Synergistic Interactions between Tamoxifen and Trastuzumab (Herceptin)

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ABSTRACT

Purpose: HER-2/neu and estrogen receptor (ER) are critical in the biology of breast carcinoma, and both are validated therapeutic targets. Extensive interactions between the signaling pathways of these receptors have been demonstrated. This suggests that targeting both receptors simultaneously may have a dramatic effect on the biology of breast cancer. This hypothesis was tested in cell culture experiments.

Experimental Design: ER-positive, HER-2/neu-overexpressing BT-474 human breast carcinoma cells were cultured in the presence of the anti-HER-2/neu therapeutic antibody trastuzumab (Herceptin), the antiestrogen tamoxifen, or both. The effects on cell growth, cell cycle distribution, clonogenicity, survival, and the level and activity of HER-2/neu were examined.

Results: The combination of tamoxifen and Herceptin resulted in synergistic growth inhibition and enhancement of cell accumulation in the G0-G1 phase of the cell cycle, with a decrease in cells in S phase. Clonogenicity was inhibited in the presence of each drug and more so by the combination, although prior exposure to drugs did not affect subsequent clonogenicity in drug-free media, and neither drug nor the combination induced apoptosis. Herceptin, but not tamoxifen, inhibited signaling by HER-2/neu.

Conclusions: The combination of tamoxifen and Herceptin is formally demonstrated to result in synergistic growth inhibition and enhancement of G0-G1 cell cycle accumulation. In vitro, the individual drugs or combination produces a cytostatic effect. These results suggest that combined inhibition of ER and HER-2/neu signaling may represent a powerful approach to the treatment of breast cancer.

INTRODUCTION

Approximately 25% of invasive human breast tumors have amplification of the HER-2/neu gene and/or overexpression of HER-2/neu protein, which has been found to be an adverse prognostic factor (reviewed in Refs. 1 and 2). Trastuzumab (Herceptin) is a humanized monoclonal antibody against the extracellular domain of HER-2/neu (3–5) that has shown significant therapeutic activity in the treatment of patients with HER-2/neu-overexpressing metastatic breast cancer, both as a single agent (6–8) and in enhancing antitumor activity in combination with cytotoxic chemotherapy (9–11). Signaling by the estrogen/estrogen receptor (ER) pathway is also important in breast cancer tumorigenesis and biology. Antiestrogen therapy has long been a cornerstone of the treatment of breast cancer. A large body of literature has now demonstrated extensive interactions between the ER signaling pathway and growth factor receptor (including HER-2/neu) signaling, both in terms of downstream effects as well as regulation of each other’s activity.

HER-2/neu and ER are capable of down-regulating each other. Clinically, an inverse relationship between HER-2/neu and ER expression is well appreciated (1, 2). Regulatory elements exist within the HER-2/neu gene which allow estrogen to negatively regulate its transcription (12–18); inhibiting estrogen action can increase HER-2/neu levels (12, 13, 17, 19–21). Conversely, cross-activation of HER-2/neu by the neuregulins (ligands for HER-3 and HER-4) results in decreased ER levels (22–25) via unknown mechanisms.

In clinical practice, it has been observed in retrospective studies that tumors that overexpress HER-2/neu, even when ER positive, may have reduced responsiveness to antiestrogen therapy (Refs. 26–33 but see Ref. 34). Although there is an inverse relationship between the degree of ER positivity and HER-2/neu expression, a not insignificant proportion of human breast cancers are both ER positive and overexpress HER-2/neu (26–28). Growth factor signaling alters steroid hormone responsiveness/sensitivity. Stimulation of growth factor signaling can lead to estrogen independence in ER-positive cells that are otherwise estrogen dependent (23, 24). Growth factor signaling can also lead to antiestrogen resistance in such cells (24, 35, 36). Such results suggest that growth factor signaling can rescue estrogen-dependent cells from the effects of estrogen deprivation.

The ER antagonist tamoxifen has been the most widely used antiestrogen in clinical practice. One component of the antiestrogen mechanism of tamoxifen is believed to be inhibition of the binding of coactivators to ER (37) and, possibly even more importantly, strengthening of the interaction of ER with corepressors such as N-CoR (38). Acquired tamoxifen resist-
ance may be related to a change in the balance of association of ER with coactivators or corepressors. One study focused on why the forced overexpression of HER-2/neu in estrogen-dependent, tamoxifen-sensitive cells results in tamoxifen resistance (39). These investigators found that a HER-2/neu tyrosine kinase inhibitor restored tamoxifen sensitivity to this cell line. The important mechanistic insight was that this was accompanied by restoration of tamoxifen-induced association of ER with the corepressor N-CoR in the transfected cells, which had been impaired compared with the parental line. Treatment with a combination of the tyrosine kinase inhibitor and tamoxifen markedly inhibited the growth of the transfected cell line as a xenograft, whereas the transfected line was known to be otherwise tamoxifen resistant in vivo.

Mechanistically, it has been found that growth factor signaling may result in ligand-independent activation of ER. Activation of the ras/mitogen-activated protein kinase pathway, known to lie downstream of growth factor receptor signaling, can cause ligand-independent activation of ER and even activation of ER in the presence of tamoxifen (40–43); this appears to involve the phosphorylation of ER Ser118 by mitogen-activated protein kinase (40, 43), a site normally phosphorylated in response to estrogen itself by a mitogen-activated protein kinase-independent mechanism (44). Another study found that the activation of ER by epidermal growth factor was mediated by phosphatidylinositol 3’-kinase/Akt signaling (45), a survival pathway also activated by HER-2/neu. These studies suggest additional mechanistic explanations for how signaling by growth factor receptors can result in hormone independence/tamoxifen resistance.

Conversely, estrogen treatment can lead to activation of growth factor signaling pathways. Estrogen is known to induce epidermal growth factor-related peptides, including transforming growth factor-α (46, 47), and medroxyprogesterone acetate induces neuregulin production (48). It therefore appears that sex hormones may exert their mitogenic response by inducing release of peptide growth factors that then activate receptors such as HER-2/neu and epidermal growth factor receptor. Direct association of ER with the cytoplasmic tyrosine kinase src has also been observed, resulting in activation of src and src-dependent activation of the ras/mitogen-activated protein kinase pathway (49, 50). The ER has also been found to bind to the p85 regulatory subunit of phosphatidylinositol 3’-kinase in a ligand-dependent manner, resulting in activation protein kinase B/Akt (51).

Hence, activation of ER leads to a rapid activation of pathways typically activated by peptide growth factors, and activation of growth factor signaling pathways by peptide growth factors can activate ER directly and independently of estrogen. This biology and apparent redundancy/positive feedback argues that targeting both ER and growth factor receptors simultaneously could be a fruitful approach to the treatment of breast cancer. Preliminary work on this concept (52, 53) has shown encouraging results. Our goal was to further explore the combined effect of antiestrogen and anti-HER-2/neu treatment in vitro, using two clinically useful drugs in the treatment of breast cancer, tamoxifen and Herceptin. Because both tamoxifen and Herceptin are active as single agents in breast cancer and have a favorable toxicity profile compared with cytotoxic chemotherapy, our results may translate into efficacious and safe therapy for this malignancy.

**MATERIALS AND METHODS**

**Drugs.** Herceptin was a gift of Genentech (South San Francisco, CA). A 5 mg/ml stock preparation was kept at 4°C and was further diluted in sterile PBS before addition to cells in culture. Tamoxifen was purchased from Sigma (St. Louis, MO) and dissolved in 100% ethanol at a stock concentration of 10 mg/ml; the stock was further diluted in PBS and/or culture media before addition to cells in culture. Tamoxifen stocks were kept light protected.

**Cell Culture.** BT-474 (54) is a human breast cancer cell line that is positive for ER (low; Ref. 23), is estrogen dependent for growth (23), and highly overexpresses HER-2/neu in association with gene amplification (55, 56). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, 10 μg/ml insulin, and penicillin at 37°C in 5% CO₂ humidified air.

**Growth Curves.** BT-474 cells in exponential growth were seeded on day −1 in 12-well plates at 2 × 10⁵ cells/well in 3 ml of culture volume and allowed to adhere overnight. Drugs were added on day 0. For time point 0 (24 h after seeding), and subsequently for all other time points, cells were washed twice in PBS, trypsinized, collected, and counted using a Coulter ZBI particle counter with a multichannel analyzer, with counts confirmed by counting with a hemocytometer. Growth curves were analyzed for cells that were treated with either 1 μM tamoxifen, 10 μg/ml Herceptin, or both. As controls, cells were also examined when grown in drug-free media (“No Addition”) or 10 μg/ml irrelevant antibody (rabbit anti-mouse IgG; Vector Laboratories, Inc., Burlingame, CA) plus ethanol (the vehicle for tamoxifen, at identical concentration; “Vehicle”). The data represent two individual experiments performed in duplicate and averaged together.

**WST-1 Colorimetric Growth Assay.** BT-474 cells were plated in 96-well plates at a density of 10⁴ cells/100-μl culture/ well. They were allowed to adhere overnight and then either left untreated or treated with Herceptin at doses of ≤10 μg/ml, tamoxifen at doses of ≤8 μM, or various combinations of the two. After a 5- or 6-day incubation period, the WST-1 tetrazolium salt colorimetric growth assay (Boehringer Mannheim Biochemicals, Mannheim, Germany) was performed by adding 1/10 volume of WST-1 solution and incubating for 2.5 h at 37°C. Absorbance at 450 nm was determined using a microplate reader (Molecular Devices Corp., Sunnyvale, CA). Results were expressed as a percentage of control (cells without drug). The mean and SE values of four or more wells in at least two experiments for each point are reported.

**Analysis of Synergy.** Data from the WST-1 colorimetric growth assay were analyzed using the method of Chou and Talalay (57), using commercially available software (CalcuSyn; Ref. 58). This method is based on the median-effect equation for dose-effect relationship: \( f / f_0 = (D/D_m)^n \), which can be linearly transformed as \( \log (f / f_0) = m \log (D) - m \log (D_m) \), where \( f_a \) = fraction affected, \( f_u \) = fraction unaffected, \( D \) = dose, \( D_m \) = dose that produces median-effect (IC₅₀), and \( m \) = coefficient signifying the sigmoidicity of the curve (or slope in linear
transformation). For the model to be applicable, the linear correlation coefficient ($r$) should be $>0.9$. For examining the effect of multiple drugs, a Combination Index (CI) is calculated based on the doses that have equivalent effects, according to the formula: $CI = (D1/D1c) + (D2/D2c)$, where $D1$ and $D2$ are the doses for each drug alone that inhibit x%, and $D1c$ and $D2c$ are the doses for each drug in a combination that inhibits the same percentage x%. The CI is a quantitative measure of the degree of interaction between two or more drugs. When it equals to 1, it denotes additivity, $>1$ antagonism, $<1$ synergism, and $<0.3$ strong synergism. The CI was calculated under the assumption of a mutually nonexclusive drug interaction.

**Analysis of Cell Cycle by Flow Cytometry.** BT-474 cells in exponential growth were seeded at a confluency of $\sim$50% in 100-mm dishes. After overnight incubation, cells were either left untreated or treated with 1 $\mu$M tamoxifen, 10 $\mu$g/ml Herceptin, or the combination. At indicated time points, cells were trypsinized, washed with cold PBS, resuspended in 2 ml of ice cold PBS, fixed by three stepwise additions of 2 ml each of 95% ethanol, and stored at 4°C for cell cycle analysis (39). Subsequently, cells were resuspended in 1 mg/ml RNase (Sigma) in PBS and stained with 0.05 mg/ml propidium iodide (Sigma P5264) for 1 h on ice. Flow cytometric analysis was performed with a FACS Vantage flow cytometer (Becton-Dickinson, San Jose, CA). A minimum of 15,000 cells was analyzed for each sample. Data analysis was performed using Modfit 5.2 analysis software (Verity Software House, Topsham, ME). Each point represents at least duplicate samples from between one and three experiments or triplicate samples from a single experiment.

**Apoptosis Assays.** To measure apoptosis, BT-474 cells were plated at $2.5 \times 10^6$ cells/100-mm dish and cultured in the presence of 1 $\mu$M tamoxifen, 10 $\mu$g/ml Herceptin, both drugs, no drug, or vehicle for 3 days. Both adherent and floating cells were harvested and used for the following apoptosis assays. For the DNA ladder assay, DNA was prepared using the Apoptotic DNA Ladder Kit (Roche Diagnostics, Mannheim, Germany) and electrophoresed through 1% agarose. For the poly(ADP-ribose) polymerase (PARP) cleavage assay, whole cell lysates were prepared by boiling in lysis buffer [100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 6 mM urea, 0.2% Bromphenol Blue, 10% $\beta$-mercaptoethanol, and 7 mM EDTA] followed by 3 min of water bath sonication. Protein concentrations of the lysates were determined by using a dencitric cell protein assay kit (Bio-Rad, Hercules, CA); 100 $\mu$g of protein from each lysate were resolved in 7.5% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. Western blotting was carried out using an anti-PARP antibody from Cell Signaling Technology (Beverly, MA). Apoptosis was detected by monitoring proteinolysis of the $M_r$ 116,000 native PARP enzyme to the apoptosis-specific $M_r$ 89,000 fragment. The flow cytometric terminal deoxynucleotidyl transferase-mediated nick end labeling assay was performed using the APO-bromodeoxyuridine kit (Phoenix Flow Systems, San Diego, CA). The percentage of FITC-positive apoptotic cells in the gated area was quantitated by flow cytometry.

**Soft Agar Colony Growth Assay.** To prepare base plates, 1 ml of RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, 10 $\mu$g/ml insulin, 10 mM HEPES, and 0.8% agarose was added into each 35-mm dish. The dishes were placed at 4°C to solidify the agarose and then transferred to a 37°C incubator. BT-474 cells were mixed with RPMI 1640 supplemented with the same components in the presence of 1 $\mu$M tamoxifen, 10 $\mu$g/ml Herceptin, both drugs, no drug (“Control”), or vehicle, then plated on top of the base at a density of $2 \times 10^4$ cells/35-mm dish in triplicate. Dishes were incubated at 37°C for 15 days. Clusters of greater than or equal to six cells were scored as colonies, and single cells as well as clusters of fewer than six cells were scored as single cells. Five microscopic fields ($\times$60) of each dish were scored, and the colony formation percentage of each dish was calculated by dividing the number of colonies by the total of colonies plus single cells. Final results were expressed as the percentage of untreated (“Control”) cells.

**Anchorage-Dependent (Monolayer) Clonogenicity Assays.** Monolayer colony assays A and AA were performed in the continuous presence of drugs, whereas assay B was performed in drug-free media after prior exposure to drugs. For monolayer colony assay A, cells were seeded at a density of $5 \times 10^3$ cells/60-mm dish in triplicate in the presence of 1 $\mu$M tamoxifen, 10 $\mu$g/ml Herceptin, both drugs, no drug, or vehicle. Dishes were incubated at 37°C for 30 days. Media were exchanged for fresh media containing drugs every 6 days. Dishes were stained with 1 ml of 0.005% crystal violet for 30 min and washed three times with double-distilled water. Solid cell clusters of $\geq 0.4$ mm in diameter were scored as colonies. Clusters of cells that were not solid, but more dispersed, were sometimes also observed. In assay AA, these “loose” clusters were also scored as colonies. The trends for the effect of drug treatment, compared with control untreated cells, were the same whether these dispersed clusters were scored as colonies or not. Results are expressed as a percentage of control (untreated) colony number.

For monolayer colony assay B, cells were seeded on day −1 in 100-mm dishes at a density of $2.5 \times 10^6$ cells/dish and allowed to adhere overnight. On day 0, cells were exposed to 1 $\mu$M tamoxifen, 10 $\mu$g/ml Herceptin, both drugs, no drug, or vehicle. After 4-day incubation in the presence of drugs, cells were trypsinized and seeded at a density of $5 \times 10^3$ cells/60-mm dish in triplicate in drug-free RPMI 1640 and incubated at 37°C for 30 days. Media were exchanged for fresh (drug free) media every 6 days. Dishes were stained with 1 ml of 0.005% crystal violet for 30 min and washed three times with double-distilled water. Solid cell clusters of $\geq 0.4$ mm in diameter were scored as colonies. Results are expressed as a percentage of control (untreated) colony number.

**Immunoblot Analysis.** Cell lysates were prepared from BT-474 cells cultured in the presence of 1 $\mu$M tamoxifen, 10 $\mu$g/ml Herceptin, both drugs, no drug, or vehicle in 100-mm plates. Media were aspirated, and cells were washed twice with ice-cold PBS. Subsequently, 0.8 ml of ice-cold lysis buffer [50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 1% NP40, 2 mM EDTA, 1 mM $\beta$-glycerol phosphate, 25 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 5 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin, and 5 $\mu$g/ml pepstatin] was added into each plate and spread evenly. After incubating the plate on ice for 10 min, cells were scraped off the plate using a rubber policeman, transferred into a 1.5-ml centrifuge tube, and lysed for another 15 min on
ice. The lysate was centrifuged at 12,000 × g for 30 min at 4°C to remove insoluble material. Protein concentrations of the lysates were determined by using a Bio-Rad protein assay kit (Bio-Rad). Protein (50 μg) was separated on a 7.5% SDS-polyacrylamide gel followed by transfer to a single nitrocellulose membrane as described previously (60). Membranes were blocked for 1 h in Tris-buffered saline Superblock (Pierce) containing 0.1% Tween 20. Primary antibodies used were either anti-HER2/neu polyclonal antibody sc-284 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-phospho-HER-2/neu monoclonal antibody clone PN2A (c-erbB-2/HER-2/neu phospho-specific Ab-18; NeoMarkers, Fremont, CA). Anti-HER2/neu and anti-phospho-HER-2/neu antibodies were diluted in blocking buffer at a concentration of 1:200 to 1:2000, respectively. Membranes were incubated at 4°C overnight and washed for 1 h at room temperature. Secondary horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:200. Membranes were washed and processed using enhanced chemiluminescence (Amersham, Little Chalfont Buckinghamshire, United Kingdom).

RESULTS

Effect of Herceptin, Tamoxifen, and the Combination on Cell Growth. Growth curves for BT-474 cells that were either untreated or cultured in the presence of 1 μM tamoxifen, 10 μg/ml Herceptin, or the combination, are shown in Fig. 1. Additionally shown are data for cells treated with irrelevant antibody at 10 μg/ml plus ethanol vehicle (“Vehicle”). Each individual drug at these physiologically relevant concentrations caused partial growth inhibition, whereas the combination resulted in nearly complete growth inhibition. The data represent two individual experiments performed in duplicate and averaged together and is reflective of multiple similar experiments.

An analysis of synergism was conducted, using the method of Chou and Talalay (Ref. 57; see “Materials and Methods”) and

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**Fig. 1** Growth curves. BT-474 cells in exponential growth were seeded on day –1 in 12-well plates at 2 × 10⁵ cells/well in 3 ml of culture volume and allowed to adhere overnight. Drugs were added on day 0. At time 0 (24 h after seeding), and subsequently for all other time points, cells were washed, trypsinized, and counted. Growth curves were analyzed for cells that were treated either with 1 μM tamoxifen, 10 μg/ml Herceptin, or both. No Addition, drug-free conditions; Vehicle, 10 μg/ml irrelevant antibody plus ethanol at equivalent concentration as used for the tamoxifen vehicle. The data represent two individual experiments performed in duplicate and averaged.

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**Fig. 2** Growth assay. After a 5–6-day continuous exposure to drug, a WST-1 tetrazolium salt colorimetric growth assay was performed. Results were expressed as a percentage of control (cells without drug) in each experiment. The mean value and SE of the mean value of four or more wells in at least two experiments is reported. Top panel, effect shown as a function of Herceptin concentration; bottom panel, effect shown as a function of tamoxifen concentration (same data for both panels).
the data from the WST-1 colorimetric growth assays. This model is valid for the data collected, as indicated by the linear coefficients ($r$) of $0.9$ for each drug, as shown in Fig. 3. All combinations tested with tamoxifen in the range of 1–8 $\mu$M and Herceptin in the range of 0.375–10 $\mu$g/ml were synergistic, most strongly so with CIs of $0.35$ (Table 1).

**Effect on Cell Cycle.** Changes in cell cycle were examined by flow cytometry for BT-474 cells that were either untreated or cultured with 1 $\mu$M tamoxifen, 10 $\mu$g/ml Herceptin, or the combination. Both tamoxifen (61, 62) and Herceptin (63–65) individually are known to cause accumulation of cells in the G0–G1 phase. Our results showed that at 24 h (Fig. 4; Table 2) and 48 h (Fig. 5; Table 3) of drug exposure, both drugs individually resulted in a statistically significant increase in the percentage of cells in G0–G1 compared with untreated cells ($P < 0.05$). The combination of tamoxifen and Herceptin produced a statistically significant further increase in the percentage of cells in G0–G1 phase compared with the effect of either drug alone after 24 h of exposure (Fig. 4). At 24 h, there was also a decrease in the percentage of cells in S phase compared with untreated cells or Herceptin-treated cells, although it was comparable with the effect of single agent tamoxifen. Single agent Herceptin caused a slight increase in the percentage of cells in S phase, which might be attributable to its partial agonist activity. At 48 h, the combination resulted in a high percentage of G0–G1 cells compared with control or tamoxifen-treated cells but which was similar to that elicited by Herceptin alone (Fig. 5). However, the reduction in S phase for the combination was statistically significant compared with untreated cells or cells treated with either agent singly.

**Assays for Apoptosis.** We hypothesized that the combination of tamoxifen and Herceptin might cause apoptosis in BT-474 cells. Cells were cultured in the presence of 1 $\mu$M...
**Synergy between Tamoxifen and Herceptin**

Compared with control (Fig. 8), apoptosis was always characteristic of cells undergoing apoptosis. Camptothecin-treated BT-474 cells were used as a positive control. No PARP cleavage was observed in BT-474 cells for any of the culture conditions (Fig. 6). To confirm this result, two additional assays of apoptosis were used. Cells were cultured as above, and protein extracts were analyzed for PARP cleavage by immunoblotting with anti-PARP antibody. Etoposide-treated HL-60 cells were used as a positive control.

**Effect on cell cycle at 48 h** (Table 2). The presence of tamoxifen, Herceptin, and the combination, under these culture conditions, results in apoptosis of BT-474 cells.

**Effect on Anchorage-Dependent and -Independent Clonogenicity.** The effect of the presence of tamoxifen, Herceptin, and the combination on the soft agar colony growth of BT-474 cells was analyzed (Table 4). For these experiments, drug was present continuously during colony growth as described in “Materials and Methods.” The presence of tamoxifen, 10 μg/ml Herceptin, both drugs, vehicle controls, or no drug for 3 days. Adherent and floating cells were harvested, and DNA was isolated and subjected to electrophoresis to assay DNA fragmentation resulting in the laddering pattern characteristic of cells undergoing apoptosis.

**Table 2** Cell cycle effect at 24 h (% cells, mean ± SE)

<table>
<thead>
<tr>
<th>Phase of cycle</th>
<th>Control</th>
<th>Tamoxifen</th>
<th>Herceptin</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>G&lt;sub&gt;0&lt;/sub&gt;-G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>50.50 ± 0.3</td>
<td>54.94 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.31 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.36 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>S</td>
<td>25.62 ± 2.2</td>
<td>22.37 ± 2.7</td>
<td>27.74 ± 3.6</td>
<td>18.99 ± 2.6</td>
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<tr>
<td>G&lt;sub&gt;2&lt;/sub&gt;-M</td>
<td>23.88 ± 2.2</td>
<td>22.69 ± 2.3</td>
<td>17.94 ± 3.3</td>
<td>19.65 ± 1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 compared with control (t test).

<sup>b</sup> P < 0.05 for all comparisons (t test).

**Table 3** Cell cycle effect at 48 h (% cells, mean ± SE)

<table>
<thead>
<tr>
<th>Phase of cycle</th>
<th>Control</th>
<th>Tamoxifen</th>
<th>Herceptin</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>G&lt;sub&gt;0&lt;/sub&gt;-G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>72.08 ± 1.2</td>
<td>77.50 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82.36 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.50 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>S</td>
<td>15.10 ± 1.2</td>
<td>11.18 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.64 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.94 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>G&lt;sub&gt;2&lt;/sub&gt;-M</td>
<td>12.81 ± 0.4</td>
<td>11.31 ± 0.6</td>
<td>8.00 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.56 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 compared with control (t test).

<sup>b</sup> P < 0.05 for all comparisons (t test).

**Fig. 5** Effect on cell cycle at 48 h (method as for Fig. 4.). *, P < 0.05 compared with control (t test); **, P < 0.05 for all comparisons (t test).

**Fig. 6** DNA electrophoresis apoptosis assay. BT-474 cells were plated at 2.5 × 10<sup>6</sup> cells/100-mm dish and cultured in the presence of 1 μM tamoxifen (T), 10 μg/ml Herceptin (H), both drugs (T/H), no drug (C, control), or vehicle (V) for 3 days. Adherent and floating cells were harvested, and the DNA was prepared using the Apoptotic DNA Ladder Kit. DNA from U937 cells treated with camptothecin, supplied by the manufacturer, was used as a positive control (P).

**Fig. 7** Poly(ADP-ribose) polymerase (PARP) cleavage apoptosis assay. BT-474 cells were plated at 2.5 × 10<sup>6</sup> cells/100-mm dish and cultured in the presence of 1 μM tamoxifen (T), 10 μg/ml Herceptin (H), both drugs (T/H), no drug (C, control), or vehicle (V) for 3 days. Adherent and floating cells were harvested, and whole cell lysates were prepared as described in “Materials and Methods.” Western blotting was carried out using an anti-PARP antibody. Whole cell extract of human HL-60 leukemia cells treated with the chemotherapeutic agent etoposide, supplied by the manufacturer, was used as a positive control (P).
produced a modest inhibition of soft agar colony formation (85.3% of control), whereas Herceptin produced a more pronounced inhibition (25.8% of control) and the combination the greatest degree of inhibition (13.5% of control).

Table 4  Soft agar colony growth and monolayer clonogenicity assays

Results are expressed as a percentage of control (no drugs or vehicles added). Concentrations used were 1 μM tamoxifen or 10 μg/ml Herceptin. For the soft agar assay and monolayer assays A and AA, drug was continuously present. For monolayer assay B, cells were precultured in the presence of drug for 4 days, then replated in drug-free media for the assay. In assay A, only solid colonies were scored as such, whereas in assay AA, loosely dispersed clusters were also scored as colonies as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Relative colony formation</th>
<th>Soft agar colony assay</th>
<th>Monolayer colony assay A</th>
<th>Monolayer colony assay AA</th>
<th>Monolayer colony assay B</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 2.61</td>
<td>100 ± 2.57</td>
<td>100 ± 2.39</td>
<td>100 ± 7.90</td>
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<tr>
<td>Vehicle</td>
<td>98.5 ± 4.28</td>
<td>99.3 ± 2.34</td>
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<td>97.4 ± 9.71</td>
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<td>TAM</td>
<td>85.3 ± 3.16</td>
<td>26.4 ± 1.12</td>
<td>60.5 ± 5.24</td>
<td>109.5 ± 9.17</td>
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<td>Herceptin</td>
<td>25.8 ± 3.21</td>
<td>6.2 ± 0.56</td>
<td>38.2 ± 3.63</td>
<td>100 ± 5.83</td>
</tr>
<tr>
<td>TAM + Herceptin</td>
<td>13.5 ± 2.77</td>
<td>1.1 ± 0.56</td>
<td>25.5 ± 2.44</td>
<td>108.2 ± 7.58</td>
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Immunoblot Analysis of HER-2/neu Expression Level and Phosphorylation State. Changes in HER-2/neu levels over time were analyzed by immunoblotting of cell lysates with anti-HER-2/neu antibody (Fig. 9, left panel). For cells that were treated with 1 μM tamoxifen, 10 μg/ml Herceptin, or the combination, HER-2/neu levels in lysates were relatively stable over the 5-day time course of the experiment. Because the activity of receptor tyrosine kinases is regulated and autophosphorylation is indicative of the signaling activity, autophosphorylated HER-2/neu levels were examined by immunoblotting with anti-phospho-HER-2/neu antibody PN2A (Fig. 9, right panel). For untreated (“C” = “Control”) and vehicle-treated (“V” = “Vehicle”) cells, an easily detectable basal level of phosphorylated receptor was found, likely caused by the high level of overexpression. Compared with control or vehicle-treated cells, tamoxifen produced no effect on the level of phosphorylated HER-2/neu in lysates. In contrast, Herceptin caused a marked decrease in level of phosphorylated HER-2/neu that was evident at the earliest time point examined (8 h) and persisted to the
same degree throughout the 5-day time course of this experiment. The addition of tamoxifen to Herceptin did not have any impact on the Herceptin effect.

**DISCUSSION**

Our results demonstrate using the ER-positive, HER-2/neu-overexpressing BT-474 cell line that the combination of tamoxifen plus Herceptin results in synergistic inhibition of cell growth in culture, accompanied by enhanced accumulation of cells in the G1-G0 phase of the cell cycle and reduction in S phase. The formal mathematical demonstration of synergy between such agents (antiestrogen and anti-HER-2/neu) has not been demonstrated previously. In the presence of drug, both agents inhibited anchorage-dependent and -independent clonogenicity, with the greatest degree of inhibition elicited by the combination. However, previous culture in drug-containing media did not adversely impact the subsequent anchorage-dependent clonogenicity in drug-free media, and neither drug nor the combination resulted in apoptosis, indicating a purely cytostatic effect in vitro.

Individually, tamoxifen (61, 62) and Herceptin/4D5 (63–65) have been reported to promote accumulation of cells in the G0-G1 phase of the cell cycle. We confirmed this and found an even greater effect of the combination (at 24 h). Differences were also apparent at 48 h, although the combination resulted in a similar percentage of cells in G0-G1, as did single agent Herceptin; of note is that even untreated cells had >70% G0-G1 cells by 48 h. The combination, however, did produce the lowest percentage of S phase at 48 h. In work with BT-474 cells, antibody 4D5 (the murine monoclonal antibody from which humanized Herceptin was derived) was found to result in increased levels of the cyclin-dependent kinase inhibitor p27, association of p27 with Cdk2, inactivation of cyclin-Cdk2 complexes, and hypophosphorylation of the retinoblastoma protein; the effect was reversible, and apoptosis was not observed (63). Similar results have been noted in the treatment of SKBR3 cells with another anti-HER-2/neu antibody (66). How the combination of anti-HER-2/neu therapy with antiestrogen impacts the cell cycle regulatory proteins is the subject of ongoing work in our laboratory.

We observed that Herceptin treatment did not affect the level of expression of HER-2/neu but did inhibit the signaling activity of HER-2/neu, as indicated by a decrease in the amount of Tyr-1248 phosphorylated HER-2/neu present. This inhibition of phosphorylation was evident at the earliest time point (8 h) examined. The inhibition of HER-2/neu activity is consistent with previous reports, although in other studies, the effect of Herceptin on total HER-2/neu levels has been varied. In experiments using 4D5, treatment has been reported to result in decreased levels of HER-2/neu using several cell lines, including SKBR3 and HER-2/neu-transfected NIH-3T3 cells (3, 67, 68). However, consistent with our results, a report in which BT-474 cells were treated with 4D5 found inhibition of phosphorylation of HER-2/neu but no decrease in level of expression (63). The differences in different studies could be caused by inherent cell line differences or the particular experimental conditions, such as time points examined or the density of the cells, which itself is known to impact HER-2/neu levels (69, 70). In our study, we followed cell changes for longer time points than in most other studies. The long time points examined in our study may better reflect steady-state changes obtained with chronic therapy in the clinical situation.

Some studies have demonstrated induction of phosphorylation of HER-2/neu by Herceptin. Herceptin, 4D5, and other anti-HER-2/neu growth-inhibiting antibodies are also known to have the properties of being partial HER-2/neu agonists, inducing phosphorylation of HER-2/neu and at times transiently activating downstream signaling events, although these agonistic properties predominate at early time points, and any signaling occurs transiently, if at all (66, 67, 71). Using SKBR3 cells, it was shown that the F(ab) fragment of 4D5 resulted in increased tyrosine phosphorylation of HER-2/neu at 15 min,
whereas treatment with 4D5 for 11–15 h resulted in reduced phosphorylation; furthermore, it was demonstrated that the decrease in phosphorylated HER-2/neu could not be attributed solely to down-regulation of the receptor but must have involved inhibition of the autophosphorylation activity itself (72), consistent with our results showing inhibition of phosphorylation without changes in total expression level. Such results may again be dependent on the experimental design, because in another study, 4D5 treatment of BT-474 cells was reported to result in HER-2/neu de-phosphorylation as early as 10 min after treatment, with decreases in phosphorylation of downstream Erk1/2 and protein kinase B occurring concomitantly or soon thereafter (63).

Estrogen is known to cause transcriptional down-regulation of HER-2/neu (12–18), and inhibiting estrogen signaling can increase HER-2/neu levels (12, 13, 17, 19–21). Tamoxifen has been reported previously to antagonize the HER-2/neu-down-regulating effect of estrogen and up-regulate levels of HER-2/neu (19), even in nonestrogen-supplemented media (13, 17). The antiestrogen toremifene also inhibited the estradiol-induced repression of HER-2/neu expression at 12 and 72 h in vitro in ZR-75-1 cells, and toremifene or tamoxifen inhibited estrogen-induced repression of HER-2/neu in vivo using ZR-75-1 xenografts when analyzed on day 10 of treatment (20). In contrast, one publication reported HER-2/neu ligand-like stimulatory effects of estradiol (73), with rapid induction of phosphorylation, down-regulation of HER-2/neu, and induction of morphological transformation (but not anchorage independence). In that study, tamoxifen was able to reverse the estradiol-induced phosphorylation and morphological transformation. An additional mechanism by which tamoxifen could inhibit activity of HER-2/neu is to antagonize the ability of estrogen to induce the autocrine secretion of epidermal growth factor family growth factors (46, 47). However, we found that tamoxifen, although enhancing the antiproliferative effect of Herceptin, did not do so via any effect on HER-2/neu expression levels or signaling activity. We hypothesize that the synergistic effects of Herceptin and tamoxifen may result from signaling pathway interactions downstream from the two targeted receptors, and experiments to address this are currently underway in our laboratory.

As suggested above, in vitro effects of these drugs may be cell line context dependent. Although it is of interest to extend these results to other cell lines, BT-474 is the only available cell line that endogenously overexpresses HER-2/neu and is also ER positive/estrogen dependent. Although it is tempting to use cells molecularly engineered to express these receptors, this may result in misleading observations, e.g., it has been found that transfection of ER into otherwise ER-negative cells produces a phenotype in which estrogen is actually growth inhibitory rather than stimulatory (74–76). A number of investigations has also studied MCF7 cells transfected with HER-2/neu and, unlike cells naturally overexpressing HER-2/neu, these transfected cells are not growth inhibited by anti-HER-2/neu antibody (35). Nonetheless, we are currently extending these experiments to other cell lines with varying endogenous levels of expression of these receptors.

A previous report using BT-474 cells investigated the combined effect of tamoxifen and 4D5 and found enhanced inhibition of cell growth using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxetrazolium bromide assay and enhanced inhibition of [3H]thymidine incorporation into DNA, compared with either drug alone (52). Another study showed that the combination of Herceptin with ICI 182,780, a pure antiestrogen that also down-regulates ER, resulted in enhanced growth inhibition over either drug alone using ML-20 cells reported to express a high level of ER and moderate level of HER-2/neu (53). An analysis of synergy was not performed. Either drug increased the percentage of cells in G0/G1, although the combination was not different from the effect of the antiestrogen alone. A measurable but small amount of apoptosis was observed with the combination treatment that was greater than that of either drug alone. Neither of these studies examined the effect on levels or activity of HER-2/neu. Although our results and those of others support a purely cytostatic effect of these drugs in vitro, under the added cellular stresses of in vivo conditions, cell destruction certainly occurs because both drugs result in tumor regressions in breast cancer patients. We are currently performing in vivo experiments to examine this combination using a murine BT-474 xenograft model.

Targeted therapy based on the well-studied biology of malignancies is resulting in advances in the treatment of cancer patients. Individual therapeutics with a defined target help to make these strides, but major impact will likely continue to come from the combinations of therapeutic agents. In clinical oncology, combinations of drugs are often chosen empirically, often simply on the basis of feasibility of safe delivery when nonoverlapping toxicities are associated. The interactions between the growth factor and ER signaling pathways known in breast carcinoma provide strong biological rationale for combining agents that target these two pathways in particular. These results may translate into improved therapy for patients with breast carcinomas in which both of these pathways are known to be of biological significance.

ACKNOWLEDGMENTS

We thank the services of the Yale Cancer Center Flow Cytometry shared resource.

REFERENCES

1418 Synergy between Tamoxifen and Herceptin


Synergistic Interactions between Tamoxifen and Trastuzumab (Herceptin)


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