

## Truncated ErbB2 Expressed in Tumor Cell Nuclei Contributes to Acquired Therapeutic Resistance to ErbB2 Kinase Inhibitors

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### Abstract

ErbB2 tyrosine kinase inhibitors (TKI) block tyrosine autophosphorylation and activation of the full-length transmembrane ErbB2 receptor (p185<sup>ErbB2</sup>). In addition to p185<sup>ErbB2</sup>, truncated forms of ErbB2 exist in breast cancer cell lines and clinical tumors. The contribution of these truncated forms, specifically those expressed in tumor cell nuclei, to the development of therapeutic resistance to ErbB2 TKIs has not been previously shown. Here, we show that expression of a 95-kDa tyrosine phosphorylated form of ErbB2, herein referred to as p95L (lapatinib-induced p95) was increased in ErbB2<sup>+</sup> breast cancer cells treated with potent ErbB2 TKIs (lapatinib, GW2974). Expressed in tumor cell nuclei, tyrosine phosphorylation of p95L was resistant to inhibition by ErbB2 TKIs. Furthermore, the expression of p95L was increased in ErbB2<sup>+</sup> breast cancer models of acquired therapeutic resistance to lapatinib that mimic the clinical setting. Pretreatment with proteasome inhibitors blocked p95L induction in response to ErbB2 TKIs, implicating the role of the proteasome in the regulation of p95L expression. In addition, tyrosine phosphorylated C-terminal fragments of ErbB2, generated by alternate initiation of translation and similar in molecular weight to p95L, were expressed in tumor cell nuclei, where they too were resistant to inhibition by ErbB2 TKIs. When expressed in the nuclei of lapatinib-sensitive ErbB2<sup>+</sup> breast cancer cells, truncated ErbB2 rendered cells resistant to lapatinib-induced apoptosis. Elucidating the function of nuclear, truncated forms of ErbB2, and developing therapeutic strategies to block their expression and/or activation may enhance the clinical efficacy of ErbB2 TKIs. *Mol Cancer Ther*; 10(8); 1367–74. ©2011 AACR.

### Introduction

ErbB2, a 185-kDa transmembrane receptor tyrosine kinase (p185<sup>ErbB2</sup>), is deregulated in 25% of all breast cancers, where it predicts for a poor clinical outcome (1). ErbB2 activation requires autophosphorylation of tyrosine (Y) residues within the cytoplasmic domain of the receptor (e.g., Y1248; ref. 2). These phosphotyrosine residues serve as docking sites for adaptor proteins that link ErbB2 to downstream mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) signaling networks that promote the growth and survival of breast cancer cells (2–6). In addition to p185<sup>ErbB2</sup>, truncated forms of ErbB2 lacking all or most of the N-terminus extracellular domain (ECD) exist in ErbB2<sup>+</sup> breast cancer cell lines and clinical tumors (7–10). The most extensively studied

truncated forms retain the transmembrane region and are expressed at the cell surface. Historically referred to as p95, truncated forms of ErbB2 expressed at the cell surface form heterodimers with other ErbB receptors (11) and interact with the p85 subunit of PI3K (12), thereby activating downstream signal transduction cascades in a manner similar to p185<sup>ErbB2</sup>. The generation of p95 has been shown to be dependent upon metalloproteinase activity (7). p95-positive breast cancers exhibit an aggressive clinical phenotype characterized by an increased incidence of lymph node involvement at the time of initial diagnosis (13, 14) and are more resistant to trastuzumab, as they lack the ECD (15, 16).

Lapatinib is a highly selective small molecule inhibitor of the ErbB2 and EGFR tyrosine kinases. Inhibition of ErbB2 tyrosine autophosphorylation by lapatinib leads to the inactivation of downstream cell growth and survival signals (17–19). Although a significant advancement in the treatment of breast cancer, the clinical efficacy of lapatinib has been limited by the development of acquired therapeutic resistance (20, 21). To address this problem, we generated clinically relevant models of acquired resistance to lapatinib using human ErbB2<sup>+</sup> breast cancer cell lines (22, 23).

We now show that treatment with ErbB2 tyrosine kinase inhibitors (TKI) increased the expression of a tyrosine phosphorylated, truncated form of ErbB2 that

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was expressed in the nuclei of ErbB2<sup>+</sup> breast cancer cells, which will herein be referred to as p95L (lapatinib-induced p95). In contrast to truncated forms of ErbB2 expressed at the cell surface, the phosphorylation of p95L, and similar truncated forms that were also expressed in tumor cell nuclei, was resistant to ErbB2 TKI. The data supporting the activation and nuclear localization of p95L in response to ErbB2 TKI, and the role of nuclear, truncated forms of ErbB2 in the development of therapeutic resistance to ErbB2 TKIs, will be discussed.

## Materials and Methods

### Cell culture and reagents

BT474, SKBR3, Au565, MCF7, and T47D breast cancer cell lines were obtained from the American Type Culture Collection. Lapatinib-resistant breast cancer cells were generated as previously described (22). All cells were cultured as previously described (11, 17, 22). No independent authentication of these cells was done by the authors. Anti-phosphotyrosine (p-tyr) antibody, GW2974, and calpain inhibitor 1 were purchased from Sigma-Aldrich. Anti-c-ErbB2 (Ab-11) monoclonal antibody was from Neo Markers. Anti-ErbB2 (AA1243-1255) and anti-phospho-ErbB2 (Y1248) antibodies were from Upstate Biotechnology. MG132,  $\gamma$ -secretase inhibitor, and lactacystin were from Calbiochem. BB94 (batimastat) was from Kimia Corp. Protein G agarose was purchased from Boehringer Ingelheim. IRDye800 conjugated affinity purified anti-rabbit IgG and anti-mouse IgG were from Rockland. Alexa Fluor 680 goat anti-rabbit IgG was obtained from Molecular Probes. Lapatinib (GW572016) and *N*-[3-chloro-4-[(3-fluorobenzyl)oxy]phenyl]-6-[5-([[(2-methylsulfonyl)ethyl]amino)methyl]-2-furyl]-4-quinazolinamine (Fig. 1A) was purchased from LC Laboratories. Lapatinib for cell culture work was dissolved in dimethyl sulfoxide (DMSO; 0.01%).

### Isolation of nuclear extracts, SDS-PAGE, and Western blot analysis

Details of cell fractionation, immunoprecipitation, SDS-PAGE, and Western blot analysis were previously described (22). Membranes were probed with specific antibodies recognizing target proteins and visualized using the Odyssey Infrared Imaging System (LI-COR, Inc.). Membranes were incubated with fluorescent-labeled secondary antibody at a 1:10,000 dilution with 3% bovine serum albumin in PBS for 60 minutes protected from light. After washing in PBS + 0.1% Tween-20, the membranes were scanned using an Odyssey imaging system.

### Human tumor xenografts, animal treatment, and human tumor biopsies

NOD.CB17-*Prkdc*<sup>scid</sup>/J (NOD/SCID) mice were purchased from Jackson Laboratory and bred in the Duke Comprehensive Cancer Center Isolation Facility. BT474

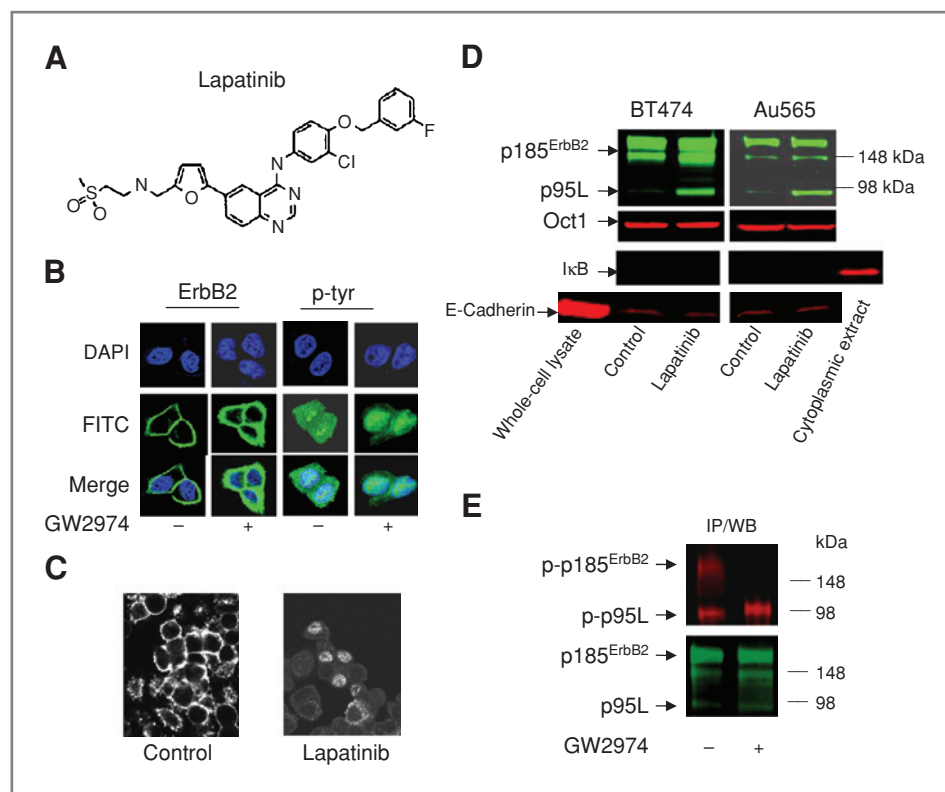
and rBT474 cells were suspended in HBSS and mixed with Matrigel (BD Biosciences) at 1:1 ratio to make final concentrations of  $1 \times 10^4$  cells per 50  $\mu$ L. A 50- $\mu$ L tumor cell suspension was inoculated into bilateral mammary fat pads of female NOD/SCID mice (5 ~ 6 weeks old, 4 mice per group). Animals were treated with lapatinib (75 mg/kg/d) by oral gavage until they were sacrificed. Tumor dimensions were measured serially, and tumor volumes calculated using the following formula: long axis  $\times$  (short axis)<sup>2</sup>  $\times$  0.52. The mice were euthanized with CO<sub>2</sub> inhalation and tumor xenografts excised 59 days after implantation of tumor cells. All animal studies were conducted in compliance with Duke animal care regulations. Human biopsies were collected from breast cancer skin metastasis after informed written consent was obtained as part of an Institutional Review Board-approved tissue collection protocol. Tumor specimens were flash frozen in liquid nitrogen and stored at -80°C. Tissue extracts were prepared for Western blot analysis by homogenization in radioimmunoprecipitation assay buffer at 4°C.

### Expression of truncated forms of ErbB2 in human breast cancer cell lines

C-terminal fragments (CTF; c-611, c-676, and c-678) were generated on the basis of ErbB2 open reading frames from LTR-2/ErbB2 (8) and subcloned into the pcDNA 3.1 (+). c-611, c-676, and c-678 were subcloned into the pcDNA3.1 vector (Invitrogen) with forward primers: 5'-ACAAGCTT ACCATGCCCATCTGGAAG-3', 5'-ACAAGCTTACCATGAAGCGACGGCAGCA-3' and 5'-ACAAGCTT ACCATGCGGAGACTGCTG-3', and reverse primer: 5'-AACTCGAG TCACACTGG-CACGTCAG-3'. MCF7 and T47D breast cancer cells were transfected with empty vector alone (controls) or the same vector containing p185<sup>ErbB2</sup> or the various CTFs using the Lipofectamine 2000 Reagent from Invitrogen according to the manufacturer's protocol. Stably transfected cells were selected using G418 (400  $\mu$ g/mL), and the expression levels of CTFs were confirmed by Western blot analysis.

### Immunofluorescence microscopy

Cells were cultured in 6-well plates with or without the indicated treatments. After washing with PBS, cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.2% Triton X-100 for 20 minutes, and blocked with 2% bovine serum albumin in PBS at room temperature followed by washing with PBS and incubated with anti-ErbB2 or anti-phosphotyrosine-specific antibodies overnight at 4°C. After extensive washings, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit or rabbit anti-mouse antibodies followed by counterstaining with 1.5  $\mu$ g/mL 4',6-diamidino-2-phenylindole (DAPI) from Vector Labs. An Olympus L Fluoview FV1000 was used for all photographs.



**Figure 1.** Phosphorylation of nuclear, truncated ErbB2 is resistant to ErbB2 TKI. **A**, the chemical structure of lapatinib. **B**, BT474 cells were treated for 48 hours with GW2974 (1  $\mu\text{mol/L}$ ) or vehicle alone (-). Total ErbB2 and phosphotyrosine (p-tyr) signals (green) were visualized by immunofluorescence microscopy as described in Materials and Methods. Cell nuclei were counterstained blue with DAPI. The bottom row merges FITC and DAPI signals. **C**, Au565 cells were treated with lapatinib (1  $\mu\text{mol/L}$ ) or vehicle alone (control) for 24 hours, and p-ErbB2 was assessed by immunofluorescence microscopy using an ErbB2 phosphotyrosine-specific primary antibody and an FITC-conjugated secondary antibody. **D**, steady-state protein levels of p185<sup>ErbB2</sup> and p95L were determined in nuclear extracts from BT474 and Au565 cells treated for 24 hours with lapatinib (500 nmol/L) or vehicle alone (control). Steady-state protein levels of Oct 1, I $\kappa$ B, and E-cadherin, which represent nuclear, cytoplasmic, and cell membrane proteins, respectively, were used to confirm the purity of nuclear extracts. **E**, Au565 cells were treated with GW2974 (1  $\mu\text{mol/L}$ ) or vehicle alone (-) for 24 hours. Steady-state levels of total and phosphorylated p185<sup>ErbB2</sup> and p95L were analyzed by ErbB2 IP/Western blot (WB) analysis from nuclear extracts. Total (green) and phosphorylated (red) forms of the indicated proteins are shown. Cells treated with vehicle alone (0.01% DMSO) served as controls for all of the experiments in Fig. 1.

### Proliferation and apoptosis assay

The proliferation assay was carried out in a 96-well plate format in a final volume of 100  $\mu\text{L}$  per well cell culture medium with the cell proliferation reagent WST-1 from Roche Diagnostics. Details of the WST-1 proliferation and Annexin V/nexin 7-AAD apoptosis assays were previously published (17, 22).

### Statistical analysis

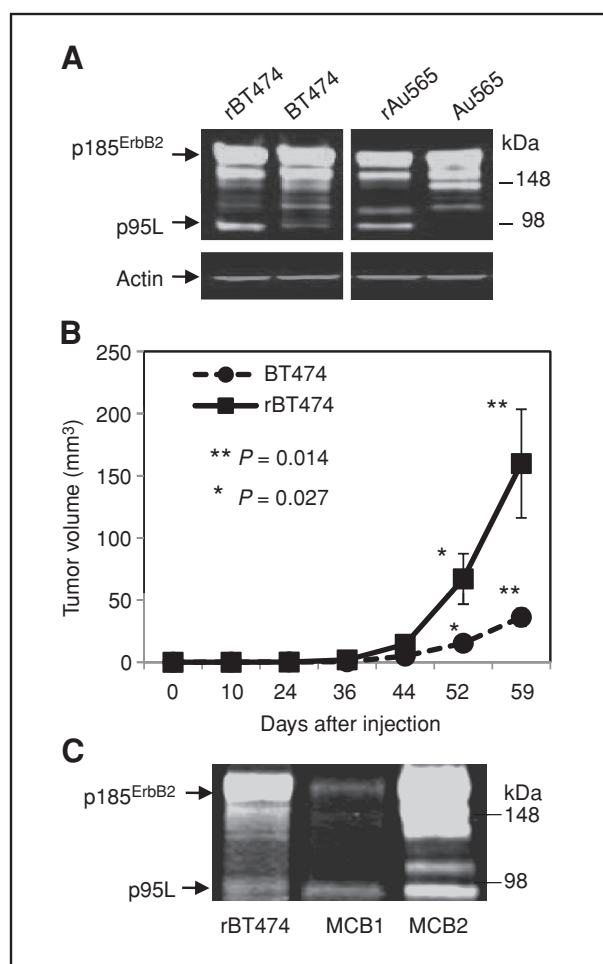
Data were expressed as means with standard error bars included. Student's *t* test was used to determine statistical significance between 2 groups.  $P < 0.05$  was considered a statistically significant difference.

### Results

#### ErbB2 TKIs increase the expression of phospho-p95L in tumor cell nuclei

The effects of ErbB2 TKI on ErbB2 tyrosine phosphorylation were determined in BT474 cells, a human ErbB2<sup>+</sup>

breast cancer cell line, using immunofluorescence microscopy. Total ErbB2 protein and phosphotyrosine expression were determined using an ErbB2-specific antibody and a phosphotyrosine (p-tyr) antibody, respectively. ErbB2 and p-tyr signals were visualized using a secondary FITC-conjugated antibody (green). Total ErbB2 expression was unchanged in response to GW2974, an ErbB2 TKI (Fig. 1B). The p-tyr signal primarily localized to the cell surface and cytoplasm in vehicle-treated controls (-). Relatively little p-tyr signal was seen in the nuclei (blue/DAPI) of control cells (Merge). Whereas cell surface and cytoplasmic p-tyr were markedly reduced in response to GW2974, nuclear p-tyr persisted (Fig. 1B, Merge). We treated another ErbB2<sup>+</sup> breast cancer cell line, Au565, with lapatinib and examined phospho-ErbB2 (p-ErbB2) expression using an ErbB2 phosphotyrosine-specific antibody and an FITC-conjugated secondary antibody (green). Similar to BT474 cells, p-ErbB2 at the cell surface, but not in the nuclei of some ErbB2 cells, was markedly reduced by lapatinib (Fig. 1C).



**Figure 2.** Increased expression of p95L in ErbB2<sup>+</sup> breast cancer models of acquired lapatinib resistance and clinical tumor samples. **A**, steady-state protein levels of p185<sup>ErbB2</sup> and p95L in rBT474 and rAu565 cells and their lapatinib-sensitive parental cell counterparts (BT474 and Au565) were determined by Western blot analysis. Actin steady-state protein levels served as a control for equal loading of protein. The results are representative of 3 independent experiments. **B**, tumor xenografts from rBT474 and BT474 cells were established bilaterally in mammary fat pads of NOD/SCID female mice treated with lapatinib 75 mg/kg/d by oral gavage for 59 days. Four mice were in each group, with 2 tumors in each mouse. Mean tumor volume (mm<sup>3</sup>) for rBT474 and BT474 are indicated (with error bars). Differences in mean tumor volumes between the 2 groups were statistically significant ( $P < 0.05$ ). **C**, total ErbB2 steady-state protein levels were analyzed in 2 metastatic sites of ErbB2<sup>+</sup> breast cancer (MCB1 and MCB2) that had progressed in women taking lapatinib. Cell lysate from rBT474 is shown with p95L indicated.

We next isolated nuclear extracts from BT474 and Au565 cells treated with vehicle alone (controls) or lapatinib. The purity of nuclear extracts was confirmed using Oct 1, IκB, and E-cadherin, which represent nuclear, cytoplasmic, and cell membrane proteins, respectively. Steady-state levels of total p95L protein increased in lapatinib-treated cells without an appreciable change in p185<sup>ErbB2</sup> (Fig. 1D). Additional molecular weight bands above 98 kDa, which have been seen previously in ErbB2 blots, were observed.

In Fig. 1E, total ErbB2 protein was immunoprecipitated (IP) from nuclear extracts isolated from Au565 cells treated with vehicle alone (control) or GW2974. Steady-state levels of total (green) and phosphorylated (red) p185<sup>ErbB2</sup> and p95L were determined by Western blot analysis. Although both p185<sup>ErbB2</sup> and p95L were expressed in a phosphorylated state, GW2974 inhibited phosphorylation of p185<sup>ErbB2</sup> but not p95L. Similar results were seen in other ErbB2<sup>+</sup> breast cancer cell lines (data not shown).

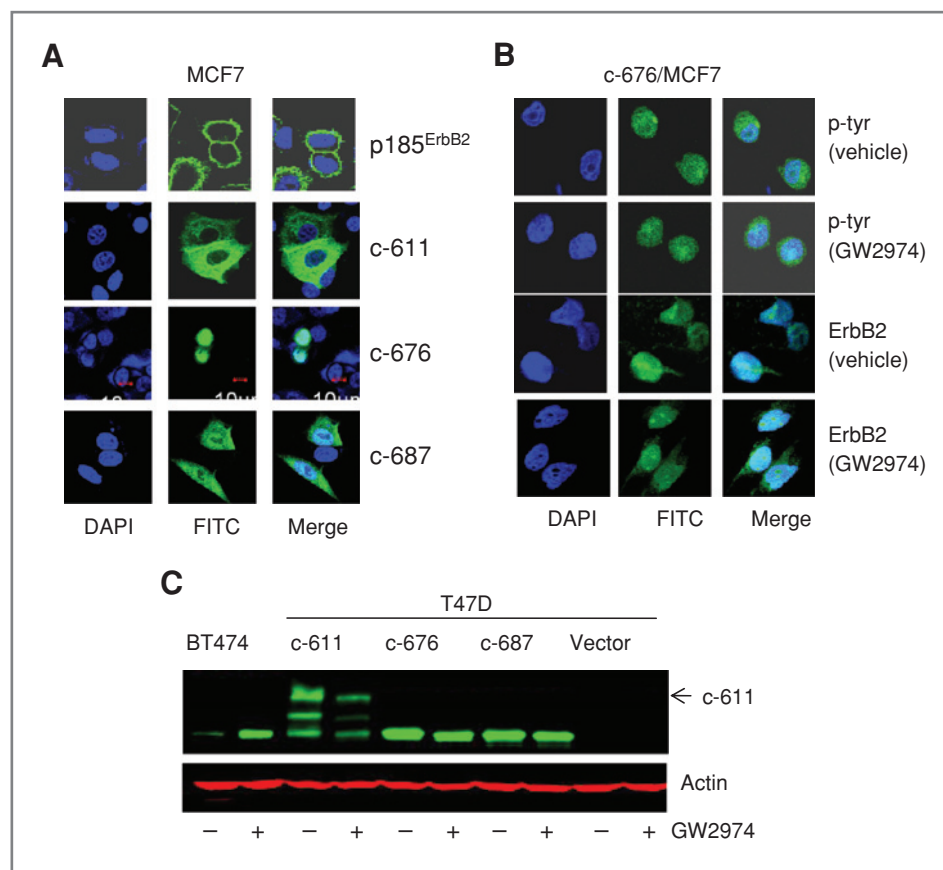
### Increased expression of p95L in lapatinib-resistant breast cancer cell lines and tumor xenografts

P95L protein levels were increased in models of acquired resistance to lapatinib (e.g., rBT474; rAu565; ref. 22) compared with their lapatinib-sensitive cell counterparts (Fig. 2A). In Fig. 2B, the growth of tumor xenografts established from resistant cells (rBT474) was significantly increased compared with tumors derived from parental cells (BT474), in animals treated with lapatinib ( $P < 0.05$ ). Steady-state p95L protein levels were increased in rBT474 compared with BT474 tumor xenografts (data not shown).

To determine whether a truncated form(s) of ErbB2 similar to p95L could be detected in clinical tumors, we analyzed steady-state ErbB2 protein levels in biopsies from metastatic breast cancer sites that had developed while patients were on lapatinib therapy. A truncated form of ErbB2, similar in molecular weight to p95L in rBT474 cells, was seen in both clinical samples (Fig. 2C, MCB1 and MCB2).

### Comparison of p95L with CTFs of ErbB2 generated by alternate initiation of translation

CTFs of ErbB2 generated by alternate initiation of translation (e.g., c-611, c-676, c-687) have been reported (8). c-611 lacks most of the ECD, whereas c-676 and c-687 lack the ECD and transmembrane regions. We synthesized CTFs by alternate initiation of translation from methionines 611 (c-611) and 676 (c-676) and used the pcDNA3.1 vector to express them in non-ErbB2-overexpressing MCF7 and T47D breast cancer cells. In Fig. 3A, the subcellular localization of p185<sup>ErbB2</sup>, c-611, c-676, and c-687 expressed in MCF7 transfected cells was determined by immunofluorescence microscopy using an ErbB2-specific primary and FITC-conjugated secondary antibody (green). While c-611 localized to the cell membrane and cytoplasm, c-676 was seen primarily in tumor cell nuclei (blue). In Fig. 3B, the effects of GW2974 on the phosphorylation of c-676 expressed in MCF7 transfected cells were examined by immunofluorescence microscopy using a phosphotyrosine (p-tyr) antibody and FITC-conjugated secondary antibody (green). Phosphorylation of nuclear c-676 was not inhibited by GW2974. The effect of GW2974 on steady-state phosphoprotein levels of the indicated CTFs was next determined by Western blot analysis using an ErbB2 phosphotyrosine-specific antibody in whole cell extracts from T47D cells transfected



**Figure 3.** Similarity of p95L induced by ErbB2 TKI and ErbB2 CTFs generated by alternate translation initiation. A, the subcellular localization of the indicated CTFs (c-611, c-676, c687) and p185<sup>ErbB2</sup> expressed in MCF7 cells was determined by immunofluorescence microscopy using an ErbB2-specific antibody and an FITC-conjugated secondary antibody (green). Nuclei were counterstained blue with DAPI and the overlap of DAPI and FITC is shown (Merge). B, MCF7 cells expressing c-676 were treated for 24 hours with GW2974 (8 μmol/L) or vehicle alone (DMSO). The subcellular localization of p-tyr and c-676 signals was determined by immunofluorescence microscopy using phosphotyrosine (p-tyr) and ErbB2-specific antibodies, respectively, and visualized with an FITC-conjugated secondary antibody (green). The overlap between DAPI and FITC is shown (Merge). C, phospho-ErbB2 steady-state protein levels (green) were determined by Western blot analysis in T47D cells transfected with the indicated CTFs following treatment with GW2974 (8 μmol/L) or vehicle (DMSO) alone for 24 hours. Actin (red) steady-state protein levels served as a control for equal loading of protein. The arrow indicates the mature form of c-611. Results shown in Fig. 3 are representative of 3 independent experiments.

with c-611, c-676, c-687, or vector alone (Fig. 3C). GW2974 inhibited tyrosine phosphorylation of c-611 but not c-676 or c-687. Expression of p95L in BT474 cells treated with GW2974 was included as a reference. Similar results were observed in MCF7 transfected cells (data not shown).

#### Proteasome inhibitors block p95L induction by ErbB2 TKI

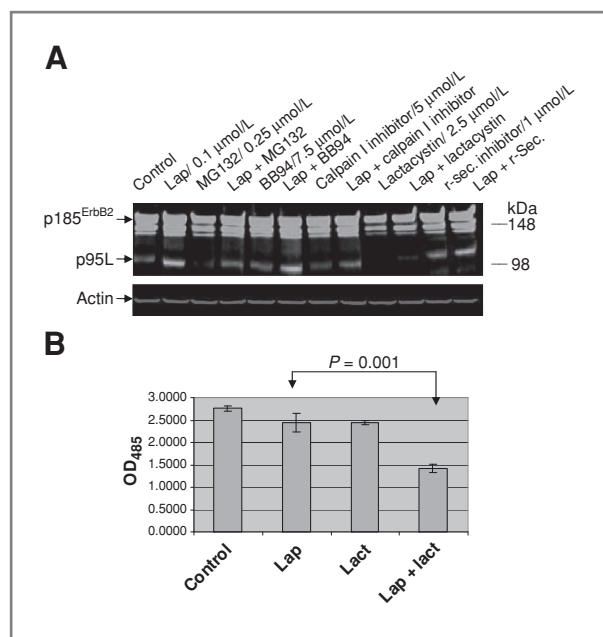
We examined the effects of protease inhibitors on p95L expression in lapatinib-treated Au565 cells. Cells were treated as indicated in Fig. 4A. Briefly, cells were treated with lapatinib alone, the indicated protease inhibitors alone, or (24) a combination of lapatinib plus protease inhibitor. Included among the protease inhibitors were BB-94, a metalloproteinase inhibitor that blocked phorbol ester-induced p95 expression (7), and a  $\gamma$ -secretase inhibitor that reduced ErbB4 truncation (25). The BB-94 and the  $\gamma$ -secretase inhibitor had little effect on the induction

of p95L by lapatinib (Fig. 4A). However, inhibitors of the 20S proteasomal subunit (lactacystin, MG132, calpain I inhibitor) blocked the induction of p95L in lapatinib-treated Au565 cells (Fig. 4A). Cells treated with vehicle alone served as controls.

Treatment with lactacystin alone, at the same concentration that blocked induction of p95L, had relatively little antitumor activity in Au565 cells (Fig. 4B). However, there was enhanced antitumor activity when lactacystin was combined with a sublethal concentration of lapatinib (0.1 μmol/L) that was otherwise sufficient to induce p95L.

#### Expression of truncated ErbB2 reduces the antitumor activity of lapatinib

To determine the impact of nuclear, truncated forms of ErbB2 on the antitumor activity of lapatinib, we expressed c-676 in BT474 cells (Fig. 5A). We chose c-676 because of its



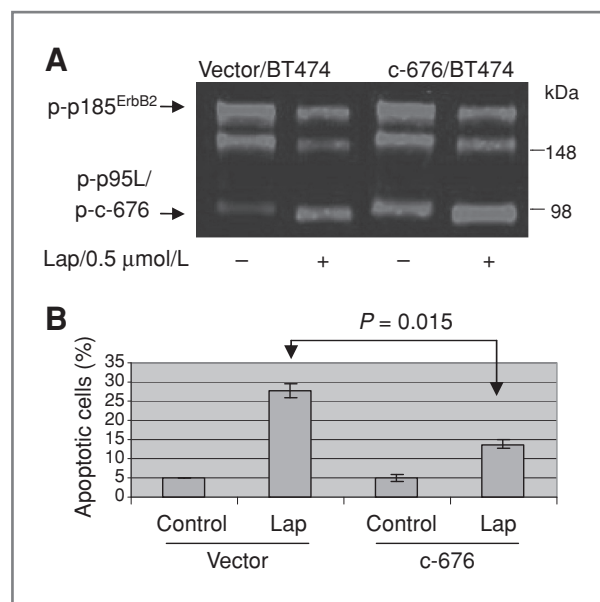
**Figure 4.** Increased p95L following treatment with ErbB2 TKI is proteasome dependent. **A**, Au565 cells were treated lapatinib in the presence or absence of the indicated protease inhibitors. The concentrations of lapatinib (lap) and individual protease inhibitors used alone are shown. The same concentrations were used in combination treatments. After 72 hours, cells were harvested and p185<sup>ErbB2</sup> and p95L steady-state protein levels were assessed. Actin steady-state protein levels served controls for equal loading of protein. **B**, effects of lapatinib (0.1  $\mu\text{mol/L}$ ) alone, lactacystin (lact; 2.5  $\mu\text{mol/L}$ ) alone, and lapatinib (0.1  $\mu\text{mol/L}$ ) + lactacystin (2.5  $\mu\text{mol/L}$ ) on Au565 cell proliferation after 24 hours. Cells treated with 0.01% DMSO served as controls. Studies were conducted in triplicates with error bars included. Differences were statistically significant ( $P = 0.001$ ). Results were confirmed in 3 independent experiments.

similarities to p95L, for example, molecular weight, nuclear localization, and resistant to ErbB2 TKI. Using an ErbB2 phosphotyrosine-specific antibody in Western blot analysis, we found that lapatinib increased steady-state p95L phosphoprotein levels in cells transfected with vector alone (Fig. 5A). In contrast, the phosphorylation of c-676 and p95L was unaffected by lapatinib. Cells transfected with vector alone served as controls.

Importantly, BT474 cells, which are normally highly sensitive to the antitumor effects of lapatinib, became significantly more resistant to lapatinib after c-676 was expressed in the nuclei of BT474 cells ( $P = 0.015$ ; Fig. 5B). Cells transfected with vector alone served as controls.

## Discussion

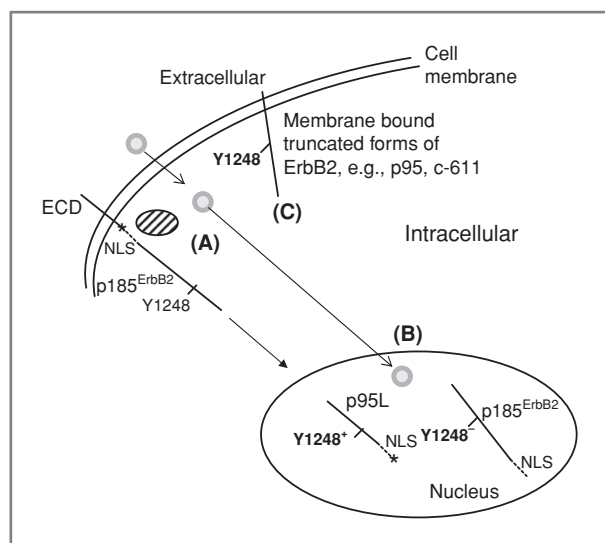
The development of acquired therapeutic resistance represents a significant barrier limiting the clinical efficacy of lapatinib. Acquired resistance to lapatinib does not appear to be related to loss of target sensitivity as tyrosine phosphorylation of p185<sup>ErbB2</sup> remains inhibited in ErbB2<sup>+</sup> breast cancer cells that have developed resis-



**Figure 5.** Expressing of c-676 reduces BT474 cells sensitivity to lapatinib. **A**, BT474 cells transiently transfected with c-676 were treated for 48 hours with 0.5  $\mu\text{mol/L}$  lapatinib or vehicle (DMSO) alone (–). Steady-state phosphoprotein levels of p185<sup>ErbB2</sup> and p95L/c-676 were determined using an ErbB2 phosphotyrosine-specific antibody. Cells transfected with vector alone served as controls. **B**, apoptosis was assessed by Annexin V staining and flow cytometry in c-676-expressing BT474 cells treated as described above. Cells transfected with vector alone served as controls. Experiments were conducted in triplicates with error bars included. Results were statistically significant ( $P = 0.015$ ). The data are representative of 3 independent experiments.

tance to lapatinib (22, 26). We now show that p95L is expressed in the nuclei of ErbB2<sup>+</sup> breast cancer cells, in a tyrosine phosphorylated, presumably activated state. In contrast to truncated forms of ErbB2 expressed at the cell surface, the phosphorylation of truncated ErbB2 in the nucleus was resistant to ErbB2 TKIs. Importantly, expression of a truncated form of ErbB2 (c-676) in the nuclei of ErbB2<sup>+</sup> breast cancer cells rendered cells resistant to the antitumor effects of lapatinib (Fig. 5).

Distinct forms of ErbB2 differing in their (i) subcellular localization, (ii) regulation of expression, and (iii) sensitivity to ErbB2 targeted therapies exist in breast cancer cells. Expressed at the cell surface, p95 and c-611 mediate resistance to trastuzumab but not ErbB2 TKIs (11, 15). In contrast, truncated forms of ErbB2 expressed in tumor cell nuclei in a tyrosine phosphorylated state are resistant to inhibition by ErbB2 TKIs. Whereas some nuclear, truncated forms of ErbB2 are generated through alternate initiation of translation (8), p95L appears to be mediated by the activation of the proteasome, as proteasome inhibitors block its induction (Fig. 4A). As summarized in Fig. 6, cleavage at a putative proteasome recognition site located within the intracellular domain (see \*) would generate a truncated form of ErbB2 with a predicted molecular weight similar to p95L. This truncated form contains tyrosine



**Figure 6.** Summary of our working model. A, the proteasome (hatched circle) is activated in ErbB2<sup>+</sup> breast cancer cells treated with lapatinib (solid circle). As a consequence, p185<sup>ErbB2</sup> is cleaved at a putative proteasome recognition site (\*), generating a truncated molecule (p95L) that retains the NLS and tyrosine autophosphorylation sites (e.g., Y1248). B, p95L localizes to tumor cell nuclei where Y1248 phosphorylation is resistant to lapatinib (Y1248<sup>+</sup>). In contrast, the tyrosine phosphorylation of nuclear p185<sup>ErbB2</sup> is inhibited by lapatinib (Y1248<sup>-</sup>). C, truncated forms of ErbB2 expressed at the cell surface (e.g., p95 and c-611) are inhibited by lapatinib (Y1248).

autophosphorylation sites (e.g., Y1248) and the nuclear localization signal (NLS). In the nucleus, tyrosine phosphorylation (Y1248) of p95L is resistant to lapatinib (Fig. 6B). In contrast, phosphorylation of p185<sup>ErbB2</sup>, which is also expressed in the nucleus, is inhibited by lapatinib. The role of the proteasome in the induction of p95L is consistent with recent findings from our laboratory showing evidence of proteasome activation in lapatinib-treated ErbB2<sup>+</sup> breast cancer cells (27). It is tempting to speculate that deregulation of intracellular calcium, which occurs in lapatinib-treated ErbB2<sup>+</sup> cells (28), leads to the accumulation of unfolded proteins, which in turn activates the proteasome.

It is not clear why the phosphorylation of truncated ErbB2 in the nucleus is resistant to ErbB2 TKI. One potential explanation is that the structural conformation of p95L and c-676 differs from p185<sup>ErbB2</sup>, preventing ErbB2 TKIs to gain access to their active sites. Studies to elucidate the structural conformation of nuclear, truncated forms of ErbB2 should help answer this question.

Although the function of nuclear, truncated forms of ErbB2 is unknown, insight from studies of p185<sup>ErbB2</sup> may be informative. When expressed at the cell surface, p185<sup>ErbB2</sup> promotes tumor growth and survival by acti-

vating downstream cell signaling cascades (2–6). In contrast, nuclear p185<sup>ErbB2</sup> directly regulates gene transcription (29–31). Nuclear p185<sup>ErbB2</sup> has been shown to activate thymidylate synthase gene transcription, which is blocked by lapatinib (29). This is consistent with our observation that phosphorylation of nuclear p185<sup>ErbB2</sup> is inhibited by lapatinib, presumably abrogating its transcriptional activity. It is tempting to speculate that phosphorylated forms of truncated ErbB2 expressed in tumor cell nuclei are also involved in regulating gene transcription, especially in light of the proteasome-dependent regulation of p95L. Although generally associated with complete proteolysis of proteins, proteasomal processing has been shown to generate biologically active proteins, particularly those involved in regulating gene transcription (32). Studies to elucidate the function(s) of nuclear, truncated forms of ErbB2 are warranted.

Patients take lapatinib on a chronic, daily basis. Our model would have predicted that this schedule would lead to the accumulation of p95L in breast cancer cells, thereby contributing to the development of acquired resistance. Results in models of acquired lapatinib resistance confirm this prediction (Fig. 2A). In addition, lower molecular weight forms of ErbB2, similar to p95L, were expressed in clinical biopsies from ErbB2<sup>+</sup> breast cancers that had progressed on lapatinib therapy (Fig. 2C). Although intriguing, these findings will require confirmation in larger studies.

Although the exact function of nuclear, truncated forms of ErbB2 remains unknown, we have provided evidence supporting their role in the development of therapeutic resistance to lapatinib and GW2974 (Fig. 5B). Strategies to enhance the clinical efficacy of ErbB2 TKIs may now include therapies that prevent induction of p95L and/or inactivate other truncated forms of ErbB2 that are expressed in tumor cell nuclei.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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