Trastuzumab Reverses Letrozole Resistance and Amplifies the Sensitivity of Breast Cancer Cells to Estrogen

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Abstract

In this study, we investigated adaptive mechanisms associated with aromatase inhibitor (AI) resistance in breast cancer cells and show that sensitivity to AIs can be extended through dual inhibition of estrogen receptor (ER) and human epidermal receptor-2 (Her-2) signaling. We used human ER-positive breast cancer cells stably transfected with the aromatase gene (MCF-7Ca). These cells grow as tumors in nude mice and are inhibited by AIs. Despite continued treatment, tumors eventually become insensitive to AI letrozole. The cells isolated from these long-term letrozole-treated tumors (LT-LT-Ca) were found to have decreased ERα levels. Our results suggest that LT-LT-Ca cells survive estrogen deprivation by activation of Her-2/mitogen-activated protein kinase (MAPK) pathway. Here, we show that trastuzumab (antibody against Her-2; IC50 = 0.4 mg/mL) was very effective in restoring the ERα levels and sensitivity of LT-LT-Ca cells to endocrine therapy by down-regulation of Her-2/MAPK pathway and up-regulation of ERα. In contrast, trastuzumab was ineffective in the parental hormone-responsive MCF-7Ca cells (IC50 = 4.28 mg/mL) and xenografts. By blocking Her-2, trastuzumab also up-regulates ERα and aromatase expression and hypersensitized MCF-7Ca cells to E2. We show that trastuzumab is beneficial in hormone-refractory cells and xenografts by restoring ER, implicating Her-2 as a negative regulator of ERα. In xenograft studies, the combination of trastuzumab plus letrozole is equally effective in inhibiting growth of MCF-7Ca tumors as letrozole alone. However, on the acquisition of resistance and increased Her-2 expression, the combination of letrozole plus trastuzumab provided superior benefit over letrozole or trastuzumab alone. [Cancer Res 2009;69(4):1416–28]

Introduction

Aromatase inhibitors (AI) such as letrozole and anastrozole that reduce estrogen production have now been shown to be more effective than antiestrogen (AE) tamoxifen in estrogen receptor–positive (ER+) breast cancer patients and have few side effects. Nevertheless, not all patients respond and resistance to treatment may eventually occur in others. To investigate the mechanisms involved in the loss of sensitivity of the tumors to AIs, we developed a cell line isolated from tumors of human ER+ breast cancer cells (MCF-7) stably transfected with aromatase (MCF-7Ca) grown in mice treated with letrozole for an extended period of time (1–3). We have previously reported that during treatment with letrozole, MCF-7Ca xenografts up-regulate human epidermal receptor-2 (Her-2) and proteins in the downstream mitogen-activated protein kinase (MAPK) signaling pathway (1). These cells also exhibited lower expression of ERα and apparent “estriol-independent” growth. We have also shown that signaling pathways, such as the Her-2/MAPK, are key regulators of the growth of letrozole-refractory cells (1, 4). Similarly, several other investigators have reported the importance of members of epidermal growth factor receptor (EGFR) family (Her-2/EGFR) in resistance to endocrine therapy (5–8). We and others have suggested that the combination of EGFR-Her-2 tyrosine kinase inhibitors or trastuzumab (monoclonal humanized antibody against Her-2, Herceptin) in combination with AIs or AIs may delay acquisition of resistance (4, 9–13).

In this study, we investigated the effects of trastuzumab on the growth of letrozole-refractory breast cancer cells. On examination, MCF-7Ca-derived tumors treated with letrozole up-regulated Her-2 4 weeks into treatment despite continued responsiveness to letrozole. Moreover, the level of Her-2 protein was found to be 6-fold higher in letrozole-refractory tumors than the control tumors (1). However, when Her-2 was inhibited, ERα levels were restored. This suggests that Her-2 is a negative regulator of ERα. This observation led to our hypothesis that inhibition of both the Her-2 and estrogen signaling pathways is required to prolong the responsiveness of the tumors to endocrine therapies (14, 15).

Thus, the combination of an AI plus Her-2 inhibitor could provide a substantial benefit in tumor growth inhibition when used after the acquisition of letrozole resistance.

Materials and Methods

Materials. DMEM, modified improved MEM (IMEM), penicillin/streptomycin solution (10,000 IU each), 0.25% trypsin–1 mmol/L EDTA solution, Dulbecco’s PBS (DPBS), and genetin (G418) were obtained from Invitrogen, Androstenedione (Δ4), tamoxifen, and Matrigel were obtained from Sigma Chemical Co. Antibodies against Her-2 and phosphorylated Her-2 (p-Her-2) were purchased from Upstate and antibodies against phosphorylated MAPK (p-MAPK), MAPK, phosphorylated Elk-1 (p-Elk-1), and phosphorylated p90RSK (p-p90RSK) were purchased from Cell Signaling Technology. Antibodies against ERα, and aromatase (CYP-19) were purchased from Santa Cruz Biotechnology. Radioactive ligands for ER-binding assay and aromatase assay, 3H-E2 (40 Ci/mmol), and 3H-Δ4 (23.5 Ci/mmol) were purchased from Perkin-Elmer.

MCF-7 human breast cancer cells stably transfected with the human aromatase gene (MCF-7Ca) were provided by Dr. S. Chen (City of Hope,
Duarte, CA). Letrozole was provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland). The pure AE fulvestrant and anastrozole were supplied by Dr. E. Anderson (AstraZeneca Pharmaceuticals, Macclesfield, United Kingdom).

**Cell culture.** MCF-7Ca cells were routinely cultured in DMEM supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, and 700 μg/ml G418. LTLT-Ca cells were developed from MCF-7Ca cells as described earlier from tumors of mice treated with letrozole for 56 wk (1). The cells were maintained in steroid-depleted medium, which consisted of phenol red-free IMEM supplemented with 5% dextran-coated charcoal-treated serum, 1% penicillin/streptomycin, and 750 μg/ml G418 and 1 μM/l letrozole. Cell proliferation assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier (4). The results were expressed as a percentage of the cell number in the ΔA-treated control wells. IC50 values for inhibitors were calculated from the linear regression line of the plot of percentage inhibition versus log inhibitor concentration.

**Tumor growth in ovariectomized female athymic nude mice.** All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland, Baltimore. Female ovariectomized BALB/c athymic nude mice 4 to 6 wk of age were obtained from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water *ad libitum*.

The tumor xenografts of MCF-7Ca cells were grown in the mice as described earlier (4A). The resulting homogenate was used for aromatase activity assay (19). The resulting homogenate was used for aromatase activity assay (19). The results were expressed as a percentage of the cell number in the ΔA-treated control wells. IC50 values for inhibitors were calculated from the linear regression line of the plot of percentage inhibition versus log inhibitor concentration.

**Western blotting.** The protein extracts from tumor tissues were prepared by homogenizing the tissue in ice-cold DPBS containing protease inhibitors (18). A total of 50 μg protein from each sample was analyzed by SDS-PAGE as described previously (4). Bands were quantitated by densitometry using Molecular Dynamics Software (ImageQuant). The densitometric values are corrected for loading control.

**Competitive binding studies.** Binding of trastuzumab to ER in MCF-7Ca cells was assessed by competitive binding assay as described before (3). MCF-7Ca cells were transferred to steroid-depleted medium for 1 d before plating for the binding assay. [3H]-E2 was used as a ligand and nonradioabeled E2 was used to determine nonspecific binding. Triamcinolone (1 μM/l) was used to saturate glucocorticoid receptors.

The radiometric [3H]-E2 release assay was performed as described previously (19). For pretreatment studies, cells were treated with indicated agent for 24 h before incubating with [3H]-E2 for 18 h. For measuring aromatase activity in tumor samples, the tumors were homogenized in ice-cold DPBS. The resulting homogenate was used for aromatase activity assay (19). The activity of the enzyme is corrected for protein concentration in the tumor homogenates and cells.

**Etxt transactivation assay.** To measure activation of ERs, an ELISA-based ER transactivation assay was performed as per manufacturer’s guidelines (Panomics). Briefly, the nuclear lysates of cells or tumors were generated as described by the manufacturer. Activated ER from nuclear extracts was allowed to bind to an ER consensus binding site (ER probe) on a biotinylated oligonucleotide. These oligonucleotides were then immobilized on a streptavidin-coated 96-well plate. The ER bound to the oligonucleotide were then detected by an antibody directed against ER. An additional horseradish peroxidase–conjugated secondary antibody provided colorimetric readout quantified by reading absorbance at 450 nm.

**RNA extraction and reverse transcription.** RNA was extracted and purified using the RNeasy Mini kit (Qiagen) as per manufacturer’s protocol. Total RNA concentration and purity were determined from 260 and 280 nm absorbances. RNA was diluted with water to 0.08 μg/μL and reverse transcription as described as described by Kazi and colleagues (20).

**PCR.** Analysis of ERs, CYP-19, and pS2 mRNA expression was done by conventional PCR. Each 30 μL reaction consisted of 3 μL reverse transcription, 3 μL 10× buffer, 2.4 μL deoxynucleotide triphosphate mix, 3 μL 5 μM/l primer mix, 0.15 μL Taq DNA polymerase (Qiagen), and 18.45 μL molecular biology-grade water. The following primers were used for the PCR analysis: human pS2, 5′-ACCTGGAGAGAGTATGTGAT′-3′ (forward) and 5′-AAATCCATCCTCTCCTGGT-3′ (reverse; ref. 21); human ERs, 5′-GATCTTCTTACGACCTTCATG-3′ (forward) and 5′-TCTTCCA-GAGTCTTACAGTGCT-3′ (reverse); human CYP-19, 5′-GATATTGGAAG-3′ (forward) and 5′-GGGTAAGAGCATCTTCAGC-3′ (reverse; ref. 19, 22); and human 18s rRNA, 5′-CAACCTTC-3′ (forward) and 5′-CGCTTATTGGCAGTGAATTAC-3′ (reverse; ref. 20).

**ChIP assay.** For in vitro ChIP assay, the treated cells were washed with DPBS and fixed with 1% formaldehyde/DPBS for 10 min at 37°C, after which the cells were washed with ice-cold DPBS containing protease inhibitors. The cells were collected into 1 mL DPBS and pelleted by centrifugation at 6,000 rpm for 5 min at 4°C. The cell pellet was resuspended in nuclear lysis buffer (ChIP kit, Upstate) and incubated on ice for 15 min.

For in vivo ChIP assay, tumor slices were immersed in 2% formaldehyde/DPBS mixture and incubated at room temperature for 15 min. Fixation was stopped by adding 1 mol/l glycine and incubating for 5 min at room temperature. The tissues were rinsed in ice-cold DPBS containing the protease inhibitor tablet and homogenized on ice in modified radio-immunoprecipitation assay buffer. The tissue homogenate was centrifuged at 15,000 rpm for 5 min at 4°C; the nuclear pellet was resuspended in nuclear lysis buffer and incubated on ice for 1 h. Samples were sonicated for 10×10 s cycles, with 20-s pauses between each cycle. The sonicated samples were centrifuged at 14,000 rpm for 10 min at 4°C. Sonicated samples were diluted 1:10 with dilution buffer (ChIP kit) before being immunocleaned in a solution containing protein A or G-Sepharose slurry (Amersham) in Tris/EDTA buffer, salmon sperm DNA (Invitrogen), and normal mouse or rabbit serum (Sigma Chemical) for 2 h at 4°C. Immunocleaned supernatants were incubated overnight at 4°C with anti-ERs antibody (Santa Cruz Biotechnology). Protein A or G-Sepharose beads and salmon sperm DNA were then added and incubated for 1 h at 4°C. The beads were then washed sequentially with 1 mL each of wash buffers. The protein-DNA complexes were then eluted by twice incubating beads in elution buffer for 10 min at room temperature with vigorous mixing. To separate immunoprecipitated protein and DNA, the pooled elutes were incubated at 65°C overnight. The DNA was purified using the QIAquick PCR Purification kit (Qiagen). Alternatively, immunoprecipitated ERs on the beads was subjected to Western immunoblotting. The boiled (denatured) protein samples were resolved by SDS-PAGE and membranes were probed for histone H3 and RNA polymerase II.

The yield of target region DNA in each sample after ChIP was analyzed by conventional PCR. The following primers were used for PCR analysis (34 cycles at 60°C annealing temperature): human pS2, 5′-GGCCATCTCT-CACTTGAATC-3′ (forward) and 5′-GGCCAGGCTCTGTTGCAAA-3′ (reverse; ref. 20); human CYP-19 Promoter L3/II, 5′-GGGATGACACCG-3′ (forward) and 5′-TCCTCCTCCATGCTGGAGA-3′ (reverse; ref. 23).

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The promoter that was analyzed was I.3/II, which is the main aromatase promoter used in breast cancer cell lines such as MCF-7 (24) and thus measures the effect of trastuzumab on endogenous aromatase in MCF-7 cells. The MCF-7Ca cells contain human placental aromatase cDNA (placental CYP-19 uses promoter I.1 and I.2; ref. 25).

Statistics. For in vivo studies, mixed-effects models were used. The tumor volumes were analyzed with S-PLUS (7.0; Insightful Corp.) to estimate and compare an exponential variable ($\beta$) controlling the growth rate for each treatment group. The original values for tumor volumes were log transformed. In the process of searching for a suitable model, we observed that the piecewise (knot at 15 wk) model fits the data reasonably well (on groups switched after week 15 to another drug). The spline model with a single knot at time = week-15 wk was used to accommodate the nonlinearity with a piece-wise linear model.

**Figure 1.** A, effect of trastuzumab on proliferation of MCF-7Ca and LTLT-Ca cells in vitro. Viability of cells was measured by MTT assay after 6-d treatment with trastuzumab as described in Materials and Methods. The treatment with trastuzumab is significantly more effective in reducing cell viability of LTLT-Ca cells compared with MCF-7Ca cells at all doses. At each dose of trastuzumab tested (0.1–10 mg/mL) in LTLT-Ca cells, growth was inhibited significantly better (*, $P < 0.0001$) than in MCF-7Ca cells. B, effect of trastuzumab treatment at various time points on the expression of protein in the Her-2/MAPK pathway, ERα, and aromatase in LTLT-Ca cells. Expression of proteins was examined using Western immunoblotting as described in Materials and Methods. Blot shows Her-2, p-Her-2 at 185 kDa, p-MAPK, MAPK at 42 to 44 kDa, p-p90RSK at 90 kDa, p-Elk-1 at 41 kDa, ERα at 66 kDa, aromatase at 55 kDa, and β-actin at 45 kDa. The blots show a single representative of three independent experiments.
Trastuzumab inhibits growth of LTLT-Ca cells and tumors and down-regulates MAPK activation. Trastuzumab was found to inhibit growth of LTLT-Ca cells in a dose-dependent manner with an IC_{50} of 400 μg/mL (2.75 μmol/L) compared with 4.28 mg/mL (29.41 μmol/L) in parental MCF-7Ca cells (Fig. 1A).

Trastuzumab Reverses Letrozole Resistance

Results

Trastuzumab inhibits growth of LTLT-Ca cells and tumors and down-regulates MAPK activation. Trastuzumab was found to inhibit growth of LTLT-Ca cells in a dose-dependent manner with an IC_{50} of 400 μg/mL (2.75 μmol/L) compared with 4.28 mg/mL (29.41 μmol/L) in parental MCF-7Ca cells (Fig. 1A).

In LTLT-Ca cells, trastuzumab (100 μg/mL) also decreased the activation of proteins of the MAPK pathway along with Her-2 in a time-dependent manner (Fig. 1B). This down-regulation was initiated as early as 15 minutes and was sustained until at least 72 hours. In addition, ERα was nearly restored to the level of the parental MCF-7Ca cells.

To study the effect of trastuzumab in vivo, LTLT-Ca cells were inoculated s.c. into ovariectomized nude mice. The following day, the mice were divided into two groups. One group received letrozole (n = 5) and the other group received vehicle only (control, n = 25).

All P values <0.05 were considered statistically significant. The graphs are represented as mean ± SE.
When the tumors of the mice in the control group reached ~300 mm³, this group was regrouped into five (n = 5) and injected with vehicle or 2, 5, 10, and 20 mg/kg/wk of trastuzumab i.p. (divided in two doses). As shown in Fig. 1C, the tumors in all of the trastuzumab-treated groups regressed over the course of the experiment. The dose of 5 mg/kg/wk trastuzumab was selected for future investigations. As shown previously (1), treatment with letrozole did not result in a statistically significant difference in tumor growth than observed in controls (P = 0.91).

**Trastuzumab restores sensitivity of LTLT-Ca cells to AEs and AIs.** As shown in our earlier studies, LTLT-Ca cells exhibit cross-resistance to growth-inhibitory effects of tamoxifen, fulvestrant, exemestane, and anastrozole (1). However, the combination of trastuzumab (100 µg/mL) with AEs or AIs produced synergistic growth inhibition. As shown in Fig. 1D, the combination of trastuzumab with AE/AI at 1 µmol/L each produced synergistic growth inhibition. The combination was statistically better than either drug alone (P < 0.0001) or control (P < 0.0001), whereas single drug treatment with AE or AI was not statistically different from control. As shown in Fig. 2A, proliferation of LTLT-Ca cells was not inhibited with letrozole (10⁻¹² to 10⁻⁵ mol/L), whereas cotreatment with trastuzumab (100 µg/mL) and letrozole inhibited the growth of LTLT-Ca cells in a dose-dependent manner. However, in MCF-7Ca cells, the combination of letrozole plus trastuzumab was not significantly different from letrozole at 10⁻⁹ to 10⁻⁵ mol/L but different from letrozole alone at 10⁻¹² to 10⁻¹⁰ mol/L (Fig. 2B). Although the IC₅₀ value of letrozole in the presence of trastuzumab was 2-log lower than that in the absence of trastuzumab, it should be noted that this effect was not seen in vivo.

The effect of combining trastuzumab plus letrozole was also examined in letrozole-refractory LTLT-Ca xenografts (data not shown).

**Figure 2.** A, effect of combination of letrozole and trastuzumab in LTLT-Ca cells. Viability of cells was measured by MTT assay after 6-d treatment with letrozole (10⁻¹² to 10⁻⁵ mol/L) alone or in the presence of trastuzumab (100 µg/mL) as described in Materials and Methods. The treatment with the combination of letrozole plus trastuzumab was significantly better than single drug treatment or control. *, P < 0.01 (10⁻¹² to 10⁻⁹ mol/L); †, P < 0.001 (10⁻⁸ to 10⁻⁵ mol/L). B, effect of combination of letrozole and trastuzumab in MCF-7Ca cells. Viability of cells was measured by MTT assay after 6-d treatment with letrozole (10⁻¹² to 10⁻⁵ mol/L) alone or in the presence of trastuzumab (100 µg/mL) as described in Materials and Methods. Combination of letrozole plus trastuzumab was significantly better than single drug treatment or control. *, P < 0.0001 (10⁻¹² to 10⁻¹⁰ mol/L).
shown). The tumors of mice receiving trastuzumab plus letrozole regressed significantly in volume. The combination was significantly better than trastuzumab alone \((P < 0.0001)\), letrozole alone \((P = 0.0001)\), and control \((P < 0.0001)\), although trastuzumab alone was significantly better than control \((P < 0.0001)\). These data suggest that combining letrozole plus trastuzumab was significantly more effective than single agent in letrozole-refractory LTLT-Ca xenografts \((26)\).

**Trastuzumab up-regulated ERα- and E2-mediated transcription in LTLT-Ca cells.** Treatment with trastuzumab up-regulated ERα in LTLT-Ca cells (Fig. 1B). The maximum increase of ERα was seen at 24 hours and was found to be 0.83-fold compared with basal level of ERα in MCF-7Ca cells. A 10-fold higher level of ERα was found in LTLT-Ca cells compared with basal levels (0.83 compared with 0.08). In addition, trastuzumab pretreatment followed by E2 treatment for 1 hour increased ERα-mediated transcription in a time-dependent manner (Fig. 2C). A 72-hour pretreatment of LTLT-Ca cells with trastuzumab followed by 1 hour of E2 treatment induced ERα transcriptional activation to the same extent as in parental MCF-7Ca cells stimulated with E2 alone.

In vitro ChIP assay and reverse transcription-PCR (RT-PCR) analysis were performed to examine the effect of trastuzumab alone or in combination with E2 on ERα-mediated transcription.
however, the combination did not increase this further. The MCF-7Ca cells may require different treatment period with trastuzumab to exhibit similar effect. These results suggest that trastuzumab activated the aromatase transcription in ER-dependent manner in LTLT-Ca cells.

**Trastuzumab amplified mitogenic effects of estradiol in vitro and in vivo.** E2 stimulation of MCF-7Ca cells resulted in a typical biphasic dose-response curve where maximum stimulation occurs at a concentration of ~10^-9 mol/L (Fig. 3C). In contrast, as reported in our earlier studies, proliferation of LTLT-Ca cells is not stimulated by E2 (1,27). However, when pretreated with trastuzumab (100 μg/mL) for 72 hours, LTLT-Ca cells exhibited a marked stimulation of proliferation at concentrations of 10^-12 to 10^-7 mol/L when compared with E2 alone (P < 0.0001). In addition, when MCF-7Ca cells were pretreated with trastuzumab, E2-stimulated proliferation was increased at concentrations of 10^-11 to 10^-6 mol/L (P = 0.02 and 0.03, respectively). The results suggest that inhibition of Her-2 restores E2α and E2 sensitivity and that Her-2 is a negative regulator of ER.

Pretreatment of MCF-7Ca, MCF-7, and LTLT-Ca cells with trastuzumab for 24 hours also increased aromatase activity in a dose-dependent manner (Fig. 3D). At the same time, aromatase protein expression was up-regulated by trastuzumab in a dose-dependent manner along with E2α (Fig. 4A). Trastuzumab caused an increase in the aromatase activity of MCF-7, suggesting that trastuzumab influences endogenous aromatase. To investigate whether suppressing Her-2 resulted in up-regulation of E2α and aromatase was limited to LTLT-Ca cells, we carried out the same experiment with ER-negative and Her-2-positive SKBr-3 cells. The results were similar, confirming that inhibition of Her-2 results in increased aromatase activity (Fig. 3D, inset) and E2α up-regulation (data not shown). However, this effect was not seen in ER-negative and Her-2-negative MDA-MB-231 cells (data not shown). Trastuzumab did not bind competitively to ERα (Fig. 4B), suggesting that the stimulatory effects were E2α dependent, not due to intrinsic estrogenic properties of trastuzumab. In addition, when MCF-7Ca cells were cotreated with increasing doses of trastuzumab for 24 hours followed by 1 nmol/L E2 for 1 hour, transcriptional activity of E2α was found to be up-regulated in a dose-dependent manner. This increase was found to be higher than E2α (1 nmol/L) treatment alone (Fig. 4C).

The measurement of uterine weight is a useful bioassay that correlates with the levels of circulating estrogens in the body of the mice. The uterus of mice bearing LTLT-Ca xenografts treated with trastuzumab were removed and weighed at the completion of the experiment. Trastuzumab induced a statistically significant (P = 0.001) dose-dependent increase in uterine wet weight compared with controls (Fig. 4D). Increased uterine weight suggests that cotreatment with trastuzumab acts to enhance ERα and effects of E2 on the uterus. Furthermore, increased tumor aromatase activity suggests that trastuzumab increases E2 synthesis from Δ4Δ (data not shown).

**Trastuzumab is ineffective in inhibiting growth of MCF-7Ca tumors.** MCF-7Ca xenografts were grown in female ovariectomized mice. When tumors reached measurable size (~300 mm^2), mice were assigned to four groups: (a) control (n = 5); (b) trastuzumab, 5 mg/kg/wk, divided in two doses (n = 5); (c) letrozole, 10 μg/d, plus trastuzumab, 5 mg/kg/wk, divided in two doses (n = 5); and (d) letrozole, 10 μg/d (n = 30). The tumors were measured weekly and volume was calculated as described in Materials and Methods. Trastuzumab did not inhibit the growth of MCF-7Ca tumors (Fig. 5A). The growth rate

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**Figure 3.** A, effect of trastuzumab and E2 alone or in combination on the ERα-mediated transcriptional activation in MCF-7Ca and LTLT-Ca cells. The in vitro ChIP assay was performed as described in Materials and Methods. E2-induced recruitment of ERα to the DNA and transcriptional activation in MCF-7Ca and LTLT-Ca cells was measured by Western blotting. The blot shows histone H3 at 15 kDa and RNA polymerase II at 300 kDa. Left, MCF-7Ca cells; right, LTLT-Ca cells. For both panels: lane 1, E2W control; lane 2, E2 (10 nmol/L); lane 3, trastuzumab (100 μg/mL); lane 4, trastuzumab plus E2. B, effect of trastuzumab and E2 alone or in combination on the ERα-mediated transcriptional activation in MCF-7Ca and LTLT-Ca cells. In the in vitro ChIP assay was performed as described earlier. E2-induced recruitment of ERα to the aromatase I/III and pS2 promoter in MCF-7Ca and LTLT-Ca cells was examined by PCR. The blot shows aromatase product at 317 bp and pS2 at 415 bp. Input indicates samples before immunoprecipitation. Left, MCF-7Ca cells; right, LTLT-Ca cells. For both panels: lane 1, E2W control; lane 2, E2 (10 nmol/L); lane 3, trastuzumab (100 μg/mL); lane 4, trastuzumab plus E2.
of control tumors ($\Delta^1A$, 100 $\mu$g/d) was $\beta I = 0.17 \pm 0.1$, which was not statistically different from that of tumors in mice treated with trastuzumab ($\beta I = 0.19 \pm 0.14$) over the first 7 weeks. The mice in both these groups (control and trastuzumab) were sacrificed at week 7 due to large tumor volume. The tumors and uteri of the treated mice were removed, weighed, and stored at $-80^\circ$C for any additional analysis. The tumor weights (Fig. 5B) of control (1.37 $\pm$ 0.57 g) and trastuzumab (2.68 $\pm$ 0.57 g) were not statistically different ($P = 0.14$). However, trastuzumab treatment alone was significantly less effective than other treatments [versus letrozole ($P = 0.0009$) or versus trastuzumab plus letrozole ($P = 0.0001$)].

As shown in Fig. 5C, uteri in mice treated with trastuzumab weighed significantly more than those treated with $\Delta^1A$ ($P = 0.008$). The increase in uterine weight again suggests amplified growth-stimulatory effects of E2 on cotreatment with trastuzumab.

**Combination of trastuzumab plus letrozole is more effective than either drug alone in letrozole-refractory tumors.** As shown in Fig. 5A, combination of letrozole plus trastuzumab was effective in reducing the tumor growth rate ($\beta I = -0.04 \pm 0.04$ over weeks 0–15; $\beta I = 0.28 \pm 0.06$ over weeks 15–27). However, the combination was no more effective than letrozole as single agent ($\beta I = -0.01 \pm 0.02$ over weeks 0–15; $\beta I = 0.1 \pm 0.08$ over weeks 15–27).

Letrozole inhibited the growth of these tumors for a prolonged period (13 weeks). Nevertheless, tumors ultimately began to grow on continued treatment and had doubled in volume by week 15. At this time, the mice were subdivided into three groups: (a) trastuzumab, 5 mg/kg/wk (two doses; $n = 10$); (b) letrozole, 10 $\mu$g/d, plus trastuzumab, 5 mg/kg/wk ($n = 10$); and (c) letrozole, 10 $\mu$g/d ($n = 10$). The experiment was terminated on week 28. Tumors in mice switched to combination therapy following letrozole resistance responded better than combination treatment from week 0 ($P < 0.0001$). This suggests that on letrozole resistance, the addition of trastuzumab to the treatment regimen is superior to combination therapy from the...
beginning. The growth rate of these tumors was significantly
different from the tumors of mice that stayed on letrozole
treatment ($P = 0.008$). Tumors treated with letrozole responded
better when switched to letrozole plus trastuzumab than those
kept on letrozole. Letrozole-refractory tumors switched to
trastuzumab alone responded to the treatment only for the first
3 weeks (weeks 16–19) but then began to regrow at the rate
similar to that of the letrozole-treated mice (weeks 15–28;
$P = 0.97$). Thus, on resistance to letrozole, the addition of
trastuzumab was more effective than the switch to trastuzumab,
suggesting that Her-2 inhibition extends sensitivity of letrozole.

Protein expression and activity of tumors treated with
letrozole and trastuzumab alone or in combination. At
28 weeks (Fig. 5A), tumors of mice bearing MCF-7Ca xenografts
were analyzed for expression of Her-2, p-MAPK, MAPK,
aromatase, and ERa protein expression by Western blotting
(Fig. 5D). Treatment with trastuzumab increased ERa and
decreased Her-2 and p-MAPK protein expression. In contrast,
letrozole reduced ERa and increased Her-2 and p-MAPK levels.
The combination of letrozole plus trastuzumab did not affect ERa
levels and caused moderate increases in Her-2 and p-MAPK
compared with letrozole. This would be consistent with
stimulation of growth due to release from aromatase inhibition,
allowing estrogen production. In mice treated with letrozole and
the addition of trastuzumab on tumor volume doubling, ERa
levels were moderately increased, p-MAPK was down-regulated,
and Her-2 was moderately reduced.

As shown in Fig. 6A, trastuzumab increased aromatase activity
in the tumors, whereas letrozole inhibited aromatase activity. This
increased activity was also accompanied by a 3.7-fold up-regulation
of aromatase protein expression (Fig. 5D). The aromatase activity in the tumor samples from letrozole, letrozole plus trastuzumab, and letrozole switched to letrozole plus trastuzumab was found to be significantly lower than control ($P < 0.001$) or trastuzumab treatment ($P < 0.001$). The aromatase activity of tumors from letrozole switched to trastuzumab alone–treated mice was, however, not significantly different from control but significantly higher than letrozole alone ($P < 0.01$). However, aromatase protein expression in all these groups was not significantly different from control. The data suggest that letrozole maintains aromatase activity in tumors.
inhibition after prolonged treatment and switching to trastuzumab therapy removes the inhibitory effect of the AI on aromatase. Furthermore, tumors of mice treated with letrozole switched to letrozole plus trastuzumab show reduced aromatase activity, suggesting that addition of trastuzumab to letrozole did not interfere directly with aromatase inhibition by letrozole.

**Trastuzumab increases transcriptional activation of ERα in MCF-7Ca xenografts.** The *in vivo* ChIP assay was used to

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**Figure 6.** A. effect of trastuzumab and letrozole alone or in combination on aromatase activity of MCF-7Ca xenografts. The tumors of mice treated with letrozole, trastuzumab, and the combinations were examined for aromatase activity as described in Materials and Methods. The activity of the enzyme after treatment is corrected for total protein amount in the tumors. Aromatase activity of MCF-7Ca xenografts treated with trastuzumab is significantly higher than control (*, *P* < 0.001). Aromatase activity of control is significantly higher compared with letrozole (*P* < 0.001), letrozole plus trastuzumab (*, *P* < 0.001), and letrozole switched to letrozole plus trastuzumab (*, *P* < 0.001). The aromatase activity of letrozole switched to trastuzumab was not significantly different from Δ4A-treated control but significantly higher than letrozole (*, *P* < 0.01). One-way ANOVA, Tukey-Kramer multiple comparisons test. B. effect of trastuzumab and letrozole alone or in combination on the ERα-mediated transcriptional activation in MCF-7Ca xenografts. The *in vivo* ChIP assay was performed as described in Materials and Methods. E2-induced recruitment of ERα to the DNA in MCF-7Ca xenografts. The blot shows histone H3 at 15 kDa and RNA polymerase II at 300 kDa. ChIP analysis was done using ERα antibody. Input indicates samples before immunoprecipitation. C. effect of trastuzumab and letrozole alone or in combination on the ERα-mediated transcriptional activation in MCF-7Ca xenografts. The *in vivo* ChIP assay was performed as described earlier. Treatment-induced recruitment of ERα to the aromatase I.3/I and pS2 promoter in MCF-7Ca xenografts was examined by PCR. Input indicates samples before immunoprecipitation. The blot shows aromatase product at 317 bp and pS2 at 415 bp.
measure the effect of trastuzumab on transcriptional activation of ERα in MCF-7Ca xenografts. ΔΔA-treated control and trastuzumab-treated tumors exhibited active transcription of ERα, as evidenced by recruitment of histone H3 and RNA polymerase II into the transcriptional complex (Fig. 6B). The treatment of MCF-7Ca tumors with trastuzumab increased recruitment of ERα to the pS2 and aromatase L3/II promoter (Fig. 6C). These findings support in vivo tumor volume data and suggest that trastuzumab treatment in a hormone-sensitive breast cancer model is agonistic via its effects on Her-2 and hence the ER. As such, Her-2 inhibition without blockade of estrogen signaling in the clinical setting could be detrimental.

Discussion

Although AI letrozole is more effective and tumor growth is suppressed over an extended period compared with tamoxifen, eventually tumors become resistant to AI therapy (1). Previous results indicate that tumor growth is maintained by activating Her-2/MAPK signaling pathways in letrozole-resistant LTLT-Ca cells and letrozole-refractory tumors. In addition, ER expression is decreased and cells become insensitive to E2, suggesting a hormone-refractory phenotype. Our previous studies also indicated that the combination of letrozole with MAPK inhibitor PD98059 up-regulated ERα expression, consistent with the reports that hyperactivation of MAPK induces loss of ERα in breast cancer cells (1, 28). Her-2 is overexpressed in about 25% to 30% of breast cancers and correlates with poor disease-free survival and overall survival (29, 30). With the development of trastuzumab, effective targeted treatment of Her-2–overexpressing cancers has been possible. Treatment with trastuzumab also resulted in up-regulation of ERα. We have shown that loss of ERα expression induced by MAPK hyperactivation is reversible as observed in findings of others (31). Our studies show that trastuzumab by blocking Her-2 can reverse the suppression of ERα. To prevent development of letrozole resistance and restore hormone sensitivity of resistant cells, we used trastuzumab to inhibit the Her-2/MAPK pathway in the present study.

The combination of trastuzumab with letrozole reversed resistance to the AI and restored responsiveness of the tumors. However, this effect was limited to letrozole-refractory and Her-2–overexpressing cells and tumors. Trastuzumab alone was only effective for a brief period of 4 weeks in letrozole-refractory tumors. In the tumors with low Her-2 levels, the letrozole plus trastuzumab combination was no more effective than letrozole single agent. However, in Her-2–overexpressing tumors, trastuzumab plus letrozole was far superior in inhibiting tumor growth compared with either single agent (Fig. 5A). These results suggest that trastuzumab restores sensitivity of LTLT-Ca cells to letrozole by inhibiting Her-2 and up-regulation of ERα. As such, blockade of the ERα pathway remains an essential component of tumor and cell growth inhibition. Up-regulation of intratumoral aromatase was also seen with trastuzumab treatment to make the cells more responsive to AIs.

In our current study, we have shown that inhibition of Her-2 with trastuzumab results in activation of ERα-mediated signaling pathway. Based on these data, we hypothesize that an inverse and compensatory relationship exists between Her-2 and ERα and inhibition of one pathway leads to activation of the other. Several reports have suggested that translocation of ERα to the membrane may be responsible for the cross-talk with EGFR family members in endocrine-resistant phenotype (32–34), whereas a few reports have also suggested that EGFR family transmembrane receptors such as Her-2 can translocate to the nucleus and act as transcription factors (35–38).

Studies performed in trastuzumab-resistant variants of breast cancer cell lines have indicated up-regulation of transforming growth factor α (TGFα) and vascular endothelial growth factor (VEGF; refs. 39, 40). Interestingly, both VEGF and TGFα are estrogen-responsive genes (41). These data are consistent with our results and suggest that Her-2 suppresses ERα, as resistance to trastuzumab is associated with up-regulation of ERα-responsive genes (39, 40). Our studies show that trastuzumab alone was not an effective treatment strategy in this model and the data suggest that it is likely to be due to up-regulation of intratumoral aromatase and ERα. To distinguish whether up-regulation of ERα was a result of increased protein synthesis or reduced degradation, cells were treated with trastuzumab in the presence or absence of cycloheximide (translational inhibitor). Western immunoblotting analysis revealed that trastuzumab treatment increased ERα to a greater extent in the absence of cycloheximide, suggesting that trastuzumab modulates protein synthesis. However, even in the presence of cycloheximide, ERα protein was up-regulated by trastuzumab treatment, suggesting that trastuzumab treatment affects protein degradation as well (data not shown). In addition, RT-PCR analysis revealed that ERα mRNA expression is increased with trastuzumab treatment in a dose-dependent manner (Fig. 2D).

In agreement with our findings, clinical studies have shown that trastuzumab as single agent has promising effects in first-line treatment of ~40% breast cancer patients (42). However, the median duration of response is only ~8 months. Furthermore, intrinsic or de novo resistance to trastuzumab in Her-2–overexpressing breast cancers has been reported. As the use of trastuzumab is now extended to adjuvant treatment of breast cancer, it is important to assess the molecular effects of trastuzumab on Her-2–overexpressing breast cancer cells and determine appropriate strategies for optimal treatment. Our studies clearly show the importance of inhibiting both estrogen signaling by AI letrozole and the growth factor receptor Her-2 pathway in overcoming resistance to therapy. However, if used for treatment of hormone-responsive tumors, trastuzumab may amplify estrogen signaling and stimulate tumor growth. The results indicate that inhibition of Her-2 via trastuzumab can restore the responsiveness of tumors to letrozole and trastuzumab, thus extending the use of AIs and delaying the need for chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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