Low-scale phosphoproteome analyses identify the mTOR effector p70 S6 kinase 1 as a specific biomarker of the dual-HER1/HER2 tyrosine kinase inhibitor lapatinib (Tykerb®) in human breast carcinoma cells

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Background: Discovery of key proliferative and/or survival cascades closely linked to the biological effects of human epidermal growth factor receptor (HER) 1 (erbB-1) and/or HER2 (erbB-2) inhibitors may identify a priori mechanisms responsible for the development of acquired resistance in breast cancer disease. Here, we took advantage of a semiquantitative protein array technology to identify intracellular oncogenic kinases that distinctively correlate with breast cancer cell sensitivity/resistance to the dual-HER1/HER2 tyrosine kinase inhibitor lapatinib (Tykerb®). Materials and methods: MCF-7 cells were forced to overexpress HER2 following stable transduction with pBABE-HER2 retroviruses. The Human Phospho-MAPK Array Proteome Profiler™ (R&D Systems) was used to molecularly assess the effects of both the mono-HER2 inhibitor trastuzumab (Herceptin™) and the dual-HER1/HER2 inhibitor lapatinib on 21 different oncogenic kinases. A model of acquired resistance to lapatinib (MCF-7/HER2-Lap10 cells) was established by chronically exposing MCF-7/HER2 cells to increasing concentrations of lapatinib for >10 months. Results: Treatment of MCF-7/HER2 cells with either trastuzumab or lapatinib similarly impaired HER2-enhanced activation status (i.e. phosphorylation) of the mitogen-activated protein kinases, c-Jun N-terminal kinases 1–3 and p38β/α/γ/δ and of the serine/threonine kinases AKT, glycogen synthase kinase-3, p90 ribosomal s6 kinase1/2, and mitogen- and stress-activated protein kinase1/2. Trastuzumab was less effective than lapatinib at blocking extracellular-signal-regulated kinase (ERK) 1/2 and, notably, it failed to deactivate the mammalian target of rapamycin (mTOR) effector p70S6K1. Conversely, lapatinib treatment caused a drastic decrease in the phosphorylation of p70S6K1 at ERK1/2-regulated sites (Thr389/Ser407) and, as a consequence, p70S6K1 activity measured by its phospho-Thr389 levels was abolished. The mTOR inhibitor rapamycin was found to supraadditively increase lapatinib efficacy in MCF-7/HER2 cells (~10-fold enhancement; combination index (CI) < 0.920 < 1.0 = additivity, P < 0.001) but not in p70S6K1 gene-amplified MCF-7 parental cells (~1.3-fold enhancement; CI = 0.920 = 1.0 = additivity). Lapatinib-resistant MCF-7/HER2-Lap10 cells, which are capable of growing in the continuous presence of 10 μM lapatinib without significant effects on cell viability, notably exhibited a lapatinib-insensitive hyperphosphorylation of p70S6K1. Rapamycin cotreatment suppressed p70S6K1 hyperactivation and synergistically sensitized MCF-7/HER2-Lap10 cells to lapatinib (<20-fold increase in lapatinib-induced cytotoxicity; CI = 0.175 < 1.0 = additivity). Conclusions: Serine-threonine kinase p70S6K1, a marker for mTOR activity that regulates protein translation, constitutes a specific biomarker for the biological effects of the dual-HER1/HER2 inhibitor lapatinib. The clinical implications of our data are that the efficacy of lapatinib might be enhanced with therapies that target the mTOR pathway. Rapamycin analogues such as CCI-779 (Temsirolimus) and RAD001 (Everolimus) may warrant further clinical evaluation to effectively delay or prevent the development of acquired resistance to lapatinib in HER2-positive breast cancer patients.

Key words: breast cancer, HER2, lapatinib, mTOR, proteomics, rapamycin, tyrosine kinases

Introduction

Human epidermal growth factor receptor 1 [(HER1)—erbB-1, EGFR] and HER2 (erbB-2) tyrosine kinase receptors promote tumor growth and survival in a variety of epithelial tumors, where their overexpression and/or hyperactivation correlates with a poor clinical outcome [1–5]. A number of treatment strategies are currently being used to target HER receptors in breast carcinomas, including mAbs directed against their extracellular domains and small molecule blockers of their tyrosine kinase activities [6–10]. Trastuzumab (Herceptin™,
Roche, Basle, Switzerland), a humanized mAb targeting the extracellular domain of HER2, is approved for treating patients whose breast cancers overexpress HER2 protein or demonstrate HER2 gene amplification [11–13]. Unfortunately, not all HER2-overexpressing breast carcinomas respond to treatment with trastuzumab and its clinical benefit is limited by the fact that resistance develops rapidly in virtually all trastuzumab-treated patients [14–17]. Indeed, 70%–80% of patients with HER2-overexpressing breast cancer do not respond to trastuzumab given as single-agent therapy due to either primary or acquired resistance. Because lapatinib (Tykerb®) inhibits the tyrosine kinase activity of HER2 itself in addition to that of the HER2 coreceptor HER1 [18–20], this small molecular inhibitor might represent a valuable strategy to effectively inhibit survival of cancer cells that have progressed on trastuzumab [21]. Indeed, lapatinib has recently been found to show activity in cultured breast cancer cells with acquired resistance to the HER2 antibody trastuzumab [22], and a phase 1 study of lapatinib has shown clinical activity in patients with trastuzumab-refractory breast cancer [23]. Unfortunately, results from early-phase trials indicate that clinical responses to lapatinib monotherapy in patients with HER2-overexpressing breast carcinomas are generally short lived. Although it has recently been indicated that lapatinib resistance is mediated, at least in part, by enhanced estrogen receptor (ER) signaling [24], the ultimate mechanisms by which breast cancer cells escape lapatinib-mediated growth inhibition and acquire resistance to lapatinib after long-term exposure remain largely unknown [25–30].

It has been hypothesized that pharmacodynamic studies should be included in clinical trials of signal transduction inhibitors in cancer patients, in order to individualize early markers of response to such agents. It has also been demonstrated that measurement of markers or proliferation or apoptosis may prove more informative than the evaluation of intracellular signaling proteins in monitoring the effects of target-based agents. In any case, regardless of the effects of HER-targeting inhibitors on the activation status of HER1 and/or HER2, it is the repercussions of HER inhibitor on downstream signaling pathways that correlate with tumor growth inhibition. Identification of these pathways and whether they are operative or not in the presence of HER inhibitors may enable therapeutic decisions to be based on tumor biology rather than histology alone. Discovery of key proliferative and/or survival cascades closely linked to the biological/clinical effects of HER1/HER2 inhibitors may also identify a priori mechanisms responsible for the development of acquired resistance. Here, we took advantage of a low-scale phosphoproteomic approach (i.e., Human Phospho-MAPK Array Proteome ProfilerTM, R&D Systems, Inc., Minneapolis, MN) to molecularly profile intracellular oncogenic kinases that distinctively correlate with breast cancer cell sensitivity/resistance to the dual-HER1/HER2 tyrosine kinase inhibitor lapatinib. Using MCF-7 breast cancer cells engineered to stably overexpress HER2 following stable transduction with pBabe/HER2 retroviruses (i.e., MCF-7/HER2 cells) and developing a model of acquired lapatinib resistance by chronically exposing lapatinib-sensitive MCF-7/ HER2 cells to high-dose lapatinib for >10 months—which simulates the clinic where patients receive lapatinib on a daily chronic basis—we here reveal that serine-threonine kinase p70S6K1, a marker for mammalian target of rapamycin (mTOR) activity that regulates protein translation [31–37], not only constitutes a specific biomarker for the biological effects of the dual-HER1/HER2 inhibitor lapatinib but also further represents a molecular target for delaying or preventing the development of lapatinib-acquired autoresistance in HER2-overexpressing breast cancer cells.

materials and methods
drugs
Trastuzumab (Herceptin™)—kindly provided by Hospital Universitari de Girona Dr Josep Trueta Pharmacy (Girona, Spain) — was solubilized in bacteriostatic water for injection containing 1.1% benzyl alcohol (stock solution at 21 mg/ml), stored at 4°C and used within 1 month. Lapatinib (GW572016; Tykerb®) was gently provided by GlaxoSmithKline and Corporate Environment, Health and Safety (Brentford, Middlesex, UK). Rapamycin was purchased from Sigma Chemicals (St Louis, MO).

Lapatinib and rapamycin were dissolved in dimethyl sulfoxide and stored in the dark as stock solutions (10 mM) at −20°C until utilization. For experimental use, trastuzumab, lapatinib, and rapamycin were prepared freshly from stock solutions and diluted with growth medium. Control cells were cultured in medium containing the same concentration (by vol) as the experimental cultures with treatments. The vehicle solutions had no noticeable influence on the proliferation of experimental cells.

cell lines and culture conditions
MCF-7 and SKBR3 breast cancer cell lines were obtained from the American Type Culture Collection, Manassas, VA, and they were routinely grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco® Cell Culture Systems, Invitrogen S.A., Barcelona, Spain) containing 10% fetal bovine serum (FBS, Bio-Whittaker, Inc., Walkersville, MD), 1% l-glutamine, 1% sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO2. Cells were screened periodically for Mycoplasma contamination.

construction of pBABE/HER2 retroviruses and retroviral infection
A full-length human HER2 complementary DNA construct in the pCMV-SPORT6 plasmid was purchased from Resource Center for Genome Research (Berlin, Germany). The insert was excised from pCMV-SPORT6 using EcoRV and NcoI sites and blunt end ligated into the pBABE-puro retroviral vector (Addgene, Inc., Cambridge, MA) at the EcoRI site. Retroviruses were generated by cotransfection of 293T-derived phoenix packaging vector (Addgene, Inc., Cambridge, MA) at the EcoRI site. To establish MCF-7/HER2-Lap10 lapatinib-acquired resistant cells, 293T cells were cultured at 5% CO2, 37°C periodically for 10 months before starting any experimental procedure. Briefly, 1098 | Vazquez-Martin et al. | Volume 19 | No. 6 | June 2008
MCF-7/HER2 cells were exposed to 5 μM lapatinib for 1 month followed by 10 μM lapatinib for another 2 months and 20 μM lapatinib for 1 month. Following this 4-month period, medium was replaced twice weekly and cells were passaged at 70% confluence always in the presence of 10 μM lapatinib. The resistant pool was maintained in medium without lapatinib for at least 2 days before each experiment.

HER2-specific ELISA

Determination of HER2 protein content was carried out with a commercially available quantitative ELISA (Human neu Quantitative ELISA System; Oncogene Science, Cambridge, MA) according to the manufacturer’s protocol. The concentrations of HER2 in test samples (in nanograms of HER2 per milligram of total protein) were determined by interpolation of the sample absorbances from the standard curve. Each experiment was carried out in duplicate wells.

phosphor-proteome profiling

MCF-7, MCF-7/HER2, and MCF-7/HER2-Lap10 cells were plated in 100-mm tissue culture dishes and cultured in DMEM with 10% FBS until they reached 75%–80% confluence. The cells were washed twice with serum-free DMEM and incubated overnight in serum-free DMEM. Cells were then cultured in low-serum (0.1% FBS) DMEM in the presence or absence of experimental agents, as specified. Cells were rinsed with cold phosphate-buffered saline (PBS) and immediately solubilized in NP-40 lysis buffer [1% NP-40, 20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin] by rocking the lysates gently at 4°C for 30 min. Following microcentrifugation at 14,000 g for 5 min, supernatants were transferred into a clean test tube and sample protein concentrations were determined using the Pierce Protein assay kit (Rockford, IL). Five hundred micrograms of lysates were diluted and incubated with the Human Phospho-MAPK Array Kit (Proteome Profiler™, R&D Systems) as per the manufacturer’s instructions. Phospho-MAPK Array data were developed on X-ray films following exposure to chemiluminescent reagents.

immunoblotting analyses: activation status of HER2 and p70S6K1

Testing for the phosphorylation (activation) status of HER2 and p70S6K1 was carried out by immunoblotting procedures using the monoclonal c-erbB-2/HER2 (phosphor specific) antibody Ab-18 (NeoMarkers, Fremont, CA) and the PhosphoPlus™ p70 S6 kinase (Thr389 and Thr421/Ser424) Antibody Kit (Cell Signalling Technology, Inc., Beverly, MA), respectively. The Ab-3 (Oncogene Research Products, Cambridge, MA) anti-HER2 mouse mAb was employed for assaysing levels of total HER2 protein. Briefly, trastuzumab-treated, lapatinib-treated, and untreated control cells were washed twice with cold PBS and then lysed as described above. Equal amounts of protein (i.e. 50 μg) were resuspended in 5x Laemli sample buffer (10 min at 70°C), resolved by electrophoresis on 3%–8% NuPAGE Tris-Acetate (HER2) or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (p70S6K1), and transferred onto nitrocellulose membranes. Nonspecific binding on the nitrocellulose filter paper was minimized by blocking for 1 h at room temperature (RT) with TBS-T buffer [25 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] containing 5% (mass/vol) nonfat dry milk. The treated filters were washed in TBS-T and then incubated with phospho-c-erbB-2/HER2 (clone PN2A), phospho-p70 S6 kinase Thr389 (clone 108D2), phospho-p70 S6 kinase Thr421/Ser424, or total p70 S6 kinase antibodies in 5% mass/vol bovine serum albumin, 1× TBS-T buffer, and 0.1% Tween 20 at 4°C with gentle shaking overnight.

For protein loading and transfer, densitometric values of protein bands were quantified using the Scion Image software (Scion Corporation, Frederick, MD).

cell proliferation assays

To test the effects of lapatinib, rapamycin, and their combination on cell proliferation, cells were plated into 96-well, flat-bottomed plates at 2–4 × 10^3 cells per 100 μl well. After the overnight incubation, triplicate wells were treated with varying concentrations of lapatinib in the absence or presence of 1 nM rapamycin for 5 days. Relative percentage of metabolically active cells relative to untreated controls was then determined on the basis of the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazone. The amount of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide that is converted to formazone indicates the number of viable cells. The results were assessed in a 96-well format plate reader by measuring the absorbance at a wavelength of 570 nM (A570 nm). The percentage of metabolically active cells was compared with the percentage of control cells growing in the absence of lapatinib and/or rapamycin in the same culture plate. The IC50s were determined by nonlinear regression analysis using the equation for a sigmoid plot.

The degree of sensitization to lapatinib by rapamycin was evaluated by dividing IC50 values of control cells (i.e. in the absence of rapamycin) by those obtained when cells were simultaneously exposed to rapamycin.

determination of synergism and antagonism: isobologram analysis

The nature of the interaction between lapatinib and rapamycin was evaluated by the isobologram technique, a dose-oriented geometric method of assessing drug interactions [38–40]. In the isobologram method, the concentration of one agent producing a desired (e.g. 50% inhibitory) effect is plotted on the horizontal axis and the concentration of another agent producing the same degree of effect is plotted on the vertical axis; a straight line joining these two points represents zero interaction (additivity) between two agents. The experimental isoeffect points are the concentrations (expressed relative to the IC50 value) of the two agents which when combined decreased cell viability by 50%. When the experimental isoeffect points fall below that line, the combination effect of the two drugs is considered to be supraadditive or synergistic, whereas antagonism occurs if the point lies above it. A quantitative index of these interactions was provided by the isobologram equation combination index (CI)50 where, for this study, A and B represent the respective concentrations of lapatinib and rapamycin required to produce a fixed level of inhibition (IC50) when administered alone, a and b represent the concentrations required for the same effect when lapatinib and rapamycin were administered in simultaneous combination, and CI50 represents an index of drug interaction (CI). CI values of <1 indicate synergy, a value of 1 represents additivity and values of >1 indicate antagonism. For all estimations of CI50, we used only isoboles where intercept data for both axes were available.

apoptosis assays

The ability of lapatinib, rapamycin, and their combination to induce apoptosis was assessed using the Cell Death Detection ELISAp pluskit obtained from Roche Diagnostics. Briefly, cells (5–10 × 10^3 per well) were grown in 96-well plates and treated, in duplicates, for 72 h with the indicated doses of lapatinib in the absence or presence of rapamycin, as specified. After treatment, the 96-well plates were centrifuged (200 g) for 10 min. The supernatant was discharged, lysis buffer was added, and samples were incubated at RT for 30 min following the manufacturer’s
instructions. Anti-histone biotin and anti-DNA peroxidase antibodies were added to each well and incubated at RT for 2 h. After three washes, the peroxidase substrate was added to each well and the plates were read at 405 nm at multiple time intervals. The enrichment of histone DNA fragments in treated cells was expressed as fold increase in absorbance as compared with control (vehicle-treated) cells.

**Statistics**

Two-group comparisons were carried out by the Student’s t-test for paired and unpaired values. Comparisons of means of three or more groups were carried out by analysis of variance (ANOVA), and the existence of individual differences, in case of significant F values at ANOVA, was tested by Scheffe’s multiple contrasts.

**Results**

phospho-proteomic profiling of MCF-7 breast carcinoma cells in the absence or presence of HER2 overexpression

MCF-7 breast cancer cells, which naturally express physiological levels of HER2, were engineered to overexpress the wild-type form of human HER2 by stable transduction with pBABE-HER2 retroviral vector, thus avoiding undesirable clone-related effects. When HER2-specific ELISA was employed to quantitatively measure the concentration of HER2, MCF-7/HER2 cells were found to express HER2 protein levels ~70 times higher than MCF-7/pBABE cells. Thus, cell lysates from empty vector-transduced MCF-7/pBABE cells and MCF-7/HER2 cells were found to stably express ~10 ng HER2 mg protein~1 and ~750 ng HER2 mg protein~1, respectively. ELISA-based characterization of HER2 overexpression in MCF-7/HER2 cells (~75-fold increase when compared with MCF-7/pBABE-matched control and MCF-7 parental cells) was further confirmed by immunoblotting procedures.

To simultaneously identify the activation status of all three major mitogen-activated protein kinases (MAPKs), the extracellular-signal regulated kinases (ERK1/ERK2), c-Jun N-terminal kinases (JNK 1–3), and different p38 MAPK isoforms (α, β, γ, and δ) and other intracellular kinases, such as AKT, glycogen synthase kinase (GSK-3), p90 ribosomal S6 kinase (RSK1/2), mitogen- and stress-activated protein kinase (MSK1/2), and p70 S6 kinase 1 (p70S6K1) in our experimental systems, we took advantage of the recently developed Human Phospho-MAPK Array (Proteome Profiler™, R&D Systems), a rapid, sensitive, and semiquantitative tool able to identify the levels of phosphorylation of multiple intracellular kinases (Figure 1A). This protein array technology allows the screening of multiple proteins without carrying out numerous immunoprecipitation and/or western blot analyses. No significant activation of any intracellular kinase could be detected when using the Human Phospho-MAPK Array in whole-cell lysates from MCF-7/pBABE control cells (Figure 1B–F). The sole exception occurred with HSP27 and p70S6K1, which were present at high levels in MCF-7/pBABE and MCF-7 parental cells (data not shown), indicating that HSP27 and p70S6K1 were highly activated in these cells. These findings agree with earlier reports demonstrating that the MCF-7 strain constitutively express high levels of the small heat shock protein HSP27 while naturally bearing a genomic amplification of the p70S6K1 gene on chromosome 17q23 [33,41,42]. Forced overexpression of HER2 significantly increased the activation (i.e. phosphorylation) status of ERK1/ERK2, JNK 1–3, p38 MAPK isoforms, AKT, GSK-3, RSK1/RSK2, and MSK1/MSK2 as assessed by the Human Phospho-MAPK Array in whole-cell lysates from MCF-7/HER2 cells (Figure 1B–F). Constitutive phosphorylation of HSP27 was slightly increased upon overexpression of HER2 in MCF-7 cells. High levels of phospho-p70S6K1 in MCF-7 parental cells were notably increased in MCF-7/HER2 cells.

The mTOR effector p70S6K1 is exclusively inhibited by the dual-HER1/HER2 inhibitor lapatinib but not by the mono-HER2 inhibitor trastuzumab

Treatment with either trastuzumab (100 µg/ml, 24 h) or lapatinib (10 µM, 24 h) similarly affected the HER2-regulated activation status of the MAPKs JNK 1–3 (Figure 1C) and p38A/β/γ/δ (Figure 1D), of the serine/threonine kinases AKT, GSK-3, RSK1/RSK2, MSK1/MSK2, and of the small heat shock protein HSP27 in MCF-7/HER2 cells (Figure 1E and F). Trastuzumab was significantly less effective than lapatinib at blocking ERK1/2 MAPK (Figure 1B) and, notably, it completely failed to deactivate p70S6K1 in MCF-7/HER2 cells (Figure 1F).

Conversely, lapatinib drastically reduced phosphor-p70S6K1 to levels even lower than those observed in p70S6K1 gene-amplified MCF-7 parental cells (Figure 1F).

Activation of p70S6K1 is a multistep process beginning with the phosphorylation of amino acid residues Ser411, Ser418, Thr389, and Ser422 within the autoinhibitory domain by proline-directed kinases such as MAPK, and this process is followed by mTOR-mediated phosphorylation of Thr389, an indicator of p70S6K1 activity [43–46]. Since Human Phospho-MAPK Array (Proteome Profiler™) membranes employ p70S6K1Thr389-specific antibodies, we further characterized the activation status of the mTOR effector p70S6K1 by immunoblotting procedures with phosphopeptide-specific antibodies. Lapatinib caused a drastic decrease in the phosphorylation of p70S6K1 on MAPK-regulated sites (Thr389/Ser344) (data not shown). Treatment with trastuzumab failed to modulate p70S6K1 activity as measured by the phospho-Thr389 status of p70S6K1. Conversely, phospho-Thr389 forms of p70S6K1 were undetectable following treatment with lapatinib (Figure 1G).

pharmacological inhibition of mTOR activity synergistically enhances lapatinib efficacy

Given that dual inhibitor treatment (i.e. lapatinib) was necessary to completely block p70S6K1 activity, the above results indicated that HER1 and HER2 receptors cooperate in the regulation of the mTOR effector p70S6K1 in MCF-7/HER2 cells. To evaluate whether this cooperation is targeted to the mTOR translational signaling pathway to drive cell proliferation, we characterized the efficacy of lapatinib in the absence or presence of the mTOR inhibitor rapamycin.

The antiproliferative effects of lapatinib and rapamycin were initially assessed by characterizing the metabolic status of MCF-7/pBABE and MCF-7/HER2 cells, following treatments with graded concentrations of the agents (Figure 2A).
HER2-overexpressing breast cancer cells likewise exhibited an exacerbated sensitivity to lapatinib. Thus, lapatinib IC$_{50}$ value (i.e. the concentration of the drug necessary to reduce cell proliferation by 50%) was as low as 0.35 ± 0.01 μM in MCF-7/HER2 cells, whereas this value was ~7-fold higher in HER2-negative MCF-7/pBABE cells (IC$_{50}$ = 2.35 ± 0.12 μM; Figure 2A). There were no statistically significant differences on the antiproliferative activity of rapamycin against MCF-7/HER2 and MCF-7/pBABE-matched control cells (7.0 ± 0.01 nM in MCF-7/HER2 cells versus 6.5 ± 0.02 nM in MCF-7/pBABE cells; Figure 2B). The sensitizing effects of rapamycin on the sensitivity of HER2-negative MCF-7 cells and HER2-overexpressing MCF-7/HER2 cells to lapatinib are shown in Figure 2C. In order to measure changes in lapatinib efficacy in the absence or presence of rapamycin, a ‘sensitization factor’ was determined by dividing the IC$_{50}$ value for lapatinib as single agent by that IC$_{50}$ value obtained upon coexposure to rapamycin. Remarkably, a statistically significant 10-fold increase in the antiproliferative effects of lapatinib was observed when MCF-7/HER2 cells were cocultured in the presence of 1 nM rapamycin ($P < 0.001$; two-sided ANOVA analysis). Coexposure to rapamycin failed to significantly increase lapatinib efficacy in HER2-negative MCF-7 cells. To further delineate the nature of the interaction (i.e. antagonism, addition, and synergism) between lapatinib and rapamycin, we carried out isobologram transformations of the lapatinib–rapamycin antiproliferative effects. A representative transformation is presented graphically (isobologram) in Figure 2D. The straight line drawn between the IC$_{50}$ value for lapatinib alone and the IC$_{50}$ value for rapamycin alone indicates the alignment of theoretical isoeffect data points for additive interactions. The true IC$_{50}$ values (i.e. the experimental concentrations of lapatinib and rapamycin which combined produced 50% reduction in cell proliferation) were plotted and compared with the additive line. Data points above the ‘additive line’ indicate antagonism and those below the

Figure 1. Phosphoproteomic analysis of trastuzumab/lapatinib-treated MCF-7/human epidermal growth factor receptor (HER) 2 cells. (A) Template showing the location of MAPK antibodies spotted onto the Human Phospho-MAPK Array Kit (Proteome Profiler; R&D Systems). (B–F) Detection of trastuzumab/lapatinib-modulated oncogenic intracellular kinases in MCF-7/HER2 breast cancer cells. Five hundred micrograms of total cell lysates from overnight serum-starved MCF-7/pBABE and MCF-7/HER2 untreated (control) cells as well as 500 μg of total cell lysates from MCF-/HER2 cells cultured in 0.1% fetal bovine serum-containing Dulbecco’s Modified Eagle’s Medium (DMEM) in the presence of either 100 μg/ml trastuzumab or 10 μM lapatinib for 24 h were incubated with membranes of the Human Phospho-MAPK Array Kit as per the manufacturer’s instructions. Phospho-MAPK Array data were developed on X-ray films following exposure to chemiluminescent reagents. Figure shows a representative phosphoproteome analysis of (B) extracellular-signal regulated kinase (ERK) 1/2 mitogen-activated protein kinases (MAPKs), (C) c-Jun N-terminal kinases (JNKs) and mitogen- and stress-activated protein kinases (MSKs), (D) p38 MAPKs, (E) p90 ribosomal S6 kinases (RSKs), glycogen synthase kinases (GSKs) and HSP27, and (F) AKTs and S6K1. Equivalent results were obtained in three independent experiments. (G) Effects of trastuzumab and lapatinib on the activation status of p70S6K1. Fifty micrograms of total protein from overnight serum-starved MCF-7/HER2 cells cultured in 0.1% FBS-containing DMEM in the presence or absence of either 100 μg/ml trastuzumab or 10 μM lapatinib for 24 h were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to immunoblotting analyses for either phosphor-p70 S6K1 (Thr421)—top—or total p70 S6K1—bottom—using the PhosphoPlus p70 S6 Kinase Antibody Kit as described in ‘materials and methods’. Figure shows a representative immunoblotting analysis. Equivalent results were obtained in three independent experiments.
Figure 2. Human epidermal growth factor receptor (HER) 2-related effects of lapatinib and rapamycin on breast cancer cell proliferation. (A and B) Figures show dose–response cell proliferation curves of MCF-7/pBARE and MCF-7/HER2 cells treated with either lapatinib (A) or rapamycin (B). Cells seeded in 96-well plates (2000–3000 per well) were cultured in triplicate in the absence or presence of graded concentrations of the agents, which were not renewed during the entire period of cell exposure. Once control—untreated—wells reached confluence, cells were exposed to 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide reagent and absorbance at 570 nm was measured in a microplate reader. The cell proliferation effects from exposure of cells to lapatinib and rapamycin were analyzed by generating concentration–effect curves as a plot of the fraction of unaffected (surviving) cells versus drug concentration. Dose–response curves were plotted as percentages of the control cells absorbance (=100%), which was obtained from wells treated with appropriate concentrations (dimethyl sulfoxide by vol) of agents vehicle that were processed simultaneously. IC50 values were designated for the concentrations of the agents decreasing absorbance values at 570 nm by 50%, as determined by interpolation. (C) Sensitizing effects of the mammalian target of rapamycin inhibitor rapamycin on lapatinib efficacy. Figure shows IC50 values of lapatinib obtained in MCF-7/pBARE and MCF-7/HER2 cells cultured in the presence or absence of 1 nM rapamycin. Data are the mean (columns) and 95% confidence intervals (bars) of three independent experiments carried out in triplicate. One-factor analysis of variance was used to analyze differences in the lapatinib IC50 values between each treatment group. All statistical tests were two-sided. (NS, not statistically significant). (D) Analysis of the nature of the interaction between lapatinib and rapamycin in MCF-7/pBARE and MCF-7/HER2 cells. The combined effect of a simultaneous exposure to lapatinib plus rapamycin was evaluated by the isobologram method using the IC50 values for MCF-7/HER2 and MCF-7/pBARE-matched control cells as described in ‘materials and methods’. When the experimental isoeffect points fall below the additivity line, the combination effect of the two drugs is considered to be supraadditive or synergistic, whereas antagonism occurs if the points lie above it. A quantitative index of these interactions was provided by the isobologram equation combination index (CI50) = (a/A) + (b/B), where, for this study, A and B represent the respective concentrations of lapatinib and rapamycin required to produce a fixed level of inhibition (IC50) when administered alone, a and b represent the concentrations required for the same effect when lapatinib and rapamycin were administered in combination, and CI50 represents an index of drug interaction (combination index). CI50 values of <1 indicate synergy, a CI50 value of 1 represents additivity, and CI50 values of >1 indicate antagonism.
diagonal indicate synergism. The experimental isoeffect for MCF-7/HER2 cells fell in the left side of the additive line, clearly denoting that a synergistic effect emerges when combining lapatinib and rapamycin in MCF-7/HER2 cells. Thus, the mean CI50 value revealed that the amount of the two agents necessary to reduce cell proliferation by 50% was only 0.243 times as much as it would be required if they demonstrated purely additive actions in MCF-7/HER2 cells. The amount of the two agents necessary to reduce cell viability by 50% was 0.920 times as much as it would be required if they demonstrated only additive actions in MCF-7/pBABE cells, thus denoting an absence of supraadditive interactions when combining lapatinib and rapamycin in HER2-negative breast cancer cells.

pharmacological inhibition of mTOR activity synergistically enhances lapatinib-induced apoptotic cell death

We speculated that increased sensitivity to the dual-HER1/HER2 inhibitor lapatinib observed in HER2-overexpressing cancer cells in the presence of the mTOR inhibitor rapamycin was not simply the result of changes in lapatinib-sensitive cell proliferation but it might actually be due to mTOR-related antiapoptotic machinery. To address this question, MCF-7/pBABE and MCF-7/HER2 cells were exposed to lapatinib in the absence or presence of rapamycin, apoptotic cell death was measured by a Cell Death ELISA kit that detects apoptosis-induced DNA histone fragmentation, and the x-fold increase in apoptosis was calculated by comparing the ELISA absorbance readings of treated samples, with the values of untreated controls cells as 1.0-fold (Figure 3). MCF-7/HER2 cells were likewise exquisitely sensitive to the proapoptotic effects of lapatinib (up to 10.5-fold increase in the presence of 5 μM lapatinib versus 1.0-fold in untreated controls; Figure 3A). Rapamycin as single agent (1 nM) increased apoptotic cell death by ~2.0 times in MCF-7/HER2 cells. Interestingly, MCF-7/HER2 cells exhibited the highest extent of apoptotic cell death following concurrent exposure to lapatinib and rapamycin (up to 17.4-fold increase).

The notion that the mTOR translational signaling pathway functionally relates to HER1/HER2-driven enhanced cell survival was further supported when analyzing this interaction in SKBR3 breast cancer cells—a widely used lapatinib-sensitive tumor cell in vitro model characterized by exhibiting natural HER2 gene amplification, HER2 receptor protein overexpression and HER2 dependency for cell proliferation and survival—and in HER2-negative MCF-7 breast cancer cells, which naturally express physiological levels of HER2 while exhibiting natural p70S6K1 gene amplification. Rapamycin and lapatinib, as single agents, increased apoptotic cell death up to ~3.0 and 12.0 times, respectively, whereas concurrent combination of lapatinib and rapamycin drastically increased apoptotic cell death up to 34.5 times in SKBR3 cells (Figure 3B). No synergistic increases in apoptotic cell death occurred when lapatinib and rapamycin were concurrently combined in MCF-7 cells (Figure 3C).

development of auto-acquired resistance to lapatinib depends on rapamycin-sensitive hyperactivation of p70S6K1

To elucidate the relevance of p70S6K1 on the acquisition of autoresistance to lapatinib, we established lapatinib-resistant MCF-7/HER2 cells (MCF-7/HER2-Lap10) through continuous exposure to this drug for a minimum of 6 months before starting any experimental procedure (see ‘materials and methods’). MCF-7/HER2-Lap10 cells are now capable of development of auto-acquired resistance to lapatinib depends on rapamycin-sensitive hyperactivation of p70S6K1

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growing in the continuous presence of 10 μM lapatinib without significant effects on cell viability. Resistance to lapatinib was initially confirmed carrying out 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based cell viability assays in the MCF-7/HER2-Lap10 pool; IC_{50} values were calculated from the percentage of viable cells after exposure to various concentrations of lapatinib. As indicated above, the IC_{50} value for the parental cells was 0.35 ± 0.01 μM, which drastically increased up to 21 ± 2 μM lapatinib (~60-fold increase) in the resistant pool (Figure 4A).

To establish whether lapatinib-mediated inhibition of p70S6K1 was altered in the resistant cells, we examined the status of p70S6K1 phosphorylation in the MCF-7/HER2-Lap10 pool in the absence or presence of lapatinib. Phospho-p70S6K1 (Thr^{389}) was drastically increased in untreated lapatinib-resistant MCF-7/HER2-Lap10 cells when compared with lapatinib-sensitive MCF-7/HER2 cells, while no changes were found on total (unphosphorylated) p70S6K1 between MCF-7/HER2-Lap10 and MCF-7/HER2 cells (data not shown). Although the phospho-p70S6K1 (Thr^{389}) (108D2) rabbit antibody detects endogenous levels of p70S6K1 only when phosphorylated at Thr^{389}, the antibody also detects activation of p85 S6 kinase when phosphorylated at the analogous site Thr^{412} and S6KII phosphorylated at Thr^{401}. Indeed, all the S6 kinase isoforms (p70S6K1, p85S6K1, and S6KII) exhibited a hyperactivation status at their Thr-sites (p70S6K1Thr^{389}, p85S6K1Thr^{412}, and S6KIIThr^{401}) in lapatinib-resistant MCF-7/HER2-Lap10 cells. Notably, this hyperactivation of all the S6 kinase isoforms remained largely unaffected following treatment with lapatinib (Figure 4B, top panel). Nonetheless, to eliminate the possibility of HER2 tyrosine kinase activity-independent mechanisms for the appearance of lapatinib-insensitive hyperphosphorylation of p70S6K1, steady-state HER2 protein levels were quantitatively determined in lapatinib-resistant MCF-7/HER2-Lap10 cells using HER2-specific ELISA. Additionally, we carried out immunoblotting analyses using a monoclonal c-erbB-2/HER2 (phosphor specific) antibody (clone PN2A), which specifically recognized the activated, tyrosine phosphorylated (P-Tyr1248) form of HER2. Acquired resistance to lapatinib was not by loss of target affinity because lapatinib-resistant MCF-7/HER2-Lap10 cells exhibited HER2 protein levels equivalent to those found in lapatinib-sensitive MCF-7/HER2 parental cells (Figure 4B, bottom panel) or was resistance related to refractoriness of HER2 tyrosine kinase activity to lapatinib because P-Tyr^{1248} HER2 inhibition was similar in MCF-7/HER2-Lap10 cells compared with that in lapatinib-treated MCF-7/HER2 parental cells (Figure 4C, bottom panel).

To further corroborate the above findings, we carried out phosphoproteome analyses of parental and resistant cell lines following short-term treatment with 10 μM lapatinib. In the parental cell line, p70S6K1 phosphorylation was rapidly inhibited following 2 h treatment with lapatinib (Figure 4C, top). In contrast, in the resistant cells, phosphorylation of ERK1/2 and ERK1/2-regulated p70S6K1 Thr^{413}/Ser^{414} phosphosites persisted at significantly higher levels in the presence of lapatinib (Figure 4C, bottom). Parallel microphotographs showing substantial differences in the antiproliferative effects of lapatinib in parental and resistant cells strongly indicate that increased p70S6K1 signaling underlies, at least in part, the acquired resistance to lapatinib in HER2-overexpressing breast cancer cells.

To determine whether mTOR activity was critical for acquired resistance to lapatinib—as reflected by lapatinib-refractory hyperphosphorylation of the mTOR effector p70S6K1—we initially assessed the appearance of changes on rapamycin sensitivity between parental and resistant cells. Rapamycin IC_{50} values slightly decreased from 7.5 ± 0.5 nM in lapatinib-sensitive MCF-7/HER2 cells to 4.5 ± 0.4 nM in lapatinib-resistant MCF-7/HER2-Lap10 cells (Figure 5A). Immunoblotting analyses of p70S6K1 phosphorylation at Thr^{389} clearly demonstrated that rapamycin treatment effectively decreases constitutive p70S6K1 hyperactivation in lapatinib-resistant cells, while cotreatments with lapatinib and rapamycin completely inhibited p70S6K1 hyperactivity in lapatinib-resistant MCF-7/HER2-Lap10 cells (Figure 4B, top panel). Moreover, cotreatment with rapamycin restored lapatinib sensitivity in the resistant pool. Thus, 0.1 nM rapamycin increased by seven-fold MCF-7/HER2-Lap10 cells sensitivity to lapatinib while 1 nM rapamycin decreased lapatinib IC_{50} value to <1 μM lapatinib (>21-fold sensitization; Figure 5B). Isobologram analyses confirmed that strong synergism occurred when lapatinib and rapamycin were concurrently combined as the CI_{50} value revealed that the amount of the two agents necessary to reduce cell proliferation by 50% was only 0.175 times as much as it would be necessary if they demonstrated purely additive actions in MCF-7/HER2-Lap10 cells (Figure 5C).

discussion

Two earlier studies have evaluated which mechanisms might account for the development of acquired resistance to HER2 tyrosine kinase inhibitors such as lapatinib in HER2-overexpressing breast cancer cells. Upon establishment of a model of acquired lapatinib resistance (i.e. HER2-overexpressing and ER-positive BT474 cells chronically exposed to 5 μM lapatinib), Xia et al. [24] employed oligonucleotide array platforms to compare gene expression in lapatinib-resistant and lapatinib-sensitive parental cells. Gene expression analysis in conjunction with protein expression validation indicated that acquired resistance to lapatinib in their model was mediated, at least in part, by a switch in cell survival dependence and regulation of a key antiapoptotic mediator (i.e. survivin) from HER2 alone to dependence upon ER and HER2 rather than loss of HER2 expression or insensitivity of HER2 signaling to lapatinib. Using phosphoprotein-based analyses of targeted pathways, Hedge et al. [30] analyzed phosphorylation changes in human breast cancer cell lines in the presence or absence of lapatinib. In their hands, cells responsive to lapatinib (i.e. HER2 gene-amplified BT474 and SKBR3 cell lines) exhibited strong differential effects on multiple genes in the AKT pathway, whereas lapatinib weakly down-regulated the AKT pathway in nonresponsive breast cancer cell lines (i.e. low HER2-expressing MDA-MB-468 and T47D cells). Microarray-based gene expression profiling confirmed Xia’s findings as they revealed that lapatinib treatment stimulates the expression of ER [30].
Figure 4. Molecular and cellular characterization of lapatinib-conditioned (high-dose resistant) MCF-7/human epidermal growth factor receptor (HER) 2-Lap10 cells. (A) (left) Figure shows dose–response cell proliferation curves of MCF-7/HER2-Lap10 cells treated with lapatinib in the presence or absence of increasing concentrations of the mammalian target of rapamycin inhibitor rapamycin. Data were analyzed as described in Figure 2A and B. (Right) IC50 values for lapatinib in MCF-7/HER2 and MCF-7/HER2-Lap10 cells were designated for the concentrations of the agent decreasing absorbance values at 570 nm by 50%, as determined by interpolation using 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide-based colorimetric cell proliferation assays. Data are the mean (columns) and 95% confidence intervals (bars) of three independent experiments carried out in triplicate. One-factor analysis of variance was used to analyze differences in the lapatinib IC50 values between each cell line. All statistical tests were two-sided. (B) (top) The effects of lapatinib and rapamycin on the activation status of p70S6K1 were assessed by immunoblotting procedures. Fifty micrograms of total protein from overnight serum-starved MCF-7/HER2-Lap10 cells cultured with 10 μM lapatinib in the presence or absence of 1 nM rapamycin (24 h) were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to immunoblotting analyses using the PhosphoPlus® p70 S6 Kinase Antibody Kit as described in ‘materials and methods’. Figure shows a representative immunoblotting analysis. Equivalent results were obtained in three independent experiments. (Bottom) The Oncogene Science HER2 microtiter enzyme-linked immunosorbent assay was used according to the manufacturer’s instructions to compare HER2 concentrations in whole-cell lysates from MCF-7/HER2 and MCF-7/HER2-Lap10 cells. Results are means (columns) and 95% confidence intervals (bars) of three independent experiments made in triplicate. (Right) The activation status of HER2 oncoprotein in the absence or presence of 10 μM lapatinib for 2 h was tested using a Phosphor-Tyr 1248-HER2 immunoblotting procedure as described in ‘materials and methods’. Figure shows a representative phosphoproteome analysis. Equivalent results were obtained in at least, three independent experiments. (C) Five hundred micrograms of total cell lysates from MCF-7/HER2 and MCF-7/HER2-Lap10 cells following a short-term treatment with 10 μM lapatinib (2 h) were incubated with membranes of the Human Phospho-MAPK Array Kit (Proteome Profiler®; R&D Systems) as per the manufacturer’s instructions. Phospho-MAPK Array data were developed on X-ray films following exposure to chemiluminescent reagents. Figure shows a representative phosphoproteome analysis. Equivalent results were obtained in three independent experiments. Representative microphotographs of untreated—control—cells (left) and experimental cultures following 48 h treatment with 10 μM lapatinib (right) are also shown.
Here, we employed a low-scale semiquantitative phosphoproteomic analysis of HER2 signaling and lapatinib-induced HER inhibition. Stably transduced cell lines overexpressing HER2 or empty vector were generated, and the effect of lapatinib on these cells was characterized prior and after development of acquired autoresistance. First, we sought to establish a lapatinib-associated biomarker capable to specifically discriminate the biological actions of the mono-HER2 inhibitor trastuzumab and the dual-HER1/HER2 inhibitor lapatinib in HER2-overexpressing breast cancer cells. Secondly, once this biomarker was identified, we then analyzed its relevance for the development of acquired resistance to lapatinib. Our findings showed that interrupting HER2 signaling using trastuzumab or lapatinib specifically and differentially affects the activity of the mTOR translational signaling pathway. When we analyzed the activation status of all three major MAPks, ERK1/ERK2, JNK 1–3, and p38 MAPks, and other intracellular kinases, such as AKT, GSK-3, RSK1/RSK2, MSK1/MSK2, and p70S6K1, the mTOR target p70S6K1 was the sole kinase activity exclusively inhibited by the dual-HER1/HER2 inhibitor lapatinib but not affected by the mono-HER2 inhibitor trastuzumab. These findings indicated that combined blockade of HER1 and HER2 receptor tyrosine kinase activities would be required to fully inhibit the activity of mTOR effector molecules in HER2-overexpressing breast cancer cells. Although previous reports have shown that signaling from HER1 or HER2 is involved in the activation of the mTOR effector [47–52], our current findings indicate that each receptor class appears to contribute to p70S6K1 activity by regulating distinct pathways. Trastuzumab-mediated inactivation of HER2 and AKT signaling failed to cause down-regulation of p70S6K1 at ERK1/2 MAPK-directed sites in the autoinhibitory domain, resulting in an incomplete block of p70S6K1 activity. Inhibition of the AKT and ERK1/2 MAPK pathways, as a consequence of dual HER1 and HER2 inhibition in the presence of lapatinib, caused a complete inhibition of p70S6K1 activity. Indeed, the inhibition of MAPK activity alone or simultaneous down-regulation of ERK1/2 MAPK and AKT activity by the selective inhibitors, U0126 and LY294002, had the same effect on p70S6K1 phosphorylation as the HER1/HER2 inhibitor (data not shown), proving the importance of these pathways in the regulation of p70S6K1 activity in HER2-overexpressing breast cancer cells. Although earlier studies indicated that the ERK1/2 MAPK pathway was not necessary for p70S6K1 activation, later reports demonstrated the importance of this pathway in p70S6K1 phosphorylation [43–47]. These findings together with our current results indicate that different pathways control p70S6K1 activation through cooperation of the HER1 and HER2 tyrosine kinase receptors, thus revealing the mTOR effector p70S6K1 as a valuable biomarker determining the efficacy of the dual-HER1/HER2 inhibitor lapatinib. Indeed, blockade of HER1/HER2 and mTOR pathways had the strongest impact on cell proliferation and apoptotic cell death, which paralleled the effects on mTOR and p70S6K1 activity.

In breast cancer cells with constitutive HER1/HER2-activated pathways, mTOR-regulated translation is an important downstream component determining the efficacy of the HER1/2-targeted tyrosine kinase inhibitor lapatinib (Figure 6). It is therefore ironic that lapatinib resistance appeared to occur as a direct consequence of its potent inhibitory effect on HER1/2-regulated mTOR pathway, its ‘sensitivity biomarker’ versus the mono-HER2 inhibitor trastuzumab. Corroborating our in vitro findings, elevated levels of p70S6K1 have been associated with lapatinib resistance.
HER2-overexpressing breast carcinomas.

Figure 6. Schematic depicting the role of p70 S6K1 in mediating Ras/Raf/MEK/extracellular-signal regulated kinase (ERK) and P70K/AKT signaling downstream of the human epidermal growth factor receptor (HER) 1/HER2 receptors in breast cancer cells. Although the mono-HER2 inhibitor trastuzumab can suppress the HER2-mediated downstream activation of AKT→p70S6K1 signaling, HER1→ERK1/2→p70S6K1 will be a resilient node in this circuit. The dual-HER1/HER2 inhibitor lapatinib can suppress both the HER1- and HER2-regulated downstream ERK and AKT signaling. In this scenario, the activation status of p70 S6K1 appears to assume a prominent role in determining acute (sensitivity) and chronic (resistance) breast cancer cell responses to lapatinib. Therefore, concurrent inhibition of p70S6K1 activity may not only enhance the efficacy of HER inhibitors but further may represent a valuable molecular avenue for delaying or preventing the development of lapatinib-acquired resistance in HER2-overexpressing breast carcinomas.

clinical response to lapatinib while linked to resistance to trastuzumab in patients with metastatic cancers overexpressing HER2 and/or expressing HER1 [23,53]. We observed that a striking lapatinib-insensitive and rapamycin-sensitive p70S6K1 hyperactivation occurs in a model of acquired autoresistance to lapatinib developed by continuously growing MCF-7/HER2 cells in the presence of high-dose lapatinib (up to 20 μM lapatinib) for >10 months. Although a noteworthy weakness of our current approach (i.e. lapatinib-resistant pool from HER2-overexpressing breast cancer cells that are initially sensitive to lapatinib-induced apoptosis) is that pools may contain cells with various degrees of resistance and that the pool developed during this study may harbor resistance acquired over time or may represent a selected subpopulation of cells with inherent resistance, pools also represent the alterations that are represented in the majority of tumor cell populations, whereas clones—another approach that would have been used to answer our research question—represent just one isolated alteration. Nonetheless, in this scenario, the HER2 signaling platform remains largely intact (i.e. HER2 protein levels remains unchanged and HER2 tyrosine kinase activity remains completely inhibited upon chronic exposure to lapatinib), whereas mTOR appears to assume a prominent role in regulating p70S6K1 activity and cell survival during the development of acquired resistance to lapatinib, thus making simultaneous inhibition of mTOR and HER2 pathways imperative to overcome lapatinib resistance. Data showing that the combination of lapatinib plus the mTOR inhibitor rapamycin (sirolimus) prevents the acquired lapatinib-resistant phenotype are consistent with the mTOR effector p70S6K1 playing a major role in this process.

In summary, our current findings strongly indicate that activation status of the serine-threonine kinase p70S6K1, a marker for mTOR activity that regulate ribosomal protein translation and ribosome biogenesis, not only constitutes a specific biomarker for the biological effects of the dual-HER1/HER2 inhibitor lapatinib but also further represents a molecular target for preventing the development of lapatinib-acquired resistance in HER2-overexpressing breast carcinomas. The clinical implications of our data are that the efficacy of lapatinib in these patients might be enhanced with therapies that target the mTOR pathway. Rapamycin analogues such as CCI-779 (also known as temsirolimus; Wyeth, Madison, NJ), RAD001 (also known as everolimus; Novartis, Basle, Switzerland), or AP23573 (Ariad Pharmaceuticals, Inc., Cambridge, MA), which are now in phase I–III oncology clinical trials [54–60], may warrant further clinical evaluation to effectively delay or prevent the development of acquired resistance to lapatinib.

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references


