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 G_0-G_1 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 G_0-G_1 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-
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The forced overexpression of HER-2/neu with coactivators or corepressors. One study focused on why 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 × 10^5 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 10^4 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: \( f_a/f_u = (D/D_m)^m \), which can be linearly transformed as \( \log (f_a/f_u) = m \log (D) - m \log (D_m) \), where \( f_a = \text{fraction affected, } f_u = \text{fraction unaffected, } D = \text{dose, } D_m = \text{dose that produces median-effect (IC}_{50}, \) and \( m = \text{coefficient signifying the sigmoidicity of the curve (or slope in linear relationship between dose and response).} \)
transformation). For the model to be applicable, the linear

\[ r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}} \]

correlation coefficient \( r \) should be \( >0.9 \). For examining the
effect of multiple drugs, a Combination Index (CI) is calculated

\[ CI = \frac{D_1}{D_{E1}} \times \frac{D_2}{D_{E2}} \]

developmental effects, according to the formula: \( D_{E1} \) and \( D_{E2} \) are the
doses for each drug alone that inhibit x%, and \( D_{E1} \) and \( D_{E2} \) 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
~50% in 100-mm dishes. After overnight incubation, cells were
either left untreated or treated with 1 \( \mu \text{M} \) tamoxifen, 10 \( \mu \text{g/ml} \) Herceptin, or the combination. At indicated time points, cells
were trypsinized, washed with cold PBS, resuspended in 2 ml of
cold PBS, fixed by three stepwise additions of 2 ml each of
95% ethanol, 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 isoleide
(Sigma PS264) 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 exper-
iment.

**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 \( \mu \text{M} \) tamoxifen, 10 \( \mu \text{g/ml} \) 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 \( \mu \text{M} \) Tris (pH 6.8),
4% SDS, 20% glycerol, 6 \( \mu \text{M} \) urea, 0.2% Bromphenol Blue, 10% \( \beta \)-mercaptoethanol, and
7 \( \mu \text{M} \) EDTA] followed by 3 min of
water bath sonication. Protein concentrations of the lysates
were determined by using a dendritic cell protein assay kit (Bio-Rad,
Hercules, CA); 100 \( \mu \text{g} \) of protein from each lysate were re-
 solved 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 proteol-
ysis of the M_116,000 native PARP enzyme to the apoptosis-
specific M_67,000 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-posi-
tive 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 \( \text{mM} \)
\( \text{L-glutamine}, 10 \mu\text{g/ml insulin, 10 mM HEPES, and 0.8% agar-}
ose 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 supple-
mented with the same components in the presence of 1 \( \mu \text{M} \)
tamoxifen, 10 \( \mu \text{g/ml} \) Herceptin, both drugs, no drug (“Control”),
or vehicle, then plated on top of the base at a density of
2 \( \times \) 10^5 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 micro-
scopic fields (\( \times60 \)) 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 As-
says.** Monolayer colony assays A and AA were performed in
the continuous presence of drugs, whereas assay B was per-
formed in drug-free media after prior exposure to drugs. For
monolayer colony assay A, cells were seeded at a density of 5 \( \times \)
10^5 cells/60-mm dish in triplicate in the presence of 1 \( \mu \text{M} \)
tamoxifen, 10 \( \mu \text{g/ml} \) Herceptin, both drugs, no drug, or vehicle.
Dishes were incubated at 37°C for 30 days. Media were ex-
changed 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 clus-
ters of \( \geq 0.4 \text{ mm} \) 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 \( \mu \text{M} \) tamoxifen, 10 \( \mu \text{g/ml} \) 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 \( \times \) 10^5 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 \( \geq 0.4 \text{ mm} \) 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 \( \mu \text{M} \) tamoxifen, 10 \( \mu \text{g/ml} \) 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
\( \text{mM Tris-HCl (pH 7.6)}, 150 \text{ mM NaCl, 1% NP40, 2}\text{ \( \mu \text{M} \) EDTA,}
1 \( \text{mM} \) \( \beta \)-glycerol phosphate, 25 \( \text{mM} \) NaF, 1 \( \text{mM NaVO_4, 1 mM}
phenylmethylsulfonyl fluoride, 5 \( \mu \text{g/ml} \) leupeptin, 10 \( \mu \text{g/ml}
aptinokin, and 5 \( \mu \text{g/ml} \) 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
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 1:2000 and 1:200, 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 1:2000. 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 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.

The effect of the addition of varying concentrations of tamoxifen or Herceptin to a given concentration of the other drug on cell growth using the WST-1 colorimetric growth assay after a 5–6-day exposure period is shown in Fig. 2. These are clinically relevant concentrations of Herceptin that can be achieved in patients (6, 7). For Herceptin alone, near maximal growth inhibition is seen at 1 μg/ml; higher concentrations did not significantly further inhibit cell growth (Fig. 2, top panel). The IC₅₀ for Herceptin was 0.4 μg/ml. The addition of tamoxifen at all doses in the range of 1–8 μM significantly enhanced the growth inhibition of any given concentration of tamoxifen. For single agent tamoxifen, increasing growth inhibition is seen in a dose-response fashion at concentrations ranging from 0 to 8 μM (Fig. 2, bottom panel). The IC₅₀ for tamoxifen was 0.4 μg/ml. The addition of tamoxifen at all doses in the range of 1–8 μM significantly enhanced the growth inhibition of any given concentration of tamoxifen.

An analysis of synergism was conducted, using the method of Chou and Talalay (Ref. 57; see “Materials and Methods”) and
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 \( r = 0.9 \) for each drug, as shown in Fig. 3. All combinations tested with tamoxifen in the range of 1–8 \( \mu \text{M} \) and Herceptin in the range of 0.375–10 \( \mu \text{g/ml} \) 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 \( \mu \text{M} \) tamoxifen, 10 \( \mu \text{g/ml} \) 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 \( \mu \text{M} \)

<table>
<thead>
<tr>
<th>Herceptin (( \mu \text{g/ml} ))</th>
<th>Tamoxifen (( \mu \text{M} ))</th>
<th>Fa</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.375</td>
<td>1</td>
<td>0.747</td>
<td>0.250</td>
</tr>
<tr>
<td>0.375</td>
<td>2</td>
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<td>1</td>
<td>0.5</td>
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<td>0.062</td>
</tr>
<tr>
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<td>1</td>
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</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.930</td>
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</tr>
<tr>
<td>1</td>
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<td>0.893</td>
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<tr>
<td>1</td>
<td>8</td>
<td>0.929</td>
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<td>3</td>
<td>1</td>
<td>0.829</td>
<td>0.337</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
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<tr>
<td>10</td>
<td>8</td>
<td>0.900</td>
<td>0.269</td>
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</table>

**Fig. 3** Median-effect plot for tamoxifen and Herceptin, used for analysis of synergy between the two. Growth inhibition data were analyzed with the method of Chou and Talalay, using commercially available software (CalcuSyn). The tamoxifen dose is in \( \mu \text{M} \), and the Herceptin dose is in \( \mu \text{g/ml} \). For the tamoxifen plot, the linear correlation coefficient was \( r = 0.98 \), and for the Herceptin plot, the linear correlation coefficient was \( r = 0.92 (\geq 0.9) \), both of which suggest goodness of fit for the data to the median-effect equation. Top panel, the untransformed data; bottom panel, the logarithmically transformed equation. A curve for the combination of the two drugs is not shown, because combinations were tested at a variety of different concentrations of the two drugs (see Table 1) that were not at a constant ratio.

**Fig. 4** Effect on cell cycle at 24 h. BT-474 cells in exponential growth were plated at a confluency of ~50% in 100-mm dishes. After overnight incubation, 1 \( \mu \text{M} \) tamoxifen, 10 \( \mu \text{g/ml} \) Herceptin, neither (Control), or the combination was added to the media. After a 24-h incubation, cells were trypsinized, washed with cold PBS, fixed in ethanol, and stored at 4°C. Subsequently, cells were resuspended in RNase and subjected to propidium iodide. Samples were analyzed by flow cytometry as described in “Materials and Methods.” * * * P < 0.05 compared with control (t test); ** * * * P < 0.05 for all comparisons (t test).
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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>G₀-G₁</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>
</tr>
<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>
</tr>
<tr>
<td>G₂-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>G₀-G₁</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>
</tr>
<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;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>G₂-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;b&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).

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 for DNA fragmentation resulting in the laddering pattern characteristic of cells undergoing apoptosis. Camptothecin-treated U937 cells were used as a positive control. No DNA fragmentation was observed for any of the conditions of treatment of BT-474 cells (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. No PARP cleavage was observed for any of the culture conditions (Fig. 6).

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 combination, under these culture conditions, results in apoptosis of BT-474 cells.

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 × 10⁶ 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 × 10⁶ 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).

Monolayer clonogenicity on plastic culture dishes was analyzed by two approaches (Table 4). In the first approach, the assay was conducted in the presence of drug. Tamoxifen resulted in a modest inhibition of colony formation, Herceptin a more pronounced inhibition, and the combination the greatest degree of inhibition. The trends were the same whether only solid clusters were scored as colonies (Assay A) or whether the dispersed clusters (described in “Materials and Methods”) were also scored as colonies (Assay AA). In the second approach, cells were precultured for 4 days in the presence of drug, and cells were then harvested by trysinization and replated at equivalent densities for assay in the absence of drug (Table 4, Monolayer colony assay B). Pre-exposure to tamoxifen, Herceptin, or the combination did not impair the subsequent clonogenicity after drug was removed.

**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>
</tr>
</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>
</tr>
<tr>
<td>Vehicle</td>
<td>98.5 ± 4.28</td>
<td>99.3 ± 2.34</td>
<td>99.2 ± 4.29</td>
<td>97.4 ± 9.71</td>
</tr>
<tr>
<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>
</tr>
<tr>
<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>
</tr>
</tbody>
</table>

**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-G2 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 G1-G2 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 G1-G2, as did single agent Herceptin; of note is that even untreated cells had >70% G1-G2 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.

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**Fig. 9** Immunoblot analysis of HER2/neu expression level and phosphorylation state. BT-474 cells were cultured in the presence of 1 μM tamoxifen (T), 10 μg/ml Herceptin (H), a combination of both drugs (T/H), no drug (C), or vehicle (V) as described in “Materials and Methods.” At the indicated time points, cell lysates were prepared, and 50 μg of protein were electrophoresed and immunoblotted with antibody to HER2 (left panel) or phosphorylated HER2 (right panel).
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-diphenyltetrazolium 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.

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Synergistic Interactions between Tamoxifen and Trastuzumab (Herceptin)
