ORIGINAL ARTICLE

Roles of BIM induction and survivin downregulation in lapatinib-induced apoptosis in breast cancer cells with HER2 amplification

J Tanizaki1, I Okamoto1, S Fumita1, W Okamoto1, K Nishio2 and K Nakagawa1

1Department of Medical Oncology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka, Japan and 2Department of Genome Biology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka, Japan

Lapatinib, a dual tyrosine kinase inhibitor of the epidermal growth factor receptor and human epidermal growth factor receptor 2 (HER2), is clinically active in patients with breast cancer positive for HER2 amplification. The mechanism of this anti-tumor action has remained unclear, however. We have now investigated the effects of lapatinib in HER2 amplification-positive breast cancer cells with or without an activating PIK3CA mutation. Lapatinib induced apoptosis in association with upregulation of the pro-apoptotic protein Bcl-2 interacting mediator of cell death (BIM) through inhibition of the MEK-ERK signaling pathway in breast cancer cells with HER2 amplification. RNA interference (RNAi)-mediated depletion of BIM inhibited lapatinib-induced apoptosis, implicating BIM induction in this process. The pro-apoptotic effect of lapatinib was less pronounced in cells with a PIK3CA mutation than in those without one. Lapatinib failed to inhibit AKT phosphorylation in PIK3CA mutant cells, likely because of hyperactivation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway by the mutation. Depletion of PIK3CA (a catalytic subunit of PI3K) revealed that survivin expression is regulated by the PI3K pathway in these cells, suggesting that insufficient inhibition of PI3K-survivin signaling is responsible for the limited pro-apoptotic effect of lapatinib in HER2 amplification-positive cells with a PIK3CA mutation. Consistent with this notion, depletion of survivin by RNAi or treatment with a PI3K inhibitor markedly increased the level of apoptosis in PIK3CA mutant cells treated with lapatinib. Our results thus suggest that inhibition of both PI3K-survivin and MEK-ERK-BIM pathways is required for effective induction of apoptosis in breast cancer cells with HER2 amplification. Oncogene advance online publication, 18 April 2011; doi:10.1038/onc.2011.111

Keywords: BIM; survivin; HER2 amplification; PIK3CA mutation; apoptosis; breast cancer

Introduction

Breast cancer is the leading cause of cancer death among women worldwide. Amplification of the human epidermal growth factor receptor 2 (HER2) gene occurs in 25–30% of breast cancers (Slamon et al., 1987, 1989), and HER2 is thus an attractive target for the development of therapeutic drugs. Lapatinib, a dual tyrosine kinase inhibitor of HER2 and the epidermal growth factor receptor (EGFR), has shown anti-tumor activity for breast cancer with HER2 amplification in pre-clinical and clinical studies (Geyer et al., 2006; Konecny et al., 2006; Gomez et al., 2008). Although lapatinib improved the overall outcome for such patients, not all patients were benefited from the treatment. Characterization of the molecular basis of the response to lapatinib will thus be important to maximize the clinical efficacy of this drug.

Mutations in PIK3CA, which encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K), have been identified in 8–40% of breast cancers (Samuels et al., 2004; Saal et al., 2005; Berns et al., 2007). Although a positive correlation between HER2 overexpression and the presence of PIK3CA mutations has been described (Saal et al., 2005), the relation between the efficacy of lapatinib and such mutations has remained unclear (Eichhorn et al., 2008; Toi et al., 2009; Kataoka et al., 2010). We have therefore now investigated the effects of lapatinib in HER2 amplification-positive breast cancer cells with or without an activating PIK3CA mutation, and we further examined the mechanism responsible for the induction of apoptosis in these cells.

Results

Lapatinib inhibits cell proliferation and induces apoptosis in breast cancer cells with HER2 amplification

We first examined the effect of lapatinib on the proliferation in vitro of breast cancer cells positive or negative for HER2 amplification (Figure 1a). All six cell lines with HER2 amplification, including SK-BR3, ZR-75-30, BT-474, MB-361, MB-453 and HCC1954, were sensitive to lapatinib, with median inhibitory concentration (IC50) values ranging from 0.05 to 0.80 μM, which are within the clinically achievable concentration range...
for this drug (LoRusso et al., 2008; Burris et al., 2009). Among these HER2 amplification-positive cells, those with an activating PIK3CA mutation (MB-361, MB-453 and HCC1954) were less sensitive to lapatinib than those without such a mutation (SK-BR3, ZR-75-30 and BT-474). Cell lines negative for HER2 amplification, including MCF-7, T47-D and MB-231, were resistant to lapatinib, with IC50 values of > 5.0 μM.

We next examined the effect of lapatinib on apoptosis in these various breast cancer cell lines (Figure 1b). An annexin V binding assay showed that lapatinib (1 μM) induced apoptosis in all HER2 amplification-positive cells, but was largely without effect in amplification-negative cells. Consistent with the IC50 values for the anti-proliferative effect of the drug, the extent of lapatinib-induced apoptosis was less pronounced in HER2 amplification-positive cells with an activating PIK3CA mutation than in those without such a mutation. We further examined the effect of lapatinib on clonogenic survival of breast cancer cells. Again, lapatinib greatly reduced the clonogenicity of HER2 amplification-positive cells without a PIK3CA mutation, whereas the reduction in the number of clones was less marked for those with a PIK3CA mutation (Figure 1c).

Differential effect of lapatinib on AKT signaling in HER2 amplification-positive breast cancer cells with or without an activating PIK3CA mutation

We examined the effects of lapatinib on the AKT and ERK (extracellular signal-regulated kinase) signaling pathways in breast cancer cell lines (Figure 2a). Immunoblot analysis showed that phosphorylation of both AKT and ERK was markedly inhibited by lapatinib in HER2 amplification-positive cells without an activating PIK3CA mutation. In HER2 amplification-positive cells harboring a PIK3CA mutation, however, lapatinib inhibited the phosphorylation of ERK but had little effect on that of AKT. Lapatinib showed little effect on the phosphorylation of AKT or ERK in HER2 amplification-negative cells. These data thus revealed that, whereas lapatinib inhibited the phosphorylation of ERK in all HER2 amplification-positive cells, its effect on that of AKT was dependent on PIK3CA mutational status.

Effects of lapatinib on apoptosis-related proteins in HER2 amplification-positive breast cancer cells with or without an activating PIK3CA mutation

Given that lapatinib induced apoptosis in cells with HER2 amplification, we examined its effects on apoptosis-related proteins in these cells (Figure 2b). Immunoblot analysis revealed that lapatinib upregulated the expression of Bcl-2 interacting mediator of cell death (BIM), a pro-apoptotic
member of the Bcl-2 family of proteins, in HER2 amplification-positive cells regardless of the PIK3CA mutational status, whereas it had little effect on the expression of other Bcl-2 family members, including Mcl-1, Bcl-2 and Bcl-xL. Quantitative reverse transcription and PCR analysis showed that lapatinib increased the amount of BIM mRNA in all HER2 amplification-positive cells in a manner independent of the PIK3CA mutational status (Supplementary Figure 1), suggesting that BIM induction by lapatinib is mediated at the transcriptional level. On the other hand, lapatinib downregulated the expression of survivin, a member of the inhibitor of apoptosis protein (IAP) family, in HER2 amplification-positive cells without an activating PIK3CA mutation but not in those with such a mutation. The expression of other IAP family members, including XIAP and c-IAP1, was not substantially affected by lapatinib in any of the cell lines examined.

To identify the signaling pathways responsible for induction of BIM and downregulation of survivin by lapatinib, we examined the effects of specific inhibitors of PI3K (BEZ235 and LY294002) and of the ERK kinase MEK (AZD6244 and U0126). Each of the MEK inhibitors induced BIM expression without affecting the expression of survivin in HER2 amplification-positive cells regardless of the PIK3CA mutational status (Figure 2c), suggesting that expression of BIM is regulated by the MEK-ERK pathway. Conversely, the PI3K inhibitors reduced the abundance of survivin without affecting that of BIM in all cells with HER2 amplification (Figure 2c). We further examined the effect of depletion of PIK3CA (p110α) by RNA interference (RNAi) on survivin expression in PIK3CA mutant cells. Introduction of two independent small interfering RNAs (siRNAs) specific for PIK3CA mRNA (PIK3CA-1 and PIK3CA-2 siRNAs) into HER2 amplification-positive cells with an activating PIK3CA mutation, resulted in a marked decrease in the expression of p110α and a concomitant decrease in the level of AKT phosphorylation. This depletion of p110α was also associated with downregulation of survivin expression in these cell lines (Figure 2d), suggesting that survivin expression was regulated through the PI3K pathway. Together, these data suggested that lapatinib induced BIM expression through inhibition of the MEK-ERK pathway in HER2 amplification-positive
cells with or without an activating PIK3CA mutation. On the other hand, lapatinib downregulated survivin expression through inhibition of the PI3K signaling pathway in HER2 amplification-positive cells without a PIK3CA mutation, but it had little effect on survivin expression in cells with such a mutation, likely as a result of activation of the PI3K pathway by the PIK3CA mutation.

Effect of inhibition of BIM induction on lapatinib-induced apoptosis in cells with HER2 amplification

To investigate the role of BIM induction in lapatinib-induced apoptosis, we transfected HER2 amplification-positive cells with two independent siRNAs specific for BIM mRNA (BIM-1 and BIM-2 siRNAs). Each BIM siRNA markedly suppressed the lapatinib-induced upregulation of BIM without affecting lapatinib-induced downregulation of survivin (Figure 3a). The annexin V binding assay showed that such transfection resulted in partial inhibition of lapatinib-induced apoptosis in HER2 amplification-positive cells without an activating PIK3CA mutation, whereas lapatinib-induced apoptosis was almost completely inhibited by BIM siRNA in cells with such a mutation (Figure 3b). Similar to the results of the annexin V binding assay, transfection with BIM siRNA resulted in partial inhibition of the lapatinib-induced activation of caspase-3 in cells without a PIK3CA mutation, whereas it resulted in almost complete inhibition of this effect of lapatinib in cells with a PIK3CA mutation (Figure 3c). The BH3-mimetic ABT737, which binds to anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xl and Bcl-w, was shown to enhance apoptosis under conditions of BIM induction (Cragg et al., 2007, 2008; Gong et al., 2007). We therefore examined the effect of the combination of lapatinib and ABT737 on induction of apoptosis in HER2-amplified breast cancer cells with or without a PIK3CA mutation. We found that ABT737 enhanced lapatinib-induced apoptosis both in HER2-positive cells without a PIK3CA mutation, and in those with a PIK3CA mutation with an average fold increase of 1.20 and 1.48, respectively (P<0.05) (Supplementary Figure 2), supporting a role for BIM induction in lapatinib-induced apoptosis. These data thus indicated that BIM induction contributes to lapatinib-induced apoptosis in cells with HER2 amplification, but that the extent of this contribution differs according to the mutational status of PIK3CA.

Combined effect of lapatinib and BEZ235 on apoptosis in HER2 amplification-positive cells with an activating PIK3CA mutation

Given that lapatinib manifested only a moderate pro-apoptotic effect in cells with an activating PIK3CA
mutation despite the preserved induction of BIM, we hypothesized that insufficient inhibition of the PI3K pathway by lapatinib might be responsible for the limited size of this effect compared with that observed in cells without such a mutation. We therefore examined whether additional inhibition of the PI3K pathway by BEZ235 might enhance the effect of lapatinib on apoptosis in PIK3CA mutant cells. Treatment with BEZ235, which was previously shown to inhibit the PI3K pathway in cells expressing activated PIK3CA (Serra et al., 2008; Brachmann et al., 2009), resulted in marked inhibition of AKT phosphorylation (but not of ERK phosphorylation) in HER2 amplification-positive cells with an activating PIK3CA mutation (Figure 4a). The combination of BEZ235 and lapatinib resulted in inhibition of both AKT and ERK phosphorylation (Figure 4a). Consistent with the notion that regulation of survivin is mediated through the PI3K pathway and that of BIM is mediated through the MEK-ERK pathway, treatment with BEZ235 alone induced downregulation of survivin expression without affecting BIM expression, whereas the combination of BEZ235 and lapatinib elicited both survivin downregulation and BIM upregulation (Figure 4a). The combination of BEZ235 and lapatinib increased the number of apoptotic cells to an extent markedly greater than that apparent with either agent alone in HER2 amplification-positive cells with a PIK3CA mutation, whereas the effect of lapatinib was similar in the absence or presence of BEZ235 in those without a PIK3CA mutation or in cells negative for HER2 amplification (Figure 4b). A similar pattern was observed for the effects of lapatinib and
BEZ235 on caspase-3 activity (Figure 4c). We further examined the effect of combined treatment with BEZ235 and lapatinib on the growth in vivo of HER2 amplification-positive breast cancer cells with a PIK3CA mutation. At the completion of the experiments, tumors treated with either control or lapatinib alone had doubled in size, whereas the combination of lapatinib and BEZ235 maintained tumor regression (P < 0.05) (Figure 4d), consistent with the combined effect of these agents observed in our in vitro experiments. All treatments were well tolerated by the mice, with no signs of toxicity or weight loss during therapy (data not shown). These results thus suggested that effective inhibition of the PI3K pathway and lapatinib treatment cooperate to elicit a substantial level of apoptosis that is accompanied by BIM induction and survivin downregulation in HER2 amplification-positive cells with an activating PIK3CA mutation.

**Combined effect of lapatinib and depletion of survivin on apoptosis in HER2 amplification-positive cells with an activating PIK3CA mutation**

Finally, to investigate the effect of downregulation of survivin expression on apoptosis in HER2 amplification-positive cells with an activating PIK3CA mutation, we depleted such cells of survivin by RNAi (Figure 5a). Each of two independent survivin siRNAs induced apoptosis in these cells, whereas the combination of survivin depletion and lapatinib increased the number of apoptotic cells to an extent significantly greater than that observed with either treatment alone (Figure 5b). These effects on the number of apoptotic cells were confirmed by measurement of caspase-3 activity (Figure 5c). These data thus suggested that down-regulation of survivin itself has a pro-apoptotic effect in cells with a PIK3CA mutation, but that survivin depletion and lapatinib cooperate to induce an enhanced level of apoptosis.

**Discussion**

HER2 amplification is a frequent molecular abnormality in breast cancer, and is associated with a poor outcome and aggressiveness of the disease (Slamon et al., 1987, 1989). Lapatinib, a dual tyrosine kinase inhibitor of EGFR and HER2, shows anti-tumor activity in HER2-overexpressing breast cancer (Geyer et al., 2006; Konecny et al., 2006; Gomez et al., 2008), but the precise mechanism of its anti-tumor effect has remained unclear. We have now investigated the downstream mediators of lapatinib-induced apoptosis in breast cancer cells with HER2 amplification. BIM is a key pro-apoptotic member of the Bel-2 family of proteins,
and initiates apoptosis signaling by binding to and antagonizing the function of pro-survival Bcl-2 family members (Chen et al., 2005). Our results indicate that lapatinib induces upregulation of BIM expression in HER2 amplification-positive cells, and that depletion of BIM by RNAi results in marked inhibition of lapatinib-induced apoptosis in these cells. These data suggest that upregulation of BIM expression contributes to the induction of apoptosis by lapatinib in breast cancer cells with HER2 amplification. We found that BIM induction by lapatinib occurred in HER2 amplification-positive cells regardless of PIK3CA mutational status and was associated with inhibition of ERK phosphorylation. With the use of specific inhibitors of MEK, we also found that regulation of BIM expression is mediated by the MEK-ERK signaling pathway. These findings are consistent with those of previous studies showing that MEK inhibitors induce BIM expression in B-RAF mutant cells (Cragg et al., 2008), and that inhibition of the MEK-ERK pathway contributes to BIM induction by EGFR tyrosine kinase inhibitors in non-small cell lung cancer (Costa et al., 2007; Cragg et al., 2007; Gong et al., 2007), and that such upregulation of BIM has an essential role in the induction of apoptosis by these agents. We also found that ABT737 enhanced the induction of apoptosis by lapatinib in cells with HER2 amplification regardless of PIK3CA mutational status, further supporting a role for BIM induction in lapatinib-induced apoptosis. To our knowledge, the present study is the first to show that induction of BIM through inhibition of the MEK-ERK pathway is required for lapatinib-induced apoptosis in breast cancer with HER2 amplification.

Although lapatinib-induced upregulation of BIM expression occurred in a manner independent of PIK3CA mutational status, the pro-apoptotic effect of lapatinib was less pronounced in cells with an activating PIK3CA mutation than in those without one. Given that such PIK3CA mutations result in hyperactivation of the PI3K signaling pathway (Isakoff et al., 2005; Zhao et al., 2005; Berns et al., 2007), we examined whether activation of this pathway was associated with this difference in the extent of apoptosis. Indeed, we found that lapatinib did not inhibit the phosphorylation of AKT in HER2 amplification-positive cells with an activating PIK3CA mutation. We therefore examined the effect of specific inhibitors of the PI3K pathway on lapatinib-induced apoptosis in cells with a PIK3CA mutation. Treatment with BEZ235 effectively inhibited AKT phosphorylation, and the combination of BEZ235 and lapatinib thus inhibited both AKT and ERK phosphorylation and had a pro-apoptotic effect that was markedly greater than that observed with either agent alone. Consistent with these in vitro experiments, the combination of lapatinib and BEZ235 exhibits an enhanced anti-tumor effect in vivo with HER2-positive xenografts with a PIK3CA mutation. These results suggest that additional inhibition of the PI3K pathway is required for effective induction of apoptosis by lapatinib in cells with a PIK3CA mutation. Lapatinib shows clinical efficacy both alone and in combination with chemotherapeutic agents, but not all patients with HER2 amplification-positive tumors respond to such treatment (Slamon et al., 1987; Slamon, 1990; Geyer et al., 2006; Di Leo et al., 2008; Gomez et al., 2008). PIK3CA mutations have been detected in 20–30% of breast cancer patients with HER2 amplification (Saal et al., 2005; Stemke-Hale et al., 2008), and our data now suggest that activation of the PI3K signaling pathway associated with the presence of a PIK3CA mutation may be responsible, at least in part, for the limited efficacy of lapatinib in patients with tumors positive for both HER2 amplification and a PIK3CA mutation. Similar to the effects of lapatinib, the MEK inhibitor AZD6244 inhibited ERK phosphorylation and increased BIM expression, without affecting AKT phosphorylation or survivin expression, and it cooperated with BEZ235 to induce apoptosis in HER2 amplification-positive cells with a PIK3CA mutation (Supplementary Figure 3). These data thus indicate the importance of simultaneous interruption of the PI3K-survivin and MEK-ERK-BIM pathways for effective induction of apoptosis in such cells. However, the extent of apoptosis induced by AZD6244 alone or in combination with BEZ235 was less pronounced than that induced by lapatinib, suggesting that the anti-tumor effect of lapatinib in these cells is not mediated exclusively through inhibition of MEK-ERK signaling. Further investigation is thus needed to clarify the relationship of PIK3CA mutational status to the efficacy of lapatinib. The development of PI3K inhibitors has advanced substantially in recent years, and clinical trials of these agents alone or in combination with other anti-tumor agents are under way. Our study therefore provides a rationale for clinical evaluation of combination therapy with lapatinib and a PI3K inhibitor in breast cancer patients with HER2 amplification and a PIK3CA mutation.

Survivin is essential for proper completion of various stages of cell division, with this protein having been found to contribute to centrosomal function, spindle formation and kinetochore attachment to spindle microtubules (Speliotes et al., 2000; Uren et al., 2000). Survivin is preferentially expressed during the mitotic phase of the cell cycle and is physically associated with the mitotic apparatus. It has also been found to be overexpressed in some tumors, with such overexpression having been associated with a poor clinical outcome (Ambrosini et al., 1997; Tanaka et al., 2000; Altiere, 2003). Like other members of the IAP family such as XIAP and c-IAP1, survivin contains a single BIR (baculoviral IAP repeats) domain. Molecular antagonists of survivin, including anti-sense and siRNA oligonucleotides as well as dominant negative mutants, have been shown to induce apoptosis (Olie et al., 2000; Kanwar et al., 2001), suggestive of an association between survivin and apoptosis. Consistent with these previous findings, we have now shown that depletion of survivin by two independent siRNAs specific for survivin mRNA increased the number of apoptotic cells and the activity of caspase-3 in HER2 amplification-positive breast cancer cells with a PIK3CA mutation. With the use of siRNAs specific for PIK3CA mRNA,
we further showed that survivin expression is regulated by the PI3K signaling pathway, consistent with previous studies linking survivin expression to this signaling pathway (McKenzie et al., 2010; Peirce et al., 2010). Our finding that survivin downregulation through inhibition of PI3K signaling was associated with the induction of apoptosis, is consistent with the key role of this signaling pathway in cell survival. We found that lapatinib downregulated survivin expression in association with the induction of apoptosis in HER2 amplification-positive cells without an activating PIK3CA mutation. In contrast, expression of survivin was not markedly affected by lapatinib in cells harboring such a PIK3CA mutation. We therefore examined the effect of inhibition of survivin expression on lapatinib-induced apoptosis in PIK3CA mutant cells. In such cells, the combination of survivin depletion by RNAi and lapatinib treatment exhibited a pro-apoptotic effect markedly greater than that observed with either approach alone, suggesting that downregulation of survivin promotes lapatinib-induced apoptosis. We also found that, unlike lapatinib, the PI3K inhibitor BEZ235 induced downregulation of survivin expression in cells with an activating PIK3CA mutation, suggesting that this effect contributes, at least in part, to the enhanced level of apoptosis induced by the combination of lapatinib and BEZ235. Insufficient inhibition of the PI3K-survivin pathway may thus account for the smaller pro-apoptotic effect of lapatinib in HER2 amplification-positive cells with an activating PIK3CA mutation compared with that observed in those without such a mutation.

In conclusion, we have shown that both induction of BIM and inhibition of survivin have a role in lapatinib-induced apoptosis in HER2 amplification-positive breast cancer cells. Moreover, both the PI3K-survivin pathway and the MEK-ERK-BIM pathway contribute independently to the induction of apoptosis in these cells regardless of PIK3CA mutational status. Our data thus show that simultaneous interruption of the PI3K-survivin and MEK-ERK-BIM pathways is required for effective induction of apoptosis in breast cancer cells with HER2 amplification. They further provide a rationale for the development of new therapeutic strategies for patients with breast tumors positive for HER2 amplification, including those with an activating PIK3CA mutation.

Materials and methods

Cell culture and reagents

The human breast cancer cell lines SK-BR3, ZR-75-30, BT-474, MB-361, MB-453, HCC1954, MCF-7, T47-D and MB-231 were obtained from American Type Culture Collection (Manassas, VA, USA). SK-BR3 cells were cultured in McCoy’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum; BT-474 cells in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum; MB-361, MB-453 and MB-231 cells in L15 medium (Invitrogen) supplemented with 10% fetal bovine serum; and the remaining cells in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum. All cells were maintained under a humidified atmosphere of 5% CO2 at 37 °C. Lapatinib was obtained from Sequoia Research Products (Pangbourne, UK), AZD6244 was from ShangHai Biochempartner (Shanghai, China) and LY294002 and U0126 were from Cell Signaling Technology (Danvers, MA, USA). BEZ235 was kindly provided by Novartis (Basel, Switzerland). MB-453 and HCC1954 cells were found to harbor an H1047 hotspot mutation, and MB-361 cells were found to contain an E545K hotspot mutation by sequencing of exons 9 and 20 of PIK3CA (Hoeflich et al., 2009; Kataoka et al., 2010; Saal et al., 2005; Samuels et al., 2004). We categorized BT-474 cells as negative for an activating PIK3CA mutation for this study on the basis of the demonstrated lack of transforming activity for the K111N mutation and its minimal effect on downstream signaling (Gymnopoulu et al., 2007; Zhang et al., 2008).

Growth inhibition assay in vitro

Cells were plated in 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of lapatinib for 72 h. TetraColor One (5 mM tetrazolium monosodium salt and 0.2 mM 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku, Tokyo, Japan) was then added to each well, and the cells were incubated for 3 h at 37 °C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA, USA). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of lapatinib resulting in 50% growth inhibition (IC50) was calculated.

Annexin V binding assay

Binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche, Basel, Switzerland). Cells were harvested by exposure to trypsin-EDTA, washed with phosphate-buffered saline and centrifuged at 200 g for 5 min. The cell pellets were resuspended in 100 μl of Annexin-V-FLUOS labeling solution, incubated for 10–15 min at 15–25 °C and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Clonogenicity assay

Cells were seeded in triplicate in six-well plates and cultured for 48 h in the presence of lapatinib (1 μM) or vehicle. They were then cultured in drug-free medium for 14 days, fixed with methanol:acetic acid (10:1, v/v) and stained with crystal violet. The mean percentage cell survival relative to controls was determined from triplicate wells.

Immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline and then lysed in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride and leupeptin (1 μg/ml). The protein concentration of cell lysates was determined with a BCA protein assay kit (Thermo Fischer Scientific, Waltham, MA, USA), and equal amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis on a 7.5 or 12% gel (Bio-Rad, Hercules, CA, USA). The separated proteins were transferred to a nitrocellulose membrane, which was then incubated with Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 20 min at room temperature before incubation overnight at 4 °C with primary antibodies. Rabbit polyclonal antibodies to human phosphorylated HER2 (pY1248), to phosphorylated AKT, to
AKT, to BIM, to Mcl-1, to Bel-2, to Bel-xL, to XIAP and to p110z were obtained from Cell Signaling Technology; those to phosphorylated ERK and to ERK were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); those to c-IAP1 were from R&D Systems (Minneapolis, MN, USA); those to HER2 were from Millipore (Billerica, MA, USA); those to survivin were from Novus (Littleton, CO, USA); and those to β-actin were from Sigma. All antibodies were used at a 1:1000 dilution, with the exception of those to β-actin (1:200). The membrane was then washed with phosphate-buffered saline containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (Sigma). Immune complexes were finally detected with chemiluminescence reagents (GE Healthcare, Little Chalfont, UK).

### Gene silencing

Cells were plated at 50–60% confluence in six-well plates or Gene silencing reagents (GE Healthcare, Little Chalfont, UK). complexes were finally detected with chemiluminescence temperature with horseradish peroxidase-conjugated goat containing 0.05% Tween 20 before incubation for 1 h at room membrane was then washed with phosphate-buffered saline were from Sigma. All antibodies were used at a 1:1000 were from Novus (Littleton, CO, USA); and those to survivin were from Millipore (Billerica, MA, USA); those to survivin from R&D Systems (Minneapolis, MN, USA); those to HER2 Biotechnology (Santa Cruz, CA, USA); those to survivin, expressed in cancer and lymphoma. (Nat Med) 3: 917–921. 

The activity of caspase-3 in cell lysates was measured with the use of a CCP32/Caspase-3 Fluorometric Protease Assay Kit (MBL, Woburn, MA, USA). Fluorescence attributable to cleavage of the Asp–Glu–Val–Asp–7-amino-4-trifluoromethyl coumarin (DEVD-AFC) substrate was measured at excitation of caspase-3 in cell lysates was measured with the activity assay in vivo experiments are presented as means ± s.e. from three independent experiments, and were analyzed with the unpaired two-tailed Student’s t-test. In vivo data are presented as means ± s.e. from six mice and were analyzed by the unpaired two-tailed Student’s t-test. A P-value of <0.05 was considered statistically significant.

### Conflict of interest

The authors declare no conflict of interest.

### Acknowledgements

We thank E Hatashita, K Kuwata, and H Yamaguchi for technical assistance.

### References


\[ \text{In vivo} \text{test.} \]

\[ \text{In vitro} \text{test.} \]

\[ \text{In vivo} \text{test.} \]
BIM and survivin in lapatinib-induced apoptosis
J Tanizaki et al


Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)