Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling

Rita Nahta,[1,3] Linda X.H. Yuan,[1,3] Yi Du,[2,4] and Francisco J. Esteva[1,2,3,4]

Departments of [1]Breast Medical Oncology and [2]Molecular and Cellular Oncology; [3]Breast Cancer Translational Research Laboratory, The University of Texas M. D. Anderson Cancer Center; and [4]The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas

Abstract

The majority of breast cancer patients who achieve an initial therapeutic response to the HER2-targeted antibody trastuzumab will show disease progression within 1 year. Thus, the identification of novel agents that effectively inhibit survival of cancer cells that have progressed on trastuzumab is critical. In the current study, we show that the dual epidermal growth factor receptor (EGFR)/human EGFR-2 (HER2) kinase inhibitor lapatinib induces apoptosis in trastuzumab-resistant cells derived from the HER2-overexpressing SKBR3 breast cancer line. Lapatinib inhibited EGFR and HER2 signaling in resistant cells, blocking activation of downstream Akt, mitogen-activated protein kinase, and S6 kinases and inducing expression of p27kip1. Importantly, lapatinib also inhibited insulin-like growth factor I (IGF-I) signaling and growth-promoting effects in parental and resistant cells, and the cytotoxic effects of lapatinib were further enhanced by the IGF-I receptor–blocking antibody αIR3. As increased IGF-I receptor signaling has been implicated in trastuzumab resistance, our data strongly support further study of lapatinib as a potential therapeutic in breast cancers that have progressed on trastuzumab. [Mol Cancer Ther 2007;6(2):667–74]

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Requests for reprints: Rita Nahta or Francisco J. Esteva, Department of Breast Medical Oncology, Unit 1354, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030-4009. Phone: 713-792-2817; Fax: 713-563-0739. E-mail: rnahta@mdanderson.org or festeva@mdanderson.org

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Introduction

The *her2* (*erbB2/neu*) gene is amplified and/or overexpressed in ~20% to 30% of invasive breast carcinomas and is associated with increased metastatic potential and decreased overall survival (1). Trastuzumab (Herceptin, Genentech, San Francisco, CA) is a recombinant humanized monoclonal antibody directed against the extracellular domain of the human epidermal growth factor receptor (EGFR) 2 (HER2) tyrosine kinase receptor (2). Clinical studies established that trastuzumab is active against HER2-overexpressing metastatic breast cancers (3–5), leading to its approval in 1998 by the U.S. Food and Drug Administration. However, the objective response rates to trastuzumab monotherapy are quite low, ranging from 12% to 34% for a median duration of 9 months (4, 5). The majority of patients who achieve an initial response to trastuzumab-based therapy acquire resistance within 1 year of treatment initiation (6, 7). Identification of novel agents that inhibit the growth of trastuzumab-resistant cells is critical to improving the survival of metastatic breast cancer patients whose tumors overexpress HER2.

Lapatinib (GW572016; GlaxoSmithKline, Research Triangle Park, NC) is a small molecule inhibitor of the EGFR and HER2 tyrosine kinase domains. Lapatinib has been shown to interrupt baseline and ligand-stimulated activity of EGFR and HER2 and to block downstream signaling through the Akt and mitogen-activated protein kinase pathways *in vitro* and *in vivo* in models of HER2-overexpressing breast cancer (8, 9). In addition to inducing significant apoptosis as a single agent (9), lapatinib also sensitized HER2-overexpressing breast cancer cells to radiation (10) and restored tamoxifen sensitivity to tamoxifen-resistant breast cancer models (11).

In the current study, we show that lapatinib induced apoptosis of HER2-overexpressing breast cancer cells that are resistant to trastuzumab. Lapatinib inhibited EGFR and HER2 signaling in the resistant cells to a similar degree as in parental cells. We previously showed that physical interaction and cross-signaling between HER2 and insulin-like growth factor I (IGF-I) receptor contributes to trastuzumab resistance (12). We show here that lapatinib blocked IGF-I receptor signaling, reducing IGF-I–stimulated phosphorylation of IGF-I receptor and inducing apoptosis of trastuzumab-resistant cells even in the presence of IGF-I. Furthermore, cotreatment of trastuzumab-resistant cells with the IGF-I receptor–targeted antibody αIR3 increased the cytotoxic effects of lapatinib, suggesting that single agent lapatinib or the combination of lapatinib with an IGF-I receptor–targeted agent may effectively inhibit breast cancers that have progressed on trastuzumab.

Materials and Methods

Materials

Trastuzumab (Genentech) was dissolved in sterile water at a stock concentration of 20 mg/mL. Lapatinib (GW572016) was provided by GlaxoSmithKline at a stock concentration of 10 mmol/L dissolved in DMSO. IGF-I (Sigma-Aldrich, St. Louis, MO) was dissolved at 100 μg/mL in PBS and used at 100 ng/mL. The anti–IGF-I receptor antibody clone αIR3 was purchased from Calbiochem (San Diego, CA).

Cell Culture

SKBR3 parental and trastuzumab-resistant breast cancer cells were maintained in DMEM supplemented with 10% FCS. Trastuzumab-resistant, SKBR3-derived cells were developed as previously described (13). Briefly, SKBR3 cells were maintained in 4 μg/mL trastuzumab for 3 months, at which point resistant pools were developed by collecting all viable cells on one plate; clones were developed by propagating a single colony of viable cells on a plate. Resistance to trastuzumab was confirmed by trypan blue exclusion assays as described below. All trastuzumab-resistant pools and clones were maintained on 4 μg/mL trastuzumab.

Dose-Response Assays

Parental SKBR3, resistant pool 2, clone 3, and clone 4 cells were treated with increasing doses of trastuzumab for 6 days or lapatinib for 72 h, at which point cell survival was determined by trypan blue exclusion. In addition, pool 2 cells were treated with lapatinib ± αIR3 for 72 h before trypan blue exclusion analysis. All experiments were done at least in duplicate. Cell survival for all experiments is expressed as the percentage of viable cells compared with untreated or serum-starved cells.

Cell Cycle Analysis

Parental SKBR3 and pool 2 cells were treated with 1 μmol/L lapatinib for 0, 6, 24, or 48 h, fixed overnight in 70% ethanol, and then resuspended in PBS containing propidium iodide (50 μg/mL) supplemented with RNase A (1 μg/mL). DNA content was measured using a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ). Alternatively, parental, pool 2, and clone 3 cells were serum starved overnight and then treated with lapatinib (1 μmol/L) in the presence of IGF-I (100 ng/mL) for 24 h before fixation and FACScan analysis.

Annexin V Staining

Parental SKBR3 cells, resistant pool 2, clone 3, and clone 4 cells were treated with 0, 0.1, 1, or 10 μmol/L lapatinib for 48 h, at which point cells were collected and stained with Annexin V-phycoerythrin and 7-aminoactinomycin D (Becton Dickinson). Apoptotic cell death was measured as cells staining positive for Annexin V-phycoerythrin as assessed by fluorescence-activated cell sorting analysis.

IGF-I Stimulation

Parental and pool 2 cells were serum starved overnight and then stimulated with IGF-I (100 ng/mL) for 10 or 20 min. Cells were either lysed for protein directly in the dish or treated with lapatinib (1 μmol/L, overnight) before protein lysis. Immunoblotting was then done for phosphorylated and total IGF-I receptor or HER2 and for cleavage of poly-ADP-ribose polymerase (PARP) as described below. In addition, parental, pool 2, and clone 3 cells were treated with lapatinib (1 μmol/L) in the presence of IGF-I (100 ng/mL) for 24 h before cell cycle analysis, which was done as described above.

Immunoprecipitation

Parental and trastuzumab-resistant cells were either untreated or treated with lapatinib (1 μmol/L, 24 h), and lysed in buffer containing 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and protease and phosphatase inhibitor cocktails (Sigma Chemical). IGF-I receptor was immunoprecipitated (polyclonal antibodies, Cell Signaling, Beverly, MA) from total protein extracts (200 μg) overnight, washed in PBS containing 0.1% Tween 20 buffer, and immunoblotted to detect HER2 (monoclonal Ab-3, Oncogene Research Products, EMD Biosciences, Inc., San Diego, CA).

Immunoblotting

Cells were lysed in buffer containing 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and protease and phosphatase inhibitor cocktails (Sigma Chemical). Total protein extracts (30 μg) were immunoblotted using the following antibodies at the indicated dilutions: EGFR (Cell Signaling) and p-Tyr^{1173} EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibodies were used at 1:1,000; HER2 monoclonal Ab-3 (Oncogene Research Products) was used at 1:1,000; polyclonal antibodies against p-Tyr^{1248} HER2 (Upstate Biotechnology, Lake Placid, NY) was used at 1:500; p-Ser^{473} Akt (monoclonal 587F11), S6 ribosomal protein (monoclonal 54D2), and polyclonal antibodies against Akt, p-Thr^{202}/Tyr^{204} p42/p44 mitogen-activated protein kinase, total p42/p44 mitogen-activated protein kinase, p-Ser$^{235/236}$ S6, IGF-I receptor, and PARP (Cell Signaling) were each used at 1:1,000; p27kip1 polyclonal and survivin monoclonal (D-8; Santa Cruz Biotechnology) antibodies were each used at 1:1,000; β-actin monoclonal antibody (Santa Cruz Biotechnology) was used at 1:5,000; polyclonal p-Tyr$^{1162/1163}$ IR/IGF-I receptor (Biosource, Camarillo, CA) was used at 1:500. Secondary antibodies were chosen according to the species of origin of the primary antibody. Protein bands were detected and quantitated using the Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE).

Results

Lapatinib Induces Apoptosis in Trastuzumab-Resistant Breast Cancer Cells

Parental HER2-overexpressing SKBR3 breast cancer cells and trastuzumab-resistant pool 2, clone 3, and clone 4 cells derived from these parental cells were treated with 2-fold serial dilutions of trastuzumab ranging from 6.25 to 100 μg/mL for 6 days or lapatinib ranging from 1 nmol/L to 10 μmol/L for 72 h. Growth inhibition was determined by trypan blue exclusion assays. Relative resistance to trastuzumab was confirmed in pool 2, clone 3, and clone 4

cells compared with parental cells (Fig. 1A). In contrast, the tyrosine kinase inhibitor lapatinib inhibited survival of the trastuzumab-resistant cells to a similar degree as parental cells (Fig. 1B).

Cell cycle analysis showed that 1 μmol/L lapatinib induced a G_0-G_1 arrest with reduced S phase within 24 h in both parental and pool 2–resistant cells (Fig. 2A). To determine if growth inhibition was a reflection of increased apoptosis, cells were treated with pharmacologically relevant doses of lapatinib (0.1–10 μmol/L) for 48 h and then stained with Annexin V-phycoerythrin to detect early apoptosis and 7-aminoactinomycin D to detect cells in the late stages of apoptosis or necrosis. Cells were then examined by fluorescence-activated cell sorting analysis (Fig. 2B), and Annexin V-phycoerythrin–positive cells were counted to measure early apoptotic cell death. Lapatinib induced significant apoptosis in both trastuzumab-sensitive and trastuzumab-resistant breast cancer cells. Lower drug doses (0.1 and 1 μmol/L) induced slightly higher levels of apoptosis in parental cells versus resistant cells (Fig. 2C). For example, 30% of parental cells treated with 0.1 μmol/L of lapatinib were apoptotic versus 15% to 20% of resistant cells. However, the highest dose of lapatinib (10 μmol/L) induced similar levels of apoptosis (30–40%) in parental and resistant cells.

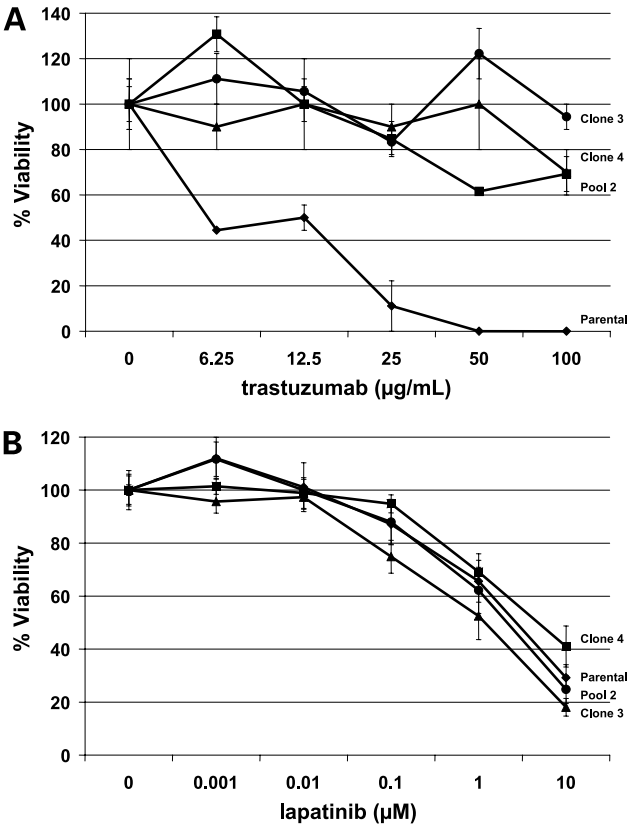


Figure 1. Lapatinib inhibits survival of trastuzumab-resistant breast cancer cells. Parental SKBR3 HER2-overexpressing breast cancer cells and trastuzumab-resistant pool 2, clone 3, and clone 4 cells derived from SKBR3 cells were treated with 2-fold serial dilutions of (**A**) trastuzumab ranging from 6.25 to 100 μg/mL for 6 d or (**B**) lapatinib ranging from 0.001 to 10 μmol/L for 72 h. Cells were trypsinized and counted by trypan blue exclusion. *Points,* cell survival for all experiments, expressed as the percentage of viable cells compared with untreated cells; *bars,* SD between duplicate or triplicate cultures. Lapatinib reduced cell survival to a similar degree in parental and trastuzumab-resistant breast cancer cells.

PARP, which is cleaved by caspases during apoptosis to produce an 89-kDa fragment from the full-length 116-kDa protein, was measured as an additional marker of apoptosis by immunoblotting. Parental and resistant cells were treated with 1 μmol/L lapatinib, and total protein was immunoblotted for PARP after 48 h of drug treatment (Fig. 2D). Lapatinib treatment increased fragmentation of PARP in parental and resistant cells consistent with induction of apoptosis. In addition, lapatinib treatment of parental and resistant cells down-regulated expression of the antiapoptotic protein survivin, a molecular mechanism previously associated with lapatinib-induced apoptosis (14).

Lapatinib Blocks EGFR and HER2 Signaling in Trastuzumab-Resistant Breast Cancer Cells

Lapatinib has been shown to inhibit signaling from EGFR and HER2 in HER2-overexpressing breast cancer cells (8, 9). In parental and trastuzumab-resistant SKBR3 cells, lapatinib (1 μmol/L, overnight) dramatically reduced phosphorylation of EGFR and HER2 (Fig. 3A). Downstream signaling from these receptors was also blocked in parental and resistant cells, as evidenced by decreased phosphorylation of Akt, mitogen-activated protein kinase, and S6 kinases (Fig. 3B). In addition, p27kip1, whose down-regulation was previously associated with trastuzumab resistance (13), was significantly induced upon treatment with lapatinib. Induction of p27kip1 is not likely to be the sole factor contributing to lapatinib-mediated G_0-G_1 arrest, however, as knockdown of p27kip1 did not significantly inhibit lapatinib-mediated cell cycle arrest (data not shown). These results show that lapatinib effectively inhibits EGFR/HER2 signaling in breast cancer cells that are no longer responsive to trastuzumab.

Inhibitory Effects of Lapatinib on IGF-I Signaling

We previously showed that IGF-I receptor interacts with and cross-talks to HER2 in trastuzumab-resistant pool 2 cells (12). This interaction was also observed in three resistant clones derived from SKBR3 cells (Fig. 4A). Lapatinib did not affect this physical interaction, as shown in pool 2 cells after immunoprecipitation of IGF-I receptor followed by immunoblotting for HER2 (Fig. 4A).

To examine the effects of lapatinib on IGF-I signaling and cross-talk, parental, and pool 2 cells were serum starved overnight and then stimulated with IGF-I (100 ng/mL) for 20 min, at which point cells were either lysed for protein or treated with lapatinib (1 μmol/L) for 24 h before protein collection. Lysates were also obtained from serum-starved cells treated with lapatinib in the absence of IGF-I. Immunoblotting showed that lapatinib reduced phosphorylation of HER2 in parental cells to 25% of the original level in the absence or presence of IGF-I (Fig. 4B). As previously shown (12), we observed cross-talk from IGF-I receptor to HER2 in trastuzumab-resistant pool 2 cells, resulting in

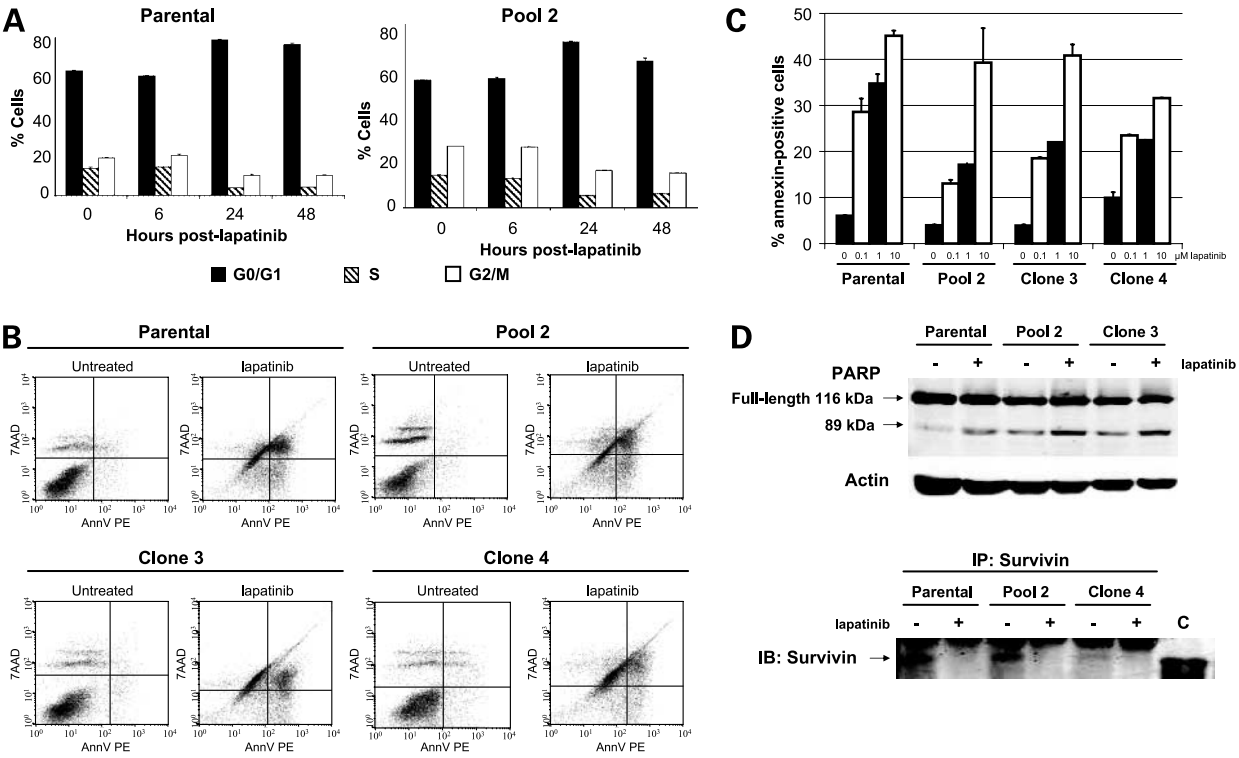


Figure 2. Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells. **A,** parental and resistant pool 2 cells were treated with 1 μmol/L lapatinib for 0, 6, 24, or 48 h, at which point cells were fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. *Columns,* percentages of cells in G_0-G_1, S, or G_2-M for each group; *bars,* SD between duplicate cultures. Lapatinib induced G_0-G_1 arrest with decreased S phase within 24 h in parental and trastuzumab-resistant breast cancer cells. **B** and **C,** parental, pool 2, clone 3, and clone 4 cells were treated with lapatinib at 0.1, 1, or 10 μmol/L for 48 h. Cells were collected, stained with Annexin V-phycoerythrin (*AnnV PE*) and 7-aminoactinomycin D (*7AAD*), and analyzed by flow cytometry. **B,** representative profiles of untreated and lapatinib-treated (10 μmol/L) groups are shown for each cell line. **C,** *columns,* percentage of cells staining positive for Annexin V-phycoerythrin (which is equal to the sum of the *bottom* and *top right quadrants* in the profiles shown in **B**), shown for each treatment group; *bars,* SD between duplicate cultures. **D,** parental, pool 2, and clone 3 or clone 4 cells were untreated or treated with lapatinib (1 μmol/L for 48 h), lysed for protein, and either immunoblotted (*IB*, 30 μg) to detect PARP cleavage or immunoprecipitated (*IP*, 100 μg) using an antisurvivin antibody and then immunoblotted for survivin. HeLa cell extract was used as a positive control (*C*) for survivin. Lapatinib induced apoptosis in trastuzumab-sensitive parental cells and in trastuzumab-resistant pool 2, clone 3, and clone 4 cells, as evidenced by increased Annexin V-phycoerythrin staining, increased PARP cleavage, and survivin down-regulation in lapatinib-treated cells.

increased HER2 receptor phosphorylation. Lapatinib blocked this cross-signaling, as phosphorylation of HER2 was inhibited even in the presence of IGF-I. IGF-I–stimulated phosphorylation of IGF-I receptor was also reduced by lapatinib in parental and pool 2 cells, indicating that lapatinib inhibits IGF-I signaling in addition to its known activity as an EGFR/HER2 inhibitor.

We next examined whether lapatinib is able to effectively inhibit growth of trastuzumab-resistant cancer cells in the presence of IGF-I signaling. Parental, pool 2, and clone 3 cells were serum starved overnight and then treated with lapatinib (1 μmol/L) and IGF-I (100 ng/mL). After 24 h, cells were fixed and stained with propidium iodide and then DNA content was analyzed by flow cytometric cell cycle analysis. Representative profiles are shown for resistant cells (Fig. 4C). Lapatinib induced G_0-G_1 arrest with reduced proliferation in the presence of IGF-I in pool 2 and clone 3 resistant cells, as well as in parental SKBR3 HER2-overexpressing breast cancer cells (Table 1). These results show that lapatinib effectively prevents IGF-I–mediated proliferative effects. Furthermore, examination of lapatinib effects on PARP cleavage in the presence of IGF-I indicated that induction of apoptosis is maintained (Fig. 4D). In parental cells, lapatinib alone mediated cleavage of the full-length 116-kDa PARP protein into the 89- and 24-kDa forms. In the presence of IGF-I, PARP cleavage was still evident in parental cells but to a lesser degree than when cells were treated in the absence of IGF-I. Resistant cells, however, maintained a high level of PARP fragmentation when treated with lapatinib in the presence of IGF-I, suggesting that lapatinib remains an effective inducer of apoptosis in trastuzumab-resistant cells even when IGF-I receptor is stimulated. Thus, lapatinib can overcome IGF-I signaling and induce apoptosis in cells that have progressed on trastuzumab.

Based on the finding that lapatinib reduced IGF-I signaling, we examined the biological effects of combining lapatinib with an IGF-I receptor inhibitor. Pool 2 cells were treated with lapatinib ± αIR3, the IGF-I receptor monoclonal antibody, for 72 h, at which time cell viability was

determined by trypan blue exclusion assay (Fig. 4E). At each of the five dose combinations shown, αIR3 increased the inhibitory effect of lapatinib on cell viability as the combination of αIR3 with lapatinib reduced cell viability to a greater degree than either agent alone. These data collectively suggest that lapatinib not only inhibits HER2 and EGFR signaling but also abrogates IGF-I receptor signaling. In addition, our data support the need for preclinical studies examining combinations of clinically relevant IGF-I receptor–inhibiting agents with lapatinib, particularly in the context of HER2-overexpressing breast cancers that have progressed on trastuzumab.

Discussion

We made the following observations during our study: (*a*) Lapatinib induced apoptosis in HER2-overexpressing breast cancer cells that are either naive (parental cells) or refractory (resistant cells) to trastuzumab. (*b*) In addition to blocking HER2 and EGFR signaling, lapatinib reduced IGF-I receptor signaling in HER2-overexpressing breast cancer cells. Inhibition of IGF-I signaling and proliferation were achieved by lapatinib in both trastuzumab-sensitive and resistant cells, and IGF-I receptor cross-talk to HER2 was blocked by lapatinib in resistant cells. (*c*) Cotreatment with an IGF-I receptor–targeted agent increased lapatinib-mediated growth inhibition. These results suggest that lapatinib may be an effective therapy in trastuzumab-resistant cancers and further suggest that lapatinib-mediated cytotoxicity may be due in part to the effects on the IGF-I signaling pathway.

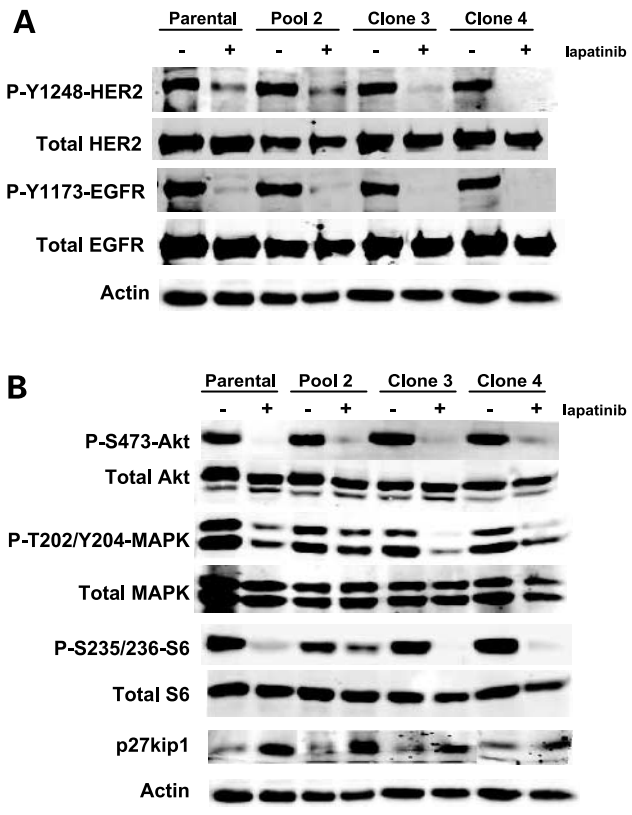
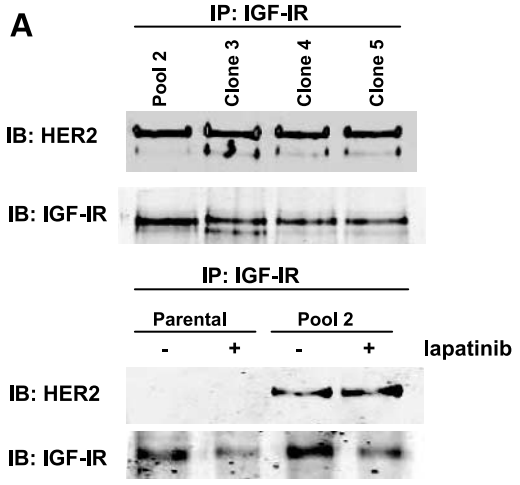


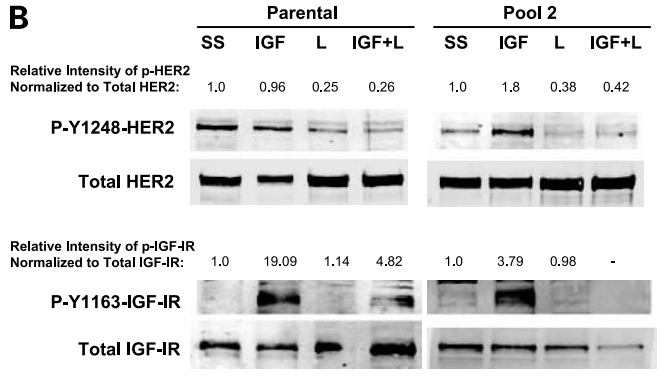
Figure 3. Lapatinib inhibits EGFR and HER2 signaling in trastuzumab-resistant cells. Parental, pool 2, clone 3, and clone 4 cells were treated with 1 μmol/L lapatinib for 24 h. Immunoblotting (30 μg) was done (**A**) for phosphorylated and total HER2 and EGFR and (**B**) for the downstream signaling molecules, phosphorylated and total Akt, mitogen-activated protein kinase, S6, and total p27kip1. Actin served as a loading control. Lapatinib inhibited EGFR and HER2 phosphorylation, blocked signaling pathway downstream of these receptors, and induced p27kip1 in parental and resistant cells.

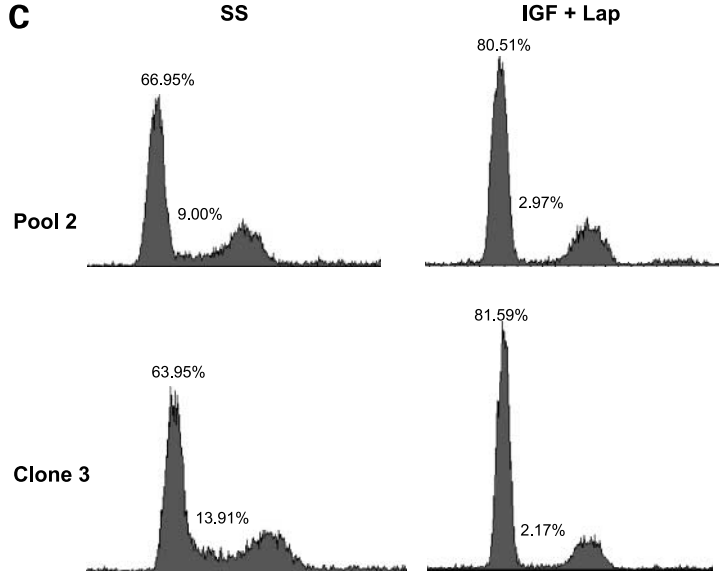
Lapatinib has been shown to inhibit baseline HER2 and EGFR signaling and to block signaling stimulated by the HER family ligands transforming growth factor (TGF)-α, heregulin, and epidermal growth factor (9, 15). We show here, for the first time, that lapatinib also inhibits signaling stimulated by IGF-I. Importantly, lapatinib blocked cellular proliferation in the presence of IGF-I and effectively induced apoptosis as measured by PARP cleavage in IGF-I-stimulated cells. Further studies examining the efficacy of lapatinib in IGF-I–dependent cell lines are warranted to determine if lapatinib may be effective in the setting of constitutive IGF-I signaling. Our results indicate that lapatinib inhibits multiple signaling pathways (EGFR/HER2 and IGF-I receptor pathways), potentially making this an unusual and highly effective therapy as it targets two signaling families that are highly implicated in human cancers. Based on these results, our current work continues to explore the hypothesis that lapatinib may be most effective in cancer cells that have constitutive signaling from both the EGFR/HER2 and the IGF-I receptor pathway. Importantly, results from a clinical trial showed that elevated baseline levels of IGF-I receptor correlated with response to lapatinib in metastatic breast cancer patients, whereas nonresponders had lower baseline levels of IGF-I receptor (16). As indicated in that study, it is interesting that increased IGF-I receptor may predict for sensitivity to lapatinib, as IGF-I receptor overexpression and signaling have been linked to resistance to trastuzumab (12, 17). Thus, one of the molecular mechanisms contributing to trastuzumab resistance (increased IGF-I signaling) may potentially increase sensitivity to another HER2-targeted agent, lapatinib. Hence, further studies examining the efficacy of lapatinib in the setting of trastuzumab resistance are warranted.

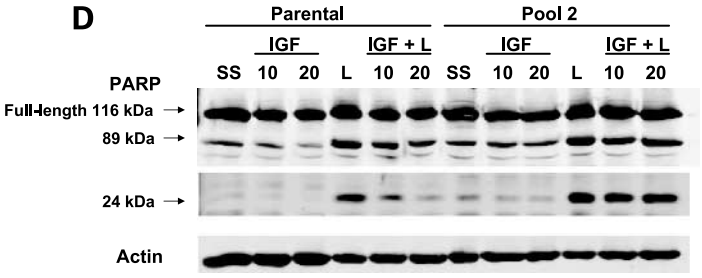
Our findings suggest that there is no cross-resistance between trastuzumab-resistant cells and the HER2-targeted tyrosine kinase inhibitor lapatinib. These results are in support of data published by Konecny et al. (15), who showed that lapatinib inhibits the viability of HER2-overexpressing breast cancer cells that were maintained for a long term on trastuzumab (15). Importantly, they also described studies in which lapatinib and trastuzumab synergistically inhibited survival of trastuzumab-naive cells, suggesting that a combination of HER2-targeted agents may be more beneficial than a single agent. Future studies should also examine whether such combination

treatments would reduce the development of resistance to HER2-targeted agents. These findings are in contrast to results suggesting that trastuzumab-resistant cells may show cross-resistance to another HER2-targeted agent, pertuzumab, which is an antibody that binds a different extracellular HER2 region than does trastuzumab (12, 18). Hence, inhibition of the tyrosine kinase region of HER2 may be a more effective strategy in breast cancers that have









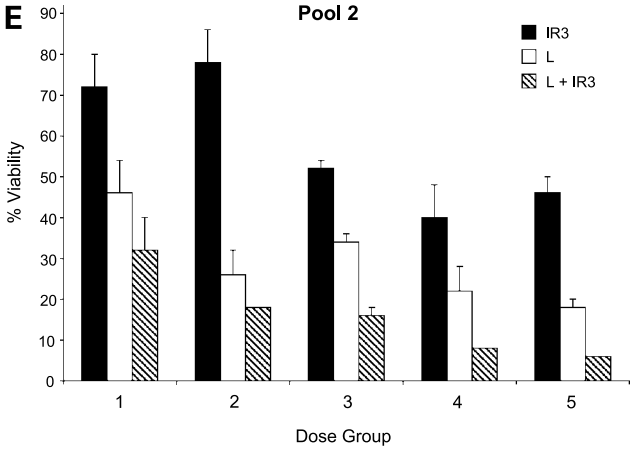


Table 1. Lapatinib induces G_0-G_1 arrest in the presence of IGF-I

Sample	Percentage of cells per cycle phase		
	G_0-G_1	S	G_2-M
Parental, serum-starved control	77.46 ± 0.29	8.8 ± 0.13	12.0 ± 0.05
Parental, IGF-I + lapatinib	88.83 ± 0.06	1.6 ± 0.04	4.9 ± 0.12
Pool 2, serum-starved control	66.87 ± 0.08	8.7 ± 0.31	21.68 ± 0.43
Pool 2, IGF-I + lapatinib	80.41 ± 0.09	3.0 ± 0.03	13.33 ± 0.1
Clone 3, serum-starved control	64.29 ± 0.34	13.55 ± 0.35	20.29 ± 0.08
Clone 3, IGF-I + lapatinib	75.57 ± 4.43	2.22 ± 0.05	14.27 ± 0.45

NOTE: Parental, pool 2, and clone 3 cells were serum starved overnight and then either fixed without further treatment or incubated with IGF-I (100 ng/mL) and lapatinib (1 μmol/L) for 24 h before fixation. Cells were stained with propidium iodide, and DNA content was examined by flow cytometric cell cycle analysis (fluorescence-activated cell sorting). Percentages of cells in each cell cycle phase are shown with SD between duplicate cultures per group. Increased percentages of cells in G_0-G_1 and reduced percentages in S phase indicate that lapatinib induces cell cycle arrest at G_0-G_1 even in the presence of exogenously added IGF-I.

progressed on HER2 antibody therapy. Alternatively, sensitivity to lapatinib but relative lack of sensitivity to pertuzumab may be due to lapatinib-mediated inhibition of IGF-I signaling which is enhanced in resistant cells, whereas pertuzumab does not seem to inhibit IGF-I signaling (12) and may, thus, be unable to inhibit the survival of trastuzumab-resistant cells.

Xia et al. (19) recently reported that lapatinib induces signaling from the estrogen receptor, which leads to decreased efficacy. The authors confirmed that tumor biopsies from HER2-overexpressing patients treated with lapatinib showed increased estrogen receptor signaling. As our cell culture model uses an estrogen receptor–negative line (SKBR3), modulation of estrogen receptor signaling is not likely to be contributing to any of the molecular changes we described here or to the trastuzumab-resistant phenotype of these cells. However, in estrogen receptor–positive HER2-overexpressing breast cancers, cotargeting of estrogen receptor, HER2, and IGF-I receptor may be an important strategy worthy of further investigation. In fact, treatment of BT474 estrogen receptor–positive HER2-overexpressing breast cancer cells or MCF7 estrogen receptor–positive IGF-I receptor–elevated breast cancer cells with a triple combination of estrogen receptor, HER2, and IGF-I receptor antagonists augmented the apoptotic effects of single agents or dual combinations (20).

Our results indicate that cotargeting of IGF-I receptor and HER2 may be beneficial in trastuzumab-refractory HER2-overexpressing breast cancers. Preclinical studies support this concept, as trastuzumab and a dominant negative IGF-I receptor construct produced synergistic growth inhibition in MCF7 cells stably transfected with HER2 (MCF7/HER18 cells) which have endogenous overexpression of IGF-I receptor (21). In addition, treatment with recombinant human IGF-binding protein 3, which inhibits IGF-I signaling, restored or potentiated the response to trastuzumab *in vitro* and in xenograft mouse models using either MCF7/HER18 cells or SKBR3 HER2-overexpressing cells stably transfected with IGF-I receptor (22).

Trastuzumab is the first targeted agent approved for use in patients with HER2-overexpressing breast cancer. Disease progression despite treatment with trastuzumab necessitates the identification of novel agents. Our studies suggest that the dual HER2/EGFR tyrosine kinase inhibitor lapatinib should be explored as a potential therapy in

Figure 4. Lapatinib reduces IGF-I signaling in trastuzumab-resistant cells. **A,** IGF-I receptor (*IGF-IR*) was immunoprecipitated from lysates (200 μg) of pool 2, clone 3, clone 4, and clone 5 cells and then immunoblotted for HER2 or IGF-I receptor to confirm pull down. The HER2 protein was pulled down with IGF-I receptor in all lysates, supporting interaction between HER2 and IGF-I receptor in trastuzumab-resistant cells. Further, parental and pool 2 cells were treated for 24 h with 1 μmol/L lapatinib and then lysed for protein. IGF-I receptor was immunoprecipitated from lysates (200 μg) and immunoblotted for HER2 or IGF-I receptor. Lapatinib did not disrupt interaction between HER2 and IGF-I receptor in resistant cells. **B,** parental and pool 2 cells were serum starved overnight and then stimulated with IGF-I (100 ng/mL) for 20 min. Cells were then either collected for protein or treated with lapatinib (1 μmol/L) for 24 h before collection. Lysates from cells treated with lapatinib (1 μmol/L, 24 h) in the absence of IGF-I were also collected. Immunoblotting (30 μg) was done for phosphorylated and total HER2 or IGF-I receptor. Bands were quantitated and normalized to total receptor levels. Lapatinib blocked IGF-I–stimulated phosphorylation of IGF-I receptor in parental and pool 2 cells with undetectable levels in IGF-I–stimulated pool 2 cells treated with lapatinib and blocked IGF-I–stimulated IGF-I receptor cross-talk to HER2. For **B** and **D**, lysates are labeled as follows: *SS,* serum-starved control; *IGF,* cells stimulated with IGF-I (100 ng/mL) for 10 or 20 min; *L,* lapatinib-treated cells (1 μmol/L, 24 h); *IGF + L,* IGF-I (100 ng/mL) stimulation for 10 or 20 min followed by lapatinib treatment (1 μmol/L, 24 h). **C,** pool 2– and clone 3–resistant cells were serum starved overnight and then treated with lapatinib (1 μmol/L) in the presence of IGF-I (100 ng/mL). After 24 h, cells were fixed overnight and stained with propidium iodide. DNA content was analyzed by flow cytometric cell cycle analysis. Representative cell cycle profiles with percentages of cells in G_0-G_1 and S phase are shown for serum starved and IGF-I + lapatinib (*IGF + Lap*)–treated cells. Lapatinib induced G_0-G_1 arrest with reduced proliferation in the presence of IGF-I. **D,** parental and pool 2 cells were serum starved overnight and then stimulated with IGF-I (100 ng/mL) for 10 or 20 min. Cells were then either collected for protein or treated with lapatinib (1 μmol/L) 24 h before collection. Lysates from cells treated with lapatinib (1 μmol/L, 24 h) in the absence of IGF-I were also collected. Lysates were immunoblotted (30 μg) to detect PARP cleavage. Lapatinib-mediated PARP cleavage was reduced in parental cells in the presence of IGF-I; however, significant PARP cleavage was observed in resistant cells even in the presence of IGF-I stimulation, suggesting that lapatinib retains its ability to induce significant apoptosis in IGF-I–stimulated trastuzumab-resistant cells. **E,** pool 2 cells were treated with lapatinib and/or αIR3 (*IR3*) for 72 h, at which time cell viability was examined by trypan blue exclusion. Dose groups are as follows: *1,* 62.5 ng/mL αIR3 ± 31.25 nmol/L lapatinib; *2,* 125 ng/mL αIR3 ± 62.5 nmol/L lapatinib; *3,* 250 ng/mL αIR3 ± 125 nmol/L lapatinib; *4,* 500 ng/mL αIR3 ± 250 nmol/L lapatinib; *5,* 1,000 ng/mL αIR3 ± 500 nmol/L lapatinib. *Columns,* cell survival for all experiments, expressed as the percentage of viable cells compared with untreated cells; *bars,* SD between duplicate cultures. αIR3 increased the inhibitory effect of lapatinib on cell viability, as the combination reduced cell viability to a greater degree than either agent alone. *Black columns,* αIR3 alone; *white columns,* lapatinib alone; *shaded columns,* combination.

HER2-overexpressing breast cancers that are refractory to trastuzumab and that strategies that cotarget the IGF-I receptor and HER2 pathways should be developed and explored in this patient population.

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