Resistance to ErbB2 Tyrosine Kinase Inhibitors in Breast Cancer Is Mediated by Calcium-Dependent Activation of RelA

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Abstract

The widespread clinical use of therapies targeting the ErbB2 receptor tyrosine kinase oncogene represents a significant advance in breast cancer treatment. However, the development of therapeutic resistance represents a dilemma limiting their clinical efficacy, particularly small-molecule tyrosine kinase inhibitors that block ErbB2 autophosphorylation and activation. Here, we show that lapatinib (GW572016), a highly selective, small-molecule inhibitor of the ErbB2 and epidermal growth factor receptor tyrosine kinases, which was recently approved for the treatment of advanced-stage ErbB2+ breast cancer, unexpectedly triggered a cytoprotective stress response in ErbB2+ breast cancer cell lines, which was mediated by the calcium-dependent activation of RelA, the prosurvival subunit of NF-κB. Abrogation of lapatinib-induced RelA activation using either small interfering RNA constructs or an intracellular calcium chelator enhanced the apoptotic effects of lapatinib in parental ErbB2+ breast cancer cells and overcame therapeutic resistance to lapatinib in ErbB2+ breast cancer lines that had been rendered resistant to lapatinib through chronic exposure to the drug, mimicking the clinical setting. In addition, analysis of changes in phospho-RelA expression in sequential clinical biopsies from ErbB2+ breast cancers treated with lapatinib monotherapy revealed marginally statistically significant differences between responders and nonresponders, which was consistent with our preclinical findings. Elucidating the regulation of RelA by lapatinib in ErbB2+ breast cancers, and showing its role in the development of therapeutic resistance to lapatinib, identifies another therapeutic target to overcome or prevent the onset of resistance to lapatinib in some women with ErbB2+ breast cancers. Mol Cancer Ther; 9(2); 292–9. ©2010 AACR.

Introduction

Overexpression of the ErbB2 receptor tyrosine kinase oncogene occurs in 20% of breast cancers where it predicts for a poor clinical outcome (1). Consequently, targeting ErbB2 using small-molecule inhibitors that compete with ATP for binding at the ErbB2 catalytic kinase domain represents an attractive treatment strategy. For example, lapatinib (GW572016) is a highly selective, reversible, small-molecule inhibitor of the ErbB2 and epidermal growth factor receptor tyrosine kinases, which was recently approved for the treatment of advanced-stage, previously treated ErbB2+ breast cancers (2, 3). Lapatinib is an equipotent inhibitor of the epidermal growth factor receptor and ErbB2 tyrosine kinases (4); however, the preponderance of evidence indicates that its antitumor activity in breast cancer is primarily mediated through inhibition of ErbB2 signaling (5–7).

Although lapatinib represents an advanced treatment for women with aggressive ErbB2+ breast cancers, the development of therapeutic resistance remains a persistent clinical dilemma limiting its clinical efficacy (3, 5). In contrast to inhibitors that target other receptor tyrosine kinases where resistance has been attributed to mutations within the targeted receptor (8, 9), ErbB2 mutations are infrequent in breast tumors (10). Therefore, to elucidate mechanisms of resistance, we developed models of lapatinib resistance by chronically exposing ErbB2+ breast cancer cell lines that were initially sensitive to lapatinib to the activation of FOXO3a transcription, which in
turn upregulated estrogen receptor signaling. These series of events resulted in a switch in the regulation of cell survival from ErbB2 to estrogen receptor. The combination of lapatinib plus an antiestrogen prevented the development of resistance in ErbB2+ breast cancer cell lines, providing the preclinical rationale for initiating ongoing clinical trials evaluating this combination (11).

However, breast cancer is a heterogeneous disease, with several subtypes distinguishable by their gene expression profiles (12). In light of the diverse repertoire of protein signaling pathways that are operative in these subtypes, it is highly unlikely that a single underlying mechanism will be responsible for lapatinib resistance. Therefore, targeting the estrogen receptor might be an effective treatment strategy for lapatinib resistance in some, but probably not all, breast cancers.

Activation of cytoprotective stress responses in lapatinib-treated ErbB2+ breast cancer cells might also contribute to the development of autoresistance. In this context, we showed that GW2974, a potent small-molecule dual ErbB2 and epidermal growth factor receptor tyrosine kinase inhibitor with a similar activity profile to lapatinib (13), induced a potent cellular metabolic stress response regulated by AMP-activated protein kinase in ErbB2-expressing cells (14). Activation of AMP-activated protein kinase switched cell metabolism from an anabolic ATP-consuming to a catabolic ATP-generating state, which served to protect cells from apoptotic stimuli (14). In addition, ErbB2+ breast cancer cells that had been rendered resistant to lapatinib (11) expressed increased phosphorylated, activated RelA, a subunit of NF-κB, the activation of which in response to a variety of cell stresses protects cells from apoptosis (15–19).

In response to certain cytotoxic chemotherapeutic agents and radiation therapy, NF-κB activates antiapoptotic genes that regulate protein products that antagonize the therapeutic, proapoptotic effects of these cancer therapies through (a) direct effects at the mitochondrial level (e.g., Bcl-xl), (b) inhibition of death receptor (CD95)-induced apoptosis (e.g., activation of FLIP), or (c) blocking caspase activation (e.g., XIAP; refs. 20–24), contributing to the development of therapeutic resistance. Relatively little is known about the effect of ErbB2+ tyrosine kinase inhibitors on RelA in breast cancer or whether RelA plays a role in the development of resistance to small-molecule inhibitors of ErbB2 kinase. This article describes the effect of lapatinib on the activation of RelA, its dependence on intracellular calcium, and the role of RelA in mediating therapeutic autoresistance to lapatinib in ErbB2+ breast cancer cells. In addition to these preclinical findings, their potential clinical relevance will be further discussed.

Materials and Methods

Cell Culture and Reagents

BT474 and SKBR3 breast cancer cell lines were obtained from the American Type Culture Collection. Lapatinib-resistant breast cancer cells (rBT474 and rSKBR3) were generated and maintained in culture as described previously (11). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and l-glutamine (Life Technologies) and maintained in a humidified atmosphere of 5% CO2 at 37°C.IRDye 800–conjugated affinity-purified anti-rabbit IgG and anti-mouse IgG were from Rockland. Alexa Fluor 680 goat anti-rabbit IgG was purchased from Molecular Probes. Antibodies to phospho-RelA (Ser529) were from Biosource. Anti-human NF-κB (RelA) antibody was purchased from Santa Cruz Biotechnology, and anti-mouse horseradish peroxidase conjugates were from Jackson ImmunoResearch Laboratories. SuperSignal West Femto Maximum Sensitivity Substrate was from Pierce. Lapatinib (2) was provided by GlaxoSmithKline. Lapatinib for cell culture work was dissolved in DMSO.

SDS-PAGE and Western Blot Analysis

Whole-cell extracts were prepared by scraping cells off Petri dishes, washing cell pellets twice in PBS, and then resuspending pellets in two packed cell volumes of radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 0.25% (w/v) deoxycholate, 1% NP-40, 5 mmol/L sodium orthovanadate, 2 mmol/L NaF, and a protease inhibitor cocktail]. Protein concentrations were determined using a modification of the Bradford method (Bio-Rad). For Western blot analysis, equal amounts of proteins (25–50 μg) were resolved by either 15%–4% or 7.5%–15% gradient SDS-PAGE under reducing conditions as described previously. Membranes were then probed with specific antibodies recognizing target proteins and IRDye 800–conjugated anti-rabbit or anti-mouse IgG or Alexa Fluor 680 anti-rabbit IgG, and proteins were visualized using the LI-COR Odyssey Infrared Imaging System.

Small Interfering RNA Transfection

Cell transfections were done in a 12-well format using 2 μL Lipofectamine 2000 (Invitrogen Life Technologies) in Opti-MEM I at 1.8 × 105 cells per well. SMART pools [containing four individual small interfering RNA (siRNA) motifs] and siControl nontargeting pool were purchased from Dharmacon. The concentration of siRNA was 100 nmol/L in a final volume of 1.1 mL according to the Invitrogen transfection protocol. After 16 to 18 h, the transfection medium was removed and replaced with complete RPMI 1640.

Apoptosis Assay

After 24 h transfection with siRNA, cells were treated in 12-well plates with lapatinib at the concentration as indicated in the figure legends. Cells were harvested with trypsin-EDTA; 5,000 cells in 50 μL were sampled on 96-well microplates. The cells were stained directly in the microplates with Annexin V-PE and Nexin 7-AAD in 1× Nexin Buffer in a 200 μL final reaction volume. After incubating at room temperature for 20 min, the reaction samples were ready to be analyzed in the
Guava PCA-96 System (Guava Technology). Anti-poly (ADP-ribose) polymerase monoclonal antibody was from BD Pharmingen. The antibody was diluted in 1:2,000 for Western blots to detect 116 kDa intact and 85 kDa cleaved forms of poly(ADP-ribose) polymerase. The development of the Western blot is the same as that described above.

Immunohistochemistry

Sequential fresh tumor biopsies were collected from patients under informed written consent as part of a protocol evaluating the efficacy of lapatinib monotherapy in women with progressive, advanced-stage ErbB2+ inflammatory breast cancer as described previously (5). Briefly, core needle tumor biopsies were processed in formalin fixative containing a cocktail of phosphatase inhibitors to preserve the phosphoprotein signal (5). Biopsies were stained with H&E to verify the presence of tumor by light microscopy. Phospho-RelA immunostaining was done using a primary antibody from Cell Signaling Technology and processed with antigen retrieval using citrate buffer (pH 6; DakoCytomation) in the “decloaker” (Biocare). Phospho-RelA was immunostained using the Autostainer (DakoCytomation). Envision+ dual-link polymer-horseradish peroxidase (DakoCytomation) was used as the detection chemistry and DAB+ (DakoCytomation) was used as the chromogen. After immunostaining, slides were counterstained manually with methyl green (DakoCytomation). Slides were analyzed by microscopy with the aid of a computer program, which is programmed to recognize areas of the biopsy containing tumor cells. Ten random fields from each slide were scored, with each field scored for the intensity of staining of the target (e.g., phospho-RelA) and attributed an absorbance value. The mean absorbance of the 10 random fields provided an overall value for target expression in that biopsy. Paraffin-embedded cell buttons from established cell lines that overexpress RelA and phospho-RelA (tumor necrosis factor-α–stimulated breast cancer lines) served as positive controls, whereas human foreskin fibroblasts cell lines lacking phospho-RelA expression served as negative controls.

Statistical Analysis

Annexin V assays were reported as the mean of three runs, shown with SD on each histogram. Ratios of day 28 to pretreatment values of phospho-RelA expression from clinical tumor biopsies were compared between responders and nonresponders using the Wilcoxon rank-sum test. Data from all patients are plotted on a log scale to accommodate the wide range of values, which necessitated excluding two whose ratio was zero.

Results

Calcium-Dependent Activation of RelA in Lapatinib-Treated ErbB2+ Breast Cancer

We recently showed increased steady-state protein levels of NF-κB/RelA in ErbB2+ breast cancer cell lines rendered resistant to lapatinib (11). Because inhibition of ErbB2 kinase activity has not been shown previously to affect RelA activity, we explored the effect of lapatinib on the regulation of RelA in ErbB2+ breast cancer cell lines. RelA phosphorylation was assessed in BT474 and SKBR3 cells before and at various time points following treatment with a clinically relevant concentration of lapatinib (25). Under

Figure 1. Time- and dose-dependent activation of RelA by lapatinib in ErbB2+ breast cancer cell lines. A, ErbB2+ breast cancer cells (BT474 and SKBR3) were treated with lapatinib (500 nmol/L) for the various times indicated. Equal amounts of protein from total cell lysate were separated by SDS-PAGE, and steady-state protein levels of phospho-RelA (Ser529) were assessed by Western blot. Actin steady-state protein levels served as a control for equal loading of protein. B, BT474 and SKBR3 cells were treated with lapatinib at the indicated concentrations. After 24 h, cells were harvested and equal amounts of protein from total cell lysate were separated by SDS-PAGE, and steady-state phospho-RelA protein levels were assessed by Western blot. Actin protein served as a control for equal loading of protein. Representative of three independent experiments.
these conditions, total RelA protein remained essentially unchanged, but steady-state levels of phospho-RelA (Ser529) protein were markedly increased after 24 and 48 h of lapatinib treatment (Fig. 1A). Lapatinib had less of an effect on phosphorylation of Ser 536 (data not shown). It is worth noting that increased RelA phosphorylation occurred in cells that were exposed to a concentration of lapatinib (100 nmol/L) that inhibited phospho-ErbB2 and downstream phospho-extracellular signal-regulated kinase 1/2 and phospho-Akt but was sublethal (26), indicating that RelA activation in response to lapatinib was not an epiphenomenon associated with apoptosis (Fig. 1B).

The effect of lapatinib on RelA resembled the activation of the intracellular metabolic stress sensor AMP-activated protein kinase by GW2974 in ErbB2-expressing cells where AMP-activated protein kinase activation by GW2974 was highly dependent on increased intracellular calcium concentrations (14). Moreover, RelA was also recently shown to be calcium dependent in glial cells (27). In light of these findings, we sought to ascertain whether the effects of lapatinib on RelA were dependent on intracellular calcium. To address this question, ErbB2+ breast cancer cells were cocultured with a sublethal concentration of BAPTA/AM (10 μmol/L), an intracellular calcium chelator, before treatment with 100 nmol/L lapatinib. Although BAPTA/AM alone had relatively little effect on constitutive steady-state levels of total RelA protein in BT474 and SKBR3 cells, it blocked RelA phosphorylation in response to lapatinib (Fig. 2A). Importantly, pretreatment with 10 μmol/L BAPTA/AM increased breast cancer cell apoptosis when combined with a sublethal concentration of lapatinib (Fig. 2B).

**Targeted Molecular Knockdown of RelA Sensitizes Therapy-Naive and Lapatinib-Resistant ErbB2+ Breast Cancer Cells to Lapatinib-Induced Apoptosis**

To show the role of RelA in the development of therapeutic resistance to lapatinib, we selectively knocked down RelA in parental ErbB2+ breast cancer cells using siRNA constructs. Targeted siRNA knockdown markedly reduced steady-state levels of total RelA protein, blocking lapatinib-induced RelA phosphorylation in BT474 and SKBR3 breast cancer cells compared with controls that were transfected with scrambled siRNA sequences.
Targeted knockdown of RelA in combination with a sublethal concentration of lapatinib (100 nmol/L) that was sufficient to inhibit phospho-ErbB2 (26) and increasing RelA phosphorylation (Fig. 1B) resulted in enhanced tumor cell apoptosis as shown by both Annexin V staining and poly(ADP-ribose) polymerase cleavage compared with either treatment alone (Fig. 3A and B).

Because phospho-RelA expression was increased in ErbB2+ breast cancer cell lines that had been rendered resistant to lapatinib (11), we next sought to determine whether molecular knockdown of RelA would overcome resistance. Targeted knockdown of RelA protein in rBT474 and rSKBR3 cells led to decreased steady-state protein levels of phospho-RelA, which enhanced tumor cell apoptosis when combined
with otherwise sublethal concentrations of lapatinib (Fig. 4A and B).

 Activation of RelA in Clinical Tumor Biopsies from Women with ErbB2+ Breast Cancer Treated with Lapatinib Monotherapy

To explore the clinical relevance of our preclinical findings, we compared phospho-RelA protein expression by quantitative immunohistochemistry in sequential biopsies of ErbB2+ breast cancer tumors obtained before (day 0) and after 28 days (day 28) of treatment with lapatinib as monotherapy. The biopsies were obtained with informed consent from patients with advanced-stage ErbB2+ inflammatory breast cancer receiving 1,500 mg once daily in a phase II clinical trial (5). Inflammatory breast cancer is characterized by an increased incidence of ErbB2+ and inherent NF-κB activation compared with noninflammatory breast cancer (5, 28). Therefore, it provided a more rigorous condition to test the clinical relevance of our preclinical findings. Thirty-five patients had sequential biopsies that contained tumor. Because these biopsies were obtained from chest wall/skin involved, which is characteristic of inflammatory breast cancer, our analysis included the best clinical response in chest wall/skin disease as determined by the investigator. Figure 5 shows two representative examples of nonresponders, the ErbB2+ breast cancers of which exhibited increased expression of phospho-RelA in response to lapatinib therapy. Ratios of day 28 to pretreatment phospho-RelA expression were variable, spanning a 3-log range. However, a borderline significant \( (P = 0.054) \) difference was observed between nonresponders and responders, with median ratios of 1.75 (a 75% increase) and 0.60 (a 40% decrease), respectively (Fig. 6).

Discussion

The development of therapeutic resistance to lapatinib remains a dilemma limiting its clinical efficacy. In contrast to other kinase inhibitors where resistance develops as a consequence of mutations within the target (8, 9), ErbB2 mutations are infrequently found in breast cancer (10), suggesting that other mechanisms play a role in the development of therapeutic resistance to lapatinib. We have shown that increased RelA phosphorylation in response to lapatinib contributes to the development of therapeutic autoresistance. By blocking phosphorylation of RelA, we sensitized treatment-naive ErbB2+ breast cancer cells to the apoptotic effects of an otherwise sublethal concentration of lapatinib. Targeting RelA in ErbB2+ breast cancers dependent on this factor for resistance offers a therapeutic strategy for improving the response to treatment with lapatinib.

Lapatinib is a highly selective, small-molecule kinase inhibitor (29); therefore, it is unlikely that its modulation of RelA phosphorylation is an off-target effect. It is also doubtful that the effects of lapatinib on RelA represent an epiphenomenon associated with cell apoptosis, as increased RelA phosphorylation occurred at concentrations of lapatinib (e.g., 100 nmol/L) that had relatively little effect on tumor cell survival (Fig. 3A and B) despite being sufficient to inhibit ErbB2 tyrosine phosphorylation (4, 21).
Interestingly, lapatinib-induced phosphorylation of RelA was dependent on intracellular calcium, as sublethal concentrations of the intracellular calcium chelator BAPTA/AM not only blocked RelA phosphorylation in response to lapatinib but also sensitized tumor cells to lapatinib-induced apoptosis (Fig. 2A and B). We showed previously that GW2974, an analogue of lapatinib, increased intracellular concentrations of calcium that contributed to the activation of AMP-activated protein kinase, which was also blocked by BAPTA/AM (14). The mechanism by which these small-molecule kinase inhibitors (lapatinib; GW2974) targeting ErbB2 elicit calcium-dependent stress responses in ErbB2-expressing cells remains to be elucidated.

The endoplasmic reticulum has been shown to be a key sensor of intracellular stress and a major repository of intracellular calcium. Under certain circumstances, increased intracellular calcium can lead to cell apoptosis (30). Activation of t- myo-inositol-1,4,5-trisphosphate receptors releases calcium from endoplasmic reticulum storage sites, and the resulting increase in intracellular calcium activates NF-κB, which constitutes negative feedback inhibiting the expression of t- myo-inositol-1,4,5-trisphosphate receptors and thereby blocking further release of calcium (31, 32). Activation of RelA in response to lapatinib may therefore represent a feedback loop to shutdown calcium release from the endoplasmic reticulum and protect against calcium-induced apoptosis. Alternatively, calcium regulates casein kinase II, and tumor necrosis factor-α has been shown to induce phosphorylation of RelA on Ser529 through a casein kinase II-dependent mechanism (33). It is possible that lapatinib could affect casein kinase II activity through increasing intracellular calcium concentrations, which in turn contribute to increased phosphorylation of RelA (Ser529). Elucidating the calcium-dependent regulation of RelA by lapatinib may identify additional tractable therapeutic targets to prevent or overcome resistance to lapatinib and possibly other ErbB2 kinase inhibitors currently in clinical development.

Breast cancer is a heterogeneous disease (12); therefore, it is highly unlikely that a single underlying mechanism will be responsible for the development of therapeutic resistance to ErbB2 kinase inhibitors. To increase efficacy without exposing women to unnecessary toxicity, therapies designed to treat or prevent resistance should target specific mechanisms involved in mediating resistance to lapatinib. As we have shown previously, combining antiestrogens with lapatinib may overcome or delay the onset of resistance to lapatinib in certain tumors where derepression of estrogen receptors contributes to lapatinib resistance (11). However, this strategy is unlikely to be effective in treating therapeutic resistance to lapatinib that is mediated by estrogen receptor–independent mechanisms.

Here, we show that RelA is activated in the presence of lapatinib, which in turn abrogates the antitumor activity of lapatinib in ErbB2+ breast cancer cells. Similar to the derepression of estrogen receptor, increased phosphorylation of RelA is a relatively early event following treatment with lapatinib. The majority of parental BT474 or SKBR3 cells undergo apoptosis, particularly within the first 72 h following exposure to therapeutic concentrations of lapatinib. However, persistent exposure to lapatinib leads to the outgrowth of resistant cells in vitro (11) and in patients, most of whom develop resistance within 1 year of initiating lapatinib therapy (3, 5). We propose that the early activation of prosurvival factors such as RelA, which may occur in a subset of tumor cells, promotes the survival of what will eventually become the resistant cell population. Moreover, activation of RelA by lapatinib promotes cell survival, whereas other effects of lapatinib may promote apoptosis; it is the aggregate balance between the activation of proapoptotic and antiapoptotic responses that will likely determine the fate of cells exposed to lapatinib. Nonetheless, using a selective, targeted knockdown strategy, we now show that targeting RelA may enhance the antitumor activity of lapatinib in some ErbB2+ breast cancers.

Targeting RelA alone will likely have limited clinical benefit in the treatment of advanced-stage breast cancer, as single-agent therapies are rarely effective in this setting. However, targeting ErbB2 and RelA simultaneously may prevent or delay the onset of therapeutic resistance to ErbB2 kinase inhibitors, similar to the combination of antiestrogens and lapatinib in certain breast cancers (11). By enhancing the antitumor activity of otherwise lower, sublethal concentrations of lapatinib, RelA targeted therapies may improve the therapeutic index of lapatinib.

We also sought to show the potential clinical relevance of our preclinical findings by analyzing changes in the expression of phospho-RelA in sequential biopsies from ErbB2+ breast cancers treated with lapatinib monotherapy as part of a phase II clinical trial (5). All of these women had ErbB2+ inflammatory breast cancer, one of the most aggressive forms of breast cancer (5). In Fig. 6, a comparison of the day 28 to pretreatment ratio of phospho-RelA expression in tumors showed borderline statistical significance between nonresponders (median 75% increase in the ratio) and responders (median 40% decrease in the ratio; Fig. 6). These clinical and preclinical findings support further investigation into the role of RelA as a mediator of resistance to lapatinib.

In light of the molecular heterogeneity of breast cancer, it is not surprising that phospho-RelA expression did not increase in some nonresponders because other mechanisms are likely to be responsible for lapatinib resistance (e.g., derepression of estrogen receptor; ref. 11). These findings resemble the multiple mechanisms implicated in the development of resistance to trastuzumab, where one factor alone (e.g., PTEN loss) does not account for all of trastuzumab resistance (34).

Treatments to prevent or overcome therapeutic resistance to lapatinib should be tailored to a specific mechanism(s) of resistance that is operative in a given tumor
(e.g., activation of RelA and derepression of estrogen receptor). Elucidating the regulation of RelA by lapatinib and showing its role in the development of therapeutic resistance will provide an opportunity to tailor treatment options to overcome or prevent resistance that is based on a specific mechanism rather than empiricism.

Disclosure of Potential Conflicts of Interest

K.M. Koch: employee, GlaxoSmithKline. N.L. Spector: honoraria, Speakers’ Bureau. No other potential conflicts of interest were disclosed.

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