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Research Article

Activity of the Dual Kinase Inhibitor Lapatinib (GW572016) against HER-2-Overexpressing and Trastuzumab-Treated Breast Cancer Cells

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Abstract

Lapatinib (GW572016) is a selective inhibitor of both epidermal growth factor receptor (EGFR) and HER-2 tyrosine kinases. Here, we explore the therapeutic potential of lapatinib by testing its effect on tumor cell growth in a panel of 31 characterized human breast cancer cell lines, including trastuzumab-conditioned HER-2-positive cell lines. We further characterize its activity in combination with trastuzumab and analyze whether EGFR and HER-2 expression or changes induced in the activation of EGFR, HER-2, Raf, AKT, or extracellular signal-regulated kinase (ERK) are markers of drug activity. We report that concentration-dependent antiproliferative effects of lapatinib were seen in all breast cancer cell lines tested but varied significantly between individual cell lines with up to 1,000-fold difference in the IC_{50}s (range, 0.010-18.6 μmol/L). Response to lapatinib was significantly correlated with HER-2 expression and its ability to inhibit HER-2, Raf, AKT, and ERK phosphorylation. Long-term *in vivo* lapatinib studies were conducted with human breast cancer xenografts in athymic mice. Treatment over 77 days resulted in a sustained and significant reduction in xenograft volume compared with untreated controls. For the combination of lapatinib plus trastuzumab, synergistic drug interactions were observed in four different HER-2-overexpressing cell lines. Moreover, lapatinib retained significant *in vitro* activity against cell lines selected for long-term outgrowth (>9 months) in trastuzumab-containing (100 μg/mL) culture medium. These observations provide a clear biological rationale to test lapatinib as a single agent or in combination with trastuzumab in HER-2-overexpressing breast cancer and in patients with clinical resistance to trastuzumab. (Cancer Res 2006; 66(3): 1630-9)

Introduction

Growth and differentiation of both normal and malignant human breast cancer cells are regulated in part by a signaling network of four members of the type 1 receptor tyrosine kinase family, which include the epidermal growth factor receptor (EGFR; HER-1), HER-2, HER-3, and HER-4 (1). Binding of receptor-specific ligands to the ectodomain of EGFR, HER-3, and HER-4 results in the formation of receptor dimers and hetero-oligomers to which HER-2 is recruited as the preferred heterodimerization partner (2–7). As HER-2 is the only HER family receptor species that does not bind known ligands, its principal biological role as a signal transducer results from recruitment into heteromeric receptor complexes with HER-1, HER-3, or HER-4 (8). Dysregulation of the HER-mediated signaling network has been implicated in the pathogenesis of breast cancer. Perhaps the best example of this in human breast cancer is provided by amplification of the *HER-2* gene, which results in HER-2 protein overexpression (9). This alteration is present in ~20% of human breast cancers and is associated with an aggressive form of the disease with significantly shortened disease-free survival and overall survival (10).

It has been suggested that HER-2 may play an important role in the oncogenic activity of EGFR, because preclinical experiments have shown that HER-2 and EGFR act synergistically to transform NIH3T3 cells (11). Being the most common heterodimerization partner of EGFR (6), HER-2 potentiates EGFR signaling in *trans* by enhancing the binding affinity of its ligand EGF (12), reducing its degradation (13), and predisposing the receptor to recycling (14). In turn, it has been shown that EGF-induced stimulation of EGFR leads to activation of HER-2 by transduction through heterodimerization (2, 15), and recent studies have shown that EGFR-specific inhibitors can reduce HER-2 signaling and growth of breast cancer cells that express high levels of HER-2 (16–18). Thus, combined inhibition of both EGFR and HER-2 may be more efficacious than targeting either one of them alone.

One successful antireceptor strategy has been the development of trastuzumab, a humanized monoclonal antibody, which targets the extracellular domain of HER-2 (19, 20). An alternative antireceptor strategy has been the development of small-molecule inhibitors that compete with ATP for the ATP-binding domain in the intracellular portion of receptor tyrosine kinases. Recently, agents have been developed that simultaneously inhibit both EGFR and HER-2 epithelial growth factor receptors (21). Lapatinib is such a synthetic small-molecule inhibitor of the HER-2 and EGFR tyrosine kinases (22). This compound is a potent ATP-competitive inhibitor in cell-free biochemical kinase assays inhibiting the recombinant EGFR and HER-2 tyrosine kinases by 50% (IC_{50}) at concentrations of 10.8 and 9.3 nmol/L, respectively (22). Lapatinib is a reversible inhibitor with estimated dissociation constant (K_i) values of 3 and 13 nmol/L for EGFR and HER-2, respectively (23). In cell-based assays, lapatinib inhibits the growth of HER-2-overexpressing BT474 breast cancer cells at comparably low concentrations (IC_{50}, 100 nmol/L; ref. 22).

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However, 30- to 40-fold higher concentrations are necessary to inhibit MCF-7 and T47D human breast cancer cells, which both express normal levels of EGFR and HER-2 (22). Thus, evaluation of additional breast cancer cell lines each expressing different levels of EGFR and HER-2 may provide more comprehensive information on the potential utility of a dual kinase inhibitor in intact breast cancer cells.

Because the *in vitro* studies were carried out for short-term periods (7 days), we further evaluated the long-term effect of lapatinib *in vivo*. BT474 human breast carcinoma xenografts, which are tumorigenic in athymic mice, served as the tumor target for the *in vivo* studies.

Because lapatinib inhibits the tyrosine kinase activity of HER-2 itself in addition to that of its coreceptor EGFR, this small-molecule inhibitor might increase the activity of trastuzumab or show activity in cells with acquired resistance to the HER-2 antibody trastuzumab. To address this possibility, we characterized the effects of combinations of lapatinib and trastuzumab in HER-2-overexpressing breast cancer cells using the median effect/combination index (CI) isobologram method for multiple drug effect analysis (24). Moreover, we assessed the activity of lapatinib against cells selected for long-term outgrowth in trastuzumab-containing medium. Such trastuzumab-conditioned HER-2-overexpressing breast cancer cells were used to study the activity of lapatinib in cells with acquired resistance to the HER-2 antibody trastuzumab.

Finally, we sought to identify downstream biomarkers associated with response to lapatinib. This was accomplished by measuring EGFR and HER-2 receptor expression and assaying the phosphorylation status of EGFR, HER-2, and signaling intermediates, such as Raf, AKT, and extracellular signal-regulated kinase (ERK), in each of the cell lines both before and after exposure to lapatinib.

Materials and Methods

Cell lines, cell culture, and reagents. The effects of lapatinib on cell growth were studied in 31 human breast cancer cell lines, including (*a*) a panel of 22 established cell lines that express varying levels of EGFR and HER-2, (*b*) 3 HER-2-transfected and empty vector control-transfected cell line pairs, and (*c*) 3 trastuzumab-conditioned HER-2-overexpressing breast cancer cell lines selected for long-term outgrowth in trastuzumab-containing medium. The cell lines MDA-MB-175, BT474, SK-BR-3, MDA-MB-361, UACC-893, UACC-812, T47D, MDA-MB-453, MDA-MB-468, CAMA1, MB157, MCF-7, MDA-MB-435, ZR-75-1, BT20, and MDA-MB-231 were obtained from American Type Culture Collection (Rockville, MD). The cell lines EFM192A, KPL1, EFM19, and CAL51 were obtained from the German Tissue Repository DSMZ (Braunschweig, Germany), and the cell lines SUM190 and SUM225 were obtained from the University of Michigan (Ann Arbor, MI). MDA-MB-175, UACC-893, UACC-812, and MDA-MB-157 cells were cultured in L15 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, and 1% penicillin G-streptomycin-fungizone solution (PSF, Irvine Scientific, Santa Ana, CA). CAL51 and KPL1 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and PSF. SUM190 and SUM225 cells were cultured in Ham's F-12 supplemented with 5% heat-inactivated FBS and PSF, 5 mg/mL insulin, and 1 mg/mL hydrocortisone. The remaining cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mmol/L glutamine, and PSF. MCF-7, T47D, and ZR-75-1 cells, each with normal levels of HER-2 expression, were stably transfected previously with a full-length cDNA of the human *HER-2* gene as described earlier (25). Trastuzumab conditioned cell lines SK-BR-3/mAbHER-2, BT474/mAbHER-2, and MDA-MB-361/mAbHER-2 were generated by culturing cells for a minimum of 9 months in RPMI 1640 supplemented with 100 μg/mL recombinant humanized monoclonal HER-2 antibody (mAbHER-2) trastuzumab (Genentech, Inc., South San Francisco, CA). Trastuzumab was removed from the medium 24 hours before treatment with lapatinib that was provided by GlaxoSmithKline as a 10 mmol/L concentrated stock solution in DMSO. EGF and heregulin β1 were obtained from Invitrogen Life Technologies (Carlsbad, CA).

Quantitation of HER-2 and EGFR expression. HER-2 and EGFR protein content was measured with a commercially available quantitative ELISA (Oncogene Research Products/Calbiochem, San Diego, CA, and R&D Systems, Minneapolis, MN, respectively) according to the manufacturer's instructions. Cell lysates were prepared as described previously (26), and HER-2 and EGFR protein levels were given in nanogram per milligram of total protein.

Proliferation assays. Cells were plated into 24-well plates at a density of 2×10^5 to 5×10^5 and grown in cell line–specific medium without or with increasing concentrations of lapatinib (ranging between 0.008 and 10 μmol/L). Cells were harvested by trypsinization on day 7 and counted using a particle counter (Z1, Beckman Coulter, Inc., Fullerton, CA). Growth inhibition was calculated as a percentage of the untreated controls. Experiments were done twice to thrice in duplicate for each cell line. The log of the fractional growth inhibition was then plotted against the log of the drug concentration, and the IC_{50}s were interpolated from the resulting linear regression curve fit (Calcusyn, Biosoft, Ferguson, MO). Comparisons of IC_{50}s between cell lines were done using the Student's t test.

Cell cycle analysis. Cells were plated in six-well tissue culture dishes and treated with vehicle (0.1% DMSO) or lapatinib at the concentrations indicated for 24 hours. Cells were then trypsinized, collected, and washed in PBS. Cells were subsequently fixed in 70% ethanol, incubated HCl/Triton X-100, washed, and stained with propidium iodide. Samples were analyzed on the FacSort (Becton Dickinson, San Jose, CA). DNA content was estimated by gating histograms generated with the FL2-area variable.

Analysis of long-term activity of lapatinib against HER-2-overexpressing breast carcinoma xenografts *in vivo*. C.B-17 severe combined immunodeficient female mice (4-6 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). The research complied with national legislation and with company policy on the Care and Use of Animals and with related codes of practice. BT474 human tumor xenografts were initiated by implantation of tumor fragments (20-100 mg) from established tumors. Tumor size was estimated using the formula: Length × Width^2 / 2 = Tumor volume (mm^3). Treatment began when tumors were palpable and 3 to 5 mm in diameter. Experimental compounds were given p.o. twice daily for 77 days in a vehicle of 0.5% hydroxypropylmethylcellulose/0.1% Tween 80. The groups treated with vehicle control contained 8 mice, and the group treated with 75 mg/kg over 77 days contained 16 mice. Statistical significance of the differences was analyzed by single-factor ANOVA of the log-transformed tumor volume data.

Immunoprecipitation and Western blots. Following treatment with lapatinib as indicated, cells were washed in PBS and lysed at 4°C in lysis buffer. Insoluble material was cleared by centrifugation at 10,000 × g for 10 minutes. Protein was quantitated using BCA (Pierce, Rockford, IL), resolved by SDS-PAGE, and transferred to nitrocellulose membranes (Invitrogen Life Technologies). HER-2, EGFR, AKT, and ERK expression were detected by monoclonal anti-HER-2 (Ab-3, Calbiochem), anti-EGFR antibodies (Pharmingen, San Diego, CA), polyclonal anti-AKT (AKT1, AKT2, and AKT3 protein kinases) and anti-ERK1/2 (p44/42 mitogen-activated protein kinase) antibodies (Cell Signaling Technology, Beverly, MA), respectively. pAKT and pERK were detected by polyclonal anti-pAKT (Ser^{473}) and anti-pERK (Tyr^{202}/Tyr^{204}) antibodies (Cell Signaling Technology). pRaf was detected by a polyclonal anti-pRaf-1 (Ser^{338}) antibody (Upstate, Charlottesville, VA). Tyrosine phosphorylation of HER-2 and EGFR was analyzed as follows. Immunoprecipitations were done by allowing 250 μg protein lysate to incubate with 3 μg monoclonal anti-HER-2 (Ab-3) or anti-EGFR antibody (Ab-1, Calbiochem) and protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight with gentle agitation. The immunoprecipitates were washed thrice in lysis buffer and then denatured in Laemmli buffer before SDS-PAGE. Immunoblotting was done using a monoclonal anti-phosphotyrosine antibody (Upstate). Detection for concentration- and time-dependent experiments were done using enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) or Dura system

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(Pierce). Densitometry of the resulting Western blots was done using the ImageQuant software (Amersham Biosciences). Detection in all other experiments was done using ECL Plus chemifluorescent reagent (Amersham Biosciences), and densitometry of the resulting Western blots was done using the chemifluorescence method by Typhoon 9400 (Amersham Biosciences). Results were controlled for equal loading and exposure time by comparison with relative tubulin content. Correlations between IC_{50}s and the percent reduction of baseline phosphorylation levels were done by calculating the Spearman's rho correlation coefficient.

Multiple drug effect analysis. Aliquots of 3×10^3 to 5×10^3 SK-BR-3, BT474, MDA-MB-361, and MDA-MB-453 cells were plated in 96-well microdilution plates. Following cell adherence (24 hours), experimental medium containing either control medium, trastuzumab, lapatinib, or the combination (trastuzumab plus lapatinib) were added to appropriate wells in duplicate, and serial 2-fold dilutions were done to span clinically relevant concentration ranges for the dose-effect analysis for trastuzumab and lapatinib or drug combination. Multiple drug effect analysis was done as described previously (24, 27). CI values were derived from variables of the median effect plots and statistical tests were applied (unpaired, two-tailed Student's *t* test) to determine whether the mean CI values at multiple effect levels were significantly different from CI = 1. In this analysis, synergy is defined as CI values significantly lower than 1.0, antagonism as CI values significantly higher than 1.0, and additivity as CI values equal to 1.0.

Results

Activity of lapatinib in human breast cancer cells. The effects of lapatinib on human breast cancer cells were evaluated using a panel of 22 established breast cancer cell lines, which express widely varying levels of EGFR and HER-2. To determine the relative effect of expression levels of HER-2 and EGFR on lapatinib response, an ELISA was used to quantitate the EGFR and HER-2 receptor content of the various cell lines used in the *in vitro* studies (Table 1). Nine of the 22 established breast cancer cell lines (BT474, SK-BR-3, MDA-MB-361, MDA-MB-453, UACC812, UACC893, EFM192A, SUM190, and SUM225) are known to have *HER-2* gene amplification and overexpress HER-2 protein (28–30). The HER-2 protein levels in these cells ranged between 108 ng/mg protein (MDA-MB-453) and 1,161 ng/mg protein (SUM225). Three of the established breast cancer cell lines (MDA-MB-468, MDA-MB-231, and BT20) are known to express EGFR at high levels (31, 32) and the EGFR protein expression in these cell lines ranged between 58 ng/mg protein (MDA-MB-231) and 908 ng/mg protein (MDA-MB-468). All of the remaining established human breast cancer cell lines express lower levels of both HER-2 and EGFR. After quantitation of EGFR and HER-2 receptor levels in the cell lines under study, growth assays were done (Fig. 1*A*). Lapatinib inhibited the proliferation of all human breast cancer cell lines investigated in a concentration-dependent fashion; however, the IC_{50}s varied widely between the individual cell lines tested. The IC_{50}s ranged between 0.010 μmol/L in HER-2-overexpressing UACC-812 breast cancer cells and 18.6 μmol/L in MDA-MB-231 breast cancer cells that express high levels of EGFR (Table 1). Human breast cancer cell lines containing *HER-2* gene amplification and high levels of HER-2 overexpression (>200 ng/mg protein), such as BT474, UACC812, SUM190, SK-BR-3, SUM225, UACC893, and MDA-MB-361, showed IC_{50}s less than 1 μmol/L (range, 0.01-0.9 μmol/L), whereas those cell lines with *HER-2* gene amplification but lower levels of HER-2 overexpression (100-200 ng/mg protein), such as EFM192A and MDA-MB-453, showed IC_{50}s equal to 1.1 and 3.9 μmol/L, respectively. In contrast, breast cancer cell lines expressing high levels of EGFR (MDA-MB-468, BT20, MDA-MB-231) showed IC_{50}s ranging between 4.7 μmol/L (MDA-MB-468) and 18.6 μmol/L (MDA-MB-231), which were higher compared with those seen in cells with HER-2 amplification/overexpression. With the exception of MDA-MB-175 cells, all breast cancer cell lines with low HER-2 and EGFR expression (EFM19, KPL1, MDA-MB-157, MCF-7, CAMA1, T47D, MDA-MB-435, and ZR-75-1)

Table 1. Lapatinib concentrations that achieve IC_{50} and the corresponding levels of HER-2 and EGFR expression as measured by ELISA

Cell line	IC_{50} (SE), μmol/L	HER-2 (SE), ng/mg	EGFR (SE), ng/mg
UACC812	0.010 (0.007)	436 (33)	25 (5.3)
MDA-MB-175	0.012 (0.011)	20 (3.3)	3.8 (0.4)
SUM190	0.018 (0.008)	396 (29)	3.1 (0.6)
BT474	0.022 (0.020)	530 (48)	7.3 (0.7)
SK-BR-3	0.037 (0.031)	913 (114)	38 (7.4)
SUM225	0.083 (0.081)	1,161 (177)	51 (9.1)
UACC893	0.433 (0.076)	577 (77)	14 (3.2)
MDA-MB-361	0.989 (0.222)	211 (25)	6.6 (1.2)
EFM192A	1.1 (0.1)	115 (22)	1.0 (1.0)
T47D	1.9 (1.4)	11 (2.9)	31 (8.0)
CAL51	1.2 (0.8)	7.1 (1.4)	11 (0.3)
MDA-MB-453	3.9 (1.2)	108 (18)	1.3 (0.4)
EFM19	4.6 (1.7)	22 (2.3)	0.8 (0.2)
MDA-MB-468	4.7 (1.6)	0.1 (0.1)	908 (73)
KPL1	5.4 (0.5)	8.4 (1.4)	3.2 (0.4)
MDA-MB-157	6.3 (3.6)	4.3 (1.2)	59 (13)
MCF-7	7.7 (0.9)	4.7 (1.6)	2.7 (0.6)
CAMA1	8.3 (0.8)	18 (3.1)	3.2 (0.8)
MDA-MB-435	8.5 (1.3)	2.2 (0.8)	4.4 (0.8)
BT20	9.8 (0.4)	36 (9.1)	295 (36)
ZR-75-1	9.9 (2.3)	40 (4.5)	8.3 (3.5)
MDA-MB-231	18.6 (9.8)	5.2 (1.9)	58 (2.5)

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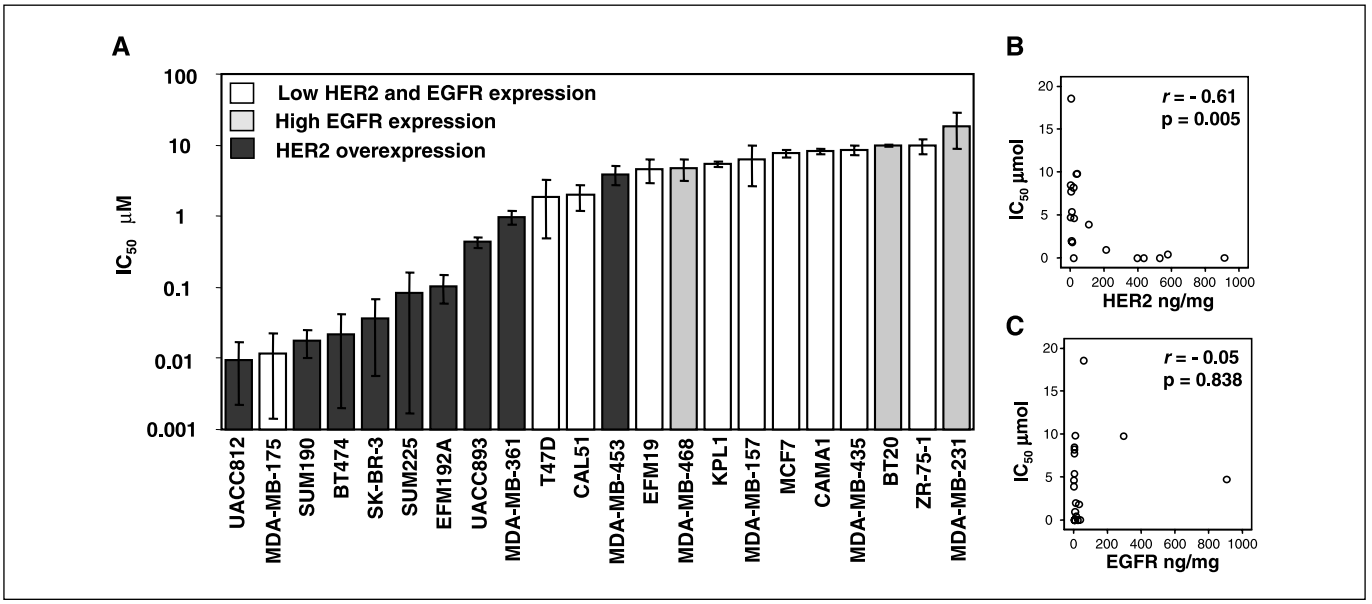

Figure 1. Growth-inhibitory effects of lapatinib were studied across a panel of human breast cancer cell lines (*A*). Cells were grown in cell line–specific medium without or with increasing doses of lapatinib (ranging between 0.008 and 10 μmol/L). Cells were trypsinized and counted after 7 days of treatment. The percent inhibition was calculated compared with untreated controls. The log of the fractional growth inhibition was plotted against the log of the drug concentration. The dose achieving IC_{50} was interpolated from the resulting linear regression curve fit. Cell lines are ordered from low to high IC_{50}s (*left* to *right*). *Bars,* SE. *B* and *C,* correlations between IC_{50}s and HER-2 (*B*) and EGFR (*C*) expression as measured by ELISA (Spearman's rho correlation for nonparametric data).

showed IC_{50}s greater than 1 μmol/L, ranging between 1.8 μmol/L (T47D) and 9.8 μmol/L (ZR-75-1). These results indicate that *HER-2* gene amplification and HER-2 overexpression is associated with a consistently higher sensitivity to lapatinib *in vitro* across various cell lines tested. Statistical analysis comparing the growth-inhibitory effect of lapatinib (IC_{50}s) with the absolute levels of HER-2 or EGFR expression reveals a direct and linear inverse correlation between sensitivity to lapatinib and HER-2 expression ($r = -0.61$, $P = 0.005$) but not EGFR expression ($r = 0.05$, $P = 0.838$; Fig. 1*B* and *C*).

Effect of engineered HER-2 overexpression on sensitivity to lapatinib. The effects of lapatinib on human breast cancer cells were further evaluated using a panel of paired control- and HER-2-transfected cells. MCF-7, T47D, and ZR-75-1 cells (none of which exhibit *HER-2* gene amplification and all of which do not overexpress HER-2) have been stably transfected previously with a full-length human *HER-2* gene and all express higher levels of HER-2 protein than control-transfected cell lines (Table 2). The IC_{50}s were statistically significantly lower in MCF-7/HER-2 ($P = 0.002$), T47D/HER-2 ($P < 0.001$), and ZR-75-1/HER-2 cells ($P < 0.001$) compared with the control-transfected MCF-7/CON, T47D/CON, and ZR-75-1/CON cells, respectively (Table 2). These results further confirm that HER-2 overexpression is associated with a consistent and predictable increase of in *in vitro* sensitivity to lapatinib.

Cell cycle analyses. Treatment of HER-2-overexpressing BT474 human breast cancer cells for 24 hours with 0.1 or 0.5 μmol/L lapatinib increases the percentage of cells with sub-2N DNA from 5% to 26% and 43%, respectively, and reduces the percentage of cells undergoing G_1 phase from 75% to 52% and 38%, respectively (Fig. 2). The increase in the number of cells with sub-2N DNA content following treatment with 0.1 or 0.5 μmol/L lapatinib for 24 hours is consistent with the results of earlier studies investigating the effects of 1 and 10 μmol/L lapatinib for 72 hours on the fraction of cells in sub-2N DNA or cell survival (22).

Table 2. Comparison of lapatinib concentrations that achieve IC_{50} and the corresponding levels of HER-2 and EGFR expression as measured by ELISA between HER-2 and control-transfected breast cancer cell lines

Cell line	IC_{50} (SE), μmol/L	HER-2 (SE), ng/mg	EGFR (SE), ng/mg
MCF-7/CON	13.6 (0.8)	3.6 (1.5)	0.6 (0.2)
MCF-7/HER-2	4.4* (1.1)	352 (46)	0.1 (0.1)
T47D/CON	4.0 (0.4)	56 (21)	0.6 (0.2)
T47D/HER-2	2.0* (0.3)	229 (23)	20 (8.6)
ZR-75-1/CON	7.1 (0.5)	69 (64)	3.1 (0.4)
ZR-75-1/HER-2	1.7* (63)	581 (63)	2.4 (0.5)

*Significant difference.

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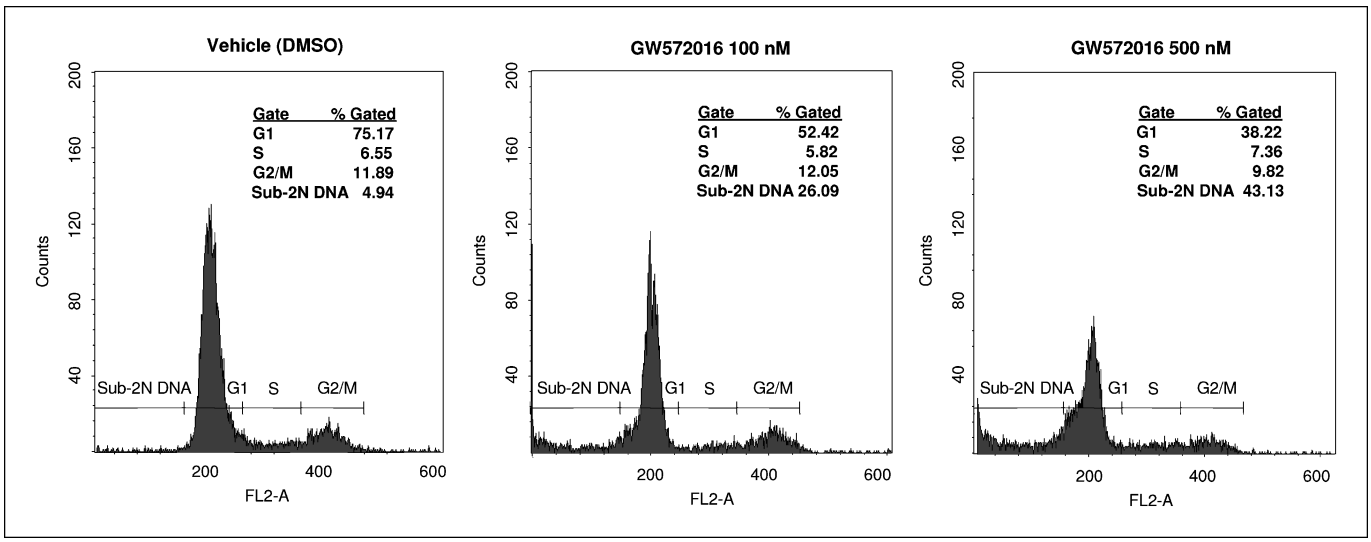

Figure 2. Cell cycle analysis of BT474 cells treated with lapatinib. Cells were treated for 24 hours with vehicle (DMSO) or lapatinib at the concentrations indicated. Cells were analyzed by flow cytometry after propidium iodine staining. The cell cycle profile was estimated by gating histograms generated with the FL2-area variable.

Long-term efficacy of lapatinib against HER-2-overexpressing breast carcinoma xenografts *in vivo*. Earlier studies have shown that 21-day twice daily dosing with lapatinib resulted in near complete inhibition of growth at 100 mg/kg and intermediate inhibition when dosed at 30 mg/kg (22). To confirm and further extend these observations, we studied the long-term *in vivo* effect of lapatinib against HER-2-overexpressing human breast cancer xenografts (Fig. 3). Lapatinib was given over 77 days at a dose of 75 mg/kg twice daily. At day 21, tumor volumes in mice that received 75 mg/kg twice daily were significantly smaller than the volumes in mice that received a vehicle control (P < 0.001). Moreover, this difference remained statistically significant for the entire duration of the experiment. Tumor volumes in mice that received 75 mg/kg lapatinib twice daily for 77 days were significantly smaller than those of mice treated with vehicle control for 21 days (P = 0.002). At day 64, however, 4 animals had reached a tumor volume of >1,000 mm^3, indicating that despite extended suppression of tumor growth in the majority of animals development of resistance did occur in individual tumors. The remaining 12 of 16 animals, nevertheless, had a median tumor burden of 303 mm^3 at day 64 compared with an initial median tumor volume of 244 mm^3.

Lapatinib in trastuzumab-conditioned HER-2-overexpressing breast cancer cells. The growth-inhibitory effects of lapatinib were evaluated in the trastuzumab-conditioned sublines BT474/mAbHER-2, SK-BR-3/mAbHER-2, and MDA-MB-361/mAbHER-2. For this purpose, the HER-2-overexpressing breast cancer cell lines BT474, SK-BR-3, and MDA-MB 361 cells were continuously grown in culture medium supplemented with 100 μmol/L trastuzumab over a time period of at least 9 months. The dose of 100 μg/mL trastuzumab was chosen because clinical trials in humans have shown trough concentrations of ~20 μg/mL and peak plasma concentrations of 185 μg/mL (33). Trastuzumab significantly inhibited growth of HER-2-overexpressing BT474 (P < 0.001), SK-BR-3 (P < 0.001), and MDA-MB-361 cells (P < 0.001) when compared with untreated controls but much less than lapatinib (Fig. 4*A*). Trastuzumab showed no growth-inhibitory effect on the trastuzumab-conditioned sublines BT474/mAbHER-2 (P = 0.34) and MDA-MB-361/mAbHER-2 (P = 0.35) when compared with untreated controls and showed less growth inhibition in SK-BR-3/mAbHER-2 when compared with untreated controls (P = 0.03; Fig. 4*B*). In contrast, lapatinib retained significant activity in the trastuzumab-conditioned sublines BT474/mAbHER-2 (P < 0.001), SK-BR-3/mAbHER-2 (P < 0.001), and MDA-MB-361/mAbHER-2 (P < 0.001) when compared with untreated controls (Fig. 4*B*).

Combination of lapatinib and trastuzumab. We next analyzed the combination of lapatinib and trastuzumab in HER-2-overexpressing breast cancer cells. Multiple drug effect analysis was done using four HER-2-overexpressing established human breast cancer cell lines to determine the nature of the interaction between lapatinib and trastuzumab (synergy, addition, or antagonism). The drug concentrations used for these experiments ranged between 0.039 and 5.0 μmol/L for lapatinib and 0.31 and 4.0 μg/mL for trastuzumab and were below the reported peak plasma concentrations achievable in humans for both drugs (33, 34).

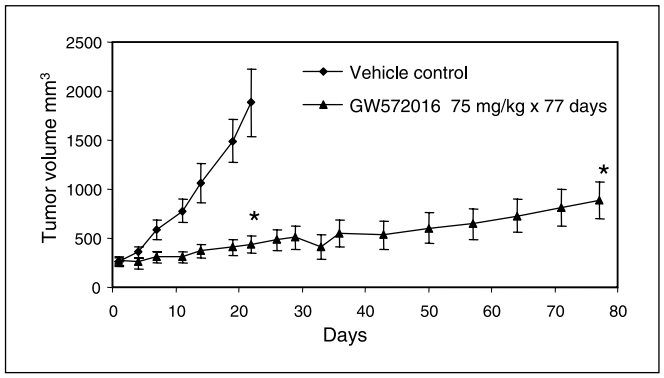

Figure 3. Tumor growth curves show the mean tumor volume as a function of time. Tumor fragments were implanted into immunocompromised mice. After establishment of tumors, animals were treated p.o. for 77 days on a twice daily schedule with lapatinib at the concentration indicated. Tumors were measured with electronic calipers. *, P < 0.001, at day 21, tumor volumes in mice that received 75 mg/kg lapatinib twice daily were significantly smaller than tumor volumes in mice that received a vehicle control. *, P = 0.002, this difference remained statistically significant for the entire duration of the experiment as volumes in mice that received 75 mg/kg lapatinib twice daily for 77 days were significantly smaller than those in mice treated with vehicle control for 21 days.

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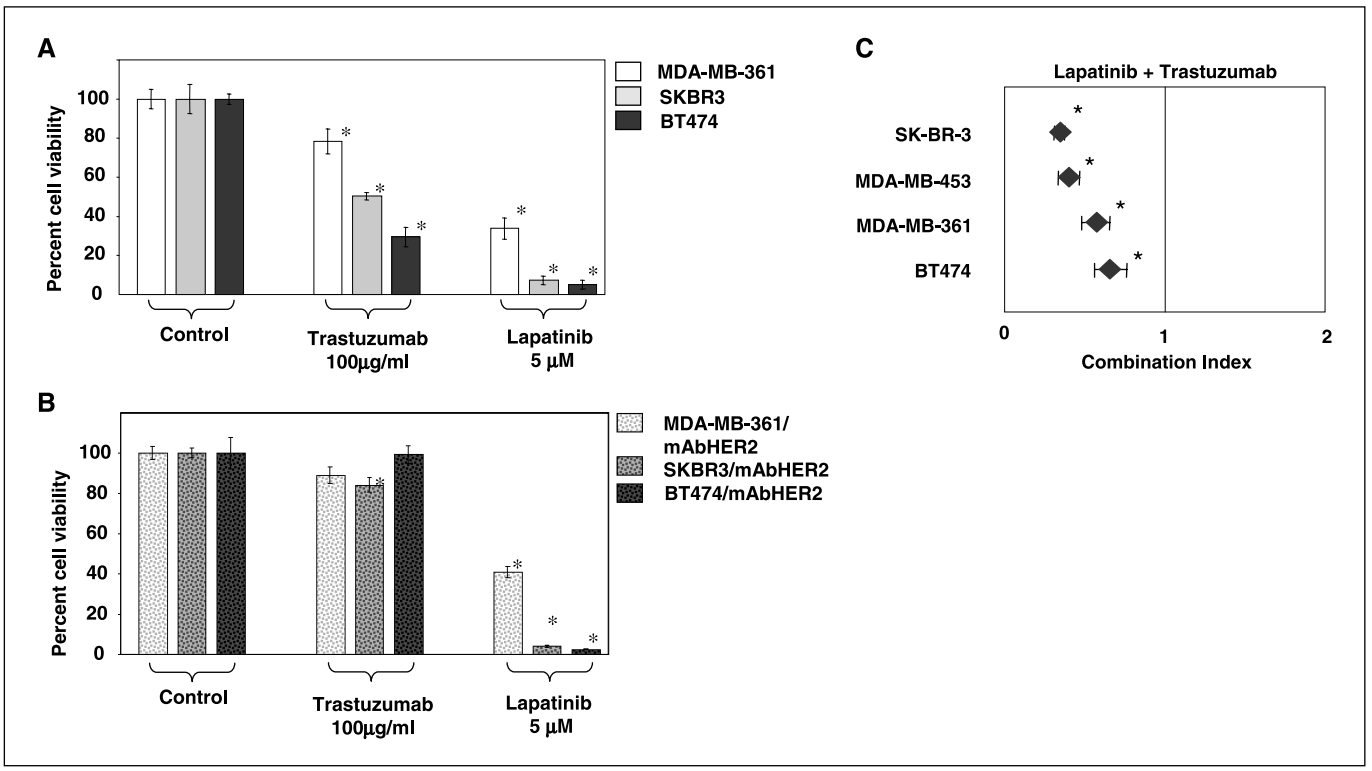

Figure 4. Growth-inhibitory effects of lapatinib were compared between established (*A*) and trastuzumab-conditioned (*B*) human breast cancer cell lines. Trastuzumab conditioned cell lines SK-BR-3/mAbHER-2, BT474/mAbHER-2, and MDA-MB-361/mAbHER-2 were generated by culturing cells for at least 9 months in RPMI 1640 supplemented with 100 μg/mL recombinant humanized monoclonal HER-2 antibody (mAbHER-2) trastuzumab. Trastuzumab was removed from the medium 24 hours before the experiments in the trastuzumab-conditioned cell lines. Cells were treated with either 5 μmol/L lapatinib or 100 μg/mL trastuzumab for 7 days. Percent cell viability was calculated compared with untreated controls. Trastuzumab significantly inhibited growth of HER-2-overexpressing BT474 ($P < 0.001$), SK-BR-3 ($P < 0.001$), and MDA-MB-361 ($P < 0.001$) cells when compared with untreated controls but much less than lapatinib. Trastuzumab showed no or less growth-inhibitory effect on the trastuzumab-conditioned sublines BT474/mAbHER-2 ($P = 0.34$), SK-BR-3/mAbHER-2 ($P = 0.03$), and MDA-MB-361/mAbHER-2 ($P = 0.35$) when compared with untreated controls, whereas lapatinib retained significant activity in the trastuzumab-conditioned sublines BT474/mAbHER-2 ($P < 0.001$), SK-BR-3/mAbHER-2 ($P < 0.001$), and MDA-MB-361/mAbHER-2 ($P < 0.001$) when compared with untreated controls. *Bars,* SE. *, significantly different percentages of cell viability compared with untreated controls. *C,* mean CI values for chemotherapeutic drug-trastuzumab combinations in four different human breast cancer cell lines. CI values were derived from variables of the median effect plots, and statistical tests were used to determine whether the CI values at multiple effect levels (IC_{20}-IC_{90}) were statistically significantly different from CI values equal to 1. CI values that are statistically significantly less than 1 indicate synergistic interactions. CI values that are statistically significantly greater than 1 indicate antagonistic interactions. CI values equal to (or not statistically significantly different from 1) indicate additive interactions. *Bars,* SE. Mean is derived from three replicates spanning clinically relevant concentration ranges sufficient to inhibit growth of control cells by 20% to 90%. *, statistically significantly less than 1 (synergistic interactions).

In vitro the two agents showed consistent strong synergistic interactions against all four cell lines with mean CI values of 0.34 ± 0.02 ($P < 0.001$) in SK-BR-3 cells, 0.40 ± 0.07 ($P < 0.001$) in MDA-MB-453 cells, 0.57 ± 0.85 ($P < 0.001$) in MDA-MB-361, and 0.66 ± 0.10 ($P < 0.001$) in BT474 cells (Fig. 4*C*).

Effects of lapatinib on HER-2 and EGFR signaling. Exposure of SK-BR-3 and BT474 human breast cancer cells to lapatinib resulted in a dose- and time-dependent reduction of phosphorylation of EGFR, HER-2, AKT, and ERK (Fig. 5*A* and *B*). We next examined the effect of lapatinib on HER-2 and EGFR receptor phosphorylation both with and without prior exposure to heregulin β1 (Fig. 5*C* and *D*). Heregulin β1 is a growth factor ligand cloned based on its ability to induce tyrosine phosphorylation of HER-2 through the formation of HER-2/HER-3 and/or HER-2/HER-4 heterodimeric complexes (1, 4). In HER-2-overexpressing breast cancer cell lines (SKBR3 and UACC-893), which constitutively express an activated HER-2 tyrosine kinase, heregulin β1 does not further increase HER-2 phosphorylation, indicating that this receptor is being maximally activated in these cell lines at baseline (35). The data indicate that heregulin β1 does not interfere with the ability of lapatinib to reduce HER-2 phosphorylation in either cell lines with constitutive HER-2 tyrosine phosphorylation or cell lines with increased HER-2 tyrosine phosphorylation following exposure to heregulin β1 (Fig. 5*C*). We next studied the ability of lapatinib to block HER-2 and EGFR receptor phosphorylation both with and without prior exposure to transforming growth factor-α (TGF-α), which is a native ligand of the EGFR. As expected, treatment with TGF-α strongly stimulated EGFR phosphorylation (Fig. 5*D*), but lapatinib consistently decreased EGFR phosphorylation back to basal levels. Importantly, however, basal EGFR phosphorylation following exposure to TGF-α and subsequent treatment with lapatinib were higher compared with EGFR phosphorylation levels following lapatinib treatment without exposure to TGF-α. These findings indicate that lapatinib did not completely inhibit phosphorylation of EGFR following stimulation with TGF-α. On ligand binding, the EGFR can form heterodimeric complexes with neighboring HER-2 receptors (36, 37) and transactivate HER-2. As expected, TGF-α induced HER-2 tyrosine phosphorylation in breast cancer cells without constitutive HER-2 phosphorylation (Fig. 5*C*). Consistent with heregulin β1 data, TGF-α-induced transactivation of HER-2 did not reverse the inhibitory effects of lapatinib on the HER-2 tyrosine kinase (Fig. 5*C*).

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Correlation between receptor signaling and *in vitro* response to lapatinib. We investigated whether the *in vitro* activity of lapatinib was associated with its ability to inhibit baseline activity (phosphorylation) of HER-2, EGFR, and the key signal transduction mediators of the HER-1/HER-2 pathways, such as AKT, Raf, and ERK (Fig. 6*A*). To quantitate EGFR and HER-2 receptor activity, we did immunoprecipitations and Western blotting to detect phosphorylated EGFR or HER-2. Additional statistical analysis comparing EGFR and HER-2 receptor phosphorylation with *in vitro* sensitivity to lapatinib (IC_{50}s) reveals that the level of phosphorylated HER-2 inhibition was inversely correlated with the IC_{50}s ($r = -0.47$, $P = 0.043$); however, a significant correlation was not seen for inhibition of EGFR phosphorylation ($r = -0.35$, $P = 0.139$; Fig. 6*B*).

We further evaluated the phosphorylation status of AKT, Raf, and ERK using phosphospecific primary antibodies (Fig. 6*A*). AKT phosphorylation has been linked to inhibition of apoptosis, thus enhancing cell survival (38). In addition, mitogenic signals transduced through growth factor receptors ultimately also converge on the common downstream effectors Raf and subsequently ERK (39). When studying the panel of breast cancer cell lines, inhibition of pRaf ($r = -0.65$. $P = 0.003$), pAKT ($r = -0.78$, $P < 0.001$), and pERK ($r = -0.78$, $P < 0.001$) was significantly inversely correlated with the IC_{50}s (Fig. 6*B*).

Discussion

Dual kinase inhibition directed against EGFR and HER-2 kinases is an attractive potential approach for treatment of breast cancer, as HER-2 and EGFR expression have both been reported to be dysregulated in breast cancer (1). Lapatinib inhibits the purified HER-2 and EGFR tyrosine kinase at nanomolar concentration in cell-free biochemical assays and effectively inhibits EGFR and HER-2 phosphorylation as well as tumor cell growth at comparably low nanomolar concentration in selected cell lines with constitutive activity of either HER-2 or EGFR (22). Evaluation of the antiproliferative effect of lapatinib in our panel of breast cancer cell lines, however, indicates that the efficacy of the dual kinase inhibitor does differ widely between individual cell lines that express different levels of HER-2 and EGFR. This variability underscores the need to evaluate multiple cell lines to determine the therapeutic potential of lapatinib for breast cancer treatment. The current data show that lapatinib is more potent in inhibiting cell growth in human breast cancer cell lines that overexpress HER-2 compared with those which

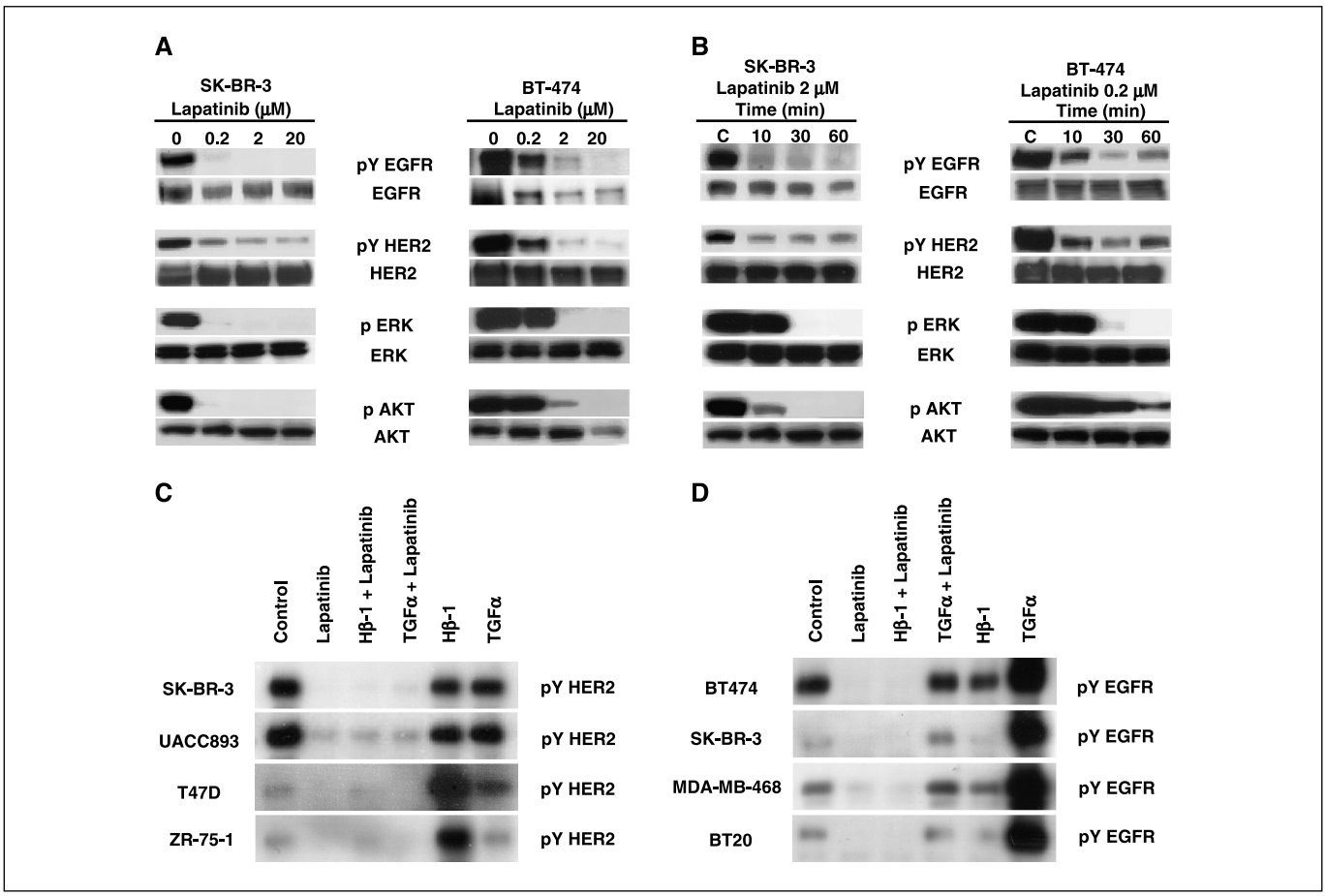

Figure 5. Dose-dependent (*A*) and time-dependent (*B*) activity of lapatinib on HER-2, EGFR, AKT, and ERK phosphorylation in SK-BR-3 and BT474 cells. Both cell lines were treated with increasing doses of lapatinib (0.2-20 μmol/L) for 1 hour (*A*) or for increasing duration (*B*; 10-60 minutes) with 2 or 0.2 μmol/L lapatinib. Immunoprecipitation and Western blotting to detect phosphorylated HER-2 or EGFR were done as described in Materials and Methods. ERK and AKT phosphorylation levels were detected using phosphospecific ERK and AKT antibodies as described in Materials and Methods. Effect of heregulin β1 and TGF-α on the activity of lapatinib on HER-2 (*C*) and EGFR (*D*) receptor phosphorylation. Cells in log-phase growth were treated with 5 μmol/L lapatinib or vehicle control (DMSO) for 1 hour before cell lysis. Cells were stimulated with ligand (10 nmol/L TGF-α or 5 nmol/L heregulin β1) 10 minutes before cell lysis. Immunoprecipitation and Western blotting to detect phosphorylated EGFR or HER-2 were done as described in Materials and Methods.

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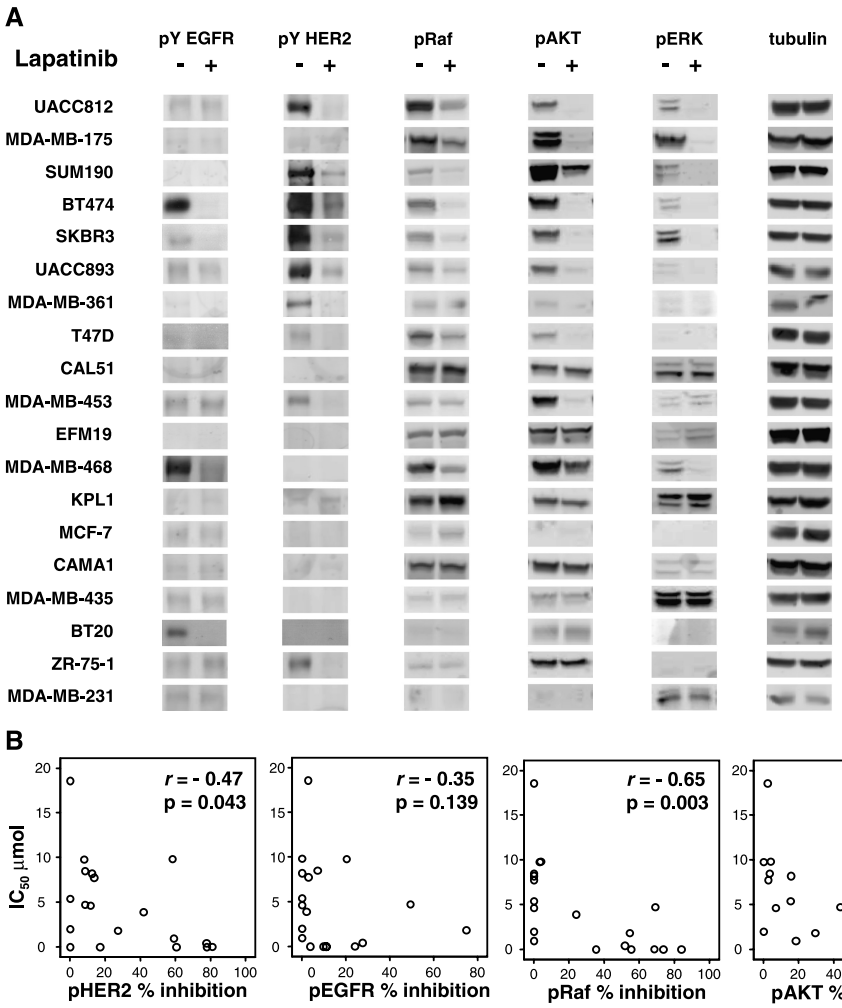

Figure 6. Comparison of the effect of lapatinib on baseline activity of HER-2, EGFR, Raf, AKT, and ERK (*A*). Cell lines are ordered from low to high IC_{50}s (*top* to *bottom*). Cells in log-phase growth were treated with 5 μmol/L lapatinib or vehicle control (DMSO) for 1 hour before cell lysis. Immunoprecipitation and Western blotting to detect phosphorylated EGFR or HER-2 were done as described in Materials and Methods. Raf, ERK, and AKT phosphorylation levels were determined using polyclonal anti-pRaf-1 (Ser^{388}), anti-pAKT (Ser^{473}), and anti-pERK (Tyr^{202}/Tyr^{204}) antibodies. The blots were incubated with a fluorescein-linked secondary antibody and detection was done by enhanced chemifluorescence (Typhoon 9400). Equal loading and exposure time were controlled for by measuring and comparing the tubulin content per sample. *B,* correlations between IC_{50}s and percent reduction of baseline HER-2, EGFR, Raf, AKT, and ERK phosphorylation. Quantitation of immunoblots was done by densitometry using the ImageQuant software. Correlations were analyzed by calculation of the nonparametric Spearman's rho correlation coefficient. All statistical tests are two sided.

express high levels of EGFR or low levels of either receptor. Despite comparable biological inhibitory activity of lapatinib on both EGFR and HER-2 tyrosine kinases, the growth-inhibitory effect of lapatinib in human breast cancer cells seems to track more closely with its anti-HER-2 effects.

The association between HER-2 overexpression and increased response to lapatinib was substantiated using a panel of paired control- and HER-2-transfected cells. This pairing of isogenic parent/daughter cells allows us to isolate HER-2 expression as a variable and directly assess the biological consequences of its overexpression on lapatinib response. These data confirm that breast cancer cells with HER-2 overexpression are significantly more sensitive to lapatinib compared with control cell lines *in vitro*. Moreover, our studies indicate that inhibition of tyrosine phosphorylation of HER-2, Raf, AKT, and ERK were also significantly associated with *in vitro* response to lapatinib, suggesting that these may also be useful markers to predict response to this molecule. This will, however, require confirmation in prospective clinical trials.

To further validate the activity of lapatinib in HER-2-overexpressing breast cancer cells, parallel *in vitro* studies were conducted measuring the fragmentation of genomic DNA into sub-2N DNA (sub-G_1 DNA), generally considered a hallmark of apoptosis, following treatment with lapatinib at concentrations of 0.1 and 0.5 μmol/L. Our results are consistent with the findings of previous studies that have shown that HER-2-overexpressing cells undergo apoptosis following treatment with lapatinib at higher concentrations of 1 and 10 μmol/L (22). We further showed that lapatinib given over 21 days at doses of 75 mg/kg twice daily nearly completely inhibits the growth of HER-2-overexpressing human breast cancer cells *in vivo* using the BT474 xenograft model. Moreover, our results of long-term *in vivo* treatment indicate that extended dosing of lapatinib over 77 days results in statistically significant suppression of tumor growth over the entire duration of the experiment.

Although it may be tempting to establish a parallel between agents inhibiting EGFR and agents inhibiting HER-2, the biology of EGFR is quite different from HER-2. EGFR is commonly expressed in breast cancer (40), but higher levels of expression per se do not define "EGFR-driven" tumors, as the effect of EGFR inhibitors is not well correlated with the levels of EGFR expression (41) and EGFR levels do not correlate with evidence of EGFR activation (42). However, in addition to its function as an individual receptor, EGFR may play an important role as a coreceptor for HER-2 (43). The cooperation that exists between EGFR and HER-2 provides a sound rationale to target EGFR particularly when HER-2 is overexpressed.

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The pure EGFR tyrosine kinase inhibitor gefitinib inhibits the growth of HER-2-overexpressing BT474, SKBR-3, and MDA-361 human breast cancer cells at low micromolar drug concentrations (17, 18). These effects may be due to inhibition in the transactivation of EGFR (rather than direct inhibition of HER-2). The cooperation that exists between EGFR and HER-2 may, however, also limit the success of agents that target individual receptors. Preclinical studies have shown that the antiproliferative activity of a HER-2 antibody can be decreased by the presence of ligand for the EGFR (44). In return, the activity of this HER-2 antibody can be restored by a tyrosine kinase inhibitor with dual activity against EGFR and HER-2 (44). Thus, simultaneous inhibition of different receptors may constitute a superior way of correcting a dysregulated signaling network.

To determine how best to use lapatinib either as a single agent or in combination with trastuzumab, we conducted a series of *in vitro* studies to evaluate its inhibitory effects in combination with trastuzumab. These preclinical studies have shown significantly enhanced activity when trastuzumab is combined with lapatinib in HER-2-positive breast cancer cells. At present, reasons for this synergy are unclear. A detailed analysis of HER-2 receptor phosphorylation following short treatment of SK-BR-3 and BT474 cells with trastuzumab, however, reveals a moderate increase of HER-2 tyrosine phosphorylation by trastuzumab treatment (45). Such an initial stimulatory effect of trastuzumab on HER-2 phosphorylation is in contrast to the pronounced immediate inhibitory effect of lapatinib on HER-2 phosphorylation as shown above in our studies (Fig. 5*B*). Thus, it may be possible that trastuzumab may initially sensitize cells to treatment with lapatinib. Furthermore, a recent study has indicated that combining lapatinib with trastuzumab enhances apoptosis in HER-2-overexpressing breast cancer cells (46). Regardless of the mechanism(s) of *in vitro* synergy, the current data indicate a consistent synergistic interaction between trastuzumab and lapatinib across all cell lines tested and clearly support further clinical evaluation of these agents in a combined regimen (47). Such strategies in the clinical setting could add more effective inhibition of the receptor tyrosine kinase to an additional important mechanism of action of trastuzumab thought to occur through immune effector cells (48).

In the current study, we were able to show activity of lapatinib against cells selected for long-term growth in trastuzumab-containing medium. These findings suggest non-cross-resistance between these two HER-2-directed agents and underscore the importance of current clinical trials evaluating lapatinib in trastuzumab-refractory breast cancer (49).

Taken together, our findings provide a rationale for clinical trials of lapatinib as a single agent or in combination with trastuzumab in HER-2-overexpressing breast cancer and suggest the potential of clinical activity in patients with resistance to trastuzumab.

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