needed for complete HER blockade indicates that HER1 signaling may be important in activating the HER pathway, even at low levels of HER1 expression. These results also indicate that when one HER pathway is blocked, tumors appear to use an alternative HER pathway. Thus, the complete initial blockade of all HER dimer pairs may be the best treatment approach.

Our data also support the idea that it is ER–HER cross talk that causes de novo tamoxifen resistance in MCF7/HER2-18 tumors. When the ER is occupied by estrogen, which activates both the genomic (nuclear activity that regulates gene transcription) and nongenomic (membrane activity that may interfere with cytoplasmic signal transduction) ER activities, even complete blockade of the HER family with all three HER inhibitors was relatively ineffective. However, when ER is bound by tamoxifen, which normally inhibits the genomic effects of ER, the combination of HER inhibitors restores the tumor's sensitivity to tamoxifen by preventing tamoxifen from activating HER2 through the nongenomic functions of ER. Consequently, the combination of tamoxifen with the three-drug combination slows proliferation, activates apoptosis, and induces complete regression of nearly all tumors.

Restoration of the sensitivity of tamoxifen-resistant tumors to the estrogen antagonist effects of tamoxifen by inhibiting the HER pathway with the three-drug combination was confirmed by the ability of tamoxifen to substantially inhibit estrogen-induced tumor growth only when the HER pathway was inhibited (Fig. 2). However, the antitumor effect of tamoxifen and HER inhibition was lower when estrogen was present than when estrogen was absent. Therefore, despite the complete HER inhibition, high levels of estrogen may reduce the potency of the ER-antagonizing activity of tamoxifen. Results from two clinical trials (53,54) indicate that, in premenopausal patients, combination treatment with ovarian ablation and tamoxifen may be associated with a better outcome than either treatment alone. It is important to emphasize that for MCF7/HER2-18 tumors, which have abundant ER and HER2, combination treatment with the three-drug regimen was ineffective unless it was combined with ER-targeted therapy such as tamoxifen or estrogen deprivation. In such tumors, both ER and HER2 appear to drive tumor cell survival, and both pathways should be blocked for optimal outcome—an observation with potential clinical importance. It is also possible that ER-positive patients who are resistant to endocrine therapy may still need to be treated with an ER inhibitor when they are treated with HER inhibitors because blocking the HER pathway may restore estrogen sensitivity. Additional studies with different model systems or in patients with ER-positive, HER2-positive tumors are required to clarify these possibilities.

BT474 cells are also ER positive, but, in contrast to the tranfected MCF7/HER2-18 cells, HER2 is naturally amplified in these cells. Although growth of BT474 cells in mice requires estrogen supplementation, tumor cells soon become estrogen independent as tumors form. Combination treatment with the three HER2 inhibitors was an effective therapy in these tumors, even in the presence of estrogen. As with MCF7/HER2-18 tumors that were treated with ER-targeted therapy and the three-drug combination, the more complete inhibition of the HER pathway resulted in rapid complete regression of all tumors. All tumor cells appear to have been eradicated by the treatment because, in mice with complete tumor regression, tumors did not regrow even after estrogen replenishment. BT474 xenograft tumors were more sensitive to anti-HER therapy than MCF7/HER2-18 tumors. No BT474 tumors regrew even after 2 months of observation. Therefore, tumors in patients that are similar to BT474 xenograft tumors may depend only on the HER pathway for survival, despite the fact that they express ER, whereas tumors similar to MCF7/HER2-18 tumors may depend on both ER and HER2 pathways for survival.

This study had several limitations. The superiority of the combined HER-targeted approach was found only in two cell lines in

an in vivo experimental model and cannot necessarily be extrapolated to other HER2-positive cell lines or to patients with HER2-amplified tumors. There are clearly other potential mechanisms for resistance to single-agent HER-targeted therapies that would not be overcome by more completely blocking the pathway at the receptor level such as activation of a different family of growth factor receptors.

Nevertheless, our findings do provide a rationale for continued studies of the three-drug strategy in other preclinical models and in patients with HER2-amplified tumors. Additional studies should also be designed to further investigate the mechanism of action and to obtain clues to the mechanisms of resistance to the three-drug combination therapy.

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Notes

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