Lapatinib inhibits the activation of NF-κB through reducing phosphorylation of IκB-α in breast cancer cells

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Abstract. Lapatinib is highly active against breast cancer with HER2 overexpression in preclinical and clinical settings. Constitutive activation of NF-κB is linked to proliferation and apoptosis in breast cancer cells. NF-κB can be activated by HER2 in breast cancer cells. However, the effect of lapatinib on NF-κB activity is not completely clear. In this study, we showed that lapatinib potently inhibited activation of NF-κB in HER2-overexpressing breast cancer cells, including SKBR3 and MDA-MB-453; but not in non-HER2-overexpressing breast cancer cells, MDA-MB-231, MDA-MB-468 and MDA-MB-435. In addition, we established a model of acquired resistance to lapatinib by chronically challenging SKBR3 breast cancer cells with increasing concentrations of lapatinib. EMSA assays showed that there was decreased NF-κB activity in the resistant cells. Western blot analysis showed that lapatinib reduced the phosphorylation of IκB-α in a time- and dose-dependent manner in SKBR3 cells. Furthermore, the expression level of p-IκB-α protein was markedly decreased in the resistant cells, compared with the parental SKBR3 cells. Additionally, treatment with the PI3K inhibitor LY294002 dramatically inhibited activation of NF-κB in HER2-overexpressing breast cancer cells. Moreover, LY294002 inhibited phosphorylation of Akt and IκB-α in SKBR3 cells. Our results suggest that lapatinib potently inhibits the activation of NF-κB in HER2-overexpressing breast cancer cells. Lapatinib appears to inactivate NF-κB through reducing phosphorylation of IκB-α via blocking the PI3K/Akt cascade.

Introduction

HER2 (human epidermal growth factor receptor 2), which is also termed ErbB2/neu, is a membrane-bound tyrosine kinase receptor of the epidermal growth factor receptor (EGFR) family including EGFR, HER2, HER3 and HER4. Ligand binding and/or receptor overexpression trigger homodimerization or heterodimerization of HER receptors, which promotes autophosphorylation of the intracellular tyrosine kinase domain, subsequently leading to activation of downstream signaling cascades, including the PI3K/Akt and Erk pathways (1). HER2 plays a vital role in cell proliferation and survival in HER2-driven breast cancer (2). Overexpression or amplification of HER2 occurs in approximately 25% of malignant breast tumors, and is associated with aggressive disease and significantly decreased disease-free survival and overall survival (3,4). Consequently, anti-HER2-targeted therapies represent an attractive strategy in clinical practice. Lapatinib, a small-molecule tyrosine kinase inhibitor of EGFR and HER2, has been shown to inhibit cell proliferation and induce apoptosis in HER2-overexpressing breast cancer cell lines (5-7). In the clinic, lapatinib in combination with capecitabine or letrozole is already successfully used in the treatment of HER2-amplified locally advanced or metastatic breast cancer patients (8,9).

Nuclear factor κB (NF-κB) is a key mediator of a variety of cellular processes involving cell cycle control, differentiation, inflammation and survival (10). The NF-κB family consists of five subunits: RelA (p65), RelB, c-Rel, p105/p50 and p100/p52. The NF-κB subunits exist as either homodimers or heterodimers, and the p65/p50 heterodimeric complex is the most abundant one in human epithelial cells. In other cell types NF-κB dimers are sequestered in the cytoplasm via association with the members of the inhibitors of NF-κB (IκB) family, which includes structurally related proteins identified as IκB-α, IκB-β, IκB-γ, IκB-ε, IκB-ζ and Bcl3. IκB-α is the most extensively studied member of the IκB family. Upon stimulation, the activated IκB kinase (IKK) phosphorylates IκB proteins or IκB-like domains, directing the IκB to ubiquitin-dependent degradation and releasing NF-κB homodimers or heterodimers to enter the nucleus where they bind to specific DNA sequences and promote the transcription of target genes (11,12). In most canonical NF-κB signaling, IκB-α is phosphorylated by IKK at Ser32 and Ser36, ubiquitylated at Lys21 and Lys22 and subsequently degraded by the 26S proteasome (11).

NF-κB-associated pathways are tightly linked to apoptosis, proliferation, invasion and angiogenesis in breast cancer cells (13-15). Increasing evidence suggests that constitutive activation of NF-κB appears to be a critical determinant of

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chemoresistance (16,17), endotherapy resistance (18) and radioresistance (19) in breast cancers.

It has been described that NF-κB can be activated by HER2 in breast cancer cells (20,21). However, the impact of lapatinib as a HER2 inhibitor on the activity of NF-κB is still not completely clear. In the present study, we aimed to investigate the effect of lapatinib on NF-κB activity in breast cancer cells. In addition, we established a lapatinib-resistant cell model by chronically exposing SKBR3 cells to increasing concentrations of lapatinib and characterized the activity of NF-κB in the resistant cells.

Materials and methods

Cell lines and cell culture. The human breast cancer cell lines, MDA-MB-435, MDA-MB-231, MDA-MB-468, SKBR3 and MDA-MB-453, were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 or DMEM supplemented with 10% fetal bovine serum at 37˚C with 5% CO_2 in a humidified incubator.

Compounds and antibodies. Lapatinib was kindly provided by GlaxoSmithKline. AZD6244 was purchased from Selleck Chemicals (Woburn, MA). LY294002 was purchased from Cell Signaling Technology (Beverly, MA). In these cases, 10 mmol/l aliquots of drug in DMSO (dimethyl sulfoxide) were stored at-20˚C and diluted just before utilization. All antibodies were purchased from commercial sources as indicated below: anti-Neu (F-11), anti-p-Neu (Tyr1248)-R, anti-ErbB-3 (C-17), anti-p-ErbB-3 (Tyr1328), anti-p-Akt1/2/3 (Ser 473)-R, anti-p-Erk (E-4), anti-Akt (Ser 473)-R, anti-Erk (E-4), NF-κB p50 and β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). EGFR, p-EGFR, phospho-NF-κB p65 (Ser536), NF-κB p65 (C22B4), IκB-α (L35A5), phospho-IκB-α (Ser32) and histone H3 antibodies were obtained from Cell Signaling Technology. HRP-conjugated goat-anti-rabbit IgG, goat-anti-mouse IgG and donkey-anti-goat IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. or Cell Signaling Technology.

Development of lapatinib-resistant breast cancer cells. To generate lapatinib-resistant SKBR3 cells (rSKBR3), parental SKBR3 cells were initially grown in medium containing 0.25 μmol/l lapatinib. The concentration of lapatinib in medium was gradually increased to 5 μmol/l over 18 months. The resistant cells were constantly pooled by collecting all viable cells of several plates onto 1 plate. Single-cell clones were isolated from the pooled resistant cells. Finally, the resistant cells were maintained in medium supplemented with 5 μmol/l lapatinib. Resistance to lapatinib was confirmed by a cell viability assay as described below. The resistant cells were cultured in medium without lapatinib for 2 or 3 days before each experiment.

Cell viability assay. Cell viability was determined using the CCK-8 (Cell Counting Kit-8; Dojindo, Japan) assay. Briefly, parental and resistant cells were seeded at a density of 3-5x10^3 cells into a 96-well microplate and incubated overnight. The cells were then treated with various concentrations of lapatinib. After incubation for 48 h, 10 μl of CCK-8 solution was added to each well, and the plates were further incubated for 3 h at 37˚C. The absorbance at 450 nm was measured with an absorbance microplate reader. Cell survival for all experiments was expressed as the percentage of viable cells compared with untreated cells.

Western blot analysis. Cells were washed twice in cold PBS and then lysed in RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25% (w/v) deoxycholate, 1% NP-40, 5 mM sodium orthovanadate, 2 mM sodium fluoride and a protease inhibitor cocktail]. The protein concentration of the supernatants was determined using a modification of the Bradford method. Equal amounts of proteins (40 μg) were resolved by SDS-PAGE. Proteins were transferred to PVDF membranes and blocked with 5% non-fat milk in PBS-T. Membranes were incubated overnight with specific antibodies. After four washes in PBS-T, membranes were then incubated with horseradish peroxidase-linked secondary antibody at a 1:2,000 dilution. Target proteins were visualized with the SuperSignal West Femto Maximum Sensitivity Substrate kit and subsequent exposure to X-OMAT X-ray film according to the manufacturer's instructions.

Nuclear extract preparation and electrophoretic mobility shift assay. Nuclear extracts were prepared using a nuclear and cytoplasmic proteins extract kit purchased from Beyotime Institute of Biotechnology (Haimen, China). Electrophoretic mobility shift assay (EMSA) was performed using a chemiluminescent assay kit according to the protocol provided by the vendor (Beyotime Institute of Biotechnology) with minor modifications. Briefly, for each reaction, 20 μg of nuclear protein extracts was incubated with biotin end-labeled probe. The protein-DNA complex was then resolved by electrophoresis on 5% native polyacrylamide gel and transferred to a nylon membrane. DNA on the membrane was immediately cross-linked for 10 min using an UV cross-linker. The biotin end-labeled DNA probe was detected using streptavidin conjugated to horseradish peroxidase (HRP) and a chemiluminescent substrate. Biotin-labeled double-strand NF-κB consensus oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and Oct-1 oligonucleotide (5'-TGT CGA ATG CAA ATC ACT AGA A-3') were obtained from Beyotime Institute of Biotechnology.

Results

Lapatinib inhibits activation of NF-κB in HER2-overexpressing breast cancer cells. To determine the effect of lapatinb on the activity of NF-κB, we performed EMSA. In SKBR3 cells, lapatinib inhibited activation of NF-κB in a dose-dependent manner (Fig. 1). Similarly, a high dose of lapatinib (5 μmol/l) markedly reduced the activity of NF-κB in another HER2-overexpressing breast cancer cell line, MDA-MB-453. In contrast, lapatinib had almost no impact on the activity of NF-κB in non-HER2-overexpressing breast cancer cell lines, including MDA-MB-231, MDA-MB-468 and MDA-MB-435 (Fig. 1). These results suggest that lapatinib inhibits the activation of NF-κB in HER2-overexpressing breast cancer cells.

To better understand how lapatinib inactivates NF-κB in HER2-positive breast cancer cells, we performed western blot

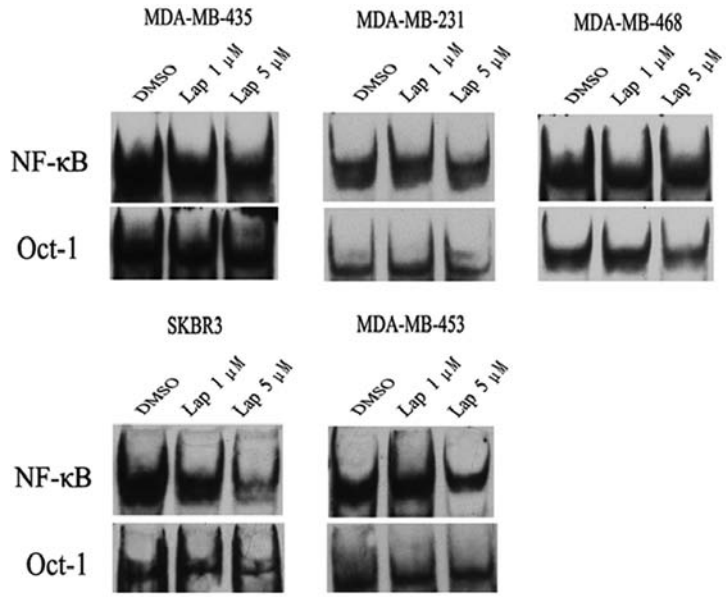

Figure 1. Effect of lapatinib on NF-κB activity in breast cancer cells. MDA-MB-435, MDA-MB-231, MDA-MB-468, SKBR3 and MDA-MB-453 breast cancer cells were treated with lapatinib (1 and 5 μmol/l) or DMSO for 24 h. EMSA for NF-κB was performed with nuclear extracts from the indicated cells. Oct-1 served as a loading control.

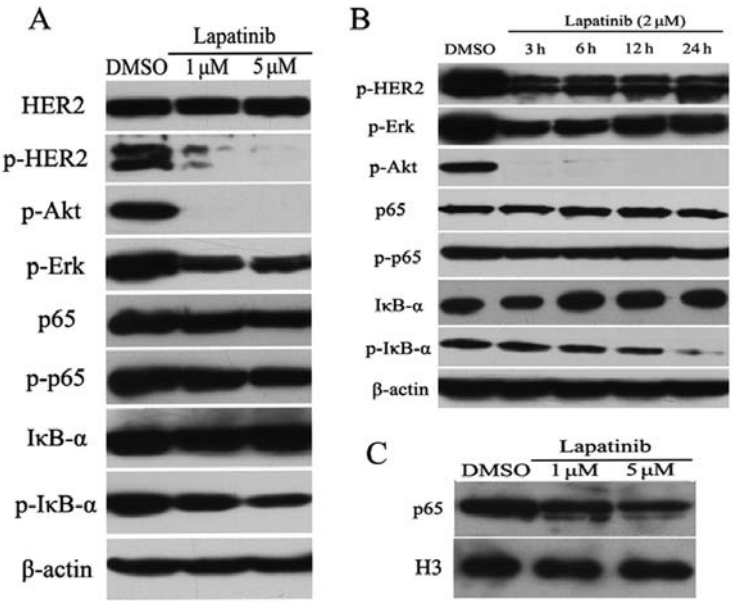

Figure 2. Effect of lapatinib on NF-κB and HER2 pathway-related protein expression in SKBR3 cells. (A) SKBR3 cells were treated with lapatinib (1 and 5 μmol/l) or DMSO for 48 h. Immunoblot analysis was carried out for the indicated proteins. (B) SKBR3 cells were treated with 2 μmol/l lapatinib for 3, 6, 12 or 24 h. Immunoblot analysis was carried put for the indicated proteins. β-actin was used as a loading control. (C) SKBR3 cells were treated with lapatinib (1 and 5 μmol/l) or DMSO for 48 h. Immunoblot analysis for p65 protein was carried out with nuclear extracts. Histone H3 was used as a loading control.

analysis. As expected, lapatinib reduced the expression levels of p-HER2, p-Akt and p-Erk proteins in a dose-dependent manner in SKBR3 cells (Fig. 2A). Importantly, treatment with lapatinib resulted in a decreased phosphorylation of IκB-α in SKBR3 cells, whereas lapatinib had almost no impact on the expression levels of total HER2, Akt, Erk, p65 and p-p65 proteins (Fig. 2A). We then treated SKBR3 cells with 2 μmol/l lapatinib for different times, and measured the protein expression levels of HER2 and NF-κB pathways by western blot analysis. As shown in Fig. 2B, lapatinib reduced the phosphorylation of IκB-α in a time-dependent manner, whereas lapatinib increased the expression level of total IκB-α protein. To further confirm the inhibitory role of lapatinib on NF-κB activity, we evaluated cellular localization of p65 by western blot analysis. Interestingly, lapatinib inhibited p65 nuclear accumulation in a dose-dependent manner in SKBR3 cells (Fig. 2C).

Establishment of lapatinib-resistant SKBR3 cells. To measure the relative resistance of parental SKBR3 and rSKBR3 cells to lapatinib, we performed CCK-8 assays. As indicated in

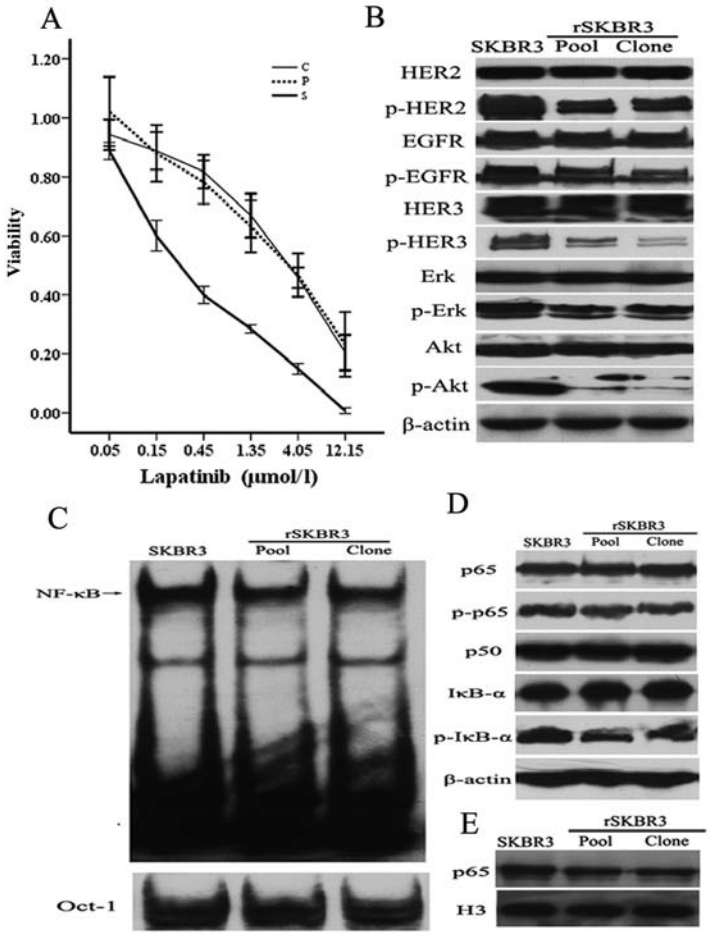

Figure 3. Effect of lapatinib on cell proliferation and protein expression in parental and resistant SKBR3 cells. (A) Parental (S) and resistant (P, pools; C, representative clone) SKBR3 cells were treated with 3-fold serial dilutions of lapatinib ranging from 0.05 to 12.15 μmol/l for 48 h, when cell viability was assayed using CCK-8. (B) Immunoblot analysis was carried out for the indicated proteins. β-actin was used as a loading control. (C) Nuclear extracts from parental and resistant (pools and representative clone) SKBR3 cells were subjected to EMSA with NF-κB or Oct-1 probes. Oct-1 was used as a loading control. (D) Immunoblot analysis was carried out for the indicated proteins. β-actin was used as a loading control. (E) Nuclear extracts were prepared from parental and resistant SKBR3 cells. Immunoblot analysis was carried out for p65 protein. Histone H3 was used as a loading control.

Fig. 3A, the 50% inhibitory concentrations (IC_{50}) of lapatinib in parental SKBR3 cells, pooled resistant cells and representative clone were ~0.3, 3.7 and 3.8 μmol/l, respectively, suggesting that SKBR3 cells gradually became insensitive to lapatinib. We next assayed the expression levels of several HER signaling pathway proteins in the parental and resistant rSKBR3 cells by immunoblot analysis. The expression levels of p-HER2, p-HER3, p-EGFR, p-Erk and p-Akt proteins decreased in rSKBR3 cells in comparison with parental SKBR3 cells, while the expression levels of total HER2, HER3, EGFR, Erk and Akt proteins had almost no change (Fig. 3B). This result demonstrated that lapatinib still retained its inhibitory role of EGFR/HER2 signaling in our resistant cell model.

Decreased NF-κB activity in lapatinib-resistant SKBR3 cells. We next examined whether the activity of NF-κB changed in our cell model. A moderately decreased NF-κB activity was observed in rSKBR3 cells, compared with parental SKBR3 cells (Fig. 3C).

We next assayed the expression levels of NF-κB pathway proteins by western blot analysis. As showed in Fig. 3D, the expression level of the p-IκB-α protein was markedly decreased in the pooled resistant cells and representative clone, when compared with the parental SKBR3 cells. The expression levels of p-p65, p65 and p50 had no significant difference between the parental and resistant rSKBR3 cells (Fig. 3D). We further evaluated the cellular localization of p65 by western blot analysis. Nuclear accumulation of p65 was decreased in the resistant cells, compared with the parental SKBR3 cells (Fig. 3E).

Lapatinib inhibits the activation of NF-κB through the PI3K/Akt pathway. We previously showed that lapatinib inhibits activation of the PI3K/Akt and Erk pathways in SKBR3 cells (6). Thus it was tempting to speculate that lapatinib may inhibit NF-κB activation through blocking these pathways. To address this possibility, we treated SKBR3 and MDA-MB-453 cells with the PI3K inhibitor LY294002 and the MEK inhibitor AZD6244, and measured the activity of NF-κB by EMSA. As indicated in Fig. 4A, a deceased NF-κB activity was observed in the SKBR3 cells treated with LY294002. In MDA-MB-453 cells, LY294002 inhibited activation of NF-κB in a dose-dependent manner (Fig. 4C). However, AZD6244

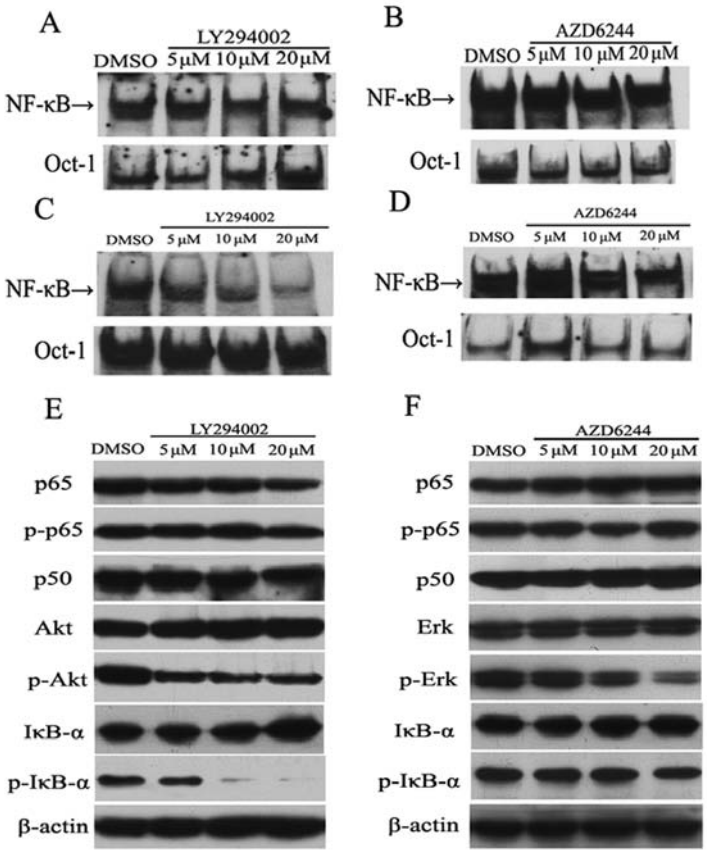

Figure 4. Effect of LY294002 and AZD6244 on NF-κB activity and pathway proteins in breast cancer cells. (A and B) SKBR3 cells were treated with LY294002 (5, 10 and 20 μmol/l) or AZD6244 (5, 10 and 20 μmol/l) or DMSO for 24 h. Nuclear extracts were used in EMSA with oligonucleotides containing elements for NF-κB or Oct-1. (C and D) MDA-MB-453 cells were treated with LY294002 (5, 10 and 20 μmol/l) or AZD6244 (5, 10 and 20 μmol/l) or DMSO for 24 h. Nuclear extracts were subjected to EMSA with NF-κB or Oct-1 probes. (E) SKBR3 cells were treated with LY294002 (5, 10 and 20 μmol/l) or DMSO for 24 h. Immunoblot analysis was carried out for the indicated proteins. (F) SKBR3 cells were treated with AZD6244 (5, 10 and 20 μmol/l) or DMSO for 24 h. Immunoblot analysis was carried for the indicated proteins.

had relatively little impact on NF-κB DNA binding in SKBR3 cells (Fig. 4B), as well as in MDA-MB-453 cells (Fig. 4D).

We next examined the impact of LY294002 and AZD6244 on the expression level of NF-κB pathway proteins by western blot analysis. In SKBR3 cells, LY294002 reduced phosphorylation of Akt in a concentration-dependent manner (Fig. 4E). Importantly, phosphorylation of IκB-α was potently inhibited by LY294002, while the expression level of total IκB-α was increased, particularly in the presence of the highest dose of LY294002 (Fig. 4E). As expected, phosphorylation of Erk was reduced by AZD6244 in a dose-dependent manner in SKBR3 cells, whereas there was no detectable change in IκB-α phosphorylation in SKBR3 cells (Fig. 4F). AZD6244 had almost no effect on the expression levels of p-p65, p65, p50 and total IκB-α.

Discussion

In the present study, we showed that lapatinib potently inhibited activation of NF-κB in two HER2-overexpressing breast cancer cell lines, SKBR3 and MDA-MB-453. We also found that there was a decreased NF-κB activity in the lapatinib-resistant SKBR3 cells. We further investigated the potential mechanism of lapatinib-induced inactivation of NF-κB. We found that lapatinib reduced the phosphorylation of IκB-α in SKBR3 cells, as well as in the rSKBR3 cells. We also demonstrated that lapatinib inhibited phosphorylation of IκB-α probably though blocking PI3K/Akt activation.

It was previously shown that there was a positive correlation between HER2 overexpression and constitutive activation of NF-κB in breast cancer cells (13,20,21). Biswas *et al* (13) revealed that herceptin, a recombinant humanized monoclonal antibody-directed HER2 receptor, blocked heregulin-induced NF-κB activation in a concentration-dependent manner in SKBR3 cells. In a separate study, it was reported that inhibition of HER-2 by AG825 or knockdown of HER-2 by RNA interference blocked TNFα-induced NF-κB activation in breast cancer cells (20). Hence it is not entirely surprising that lapatinib, a small-molecule inhibitor of HER2, has the ability to inhibit the activation of NF-κB in HER2-overexpressing breast cancer cells. Our finding is in keeping with a previous study showing that lapatinib induced a 3-fold downregulation of NF-κB1 mRNA expression in LoVo cells (22).

Evidence suggests that the primary mechanism of action of lapatinib is not compromised in cells with acquired resistance (23-25). Liu *et al* (23) previously showed that lapatinib inhibited tyrosine phosphorylation of HER1, HER2 and HER3 in both lapatinib-sensitive BT474 cells and lapatinib-resistant BT474J4 cells. In this study, we showed that lapatinib still retained its ability to inhibit phosphorylation of HER2, EGFR,

Erk and Akt in the rSKBR3 cells. Furthermore, we indicated that there was decreased NF-κB activity and nuclear accumulation of p65 in our rSKBR3 cells. Our findings in the resistant cell model further confirmed the inhibitory role of lapatinib in NF-κB activation.

NF-κB activation is regulated primarily by two pathways, the classical and the alternative pathways (26). In the canonical pathway, NF-κB is retained in the cytoplasm as an inactive heterotrimer consisting of three subunits: p50, p65 and IκB-α. On activation, IκB-α is phosphorylated by the IKK holo-complex, leading to its degradation by the ubiquitin/proteasome system, thereby releasing NF-κB dimers to the nucleus (11,12). Degradation of IκB-α alters the dynamic balance between cytosolic and nuclear localization signals to favor nuclear localization of NF-κB (11). It has been reported that inhibition of IκB-α phosphorylation leads to inactivation of NF-κB in glioma cell lines (27). Additionally, Wu *et al* (28) reported that paeoniflorin inhibited NF-κB activation by reducing the degradation of IκB-α through preventing its phosphorylation in gastric carcinoma cells. In the present study, we convincingly demonstrated that lapatinib reduced phosphorylation of IκB-α in a time- and dose-dependent manner in SKBR3 cells. Furthermore, we revealed that there was a decreased phosphorylation of IκB-α with subsequent reduction in NF-κB activity in the lapatinib-resistant SKBR3 cells. Thus, lapatinib appears to inhibit activation of NF-κB by reducing phosphorylation of IκB-α.

Overwhelming evidence has indicated that PI3K/Akt signaling triggers the activation of NF-κB and the underlying molecular mechanisms are multifactorial (29,30). Madrid *et al* (31) demonstrated that the Akt-induced potentiation of p65 transactivation capacity requires the activation of IKKβ kinase and MAPK p38. Recent data also described the ability of Akt to stimulate IKK activity directed toward the phosphorylation of IκB-α and RelA/p65 in prostate cancer cells (32). In the present study, we indicated that the PI3K inhibitor LY294002 reduced phosphorylation of Akt, and inhibited activation of NF-κB in HER2-overexpressing breast cancer cells. Furthermore, we showed that LY294002 treatment reduced phosphorylation of IκB-α in SKBR3 cells. Importantly, we revealed that the MEK inhibitor AZD6244 had no ability to inhibit phosphorylation of IκB-α in SKBR3 cells. The PI3K/Akt and Erk pathways are putative downstream signals of HER2 (1,2). As mentioned above, we convincingly demonstrated that lapatinib reduced the phosphorylation of Akt and IκB-α in SKBR3 cells. Hence, it is likely that lapatinib inhibits NF-κB activation through reduction of IκB-α phosphorylation via blocking of the PI3K/Akt cascade.

In conclusion, the current data presented herein indicate that lapatinib potently inhibited the activation of NF-κB in HER2-overexpressing breast cancer cells, and there was a decreased NF-κB activity in lapatinib-resistant SKBR3 cells. Lapatinib appears to inactivate NF-κB through reduction of the phosphorylation of IκB-α via blocking of the PI3K/Akt cascade.

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