Development and Therapeutic Options for the Treatment of Raloxifene-Stimulated Breast Cancer in Athymic Mice

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Abstract

Purpose: Selective estrogen receptor modulators (SERM) are used for the treatment and prevention of breast cancer (tamoxifen) and osteoporosis (raloxifene). Mechanisms of tamoxifen-resistance in breast cancer are incompletely understood but current research is focused on crosstalk between growth factor receptors and the estrogen receptor α (ERα) pathway. There is increasing clinical use of raloxifene for the treatment of osteoporosis, but the widespread use of this SERM will have consequences for the treatment of breast cancer in raloxifene-exposed women.

Experimental Design: We took the strategic step of developing a raloxifene-resistant tumor (MCF-7RALT) model and investigating the mechanisms responsible for resistance.

Results: MCF-7RALT tumors exhibited phase I SERM resistance, growing in response to SERMs and estradiol. Epidermal growth factor receptor/HER1 and HER2/neu mRNAs were increased in MCF-7RALT tumors. The HER2/neu blocker, trastuzumab, but not the epidermal growth factor receptor blocker, gefitinib, decreased the growth of MCF-7RALT tumors in vivo. Consequently, trastuzumab decreased pro-survival/proliferative proteins: phospho-HER2/neu, total HER2/neu, phospho-Akt (protein kinase B), glycogen synthetase kinase-3, cyclin D1, and the anti-apoptotic protein X chromosome-linked inhibitor of apoptosis, whereas increasing the pro-apoptotic protein, caspase-7, in raloxifene-treated MCF-7RALT tumors. Interestingly, ERα protein was overexpressed in untreated MCF-7RALT tumors and hyperactivated in cells derived from these tumors. Only fulvestrant completely inhibited the growth and ERα activity of MCF-7RALT tumors. The co-activator of ERα, amplified in breast-cancer-1 protein was modestly increased in the raloxifene-treated MCF-7RALT tumors and increased both basal and estradiol-induced activity of ERα in cells derived from the MCF-7RALT tumors.

Conclusions: These results suggest that overexpression and increased activity of HER2/neu might be responsible for the development of raloxifene-resistant breast cancer. The results also suggest that increased expression of basal activity of ERα could contribute to the hypersensitivity of MCF-7RALT tumors in response to estradiol because only fulvestrant blocked growth and ERα activity.

Tamoxifen, a selective estrogen receptor modulator or SERM, has been used for >30 years in the treatment of breast cancer (1), and, more recently, was found to reduce the incidence of estrogen receptor (ER)-positive breast cancers in women at high risk (2). Despite the success of tamoxifen in the treatment of early stage breast cancer, and as a palliative therapy for stage IV breast cancer, many patients eventually develop resistance to the drug, resulting in breast cancer progression. We have previously shown three phases of resistance to tamoxifen in vivo (3–6). Short-term tamoxifen exposure (1-3 years) results in phase I resistance, in which estradiol (E2) and SERMs stimulate tumor growth, whereas growth is inhibited by the ER down-regulator, fulvestrant (3, 7). Longer exposure to tamoxifen results in phase II resistance, where SERMs stimulate tumor growth, but either E2 or fulvestrant inhibit tumor growth (4, 5). Tumors exhibiting phase III resistance act in a hormone-independent or refractory fashion, where tumor growth is not inhibited by SERMs or fulvestrant, but is paradoxically inhibited by E2, which induces apoptosis (6).

The mechanism of acquired resistance remains poorly understood, and is probably multifactorial. We, and others (8–10), have shown an increase in the expression of members of the epidermal growth factor receptor (EGFR) family in tamoxifen-resistant tumors, which could result in preferential phosphorylation and activation of ER via a
growth factor–driven pathway, such as the mitogen-activated pathway kinase, rather than via the classical ER pathway. This hypothesis is supported by the finding that MCF-7 breast cancer cells transfected with the EGFR family member, HER2/neu (HER2), are resistant to the inhibitory effects of tamoxifen in vivo (11). However, there is conflicting data from clinical studies examining the association between tamoxifen-resistance and expression of HER2 (12–15). A possible explanation for these controversial findings was recently shown by Osborne et al. (16), who noted that the coexpression of HER2 and of the coactivator, amplified in breast cancer-1 (AIB-1), resulted in a significantly poorer disease-free survival, compared with tumors that expressed neither HER2 nor AIB-1, and to tumors that were HER2-positive but did not express AIB-1, in patients with early stage breast cancer treated with tamoxifen. Therefore, the presence of HER2 alone may be insufficient to render a tumor intrinsically resistant to tamoxifen, and the presence of the coactivator AIB-1 and ERα downstream may be required to fully activate the HER2-driven pathway (17). There are now two clinical trials (18, 19), which have shown higher response rates in patients with HER2-positive breast tumors treated with aromatase inhibitors, compared with tamoxifen, in the preoperative setting, which may relate to the fact that tamoxifen acts primarily on nuclear ER and has been shown to activate membrane, nongenomic ER (20) versus the aromatase inhibitors, which reduce ligand activation of both nuclear and membrane ER (21).

Raloxifene is another SERM which was initially developed as a breast cancer treatment in the 1980s, but was not pursued further because of cross-resistance with tamoxifen (22). There was no perceived advantage of raloxifene compared with tamoxifen at that time. Based on preclinical findings (23, 24), raloxifene was subsequently evaluated as an agent to maintain bone density (25), and was approved for the prevention and treatment of osteoporosis. Review of the large databases (26–28) evaluating raloxifene as a preventive for osteoporosis showed that the SERM reduced the incidence of breast cancer compared with untreated women. These data are the basis for the Study of Tamoxifen and Raloxifene or STAR trial, which has randomized 19,000 postmenopausal women at high risk of breast cancer to raloxifene (60 mg) or tamoxifen (20 mg) for 5 years. However, the long-term use of raloxifene as a preventive for osteoporosis, and potentially breast cancer, has consequences for the eventual development of raloxifene-resistant breast cancers. The early gains as a breast cancer preventive will undoubtedly create an expanding pool of patients with SERM-resistant primary tumors.

Compared with tamoxifen, little is known about raloxifene-resistance, or how to optimally treat patients who develop breast cancer while receiving raloxifene. We now report the development of a raloxifene-stimulated breast tumor model (MCF-7E2) by exposing MCF-7 breast tumors to raloxifene in vivo. We have examined the potential role of ER, cell surface signaling (HER1/2), and the coactivator, AIB1, in the development of raloxifene-stimulated growth. Our goal was to study the natural process of acquired raloxifene resistance to expose potential therapeutic targets for further evaluation.

**Materials and Methods**

**Development of breast tumors.** SERM-naive breast tumors (MCF-7E2) were developed by injecting $1 \times 10^7$ MCF-7 human breast cancer cells into each mammary fat-pad of ovariectomized, BALB/c athymic mice (Harlan Sprague-Dawley, Madison, WI), and implanting the mice with a silastic capsule containing estrogen as previously described (3, 4, 29). These MCF-7E2 breast tumors have been serially passaged by implanting established MCF-7E2 tumors into the mammary fat-pads of athymic mice as previously described (4), and treating the mice with estrogen via silastic capsules (29).

**Tumor implantation.** In each experiment, a minimum of 10 athymic ovariectomized mice per treatment group were bitransplanted with either MCF-7E2 or MCF-7/RACT breast tumor pieces (1 mm³) in mammary fat-pads (resulting 20 breast tumors per treatment group). Established tumors were used in all experiments.

**Drug administration.** In all experiments, mice bearing transplanted tumors were divided into groups of 10 and treated with different combinations of E₂, tamoxifen, raloxifene, fulvestrant or were untreated (control group). E₂ was given to the mice via 0.3 cm silastic E₂ capsules that were made as described previously (29), implanted s.c., and replaced after 6 to 8 weeks of treatment. We have previously shown that these 0.3 cm E₂ capsules produce low E₂ levels in mice (mean serum level ± SD, 83.8 ± 34.6 pg/mL) similar to those E₂ levels found in perimenopausal women (29). Tamoxifen was purchased from Sigma Chemical Co. (St. Louis, MO), and raloxifene (Evista) tablets are commercially available (Eli Lilly Pharmaceuticals, Indianapolis, IN). Fulvestrant was obtained as a generous gift from AstraZeneca (Macclesfield, United Kingdom). Tamoxifen was suspended in a solution of 90% carboxymethylcellulose (1% carboxymethylcellulose in double-distilled water) and 10% polyethylene glycol 400/Tween 80 (99.5% polyethylene glycol 400/Tween 80) at a final concentration of 10 mg/mL. Tamoxifen was given orally 5 days a week at a dose of 1.5 mg (0.15 mL) per mouse per day. Raloxifene tablets (five tablets at 60 mg/tablet) were ground into a powder using a mortar and pestle, and the powder was dissolved in 27 mL of double-distilled water. Three milliliters of 90% carboxymethylcellulose and 10% polyethylene glycol 400/Tween 80 were added to the raloxifene solution for a final concentration of 10 mg/mL. Raloxifene was given orally or by i.p. injection 5 days each week at 1.5 mg (0.15 mL) per mouse per day. Fulvestrant was dissolved in alcohol to form a clear suspension. Peanut oil was added to a final dilution of fulvestrant 50 mg/mL. Fulvestrant was given at a dose of 5 mg (0.1 mL) twice weekly by s.c. injection given in the nape of the neck following cleaning of the skin with alcohol.

Trastuzumab (Herceptin) is commercially available. Trastuzumab (21 mg/mL) was diluted 10-fold in sterile PBS to a final concentration of 2.1 mg/mL. Trastuzumab was given twice weekly by i.p. injection at a dose of 30 mg/kg (0.75 mg in 0.36 mL) for a total weekly dose of 60 mg/kg, based on prior studies (30, 31).

Gefitinib (10 mg: Iressa) was obtained as a generous gift from AstraZeneca for studies in vitro. This was used as a companion for gefitinib synthesized at Leeds University using published methods (32). Gefitinib was dissolved in 5% glucose solution (4 g/100 mL), and left stirring overnight at room temperature. Gefitinib was kept in 50 mL aliquots at room temperature. Athymic mice were treated daily with gefitinib at a dose of 160 mg/kg (4 mg in 0.1 mL) daily by gavage for 5 days each week, based on prior studies (20).

**Tumor measurement.** Tumors were measured weekly with calipers and the cross-sectional area (in cm²) was determined using the formula: length (cm) × width (cm) × π/4. All animal procedures were approved by the Animal Care and Use Committee of Northwestern University.

**Western blot analysis.** Tumors were homogenized by grinding in liquid nitrogen and resuspending in lysis buffer [1% Triton X-100, 1 mmol/L EDTA, 150 mmol/L NaCl, 50 mmol/L Tris base (pH 7.4), 25 mg/mL phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 10 μg/mL tosyl-L-lysine chloromethyl ketone, 10 μg/mL tosyl-L-phenylalanine chloromethyl ketone, 100 mmol/L NaF, 10 mmol/L orthovanadate; Sigma]. The extract was subsequently sonicated and then centrifuged for 5 minutes at 5,000 × g at 4°C. The supernatant was collected and protein concentration was measured using the Bradford assay (Bio-Rad Laboratories, Inc., Santa
Equal amounts of protein (25-50 µg) were loaded onto a 7% polyacrylamide/bisacrylamide gel for SDS-PAGE followed by Western blotting. The following proteins were detected by Western blot: phosphorylated-EGFR (1:1,000, rabbit polyclonal antibody; Cell Signaling Technology), phosphorylated-HER2/neu (1:1,000, rabbit anti-human phosphorylated residue Y1248; Upstate Biotechnology), HER2/neu (1:200, mouse anti-human HER2/neu AB-11; Neomarkers, Fremont, CA), ERα (1:200, rabbit anti-human, clone G20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), AIB-1 (2 µg/mL, rabbit polyclonal antibody, PAI-845; Affinity Bioreagents, Golden, Co.), phosphorylated Akt (1:1,000, Ser473), rabbit polyclonal antibody; Cell Signaling Technology, Beverly, MA), total Akt (1:1,000, rabbit polyclonal antibody; Cell Signaling Technology), phospho-glycogen synthetase kinase-3β (GSK3β; 1:1,000, Ser 9, rabbit polyclonal antibody; Cell Signaling Technology), X chromosome-Linked IAP (XIAP; 1:1,000, rabbit polyclonal antibody; Cell Signaling Technology), caspase-7 (1:1,000, rabbit polyclonal antibody; Cell Signaling Technology), cyclin D1 (1:1,000, mouse monoclonal antibody, sc-20044; Santa Cruz Biotechnology), and β-actin (1:20,000, mouse anti-human, clone A15; Sigma). The appropriate secondary antibody conjugated to horseradish peroxidase was used to detect the primary antibody (either goat anti-rabbit or goat anti-mouse IgG-horseradish peroxidase, Santa Cruz Biotechnology). The blot was developed using an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL). The membrane was exposed to Kodak X-OMAT film for 10 to 30 seconds.

Real-time reverse transcription-PCR for human EGFR and HER2/neu mRNAs in tumors. Total RNA was extracted from the tumors using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The total RNA was reverse-transcribed using TaqMan reverse transcription reagents (PE Applied Biosystems, Hayward, CA) with the use of random hexamers as the primers according to the manufacturer's instructions. Primers and probes for human EGFR and HER2/neu were designed using Primer Express 1.5 software set at default variables to select the most optimized primer and probe sets for this system. The sequences for the forward and reverse primers for human EGFR are 5'-TCCAGAGTTGCTGTTAT-3' and 5'-TGCAAGTTTTTCACAAAGGA-ATTC-3', respectively. The sequence for the human EGFR probe is 5'-FAM-TCCTCATTGCCCTCAA-CACAGTGGAG-QSY7-3', in which FAM is the reporter and QSY7 is the quencher (MegaBases, Inc., Evanston, IL). The sequences for the
forward and reverse primers for human HER2/neu are 5′-CTCGAGA-GCCTCAGG-ATT3′ and 5′-AGCGGACGGCATCTGTGCA-3′, respectively. The sequence for the human HER2/neu probe is 5′-TCCGAGCACCAGCTTGA-3′ where FAM is the reporter and QSV7 is the quencher (MegaBases). The quantity of human glyceraldehyde-3-phosphate dehydrogenase mRNA was also measured in each total cDNA sample for normalization purposes. The probe and primers for glyceraldehyde-3-phosphate dehydrogenase were purchased from Perkin-Elmer Applied Biosystems (Stanford, California, CA). The PCR portion of the reaction was done with the use of the TaqMan PCR Core Reagent Kit (Perkin-Elmer Applied Biosystems). In a total volume of 25 µL, 50 ng of total cDNA, 100 nmol/l probe, and 200 nmol/l primers were used in the PCR reaction. Real-time PCR was done using the ABI-PRISM 7700 sequence detection system. The PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Tumor dispersion and transfection for reporter gene assays. Tumors (four per group) were resected from the animals and immediately placed in ice-cold serum-free media (phenol red–free RPMI 1640 supplemented with 2 mMol/L l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B; Invitrogen, Carlsbad, California). Tissues were transfected to cell culture dishes containing 10 ml/tumor of 2 mg/ml collagenase type III (186 units/mg; Worthington Biochemical, Lakewood, New Jersey) in serum-free media and containing 10 ml/tumor of 2 mg/ml collagenase type III (186 units/mg; Worthington Biochemical, Lakewood, New Jersey) in serum-free media. The next day, the cells were transfected using Effectene (Qiagen) at a 4:1 ratio of transfection reagent in microliters versus micrograms of DNA following the manufacturer’s directions. The cells were then transfected with a plasmid DNA mixture containing 0.2 µg of pERE (3×) thymidine kinase–luc (containing three copies of a consensus estrogen response element (ERE) linked to the herpes simplex virus minimal thymidine kinase promoter regulating the expression of firefly luciferase; ref. 33), 0.05 µg of pRL-thymidine kinase (containing a minimal TK promoter driving expression of renilla luciferase; Promega, Madison, Wisconsin), and either 0.5 µg of pcDNA3.1 (empty expression plasmid; Invitrogen), or 0.5 µg pcDNA3.1 + AIB1 (plasmid expressing the coactivator AIB1 under the control of the cytomegalovirus promoter; ref. 34). Four hours following the transfection procedure, the medium was exchanged with fresh medium containing the indicated test agents dissolved in ethanol and diluted at 1:1,000. Forty hours following treatment with the test agents, the cells were harvested and processed for dual-luciferase reporter assays (Promega), in which the firefly luciferase activity was normalized by renilla luciferase activity.

Each treatment group consisted of six replicate wells. Each transfection experiment was done using tumor cells dispersed from four tumors per tumor type (MCF-7/E2 or MCF-7/RALT), with each experiment being done on three separate occasions. Numbers shown are means ± SD of data obtained from a single representative experiment.

Statistical methods. Analyses of time-specific comparisons of mean tumor size across groups were done with the use of pairwise Student’s t tests. All statistical tests were two-sided.

Results

Development and growth characteristics of raloxifene-stimulated MCF-7 tumors. After approximately 18 weeks of treatment with raloxifene, the MCF-7/E2 tumors started to grow in the presence of raloxifene, whereas the control tumors did not (Fig. 1A). These raloxifene-stimulated tumors (MCF-7/RALT) were then transplanted into another set of athymic mice and were again treated with vehicle control, or with raloxifene (1.5 mg daily by gavage). The MCF-7/RALT tumors grew with raloxifene compared with control tumors (Fig. 1A, insert).

Next, we did two experiments to classify the phase of SERM resistance for MCF-7/RALT tumors. In the first experiment, both tamoxifen and raloxifene stimulated MCF-7/RALT tumor growth, and there was no significant difference between the two SERMs on tumor growth (Fig. 1B). Interestingly, tumors in the control animals grew modestly. In contrast, fulvestrant did not stimulate MCF-7/RALT tumor growth, compared with the control animals. In the next experiment, estradiol (E2) robustly stimulated tumor growth compared with vehicle-treated control animals (Fig. 1C). In addition, raloxifene-treated, tamoxifen-treated, and to a lesser degree, control tumors grew, whereas fulvestrant completely inhibited tumor growth (Fig. 1C). From these two experiments, it was clear that the MCF-7/RALT tumors exhibited phase I resistance, as they are robustly stimulated to grow with E2 or SERMs, and also grow modestly in the absence of ligand. These findings likely reflect the fact that the MCF-7/RALT tumors have been exposed to raloxifene for <5 years (3, 4) and their growth might still be dependent on the ERs as fulvestrant completely inhibited the growth of MCF-7/RALT tumors.
Expression of EGFR and HER2 in MCF-7RALT tumors. We have previously shown an increase in HER2/neu mRNA expression in tamoxifen-resistant MCF-7 breast tumors compared with tamoxifen-sensitive tumors (8), which may play a part in the resistance process. We measured the levels of EGFR and HER2/neu mRNAs in the MCF-7RALT tumors by real-time PCR. We found a 2.5-fold increase in EGFR mRNA in MCF-7RALT compared with the parental MCF-7E2 tumors (Fig. 2A). Interestingly, fulvestrant-treated MCF-7RALT tumors expressed >20-fold EGFR mRNA compared with the parental MCF-7E2 tumors (Fig. 2A). Raloxifene-treated MCF-7RALT tumors expressed 2.5-fold HER2/neu mRNA compared with parental raloxifene-treated MCF-7E2 tumors (Fig. 2B).

Effects of trastuzumab and gefitinib on the growth of MCF-7RALT breast tumors. In order to determine if this increase of EGFR and HER2/neu mRNAs was of any functional significance in terms of growth in the MCF-7RALT breast tumors, we determined if raloxifene-stimulated tumor growth could be blocked by trastuzumab, a monoclonal antibody specifically targeted against the ectodomain of HER2/neu, or by gefitinib, a small molecule, which targets the tyrosine kinase domain of EGFR. Trastuzumab alone (P < 0.01), or when given with gefitinib (P < 0.001) effectively blocked raloxifene-stimulated MCF-7RALT breast tumor growth by almost 50% compared with animals treated with raloxifene alone (Fig. 3). In contrast, gefitinib alone was unable to block raloxifene-stimulated growth of MCF-7RALT tumors (Fig. 3).

In order to determine if trastuzumab and/or gefitinib were acting effectively on their respective targets, we measured the levels of EGFR and HER2/neu proteins by Western blot in the MCF-7RALT tumors from each group from the previous growth study (Fig. 3). We found a reduction in both tyrosine-phosphorylated and total HER2 protein in the tumors from mice treated with raloxifene plus trastuzumab (P < 0.01), and with raloxifene in combination with both trastuzumab and gefitinib (P < 0.01) (Fig. 4A), indicating that trastuzumab was exerting its inhibitory effects on tumor growth via HER2/neu. In contrast, we did not see a decrease in phosphorylated EGFR levels in tumors from mice treated with raloxifene and gefitinib, either alone or when given with trastuzumab (Fig. 4A).

We then examined the effects of raloxifene plus trastuzumab and/or gefitinib on the levels of prosurvival, antiapoptotic proteins in MCF-7RALT tumors. Levels of phosphorylated Akt were increased in tumors exposed to raloxifene (P < 0.01), compared with untreated tumors (Fig. 4A). Levels of phosphorylated Akt were decreased in tumors exposed to raloxifene in combination with trastuzumab (P < 0.001), and to lesser degree, in combination with trastuzumab and gefitinib (P < 0.01; Fig. 4A). Levels of total Akt were similar in all treatment groups (Fig. 4A). In order to determine if this decrease in

![Fig. 4.](image-url)

**A.** Protein was extracted and Western blots were done as described in Materials and Methods from harvested MCF-7RALT tumors after 7 weeks of treatment (Fig. 3). Western blots were probed with antibodies against phosphorylated EGFR (HER1), phosphorylated HER2/neu, total HER2, and phosphorylated and total Akt. β-Actin was measured as a loading control (left). Percentage of phospho-HER2/neu and phospho-Akt proteins were calculated by first determining the density of each protein band from three independent experiments using densitometry and dividing phosphoprotein by total protein and multiplying the product by 100 (right); *, statistical significance of the RAL-treated group to (C) control; **, statistical significance of the RAL + gef (gefitinib) or the RAL + g (gefitinib) + t (trastuzumab) treated group to the RAL group. B. Western blots were probed with antibodies against XIAP, GSK3, cyclin D1, and caspase-7 with β-actin as a loading control.
alone and this basal level of activity was further increased to 10-fold higher ERE-luciferase activity with vehicle and ERα activity is elevated in MCF-7RALT tumors compared with the parental MCF-7E2 tumors (Fig. 5B). In addition, MCF-7RALT tumors treated with vehicle, E2, or E2 + RAL and MCF-7RALT tumors treated with vehicle control, E2, or RAL. β-Actin was measured as a loading control.

Expression and function of ERα in raloxifene-stimulated tumors. A recent report has shown that there is crosstalk between ERα, HER2/neu, and AIB1 in tamoxifen-resistance (16). Therefore, we investigated both the function and expression of ERα and AIB1 in MCF-7RALT tumors. First, the endogenous activity of ERα was measured by expanding the MCF-7RALT tumor cells in vitro that were derived from the tumors developed in vivo and performing an ERE-luciferase assay. The results showed that MCF-7RALT tumor cells had 10-fold higher ERE-luciferase activity with vehicle alone and this basal level of activity was further increased to ~20-fold with the overexpression of AIB1 (Fig. 5A). E2 induced ERE-luciferase activity 3-fold in the parental MCF-7E2 tumor cells and 2-fold in the MCF-7RALT tumor cells versus vehicle and overexpression of AIB1 enhanced this activity by 6-fold in the MCF-7E2, and by 2.5-fold in the MCF-7RALT (Fig. 5A). Fulvestrant treatment, either alone or in combination with E2, abolished the ERE-luciferase activities in both MCF-7E2 and MCF-7RALT tumor cells (Fig. 5A). The ERE-luciferase results show that endogenous ERα activity is elevated in MCF-7RALT tumors compared with the parental MCF-7E2 tumors which might explain why control tumors grew slowly, E2 further increased ERα activity and robustly stimulated growth, and fulvestrant completely blocked ERα activity and inhibited MCF-7RALT tumor growth in vivo (Fig. 1B and C).

In order to determine if the elevated ERα activity in the MCF-7RALT tumor cells were due to altered protein expression of ERα and/or its coactivator, AIB1, Western blots were done. E2 decreased both AIB1 and ERα proteins dramatically in the parental MCF-7E2 tumors compared with control (Fig. 5B). Raloxifene partially reversed the effects of E2 by elevating the protein levels of AIB1 and ERα in MCF-7E2 tumors (Fig. 5B). Interestingly, AIB1 protein levels were decreased whereas ERα protein expression was increased in control MCF-7RALT tumors compared with the parental MCF-7E2 tumors (Fig. 5B). Treatment with E2 had little effect on AIB1 protein levels but decreased expression of ERα protein dramatically in the MCF-7RALT tumors (Fig. 5B). In addition, MCF-7RALT tumors treated with raloxifene expressed modestly higher AIB1 protein compared with the raloxifene-treated MCF-7E2 tumors (Fig. 5B). These data indicate that the elevated ERα activity and increased growth rate of MCF-7RALT control and E2-stimulated tumors could be due to the increased expression of ERα protein.

Discussion

In order to investigate the effect of long-term exposure to raloxifene on breast cancer and to further elucidate the mechanisms of SERM resistance, we have developed a raloxifene-resistant MCF-7 model in vivo. These raloxifene-resistant tumors overexpress ERα, and have increased expression of EGFR and HER2/neu compared with the parental SERM-sensitive MCF-7E2 breast tumors. Despite the fact that these raloxifene-resistant tumors do not have HER2/neu gene amplification (data not shown), trastuzumab inhibited their growth by 50% (Fig. 3) through a decrease of prosurvival/
proliferative proteins, HER2/neu, phosho-Akt, phospho-GSK3β, cyclin D1, and XIAP, and the simultaneous increase in the proapoptotic protein, caspase-7 (Fig. 4). In addition, transfection of AIB1 into raloxifene-resistant MCF-7 breast cancer cells that overexpress ERα protein supersensitized the cells to E2 with regard to ERα activity (Fig. 5A), explaining why MCF-7RALT tumors were robustly stimulated to grow in response to E2 (Fig. 1C). Taken together, these data suggest that crosstalk between multiple signaling pathways including HER2/neu, AIB1, and ERα could contribute to the development of acquired resistance to raloxifene.

We have previously described three phases of tamoxifen-resistance, based on whether E2 has agonist or antagonist properties on SERM-resistant tumor growth (3–6). The agonist/antagonistic effects of E2 seem to depend on length of exposure to tamoxifen. With short-term tamoxifen exposure, E2 acts as an agonist, which we have termed phase I resistance (3). However, we noted that after prolonged exposure to tamoxifen (5 years or more), E2 acts as an antagonist and inhibits tumor growth (4). Similar effects occur with E2 inducing apoptosis following long-term deprivation of MCF-7 cells (35, 36). Tumors exhibiting phase III resistance to SERMs grow in a hormone-independent manner, in which neither SERMs nor fulvestrant inhibit growth, but as in phase II resistance E2 again inhibits tumor growth (6). The raloxifene-resistant tumors described in this article clearly exhibit phase I resistance (3), as both SERMs and E2 act as agonists (3). We have previously reported that long-term exposure of MCF-7 cells to raloxifene in vitro, in the absence of E2, results in a resistant phenotype (37). Interestingly, this raloxifene-resistant model (37) exhibits phase II resistance in vivo (4, 5), as both raloxifene and tamoxifen stimulate growth, whereas E2 inhibits growth in vitro and in vivo. It is possible that longer exposure to raloxifene in the current model will, similar to the tamoxifen-resistant tumors, eventually result in phase II resistance (4).

The expression of EGFR and HER2/neu receptors is increased in raloxifene-resistant tumors, in much the same way as we have found with tamoxifen-resistant tumors (8). There is an increase in EGFR and HER2 mRNA in MCF-7RALT tumors compared with MCF-7E2 tumors, suggesting that EGFR-driven pathways may play an important role in raloxifene-resistant tumors. Although the increase in HER2 protein expression would be considered only 1+ or 2+ by immunohistochemistry (data not shown), and HER2 gene amplification was not found in the raloxifene-resistant tumors by fluorescence in situ hybridization (data not shown), trastuzumab effectively blocked the growth-stimulatory effects of raloxifene. Consequently, trastuzumab acted through HER2/neu, as levels of phosphorylated and total HER2/neu were decreased in tumors (Fig. 4A). We also showed that the inhibition of raloxifene-resistant tumor growth by trastuzumab was due to a decrease in prosurvival factors including phospho-Akt, XIAP, phospho-GSK3β, and cyclin D1, and the simultaneous increase in the proapoptotic enzyme, caspase-7 (Fig. 4A and B). These data support the re-testing of metastatic breast cancers for HER2/neu in patients who have been treated with a SERM, and suggest a possible role for trastuzumab in breast cancers with overexpression of active HER2/neu, in the absence of gene amplification.

Osborne and coworkers (16) have previously proposed that increased expression of both AIB1 and HER2/neu is necessary for breast tumors to exhibit de novo tamoxifen-resistance. In the current study, we did not find a large increase in levels of AIB1 protein in MCF-7RALT tumors. However, expression of ERα protein was dramatically elevated in the MCF-7RALT tumors compared with the parental MCF-7E2 tumors (Fig. 5B). More importantly, the results showed that endogenous activity of ERα was elevated in MCF-7RALT tumors and overexpression of AIB1 increased this activity further in response to vehicle or E2 (Fig. 5A), suggesting that the growth hypersensitivity of MCF-7RALT tumors to vehicle or E2 could be due to the increased expression and activity of ERα. This hypothesis was confirmed by treatment with the selective ER down-regulator, fulvestrant. Fulvestrant completely inhibited the growth of MCF-7RALT tumors (Fig. 1B and C) and ERα activity in cells derived from MCF-7RALT tumors (Fig. 5A). Interestingly, fulvestrant increased the expression of EGFR mRNA by almost 4-fold over controls in MCF-7RALT tumors (Fig. 2A). The functional significance of this large increase in EGFR mRNA is not yet clear as gefitinib, the small-molecule inhibitor of active EGFR, had little effect on the growth of MCF-7RALT tumors (Fig. 3). However, previous studies have shown that the development of resistance to fulvestrant could be due to increased expression and activity of EGFR.5 Thus, it is possible that long-term treatment of MCF-7RALT tumors with fulvestrant could lead to the cross-resistance to fulvestrant through an EGFR-regulated pathway. This hypothesis is currently under investigation.

We have previously proposed that a triumvirate of ERα, AIB1, and HER2/neu must interact to explain tamoxifen-resistance in breast cancers (17). Our current data supports this hypothesis to explain raloxifene-resistant breast cancer growth. The shift from SERM-sensitive to SERM-resistant growth seems to involve an increase in the expression of ERα, HER2/neu, and possibly AIB1. Therefore, inhibition of SERM-resistant tumor growth could potentially be accomplished by several different approaches: by removing the SERM ligand, by destroying ERα with fulvestrant, or by blocking HER2/neu or EGFR-driven pathways, with either trastuzumab or EGFR tyrosine kinase inhibitors. Our data support each of these potential strategic therapeutic approaches, except perhaps the use of gefitinib. Clinical application of these principles is currently obtaining mixed successes. It is true that tamoxifen-withdrawal responses have been described in patients with tamoxifen-refractory metastatic breast cancer (38). Fulvestrant or aromatase inhibitors have been shown clinically to be an effective treatment for tamoxifen-refractory breast cancers (39, 40). However, the combination of the aromatase inhibitor, letrozole and trastuzumab, did not seem to result in improved response rates, in patients with ER-positive, HER2-positive, tamoxifen-refractory breast cancer, compared with historical trials, in which either agent was used alone in this setting (41). Likewise, trials examining the use of EGFR tyrosine kinase inhibitors in metastatic breast cancer have produced disappointing results (42–44). Interestingly, however, a small phase II trial (42) showed responses in two-thirds of patients with hormone-refractory metastatic breast cancer treated with the single agent gefitinib.

Previous studies (43, 44) have noted that breast cancers deprived of E2 for long periods develop supersensitivity to E2-
showed that E₂ robustly stimulated the growth of our raloxifene-resistant tumors in vivo (Fig. 1C), and that ERE activity was increased in response to E₂ in the raloxifene-resistant cells in vitro (Fig. 5A). These data suggest that breast cancers resistant to SERMs may become supersensitive to a patient's endogenous E₂, and raise the possibility that breast cancers in patients stopping SERMs may experience accelerated tumor growth, once the patient's own E₂ interacts with tumor ERs. This concept mandates the use of immediate second-line therapy. We noted that there was modest growth in our untreated raloxifene-resistant tumors in vivo, but that the basal activity of ERE in the raloxifene-resistant cells in vitro was high (Fig. 5B). The raloxifene-resistant tumor cells were very responsive to E₂ in vitro, with enhanced activation of the ERE reporter with or without supplemental AIB1. However, fulvestrant completely blocked the effects of E₂ on raloxifene-stimulated growth in vivo (Fig. 1B and C), and inhibited E₂-induced ERE transcriptional activity in vitro (Fig. 5A). Taken together, these data suggest that fulvestrant may be a better second-line therapy choice, in patients with SERM-resistant breast cancers, than an aromatase inhibitor, that does not remove all endogenous estrogen.

Previous investigators have shown that the growth of MCF-7 breast cancer cells transfected with HER2/neu can be blocked, and that tamoxifen resistance can be delayed or reversed by gefitinib in vivo (20, 46, 47). We were, therefore, surprised that gefitinib was unable to block the growth effects of raloxifene in a model of natural drug resistance. EGFR and HER2/neu mRNAs were increased in raloxifene-resistant tumors, but our results may reflect the sophistication of supplemental survival systems during the development of SERM resistance, by a process of natural selection. Several points of clinical importance arise from our findings. First, it would seem prudent to measure the expression of both HER2/neu, along with ERs, in patients developing breast tumors, whereas being treated with SERMs, as this may allow the use of trastuzumab, which has been shown to improve outcome in both early stage (48) and metastatic breast cancers (49). Second, because the raloxifene-resistant tumors express high levels of ERs, grow robustly with E₂ and modestly in untreated animals, but are growth-inhibited by fulvestrant, we feel that the ER down-regulator may be a better choice than an aromatase inhibitor in patients with SERM-resistant breast cancers. Lastly, it would not be unreasonable to consider a trial of trastuzumab in patients developing breast cancers while on raloxifene, if they exhibit even a low level of HER2/neu expression but high activity.

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Development and Therapeutic Options for the Treatment of Raloxifene-Stimulated Breast Cancer in Athymic Mice

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