

Mechanisms of Tamoxifen Resistance: Increased Estrogen Receptor-HER2/neu Cross-Talk in ER/HER2-Positive Breast Cancer

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Background: Patients receiving adjuvant tamoxifen whose tumors express high levels of both HER2/neu (HER2) and the estrogen receptor (ER) coactivator AIB1 often develop tamoxifen resistance. We used a breast cancer model system with high expression of AIB1 and HER2 to investigate the possible mechanisms underlying this resistance. **Methods:** MCF-7 breast cancer cells, which express high levels of AIB1, and a tamoxifen-resistant derivative cell line engineered to overexpress HER2 (MCF-7/HER2-18) were treated with estrogen, tamoxifen, epidermal growth factor (EGF), or heregulin in the absence or presence of the EGF receptor (EGFR) tyrosine kinase inhibitor gefitinib. We analyzed phosphorylation of signaling intermediates by immunoblotting, ER transcriptional activity with reporter gene constructs and immunoblot analysis of endogenous gene products, promoter assembly by chromatin immunoprecipitation (ChIP) assay, and tumor cell growth *in vitro* by anchorage-independent colony formation and *in vivo* using xenografts in nude mice. **Results:** MCF-7/HER2-18 tumors were completely growth inhibited by estrogen deprivation but were growth stimulated by tamoxifen. Molecular cross-talk between the ER and HER2 pathways was increased in the MCF-7/HER2-18 cells compared with MCF-7 cells, with cross-phosphorylation and activation of both the ER and the EGFR/HER2 receptors, the signaling molecules AKT and ERK 1,2 mitogen-activated protein kinase (MAPK), and AIB1 itself with both estrogen and tamoxifen treatment. Tamoxifen recruited coactivator complexes (ER, AIB1, CBP, p300) to the ER-regulated pS2 gene promoter in MCF-7/HER2-18 cells and corepressor complexes (NCoR, histone deacetylase 3) in MCF-7 cells. Gefitinib pretreatment blocked receptor cross-talk, reestablished corepressor complexes with tamoxifen-bound ER on target gene promoters, eliminated tamoxifen's agonist effects, and restored its anti-tumor activity both *in vitro* and *in vivo* in MCF-7/HER2-18 cells. **Conclusions:** Tamoxifen behaves as an estrogen agonist in breast cancer cells that express high levels of AIB1 and HER2, resulting in *de novo* resistance. Gefitinib's ability to eliminate this cross-talk and to restore tamoxifen's anti-tumor effects should be tested in the clinic. [J Natl Cancer Inst 2004; 96:926–35]

The binding of estrogen to the estrogen receptor (ER) induces receptor phosphorylation, alters its conformation, triggers receptor dimerization, and facilitates binding of the receptor complex to the promoter region of target genes to activate transcription (1). These transcriptional effects of the ER are modulated by interactions with coregulatory proteins that function as either coactivators or corepressors (2). The conformation of estrogen-bound ER favors the recruitment of coactivators that augment

transcriptional activity. By contrast, the ER conformation induced by binding of the selective estrogen receptor modulator (SERM) tamoxifen favors the recruitment of corepressors that inhibit transcriptional activity. Tamoxifen has different partial agonist–antagonist activities in different tissues, however, and the differences may be related in part to the milieu of ER coactivators and corepressors in these tissues (3). For example, increased levels of coactivators such as AIB1 (SRC3) or SRC1 enhance the estrogen agonist properties of tamoxifen *in vitro* (4).

ER can generate multiple growth-promoting signals both inside and outside the nucleus. Estrogen-induced expression of genes encoding growth factors, their receptors, and other signaling molecules can provide cell proliferation and survival stimuli (5,6). ER can also complex with other transcription factors, such as Fos and Jun proteins on AP1 response elements, to alter the transcription of genes not normally thought to be classical estrogen targets, such as cyclin D1, insulin-like growth factor 1, and collagenase (7). Finally, new evidence also indicates that ER located in or near the cell membrane can activate growth factor receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) and HER2/neu (HER2), providing another mechanism for the growth-promoting effects of estrogen (8).

The receptor cross-talk between the ER and growth factor receptors travels in both directions. For example, ERK1,2 mitogen-activated protein kinase (MAPK) that has been activated by signaling from the EGFR or HER2 phosphorylates both ER and the ER coactivator AIB1 (9). The resulting ER phosphorylation in the N-terminal region, which can also be induced by estrogen binding, increases transcription arising from the AF-1 domain of the ER; the resulting phosphorylation of AIB1, which is not directly induced by estrogen, augments its coactivator activity (9,10).

These data raise the possibility that high tumor levels of ER coactivators, such as AIB1, could cause tamoxifen resistance and that HER2 cross-talk with ER could enhance the estrogen agonist activity of tamoxifen-bound ER. Tamoxifen's agonist properties might require both high AIB1 levels and growth

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factor receptor cross-talk, which phosphorylates and further activates both AIB1 and ER. In support of this hypothesis, we recently reported that tamoxifen-treated breast cancer patients whose ER-positive tumors express high levels of AIB1 and HER2 experience substantially more recurrences than those with ER-positive tumors that have lower expression of one or both proteins (11). Furthermore, there was a strong correlation between overexpression of AIB1 and overexpression of HER2 in these tumors, suggesting that high levels of the two proteins provide a strong selective growth advantage for tumor cells with this genotype.

The goal of the present study was to identify the mechanism for the tamoxifen resistance displayed by ER-positive tumors that express high levels of both AIB1 and HER2. We studied as an experimental model MCF-7 breast cancer cells, which express high levels of AIB1, and a derivative line, MCF-7/HER2-18, which expresses high levels of both AIB1 and HER2. We compared the estrogen agonist activity of tamoxifen-bound ER on *in vivo* tumor growth induced by estrogen and tamoxifen in the two cell lines, and we examined cross-talk between the HER2 and ER signaling pathways. We also investigated the components of the coregulatory complexes recruited by tamoxifen-bound ER to the promoter of a target gene. Finally, we examined the effect of a growth factor receptor tyrosine kinase inhibitor, gefitinib (ZD1839 or Iressa), on tamoxifen's effects in both cell lines.

MATERIALS AND METHODS

Reagents, Hormones, and Antibodies

EGF and heregulin were from Invitrogen (Carlsbad, CA) and R&D Systems (Minneapolis, MN), respectively. 17 β -Estradiol (E2), 4-hydroxy-tamoxifen (for all *in vitro* studies) and tamoxifen citrate (for *in vivo* studies), and all other reagents were from Sigma (St. Louis, MO) unless otherwise indicated. Gefitinib was provided by AstraZeneca (Macclesfield, UK). Antibodies used for immunoblotting were to phospho-Ser118-ER and AIB1 (11,12); ER α and HSP27 (NeoMarkers, Fremont, CA); progesterone receptor (PR) (DAKO, Carpinteria, CA); cathepsin D (BD Biosciences, San Diego, CA); Bcl-2 (Oncogene, Cambridge, MA); total and different phospho-forms of EGFR (Tyr⁸⁴⁵, Tyr⁹⁹², Tyr¹⁰⁴⁵, or Tyr¹⁰⁶⁸), HER2 (Tyr⁸⁸⁷, Tyr¹¹¹², or Tyr¹²⁴⁸), Akt (Ser⁴³⁷), and ERK1,2 MAPK (Thr²⁰²/Tyr²⁰⁴) (Cell Signaling Technology, Beverly, MA); IRS-1 (Upstate Biotechnology, Lake Placid, NY); cyclin D1 (Santa Cruz, Santa Cruz, CA); and β -actin (Chemicon, Temecula, CA). Antibodies used for chromatin immunoprecipitation (ChIP) assays were to AIB1 and NCoR (Affinity Bioreagents, Golden, CO), ER α and histone deacetylase 3 (HDAC3) (Santa Cruz), p300 and acetylated histone 3 (Upstate Biotechnology), and CREB binding protein (CBP) (NeoMarkers).

Xenograft Studies

MCF-7/HER2-18 xenografts were established in ovariectomized 5- to 6-week-old BALB/c athymic nude mice (Harlan Sprague Dawley, Madison, WI) supplemented with 0.25-mg 21-day-release estrogen pellets (Innovative Research, Sarasota, FL) by inoculating the mice subcutaneously with 5×10^6 cells, as described previously (17). When tumors reached 150–200 mm³ (i.e., in 2–4 weeks), the animals were randomly allocated

($n = 12$ per group) to continued estrogen (E2), estrogen withdrawal alone (–E2; by removal of the estrogen pellets), or to estrogen withdrawal plus tamoxifen citrate (Tam; 500 μ g/animal given subcutaneously in peanut oil, 5 days/week) (17), with either gefitinib (100 mg/kg of body weight, 5 days/week) or vehicle (1% Tween 80) administered via gavage. Tumor growth was assessed and tumor volumes were measured as described previously (17). Tumors were harvested either after 3 weeks of treatment ($n = 4$ per group) or when they reached 1000 mm³ ($n = 8$ per group). Mice were anesthetized with isoflurane before tumor removal. Each tumor analyzed was from a different mouse; tumor tissues were removed from each individual mouse and kept at –190 °C for later analyses. Animal care was in accordance with institutional guidelines.

Cells and Treatment

MCF-7 breast cancer cells, the derivative MCF-7/neo and MCF/HER2-18 cell lines (stably transfected with the control vector alone and a HER2 overexpression vector, respectively), and BT-474 breast cancer cells were maintained as described previously (13,14). Before treatment, tumor cells, in 10-cm dishes, were starved in phenol red-free (PRF), serum-free improved modified Eagle medium (IMEM) for 24 hours. For phosphorylation studies, cells were pretreated for 3 hours with gefitinib (1 μ M) or vehicle (dimethyl sulfoxide [DMSO] [0.001%]) followed by short-term treatment with vehicle (ethanol), estrogen (1 nM, 20 minutes), tamoxifen (100 nM, 20 minutes), EGF (100 ng/mL, 10 minutes), or heregulin (10 ng/mL, 20 minutes) in the presence or absence of gefitinib (1 μ M). For long-term induction studies, the cells were pretreated for 3 hours with gefitinib (1 μ M) or vehicle (DMSO) and were then treated for 48 hours with vehicle (ethanol), estrogen, tamoxifen, EGF, or heregulin at the same concentrations as used in the short-term treatments. Endogenous gene expression was analyzed in cells treated in the same way but for 12 hours. All of the experiments involving parental cells were carried out with both MCF-7 and MCF-7/neo cells. Because similar results were obtained with both cell lines (data not shown), only results for MCF-7 cells are reported.

Cell and Tumor Extracts and Immunoblots

After treatment, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and were then lysed immediately with 0.5 mL of lysis buffer/10-cm plate. The lysis buffer (Cell Signaling Technology) was supplemented with 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 \times protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), 1 μ M okadaic acid, and 10 μ g of Microcystin per milliliter. Cell lysates were collected, sonicated (five times for 5 seconds on ice), and microcentrifuged at 15 300g for 10 minutes at 4 °C. Cell supernatants were aliquoted and stored at –70 °C. Frozen tumor samples from the different treatment groups ($n \geq 4$ /group) were pulverized by a tissue pulverizer (Biospec Products, Bartlesville, OK) that was precooled with liquid nitrogen. Tumor powders were manually homogenized in the same supplemented lysis buffer (50 mg/0.5 mL) and extracted as above. Protein concentration was measured with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's directions. Aliquots (20–25 μ g) of protein from each sample were separated under denaturing conditions by

electrophoresis in 8%–10% polyacrylamide gels containing sodium dodecyl sulfate (SDS–PAGE) and transferred to nitrocellulose membranes by electroblotting (Schleicher & Schuell, Keene, NH). The blots were first stained with Ponceau S to confirm uniform transfer of all samples and were then incubated with specific antibodies according to the manufacturers' directions. Briefly, blots were blocked with blocking buffer (5% [wt/vol] nonfat dry milk in Tris-buffered saline [TBS; 100 mM Tris, pH 7.5, and 0.9% NaCl] containing 0.1% Tween 20 [TBS–T]) for 1 hour and then incubated with primary antibodies at dilutions per the manufacturers' directions. For all phospho antibodies, the incubations were done in 5% bovine serum albumin in TBS–T overnight at 4 °C; for other antibodies, the incubations were done in blocking buffer for 2 hours at room temperature. The blots were washed three times in TBS–T and then incubated for 1 hour at room temperature in 5% nonfat dry milk in TBS–T with horseradish peroxidase–labeled anti–rabbit or anti–mouse immunoglobulin G (IgG) (1:2000 and 1:4000 dilutions, respectively) secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were then washed in TBS–T, and the labeled proteins were detected with an enhanced chemiluminescence detection system (Amersham Pharmacia) and exposure of the membranes to X-ray film (Kodak, Rochester, NY). To detect the mobility shift in AIB1 due to phosphorylation, 10 µg of cell or tumor extracts was first preincubated in the presence or absence of protein λ-phosphatase (λ-PPase) (New England BioLabs, Beverly, MA) as previously described (9) and then electrophoresed on SDS–6% polyacrylamide gels. All experiments were repeated three times, and representative blots are presented.

Cell Cycle Analysis

Proliferating MCF-7 and MCF-7/HER2-18 cells were serum starved for 24 hours, were pretreated for 3 hours with gefitinib (1 µM) or vehicle (DMSO), and were then treated, in the presence or absence of gefitinib, for 16 hours with vehicle (ethanol), E2, tamoxifen, or heregulin, as mentioned above. Cells were then subjected to flow cytometric analysis as described previously (14), using a Beckman Coulter EPICS XL-MCS (Fullerton, CA).

Transient Transfection and Luciferase ER Reporter Assays

A total of 1.5×10^6 cells/10-cm dish were serum starved for 24 hours and transfected for 12 hours with 7.5 µg of a 2× estrogen response element (ERE)–luciferase construct (15) using LipofectAmine (Invitrogen) in PRF Opti-MEM reduced-serum medium (Invitrogen) containing 1% charcoal-stripped fetal calf serum (CS-FCS) (HyClone, Logan, UT) according to the manufacturer's directions. Cells were then pooled (two dishes per cell line) and split into 12-well plates in PRF IMEM supplemented with 0.5% CS-FCS. After 12 hours, the cells were treated for an additional 16 hours with estrogen or tamoxifen, with or without heregulin and with or without gefitinib, at the concentrations given above. Activity of the luciferase reporter gene was determined by using the Luciferase Assay System (Promega, Madison, WI).

ChIP Assays

ChIP assays were carried out with the ChIP assay kit (Upstate Biotechnology) as previously described (16), with minor modi-

fications. All reagents and buffers were from the kit unless otherwise indicated. Cells were grown on 15-cm plates to 90% confluence in steroid-starved medium (PRF IMEM containing 10% CS-FCS [HyClone]) for 7 days and then were serum starved for 24 hours. Cells were pretreated for 3 hours with gefitinib (1 µM) or vehicle (DMSO) and then treated for 45 minutes with vehicle (ethanol), 100 nM estradiol, or 1 µM tamoxifen with or without gefitinib, and immediately cross-linked with 1% formaldehyde (final concentration) added directly to the cell medium for 10 minutes at 37 °C. Cells were washed with cold PBS containing 1× protease inhibitor cocktail and 1 mM PMSF, scraped, microcentrifuged for 4 minutes at 4 °C, 420g, and lysed by incubation with 1 mL of 1× SDS lysis buffer for 10 minutes on ice. Lysates were sonicated three times for 10 seconds each at 50% duty cycle using an Out Control intensity of 3 (Sonifier 450; Branson, Danbury, CT) followed by microcentrifugation at 4 °C, 18 000g for 10 minutes. Protein concentration was measured with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Two hundred micrograms of supernatant protein was diluted 10-fold with ChIP dilution buffer and immunocleared with 80 µL of salmon sperm DNA–Protein A/G agarose in the presence of preimmune serum (Santa Cruz). Collected agarose beads were saved as control IgG. Immunoprecipitation was performed overnight at 4 °C with specific antibodies (2 µg/1 mL of precleared supernatant). Immunocomplexes were extracted by adding 80 µL of salmon sperm DNA–Protein A/G agarose for 1 hour at 4 °C followed by gentle centrifugation (110g, 1 minute, 4 °C). Precipitates were washed sequentially with 1 mL of low-salt wash buffer, high-salt wash buffer, and LiCl wash buffer and were washed twice with 1 mL of TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and extracted twice sequentially by 15 minutes of incubation at room temperature with 250 µL of freshly made elution buffer (1% SDS, 0.1 M NaHCO₃). The two sequential eluates (×2) were pooled in a total volume of 500 µL, and after the addition of 20 µL of 5 M NaCl, were heated at 65 °C for 4 hours to reverse the formaldehyde cross-linking. DNA fragments were then purified with a Qiagen Gel Extraction kit (Qiagen, Valencia, CA) in a final volume of 30 µL.

Five microliters from the 30-µL DNA extraction was amplified by polymerase chain reaction (PCR) with the following pairs of primers for the promoter region of the PS2 gene: 5'-GGC CATCTCTCACTATGAATCACTTCTGCA-3' (forward) and 5'-GGCAGGCTCTGTTTGCTTAAAGAGCGTTAGATA-3' (reverse). The PCR amplification was carried out with Sigma reagents in a final reaction volume of 20 µL containing 1× PCR buffer, all four deoxynucleoside triphosphates (each at 0.2 mM), 1 U of *Taq* DNA polymerase, and 1.25 pM primers. The reactions were performed in an MJ Research PTC-200 Peltier thermal cycler (MJ Research, Reno, NV), with the following program: initial 2 minutes of denaturing at 94 °C followed by 28 cycles of denaturing for 30 seconds at 94 °C, annealing for 30 seconds at 63 °C, and elongating for 1 minute at 72 °C; the final extension took place at 72 °C for 10 minutes. Equal volumes of each PCR sample were subjected to electrophoresis in a 2% agarose gel, which was then stained with ethidium bromide and photographed under UV illumination.

Anchorage-Independent Growth Assays

Cells were steroid starved for 7 days in PRF IMEM containing 10% CS-FCS (HyClone) and then treated for 3 hours with gefitinib at the indicated concentrations or vehicle. Colony-forming assays in soft agarose were performed as described previously (14). In brief, tumor cells in top soft agar in steroid starvation medium-containing vehicle, 10 nM E2, or 100 nM tamoxifen with gefitinib at the original treatment concentration were plated on solidified agar in steroid starvation medium. After 3 weeks, tumor cell colonies measuring at least 50 μm were counted from six replicates per treatment under a dissecting microscope.

Statistical Analysis

Values are expressed as means with 95% confidence intervals (CIs). Tumor growth curves were constructed from the mean tumor volume at each time point of measurement, with error bars representing 95% CI of the mean. The two-sample *t* test was used for two-group comparisons of relative luciferase activity and percentage of cells in S phase. Major analyses, unless otherwise specified, were for gefitinib effects (i.e., comparison of with versus without gefitinib for each specific hormonal treatment and cell group). For the anchorage-independent colony growth assays, effects of increasing concentrations of gefitinib were tested with analysis of variance. After statistically significant differences across various concentrations were established, contrasts were generated in the analysis of variance model to perform pairwise comparisons between control and each concentration of gefitinib in each treatment and cell group. In addition, to assess the magnitude of gefitinib inhibition in the tamoxifen-treated groups versus that of the estrogen-treated groups, we used linear regression models to compare the decrease in number of colonies from that of vehicle-treated cells to that of cells treated with each concentration of gefitinib between these two groups.

RESULTS

HER2 Overexpression and Tamoxifen-Stimulated Growth

We previously showed that growth of wild-type MCF-7 xenografts, which express moderate levels of the ER, low levels of EGFR and HER2, and high levels of AIB1, is stimulated by estrogen and inhibited by estrogen deprivation, either alone or in the presence of tamoxifen (17). Stable MCF-7 transfectants overexpressing HER2 (MCF-7/HER2-18 cells) are tamoxifen resistant and rapidly form xenografts in mice treated with tamoxifen or estrogen (13). To analyze the endocrine sensitivity of HER2-overexpressing breast tumors in more detail, we established MCF-7/HER2-18 xenografts in ovariectomized athymic nude mice in the presence of estrogen. Mice were then randomized to continued treatment with estrogen supplementation, estrogen withdrawal, or estrogen withdrawal plus the antiestrogen tamoxifen, and tumor growth was monitored over time (Fig. 1, A). Growth of MCF-7/HER2-18 tumors was stimulated by estrogen and completely inhibited by estrogen withdrawal nearly 60 days after removal of the estrogen pellet, indicating absolute estrogen dependence. Growth of these tumors was also stimulated when tamoxifen was added to estrogen withdrawal. This result is in contrast to the findings we previously reported for

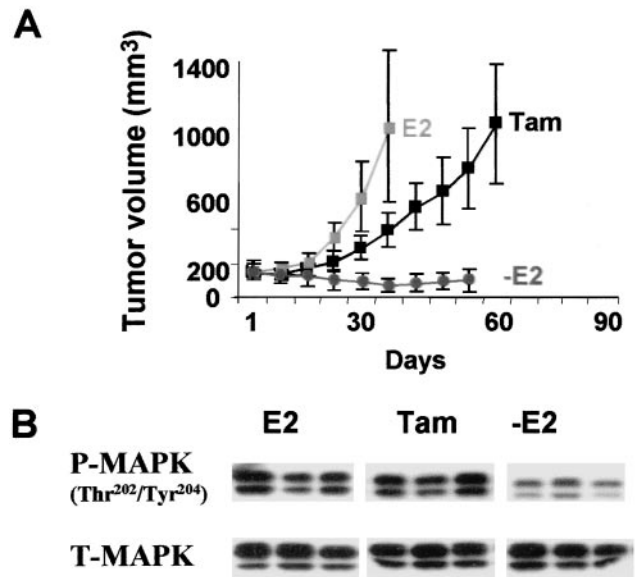


Fig. 1. Effect of estrogen and tamoxifen on HER2-overexpressing tumors. **A**) MCF-7 cells stably transfected with HER2 (MCF-7/HER2-18 clone) were established as xenografts in ovariectomized athymic nude mice in the presence of estrogen. Mice were assigned randomly (day 1), in groups of 12, to continued estrogen supplementation (E2), estrogen withdrawal (-E2), or estrogen withdrawal plus the antiestrogen tamoxifen (Tam), and tumor growth was monitored over time. Four mice from each group were harvested after 3 weeks of treatment. Tumor growth of the remaining eight mice in each group appears as the mean of tumor volumes in each treatment group; bars show 95% confidence intervals. **B**) Levels of phospho Thr²⁰²/Tyr²⁰⁴-ERK1,2 mitogen-activated protein kinase (MAPK) (P-MAPK) and total ERK1,2 MAPK (T-MAPK, doublets represent the 42- and 44-kd forms) in three individual tumor extracts from the different treatment groups (after 3 weeks of treatment) assessed by immunoblot analysis. Blots were repeated three times.

parental MCF-7 tumors, whose growth is inhibited by the combination of tamoxifen plus estrogen withdrawal (17). These data indicate that tamoxifen, like estrogen, functions like an estrogen agonist to enhance tumor growth when HER2 is overexpressed.

To investigate the effects of estrogen and tamoxifen on growth factor receptor downstream signaling, we analyzed the levels of phosphorylated ERK 1,2 MAPK in tumor extracts (Fig. 1, B). The level of this activated kinase was increased by tamoxifen as well as by estrogen compared with estrogen withdrawal alone (Fig. 1, B).

Cross-Talk Between Growth Factor Receptor and ER Pathways

To further investigate the mechanism by which the estrogen agonist properties of tamoxifen are increased in MCF-7 cells when HER2 is overexpressed, we examined the short-term effects of estrogen, EGF, heregulin, and tamoxifen on phosphorylation of ER, EGFR, HER2, ERK1,2 MAPK, and AKT in the presence or absence of the receptor tyrosine kinase inhibitor gefitinib, which has been shown to inhibit signaling from both the EGFR and HER2 (18). Treatment of parental MCF-7 cells with either estrogen or tamoxifen led to phosphorylation of the ER via a process that is independent of EGFR or HER2 signaling because it was not inhibited by gefitinib (Fig. 2, A). Treatment with heregulin, and to a much lesser extent, EGF, also caused ER phosphorylation via a process that was inhibited by gefitinib. We did not detect phosphorylation of EGFR or HER2 with EGF

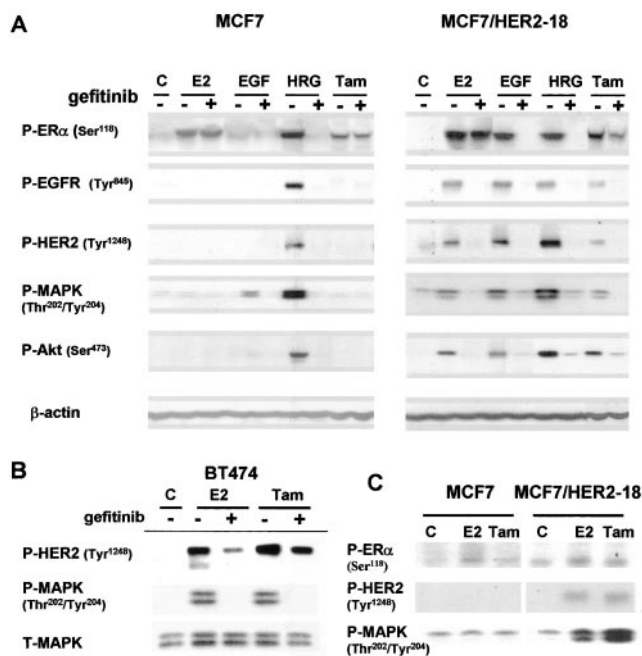


Fig. 2. Effect of HER2 overexpression and gefitinib on cross-phosphorylation between ER and EGFR/HER2 signaling components in MCF-7 and MCF-7/HER2-18 cells. **A**) MCF-7 and MCF-7/HER2-18 cells in serum-free medium, after 3 hours of preincubation with vehicle (dimethyl sulfoxide) or with the tyrosine kinase inhibitor gefitinib, were subjected to a 10–20 minute treatment with vehicle (ethanol [C]), estrogen (E2), EGF, heregulin (HRG), or 4-OH tamoxifen (Tam) in the continuous presence or absence of gefitinib. Levels of phosphorylated (P) ER α , EGF receptor (EGFR), HER2, ERK1,2 mitogen-activated protein kinase (MAPK), and AKT, at the indicated residues, were measured in cellular extracts by immunoblot analysis. Blots are representative of three experiments. β -Actin was used as a control for equal loading and transfer. **B**) BT474 cells, which naturally express high levels of AIB1 and HER2, were treated and analyzed as in panel A. Blots are representative of three experiments. β -Actin was used as a control for equal loading and transfer (not shown). **C**) Parental MCF-7 and MCF-7/HER2-18 cells were induced for long-term (48 hours) as in panel A and phosphorylation levels of ER, HER2, and ERK1,2 MAPK in cellular extracts were measured as in panel A. Blots are representative of three experiments. β -Actin was used as a control for equal loading and transfer (not shown).

treatment in MCF-7 cells under these conditions, although EGF treatment induced slightly higher levels of phosphorylated MAPK and phosphorylated AKT (Fig. 2, A), indicating that EGFR is functional in these cells. Heregulin treatment, on the other hand, led to strong phosphorylation of ER, EGFR, HER2, AKT, and ERK1,2 MAPK, and phosphorylation of all of these targets was inhibited by gefitinib. We did not detect phosphorylation of EGFR, HER2, ERK1,2 MAPK, or AKT in MCF-7 cells treated with estrogen or tamoxifen.

In contrast with parental MCF-7 cells, MCF-7/HER2-18 cells showed substantial bidirectional cross-talk between ER, on the one hand, and EGFR and HER2, on the other (Fig. 2, A). Treatment of MCF-7/HER2-18 cells with estrogen, EGF, heregulin, and tamoxifen all led to ER phosphorylation on Ser-118, as was the case in MCF-7 cells. Gefitinib inhibited the effect of EGF and heregulin on MCF-7/HER2-18 cells, and it partially inhibited the ER phosphorylation induced by tamoxifen but not that induced by estrogen, again as in MCF-7 cells. However, in the MCF-7/HER2-18 cells, both estrogen and tamoxifen induced phosphorylation of EGFR, HER2, AKT, and ERK1,2 MAPK, similar to the phosphorylation induced by EGF

and heregulin. Similar results were obtained in both cell lines by using other antibodies specific for detecting phosphorylation of Tyr-992, -1045, or -1068 on EGFR, and Tyr-887 or -1112 on HER2 (data not shown). Phosphorylation of all of these growth factor signaling molecules was abolished by gefitinib. Effects of estrogen and tamoxifen on phosphorylation of downstream effectors of EGFR and HER2 similar to those in MCF-7/HER2-18 cells were observed in another cell line, BT474, which expresses ER and is naturally gene amplified for HER2 and AIB1 (19,20) (Fig. 2, B).

All of these experiments in both cell lines involved a maximum of a 20-minute treatment with estrogen, EGF, heregulin, and tamoxifen. The effects of estrogen and tamoxifen treatments on phosphorylated levels of HER2 and MAPK were observed as early as 3 minutes (BT474 cells, data not shown) and were still evident after 48 hours (Fig. 2, C), indicating that they were not transient. Thus, in these HER2-overexpressing cells, estrogen and tamoxifen activate growth factor signaling, while at the same time growth factor signaling activates ER. The receptor tyrosine kinase inhibitor gefitinib eliminated the cross-talk in both directions.

Phosphorylation of AIB1 and Effects of Gefitinib

Because ERK 1,2 MAPK, a downstream target of HER2, phosphorylates the ER coactivator AIB1 (9), we reasoned that tamoxifen-stimulated growth in the presence of high levels of HER2 may be mediated, in part, by the functional activation of AIB1 by HER2 signaling, which would in turn enhance tamoxifen's estrogen agonist activity. In parental MCF-7 cells, treatment with heregulin but not estrogen or tamoxifen led to AIB1 phosphorylation (Fig. 3), and this AIB1 phosphorylation was completely prevented by gefitinib. In contrast, in MCF-7/HER2-18 cells, phosphorylation of AIB1 was observed not only in cells treated with heregulin but also in cells treated with estrogen and tamoxifen. Gefitinib blocked these effects, suggesting that the AIB1 phosphorylation by estrogen and tamoxifen in MCF-7/HER2-18 cells was due to ER-mediated activation of the EGFR and/or HER2 pathway.

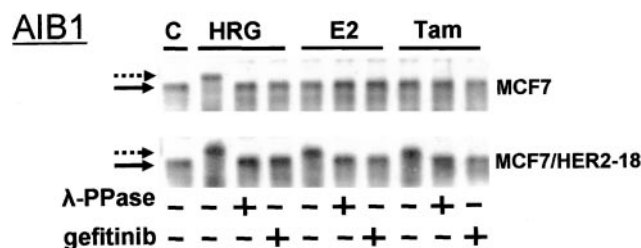


Fig. 3. Effects of HER2 overexpression and gefitinib on AIB1 phosphorylation by estrogen and tamoxifen in MCF-7 and MCF-7/HER2-18 cells. MCF-7 and MCF-7/HER2-18 cells were treated for 20 minutes with vehicle (C), heregulin (HRG), estrogen (E2), or 4-OH tamoxifen (Tam) in the presence or absence of the tyrosine kinase inhibitor gefitinib as in Fig 1, A. AIB1 expression was examined by immunoblot analysis of cell extracts, some of which had been preincubated with protein λ -phosphatase (λ -PPase) prior to electrophoresis. More slowly migrating AIB1 bands (marked by **dashed arrows**) represent the phosphorylated form of the protein, because this change in mobility was reversed by pretreatment of the extracts with λ -PPase to restore the dephosphorylated form (**solid arrows**). Blots are representative of three experiments.

Tamoxifen Agonist Effects and ER-Dependent Gene Transcription in MCF-7/HER2-18 Cells

To determine if the activation of growth factor signaling, ER phosphorylation, and AIB1 phosphorylation by tamoxifen has functional significance, we examined tamoxifen's effects on ER-dependent gene transcription. Mean luciferase activity observed with estrogen induction in each cell line was not set to 1.0. In parental MCF-7 cells, gefitinib had no effect on ER-dependent transcription of an ERE-luciferase reporter gene induced by estrogen (Fig. 4, A). The addition of heregulin, however, increased luciferase activity by more than twofold (to levels of 2.2, 95% CI = 2.05 to 2.35), and the effect was growth factor receptor dependent, as reflected by gefitinib inhibition. Relative luciferase activity was reduced by more than fourfold (to levels of 0.49, 95% CI = 0.4 to 0.58) when gefitinib was combined with heregulin ($P < .001$). As expected, tamoxifen had no agonist activity in MCF-7 cells; i.e., ERE-luciferase transcription was not induced by tamoxifen (relative activity = 0.18, 95% CI = 0.17 to 0.19), although the combination of heregulin and tamoxifen did increase luciferase activity by a small amount. By contrast, in the MCF-7/HER2-18 cells, tamoxifen-induced-luciferase activity was similar to that induced by estrogen (relative activity = 0.82, 95% CI = 0.63 to 1.01) and gefitinib treatment resulted in statistically significant inhibition of both estrogen and tamoxifen-induced activity (Fig. 4, A; 55%, $P = .003$ and 40%, $P = .008$ for gefitinib inhibition effect on estrogen and tamoxifen treatments, respectively). Heregulin treatment further enhanced luciferase activity in response to estrogen (by more than fourfold, relative activity = 4.37, 95% CI = 4.13 to 4.61) and tamoxifen (by more than 2.5-fold, relative activity = 2.24, 95% CI = 2.06 to 2.42), an effect that was statistically significantly inhibited by more than 50% by gefitinib ($P < .001$).

To confirm the agonist properties of tamoxifen on gene expression in a more physiologically relevant context, we examined a panel of endogenous estrogen-responsive gene products (Fig. 4, B). In MCF-7 cells, estrogen treatment resulted in variable increases in the expression of all the proteins examined. Most of these effects were partially inhibited by gefitinib, with the notable exception of the progesterone receptor (both A and B isoforms), which showed no reduction with the addition of gefitinib. As expected, tamoxifen demonstrated no agonist activity on any of these genes in MCF-7 cells. Heregulin by itself induced the expression of all of the genes, again except for the progesterone receptor A and B isoforms, and these effects were abolished by gefitinib. Whether the induction by heregulin is mediated through its activation of ER or by some other mechanism remains to be determined.

In the MCF-7/HER2-18 cells, estrogen had similar effects on the expression of endogenous estrogen-responsive genes, and gefitinib reduced these effects (Fig. 4, B), indicating a contribution from the growth factor signaling pathway, as was evident in the luciferase assays (Fig. 4, A). However, in contrast to its behavior in MCF-7 cells, tamoxifen also behaved as an estrogen agonist on all of the genes examined. This agonist activity was completely dependent on growth factor receptor activity—that is, it was abolished by gefitinib. Heregulin also had dramatic effects on several of these genes. Thus, in the setting of HER2 overexpression, tamoxifen behaves as an estrogen agonist on a variety of estrogen-dependent genes, and in distinct contrast to the agonist effects of estrogen, those of tamoxifen are com-

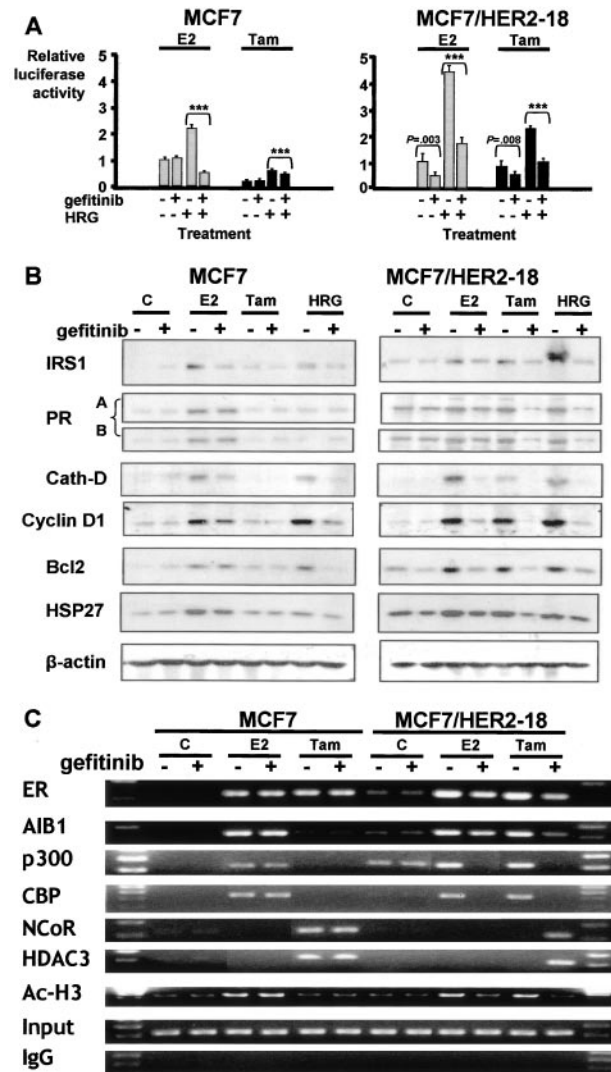


Fig. 4. Effect of HER2 overexpression and gefitinib on tamoxifen agonist activity in MCF-7 and MCF-7/HER2-18 cells. **A)** Serum-starved MCF-7 and MCF-7/HER2-18 cells were transiently transfected with a vector containing a luciferase reporter gene under the control of two estrogen response elements (15). Transfected cells were replated and 12 hours later were treated for 16 hours with estrogen (E2, gray bars) or 4-OH tamoxifen (Tam, solid bars) in the absence or presence of heregulin (HRG) and/or gefitinib. Mean luciferase activity observed with E2 induction in each cell line was set to 1.0. Each column represents the mean luciferase activity relative to estrogen induction of six replicate dishes. Bars indicate 95% confidence intervals. P values indicate statistical significance of inhibition in the presence of gefitinib in each treatment group (two-sample t test); *** = $P < .001$. **B)** Cells in serum-free medium were treated for 12 hours with control vehicle (C), E2, Tam, or heregulin (HRG), in the absence or presence of gefitinib. Expression of endogenous estrogen-responsive genes was assessed in immunoblot assays of whole-cell extracts by using antibodies against the following proteins: IRS-1, insulin receptor substrate 1; PR, progesterone receptor (which recognizes the A and B isoforms); Cath-D, cathepsin D; HSP27, heat shock protein 27, cyclin D1; and Bcl2. Blots are representative of three experiments. **C)** Steroid-starved, serum-starved cells were treated with vehicle (C), E2, or Tam in the presence or absence of gefitinib for 45 minutes. Occupancy of the estrogen-responsive pS2 promoter by ER α ; the coactivators AIB1, p300, and CBP; the corepressors NCoR and histone deacetylase H3 (HDAC3); and acetylated histone 3 (Ac-H3) was examined by chromatin immunoprecipitation (ChIP) assay. Relevant pS2 promoter sequences were PCR amplified from complexes immunoprecipitated with each antibody. Input lane shows DNA that was PCR amplified from extracts before immunoprecipitation. In the control immunoglobulin G (IgG), PCR was done in eluates from beads collected after preclearing of these extracts by using preimmune (normal) serum. Gels are representative of three experiments.

pletely dependent on cross-talk with the EGFR and/or HER2 pathway.

Having shown that tamoxifen has estrogen agonist activity on estrogen target genes in MCF-7/HER2-18 cells, we next examined the assembly of ER transcription complex components on the well-characterized estrogen-responsive pS2 promoter by using ChIP assays (Fig. 4, C). We reasoned that, under conditions of enhanced EGFR and HER2 signaling and activation of ER and AIB1, tamoxifen-bound ER may recruit coactivators instead of corepressors as previously reported (21). In MCF-7 cells, estrogen treatment induced occupancy of the pS2 promoter by ER and coactivator complexes, including AIB1 and the histone acetylases P300 and CBP, leading to acetylated histones. Tamoxifen, by contrast, induced occupancy by ER, the corepressor NCoR, and histone deacetylase (HDAC3). In MCF-7/HER2-18 cells, both estrogen and tamoxifen induced occupancy by ER, AIB1, P300, and CBP, resulting in the formation of acetylated histones. NCoR and HDAC3 replaced the coactivator complex when gefitinib was added to tamoxifen-treated cells to block growth factor signaling. The presence of coactivator complexes on the promoter when the receptor is bound by tamoxifen may explain its agonist properties on target gene expression in the MCF-7/HER2-18 cells.

Gefitinib Effects on Tamoxifen Stimulation of Tumor Growth

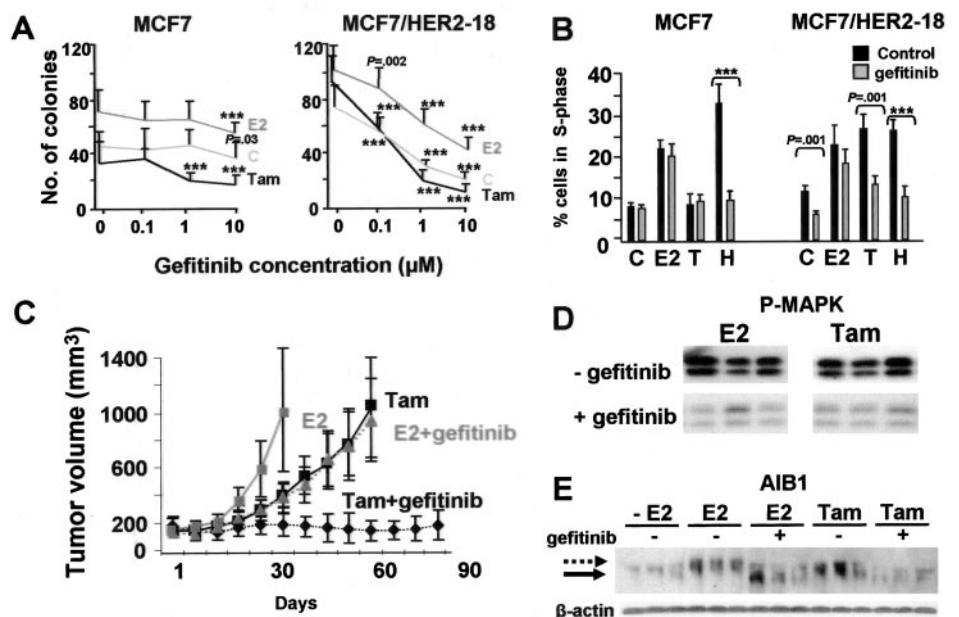
Because gefitinib blocked EGFR and HER2 cross-talk with ER, dissociated coactivator complexes from tamoxifen-bound ER on the promoters of target genes, and restored tamoxifen's antagonist effects on gene expression, we investigated its effects on tamoxifen stimulation of tumor growth. As expected, treat-

ment of MCF-7 cells with estrogen increased anchorage-independent colony formation, and treatment with tamoxifen reduced it (Fig. 5, A). Tamoxifen's antagonist properties on colony formation were further enhanced by gefitinib, because the number of colonies grown in the presence of tamoxifen was further reduced by gefitinib by more than 40% ($P < 0.001$, F test) at 1 μM and by more than 50% ($P < 0.001$, F test) at 10 μM . Gefitinib only slightly reduced the effects of estrogen on colony formation, and only at the highest concentration (10 μM). Thus, even in cells with low levels of EGFR and HER2—i.e., MCF-7 cells—tamoxifen's antagonist activity can be strengthened further by inhibiting growth factor signaling with the tyrosine kinase inhibitor.

In MCF-7/HER2-18 cells, by contrast, both tamoxifen and estrogen stimulated colony formation, although colony growth even in control conditions was higher than that of MCF-7 cells (Fig. 5, A). The agonist activity of tamoxifen was entirely dependent on growth factor signaling because it was partially inhibited by even a low concentration of gefitinib (0.1 μM) and eliminated at the higher concentrations ($\geq 1 \mu\text{M}$) that restored tamoxifen's antagonist activity ($P < .001$). Estrogen-induced colony formation was also partially inhibited by gefitinib in these MCF7/HER2-18 cells (50% at 10 μM , $P < 0.001$, F test). Yet, in these cells, the magnitude of the decrease in number of colonies caused by gefitinib at all concentrations was statistically significantly larger in the tamoxifen-treated group than in the estrogen-treated group ($P < .001$, F test from a linear regression model), suggesting that the tamoxifen agonist activity is more dependent on the growth factor signaling than that of estrogen.

We also examined the S-phase fraction as another way to assess cell proliferation. Both the basal S-phase fraction in

Fig. 5. Effect of gefitinib on tamoxifen antitumor activity *in vitro* and *in vivo*. **A)** Anchorage-independent growth of steroid-starved MCF-7 or MCF-7/HER2-18 cells was assessed by soft agarose colony-forming assays in the presence of vehicle (C), estrogen (E2), or 4-OH tamoxifen (Tam) in combination with gefitinib at the indicated concentrations. Graphs represent the mean colony number of six dishes. Bars indicate 95% confidence intervals (CIs). P values, based on the F test from contrasts generated in an analysis of variance model, indicate statistical significance of inhibition between control and each concentration of gefitinib within each treatment group; *** = $P < .001$. Only statistically significant P values are shown. **B)** Serum-starved MCF-7 and HER2-18 cells were treated with vehicle (C), estrogen (E2), 4-OH tamoxifen (T), or heregulin (H) for 16 hours and analyzed by DNA flow cytometry. Each column represents the mean of the percentage of cells in S phase of triplicate plates; bars indicate 95% CI; P values indicate statistical significance of the reduction in S-phase fraction in the presence of gefitinib compared with its absence in each treatment group (a two-sample t test); *** = $P < .001$. Only statistically significant P values are shown. **C)** Athymic nude mice bearing MCF-7/HER2-18 tumors were treated with estrogen (E2) or tamoxifen (Tam), either alone or in combination with gefitinib, and tumor growth was monitored. Graph shows the mean tumor volume in each group ($n = 8$); bars show 95% CI. **D)** Immunoblot analysis of phospho-Thr²⁰²/Tyr²⁰⁴-ERK1,2 mitogen-activated protein kinase (MAPK) (P-MAPK) in three individual tumor extracts from the different treatment groups in panel



C (after 3 weeks of treatment). Total ERK 1,2 MAPK levels were unchanged (not shown). Blots were repeated three times. **E)** Immunoblot analysis of AIB1 in tumor extracts from the different tumor treatment groups (including E2 withdrawal [-E2]). More slowly migrating AIB1 bands (dashed arrow) represent the phosphorylated form of the protein, and the unphosphorylated form is marked by a solid arrow. Blots are representative of three experiments.

untreated MCF-7/HER2-18 cells and the S-phase fraction in MCF-7/HER2-18 cells treated with tamoxifen were higher than those in MCF-7 cells (by 1.4- and 3.1-fold; $P = .005$ and $<.001$ for control untreated and tamoxifen-treated cells, respectively). However, the pronounced induction in S-phase fraction by tamoxifen compared with that of control in the MCF-7/HER2-18 cells (more than twofold) suggests that tamoxifen behaves as a strong estrogen agonist on cell proliferation in these HER2-overexpressing cells (Fig. 5, B). Heregulin also substantially increased the S-phase fraction above that of control treatment in these cells (more than twofold). Gefitinib inhibited the stimulatory effect of both tamoxifen ($P = 0.001$) and heregulin ($P < 0.001$) in the MCF-7/HER2-18 cells by more than 50% but only modestly inhibited the effects of estrogen, again demonstrating the complete growth factor receptor dependence of tamoxifen's agonist activity, in contrast with that of estrogen, which is only minimally dependent on this pathway.

Similar effects of tamoxifen and gefitinib on tumor growth were observed *in vivo* (Fig. 5, C). In contrast with parental MCF-7 tumors, the estrogen-mediated growth of which was unaffected by gefitinib (data not shown), gefitinib modestly slowed estrogen-induced growth of MCF-7/HER2-18 tumors and totally blocked their tamoxifen-stimulated growth. Despite its limited effects on estrogen-induced growth of MCF-7/HER2-18 tumors, gefitinib also strikingly reduced the levels of phosphorylated ERK1,2 MAPK in tumors from mice treated with either estrogen or tamoxifen (Fig. 1, B and 5, D). These data again indicate that estrogen-induced tumor growth is only partially dependent on EGFR- and HER2-mediated activation of ERK1,2 MAPK, whereas that induced by tamoxifen is totally dependent on this pathway. Estrogen and tamoxifen treatment of mice with MCF-7/HER2-18 xenografts also led to increases in levels of both total and phosphorylated AIB1, effects that were inhibited by gefitinib, consistent with gefitinib inhibition of tamoxifen's agonist activity (Fig. 5, E).

DISCUSSION

We have previously reported that ER-positive human breast cancers that overexpress AIB1, especially those that also overexpress HER2, are resistant to adjuvant therapy with tamoxifen (11). Given the interaction between HER2 and the ER coactivator AIB1, which are often overexpressed together in ER-positive tumors, we hypothesized that the antagonist effects of tamoxifen-bound ER in such overexpressing tumors might be reduced, and the agonist properties increased, as a mechanism for tamoxifen resistance (11). Here, we have demonstrated a plausible mechanism for this resistance in an experimental model system using ER-positive breast cancer cells that also overexpress both AIB1 and HER2. Although we did not address the exclusive role of coactivators and especially AIB1, the current data, together with the clinical data (11), strongly suggest that both HER2 and AIB1 are needed for the resistant phenotype. The agonist activity of tamoxifen on gene expression and cell proliferation was strongly increased in MCF-7/HER2-18 cells, which express high levels of both HER2 and AIB1, resulting in tumor growth stimulation by the drug. These effects are in stark contrast to tamoxifen's antagonist effects in parental MCF-7 cells, which have high AIB1 but low HER2 expression (17).

Our results provide an explanation for tamoxifen's estrogen-like activity in MCF-7/HER2-18 cells (Fig. 6). Enhanced signaling from the EGFR and HER2 tyrosine kinases activates ERK1,2 MAPK, which then phosphorylates and functionally activates both ER and AIB1, as has also been found in prior reports (9,22–24). Activation of AKT by these same tyrosine kinase receptors may have a similar effect (25). In addition, both estrogen and tamoxifen rapidly activate EGFR, HER2, AKT, and ERK1,2 MAPK in these cells, thereby establishing bidirectional cross-talk and a vicious cycle of cell survival and proliferative stimuli even when the ER is bound by tamoxifen. This cross-talk is less evident, and tamoxifen acts an antagonist, in cells with low growth factor receptor levels (13,17) or in cells with high growth factor levels in the presence of gefitinib.

In agreement with previous reports (12), we found that tamoxifen, like estrogen, induces phosphorylation of the ER on Ser-118, even in the parental cells in which it behaves as an antagonist. This interesting observation indicates that Ser-118 phosphorylation of the ER alone does not increase the agonist effects of tamoxifen sufficiently to cause resistance to the drug. Our data do suggest, however, that phosphorylation of the ER coactivator AIB1 by HER2 signaling is important for the development of resistance. In the HER2-overexpressing MCF-7/HER2-18 cells, both estrogen and tamoxifen recruited ER and AIB1 coactivator complexes to the promoter of an estrogen target gene, whereas in the parental MCF-7 cells, tamoxifen-bound ER recruited corepressor complexes with NCoR and HDAC3. In MCF-7/HER2-18 cells, gefitinib restored the recruitment of corepressor complexes with tamoxifen-bound ER

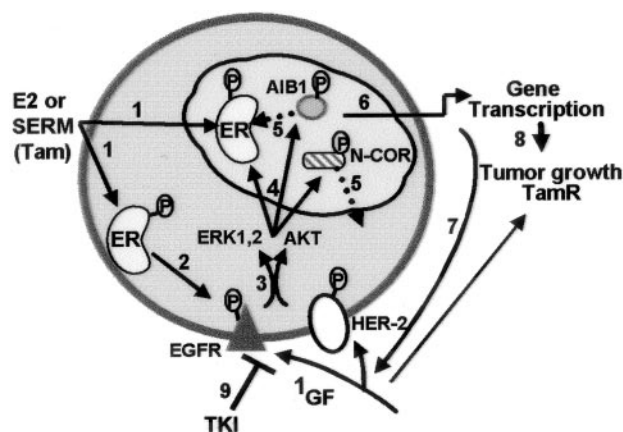


Fig. 6. Tamoxifen resistance model: Increased bidirectional ER/HER2 cross-talk converts tamoxifen into an agonist. In HER2-overexpressing tumors, peptide growth factors (1), as well as estrogen and tamoxifen via nongenomic activities (1 and 2), activate EGFR and HER2 signaling pathways (3). Activated downstream kinases, including ERK 1,2 mitogen-activated protein kinase (MAPK) and AKT, then phosphorylate both the ER and its accessory proteins (4). Corepressor complexes with NCoR are inactivated and dismissed from Tam-bound ER-promoter complexes, allowing instead the recruitment of the phosphorylated/activated coactivator complexes with AIB1 (5). This results in an increase in the agonist versus the antagonist activity of tamoxifen on gene transcription (6), which can lead to even further activation of the growth factor signaling pathways (7), thereby establishing bidirectional cross-talk and a vicious cycle of cell survival and proliferative stimulation even when ER is bound by tamoxifen. The net effect is tamoxifen resistance and tumor growth (8). This cross-talk is less evident, and tamoxifen remains an antagonist, in tumor cells with low growth factor receptor levels. In tumor cells with high levels of EGFR and/or HER2, this cross-talk can be blocked by gefitinib and presumably by other growth factor pathway inhibitors (9).

on the promoter. Blocking ERK1,2 MAPK also blocks phosphorylation of NCoR and restores its nuclear localization (26), thereby increasing its availability to bind and repress ER (27,28). The presence on the promoter of coactivator complexes explains why tamoxifen behaves as an estrogen agonist on a panel of endogenous target genes and on *in vitro* and *in vivo* proliferation of MCF-7/HER2-18 cells. Thus, in these HER2-overexpressing cells, the classical action of ER on transcription is enhanced by its nongenomic effects to activate the growth factor receptor tyrosine kinases EGFR and HER2 in response to binding of estrogen or tamoxifen. The relative contribution of classical effects of ER on gene transcription versus the rapid nonclassical (nongenomic) ER activities on the growth factor receptors in the membrane to this and other forms of tamoxifen resistance requires further investigation.

It is not clear from our data how AIB1 interacts with tamoxifen-bound ER. Coactivators like AIB1 are thought to bind ER in the hydrophobic groove in its AF-2 domain (29). When estrogen, but not tamoxifen, is bound to ER, helix 12 in the AF2 domain opens to allow access by coactivators to their binding site (29). Whether the conformation of ER is somehow altered in the presence of growth factor signaling to allow the interaction of AIB1 with this region, or whether phosphorylated AIB1 binds there or to another site on the receptor, remains to be clarified. Coactivators can bind to other sites on the ER, including the AF-1 domain, causing enhanced transactivation arising from this domain (27,30,31), and it is possible that AIB1 binds to one of these other sites in response to activation of the ER and AIB1 by growth factor signaling.

Although ligand-independent activation of the ER by ERK1,2 MAPK has been implicated as a mechanism for resistance to therapies designed to reduce endogenous estrogen levels, such as ovarian ablation or aromatase inhibitors (32), we detected only very low levels of ER and no coactivator complexes on the pS2 promoter in the absence of estrogen or tamoxifen in MCF-7/HER2-18 cells. Furthermore, in MCF-7/HER2-18 tumors from mice treated by estrogen withdrawal alone, phosphorylated ERK1,2 MAPK levels fell markedly compared with those in mice treated with estrogen or tamoxifen, and we could no longer detect phosphorylated AIB1. Finally, estrogen withdrawal was a strikingly effective inhibitor of MCF-7/HER2-18 tumor growth. Together, these observations suggest that ligand-independent activation of ER in the absence of estrogen or tamoxifen may be insufficient to trigger tumor growth and, consequently, that ovariectomy in premenopausal women and aromatase inhibition in postmenopausal women may still be worthwhile endocrine therapies for women with ER-positive tumors that overexpress EGFR, HER2, and/or AIB1. A recent clinical trial reporting a high rate of regression of primary tumors overexpressing EGFR/HER2 in patients treated with an aromatase inhibitor but not with tamoxifen supports this idea (33).

In summary, our data suggest that tamoxifen-stimulated growth of MCF-7/HER2-18 tumors is highly dependent on bidirectional cross-talk between ER and HER2. Treatment with the receptor tyrosine kinase inhibitor gefitinib blocked the cross-talk, prevented activation of ER and AIB1, reduced the recruitment of coactivator complexes, and enhanced recruitment of corepressor complexes to tamoxifen-bound ER on gene promoters. Tamoxifen's estrogen antagonist effects on gene expression and tumor growth were then restored. Gefitinib also blocked the rapid nongenomic effects of ER by which tamoxifen can activate

EGFR and HER2. Other studies have reported that receptor-blocking antibodies and an inhibitor of ERK1,2 MAPK can also inhibit growth of HER2-overexpressing breast tumor cells when given together with tamoxifen (14,34). *In vitro* (35) and *in vivo* studies of acquired tamoxifen resistance in parental MCF-7 cells also implicate enhanced EGFR and HER2 signaling cross-talk with ER as a mechanism for resistance (36). Gefitinib only minimally slowed MCF-7/HER2-18 tumor growth in estrogen-treated mice but totally blocked tamoxifen-stimulated growth. This finding indicates that estrogen enhances tumor growth largely by growth factor receptor-independent mechanisms, whereas the growth-stimulatory effects of tamoxifen are totally growth factor receptor dependent. Our data imply that monotherapy with growth factor pathway inhibitors like gefitinib may have little or only modest benefits on ER-positive, HER2-overexpressing breast cancer, but the results do provide a strong rationale for combining tamoxifen with gefitinib or other EGFR/HER2 pathway inhibitors to overcome *de novo* resistance in such tumors. Clinical trials of this new strategy are under way.

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NOTES

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