[CANCER RESEARCH 60, 5887–5894, October 15, 2000]

Inhibition of HER2/*neu* (*erb*B-2) and Mitogen-activated Protein Kinases Enhances Tamoxifen Action against HER2-overexpressing, Tamoxifen-resistant Breast Cancer Cells[1]

Hirokazu Kurokawa, Anne E. G. Lenferink, Jean F. Simpson, Paul I. Pisacane, Mark X. Sliwkowski, James T. Forbes, and Carlos L. Arteaga[2]

Departments of Medicine [H. K., A. E. G. L., J. T. F., C. L. A.], Cell Biology [C. L. A.], and Pathology [J. F. S., J. T. F.], Vanderbilt University School of Medicine, Department of Veteran Affairs Medical Center [C. L. A.] and Vanderbilt-Ingram Cancer Center [J. F. S., C. L. A.], Nashville, Tennessee 37232, and Genentech, Inc. South San Francisco, California 94080 [P. I. P., M. X. S.]

ABSTRACT

HER2/*neu* (*erb*B-2) overexpression has been causally associated with tamoxifen resistance in human breast cancer cells. Forced expression of HER2 in MCF-7 breast cancer cells resulted in mitogen-activated protein kinase (MAPK) hyperactivity and tamoxifen resistance. Inhibition of HER2 and MAPKs with AG1478 and U0126, respectively, as well as dominant-negative MEK-1/2 constructs restored the inhibitory effect of tamoxifen on estrogen receptor (ER)-mediated transcription and cell proliferation. Both AG1478 and U0126 also restored the tamoxifen-mediated association of ER with nuclear receptor corepressor (N-CoR) in the antiestrogen-resistant MCF-7 cells. Treatment with a combination of tamoxifen and a HER2 kinase inhibitor reduced tumor MAPK activity and markedly prevented growth of HER2-overexpressing MCF-7 xenografts in athymic mice. Thus, blockade of HER2 and MAPK signaling may enhance tamoxifen action and abrogate antiestrogen resistance in human breast cancer.

INTRODUCTION

Estrogen-dependent breast cancer usually progresses from an antiestrogen-sensitive to an antiestrogen-resistant state. Several molecular and cellular mechanisms have been proposed as contributors to the acquisition of this resistant phenotype including the (infrequent) loss of ER,[3] the selection of ER mutants, alterations in the intracellular pharmacology and/or binding of the antiestrogen tamoxifen to breast tumor cells, ligand-independent ER-mediated transcription, and perturbation of the interaction of ER with corepressors of transcription, among others (reviewed in Refs. 1–3). We have studied the role of HER2/*neu* (*erb*B-2) signaling in antiestrogen resistance. The HER2 receptor is the protein product of the *HER2* proto-oncogene and a member of the EGFR (HER1) family of transmembrane tyrosine kinases, which also includes HER3 and HER4. Upon binding of ligands to the EGFR, HER3, or HER4, HER2 is recruited as the preferred partner of these ligand-bound receptors into active, phosphorylated heterodimeric complexes that activate several signaling pathways involved in the proliferation and survival of tumor cells (Refs. 4 and 5 and Refs. therein). HER2 can transform normal mammary epithelial cells and is overexpressed in a cohort of breast tumors, where it is associated with a more virulent behavior and poor patient prognosis (5).

Multiple lines of experimental evidence suggest that overexpression of HER2 confers antiestrogen resistance to breast tumor cells. MCF-7 human breast cancer cells transfected with either a full-length HER2 cDNA or with ectopic heregulin-β1, the HER3/4 ligand that activates HER2, lose sensitivity to tamoxifen or estrogen dependence (6–8). Several clinical studies have shown that tumors with high HER2 expression and/or with high circulating levels of the HER2 ectodomain exhibit a statistically lower clinical response rate and/or shorter durations of response after antiestrogen therapy (9–17), further suggesting an association between high tumor levels of the proto-oncogene and resistance to endocrine therapy. Two studies, however, failed to show a reduced response to tamoxifen in HER2-overexpressing tumors (18, 19). These discrepancies may be explained by the variable number of patients in these studies, different antiestrogen therapies used, variable methods to assess HER2 overexpression, among others.

The mechanisms by which HER2 potentially mediates tamoxifen resistance are unclear. However, HER2 overexpression results in activation of the Ras/MAPK signaling pathway in breast tumor cell lines and carcinomas (4, 20). MAPK has been shown to phosphorylate Ser-118 in the ER, leading to ligand-independent ER activation with loss of the inhibitory effect of tamoxifen on ER-mediated transcription (21, 22), providing a viable mechanism to explain the association of HER2 with tamoxifen resistance. Finally, an antibody against the ectodomain of HER2 increased the inhibitory effect of tamoxifen against HER2-overexpressing BT-474 breast tumor cells in culture (23). Therefore, we have studied the effect of blocking HER2 and MAPK in antiestrogen-resistant breast tumor cells. Interruption of these signaling pathways with small molecule inhibitors or dominant-negative mutants of MAPK enhanced the inhibitory effect of tamoxifen on both ER-mediated transcription *in vitro* and on tumor cell proliferation *in vitro* and *in vivo*. Taken together, our results support a role for MAPK in HER2-induced tamoxifen resistance in ER-positive breast tumor cells, thus providing a mechanistic target for future research and treatment approaches.

MATERIALS AND METHODS

Cell Lines and Inhibitors. The human breast carcinoma cell lines MCF-7 and BT-474 were obtained from the American Type Culture Collection and maintained in IMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (Intergen, Purchase, NY). MCF-7 cells stably transfected with full-length HER2 cDNA (MCF-7/HER2–18) or control vector (MCF-7/neo) were provided by C. K. Osborne (Baylor College of Medicine, Houston, TX) and have been described previously (6). 17β-Estradiol and 4-OH tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO). The MEK-1/MEK-2 inhibitor U0126 was from Calbiochem (San Diego, CA). The quinaz-

Received 5/22/00; accepted 8/23/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by NIH Grant R01 CA80195, Department of Defense Breast Cancer Program Grant DAMD17-98-1-8262, a Clinical Investigator Award from the Department of Veteran Affairs (all to C. L. A.), and Vanderbilt-Ingram Cancer Center Support Grant CA68485. A. E. G. L. is the recipient of a Susan G. Komen Breast Cancer Foundation Fellowship Award.

[2] To whom requests for reprints should be addressed, at Division of Oncology/Vanderbilt University Medical Center, 22nd Avenue South, 1956 TVC, Nashville, TN 37232-5536. Phone: (615) 936-3524 or (615) 936-1919; Fax: (615) 936-1790; E-mail: carlos.arteaga@mcmail.vanderbilt.edu.

[3] The abbreviations used are: ER, estrogen receptor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; ERE, estrogen response element; N-CoR, nuclear receptor corepressor; P-Tyr, phosphotyrosine; BrdUrd, 8-bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PMSF, phenylmethylsulfonyl fluoride; FAM, 6-carboxy-fluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.

oline EGFR/HER2 kinase inhibitor AG1478 was provided by J. Cherrington (Sugen, Inc., Redwood City, CA).

Colony-forming Assay. Growth effects of the different inhibitors were tested in an anchorage-independent soft agarose colony-forming assay. Tumor cells were plated at a density of 3×10^4 cells/35-mm dish in IMEM, 10% FCS, 0.8% agarose, 10 mM HEPES in the absence or presence of 4-OH tamoxifen, U0126, and AG1478, either alone or in combination. Dishes were incubated in a humidified CO_2 incubator at 37°C and colonies measuring ≥ 70 μm in diameter were counted after 7 days using an OMNICON 3800 Tumor Colony Analyzer (Biologicals, Gainesville, VA).

Plasmids and Luciferase ER Reporter Assays. The pGLB-MERE and pGLB-MNON plasmids (24) were provided by D. El-Ashry (Georgetown University, Washington, DC). Each plasmid contains either a double consensus ERE (MERE) or the same sequence with the ERE palindromes scrambled (MNON) into the *Hin*dIII site of pGLB (Promega Corp., Madison, WI). The dominant-negative MEK-1 (pREP4-K97A MEK1) and dominant-negative MEK-2 (pREP4-K101A MEK2) plasmids (25) were provided by J. T. Holt (Vanderbilt University, Nashville, TN). MCF-7/HER2–18 and MCF-7/neo cells in 24-well plates were transiently transfected with 0.25 μg/well pGLB-MERE or pGLB-MNON using 2.5 μl/well Lipofectamine reagent (Life Technologies, Inc.) in phenol red-free Opti-MEM I reduced serum medium (Life Technologies, Inc.) for 8 h. In some cases, 0.25 μg/well pREP4-K97A MEK1, pREP4-K101A MEK2, or pREP4 plasmids (Invitrogen, Carlsbad, CA) were cotransfected with the ERE-containing plasmids. After an additional 24-h incubation in phenol red-free IMEM supplemented with 10% charcoal-stripped FCS (Cocalico Biologicals, Reamstown, PA), each well was replenished with fresh medium containing 1 nM 17β-estradiol with or without 1 μM 4-OH tamoxifen in the presence or absence of kinase inhibitors for 16 h. Firefly luciferase and *Renilla reniformis* luciferase activities in cell lysates were determined using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Firefly luciferase activity was normalized to *R. reniformis* luciferase activity and expressed as relative luciferase units.

Immunoprecipitation and Immunoblot Analysis. After washes with ice-cold PBS, cells were lysed in EBC buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP40, 100 mM NaF, 200 μM Na_3VO_4, 1 mM PMSF, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin] for 20 min at 4°C. For analysis of ER and N-CoR, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% NP40, 0.2% Sarcosyl, 0.4 M NaCl, 20 mM NaF, 100 μM Na_3VO_4, 10 mM sodium molybdate, 1 mM PMSF, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin as described previously (26). Cell lysates were cleared by centrifugation (14,000 rpm for 10 min at 4°C), and the protein content in the supernatants was determined by the BCA method (Pierce, Rockford, IL). Equal amounts of total protein were resolved by SDS-PAGE and then subjected to immunoblot analysis using polyclonal antibodies against erbB-2 (NeoMarkers, Freemont, CA), phospho-MAPK (Promega), total MAPK (New England BioLabs, Beverly MA), or N-CoR (Santa Cruz Biotechnology, Santa Cruz, CA), and an ERα monoclonal antibody (Novocastra laboratories, Burlingame, CA). In some cases, cell lysates were precipitated at 4°C with the erbB-2 antibody (10 μg/mg protein) and protein A-Sepharose CL-4B (Sigma). Immune complexes were then subjected to immunoblot analysis, as described, for HER2 or phosphotyrosine (P-Tyr; monoclonal antibody, Upstate Biotechnology, Lake Placid, NY). To detect an ER/N-CoR association, cell lysates were first precipitated with the N-CoR antibody (2.0 μg/mg protein) and protein G-Sepharose (Sigma), followed by immunoblot analysis for N-CoR and ER.

Chemical Cross-Linking. Chemical cross-linking in intact cells was performed as described previously (27). In brief, after a 1-h treatment with 1–10 μM AG1478 at 37°C, MCF-7/HER2–18 cells were washed with PBS and incubated in PBS containing 1 mM of the nonpermeable cross-linker bis(sulfosuccinimidyl) suberate (Pierce) for 20 min at 4°C. The reaction was terminated by adding 250 mM glycine for 5 min while rocking. Cells were then lysed in EBC buffer, and equivalent amounts of protein were resolved by 5–7% gradient SDS-PAGE, followed by immunoblot analyses for HER2 and P-Tyr.

***In Vitro* MEK Activity Assay.** MEK activity was assayed *in vitro* as described by Favata *et al.* (28). MEK-1 was precipitated overnight at 4°C from MCF-7/HER2–18 cell lysates (EBC buffer) with a MEK-1 monoclonal antibody (2.5 μg/mg protein; Transduction Laboratories, San Diego, CA) and protein A-Sepharose. The immune complexes were washed three times with EBC buffer and resuspended in 40 μl of kinase assay buffer [20 mM HEPES (pH 7.0), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 0.1 mg/ml BSA] containing 5 μg of GST-(K71A)ERK-1 (Upstate Biotechnology, eluted from agarose beads with 10 mM glutathione), 10 μM ATP, and 10 μCi of [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol; Amersham, Piscataway, NJ) at 25°C for 30 min. Kinase reactions were conducted in the presence or absence of exogenous U0126 or AG1478 and terminated by adding Laemmli sample buffer. Phosphorylated ERK-1 species were resolved by SDS-PAGE and visualized by autoradiography.

Real-Time Quantitative PCR. Gene expression was quantified using real-time quantitative PCR or TaqMan technique as described previously (29–31). Poly(A) RNAs from MCF-7, MCF-7/neo, and MCF-7/HER2–18 were prepared by Oligotex mRNA kit (Qiagen, Valencia, CA). The sequences of the primer/probe sets used for each analysis are: EGFR: F, 5′-TTCCTGTGGATCCA-GAGGA-3′; R, 5′-AGCGTAATCCCAAGGATGT-3′; and P, 5′-FAM-AGGACGGACCTCCATGCCTTTGAGAA-TAMRA-p-3′; HER2: F, 5′-TCTGGACGTGCCAGTGTGAA-3′; R, 5′-TGCTCCCTGAGGACACATCA-3′; and P, 5′-FAM- CAGAAGGCCAAGTCCGCAGAAGCC-TAMRA-p-3′; HER3: F, 5′-TTCTCTACTCTACCATTGCCCAAC-3′; R, 5′-CACCAC-TATCTCAGCATCTCGGTC-3′; and P, 5′-FAM-ACACCAACTCCAGC-CACGCTCTGC-TAMRA-p-3′; HER4: F, 5′-GAGATAACCAGCATTGAG-CACAAC-3′; R, 5′-AGAGGCAGGTAACGAAACTGATTA-3′; and P, 5′-FAM-CCTCTCCTTCCTGCGGTCTGTTCGA-TAMRA-p-3′, where F and R are the forward and reverse primers, respectively, and P is the fluorescent labeled probe. Ribosomal protein 19 (*RPL19*) was used as a housekeeping gene. Primer/probe sets for RPL19 are: F, 5′-ATGTATCACAGCCTGTACCTG-3′; R, 5′-TTCTTGGTCTCTTCCTCCTTG-3′; and P, 5′-FAM-AGGTCTAAGACCAAG-GAAGCACGCAA-TAMRA-p-3′. TaqMan analysis was performed in a standard 96-well plate format. Standard curves were constructed using 3.9–1000 ng of total mRNA prepared from cell lysates of T47D human breast cancer cells. Standard curves for RPL19 were constructed using 31.25–500 ng of total mRNA. Each dilution was run in duplicate. All samples were run in triplicate using 100 ng of mRNA for each reaction.

Xenograft Studies in Athymic Mice. Female athymic mice (Harlan Sprague Dawley, Madison, WI), 4–5 weeks of age, were supplemented with a 0.25-mg, 21-day release, 17β-estradiol pellet (Innovative Research, Toledo, OH) in the dorsal space and then injected s.c. at a distant site with 5×10^6 MCF-7/HER2–18 cells. Once tumors reached an approximate volume of 200 mm^3, 10 mice/group were randomly allocated to treatment with: (*a*) vehicle (DMSO) alone; (*b*) tamoxifen delivered as a 25-mg, 60-day release s.c. pellet (Innovative Research) plus DMSO; (*c*) AG1478 (50 mg/kg/day) given by i.p. injection; or (*d*) tamoxifen plus AG1478. Tumor diameters were serially measured with calipers and tumor volumes calculated by the formula: volume = width^2 $\times$ length/2. On day 30 after tumor cell inoculation (2 weeks of treatment), three mice/group were injected with the daily dose of AG1478 or DMSO, followed by i.p. injection with BrdUrd (50 mg/kg), and their tumors were harvested 2 h later. Tumors were homogenized using a Polytron homogenizer (Brinkmann, Westbury, NY) in TNE lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin]. After homogenization, NP40 was added (1%, v/v). Equivalent amounts of protein from the tumor lysates were then subjected to HER2, P-MAPK, and total MAPK immunoblot analysis or to precipitation with HER2 antibodies, followed by P-Tyr immunoblotting. For histological analysis, portions of the excised tumors were fixed overnight in Histoprep (Fisher Scientific, Pittsburgh, PA), sectioned, and stained with H&E. BrdUrd labeling of tumor cell nuclei was visualized by staining the sections with BrdUrd antibody (Biogenics, San Ramon, CA), as indicated by the Histo-mouse kit (Zymed Laboratories, Inc., South San Francisco, CA). Additional sections were used to stain for apoptotic cells using the DNA *in situ* nick end-labeling (TUNEL) method (Intergen).

RESULTS

HER2 Overexpression Inhibits Tamoxifen Action against ER-positive MCF-7 Cells. We have examined the mechanisms by which overexpression of HER2 confers antiestrogen resistance to breast tumor cells. We used MCF-7 cells stably transfected with a full-length

HER2 cDNA (MCF-7/HER2–18), which exhibit 45-fold higher levels of HER2 protein and acquire tamoxifen resistance relative to control cells (6). Confirming previous reports, the antiestrogen 4-OH tamoxifen inhibited anchorage-independent growth of MCF-7 control cells transfected with vector alone (MCF-7/neo) in a dose-dependent manner. In a colony-forming assay, control cells exhibited 80–83% inhibition of colony formation, whereas MCF-7 cells with high HER2 levels were modestly inhibited by 0.1–1 μM 4-OH tamoxifen (Fig. 1*A*). By immunoblot analysis, ER levels in MCF-7/HER2–18 cells and in the tamoxifen-sensitive MCF-7/neo and parental MCF-7 cell lines were similar (Fig. 1*B*). To rule whether transfection of HER2 had modified the levels of homologous EGF receptor-type kinases, we used a quantitative PCR (TaqMan; Refs. 29 and 30). Relative to a housekeeping ribosomal protein L19 (*RPL19*) gene, only the HER2 mRNA (13-fold), but not EGFR (HER1), HER3, or HER4, was found to be in excess in MCF-7/HER2–18 cells (Fig. 1*C*). Antiestrogen resistance was also documented by examining transcriptional responses of luciferase reporter constructs containing ERE. Addition of 1 nM estradiol activated ERE-luciferase transcription 18.7-fold in MCF-7/HER2–18 and 5.3-fold in MCF-7/neo cells above each control (Fig. 2). Because of different transfection efficiencies, a comparison of the constitutive ER-mediated transcription between both lines with high and low HER2 levels cannot be done. The difference in the level of estrogen-induced transcription did not translate into a difference in estrogen-stimulated cell proliferation in that both cell lines exhibited a similar 2–3-fold increase in cell number in response to estradiol in estrogen-free medium after 4 days (data not shown). Addition of 1 μM 4-OH tamoxifen suppressed estradiol-induced ERE-luciferase transcription 26% ($P = 0.28$) and 58% ($P < 0.001$) in MCF-7/HER2–18 and MCF-7/neo cells, respectively (Fig. 2). Of note, tamoxifen alone increased ERE-luciferase transcription 1.5-fold above basal in MCF-7/HER2–18 but not in control cells. These data indicate that both HER2-overexpressing and control MCF-7 cells exhibit wild-type ER function in response to exogenous ligand but a clear difference in sensitivity to tamoxifen in both growth and transcription assays.

Blockade of HER2 and MAPK Restores Transcriptional and Cellular Responses to Tamoxifen *in Vitro*. We next examined whether blocking HER2 signaling with pharmacological and genetic approaches would abrogate antiestrogen resistance. For this purpose, we used: (*a*) AG1478, a small molecule quinazoline that inhibits the EGFR and HER2 kinases with an *in vitro* IC_{50} of 3 nM and 1.4 μM, respectively (27)[4]; and (*b*) U0126, a noncompetitive inhibitor of the dual specificity MAP kinase kinases (MEK-1/MEK-2), the enzymes that activate MAPK (28). By immunoblot analysis, constitutively phosphorylated HER2 and active MAPK were only detectable in MCF-7/HER2–18 but not in MCF-7 and MCF-7/neo cells. 4-OH tamoxifen had no effect on phosphorylated HER2 or active MAPK. However, AG1478 eliminated both HER2 phosphorylation and active MAPK, whereas U0126 eliminated only active MAPK in MCF-7/HER2–18 without altering total HER2 or total MAPK protein levels (Fig. 3*A*). To explain the inhibitory effect of AG1478, a relatively specific EGFR kinase inhibitor, on HER2 phosphorylation, we examined whether AG1478 would interact with HER2 in MCF-7/HER2–18 cells. A brief 1-h incubation with 1–10 μM AG1478 induced the formation of HER2 dimers. At the higher dose of AG1478, these HER2 dimers lacked a P-Tyr signal (Fig. 3*B*), potentially explaining the inhibitory effect of AG1478 on HER2 phosphorylation shown in Fig. 3*A*. Finally, we examined the target specificity of the kinase inhibitors in an *in vitro* kinase assay using exogenous ERK-1 (p44/MAPK) as a substrate for MEK-1 immunoprecipitated from MCF-7/HER2–18 cells. U0126 but not AG1478 inhibited MEK-1 induced phosphorylation of ERK-1 *in vitro* (Fig. 3*C*), suggesting that the inhibitory effect of AG1478 on MAPK activity in intact cells (Fig. 3*A*) is not attributable to a nonspecific interaction of AG1478 with MEK-1.

We next determined whether inhibition of HER2 and MAPK would restore tamoxifen effects on ER-mediated transcription and cell

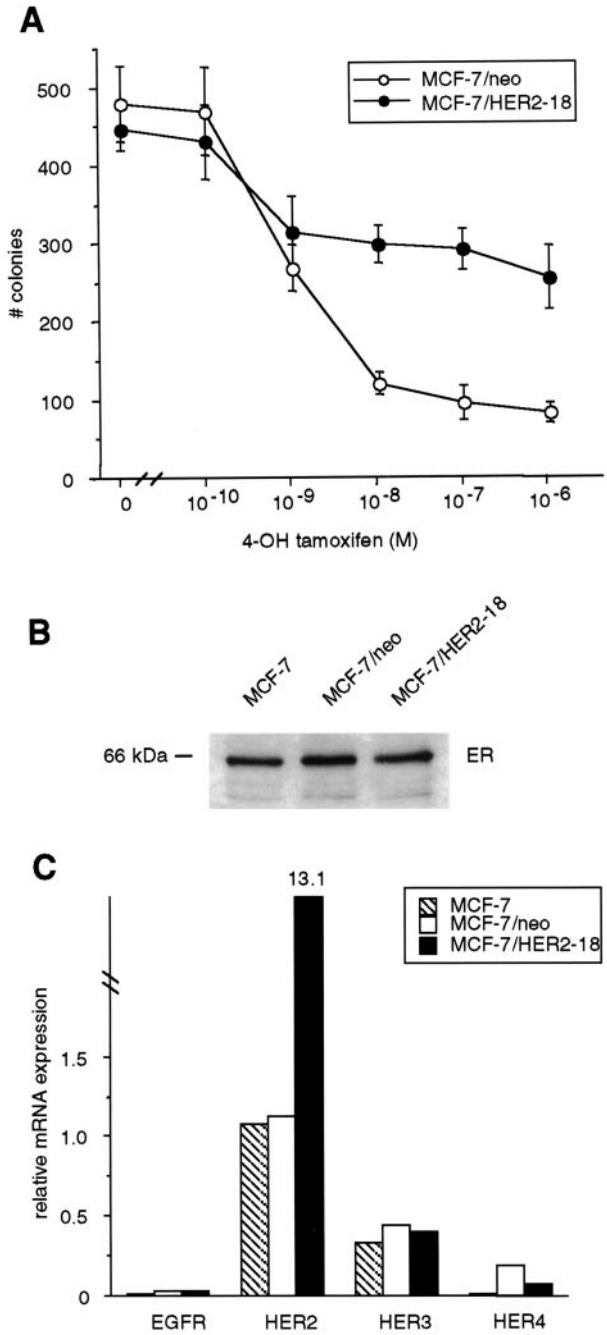

Fig. 1. Tamoxifen response and expression of ER and receptors of the EGF family. *A*, MCF-7/HER2–18 (●) and MCF-7/neo (○) cells were plated in 35-mm dishes in IMEM, 10% FCS, 0.8% agarose, and 10 mM HEPES in the presence or absence of the indicated concentrations of 4-OH tamoxifen. Colonies measuring ≥70 μm were counted 7 days later. Each data point represents the mean of triplicate dishes; *bars*, SE. *B*, MCF-7, MCF-7/neo, and MCF-7/HER2–18 cell lysates were prepared and tested for ER content by immunoblot analysis as described in "Materials and Methods." *C*, mRNAs of MCF-7 (▨), MCF-7/neo (□) and MCF-7/HER2–18 (■) were analyzed for EGFR, HER2, HER3, or HER4 expression by the quantitative RT-PCR technique as described in "Materials and Methods." Each signal was normalized with the signal by a control gene, *RPL19*, and expressed as relative mRNA expression.

[4] L. K. Shawver, Sugen, Inc., personal communication.

growth. 4-OH tamoxifen, AG1478, or U0126 did not appreciably inhibit estradiol-stimulated ERE-luciferase reporter activity in MCF-7/HER2–18 cells. However, both AG1478 and U0126 significantly enhanced the inhibitory effect of tamoxifen on ER-mediated luciferase expression in a dose-dependent fashion (Fig. 4*A*). To support that this enhancement of tamoxifen action was attributable to the interruption of MAPK activity, we used constructs encoding dominant-negative MEK-1 and MEK-2 (25). Relative to control vector, transiently transfected dominant-negative MEK-2 or the combination of dominant-negative MEK-2 and MEK-1 mutants significantly enhanced tamoxifen-mediated inhibition of ER-induced luciferase expression ($P < 0.05$; Fig. 4*B*), whereas the effect of dominant-negative MEK-1 approached statistical significance ($P = 0.1$; Fig. 4*B*). It has been proposed that tamoxifen blocks estrogen action by recruiting transcriptional corepressors to the ligand-binding domain in the ER (32). Hence, we examined the association of ER with the N-CoR in both MCF-7/neo and MCF-7/HER2–18 cells. ER and N-CoR content in these cells was similar. Tamoxifen induced a strong association of ER with N-CoR in the antiestrogen-sensitive MCF-7/neo cells, whereas this effect was markedly reduced in MCF-7/HER2–18 cells (Fig. 5). However, treatment of the tamoxifen-resistant cells with AG1478 or U0126 enhanced tamoxifen-mediated association of N-CoR with ER without any alterations in ER and N-CoR levels. These results are consistent with the transcriptional responses shown in Fig. 4.

Finally, we tested whether the transcription data correlated with tumor cell growth. 4-OH tamoxifen (0.1 μM) inhibited MCF-7/HER2–18 colony formation 40% compared with untreated controls. Addition of AG1478 or U0126 increased tamoxifen-mediated growth inhibition to similar levels (80% below control) to those seen in MCF-7/neo cells (Fig. 6*A*). Similar data were obtained with ER-positive BT-474 cells that exhibit gene amplification and constitutive phosphorylation of the HER2 receptor (33) with an IC_{50} to tamoxifen comparable with that in MCF-7/HER2–18 cells. In both cell lines, the synergistic inhibitory effect of AG1478 with tamoxifen appeared to be dose dependent. In BT-474 cells, however, the higher concentration of U0126 was markedly inhibitory *per se* (Fig. 6*B*). By immunoblot, neither AG1478 nor U1026 altered ER levels in BT-474 cells (not shown). Thus, in two HER2-overexpressing systems, blockade of HER2 and MAPK partially abrogates antiestrogen resistance.

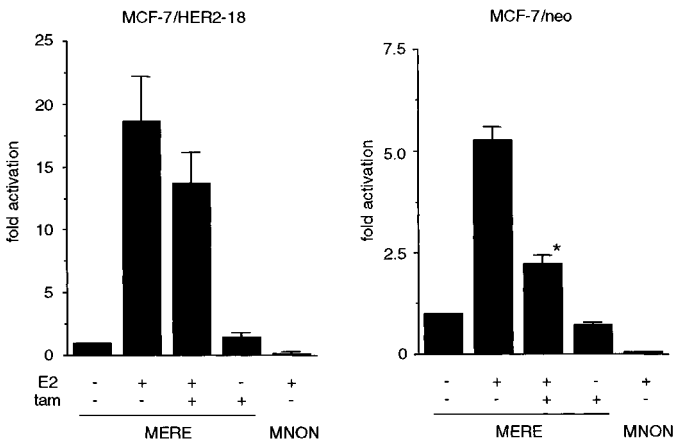

Fig. 2. Tamoxifen effect on ER-mediated transcription as a function of HER2 overexpression. MCF-7/HER2–18 and MCF-7/neo cells were transiently transfected with an ERE-containing luciferase reporter construct (*MERE*) or control vector (*MNON*) in estrogen-free medium, followed by a 16-h incubation in the presence or absence of 1 nM 17β-estradiol (*E2*) with or without 1 μM 4-OH tamoxifen (*tam*). Firefly luciferase activity was internally normalized by *Renilla* luciferase activity from a cotransfected pRL construct (Promega). Normalized luciferase activity from triplicate wells was expressed relative to the luciferase activity of each cell line in the absence of E2 and tamoxifen; *bars*, SE. *, $P < 0.001$, compared with E2 alone by Student's unpaired *t* test. These results were confirmed in three independent experiments.

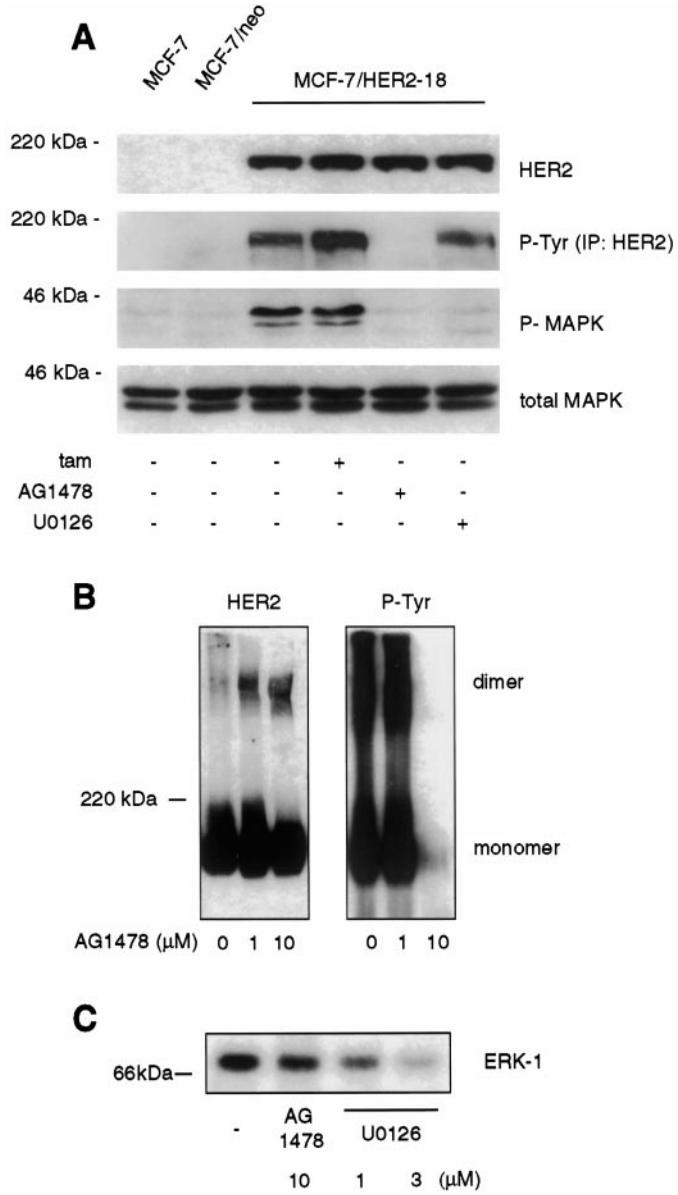

Fig. 3. Blockade of HER2 and MAPK activities by AG1478 or U0126. *A*, MCF-7, MCF-7/neo, and MCF-7/HER2–18 were lysed in EBC buffer and tested for HER2, P-MAPK, and total MAPK by immunoblot analyses. HER2 immunoprecipitates (*IP*) were analyzed by P-Tyr immunoblot. Where indicated, MCF-7/HER2–18 cells were treated for 24 h with 1 μM 4-OH tamoxifen (*tam*), 10 μM AG1478, or 3 μM U0126 prior to cell lysis. *B*, chemical cross-linking of intact MCF-7/HER2–18 cells. After a 1-h treatment with 1–10 μM AG1478, cell surface proteins were cross-linked with 1 mM bis (sulfosuccinimidyl) suberate dissolved in PBS as indicated in "Materials and Methods." After quenching the cross-linking reaction, cell lysates were prepared and subjected to immunoblot analysis of HER2 and P-Tyr. *C*, *in vitro* MEK activity assay. MEK-1 was precipitated from MCF-7/HER2–18 and incubated with GST-(K71A)ERK-1 and [γ-^{32}P]ATP at 25°C for 30 min in the presence of U0126 or AG1478 at the indicated concentrations. ERK-1 phosphorylated species were resolved by SDS-PAGE and visualized by autoradiography.

Inhibition of HER2 Signaling *in Vivo* Restores Tamoxifen Sensitivity. The effect of HER2 blockade with AG1478 on tamoxifen action was next tested against MCF-7/HER2–18 xenografts in athymic mice. Although estrogen responsive, these cells form tumors in the absence of added estradiol (6). To accelerate tumor establishment, mice were supplemented with 17β-estradiol delivered as a s.c. pellet. Once xenografts had reached a volume $\geq$200 mm^3, tumor-bearing mice were randomly allocated to no treatment or treatment with tamoxifen, AG-1478, or both. Tamoxifen minimally delayed tumor growth ($P = 0.23$), whereas AG1478 had no effect. However, the

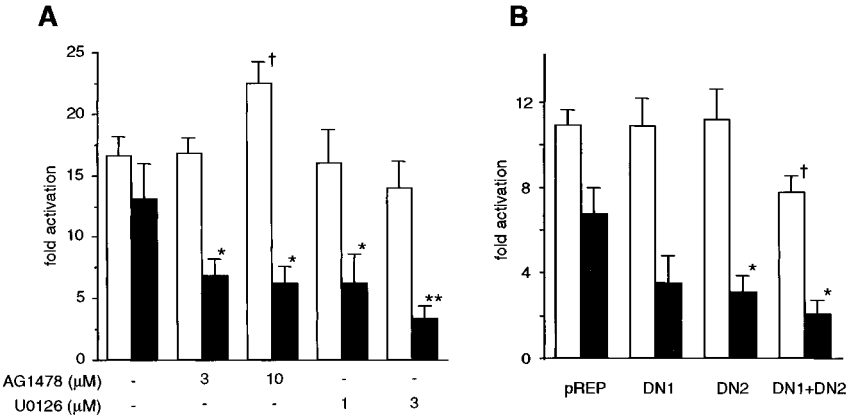

Fig. 4. HER2 and MAPK blockade facilitates antiestrogen-mediated inhibition of ERE-luciferase transcription. For both panels (*A* and *B*), MCF-7/HER2–18 cells were transiently transfected with an ERE-containing luciferase reporter construct, followed by a 16-h incubation with 1 nM 17β-estradiol in the absence (□) or presence (■) of 1 μM 4-OH tamoxifen. *A*, AG1478 and U0126 were added during this incubation period at the indicated concentrations. *B*, ERE-containing plasmid was cotransfected with dominant-negative MEK-1 (*DN1*), dominant-negative MEK-2 (*DN2*), or control vector (*pREP*). For both panels, firefly luciferase activity was internally normalized to *Renilla* luciferase as described in "Materials and Methods" and presented similar to Fig. 2 as fold-activation relative to normalized luciferase activity in the absence of estradiol (not shown). Each data point represents the mean relative luciferase activity from triplicate wells; *bars*, SE. *, $P < 0.05$; **, $P < 0.01$, compared with 4-OH tamoxifen (+); †, $P < 0.05$, compared with 4-OH tamoxifen (−) control, Student's unpaired *t* test. These results were confirmed in two additional experiments.

combination of tamoxifen and AG1478 almost completely arrested tumor growth relative to untreated controls ($P < 0.01$; Fig. 7*A*). To determine whether the effect of this combination was antiproliferative *versus* cytotoxic, three mice/group were pulsed with BrdUrd prior to tumor harvesting. Compared with control tumors, there was a significant reduction in the number of BrdUrd-labeled nuclei in the tumors treated with tamoxifen plus AG1478 ($P < 0.01$) but not in the other two experimental groups (Fig. 7*B*). Histologically, tumors from all groups were identical. To evaluate whether programmed cell death was occurring as a result of treatment, tumor sections were evaluated for the presence of apoptotic cells *in situ* as measured by TUNEL assay. A modest increase in TUNEL-positive tumor cells was only observed in the sections from xenografts treated with the combination compared with control tumor cells ($P = 0.06$; Fig. 7*B*). Finally, to validate our molecular targets *in vivo*, we examined whether HER2 signaling had been inhibited in control and treated tumors. A representative cohort is shown in Fig. 7*C*. Levels of HER2 and total MAPK were similar in tumors from all four experimental groups. However, phosphorylated HER2 and active MAPK were markedly reduced in the tumors treated with AG1478, suggesting that HER2 signaling had been disabled by AG1478 *in vivo*. Overall, these data suggest that interference of the HER2 signaling pathway enhances tamoxifen action against antiestrogen-resistant cells both *in vitro* and *in vivo*.

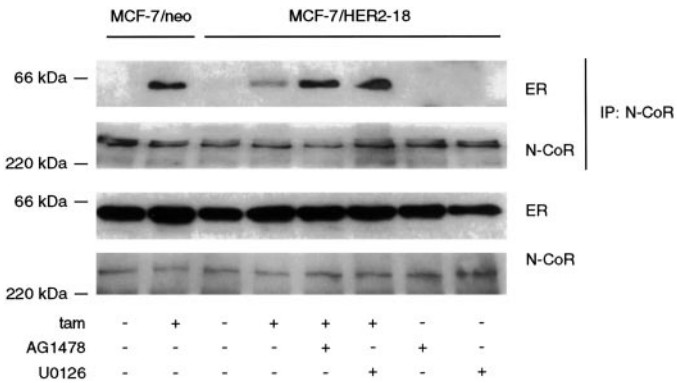

Fig. 5. Inhibition of HER2 or MAPK enhances tamoxifen-induced association of ER with N-CoR. MCF-7/neo and MCF-7/HER2–18 cell lysates were prepared from monolayers that had been treated or not with 1 μM 4-OH tamoxifen (*tam*) for 4 h. Cell lysates were tested for ER and N-CoR content by immunoblot analysis. To document the association of ER with N-CoR, in some cases N-CoR was first immunoprecipitated (*IP*), and immune complexes were subjected to ER or N-CoR immunoblot procedures (*top two rows*). Where indicated, MCF-7/HER2–18 cells were treated for 24 h with 10 μM AG1478 or 3 μM U0126 either alone or with 1 μM 4-OH tamoxifen for the last 4 h of the 24-h period. Data shown are representative of three independent experiments.

DISCUSSION

We have studied the role of HER2 signaling in antiestrogen resistance in an MCF-7 subline that overexpresses the *HER2* proto-oncogene. The cells exhibit constitutively active MAPK and an ~100-fold higher IC_{50} to tamoxifen compared with MCF-7 cells with a single copy of the *HER2* gene. In this model, ER content and function, as measured by estradiol-induced responses of ER reporters and cell proliferation, are maintained. These data suggest that the differences in tamoxifen response in this model are not attributable to differences in ER levels but to the potential interaction of signal transduction elements downstream from HER2 with the ER. To disengage HER2 function, we used the small molecule kinase inhibitor AG1478, which displays an IC_{50} against EGF-stimulated phosphorylation of an

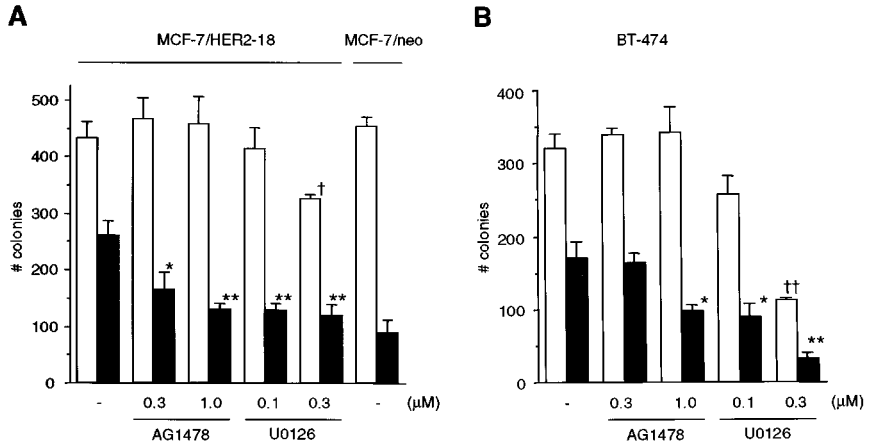

Fig. 6. AG1478 and U0126 enhance tamoxifen-mediated inhibition of tumor cell colony survival. MCF-7/HER2–18 (*A*) and BT-474 cells (*B*) were plated in a soft agarose colony-forming assay in the absence (□) or presence (■) of 0.1 μM 4-OH tamoxifen, with or without AG1478 or U0126 at the indicated concentrations as described in "Materials and Methods." *, $P < 0.05$; **, $P < 0.01$, compared with 4-OH tamoxifen (+); †, $P < 0.05$; ††, $P < 0.01$, compared with 4-OH tamoxifen (−) control, Student's unpaired *t* test. Each *column* represents the mean colony number of triplicate dishes; *bars*, SE. These results were reproduced in two independent experiments.

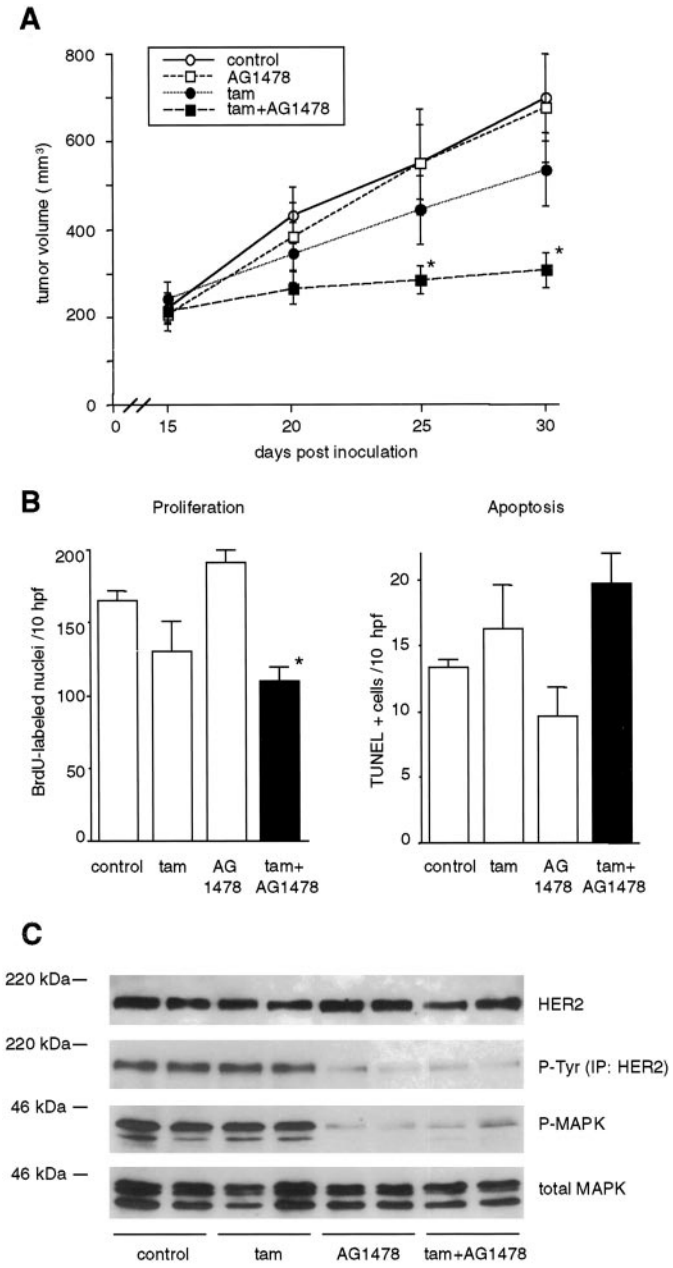

Fig. 7. Systemic administration of AG1478 inhibits MAPK and enhances tamoxifen action against MCF-7/HER2–18 xenografts. *A*, nude mice bearing ≥200 mm^3 s.c. tumors were allocated to treatment with DMSO daily (*control*, ○), tamoxifen delivered as a s.c. pellet plus DMSO (*tam*, ●), AG1478 given i.p. daily (□), or tamoxifen plus AG1478 (■) as indicated in "Materials and Methods." Each data point represents the mean tumor volume of 10 mice per group; *bars*, SE. ∗, $P < 0.01$, compared with DMSO-treated controls, Student's unpaired *t* test. *B*, assessment of tumor cell proliferation and apoptosis *in situ*. On day 30 after tumor cell inoculation (2 weeks of treatment), three mice were randomly selected from each experimental group and pulsed with BrdUrd prior to tumor harvesting. Tumor sections were subjected to immunohistochemical analysis with BrdUrd antibody or TUNEL assay as described in "Materials and Methods." Data are shown as the mean number of BrdUrd-positive nuclei or TUNEL-positive cells ($n = 3$) in 10 high-power microscopic fields (*hpf*); *bars*, SE. ∗, $P < 0.01$, compared with control group, Student's unpaired *t* test. *C*, on day 30 after tumor cell inoculation, tumors were snap-frozen and later homogenized in a buffer containing phosphatase and protease inhibitors as indicated in "Materials and Methods." Equivalent amounts of protein from the tumor lysates were subjected to HER2, P-MAPK, and total MAPK immunoblot analysis or to precipitation with HER2 antibodies, followed by P-Tyr immunoblot.

EGFR/HER2 chimeric receptor in NIH 3T3 cells of 1.4 μM.[4] AG1478 has been shown to induce the formation of inactive, unphosphorylated EGFR/HER2 heterodimers in tumor cells that naturally overexpress HER2 (27). In MCF-7/HER2–18 cells, AG1478 also induces inactive HER2 dimers (Fig. 3*B*), suggesting that it may interact directly with the HER2 kinase. By inducing the formation of inactive, unphosphorylated HER2 dimers, it can sequester the receptor from functional interactions with other HER receptor partners and, therefore, disrupt signaling. Treatment of intact cells with AG1478 eliminated active MAPK (Fig. 3*A*) but had no effect on MEK-1 activity *in vitro* (Fig. 3*C*), suggesting that the effect of the quinazoline on MAPK occurred as a result of the interruption of HER2 signaling upstream.

Several issues suggested the possibility that the EGFR kinase may have been involved in the antiestrogen-resistant phenotype of MCF-7/HER2–18 cells: (*a*) AG1478 has a predominant effect against the EGFR kinase (IC_{50}, 3 nM; Ref. 27); and (*b*) transfection of EGFR into hormone-dependent breast cancer cells also induces tamoxifen resistance (34, 35). Moreover, HER2 can prolong EGFR stability and both receptors contribute to transformation in a cooperative manner (4, 5, 36). However, we were unable to detect EGFR by immunoblot analysis of EGFR precipitates from MCF-7/HER2–18 cell lysates. In addition, the C225 antibody against the EGFR ectodomain (37) had no effect on colony survival of the HER2-transfected MCF-7 cells (data not shown). Moreover, EGFR mRNA levels, as measured by real-time quantitative PCR, were low in HER2-transfected and control cells (Fig. 1*C*). Hence, although we cannot rule out some contribution of the EGFR to tamoxifen resistance in our experimental system, we do not feel that this kinase is playing a major causal role. There are no published reports implicating the homologous family members HER3 and/or HER4 with loss of estrogen dependence or antiestrogen resistance in breast cancer cells. Nonetheless, the levels of these mRNAs were not altered by HER2 transfection into MCF-7 cells (Fig. 1*C*).

The following results implicate MAPK signaling as the mediator of antiestrogen resistance in HER2-overexpressing tumor cells: (*a*) overexpression of HER2 in MCF-7 cells results in both activation of MAPK and antiestrogen resistance; (*b*) inhibition of MAPK with U0126 enhances the ability of tamoxifen to inhibit both ER-mediated transcription and cell growth; (*c*) dominant-negative mutants of MEK-1 and MEK-2 also enhance the inhibitory effect of tamoxifen on ER-mediated transcription; and (*d*) AG1478 markedly reduces active MAPK in MCF-7/HER2–18 xenografts, and this reduction is temporally associated with tamoxifen-induced growth restraint of tumors *in vivo*. Neither HER2 overexpression (in MCF-7/HER2–18 cells) nor inhibition of HER2 and MAPK altered ER levels or reporter activity, suggesting that the restoration of tamoxifen action was independent of ER levels and function. In a more recent report, however, another HER2-transfected MCF-7 cell line (8) exhibited significant down-regulation of ER protein. Inhibition of MEK or MAPK restored ER expression and, presumably, sensitivity to antiestrogens (38). This result suggests that HER2 hyperactivity may subvert the antiestrogen response by multiple potential mechanisms.

Notably, treatment with AG1478 alone inhibited tumor MAPK but not tumor growth (Fig. 7). This result raises the intriguing possibility that estrogen-primed tumor growth is not dependent on MAPK *in vivo* and that, by inhibiting HER2, AG1478 is inhibiting other HER2-dependent signaling pathways that mediate antiestrogen resistance. This possibility will require further studies with MAPK inhibitors in combination with tamoxifen. In addition, tumors treated with AG1478 alone and in which P-MAPK was reduced (Fig. 7*C*) were harvested 2 h after treatment with AG1478. Thus, it is conceivable that the combination of tamoxifen plus AG1478 induced a more sustained down-regulation of MAPK function compared with treatment with the kinase inhibitor alone. This speculation requires further investigation beyond the scope of this report. Our *in vitro* studies on the role of MAPK would seem to be in some disagreement with a recent report by Lobenhofer *et al.* (39) in which estrogen-dependent cell proliferation and transcription were inhibited by 25 μM U0126 over a 24-h

period. It should be noted, however, that these concentrations were ~10-fold higher than those of U0126 that inhibited ERK-1 activity (Fig. 3*C*) and P-MAPK content (Fig. 3*A*), which enhanced the inhibitory effect of tamoxifen on ER reporter activity (Fig. 4*A*) in our study. Therefore, these discrepancies could be explained by the different concentrations of U0126 that were used.

MAPK has been shown to posttranslationally modify the ER by phosphorylating Ser-118. The evidence that p42/44 MAPK directly phosphorylates Ser-118 is based on the *in vitro* observation that activated MAPK phosphorylates the AF-1 domain in the NH_2 terminus of the ER but not a mutant in which Ser-118 was replaced by Ala (21, 22, 40). In some cell types, this mutation causes a reduction in estradiol-dependent transcriptional activation (21, 41). The robust activation of ERE-luciferase expression in MCF-7/HER2–18 cells (Fig. 2) would argue against a mutation in AF-1 in the HER2-overexpressing cells. In addition, we have not been able to detect phosphorylated ER bands in MCF-7/HER2–18 or BT-474 cells maintained in estrogen-containing medium, despite the use of optimal conditions to inhibit cellular phosphatases (42), implying that under conditions in which MAPK is hyperactive (Fig. 3), Ser phosphorylation may not be abundant in HER2-overexpressing tumor cells.

Inhibition of HER2 and MAPK also sensitized BT-474 human breast cancer cells to tamoxifen (Fig. 6). These cells exhibit *HER2* gene amplification, a constitutively active HER2 kinase, and at best a 50% growth inhibition in the presence of high (1 μM) concentrations of 4-OH tamoxifen. Preliminarily, we have also observed a modest synergistic inhibitory effect of tamoxifen and AG1478 in ER-positive MDA-361 human breast tumor cells, which exhibit HER2 overexpression (33) but no *HER2* gene amplification by fluorescent *in situ* hybridization analysis (data not shown). Hence, we cannot rule out that inhibition of HER2 signaling may also enhance tamoxifen action on hormone-dependent, tamoxifen-sensitive breast tumor cells that do not overexpress HER2, as suggested recently by Kunisue *et al.* (43) in studies using the pure antiestrogen ICI 182,780 and another humanized HER2 antibody. The exquisite sensitivity of wild-type and MCF-7/neo (control) cells to concentrations as low as 0.01 μM tamoxifen makes these cell lines unsuitable to address this important question. Be that as it may, the relevance of our results to breast tumor cells with normal levels of HER2 or more modest levels of HER2 overexpression will require further research.

It has been proposed that tamoxifen recruits transcriptional corepressors to the AF-2 region in the hormone-binding domain of the ER to block ER-mediated transcription (3, 32). Mitogens such as protein kinase A, dopamine, or EGF can decrease this antiestrogen-induced receptor-corepressor interaction and limit tamoxifen action (44–46). Thus, we examined whether HER2 signaling, which activates the same signaling pathways activated by EGF (4, 5), can also disrupt this interaction. Notably, tamoxifen-induced association of ER with N-CoR was reduced in HER2-overexpressing cells. Inhibition of HER2 and MAPK markedly increased the antiestrogen-mediated association of ER with N-CoR. This result implies that antiestrogen resistance in HER2-overexpressing tumor cells can, at least in part, be attributed to unrestrained MAPK signaling that alters the ER association with corepressors of transcription. The molecular mechanisms by which MAPK regulates these protein-protein associations require further study. Nonetheless, the data presented imply that: (*a*) MAPK may play a causal role in HER2-induced tamoxifen resistance in ER-positive breast tumor cells; and (*b*) that exogenous inhibitors of HER2 signal transduction can partially abrogate the antiestrogen-resistant phenotype, providing a testable therapeutic strategy in hormone-dependent human breast cancer.

ACKNOWLEDGMENTS

We thank Teresa C. Dugger, Mary Beth Carter, and Sandy Olson for outstanding technical assistance.

REFERENCES

1. Wiebe, V. J., Osborne, C. K., Fuqua, S. A. W., and DeGregorio, M. W. Tamoxifen resistance in breast cancer. Crit. Rev. Oncol. Hematol., *14:* 173–188, 1993.
2. Clarke, R., and Brünner, N. Acquired estrogen independence and antiestrogen resistance in breast cancer–estrogen receptor driven phenotypes? Trends Endocrinol. Metab., *7:* 25–35, 1996.
3. Takimoto, G. S., Graham, J. D., Jackson, T. A., Tung, L., Powell, R. L., Horwitz, L. D., and Horwitz, K. B. Tamoxifen resistant breast cancer: coregulators determine the direction of transcription by antagonist-occupied steroid receptors. J. Steroid Biochem. Mol. Biol., *69:* 45–50, 1999.
4. Tzahar, E., and Yarden, Y. The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. Biochim. Biophys. Acta, *1377:* M25–M37, 1998.
5. Riese, D. J., II, and Stern, D. F. Specificity within the EGF family/ErbB receptor family signaling network. Bioessays, *20:* 41–48, 1998.
6. Benz, C. C., Scott, G. K., Sarup, J. C., Johnson, R. M., Tripathy, D., Coronado, E., Shepard, H. M., and Osborne, C. K. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/*neu*. Breast Cancer Res. Treat., *24:* 85–95, 1992.
7. Pietras, R. J., Arboleda, J., Reese, D. M., Wongvipat, N., Pegram, M. D., Ramos, L., Gorman, C. M., Parker, M. G., Sliwkowski, M. X., and Slamon, D. J. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene, *10:* 2435–2446, 1995.
8. Liu, Y., El-Ashry, D., Chen, D., Ding, I. Y. F., and Kern, F. G. MCF-7 breast cancer cells overexpressing transfected c-*erb*B-2 have an *in vitro* growth advantage in estrogen-depleted conditions and reduced estrogen-dependence and tamoxifen-sensitivity *in vivo*. Breast Cancer Res. Treat., *34:* 97–117, 1995.
9. Harris, A. L., Nicholson, S., Sainsbury, J. R. C., Farndon, J., and Wright, C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. J. Steroid Biochem., *34:* 123–131, 1989.
10. Wright, C., Nicholson, S., Angus, B., Sainsbury, J. R., Farndon, J., Cairns, J., Harris, A. L., and Horne, C. H. Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. Br. J. Cancer, *65:* 118–121, 1992.
11. Borg, A., Baldetorp, B., Fernö, M., Killander, D., Olsson, H., Rydén, S., and Sigurdsson, H. *ERBB2* amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. Cancer Lett., *81:* 137–144, 1994.
12. Leitzel, K., Teramoto, Y., Konrad, K., Chinchilli, V. M., Volas, G., Grossberg, H., Harvey, H., Demers, L., and Lipton, A. Elevated serum c-*erb*B-2 antigen levels and decreased response to hormone therapy of breast cancer. J. Clin. Oncol., *13:* 1129–1135, 1995.
13. Carlomagno, C., Perrone, F., Gallo, C., De Laurentiis, M., Lauria, R., Morabito, A., Pettinato, G., Panico, L., D'Antonio, A., Bianco, A. R., and De Placido, S. c-*erb*B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. J. Clin. Oncol., *14:* 2702–2708, 1996.
14. Yamauchi, H., O'Neill, A., Gelman, R., Carney, W., Tenney, D. Y., Hosch, S., and Hayes, D. F. Prediction of response to antiestrogen therapy in advanced breast cancer patients by pretreatment circulating levels of extracellular domain of the HER-2/c-*neu* protein. J. Clin. Oncol., *15:* 2518–2525, 1997.
15. Berns, E. M., Foekens, J. A., van Staveren, I. L., van Putten, W. L., de Koning, H. Y., Portengen, H., and Klijn, J. G. Oncogene amplification and prognosis in breast cancer: relationship with systemic treatment. Gene (Amst.), *159:* 11–18, 1995.
16. Archer, S. G., Eliopoulos, A., Spandidos, D., Barnes, D., Ellis, I. O., Blamey, R. W., Nicholson, R. I., and Robertson, J. F. Expression of *ras* p21, p53 and c-*erb*B-2 in advanced breast cancer and response to first line hormonal therapy. Br. J. Cancer, *72:* 1259–1266, 1995.
17. Houston, S. J., Plunkett, T. A., Barnes, D. M., Smith, P., Rubens, R. D., and Miles, D. W. Overexpression of c-erbB2 is an independent marker of resistance to endocrine therapy in advanced breast cancer. Br. J. Cancer, *79:* 1220–1226, 1999.
18. Elledge, R. M., Green, S., Ciocca, D., Pugh, R., Allred, D. C., Clark, G. M., Hill, J., Ravdin, P., O'Sullivan, J., Martino, S., and Osborne, C. K. HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a Southwest Oncology Group Study. Clin. Cancer Res., *4:* 7–12, 1998.
19. Berry, D. A., Muss, H. B., Thor, A. D., Dressler, L., Liu, E. T., Broadwater, G., Budman, D. R., Henderson, I. C., Barcos, M., Hayes, D., and Norton, L. HER-2/*neu* and p53 expression *vs* tamoxifen resistance in estrogen-receptor-positive, node-positive breast cancer. J. Clin. Oncol., in press, 2000.
20. Janes, P. W., Daly, R. J., deFazio, A., and Sutherland, R. L. Activation of the Ras signalling pathway in human breast cancer cells overexpressing erbB-2. Oncogene, *9:* 3601–3608, 1994.
21. Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science (Washington DC), *270:* 1491–1494, 1995.
22. Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J., *15:* 2174–2183, 1996.

23. Witters, L. M., Kumar, R., Chinchilli, V. M., and Lipton, A. Enhanced anti-proliferative activity of the combination of tamoxifen plus HER-2-neu antibody. Breast Cancer Res. Treat., *42:* 1–5, 1997.
24. McLeskey, S. W., Zhang, L., El-Ashry, D., Trock, B. J., Lopez, C. A., Kharbanda, S., Tobias, C. A., Lorant, L. A., Hannum, R. S., Dickson, R. B., and Kern, F. G. Tamoxifen-resistant fibroblast growth factor-transfected MCF-7 cells are cross-resistant *in vivo* to the antiestrogen ICI 182,780 and two aromatase inhibitors. Clin. Cancer Res., *4:* 697–711, 1998.
25. Abbott, D. W., and Holt, J. T. Mitogen-activated protein kinase kinase 2 activation is essential for progression through the G2/M checkpoint arrest in cells exposed to ionizing radiation. J. Biol. Chem., *274:* 2732–2742, 1999.
26. Nawaz, Z., Lonard, D. M., Dennis, A. P., Smith, C. L., and O'Malley, B. W. Proteasome-dependent degradation of the human estrogen receptor. Proc. Natl. Acad. Sci. USA, *96:* 1858–1862, 1999.
27. Arteaga, C. L., Ramsey, T. T., Shawver, L. K., and Guyer, C. A. Unliganded epidermal growth factor receptor dimerization induced by direct interaction of quinazolines with the ATP binding site. J. Biol. Chem., *272:* 23247–23254, 1997.
28. Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem., *273:* 18623–18632, 1998.
29. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. Real time quantitative PCR. Genome Res., *6:* 986–994, 1996.
30. Gibson, U. E., Heid, C. A., and Williams, P. M. A novel method for real time quantitative RT-PCR. Genome Res., *6:* 995–1001, 1996.
31. Aguilar, Z., Akita, R. W., Finn, R. S., Ramos, B. L., Pegram, M. D., Kabbinavar, F. F., Pietras, R. J., Pisacane, P., Sliwkowski, M. X., and Slamon, D. J. Biologic effects of heregulin/*neu* differentiation factor on normal and malignant human breast and ovarian epithelial cells. Oncogene, *18:* 6050–6062, 1999.
32. Jackson, T. A., Richer, J. K., Bain, D. L., Takimoto, G. S., Tung, L., and Horwitz, K. B. The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. Mol. Endocrinol., *11:* 693–705, 1997.
33. Alimandi, M., Romano, A., Curia, M. C., Muraro, R., Fedi, P., Aaronson, S. A., Di Fiore, P. P., and Kraus, M. H. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene, *10:* 1813–1821, 1995.
34. Miller, D. L., El-Ashry, D., Cheville, A. L., Liu, Y., McLeskey, S. W., and Kern, F. G. Emergence of MCF-7 cells overexpressing a transfected epidermal growth factor receptor (EGFR) under estrogen-depleted conditions: evidence for a role of EGFR in breast cancer growth and progression. Cell Growth Differ., *5:* 1263–1274, 1994.
35. van Agthoven, T., van Agthoven, T. L., Portengen, H., Foekens, J. A., and Dorssers, L. C. Ectopic expression of epidermal growth factor receptors induces hormone independence in ZR-75-1 human breast cancer cells. Cancer Res., *52:* 5082–5088, 1992.
36. Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J., *16:* 1647–1655, 1997.
37. Goldstein, N. I., Prewett, M., Zuklys, K., Rockwell, P., and Mendelsohn, J. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. Clin. Cancer Res., *1:* 1311–1318, 1995.
38. Oh, A. S., Holloway, J. N., and El-Ashry, D. Increased MAPK activity in EGFR or erbB-2 overexpressing MCF-7 cells induces down-regulation of estrogen receptor (ER) expression. Proc. Am. Assoc. Cancer Res., *41:* 72, 2000.
39. Lobenhofer, E. K., Huper, G., Iglehart, J. D., and Marks, J. R. Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. Cell Growth Differ., *11:* 99–110, 2000.
40. Ali, S., Metzger, D., Bornert, J. M., and Chambon, P. Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. EMBO J., *12:* 1153–1160, 1993.
41. Joel, P. B., Smith, J., Sturgill, T. W., Fisher, T. L., Blenis, J., and Lannigan, D. A. $pp90^{rsk1}$ regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. Mol. Cell. Biol., *18:* 1978–1984, 1998.
42. Joel, P. B., Traish, A. M., and Lannigan, D. A. Estradiol-induced phosphorylation of serine 118 in the estrogen receptor is independent of p42/p44 mitogen-activated protein kinase. J. Biol. Chem., *273:* 13317–13323, 1998.
43. Kunisue, H., Kurebayashi, J., Otsuki, T., Tang, C. K., Kurosumi, M., Yamamoto, S., Tanaka, K., Doihara, H., Shimizu, N., and Sonoo, H. Anti-HER2 antibody enhances the growth inhibitory effect of anti-oestrogen on breast cancer cells expressing both oestrogen receptors and HER2. Br. J. Cancer, *82:* 46–51, 2000.
44. Fujimoto, N., and Katzenellenbogen, B. S. Alteration in the agonist/antagonist balance of antiestrogens by activation of protein kinase A signaling pathways in breast cancer cells: antiestrogen selectivity and promoter dependence. Mol. Endocrinol., *8:* 296–304, 1994.
45. Smith, C. L., Conneely, O. M., and O'Malley, B. W. Modulation of the ligand-independent activation of the human estrogen receptor by hormone and antihormone. Proc. Natl. Acad. Sci. USA, *90:* 6120–6124, 1993.
46. Lavinsky, R. M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T. M., Schiff, R., Del-Rio, A. L., Ricote, M., Ngo, S., Gemsch, J., Hilsenbeck, S. G., Osborne, C. K., Glass, C. K., Rosenfeld, M. G., and Rose, D. W. Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc. Natl. Acad. Sci. USA, *95:* 2920–2925, 1998.

Inhibition of HER2/*neu* (*erb*B-2) and Mitogen-activated Protein Kinases Enhances Tamoxifen Action against HER2-overexpressing, Tamoxifen-resistant Breast Cancer Cells

Hirokazu Kurokawa, Anne E. G. Lenferink, Jean F. Simpson, et al.

Cancer Res 2000;60:5887-5894.