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14. ABSTRACT Our research has elucidated a novel mechanism of therapeutic resistant to ErbB2 tyrosine kinase inhibitors (TKI) mediated by truncated, activated forms of ErbB2 that are expressed in the nuclei of ErbB2+ breast cancer cells. ErbB2 TKI (e.g. lapatinib) induce the expression of a truncated form of ErbB2 that we refer to as p95L, which is expressed in a tyrosine phosphorylated state in the nuclei of ErbB2+ breast cancer cells, where it is resistant to inhibition by ErbB2 TKI. Induction of p95L is proteasome-dependent, and is blocked by proteasome inhibitors, the latter representing a potential therapeutic strategy to inhibit p95L expression and potentially overcome resistance. Similar to p95L, c-611 is a truncated form of ErbB2 generated by alternate initiation of translation, which is expressed in tumor cell nuclei in a phosphorylated state, resistant to ErbB2 TKI. Importantly, expression of c-676 in the nuclei of ErbB2+ breast cancer cells that are normally sensitive to lapatinib-induced apoptosis, rendered cells resistant to ErbB2 TKI. Studies to determine the function of nuclear truncated forms of ErbB2 are underway.					
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PROGRESS REPORT (YEAR 2) SUMMARY: PROJECT W81XWH-09-1-0065

A. INTRODUCTION

Lapatinib, a highly specific inhibitor of the ErbB2 and EGFR tyrosine kinases, is approved for the treatment of advanced stage ErbB2+ breast cancers that have progressed on prior trastuzumab-containing regimens. It is currently being evaluated as a neoadjuvant and adjuvant treatment for early stage ErbB2+ breast cancers. Although lapatinib represents a significant advance in the treatment of breast cancer, its clinical efficacy has been limited by therapeutic resistance and disease progression that generally occurs within one year following initiation of therapy.^{1,2} To better understand the mechanisms responsible for acquired resistance to lapatinib, we established *in vitro* and *in vivo* models of acquired lapatinib resistance.^{3,4} Considering the molecular heterogeneity of breast cancer, one underlying mechanism does not appear to account for the development of acquired therapeutic resistance to lapatinib, and similar drugs in class. The clinical relevance of these models has already been demonstrated.^{3,4}

The rationale for the DoD proposal was based on our working hypothesis that a 95 kDa truncated form of ErbB2 was involved in mediating therapeutic resistance to ErbB2 kinase inhibitors such as lapatinib. Our **objective** is to gain a deeper understanding of the regulation of p95 by lapatinib, and the biological effects of p95 on the regulation of cell growth and survival in ErbB2+ breast cancer cells. To achieve our objective, we had proposed the following two Specific Aims:

Aim 1: To determine how HRG regulates p95 and why it is resistant to lapatinib kinase inhibition.

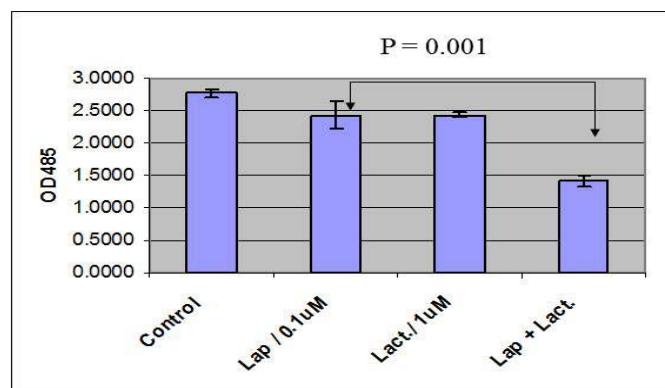
Aim 2: To identify signaling pathways that mediate the tumor promoting activities of p95 and then determine whether these pathways represent targets for therapeutic intervention.

B. BODY

Year 1 we demonstrated that ErbB2 tyrosine kinase inhibitors (TKI) including lapatinib, induced a truncated form of ErbB2 that is preferentially expressed in the nuclei of ErbB2+ breast cancer cells. We refer to this form as p95L (lapatinib inducible p95) to distinguish it from other truncated forms of ErbB2, collectively referred to as p95, that have been described in the literature. Historically, p95 refers to a truncated form that lacks the ErbB2 extracellular domain (ECD) while retaining the transmembrane region, and is therefore cell membrane bound. Cell membrane bound p95 has been shown to be generated by metalloproteinase (MMP) activity, which cleaves the ECD from full-length ErbB2, leaving a 95 kD truncated form of the receptor.⁵

During this past year, we sought to identify the mechanism(s) involved in the generation of p95L. We investigated the effects of various protease inhibitors on the induction of p95L (see manuscript in Appendix for methodology). The following protease inhibitors were examined: (i) BB-94/batimastat, an MMP inhibitor that has been shown to reduce the expression of cell membrane bound p95;⁵ (ii) a gamma secretase inhibitor that blocked ErbB4 truncation;⁶ (iii) a calpain I inhibitor; and (iv) two inhibitors of the 20S subunit of the proteasome. We found that the MMP and gamma secretase inhibitors had relatively little effect on the generation of p95L following treatment with lapatinib (see Figure 5A in the manuscript in Appendix). In contrast, lactacystin, a highly specific inhibitor of the 20S subunit of the proteasome blocked p95L induction in lapatinib-treated ErbB2+ breast cancer cells. Similarly, MG132 and the calpain I inhibitor, which also block proteasome activity, reduced the expression of p95L in lapatinib-treated cells. Since lactacystin blocked the induction of p95L, we next asked whether that same concentration of lactacystin had an effect on cell survival. ErbB2+ breast cancer cells were treated with lactacystin alone, or in combination with a sub-lethal concentration of lapatinib that was still sufficient to induce expression of p95L. All experimental treatment conditions were conducted in triplicate with data expressed as means with standard error bars included. Student's *t*-test was used to determine statistical significance between two groups. $P < 0.05$ was considered a statistically significant difference. As shown in **Figure 1**, lactacystin alone had no effect on cell survival. However, when lactacystin was combined with an otherwise sub-lethal concentration of lapatinib, there was a significant increase in cell death ($p < 0.05$).

Figure 1. Enhanced anti-tumor effect of the combination of lapatinib plus lactacystin. BT474 cells were subject to the indicated treatment conditions and cell proliferation and survival were determined after 48 h. Each treatment condition was conducted in triplicates with standard error bars indicated. The difference between lapatinib alone and the combination with lactacystin was statistically significant ($p = 0.001$).



Our working hypothesis is that p95L is involved in the development of acquired therapeutic resistance to lapatinib and similar ErbB2 TKIs (e.g. GW2974). To test our hypothesis, we needed to constitutively express p95L in otherwise lapatinib sensitive ErbB2+ breast cancer cells. Since we have not yet identified and sequenced p95L, we chose instead to express a truncated form of ErbB2 that shared common properties with p95L. C-676 is one of three ErbB2 c-terminal fragments (CTF) that are generated by alternate initiation of translation.⁷ Similar to p95L, c-676 localizes to tumor cell nuclei and shares a similar pattern on gel electrophoresis. It is also tyrosine phosphorylated on Y1248 and is resistant to inhibition by lapatinib or GW2974 (see manuscript in Appendix). We transiently transfected c-676 into BT474 cells, an ErbB2+ human breast cancer cell line that is otherwise highly sensitive to lapatinib-induced apoptosis. C-676 expression was under the control of a heterologous CMV promoter (pcDNA3.1 vector). The level of c-676 expressed in transfected BT474 cells was quite good (see Figure 5A in the manuscript in Appendix). Cells transfected with vector alone served as controls. Cells transiently transfected with c-676 and vector alone (controls) were then treated with 500 nM or vehicle alone, and after 48 h induction of apoptosis was assessed using annexin V staining and FACS analysis (details of the methodology are described in the manuscript in the Appendix). Treatment with lapatinib predictably resulted in marked apoptosis of control BT474 cells compared with controls treated with vehicle. In contrast, expression of c-676 resulted in less apoptosis in response to lapatinib compared with lapatinib-treated controls, with the difference being statistically significant ($p=0.015$) (see Figure 5B in the manuscript provide in the Appendix). The results shown are representative of three independent experiments.

We next sought to directly demonstrate the link between the expression of p95L in tumor cell nuclei and proteasomal processing of full-length ErbB2. We have generated a full-length ErbB2 construct with a His-tag at the c-terminus. The plan is to express this construct in breast cancer cells and then treat with lapatinib. If we can immunoprecipitate nuclear p95L using a His tag antibody, then we can assume it is derived from processing of full-length ErbB2. We do not anticipate problems expressing His tagged ErbB2 in cells; this has already been accomplished.

The regulation of p95L by the proteasome is consistent with our previous work demonstrating proteasome activation in lapatinib-treated ErbB2+ breast cancer cells. Directly demonstrating the involvement of the proteasome in the generation of p95L has clinical implications. To accomplish this goal, we have designed several constructs that contain mutations at putative proteasome recognition sites within full-length ErbB2. The c-terminus has been His-tagged in order to identify truncated forms generated in response to lapatinib. We are now in the process of trying to express these mutated forms of ErbB2 in breast cancer cell lines that do not constitutively express ErbB2. These putative proteasome sites predict for truncated forms of ErbB2 consistent with the molecular weight of p95L approximated by SDS-PAGE.

To identify the biological role of truncated forms of ErbB2 found in tumor cell nuclei (e.g. p95L, c-676) we first tried to establish stable transfected ErbB2 negative cell lines (MCF7; T47D) that express c-676. Initially, we were able to generate high levels of c-676 expression only transient, as cells would lose c-676 expression over time. By optimizing the selection pressure, we now have stable transfected cell lines that express c-676.

We examined the effects of expressing c-676 in MCF7 and T47D cells with and without heregulin beta 1 (HRG), a ligand for ErbB3, the latter being expressed in both MCF7 and T47D cells. As a first pass, we looked at downstream mediators of PI3K-Akt and

MAPK-Erk1/2 signaling. There were no apparent effects of expressing c-676 in the presence or absence of HRG stimulation, on these pathways.

C. KEY RESEARCH ACCOMPLISHMENTS (Year 2)

- Induction of p95L by ErbB2 TKIs is proteasome-dependent as proteasome inhibitors block p95L expression in lapatinib-treated ErbB2+ breast cancer cells.
- Demonstrating that expression of a truncated form of ErbB2 expressed in the nuclei of ErbB2+ breast cancer cells render cells resistant to the anti-tumor effects of lapatinib.
- Establishing stable transfected breast cancer cell lines that express a truncated form of ErbB2 (c-676) expressed in tumor cell nuclei.
- Generation of constructs that contain site specific deletions in putative proteasome recognition sites within the cytoplasmic domain of full-length ErbB2.

D. REPORTABLE OUTCOMES (Year 2)

Manuscript: Xia W, Liu Z, Zong R, Liu L, Zhao S, Bacus S, Mao Y, He J, Wulfkühle JD, Petricoin EF III, Osada T, Yang X, Hartman Z, Clay T, Blackwell K, Lyerly K, and Spector NL. Truncated ErbB2 expressed in tumor cell nuclei contributes to acquired therapeutic resistance to ErbB2 kinase inhibitors. *Molecular Cancer Therapeutics* (in press).

Patent: Truncated nuclear forms of ErbB family members as mediators of therapeutic resistance and as targets for therapeutic intervention (working on a provisional patent).

E. CONCLUSION

We have shown that ErbB2 TKI induce the expression of a truncated form of ErbB2 in the nuclei of ErbB2+ breast cancer cell lines. We refer to this form as p95L (lapatinib inducible p95), to distinguish it from other reported truncated forms of ErbB2 that have been collectively referred to as “p95”. There appear to be a family of truncated forms of ErbB2 that are preferentially expressed in the tumor cell nuclei. These include not only p95L, but also c-676, a CTF generated by alternate initiation of translation.⁷ We have shown that truncated forms of ErbB2 expressed in tumor cell nuclei are tyrosine phosphorylated (Y1248), which is also resistant to inhibition by ErbB2 TKI. Although one form of “p95” generated in response to phorbol ester, remains cell membrane bound and generated by MMP proteolysis,⁵ we now show the 20S proteasomal subunit plays a role in the generation of p95L in lapatinib (or GW2974) treated ErbB2+ breast cancer cells, as p95L expression was blocked by co-incubation with proteasome inhibitors. Importantly, we were able to show that expression of a c-611, which shares common properties with p95L, in the nuclei of otherwise lapatinib-sensitive ErbB2+ breast cancer cells, rendered cells resistant to lapatinib.

The development of therapeutic resistance to lapatinib represents a significant barrier limiting its clinical efficacy.^{1,2} Therapies targeting mechanisms of resistance to lapatinib and similar TKIs are likely to improve clinical outcomes in women with ErbB2+ breast cancer. Since it now appears that truncated forms of ErbB2 expressed in tumor cell nuclei are tyrosine phosphorylated and therefore presumably functionally active, gaining a deeper understanding of their regulation, and their function, will lead to therapeutic strategies to overcome, delay, or ideally prevent, the development of acquired therapeutic resistance to ErbB2 targeted therapies.

We will pursue studies to determine the functional role of truncated ErbB2 expressed in tumor cell nuclei. In this regard, there are lessons to be learned from functional studies of full-length ErbB2, which have shown that the function differs depending upon whether ErbB2 is expressed at the cell membrane or in the nucleus. When expressed at the cell membrane, full-length ErbB2 elicits its effects via cell signaling networks. However, when expressed in tumor cell nuclei, full-length ErbB2 regulates gene

transcription. We are speculating that truncated ErbB2 expressed in tumor cell nuclei is regulating gene expression. As a first step, we will analyze the effects of stably expressing c-676 in MCF7 and T47D on gene expression analysis, using cells transfected with vector alone as controls. The goal of these initial studies will address whether there are different gene expression patterns between controls and c-676 expressing cells. If there are differences in gene expression profiles, we will conduct a pathway analysis mapping to determine what cell signaling pathways are associated with the gene expression profiles, and then confirm the results by Western blot analysis. Our intent is to use this information to identify cell signaling pathways to target through targeted molecular knockdowns, and/or where available, targeted therapies. This approach will enable a deeper understanding of how truncated nuclear forms of ErbB2 mediate therapeutic resistance to ErbB2 targeted therapies, and their potential role in regulating the growth and survival of breast cancer cells.

F. REFERENCES

1. Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, *et al.* Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med.* 2006; 355: 2733-43.
2. Johnston S, Trudeau M, Kaufman B, Boussen H, Blackwell K, LoRusso P, *et al.* Phase II study of predictive biomarker profiles for response targeting human epidermal growth factor receptor 2 (HER-2) in advanced inflammatory breast cancer with lapatinib monotherapy. *J Clin Oncol.* 2008; 26: 1066-72.
3. Xia W, Bacus S, Hegde P, Husain I, Strum J, Liu L, *et al.* A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc Natl Acad Sci U S A.* 2006; 103: 7795-800.
4. Xia W, Bacus S, Husain I, Liu L, Zhao S, Liu Z, *et al.* Resistance to ErbB2 tyrosine kinase inhibitors in breast cancer is mediated by calcium-dependent activation of RelA. *Mol Cancer Ther.* 2010; 9: 292-9.
5. Codony-Servat J, Albanell J, Lopez-Talavera JC, Arribas J, Baselga J. Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. *Cancer Res.* 1999; 59: 1196-201.
6. Ni CY, Murphy MP, Golde TE, Carpenter G. gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science.* 2001; 294: 2179-81.
7. Anido J, Scaltriti M, Bech Serra JJ, Santiago Josef B, Todo FR, Baselga J, *et al.* Biosynthesis of tumorigenic HER2 C-terminal fragments by alternative initiation of translation. *EMBO J.* 2006; 25: 3234-44.

Truncated ErbB2 expressed in tumor cell nuclei contributes to acquired therapeutic resistance to ErbB2 kinase inhibitors

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Abbreviations list: TKI (tyrosine kinase inhibitors), p95L (lapatinib-induced p95).

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Abstract

ErbB2 tyrosine kinase inhibitors (TKI) block tyrosine autophosphorylation and activation of the full-length transmembrane ErbB2 receptor (p185^{ErbB2}). In addition to p185^{ErbB2} 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 demonstrated. 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+ 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+ 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+ 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.

Introduction

ErbB2, a 185 kDa transmembrane receptor tyrosine kinase (p185^{ErbB2}), 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.(2) These phosphotyrosine residues serve as docking sites for adaptor proteins that link ErbB2 to downstream mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) signaling networks that promote the growth and survival of breast cancer cells.(2-6) In addition to p185^{ErbB2}, truncated forms of ErbB2 lacking all or most of the N-terminus extracellular domain (ECD) exist in ErbB2+ 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^{ErbB2}. 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 since 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+ breast cancer cell lines.(22, 23)

We now show that treatment with ErbB2 TKIs increased the expression of a tyrosine phosphorylated, truncated form of ErbB2 that was expressed in the nuclei of ErbB2+ 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 & Methods

Cell culture and reagents

BT474, SKBR3, Au565, MCF7, and T47D breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). 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 (St. Louis, MO). Anti-c-ErbB2 (Ab -11) monoclonal antibody was from Neo Markers (Union City, CA). Anti-ErbB2 (AA1243-1255) and anti-phospho-ErbB2 (Y1248) antibodies were from Upstate Biotechnology (Lake Placid, NY). MG132, gamma-secretase inhibitor, and lactacystin were from Calbiochem (San Diego, CA). BB94 (Batimastat) was from Kimia Corp (Santa Clara, CA). Protein G agarose was purchased from Boehringer Mannheim (Germany). IRDye800 conjugated affinity purified anti-rabbit IgG and anti-mouse IgG were from Rockland (Gilbertsville, PA). Alexa Fluor680 goat anti-rabbit IgG was obtained from Molecular Probes (Eugene, OR). Lapatinib (GW572016), N-{3-Chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2(methylsulfonyl)ethyl]amino}methyl)-2-furyl]-4-quinazolinamine, was purchased from LC Laboratories (Woburn, CA). Lapatinib for cell culture work was dissolved in 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., Lincoln, NE). Membranes were incubated with fluorescent-labeled secondary antibody at a 1:10000 dilution with 3% BSA in PBS for 60 min 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*^{scid}/J (NOD/SCID) mice were purchased from Jackson Labs (Bar Harbor, ME) and bred in the Duke Comprehensive Cancer Center Isolation Facility. BT474 and rBT474 cells were suspended in Hank's Balanced Salt Solution and mixed with Matrigel (BD Biosciences, San Jose, CA) at 1:1 ratio to make final concentrations of 1×10^4 cells/50 μ l. Fifty μ l of tumor cell suspension was inoculated into bilateral mammary fat pads of female NOD/SCID mice (5~6 weeks old, 4 mice/group). Animals were treated with lapatinib (75 mg/kg/day) by oral gavage until they were sacrificed. Tumor dimensions were measured serially, and tumor volumes calculated using the following formula: long axis x (short axis)² x 0.52. The mice were euthanized with CO₂ 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 IRB 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 RIPA buffer at 4°C .

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

C-terminal fragments (c-611; c-676 and c-678) were generated based on 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 TCACACTGGCACGTCCAG-3'. MCF-7 and T47D breast cancer cells were transfected with empty vector alone (controls) or the same vector containing p185^{ErbB2} or the various CTF's using the Lipofectamine™ 2000 Reagent from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. Stably transfected cells were selected using G418 (400 $\mu\text{g}/\text{ml}$) and the expression levels of CTF's 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 min, permeabilized with 0.2% Triton X-100 for 20 min, and blocked with 2% BSA 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 FITC-conjugated swine anti-rabbit or rabbit anti-mouse antibodies followed by counterstaining with 1.5 µg/ml DAPI from Vector Labs (Burlingame, CA). An Olympus L Fluoview FV1000 was used for all photographs.

Proliferation and apoptosis assay

The proliferation assay was carried out in a 96 well plate format in a final volume of 100 µl/well cell culture medium with the cell proliferation reagent WST-1 from Roche Diagnostics (Mannheim, Germany). Details of the WST-1 proliferation and annexin V/annexin 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+ breast cancer cell line, using immunofluorescence microscopy (IF). 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 (Figure 1A). 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 (Figure 1A, Merge). We treated another ErbB2+ breast cancer cell line, Au565, with lapatinib and examined phospho-ErbB2 (p-ErbB2) expression using an ErbB2 phosphotyrosine specific antibody and a 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, (Figure 1B).

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, I κ B, 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^{ErbB2} (Figure 1C). Additional

molecular weight bands >98 kDa, which have been seen previously in ErbB2 blots, were observed.

In Figure 1D, 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^{ErbB2} and p95L were determined by Western blot. Although both p185^{ErbB2} and p95L were expressed in a phosphorylated state, GW2974 inhibited phosphorylation of p185^{ErbB2} but not p95L. Similar results were seen in other ErbB2+ 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)(22) compared to their lapatinib-sensitive cell counterparts (Figure 2A). In Figure 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 (Figure 2C, MCB1 and 2).

Comparison of p95L with c-terminal fragments of ErbB2 generated by alternate initiation of translation. C-terminal fragments (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, while c-676 and c-687 lack the ECD and transmembrane regions. We synthesized CTF's 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 Figure 3A, the subcellular localization of p185^{ErbB2}, c-611, c-676, and c-687 expressed in MCF7 transfected cells was determined by IF 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 Figure 3B, the effects of GW2974 on the phosphorylation of c-676 expressed in MCF7 transfected cells were examined by IF 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 using an ErbB2 phosphotyrosine specific antibody in whole cell extracts from T47D cells transfected with c-611, c-676, c-687, or vector alone (Figure 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 Figure 4A. Briefly, cells were treated with (i) lapatinib alone, (ii) 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 γ secretase inhibitor that reduced ErbB4 truncation.(25) BB-94 and the γ secretase inhibitor had little effect on the induction of p95L by lapatinib (Figure 4A). However, inhibitors of the 20S proteasomal subunit (lactacystin, MG132, calpain I inhibitor) blocked the induction of p95L in lapatinib-treated Au565 cells (Figure 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 (Figure 4B). However, there was enhanced antitumor activity when lactacystin was combined with a sub-lethal concentration of lapatinib (0.1 μ M) 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 (Figure 5A). We chose c-676 because of its similarities to p95L e.g. molecular weight, nuclear localization, 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 (Figure 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 ($p= 0.015$) (Figure 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^{ErbB2} remains inhibited in ErbB2+ breast cancer cells that have developed resistance to lapatinib.(22, 26) We now show that p95L is expressed in the nuclei of ErbB2+ 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+ breast cancer cells rendered cells resistant to the antitumor effects of lapatinib (Figure 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 (Figure 4A). As summarized in Figure 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 autophosphorylation sites (e.g. Y1248) and the nuclear localization signal (NLS). In the

nucleus, tyrosine phosphorylation (Y1248) of p95L is resistant to lapatinib (B). In contrast, phosphorylation of p185^{ErbB2}, 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+ breast cancer cells.(27) It is tempting to speculate that deregulation of intracellular calcium, which occurs in lapatinib-treated ErbB2+ 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^{ErbB2}, 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^{ErbB2} may be informative. When expressed at the cell surface, p185^{ErbB2} promotes tumor growth and survival by activating downstream cell signaling cascades.(2-6) In contrast, nuclear p185^{ErbB2} directly regulates gene transcription.(29-31) Nuclear p185^{ErbB2} 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^{ErbB2} 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, 33) Studies to elucidate the function(s) of truncated nuclear 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 (Figure 2A). In addition, lower molecular weight forms of ErbB2, similar to p95L, were expressed in clinical biopsies from ErbB2+ breast cancers that had progressed on lapatinib therapy (Figure 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 (Figure 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.

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References:

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987; 235: 177-82.
2. Dankort D, Jeyabalan N, Jones N, Dumont DJ, Muller WJ. Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins. *J Biol Chem*. 2001; 276: 38921-8.
3. Dougall WC, Qian X, Peterson NC, Miller MJ, Samanta A, Greene MI. The neu-oncogene: signal transduction pathways, transformation mechanisms and evolving therapies. *Oncogene*. 1994; 9: 2109-23.
4. Olayioye MA, Graus-Porta D, Beerli RR, Rohrer J, Gay B, Hynes NE. ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. *Mol Cell Biol*. 1998; 18: 5042-51.
5. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J*. 2000; 19: 3159-67.
6. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2001; 2: 127-37.
7. Codony-Servat J, Albanell J, Lopez-Talavera JC, Arribas J, Baselga J. Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases-1 in breast cancer cells. *Cancer Res*. 1999; 59: 1196-201.
8. Anido J, Scaltriti M, Bech Serra JJ, Santiago Josef B, Todo FR, Baselga J, *et al*. Biosynthesis of tumorigenic HER2 C-terminal fragments by alternative initiation of translation. *EMBO J*. 2006; 25: 3234-44.
9. Lin YZ, Clinton GM. A soluble protein related to the HER-2 proto-oncogene product is released from human breast carcinoma cells. *Oncogene*. 1991; 6: 639-43.
10. Zabrecky JR, Lam T, McKenzie SJ, Carney W. The extracellular domain of p185/neu is released from the surface of human breast carcinoma cells, SK-BR-3. *J Biol Chem*. 1991; 266: 1716-20.
11. Xia W, Liu LH, Ho P, Spector NL. Truncated ErbB2 receptor (p95ErbB2) is regulated by heregulin through heterodimer formation with ErbB3 yet remains sensitive to the dual EGFR/ErbB2 kinase inhibitor GW572016. *Oncogene*. 2004; 23: 646-53.

12. Chandarlapaty S, Scaltriti M, Angelini P, Ye Q, Guzman M, Hudis CA, *et al.* Inhibitors of HSP90 block p95-HER2 signaling in Trastuzumab-resistant tumors and suppress their growth. *Oncogene*. 2010; 29: 325-34.
13. Saez R, Molina MA, Ramsey EE, Rojo F, Keenan EJ, Albanell J, *et al.* p95HER-2 predicts worse outcome in patients with HER-2-positive breast cancer. *Clin Cancer Res*. 2006; 12: 424-31.
14. Christianson TA, Doherty JK, Lin YJ, Ramsey EE, Holmes R, Keenan EJ, *et al.* NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer. *Cancer Res*. 1998; 58: 5123-9.
15. Scaltriti M, Rojo F, Ocana A, Anido J, Guzman M, Cortes J, *et al.* Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer. *J Natl Cancer Inst*. 2007; 99: 628-38.
16. Pedersen K, Angelini PD, Laos S, Bach-Faig A, Cunningham MP, Ferrer-Ramon C, *et al.* A naturally occurring HER2 carboxy-terminal fragment promotes mammary tumor growth and metastasis. *Mol Cell Biol*. 2009; 29: 3319-31.
17. Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, *et al.* Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene*. 2002; 21: 6255-63.
18. Rusnak DW, Lackey K, Affleck K, Wood ER, Alligood KJ, Rhodes N, *et al.* The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. *Mol Cancer Ther*. 2001; 1: 85-94.
19. Konecny GE, Pegram MD, Venkatesan N, Finn R, Yang G, Rahmeh M, *et al.* Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells. *Cancer Res*. 2006; 66: 1630-9.
20. Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, *et al.* Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med*. 2006; 355: 2733-43.
21. Johnston S, Trudeau M, Kaufman B, Boussen H, Blackwell K, LoRusso P, *et al.* Phase II study of predictive biomarker profiles for response targeting human epidermal growth factor receptor 2 (HER-2) in advanced inflammatory breast cancer with lapatinib monotherapy. *J Clin Oncol*. 2008; 26: 1066-72.

22. Xia W, Bacus S, Hegde P, Husain I, Strum J, Liu L, *et al.* A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc Natl Acad Sci U S A.* 2006; 103: 7795-800.
23. Xia W, Bacus S, Husain I, Liu L, Zhao S, Liu Z, *et al.* Resistance to ErbB2 tyrosine kinase inhibitors in breast cancer is mediated by calcium-dependent activation of RelA. *Mol Cancer Ther.* 2010; 9: 292-9.
24. Ni CY, Murphy MP, Golde TE, Carpenter G. gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. *Science.* 2001; 294: 2179-81.
25. Spector NL, Xia W, Burris H, 3rd, Hurwitz H, Dees EC, Dowlati A, *et al.* Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies. *J Clin Oncol.* 2005; 23: 2502-12.
26. Xia W, Bisi J, Strum J, Liu L, Carrick K, Graham KM, *et al.* Regulation of survivin by ErbB2 signaling: therapeutic implications for ErbB2-overexpressing breast cancers. *Cancer Res.* 2006; 66: 1640-7.
27. Spector NL, Yarden Y, Smith B, Lyass L, Trusk P, Pry K, *et al.* Activation of AMP-activated protein kinase by human EGF receptor 2/EGF receptor tyrosine kinase inhibitor protects cardiac cells. *Proc Natl Acad Sci U S A.* 2007; 104: 10607-12.
28. Kim HP, Yoon YK, Kim JW, Han SW, Hur HS, Park J, *et al.* Lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor, downregulates thymidylate synthase by inhibiting the nuclear translocation of EGFR and HER2. *PLoS One.* 2009; 4: e5933.
29. Wang SC, Lien HC, Xia W, Chen IF, Lo HW, Wang Z, *et al.* Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. *Cancer Cell.* 2004; 6: 251-61.
30. Xie Y, Hung MC. Nuclear localization of p185neu tyrosine kinase and its association with transcriptional transactivation. *Biochem Biophys Res Commun.* 1994; 203: 1589-98.
31. Hervas-Aguilar A, Rodriguez JM, Tilburn J, Arst HN, Jr., Penalva MA. Evidence for the direct involvement of the proteasome in the proteolytic processing of the *Aspergillus nidulans* zinc finger transcription factor PacC. *J Biol Chem.* 2007; 282: 34735-47.
32. Tian L, Holmgren RA, Matouschek A. A conserved processing mechanism regulates the activity of transcription factors Cubitus interruptus and NF-kappaB. *Nat Struct Mol Biol.* 2005; 12: 1045-53.

Legends

Figure 1. Phosphorylation of nuclear truncated ErbB2 is resistant to ErbB2 TKI.

(A) BT474 cells were treated for 48 h with GW2974 (1 μ M) or vehicle alone (-). Total ErbB2 and phosphotyrosine (p-tyr) signals (green) were visualized by IF microscopy as described in Methods. Cell nuclei were counterstained blue with DAPI. The lower row merges FITC and DAPI signals. (B) Au565 cells were treated with lapatinib (1 μ M) or vehicle alone (control) for 24 h, and p-ErbB2 was assessed by IF microscopy using an ErbB2 phosphotyrosine specific primary antibody and a FITC-conjugated secondary antibody. (C) Steady-state protein levels of p185^{ErbB2} and p95L were determined in nuclear extracts from BT474 and Au565 cells treated for 24 h with lapatinib (500 nM) or vehicle alone (control). Steady-state protein levels of Oct 1, I κ B, and E-cadherin, which represent nuclear, cytoplasmic, and cell membrane proteins, respectively, were used to confirm the purity of nuclear extracts. (D) Au565 cells were treated with GW2974 (1 μ M) or vehicle alone (-) for 24 h. Steady-state levels of total and phosphorylated p185^{ErbB2} and p95L were analyzed by ErbB2 IP/Western blot 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 Figure 1.

Figure 2. Increased expression of p95L in ErbB2+ breast cancer models of acquired lapatinib resistance and clinical tumor samples. (A) Steady-state protein levels of p185^{ErbB2} and p95L in rBT474 and rAu565 cells and their lapatinib-sensitive parental cell counterparts (BT474, Au565) were determined by Western blot. Actin steady-state

protein levels served as a control for equal loading of protein. The results are representative of three 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/day by oral gavage for 59 days. Four mice were in each group, with 2 tumors in each mouse. Mean tumor volume (mm^3) for rBT474 and BT474 are indicated (with error bars). Differences in mean tumor volumes between the two groups were statistically significant ($p < 0.05$). (C) Total ErbB2 steady-state protein levels were analyzed in two metastatic sites of ErbB2+ breast cancer (MCB1, MCB2) that had progressed in women taking lapatinib. Cell lysate from rBT474 is shown with p95L indicated.

Figure 3. Similarity of p95L induced by ErbB2 TKI and ErbB2 c-terminal fragments (CTFs) generated by alternate translation initiation. (A) The subcellular localization of the indicated CTF (c-611; c-676; c687) and p185^{ErbB2} expressed in MCF7 cells was determined by IF microscopy using an ErbB2 specific antibody and a 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 h with GW2974 (8 μM) or vehicle alone (DMSO). The subcellular localization of p-tyr and c-676 signals was determined by IF microscopy using phosphotyrosine (p-tyr) and ErbB2 specific antibodies, respectively, and visualized with a 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 in T47D cells transfected with the indicated CTF's following treatment

with GW2974 (8 μ M) or vehicle (DMSO) alone for 24 h. 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 Figure 3 are representative of three independent experiments.

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 and individual protease inhibitors used alone, are shown. The same concentrations were used in combination treatments. After 72 h, cells were harvested and p185^{ErbB2} and p95L steady-state protein levels were assessed (green). Actin steady-state protein levels served controls for equal loading of protein. (B) Effects of lapatinib (0.1 μ M) alone, lactacystin (2.5 μ M) alone, and lapatinib (0.1 μ M) + lactacystin (2.5 μ M) on Au565 cell proliferation after 24 h. 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 three independent experiments.

Figure 5. Expressing of c-676 reduces BT474 cells sensitivity to lapatinib. (A) BT474 cells transiently transfected with c-676 were treated for 48 h with 0.5 μ M lapatinib or vehicle (DMSO) alone (-). Steady-state phosphoprotein levels of p185^{ErbB2} 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 is representative of three independent experiments.

Figure 6. Summary of our working model. (A) The proteasome (hatched circle) is activated in ErbB2+ breast cancer cells treated with lapatinib (solid circle). As a consequence, $p185^{\text{ErbB2}}$ is cleaved at a putative proteasome recognition site (*), generating a truncated molecule (p95L) that retains the nuclear localization signal (NLS) and tyrosine autophosphorylation sites (e.g. Y1248). (B) P95L localizes to tumor cell nuclei where Y1248 phosphorylation is resistant to lapatinib (Y1248 +). In contrast, the tyrosine phosphorylation of nuclear $p185^{\text{ErbB2}}$ is inhibited by lapatinib (Y1248 -). (C) Truncated forms of ErbB2 expressed at the cell surface e.g. p95, c-611 are inhibited by lapatinib (Y1248).

Fig. 1

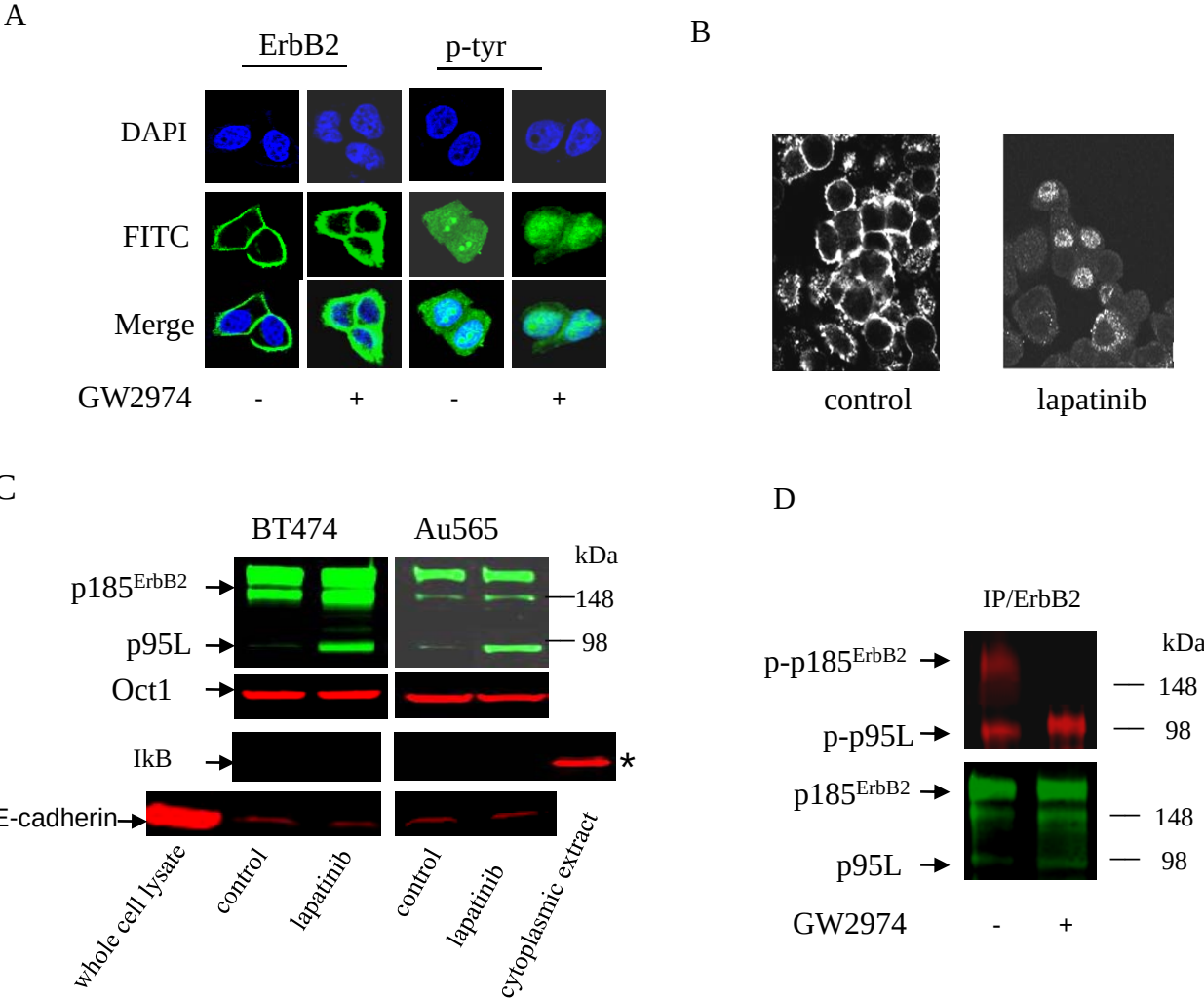


Fig. 2

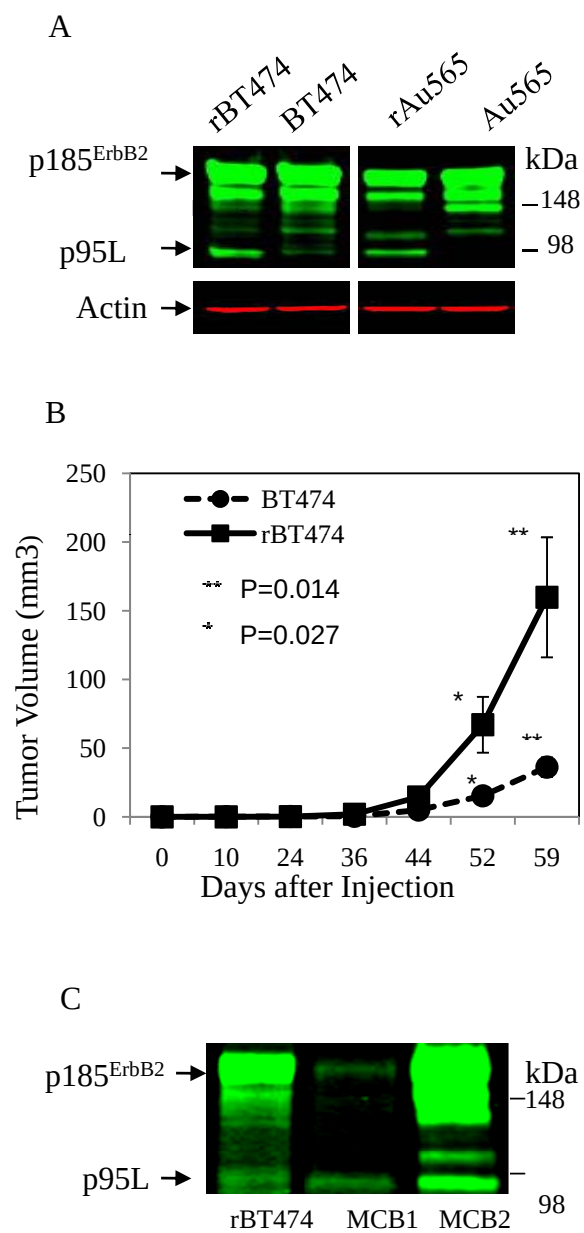


Fig. 3

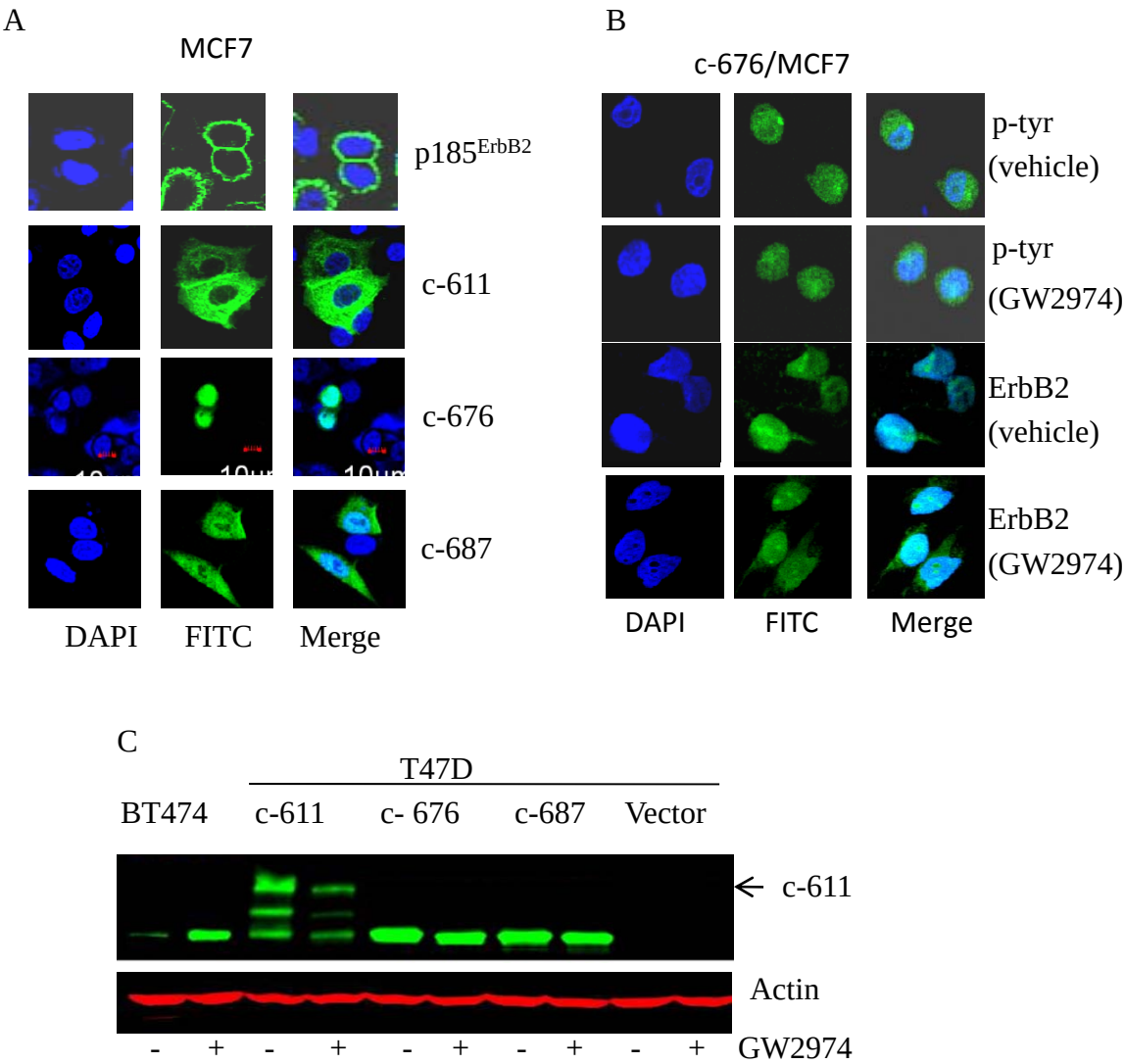


Fig. 4

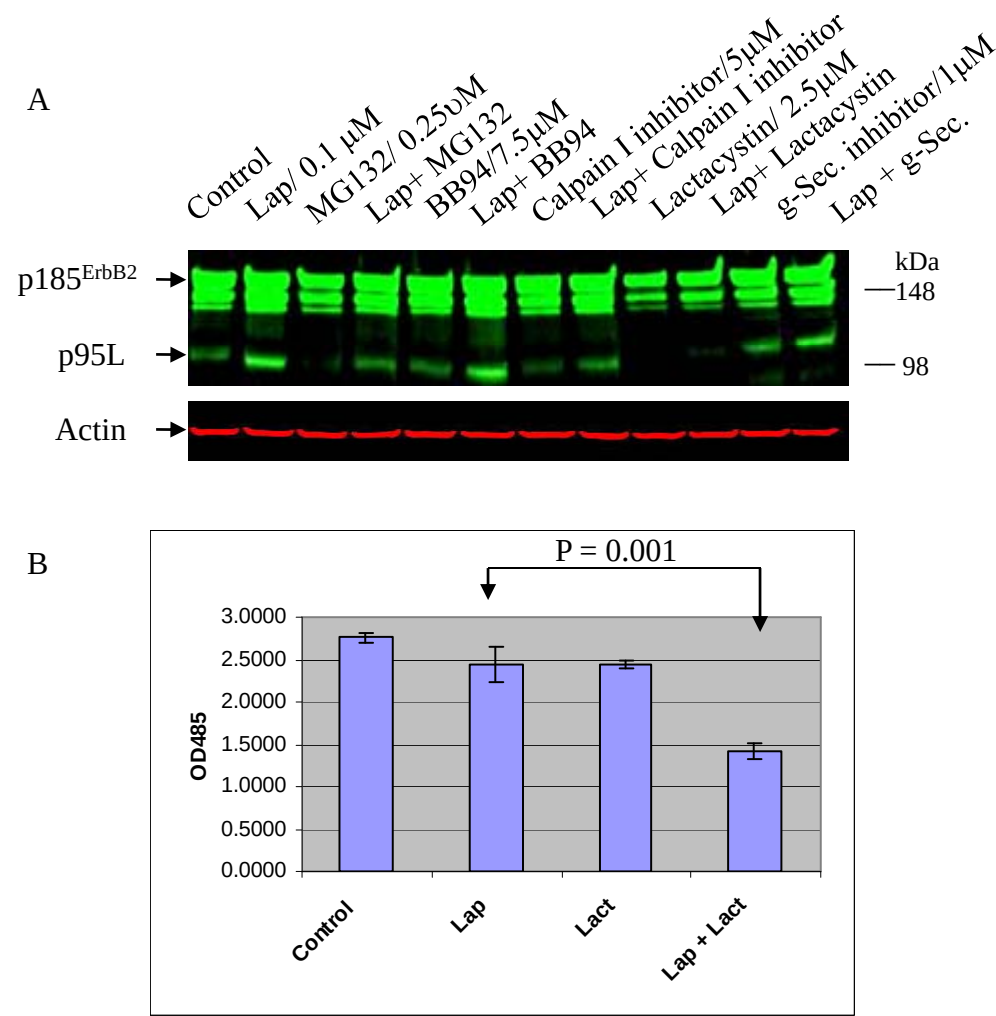


Fig. 5

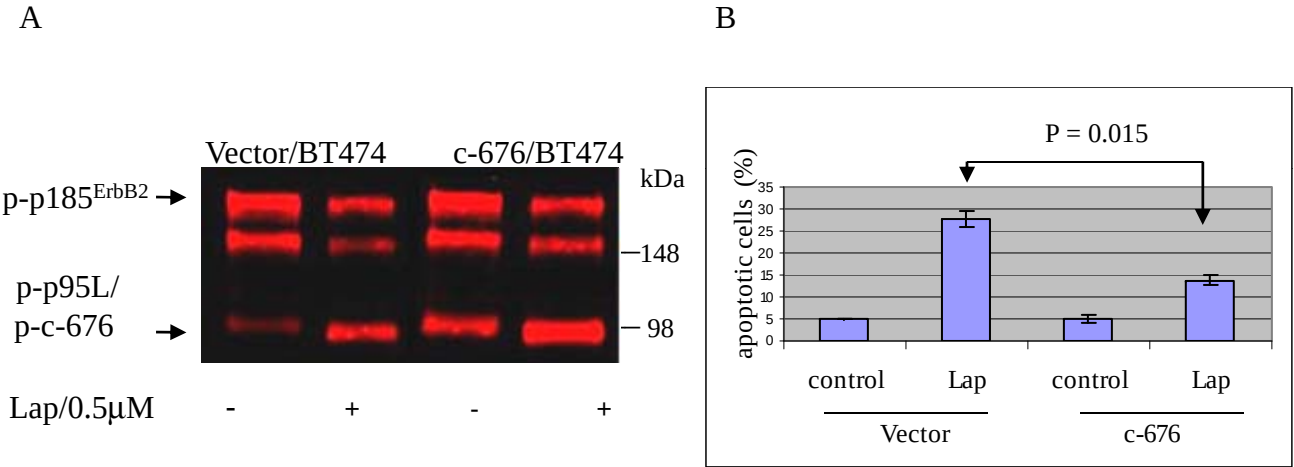


Fig. 6

