Lapatinib, a Preventive/Therapeutic Agent against Mammary Cancer, Suppresses RTK-Mediated Signaling through Multiple Signaling Pathways

Jixia Li, Yeon-Yong Cho, Alyssa Langfald, Andria Carper, Ronald A. Lubet, Clinton J. Grubbs, Marna E. Ericson, and Ann M. Bode

Abstract

Activation of receptor tyrosine kinases (RTK) plays a key role in the prognosis of mammary cancer. Lapatinib is a small molecule dual RTK inhibitor that targets epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2). Identifying the protein targets involved in the effects of lapatinib and other RTK inhibitors might help determine why preventive efficacy varies. In this study, female Sprague-Dawley rats were given methylnitrosourea (MNU) by intravenous injection resulting in the development of multiple estrogen receptor–positive tumors. Treatment with lapatinib beginning 5 days after MNU was highly effective in preventing cancer development. In addition, we treated rats with palpable mammary tumors with lapatinib daily. In these tumor-bearing animals, treatment continued for 42 days and therapeutic results were obtained. Some rats bearing cancers were treated for 5 days, and the resulting lesions were examined for biomarker modulation. Lapatinib effectively suppressed the abundance of HER2, phosphorylated HER2 (Tyr1221/1222), and phosphorylated EGFR (Tyr1173, Tyr1110) compared with tumors from untreated rats. Protein array analyses allowed parallel determination of the effect of lapatinib on the relative levels of protein phosphorylation and proteins associated with apoptosis. These results combined with immunoreactivity data indicated that, in addition to EGFR and HER2, lapatinib treatment was associated with changes in a number of other signaling molecules, including IGF-1R, Akt, and downstream targets such as GSK3, p27, p53, and cyclin D1 presumably leading to impaired proliferation, apoptosis, or cell-cycle arrest. Cancer Prev Res; 4(8); 1–8. ©2011 AACR.

Introduction

The type I family of receptor tyrosine kinases (RTK) consists of 4 homologous members including ErbB1 [i.e., epidermal growth factor receptor (EGFR)], ErbB2 [human epidermal growth factor receptor 2 (HER2)], ErbB3, and ErbB4 (1). The ErbB family members contain multiple domains, including an extracellular ligand–binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain (2). Each member of the ErbB receptor family, except HER2, is activated through ligand binding leading to downstream signaling (1). However, the ErbB receptors dimerize on ligand binding and HER2 is the preferred dimerization partner for the other 3 ErbB family members (1). Activation of the ErbB receptors plays a key role in the pathogenesis and prognosis of mammary cancer. HER2 is an important regulator of cell growth and differentiation for mammary development during puberty, and deregulation of HER2 signaling in mammary cells promotes breast tumorigenesis (3). Amplification of the her2 gene is apparently the driving oncogenic event in roughly 20% of human breast cancers and is a significant predictor of reduced overall survival and a decreased time to relapse in many patients with early-stage breast cancer (4). Overexpression of the EGFR and HER2 occurs in many breast cancers (3, 5). Hence, EGFR and HER2 are important therapeutic targets for breast cancers.

Lapatinib (Fig. 1A) is an oral, small-molecule, reversible inhibitor of both the EGFR and HER2 tyrosine kinases and suppresses the activation of both receptors in the nanomolar range. In March 2007, the U.S. Food and Drug Administration approved lapatinib for use in combination with capecitabine in the treatment of advanced breast cancers overexpressing HER2 (HER2+; ref. 3). The methyl-nitrosourea (MNU)-induced estrogen receptor–positive (ER+) rat model produces tumors with a histopathology and gene array expression pattern similar to well-differentiated ER+ human tumors (6). Prior studies showed that a variety of hormonal manipulations, including SERMS,
aromatase inhibitors, and pregnancy decreased the development of these mammary tumors similarly to what is expressed in ER$^+$ human breast cancer (7, 8).

Even though EGFR and HER2 are validated therapeutic targets, only a small percentage of patients with EGFR or HER2-overexpressing tumors respond to therapy and resistance develops even in responsive patients. So, a more complete understanding of the molecular mechanisms and protein targets involved in the effects of lapatinib and other RTK inhibitors can help determine why efficacy varies and might offer short-term biomarkers of efficacy. This requires the simultaneous identification of specific molecular markers in the complex network of signaling pathways that are modulated by lapatinib in mammary cancer. Overall, our results indicate that lapatinib strongly suppresses tumor development in the MNU rat mammary breast cancer model presumably by acting through multiple protein targets. Besides EGFR1 and HER2 Neu phosphorylation, other proteins that showed associated changes include the Src family of non-RTKs, IGF-1R, Akt, and downstream targets of Akt.

Materials and Methods

Animals

In this study, female Sprague-Dawley rats (50 days old) were given MNU by intravenous injection through the jugular vein [75 mg/kg body weight (BW)]. Two different approaches were taken to determine the preventive or therapeutic effects of lapatinib. To study the preventive effect of lapatinib on MNU-induced mammary cancer development, rats were treated with lapatinib (0, 25, or 75 mg/kg BW/d, 7 d/wk by gavage) for 148 days beginning at 55 days of age. The vehicle for lapatinib was 10% ethanol: 90% PEG-400. For the duration of the study, animals were palpated twice a week for development of tumors as described previously (9). In the therapeutic study, rats exhibiting palpable MNU-induced tumors (∼150–200 mm$^3$) were treated with lapatinib (75 mg/kg BW/d) for 42 days. Cancers were measured twice a week with calipers. In a separate study, rats bearing established mammary cancers (∼150–200 mm$^3$) were either treated or not treated with lapatinib (75 mg/kg BW/d) for 5 days to compare biomarker modulation. At sacrifice, mammary cancers were rapidly removed and fixed for immunostaining or frozen for Western blot or protein array analysis. In a separate study, rats were treated with IRESSA (10 mg/kg BW) as described (9) and extracellular signal–regulated kinase (ERK) expression was analyzed by Western blotting and immunostaining.

Immunostaining and confocal microscopy

Mammary tumors were excised and drop fixed in Zamboni’s fixative [0.03% picric acid (w/v) and 2% paraformaldehyde (w/v)] for 48 hours at 4°C and then transferred to a 20% sucrose solution with 0.05% sodium azide in PBS for storage. Processing and staining of tumors were carried out according to a published procedure (10). Whole tumors were cryosectioned into 80-μm sections. Floating sections were incubated first with the primary antibody and then incubated in 1:1,000 anti-goat IgG, conjugated to Cy2 (green color), Cy3 (red color), or Cy5 (blue color; Jackson ImmunoResearch), raised in donkey. Washed samples were mounted in agar, dehydrated in ethanol, cleared with methyl salicylate, and mounted in DEPEX (Electron Microscopy Science). Optical sections were captured by laser
scanning confocal microscopy (NIKON C1si Confocal Spectral Imaging System, NIKON Instruments Co.).

**Protein array analysis**

Frozen (−80°C) tissues harvested from 4 untreated and 4 lapatinib-treated rats were homogenized in the lysis buffer included with the Proteome Profiler Array kit (R&D). The total protein concentration of each tissue lysate was determined using the Bio-Rad Protein Assay solution with bovine albumin serum as a standard. Following the instructions provided with the Proteome Profiler Array Kit, equal amounts of proteins from each tissue lysate were then analyzed using the protein array kits. The kits included antibodies for detection of phosphorylated mitogen-activated protein kinases (MAPK), antibodies to detect a variety of phosphorylated kinases, or antibodies to detect changes in proteins associated with apoptosis.

**Western blot**

Lysate proteins (200 μg) were subjected to 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (GE Healthcare). After blotting, the membrane was incubated with a specific primary antibody at 4°C overnight. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare) after hybridization with an alkaline phosphatase–linked secondary antibody.

**Results**

**Lapatinib effectively prevents MNU-induced cancer growth**

In the preventive study, rats (N = 15) were administered MNU (75 mg/kg BW) at 50 days of age and lapatinib (0, 25, or 75 mg/kg BW/d) was then initiated at 55 days of age. Lapatinib at 75 mg/kg BW was highly effective in preventing MNU-induced mammary tumor development, whereas the lower dose was less effective (Table 1 and Fig. 1B).

**Lapatinib reduces established MNU-induced tumor size**

Based on the striking preventive efficacy of the high dose of lapatinib, we examined its ability to be therapeutically effective in the MNU rat model. Lapatinib was administered by intragastric gavage (75 mg/kg BW/d) or vehicle beginning when palpable tumors (~150–210 mm³) were observed. Rats treated with lapatinib for 42 days exhibited an average decrease of 87% in mammary tumor size (Fig. 1C and D), indicating that lapatinib is highly effective as a therapeutic treatment against MNU-induced mammary cancer development in rats.

**Lapatinib suppresses total and phosphorylated HER2 (Tyr1221/1222) and phosphorylation of EGFR (Tyr1173, Tyr1110)**

Lapatinib is a known small-molecule, reversible, and potent inhibitor of both the EGFR and HER2 tyrosine kinases. This was confirmed by results indicating that lapatinib very effectively suppressed the abundance of both total and phosphorylated HER2 (Tyr1221/1222) and phosphorylated EGFR (Tyr1173, Tyr1110) compared with tumors from untreated control rats (Fig. 2A and B). The fluorescence density analysis revealed that total and phosphorylated HER2 protein levels were decreased to less than 15% of the untreated control (Fig. 2B). Furthermore, although total EGFR protein abundance was somewhat decreased by lapatinib treatment, phosphorylation of EGFR at Tyr1173 or Tyr1110 was almost totally blocked or reduced to about 25% of the untreated control, respectively (Fig. 2B). The phosphorylation of EGFR at Tyr1173 and Tyr1110 and HER2 at Tyr1221/1222 plays an important role in the activation of downstream signaling pathways including mitogen-activated protein kinases, phospholipase C/protein kinase C, and Akt, which also play critical roles in breast cancer development and maintenance (11–13).

**Lapatinib treatment is associated with attenuated expression of Src-related proteins, IGF-1R, Akt, and GSK3**

The protein array analyses allowed parallel determination of the effect of lapatinib on the relative levels of protein phosphorylation and apoptosis-associated proteins. These data together with immunofluorescence and Western blot analysis of tumor samples confirmed that, unlike gefitinib (or IRESSA), which might target the EGFR and EGFR signaling mediated primarily through MAP/ERK kinases, lapatinib has little effect on expression of ERK

<table>
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<tr>
<th>Group</th>
<th>Treatment</th>
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<tr>
<td></td>
<td>Incidence</td>
<td>Number/rat</td>
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<tr>
<td>1</td>
<td>Lapatinib, 75 mg/kg BW/d</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>Lapatinib, 25 mg/kg BW/d</td>
<td>93</td>
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<tr>
<td>3</td>
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*MNU administered to rats (15 per group) at 50 days of age. Lapatinib was initiated at 55 days of age.

*Incidence, number, and weight of mammary tumors at end of study (148 days after MNU).
regulated (Fig. 4A). Some of these results were confirmed by immunofluorescence staining of tissue (Fig. 4B), including a marked decrease in phosphorylated Akt (S473) and GSK3α/β (S21/S9). In addition, fluorescence density analysis indicated substantial decreases in IGF-1R and cyclin D1. These results indicate that lapatinib targets EGFR/HER2 as expected but also is associated with decreases in phosphorylation of Src family nonreceptor kinases, Lyn and Lck, and changes in the downstream signaling cascade of Akt, which might result in the activation of GSK3, p53, and p27. These signals also corresponded with suppression of antiapoptotic Bcl-xL and cyclin D1 along with an increased release of cytochrome c, which suggests induction of cell-cycle arrest and mitochondria-mediated apoptosis.

Discussion

Female Sprague-Dawley rats treated with MNU have been shown to develop multiple hormonally responsive mammary cancers starting within 5 weeks after carcinogen administration (9). These cancers seem to be histologically similar to ER+ mammary cancers in humans (6). In this study, we found that the small molecular EGFR/HER2 inhibitor, lapatinib, effectively prevented MNU-induced mammary tumor growth and was an effective therapeutic agent in reducing the size of MNU-induced mammary cancers.

The EGFR is a tyrosine kinase and ligand binding results in receptor dimerization, autophosphorylation, activation of downstream signaling, and lysosomal degradation (14, 15). EGFR downstream signaling controls cell proliferation, differentiation, cell cycle, and apoptosis (16). ErbB2 (or HER2) is a receptor-like glycoprotein with intrinsic tyrosine kinase activity. Although no activating ligands have been identified (17), the kinase activity of ErbB2 can be activated without ligand binding when it is overexpressed and by heteromeric association with other members of the ErB family (18). Deregulation of EGFR/HER2 can increase tumor promotion and progression in many human malignancies including breast cancer. Our data showed that lapatinib was highly effective both in preventing MNU-induced mammary tumor growth and in reducing the size of established mammary tumors by suppressing phosphorylation and abundance of both EGFR and HER2. We had previously shown that a pure EGFR inhibitor, IRESSA, was similarly highly effective as a preventive and therapeutic agent in this model (9).

The Ras/Raf/MEK/ERKs pathway is a major downstream signaling route of the ErbB family of tyrosine kinase receptors (19) and other RTK inhibitors like gefitinib (or IRESSA), most likely exert their effects by mediating the signaling from the EGFR through ERKs. Activated ERKs are critically involved in many cellular processes including proliferation, differentiation, motility, and death (20). In the present study, we found that lapatinib treatment corresponded with decreased expression of phosphorylated EGFR and HER2 as expected. However, lapatinib treatment

Figure 2. Lapatinib suppresses HER2, phosphorylated HER2 (Y1221/1222), and phosphorylated EGFR (Y1173, Y1110). Rats bearing established mammary cancers (~100–200 mm³) were treated with vehicle or lapatinib (75 mg/kg BW) for 5 days. The mammary cancers were rapidly removed and fixed for immunoreactivity analysis to determine possible molecular mechanisms or protein targets of lapatinib. A, fixed samples were subjected to immunofluorescence staining to detect total HER2, phosphorylated HER2 (Y1221/1222), total EGFR, phosphorylated EGFR (Y1173 and Y1110) by incubating with specific primary antibodies and secondary antibodies conjugated to a fluorescence dye (Cy2, green; Cy3, red; Cy5, blue). Samples were observed by confocal microscopy. Representative images are shown from a minimum of 5 tumors with similar results. B, relative immunofluorescence was quantified using Image J software (NIH). Data are shown as the means ± standard error (SE) of a minimum of 5 tumor samples.
also correlated with attenuated phosphorylation of the Src family, Akt, and GSK3 proteins but did not affect ERKs expression. The Src family of proteins is a 10-gene family (src, fyn, yes, blk, fgr, fgr, hck, lck, lyn, and srms) of non-RTKs that play an important role in the regulation of proliferation, migration, adhesion, and tumor angiogenesis (19). For many years, the Src family of tyrosine kinases has been known to be linked with the development and progression of cancer (21). Src has been known to interact with RTKs such as the EGFR, which might enhance ErbB signaling (22). In addition, the Src family is also cross-connected with the Akt pathway (19) and the IGF-1R–induced phosphoinositide 3-kinase (PI3K)/Akt signal transduction cascade also has critical roles in prevention of apoptosis and regulation of cell-cycle progression (23). Furthermore, the IGF-1R is known to interact with the EGFR (24). The Akt pathway plays a crucial role in the regulation of cell growth and apoptosis and can be activated by HER2 (25). HER2 overexpression directly corresponds with increased Akt activation and resistance to treatment in breast cancer (26, 27). Conversely, inhibition of HER2 decreases the degree of Akt activation (25), indicating that HER2 is an upstream activator of the Akt pathway. Both our protein array data and immunofluorescence analysis indicated that lapatinib suppressed EGFR/HER2 signaling and was also associated with inhibition of phosphorylation of the Src family (i.e., Lck and Lyn) and its downstream target, Akt. The Src family of kinases and EGFR and HER2 are known to interact and synergize to enhance neoplastic growth of mammary epithelial cells (28). In addition, the interaction between the SH3 domain of Src family kinases and the proline-rich motif in the C-terminal regulatory region of Akt are reportedly required for tyrosine phosphorylation of Akt and its subsequent activation (29). Furthermore, we found that IGF-1R abundance was also substantially down-regulated, which could also be related to the decreased Akt activation.

Akt has multiple downstream direct and indirect signaling targets including mediators of apoptosis and cell cycle such as GSK3, p53, and p27. GSK3 or glycogen synthase kinase 3 is a constitutively active serine/threonine kinase that phosphorylates and subsequently inactivates a key metabolic enzyme, glycogen synthase, inducing apoptosis (30). Phosphorylation of GSK3α at S21 and GSK3β at S9 by
Akt inactivates GSK3 (30). Our results indicated that lapatinib suppresses Akt activity, which could increase GSK3 activity by inhibiting Akt’s phosphorylation of GSK3α/β (S21/S9). Despite the increased activation of GSK3, no change was observed in phosphorylation or levels of β-catenin, a well-known substrate of GSK3 (31), suggesting alternative targets for GSK3 activation. GSK3 has been reported to phosphorylate and target cyclin D1 for degradation (32, 33). Thus, GSK3 activation by lapatinib could result in reduced levels of cyclin D1 as our results revealed. Because inhibition of GSK3 activity reduced cytochrome c release, GSK3 is believed to promote apoptosis through the mitochondrial intrinsic apoptosis signaling pathway (30). Furthermore, apoptosis is enhanced by the reported binding and interaction of GSK3 with p53 (34). Our results showed that lapatinib increased phosphorylation of GSK3, cytochrome c release, and p53 phosphorylation, suggesting that lapatinib treatment is associated with mitochondrial p53 apoptosis signaling. Activation of the p53 tumor suppressor protein can lead to either cell-cycle arrest and

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DNA repair or apoptosis (35), and p53 is a critical indirect downstream target of Akt signaling. Akt negatively regulates p53 through its phosphorylation of MDM2 (36–38), which enhances the degradation of p53. Phosphorylation of p53 at Ser15 is associated with DNA damage and phosphorylation of p53 at Ser342 has been reported to influence the growth suppressor function, DNA binding, and transcriptional activation of p53 (39–41). Finally, phosphorylation of p53 at Ser46 is important in regulating the ability of p53 to induce apoptosis (42, 43). After exposure to lapatinib, phosphorylation of p53 at Ser392, Ser46, and Ser15 increased, further indicating that lapatinib might induce cell-cycle arrest and apoptosis by indirectly protecting p53 from degradation. The Cdk inhibitor, p27, which might function as a putative tumor suppressor, can also be negatively regulated by Akt (44). The nuclear localization signal of p27 contains an Akt consensus site at Thr157, and p27 phosphorylation by Akt impairs its nuclear import (44) and loss of its inhibitory actions against Cdk2 (45). Hence, lapatinib treatment might cause increased phosphorylation of p27 by inhibiting Akt (see Fig. 5 for proposed scheme of lapatinib action). These results were in good agreement with the reported effects of lapatinib on cyclin D1 and p27 in MMTV-erbB2 transgenic mice, which is a mouse model that spontaneously develops estrogen receptor–negative and ErbB2-positive mammary tumors within 14 months of age (46). In this study, mice were treated from age 3 to 15 months with vehicle or lapatinib (30 or 75 mg/kg BW) by oral gavage twice daily 6 d/wk. Investigators observed blockade with vehicle or lapatinib (30 or 75 mg/kg BW) by oral gavage twice daily 6 d/wk. Investigators observed blockade of EGFR/HER2 signaling that corresponded with decreased cyclin D1 and epiregulin mRNA levels and increased p27 mRNA expression (46). Others have shown that lapatinib in combination with tamoxifen effectively inhibited the growth of tamoxifen-resistant ErbB2 overexpressing MCF-7 mammary tumor xenografts and the drug combination was associated with a greater reduction of cyclin D1 and a larger p27 increase and cyclin E/Cdk2 inhibition than was observed with either drug alone (47). Lapatinib has also been reported to inhibit the formation of brain metastases in a well-established preclinical metastatic breast cancer xenograft model that is also resistant to trastuzumab. In this model, inhibition of ErbB2 phosphorylation by lapatinib corresponded with the reduction in the size of brain metastases (48).

In summary, our results showed that application of lapatinib markedly inhibits tumor growth in the MNU-induced mammary cancer model both as a preventive agent and as a therapeutic agent. The inhibition apparently occurs through the suppression of EGFR/HER2 signaling, which was associated with decreases in phosphorylated Src family members and IGF-1R. All 3 groups of receptors have known interactions with each other and Akt as a common target. The inhibition of Akt phosphorylation is linked to a number of downstream consequences. Suppressing phosphorylation of Akt is associated with increased phosphorylation of p53 and p27 and decreased phosphorylation and activation of GSK3, which corresponds with an increased release of cytochrome c and decreased cyclin D1 expression. These events are all characteristics linked with the induction of apoptosis and cell-cycle arrest (Fig. 5). Overall, these data suggest that a combination of lapatinib and an inhibitor of Akt might prove beneficial in RTK-resistant breast tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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