The Dual ErbB1/ErbB2 Inhibitor, Lapatinib (GW572016), Cooperates with Tamoxifen to Inhibit Both Cell Proliferation- and Estrogen-Dependent Gene Expression in Antiestrogen-Resistant Breast Cancer

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Abstract

Effective treatment of estrogen receptor (ER)-positive breast cancers with tamoxifen is often curtailed by the development of drug resistance. Antiestrogen-resistant breast cancers often show increased expression of the epidermal growth factor receptor family members, ErbB1 and ErbB2. Tamoxifen activates the cyclin-dependent kinase inhibitor, p27 to mediate G1 arrest. ErbB2 or ErbB1 overexpression can abrogate tamoxifen sensitivity in breast cancer lines through both reduction in p27 levels and inhibition of its function. Here we show that the dual ErbB1/ErbB2 inhibitor, lapatinib (GW572016), can restore tamoxifen sensitivity in ER-positive, antiestrogen-resistant breast cancer models. Treatment of MCF-7pr, T-47D, and ZR-75 cells with lapatinib or tamoxifen alone caused an incomplete cell cycle arrest. Treatment with both drugs led to a more rapid and profound cell cycle arrest in all three lines. Mitogen-activated protein kinase and protein kinase B were inhibited by lapatinib. The two drugs together caused a greater reduction of cyclin D1 and a greater p27 increase and cyclin E-cdk2 inhibition than observed with either drug alone. In addition to inhibiting mitogenic signaling and cell cycle progression, lapatinib inhibited estrogen-stimulated ER transcriptional activity and cooperated with tamoxifen to further reduce ER-dependent transcription. Lapatinib in combination with tamoxifen effectively inhibited the growth of tamoxifen-resistant ErbB2 overexpressing MCF-7 mammary tumor xenografts. These data provide strong preclinical data to support clinical trials of ErbB1/ErbB2 inhibitors in combination with tamoxifen in the treatment of human breast cancer. (Cancer Res 2005; 65(1): 18-25)

Introduction

The epidermal growth factor receptor or ErbB family of receptor tyrosine kinases consists of four members including epidermal growth factor receptor factor receptor (also called Her1 or ErbB1), Her2 (ErbB2 or neu), Her3 (ErbB3), and Her4 (ErbB4). Upon ligand binding, ErbB family members homodimerize and heterodimerize resulting in the phosphorylation of their intracellular kinase domains (1). Once ErbB1 and ErbB2 are activated, the phosphotyrosylated sites in their Src-homology 2 (SH2) domains serve as docking sites for adaptor proteins such as Shc, Grb2, and Sos resulting in the activation of the Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK and PI3K/protein kinase B (PKB) pathways and promotion of proliferation and mitogenesis (1, 2). The ErbB2 gene is amplified and overexpressed in up to 30% of primary breast cancers (3) and this is associated with poor patient prognosis (4, 5). ErbB1 is also overexpressed in up to 30% of primary invasive breast cancers and this is correlated with reduced overall survival, proliferation, and higher metastatic potential (6, 7). Inhibition of ErbB1 signaling reduces both ErbB1 and ErbB2 activity and delays tumorigenesis in MMTV/Neu mice (8). The cooperative activation of proliferative pathways by these two receptors has stimulated the development of a number of small molecule inhibitors of members of the ErbB family for use as anticancer agents.

Upon estrogen binding, estrogen receptor α (ERα) recruits and activates Src leading to activation of Shc, MEK/MAPK, and PI3K/PKB (9). Activation of these signaling kinases has a dual effect on cell proliferation: it modulates cell cycle regulators to stimulate cell cycle progression (10, 11), and these activated kinases phosphorylate ERα to positively regulate its transcriptional activity (12, 13). Liganded ER dimerizes and associates with coactivators resulting in transcriptional activation of estrogen-responsive genes (14). Newly diagnosed ER-positive breast cancers are commonly treated with the antiestrogen tamoxifen. Tamoxifen competes with estrogen for ER binding, leading to inhibition of cell cycle progression and G1 arrest (10, 11). However, tamoxifen treatment is often limited by the development of resistance and disease relapse (15). In ER-positive primary breast cancers, overexpression of both ErbB1 and ErbB2 is associated with resistance to tamoxifen therapy (7, 16). Overexpression of ErbB2 in MCF-7 causes a loss of sensitivity to tamoxifen (17, 18). Moreover, MCF-7 cells grown in the continuous presence of tamoxifen not only develop tamoxifen resistance but also show elevated total and phosphorylated ErbB1 and ErbB2 (19).

In ER-positive breast cancer cells, estrogens recruit quiescent cells into cell cycle and stimulate G1 cell cycle progression. Tamoxifen causes G0/G1 cell cycle arrest. Cell cycle progression is governed by a family of cyclin-dependent kinase (cdk) that are regulated by cyclin binding, by cdk phosphorylation, and by association with specific cdk inhibitors (20). In quiescent cells, levels of the kinase inhibitor protein, p27, are elevated and p27 binds tightly and inhibits cyclin E-cdk2. In normal and malignant mammary epithelial cells, estrogens and growth factors stimulate p27 phosphorylation and loss of p27 from cyclin E-cdk2 complexes, with resulting cyclin E-cdk2 activation and p27 proteolysis promoting S-phase entry and cell cycle progression (21, 22). We have shown that p27 is essential...
for G₁ arrest by tamoxifen (22). Moreover, transfection of constitutively activated MEK caused tamoxifen resistance through changes in p27 phosphorylation and a loss of its inhibitory function (23). MEK inhibition restored responsiveness to tamoxifen in ErbB2 or MEK overexpressing MCF-7 models (23).

Considerable attention has been directed to the development of therapeutic inhibitors of ErbB1 and ErbB2 for breast cancer treatment (24). Trastuzumab is a humznized anti-ErbB2 antibody that is approved for the treatment of ErbB2 amplified metastatic breast cancer. Several new quinazoline drugs that target ErbB1 are under investigation in clinical trials in cancer patients. However, cooperative activation of different ErbB family members through heterodimerization could circumvent the therapeutic efficacy of inhibition of a single receptor. Thus, small molecule inhibitors that inhibit both ErbB1 and ErbB2 would be therapeutically advantageous. Because both ErbB1 and ErbB2 activate MEK/PI3K/Akt leading to loss of p27 function and tamoxifen resistance, we investigated the potential for a reversible dual inhibitor of both ErbB1 and ErbB2, lapatinib (GW572016), to restore tamoxifen-mediated cell cycle arrest and to overcome tamoxifen-resistant breast tumor growth. Lapatinib and tamoxifen together led to a more profound cell cycle arrest than tamoxifen alone through an increase in p27 levels, increased p27 binding to and inhibition of cyclin E-cdk2 and down-regulation of ER transcriptional activity.

Materials and Methods

Cell Culture. MCF-7 were grown in Improved Modified Eagle’s medium (option Zn²⁺) supplemented with 5% fetal bovine serum and insulin as in ref. (11). ZR-75 (provided by P. Darbelle, Cell and Molecular Biology, University of Reading, Berkshire, UK) and T-47D (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum and 1 mmol/L sodium pyruvate. Relatively early passage MCF-7 cells were initially obtained from the Osborne Lab. These cells were initially very sensitive to G₁ arrest by 4-hydroxy-tamoxifen (4-OH-TAM), with a reduction in % S-phase cells from 40% to 2% to 4% and an increase in the % G₂/M cells to over 90% within 48 hours of drug treatment (22). Within 30 to 40 passages in the above media, a gradual loss of tamoxifen responsiveness was noted. The variant of MCF-7 used in these studies, MCF-7h, had been cultured for >50 passages and had acquired partial tamoxifen resistance compared with our earlier passage MCF-7. This line retained estrogen dependence for proliferation.

Cell Cycle Effects of Tamoxifen and Lapatinib. MCF-7h, ZR-75 and T-47D were treated with 10 nmol/L 4-OH-TAM (Sigma, St. Louis, MO) or 10 μmol/L lapatinib (GW572016; Glaxo SmithKline, Research Triangle Park, NC) or both. Asynchronously proliferating untreated and drug treated cells were collected 48 hours later for flow cytometry and lysates were prepared at the same times for immunoblotting. MCF-7h cells were synchronized in quiescence by estrogen deprivation for 48 hours as described in (11). Cells were stimulated to reenter the cell cycle by addition of 10 nmol/L 17β-estradiol and then recovered for protein and cell cycle analysis.

Flow Cytometric Analysis. Cells were pulse-labeled with 10 μmol/L bromodeoxyuridine for 2 hours and then fixed and stained with anti-bromodeoxyuridine–conjugated FITC (Becton Dickinson, Franklin Lakes, NJ) and propidium iodide. Cell cycle analysis was carried out using a Becton Dickinson FACScan, with Quest software as described in ref. (11).

Antibodies. Antibodies against MAPK, phosphorylated, activated MAPK (MAPK-P), PKB and phosphorylated activated PKB (PKB-P) were obtained from New England Biolabs (Beverly, MA), ErbB1 and ErbB2 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA) and β-actin from Sigma. p27 antibody was obtained from Transduction Laboratories (Lexington, KY), p21 antibody from Santa Cruz anti-PSTAIRE was used for detection of cdk2 in cyclin E immunoprecipitates; cyclin E1 monoclonal antibodies E12 and E172 were from E. Harlow (Mass. General, MA; ref. 25).

Immunoblotting and Immunoprecipitation. Cells were lysed in ice cold 0.1% or 1% NP40 lysis buffer. Protein was quantitated by Bradford analysis and Western analysis of cyclin E, p27, p21, cdk2, MAPK, PKB, MAPK-P and PKB-P was carried out as described in ref. (23). Equal protein loading was confirmed by probing for β-actin.

Cyclin E was immunoprecipitated from 200 μg of protein lysate with anti-cyclin E monoclonal antibody 172. Cyclin E-associated proteins were detected by immunoblotting and cyclin E-cdk2 activity was assayed. Brieﬂy, for kinase assays immunoprecipitated cyclin E was incubated with [γ-32P]ATP (Amersham, Piscataway, NJ) and histone H1 (Roche, Indianapolis, IN) at 30°C and radioactivity in histone H1 substrate was visualized by autoradiography.

Luciferase Assays. Cells were seeded in 24-well plates and transfectted with a plasmid encoding the ﬁrely luciferase gene driven by a promoter bearing two-tandem estrogen response elements (2xEREluc) and a cytomegalovirus-driven Renilla luciferase construct (phRL-TK-luc) using lipofectamine PLUS (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Cells were treated with 1 mmol/L 4-OH-TAM and 5 mol/L lapatinib for 24 hours before recovery for luciferase assays. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, San Luis Obispo, CA) and a dual-channel luminometer from Thermo LabSystems (Needham, MA).

Growth Inhibition of Tamoxifen-Resistant MCF-7 Tumor Xenografts. Ovariectomized athymic BALB/c nude mice were purchased from Taconic (Germantown, NY) and used for the xenograft studies. Tamoxifen-resistant MCF-7 tumors (MCF-TAMR) were derived by serial passage of tumor xenografts into animals treated with both tamoxifen (5 mg s.c. continuous release 60-day pellets; Innovative Research, Sarasota, FL) and estradiol (0.36 mg s.c. continuous release 60-day pellets, Innovative Research). After at least six serial xenograft passages of tumor that sustained growth in the presence of tamoxifen and estradiol, tumors were designated tamoxifen-resistant. Twenty-seven nude mice with MCF-TAMR established xenografts (median size at initiation of treatment was 40.0 mm³) were randomized to either placebo (vehicle) or treatment with lapatinib (100 mg/kg daily) by oral gavage. All mice continued to receive estradiol and tamoxifen treatment. Tamoxifen treatment was maintained in all animals to maintain selection pressure for continued resistance and because tumors regress when the tamoxifen is withdrawn. Tumors were measured thrice per week and tumor volume was determined using the formula: length × width² × (π/6). The primary objective in the xenograft study was the comparison of time with reach five times the initial tumor volume between the tamoxifen plus placebo-treated MCF-TAMR tumors and the tamoxifen plus lapatinib-treated MCF-TAMR tumors. Tumor lysates were collected from 2nd passage for tamoxifen-sensitive MCF-7 tumors and from 10th passage for MCF-TAMR tumors.

Statistical Analysis. Effects of drug treatment (lapatinib, tamoxifen or both) were compared with the % S phase of asynchronous cells in three repeat experiments. The % S-phase means for the four conditions were compared by ANOVA followed by mean pairwise comparison using Bonferroni multiple comparison approach. P < 0.05 was set as the criteria for statistical significance.

Results

Lapatinib and Tamoxifen Cooperate to Inhibit Cell Cycle Progression. We observed a progressive development of resistance to cell cycle arrest by tamoxifen with long-term passage (n > 50) of MCF-7. Treatment (48 hours) of early passage MCF-7 with 4-OHTAM caused G₁ arrest, with a decrease in the % S phase from 37% to 2% and an increase in the % cells with 2N DNA content to over 90% (22). In late passage MCF-7 (MCF-7h), treatment with 4-OHT-TAM for 48 hours failed to cause complete cell cycle arrest: the % S-phase cells decreased from 40% to only 16% in MCF-7h. Lapatinib treatment (10 μmol/L) reduced the % S from 40% to 25% with a concomitant increase in the G₁ fraction. With both 4-OHTAM...
and lapatinib together, the % S phase of MCF-7pr fell dramatically to 2% as compared with each treatment alone (P < 0.05; Figs. 1A and 2). Two other ER-positive lines, T-47D and ZR-75 obtained from American Type Culture Collection, and ZR-75 obtained from the Darbre lab, did not undergo complete G1 arrest in response to 48 hours of 4-OH-TAM, exhibiting a degree of tamoxifen resistance similar to that of MCF-7. Treatment of asynchronous T-47D cultures with either lapatinib or 4-OH-TAM alone, caused a partial arrest with the % S-phase cells falling to 25% or 17%, respectively, whereas combined treatment with lapatinib and 4-OH-TAM reduced the % S-phase cells to 2% (P < 0.05; Figs. 1A and 2). A similar pattern was observed in ZR-75 cells (Figs. 1A and 2). Thus, ErbB1/ErbB2 inhibition and ER blockade can cooperate to inhibit cell cycle progression, with the two drugs together having at least additive effects on cell cycle progression. It is noteworthy that we did not observe increased ErbB1 or ErbB2 expression in MCF-7pr compared with parental MCF-7 cells (Fig. 1C). Therefore, partial 4-OH-TAM-resistant phenotype observed in MCF-7pr does not arise from elevated ErbB1 or ErbB2 expression. Furthermore, we observed that MCF-7, T-47D, and ZR-75 expressed barely detectable ErbB1 protein levels compared with ErbB1 overexpressing BT-20 cells and relatively low levels of ErbB2 compared with ErbB2 overexpressing MDA-MB-361 (Fig. 1C).

**Effects of Lapatinib and Tamoxifen on MAPK and PKB.** Lapatinib has been shown to effectively inhibit ErbB1 and ErbB2 (26, 27). Both ErbB1 and ErbB2 activate the PI3K/PKB and Ras/Raf/MAPK pathways (1). Inhibition of either MEK or PI3K results in cell cycle arrest in MCF-7 cells (23, 28). Furthermore, constitutive MEK activation in MCF-7 has been shown to confer tamoxifen resistance (23, 29). In MCF-7, 48 hours treatment with 10 μmol/L lapatinib decreased the activating phosphorylation of both MAPK and PKB as shown by immunoblotting for MAPK-P and PKB-P, whereas total MAPK and PKB levels were unchanged (Fig. 1B). 4-OH-TAM stimulated MAPK activity in MCF-7 as shown previously (30). Whereas this may reflect a partial estrogen agonistic effect of 4-OH-TAM in MCF-7, certain mammary cell types can show sustained MAPK activation when arrested by inhibitory cytokines and by differentiation. Treatment with lapatinib and 4-OH-TAM together inhibited MAPK and PKB activities more notably than either drug alone. Note that unlike MCF-7, in which 4-OH-TAM stimulated MAPK activity, in T-47D and ZR-75, 4-OH-TAM partially inhibited MAPK. Lapatinib inhibited both MAPK and PKB when added alone. MAPK-P was more profoundly inhibited by both drugs together in T-47D but not in ZR-75 (Fig. 1B). These data underline the heterogeneity in the response of MAPK to epidermal growth factor receptor family and ER blockade among ER-positive breast cancer lines. Thus, inhibition of MAPK phosphorylation may not be the best surrogate end point for growth arrest by receptor tyrosine kinase inhibitors.

**Lapatinib and Tamoxifen Effects on p27 and Cyclin E-cdk2 Inhibition.** Our flow cytometry data showed that lapatinib and 4-OH-TAM can act together to arrest cells in G1. Because both MEK and PI3K regulate p27 levels and function in breast cells (23, 29, 31), we assayed drug effects on p27 and other cell cycle regulators, p27 binding to cyclin E and cyclin E-cdk2 activity. In MCF-7, treatment with either lapatinib or 4-OH-TAM alone modestly reduced cyclin D1 levels by less than 2-fold, whereas treatment with both inhibitors resulted in a synergistic 5-fold reduction in cyclin D1 levels (Fig. 2A). The two drugs together also had a more profound effect on cyclin D1 levels than did either drug alone in T-47D and ZR-75 (Fig. 2B and C). p27 levels increased by 1.4- to 2-fold with either drug alone and by ~2.5- to 5-fold in cells treated with both, with less dramatic increases observed in T-47D.

When asynchronous cells were treated with drug for 48 hours, immunoprecipitation followed by Western blotting showed that p27 bound to cyclin E-cdk2 increased by 2.0-fold in MCF-7 treated with lapatinib and by 1.8-fold following 4-OH-TAM alone (Fig. 2A). Treatment with lapatinib and 4-OH-TAM resulted in a 5-fold increase in p27 bound to cyclin E-cdk2. Similar effects were observed in T-47D and ZR-75 (Fig. 2B and C). The increased p27 binding to cyclin E-cdk2 mediated cyclin E-cdk2 inhibition (Fig. 3). Lapatinib or 4-OH-TAM treatment each partly inhibited cyclin E-cdk2, with more profound kinase inhibition following treatment with both drugs together (representative data for ZR-75 in Fig. 3).

**Lapatinib and Tamoxifen Inhibit Estrogen-Dependent G1-S Progression.** Although they exhibit partial tamoxifen resistance, our MCF-7 cells are estrogen-dependent for proliferation. Stimulation of estrogen-deprived quiescent MCF-7 cells with estrogen for 18 hours activated MAPK (Fig. 4A). Moreover, it stimulated cell cycle reentry with 54% of cells in S phase after 18 hours of estradiol treatment (Fig. 4B). Consistent with prior data (11), estrogen

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**Figure 1.** Lapatinib and tamoxifen cooperate to induce G1 arrest in ER-positive breast cancer lines. A, asynchronous MCF-7, T-47D, and ZR-75 cultures were treated with 10 μmol/L lapatinib (GWS272616), 10 nmol/L 4-OH-TAM or both inhibitors for 48 hours before flow cytometric analysis. Bars, SE. B, Western analyses for MAPK-P, MAPK, PKB, P-PKB, and β-actin used cells treated as in A. C, Western blot analysis for ErbB1 and ErbB2 in asynchronous BT-20, MDA-MB-361, early passage MCF-7 and MCF-7pr, T-47D, and ZR-75.
reduced p27 levels (Fig. 4A), notably decreased cyclin E-bound p27 (Fig. 4B) and activated cyclin E-cdk2. These effects were partially abrogated by treatment with either 4-OH-TAM or lapatinib alone (Fig. 4A-C). Treatment with both drugs together prevented the loss of p27 and its release from cyclin E-cdk2 activation and cell cycle reentry (Fig. 4A-C). Taken together, these findings suggest that activation of ErbB1/ErbB2 following estrogen-ER binding is required for estrogen-dependent G1-to-S phase cell cycle progression. Moreover, they suggest that therapeutic ER blockade and receptor tyrosine kinase inhibition may have greater efficacy in abrogating estrogen-stimulated breast cancer cell proliferation than that observed with either treatment modality alone.

Tamoxifen and Lapatinib Inhibit ER Transcriptional Activity. For many years, the therapeutic effect of tamoxifen was thought to result from its inhibition of estrogen-dependent ER transcriptional activity. However, liganded ER is also known to bind and activate Src, PI3K, and MAPK, leading to activation of mitogenic signaling and cell cycle progression. MAPK once activated following estrogen stimulation can in turn phosphorylate the ER in a manner that activates its transcriptional activity (12, 13). Here, we show that the therapeutic efficacy of the dual ErbB1/ErbB2 inhibitor, lapatinib (GW572016) results not only from inhibition of signaling via MAPK and PKB but also involves inhibition of estrogen ER transcriptional activity in ER-positive breast cancer cells.

We assayed ER transcriptional activity using an ERE reporter construct (2xERE-luc). Treatment with lapatinib alone reduced ER transcriptional activity by 38% and 39% in MCF-7pr and ZR-75, respectively (Fig. 5A and B). 4-OH-TAM reduced ERE-luciferase activities by 76% and 71% for MCF-7pr and ZR-75, respectively. Treatment with both drugs resulted in a further reduction in ER activity in both cell lines. Thus, in addition to antiproliferative effects resulting from inhibition of mitogenic signaling and G1 cyclin-cdk2, lapatinib can work together with 4-OH-TAM to inhibit estrogen activated ER transcriptional activity.

Lapatinib Inhibits Growth of Tamoxifen-Resistant Breast Cancer Xenograft Tumors. We next tested the ability of lapatinib to inhibit growth or cause regression of tamoxifen-resistant MCF-TAMR xenografts in vivo. In contrast to the parental MCF-7 line and early xenograft tumors, late passage serial explant xenograft MCF-TAMR tumors are not only resistant to growth inhibition by tamoxifen, they are growth stimulated by tamoxifen (32). The MCF-TAMR xenograft tumors require estradiol for tumor establishment and growth, and all ovariectomized animals were treated with estradiol implants throughout these experiments.
Treatment of MCF-TAMR tumor xenografts with 4-OH-TAM and lapatinib significantly inhibited tumor growth when compared with 4-OH-TAM treatment alone (Fig. 6B). Because these tumors regress when 4-OH-TAM is withdrawn (32), the effect of lapatinib alone was not tested. No toxicity due to lapatinib was seen in any of the 13 animals treated with lapatinib and 4-OH-TAM. There were three complete tumor regressions (23%) in the lapatinib/4-OH-TAM treated group compared with continued growth of all 14 4-OH-TAM–treated xenografts in control animals. There was a significant delay in the time to reach a 5-fold increase in the initial tumor volume in lapatinib/4-OH-TAM–treated animals versus 4-OH-TAM alone (median, 38 versus 25 days; Wilcoxon sum-rank test; \( P = 0.0026 \)). Consistent with previous findings, ErbB2 and ErbB1 levels were increased in the MCF-TAMR tumor samples compared with MCF-7 cells (Fig. 6A).

Discussion

Tamoxifen has been widely used for breast cancer prevention, for treatment of breast cancer in the adjuvant setting to prevent recurrence after tumor removal, and for metastatic disease (15). Tamoxifen resistance presents a major clinical problem. The mechanisms whereby ER-positive breast cancers develop resistance to tamoxifen are not fully understood. However, there is considerable evidence from cell lines and mouse models that constitutive activation of receptor tyrosine kinase pathways, including those downstream of ErbB1 and ErbB2 can induce tamoxifen resistance (16–19, 23).

Lapatinib is a potent inhibitor of ErbB1 and ErbB2 signaling. It binds to the catalytic domain of ErbB1 and ErbB2 and inhibits autophosphorylation of the receptors (26, 27). Lapatinib has been shown to be effective as an anticancer agent in both preclinical models (26, 27) and in Phase I/II trials in cancer patients (33). The present study was undertaken to assay the potential efficacy of lapatinib to restore tamoxifen-mediated growth arrest in breast cancer cell lines and xenograft tumors that have developed tamoxifen resistance. Tamoxifen has a cytostatic effect on breast cancer growth and causes G1 cell cycle arrest in ER-positive breast cancer cells (10). Because prior work from our group and others has shown that tamoxifen-dependent G1 arrest requires intact kinase inhibitor protein function (11, 34), we assayed the effects of these agents on p27 and its target cdk2.

Although p27 is strongly expressed in quiescent mammary epithelial cells, p27 levels are reduced in up to 60% of primary human breast cancers and this has been correlated with poor patient prognosis (35, 36). p27 is required for antiestrogen-mediated cell cycle arrest and deregulation of p27 in ErbB2 transfected cells is causally linked to antiestrogen resistance (11, 23, 37, 38). Lapatinib, when added to 4-OH-TAM effectively inhibited ErbB-dependent MAPK and PKB activation, and increased the binding and inhibition of cyclin E-ckd2 by p27 more effectively than either drug alone. It is noteworthy that the increase in p27 binding to cyclin E-ckd2 following lapatinib and 4-OH-TAM was consistently higher in all three cell lines than the up-regulation of p27 protein levels. Thus, ErbB1 and ErbB2 signaling seems to alter the affinity of p27 for its target cdk2. This is consistent with earlier work showing that p27 redistribution onto cdk2 complexes preceded the increase in p27 levels in SKBR3 and BT474 cells after ErbB2 inhibition (38).

Both MEK/MAPK and PKB alter p27 phosphorylation and oppose the cdk inhibitory effects of p27 (23, 31, 37, 39). Transfection of activated MEK reduces the cyclin E-ckd2 inhibitory activity of p27 (23) and activates p27 proteolysis (23, 38). Moreover, constitutive PKB activation shifts p27 into the cytoplasm away from nuclear cdk targets (31, 37, 39). PI3K/PKB–dependent p27 phosphorylation also increases p27 assembly into cyclin D1-ckd4 complexes and a loss of p27 binding to cyclin E-ckd2 (23). Thus, lapatinib has the potential to oppose p27 proteolysis, and to reverse the aberrant cytoplasmic sequestration of p27 seen in many cancers by causing p27 redistribution from cyclin D1-ckd4 to cyclin E-ckd2 complexes.

One of the cell cycle effectors most dramatically affected by the combination treatment with lapatinib and 4-OH-TAM was cyclin D1. Cyclin D1 is a transcriptional target of the ER (40), and in some but not all studies, its overexpression in ER-positive breast cancer cells can sequester p27, leading to G1 cell cycle arrest in ER-positive breast cancer cells (10, 23).

Figure 4. Effects of ER blockade and ErbB1/ErbB2 inhibition on estrogen-stimulated cell cycle entry. Estradiol-deprived, quiescent MCF-7 Pr were treated with lapatinib (GW572016) and/or 4-OH-TAM 30 minutes before stimulation with estradiol as described in Materials and Methods. Eighteen hours after estradiol addition, cells were harvested for protein and flow cytometric analysis. A, MAPK-P, MAPK, p27, and \( \beta \)-actin were assayed by Western blot. B, cell cycle profiles following different treatments. Cyclic E immunoprecipitates were resolved, transferred to membrane and associated proteins detected by immunoblotting. 2C, cyclic E-ckd2 activity was assayed and results graphed as % maximum. Autorad inset, radioactivity in histone H1 for nonspecific immunoglobulin G control (G) and cyclic E immunoprecipitates following different drug treatments indicated (1-5).
cancers has been associated with poor prognosis (41, 42). Cyclin D1 deficient mice are resistant to breast cancers induced by transgenic ErbB2/neu (43) suggesting a critical role of cyclin D1 in ErbB2 mediated tumorigenesis. Furthermore, overexpression of cyclin D1 can mediate antiestrogen resistance in breast cancer cell lines (44). ER blockade and ErbB1/ErbB2 inhibition reduced cyclin D1 levels most notably in MCF-7pr and in T-47D. This may reflect both transcriptional repression (40) and a loss of cyclin D1 stability, because PKB inhibition leads to cyclin D1 proteolysis (45, 46). The reduced effect of lapatinib on PKB in ZR-75 may in part account for the less dramatic loss of cyclin D1 in ZR-75 than in MCF-7pr and T-47D.

The effects of lapatinib and 4-OH-TAM on MAPK and PKB activities varied within the three cell lines tested. Inhibition of MAPK and PKB by both drugs together was not much greater than that achieved with lapatinib alone in MCF-7pr and ZR-75, yet cell cycle inhibition was significantly greater with both drugs together. It is noteworthy that in a recent Phase I clinical trial of this agent, MAPK and PKB inhibition was not always correlated with tumor response and may not be the most useful surrogate end point (47). Other cellular pathways in addition to MAPK and PKB need to be inhibited to achieve maximal growth arrest.

Although the antiproliferative effects of ErbB1/ErbB2 inhibitors are thought to result from inhibition of mitogenic signaling and effects on cell cycle regulators, our data suggest that in ER-positive cancers, they also inhibit estrogen stimulated ER transcriptional activity. Lapatinib and 4-OH-TAM both inhibited ER-dependent transcription. Lapatinib treatment alone resulted in 40% reduction in ER luciferase activity. The repression of ERE-dependent transcription was even more profound in 4-OH-TAM and lapatinib-treated cells. This reduction could in part result from inhibition of ErbB1/2 by lapatinib, reducing input to MAPK and PKB. Estrogen binding to the ER activates Src and leads to Shc, Ras and MAPK and PKB activation (48–50). Both MAPK and PKB once activated can phosphorylate the ER at specific sites to increase its transcriptional activity (12, 49, 51, 52). The ER can also be activated in a ligand-independent manner through oncogenic receptor tyrosine kinase activation (53, 54). Inhibition of signaling cross-talk and ER phosphorylation is an additional mechanism whereby by lapatinib may cooperate with 4-OH-TAM to impair both estrogen-dependent and ligand-independent ER transcriptional activity. Although there is evidence that insulin-like growth factor-I receptor can cross-talk with liganded ER, lapatinib does not inhibit the insulin-like growth factor-I receptor either in vitro or in cell-based assays (data not shown). Thus, lapatinib-mediated restoration of growth arrest in these tamoxifen-resistant models does not likely involve insulin-like growth factor-I receptor inhibition.

In some breast cancers and in MCF-7/HER2-18 cells, tamoxifen resistance may arise through altered effects of tamoxifen on ER activated transcription. In these cells, tamoxifen-bound ER may recruit coactivators to the ERE rather than corepressors as is observed in sensitive cells (55). Shou et al. (55) recently showed that pretreatment with the pure ErbB1 inhibitor, gefitinib, restored corepressor binding to the 4-OH-TAM–bound ER at ERE response elements in the PS2 promoter in MCF-7/HER2-18 cells. These findings suggest that ErbB1/ErbB2 signaling may modulate ER phosphorylation and conformation leading to altered ER-coactivator/corepressor complexes formation.

Figure 5. Both lapatinib and tamoxifen inhibit ER transcriptional activity. Asynchronous growing (A) MCF-7pr and (B) ZR-75 were transfected with 2xERELuc for 24 hours before treatment with DMSO control, 5 μmol/L lapatinib (GW572016), 1 μmol/L 4-OH-TAM or both drugs together for an additional 24 hours. Columns, mean of three independent experiments; bars, SE. Fold inhibition of ERE luciferase activity in untreated asynchronous control cells (asyn).

Figure 6. Lapatinib inhibits the growth of tamoxifen-resistant mammary tumor xenografts. A, Western blot analysis for ErbB1 and ErbB2 of tumor lysates from MCF-7 and MCF-TAMR tumors. B, tamoxifen-resistant tumor xenografts were implanted into the flanks of nude mice and continuously grown with tamoxifen. When the tumor volumes reached 40 mm³, 13 mice were treated with lapatinib (GW572016) as described in Materials and Methods, and the remaining 14 animals were maintained as controls. All mice were maintained on tamoxifen. Points, mean tumor volumes.

5 J. Liang and J. Slingerland, manuscript in preparation.
In many cancer-derived lines, higher basal levels of ErbB2 or ErbB1 are associated with a greater dependence on these receptors for cell survival and proliferation. The antitumor effects of ErbB1 or ErbB2 inhibitors have been mostly assayed in lines with constitutive ErbB1 or ErbB2 activation (8, 19, 29, 37, 56). Preclinical and clinical studies of ErbB1 inhibitors show the greatest efficacy in cells with increased expression or activation of the ErbB family member targeted by the drug (24, 57). Indeed, cells with the highest ErbB2 or ErbB1 activation are most sensitive to lapatinib (26, 27). These data raise the concern that breast and other cancers that do not have activated ErbB1 or ErbB2 will not be optimally responsive to these drugs.

We and others have shown that MCF-7*, T-47D, and ZR75 lines do not have ErbB2 amplification and have low ErbB1 and ErbB2 protein levels (58, 59). In these lines, lapatinib alone caused only partial cell cycle blockade. It is noteworthy in this regard that in tamoxifen-sensitive MCF-7 xenografts, lapatinib alone has minimal antitumor efficacy. Previous studies have shown that ErbB2 or ErbB1 inhibitors can facilitate the antiproliferative effects of tamoxifen (29, 56). Here we show that lapatinib together with tamoxifen has significant antitumor activity in vivo in tamoxifen-resistant MCF-TAMR xenografts that have elevated ErbB2 expression and a modest increase in ErbB1. Moreover, in three independent ER-positive cell lines with low ErbB1 and ErbB2 levels, lapatinib had only modest antiproliferative activity when used alone, 4-OH-TAM and lapatinib effectively blocked cell cycle progression. These data show the potential efficacy of tamoxifen together with lapatinib in treatment of ER-positive breast cancers that do not have high intrinsic activities of ErbB2 or ErbB1. The cell cycle inhibitory function of p27 and inhibition of ER-dependent transcription were both enhanced by the combination of lapatinib and 4-OH-TAM in MCF-7*, T-47D, and ZR-75.

Our mouse xenograft data and data from breast cell indicate that the combination of lapatinib and tamoxifen has the potential to abrogate tamoxifen resistance or delay its development in ER-positive breast cancer in the metastatic and adjuvant settings. Moreover, because the combination of these two drugs was more effective than either alone in the context of low basal ErbB1/ErbB2 in the cell culture studies presented here, lapatinib in combination with tamoxifen may be beneficial in patients with ER-positive breast cancer irrespective of the ErbB1/ErbB2 status of the tumor. Clinical trials to investigate and optimize combinations of tamoxifen and receptor tyrosine kinase inhibitors, such as lapatinib, are clearly warranted.

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References


N. Spector, unpublished results.

4\footnote{N. Spector, unpublished results.}


The Dual ErbB1/ErbB2 Inhibitor, Lapatinib (GW572016), Cooperates with Tamoxifen to Inhibit Both Cell Proliferation- and Estrogen-Dependent Gene Expression in Antiestrogen-Resistant Breast Cancer

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