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ORIGINAL ARTICLE

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

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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.
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Keywords: BIM; survivin; *HER2* amplification; *PIK3-CA* mutation; apoptosis; breast cancer

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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 (IC_{50}) values ranging from 0.05 to 0.80 μM, which are within the clinically achievable concentration range

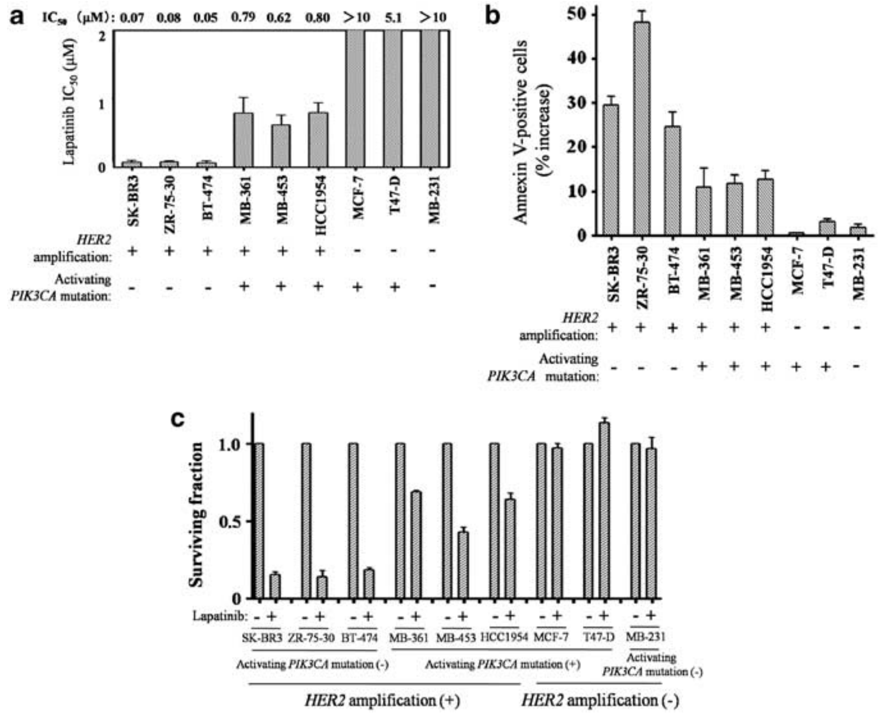

Figure 1 Effects of lapatinib on cell proliferation and apoptosis in breast cancer cells classified according to *HER2* and *PIK3CA* status. (**a**) The indicated cell lines were cultured for 72 h in complete culture medium containing various concentrations of lapatinib, after which the number of viable cells was determined and the IC_{50} value of lapatinib for inhibition of cell proliferation was calculated. (**b**) The indicated cell lines were incubated for 72 h with lapatinib (1 μM), after which the number of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. The percentage increase in the number of apoptotic cells relative to the corresponding value for cells incubated without lapatinib is shown. (**c**) The indicated cell lines were cultured for 14 days in the presence of lapatinib (1 μM) before determination of the number of colonies for calculation of the surviving fraction relative to that of control cells incubated without lapatinib. All data are means ± s.e. from three independent experiments.

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 were 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 IC_{50} 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 IC_{50} 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). These data thus revealed that lapatinib exerts anti-proliferative and anti-survival effects in cells positive for *HER2* amplification, but the extent of these effects is smaller for such cells with a *PIK3CA* mutation than for those without this genetic change.

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

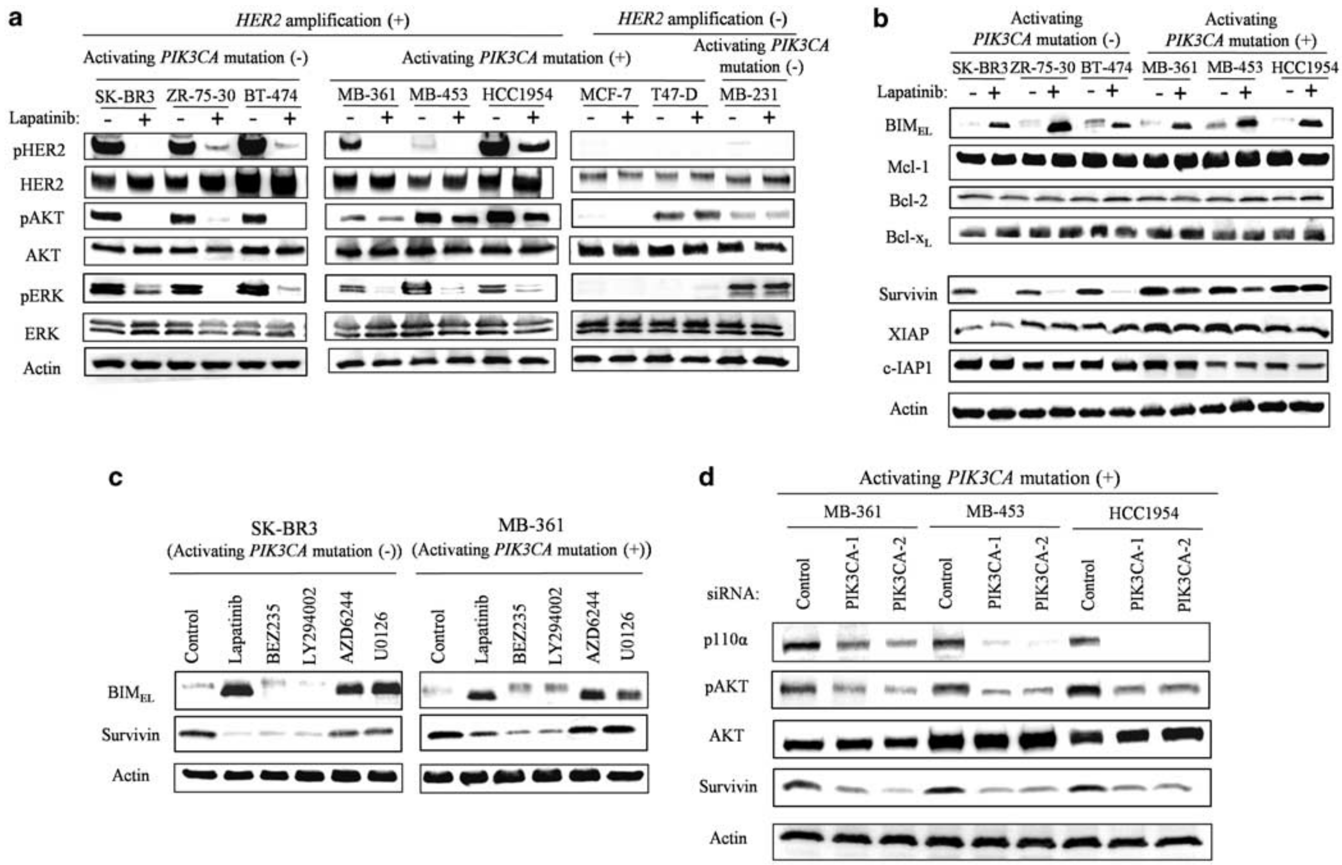

Figure 2 Effects of lapatinib on HER2, AKT and ERK phosphorylation as well as on apoptosis-related protein expression in breast cancer cell lines. (**a**, **b**) The indicated cell lines were incubated with or without lapatinib (1 μM) for 24 h (**a**) or 48 h (**b**), after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of HER2, AKT or ERK as well as with those to the indicated apoptosis-related proteins or to β-actin (loading control). The position of the band corresponding to BIM_{EL} is indicated. (**c**) SK-BR3 or MB-361 cells were incubated in the absence (control, 0.1% dimethyl sulfoxide) or presence of lapatinib (1 μM), BEZ235 (0.3 μM), LY294002 (20 μM), AZD6244 (0.3 μM) or U0126 (20 μM) for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to BIM, to survivin or to β-actin. (**d**) The indicated cell lines were transfected with non-specific (control), PIK3CA-1 or PIK3CA-2 siRNAs for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins.

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-x_L. 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

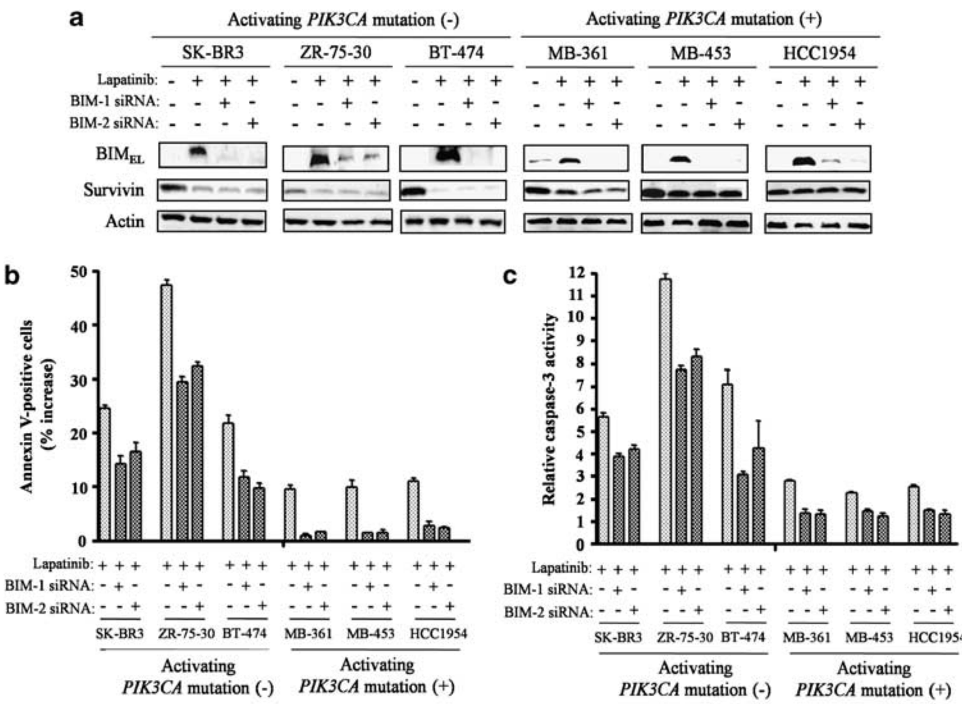

Figure 3 Effect of inhibition of BIM induction on lapatinib-induced apoptosis in *HER2* amplification-positive breast cancer cells with or without an activating *PIK3CA* mutation. (**a**) The indicated cell lines were transfected with BIM-1, BIM-2 or non-specific siRNAs for 24 h and then incubated for 48 h in complete medium with or without lapatinib (1 μM). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to BIM, to survivin or to β-actin. (**b**) Cells transfected as in a were incubated for 72 h with or without lapatinib (1 μM), and then evaluated for the proportion of apoptotic cells by staining with annexin V and propidium iodide followed by flow cytometry. The percentage increase in the number of apoptotic cells relative to the corresponding value for cells transfected with the control siRNA and incubated without lapatinib is shown. (**c**) Lysates prepared from cells treated as in (**a**) were assayed for caspase-3 activity, which is expressed relative to the corresponding value for cells transfected with the control siRNA and incubated without lapatinib. Data in b and c are means ± s.e. from three independent experiments.

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*

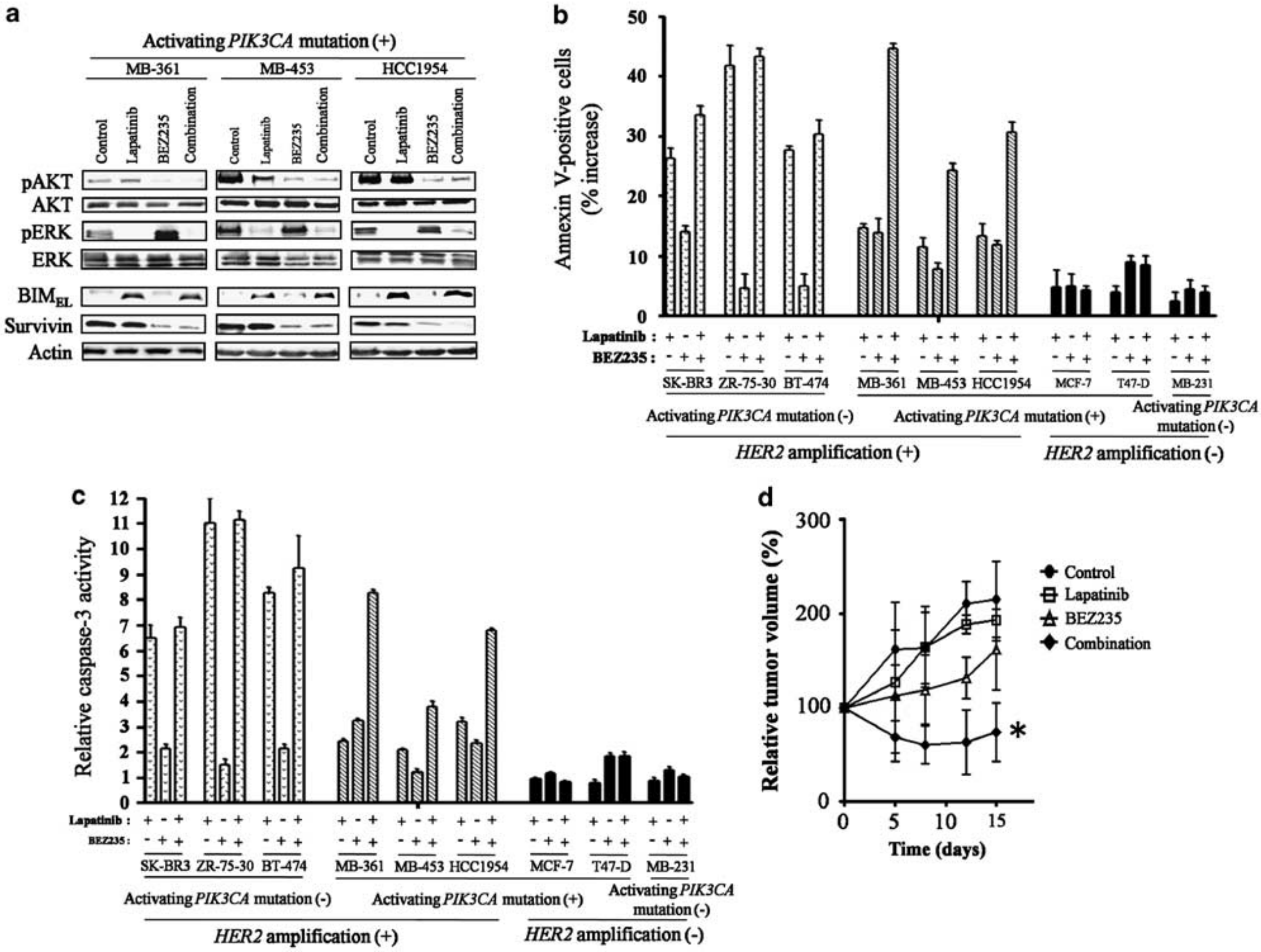

Figure 4 Effects of the combination of BEZ235 and lapatinib in *HER2* amplification-positive cells with an activating *PIK3CA* mutation. (**a**) The indicated cell lines were incubated in the absence (control, 0.1% dimethyl sulfoxide) or presence of lapatinib (1 μM), BEZ235 (0.03 μM) or both agents (combination) for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins. (**b**) Cells were incubated in the absence or presence of lapatinib (1 μM) or BEZ235 (0.03 μM), as indicated, for 72 h, after which the proportion of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. The percentage increase in the number of apoptotic cells relative to the corresponding value for cells incubated without addition is shown. (**c**) Cells treated as in (**a**) were lysed and assayed for caspase-3 activity, which is expressed relative to the corresponding value for cells incubated without addition. Data in (**b**, **c**) are means ± s.e. from three independent experiments. (**d**) Nude mice with tumor xenografts established by subcutaneous injection of HCC1954 cells were treated daily for 2 weeks with vehicle (control), BEZ235 (15 mg/kg per day), lapatinib (100 mg/kg per day) or the combination of both drugs. Tumor size was determined at the indicated times after treatment onset and is expressed as a percentage of that at time 0. Data are means ± s.e. for six mice per group. $*P<0.05$ for the combination of BEZ235 and lapatinib versus either BEZ235 or lapatinib alone.

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

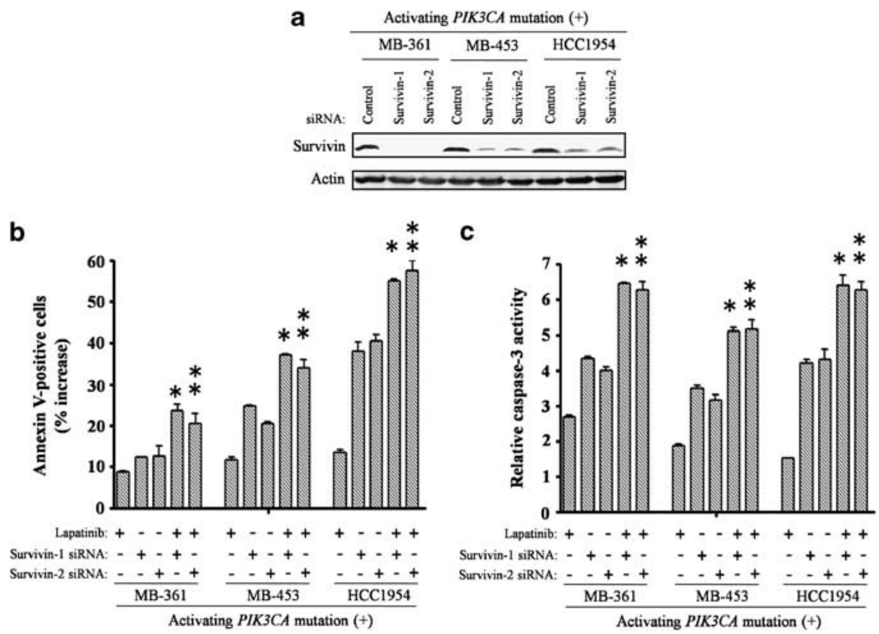

Figure 5 Effect of survivin depletion on apoptosis in *HER2* amplification-positive cells with an activating *PIK3CA* mutation. (**a**) The indicated cell lines were transfected with non-specific (control), survivin-1 or survivin-2 siRNAs for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to survivin or to β-actin. (**b**) Cells transfected as in (**a**) were incubated in complete medium with or without lapatinib (1 μM) for 72 h, after which the proportion of apoptotic cells was determined by staining with annexin V and propidium iodide followed by flow cytometry. The percentage increase in the number of apoptotic cells relative to the corresponding value for cells transfected with the control siRNA and incubated without lapatinib is shown. (**c**) Cells transfected as in (**a**) were incubated with or without lapatinib (1 μM) for 48 h, lysed and assayed for caspase-3 activity, which is expressed relative to the corresponding value for cells transfected with the control siRNA and incubated without lapatinib. Data in (**b**, **c**) are means ± s.e. from three independent experiments. *$P<0.05$ for the combination of lapatinib plus transfection with survivin-1 siRNA versus either treatment alone. **$P<0.05$ for the combination of lapatinib plus transfection with survivin-2 siRNA versus either treatment alone.

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 downregulation 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 Bcl-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; Altieri, 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% CO_2 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 (Gymnopoulos *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 (IC_{50}) 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 Bcl-2, to Bcl-x_L, to XIAP and to p110α 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 25-cm^2 flasks and then incubated for 24 h before transient transfection for the indicated times with siRNAs mixed with the Lipofectamine reagent (Invitrogen). The siRNAs specific for PIK3CA (PIK3CA-1, 5′-UCAACUUCUUCAAGAUG AA-3′; PIK3CA-2, 5′-GUAGAAUGUUUACUACCAA-3′), BIM (BIM-1, 5′-GGAGGGUAUUUUUGAAUAA-3′; BIM-2, 5′-AGGAGGGUAUUUUUGAAUA-3′), or survivin (survivin-1, 5′-GAAGCAGUUUGAAGAAUUA-3′; survivin-2, 5′-AGAAGCAGUUUGAAGAAUU-3′) mRNAs as well as non-specific (control) siRNAs were obtained from Nippon EGT (Toyama, Japan).

Assay of caspase-3 activity
The activity of caspase-3 in cell lysates was measured with the use of a CCP32/Caspase-3 Fluometric 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 and emission wavelengths of 390 and 460 nm, respectively.

Growth inhibition assay in vivo
All animal studies were done with the Recommendations for Handling of Laboratory Animals for biochemical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University. Cubic fragments of tumor tissue (∼2 by 2 by 2 mm) formed by HCC1954 cells were implanted subcutaneously into the axilla of 5–6-week-old male athymic nude mice. When their tumors became palpable, mice were divided in to four groups and treated with vehicle, BEZ235 alone, lapatinib alone and the combination of BEZ235 and lapatinib. Each treatment group contained six mice. BEZ235 and lapatinib were administered by oral gavage daily for 14 days; control animals received a 0.5% (w/v) aqueous solution of hydroxypropylmethylcellulose as vehicle. Tumor volume was determined from caliper measurements of tumor length (L) and width (W) according to the formula $LW^2/2$. Both tumor size and body weight were measured twice per week.

Statistical analysis
Quantitative data from *in vitro* 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.

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