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Resiliency and vulnerability in the HER2-HER3 tumorigenic driver

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Abstract

About 25% of breast cancers harbor the amplified oncogene HER2 and are dependent on HER2 kinase function, identifying HER2 as a vulnerable target for therapy. However,. HER2-HER3 activation is buffered so that it is protected against a nearly two log inhibition of HER2 catalytic activity; this buffering is driven by the negative regulation of HER3 by Akt. We have now further characterized HER2-HER3 signaling activity and shown that the compensatory buffering prevents apoptotic tumor cell death from occurring as a result of the combined loss of MAPK and Akt signaling. To overcome the cancer cells' compensatory mechanisms, we co-administered a PI3K/mTor inhibitor and a HER2 tyrosinc kinase inhibitor. This treatment strategy proved suboptimal because it induced both tyrosine kinase inhibitor sensitizing and desensitizing effects and robust cross-compensation of MAPK and Akt signaling pathways. Noting that HER2-HER3 activity was completely inhibited by higher, fully inactivating doses of TKI, we then attempted to overcome the cells compensatory buffering with this higher dose. This treatment crippled all downstream signaling and induced tumor apoptosis. Although such high doses of TKI are toxic in vivo when given continuously, we found that intermittent doses of TKI administered to mice produced sequential cycles of tumor apoptosis and ultimately complete tumor regression in mouse models, with much less toxicity. This strategy for inactivation of HER2-HER3 tumorigenic activity is proposed for clinical testing.

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Keywords

Lapatinib; HER2; HER3; ErbB2; ErbB3; breast cancer

Introduction

A subset of human cancers is characterized by amplification and overexpression of the human epidermal growth factor-2 (HER2) oncogene. HER2 has been best studied in the context of breast cancers where it accounts for approximately 25% of all instances and causes aggressive disease (1). In mice, overactivity of Neu (rodent homolog of HER2) leads to the development of aggressive mammary tumors that progress to metastastic disease (2). HER2 likely promotes tumorigenesis through multiple complex downstream signaling pathways (3). HER2-driven tumors continue to be highly dependent on HER2 function during their progression to invasive and metastatic disease. Indeed Neu overexpression in mice induces metastatic mammary tumors, and the subsequent reversal of Neu oncogene expression leads to complete regression of the disease at both primary and metastatic sites (4). The absolute dependency of HER2-induced tumors on continued HER2 function points to HER2 as an Achilles heel for this type of cancer and a potential target for highly effective therapies.

This evidence suggests that HER2-driven breast cancers could be effectively treated through the pharmacological inactivation of tumor HER2 in patients. Nevertheless, tyrosine kinase inhibitors (TKIs) that target the HER family show only limited clinical activity in patients with HER2-amplified breast cancer, producing only partial short-lived responses in a subset of patients. For the potent and highly selective HER family TKI lapatinib (GW572016/Tykerb), the clinical response rates range from 4 to 39% (5-9). More limited data for the irreversible inhibitor HER family TKI neratinib (HKI272) show clinical response rates ranging from 26 to 51% (10,11). Although these drugs provide options for patients with this disease, they fail to fulfill the expectation that they would show a much higher efficacy, complete responses, and possible disease eradication because of the complete dependence of these tumors on HER2. Targeting the HER2 oncoprotein in HER2-amplified breast cancers has proven to be more challenging than simple mechanistic models would predict, and the hypothesis that this disease can be eradicated through the inactivation of HER2 requires further mechanistic insight before it can be reconsidered.

HER2 is a member of the HER (or epidermal growth Factor Receptor; EGFR) family of receptor tyrosine kinases comprised of HER1 (EGFR), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). These homologous receptors share a common structure consisting of an extracellular ligand-binding domain, an intracellular tyrosine kinase domain, and a carboxyl-terminal signaling tail. The intracellular signal is generated as a consequence of receptor dimerization and transphosphorylation. With the exception of HER2, the extracellular domains do not permit dimerization unless they are structurally reconfigured by ligand binding. Dimerization among different members constitutes the principle mode of signaling in this family, and in fact structural features mandate a functional interdependency among different family members.

This interdependence is best exemplified by HER2 and HER3. HER2 has the strongest catalytic kinase activity and its extracellular domain permits dimerization without ligand binding. In fact, HER2 is the preferred dimerization partner for most other family members (12,13). On the other hand, although HER3 can bind ligand, the HER3 kinase domain lacks catalytic activity, and HER3 depends on a heterodimeric partner for signaling (14,15). The functions of HER2 and HER3 are complementary to one another, and the HER2-HER3

heterodimer forms the strongest signaling dimer among all possible dimeric combinations (12). The interdependent functions of HER2 and HER3 are evident from their behavior in cancer models. HER2 can transform cells by overexpression alone, and is one of the most potently transforming oncogenes. Although HER3 can not transform cells by itself, its co-expression synergistically enhances HER2 transformation (16,17). In fact HER3 expression is rate-limiting for transformation in HER2-amplified breast cancers, and the knockdown of HER3 reverses transformation in HER2-amplified tumors and induces tumor apoptosis (18,19).

The close mechanistic relationship between HER2 and HER3 provides insight into the limited clinical success of HER2-targeted therapies. When HER2-amplified tumors are treated with HER-targeting TKIs, the transphosphorylation of HER3 is only transiently inhibited, and HER3 signaling resumes despite continued TKI therapy. This is due to compensatory mechanisms that can enhance HER2-HER3 signaling, providing a buffering capacity that desensitizes HER2-HER3 signaling to TKIs and presents a barrier to this treatment strategy (20). Here we explore the nature, depth, and vulnerability of this signal buffering capacity by using the potent, safe, clinically approved and widely used HER2 TKI, lapatinib, and propose a new strategy for treatment of HER2-amplified breast cancers by totally inactivating HER2 kinase with an intermittent dosing schedule.

Results

Lapatinib is a reversible TKI that inhibits the EGFR and HER2 enzymes with *in vitro* IC_{50}s of approximately 10 nM (21). Lapatinib is selective for the HER family and has little interaction with the entire rest of the human kinome, making it an ideal agent to interrogate HER family signaling with minimal impact from off-target effects (22). Treatment of HER2-amplified human breast cancer cells with lapatinib initially induces a potent and rapid inactivation of HER2-HER3 and downstream Akt and MAPK signaling with inhibition apparent at 50 nM (figure 1A,B). HER2-HER3 ultimately escapes inhibition at these nanomolar concentration ranges, although durable inhibition of HER2-HER3 signaling occurs at approximately 5uM (figure 1A,B). When downstream p-Akt and MAPK pathway signaling recovers after about 48 h at the lower dose of lapatinib, the amount of HER2 and HER3 protein is increased (figure 1A). The upregulation of HER3 is, at least in part, due to its transcriptional upregulation (figure 1C). The restoration of MAPK and AKT signaling after 48 hours at nanomolar concentrations of drug is also associated with cell survival and followed by partial resumption of cell division despite continued lapatinib treatment (figure 1D). In contrast, treatment with lapatinib at concentrations that durably suppress downstream signaling induces apoptotic cell death (discussed below). HER2-HER3 signaling is therefore a dominant nodal point for receptor tyrosine kinase (RTK) signaling in these HER2-amplified tumor cells; its suppression and inactivation by lapatinib does not appear to be compensated by the activation of other RTKs (figure 1E).

These data indicated that the HER2-HER3 complex is endowed with a robust signal buffering capacity that allows it to recover from a two log inhibition of HER2 catalytic function (schematically described in figure 2). This buffering capacity is not inherent in the HER2 tyrosine kinase enzyme since in *in vitro* assays with the purified enzyme, the HER2 kinase is inhibited in linear fashion by TKIs (23,24). Treatment of SkBr3 cells with the less potent HER2 inhibitor erlotinib reveals a similar signal buffering capacity, and although the effective concentrations are higher with erlotinib, these data show that the signal buffering capacity resides in the cellular circuitry and does not depend on the specific TKI (figure 2B). HER2-HER3 signaling is similarly buffered in other HER2-amplified breast cancer cells (supplementary materials figure 1).

To test whether Akt was the driver of HER2-HER3 signal buffering, we engineered SkBr3 cells expressing myrAktΔER, a 4-OH tamoxifen (4HT)-inducible form of Akt and the relevant non-inducible control (myr*AktΔER, described in methods). The myr*Akt-ΔER fusion product (Fig. 3A, left panel) is not activated by 4HT due to the mutated myristylation domain, whereas the myrAkt-ΔER fusion product (Fig. 3A, right panel) is activated rapidly after 4HT treatment. 4HT-induced activation of Akt in SkBr3/myrAktΔER cells leads to downregulation of HER3 protein abundance revealing that steady state HER3 signaling is under Akt control (figure 3A, lanes 8,9,10). The lapatinib-induced compensatory up-regulation of HER3 expression and restoration of HER2-HER3 activation were prevented by the 4HT-induced activation of Akt (figure 3A; compare lanes 4-7 with 11-14). The lapatinib induction of HER3 expression is prevented by activated Akt (lanes 12,14), but is not inhibited as much as in cells not treated with lapatinib (lane 10). Thus Akt exerts a relative, not an absolute suppression of HER3 expression. In contrast, the lapatinib-induced upregulation of HER2 is not mediated through Akt (figure 3A, compare lanes 4-7 with 11-14). This upregulation of HER2 has been previously described and suggested to be due to decreased ubiquitination and prolonged protein half-life (25). Therefore the signal buffering capacity that protects HER2-HER3 signaling from kinase inhibitors is likely driven by the loss of Akt activity. Akt appears to regulate HER3 expression through both transcriptional and post-transcriptional mechanisms. Although the suppression of steady state HER3 protein concentrations with the 4HT-induced activation of Akt (figure 3A, lanes 1-3,8-10) is not associated with reduction of HER3 mRNA expression (figure 3B, 4HT/DMSO), the prevention of the lapatinib-induced increase in HER3 protein (figure 3A, lanes 4-8, 11-14) is associated with the prevention of the lapatinib-induced increase in HER3 mRNA expression (figure 3B, 4HT/lapatinib).

Because HER3 promotes downstream signaling predominantly through activation of PI3K, more effective inactivation of HER2-HER3 signaling could possibly be achieved by the addition of a PI3K or PI3K/mTor inhibitor to HER2 TKI treatment. Although apparently straightforward, this suggestion was undermined by significant complexity in downstream signaling. PI3K inhibitors triggered downstream negative feedback signaling that rescued Akt activity within 3 hours of inactivation while simultaneously inducing MAPK activity (figure 4A). This feedback signaling can be overcome at a higher concentrations of the PI3K/mTor inhibitor BEZ235, leading to durable inactivation of Akt, although MAPK induction remains high. The link between the PI3K and MAPK pathways is reciprocal as inhibition of MAPK signaling by the MEK inhibitor U0126 similarly upregulates Akt signaling (figure 4B). This cross-coupling of MAPK and Akt signaling reveals a circuit that could protect against the loss of both signaling pathways. Indeed, inhibition of either pathway alone, such as with U0126 or BEZ235, merely inhibits growth, whereas the complete inhibition of both pathways with a combination of 200nM lapatinib and 250nM BEZ235 induces tumor cell apoptosis (figure 4C).

Because lapatinib and BEZ235 can induce opposing effects on the downstream MAPK pathway, we tested their net effect in combination in SkBr3 cells at two concentrations of BEZ235 (figure 5A). At 100 nM, BEZ235 induces increased HER3 phosphorylation, and the induction of cross-talk signaling results in robust activation of MAPK signaling. These effects of BEZ235 at 100 nM provide little net benefit when added together with lapatinib, and the combination of BEZ235 and lapatinib is ineffective at suppressing MAPK signaling or completely and durably suppressing Akt signaling (figure 5A). At 250 nM BEZ235, the effects of both drugs provide a net benefit, and suppress MAPK and Akt pathway signaling and induce apoptotic tumor cell death (figure 5A,B).

This drug combination also reveals complexities in feedback signaling that are not explained by our current models of this circuit and require further mechanistic exploration. Although

lapatinib induces a compensatory increase in HER3 expression and restoration of its phosphorylation, BEZ235 induces the phosphorylation of HER3 without an increase in HER3 expression (figure 5A). This is also seen in BT474 cells treated with BEZ235 (supplementary materials, figure 2). This reveals that HER3 signaling is protected by multiple, redundant mechanisms. Furthermore, although lapatinib by itself induces the expression of HER3, and BEZ235 by itself induces the phosphorylation of HER3, the combination of both drugs paradoxically inhibits HER3 phosphorylation (figure 5A, compare 250 nM BEZ235 alone with 250 nM BEZ235 + lapatinib). The overall beneficial effect of BEZ235 is only seen at 250 nM, at which concentration there is a net beneficial, albeit incomplete, inactivation of MAPK, and a beneficial inactivation of HER3 and Akt signaling. Although the combination of these two drugs may have therapeutic benefits, they may not be effective at all doses and the net benefit of such combinations will ultimately need to be determined in clinical studies.

Another potential strategy for more effective treatment of HER2-driven cancers would be the complete inactivation of HER2 catalytic function. Since HER2-HER3 signal buffering depends on residual HER2 kinase function (20), it can be overcome by TKI therapy that fully inactivates HER2 kinase, leaving no residual HER2 catalytic function to drive signaling. In cell culture, lapatinib completely inhibits HER2 kinase at 5 μM and overcomes HER2-HER3 signal buffering, inducing a durable suppression of MAPK and PI3K/Akt pathways despite causing maximal upregulation of HER3 (figure 1A) and apoptotic tumor cell death (figure 4C). This effect requires high doses of the TKI to exceed the signal buffering capacity of the HER2-HER3 tumorigenic driver. Such high doses are not clinically feasible due to toxicities directly related to the inhibition of EGFR and HER2. Such toxicities are seen with all classes of HER targeting agents and include diarrhea, rash, and potentially cardiomyopathies (26-28). However, higher doses of TKI may possibly be safely administered if continuous dosing is replaced with intermittent dosing. Since HER2-inactivating TKI therapy is expected to induce potent tumor apoptosis, the intermittent administration of TKI might be expected to induce significant anti-tumor effects when given in repeated cycles. To test this treatment hypothesis, we determined the maximal tolerated dose (MTD) of lapatinib given as a 5-day course repeated every two weeks in mice. The MTD was 800 mg/kg/day, which is 8-fold higher than the MTD of lapatinib administered continuously. In mice bearing HCC1569 HER2-amplified tumors, treatment with lapatinib at the continuous dosing MTD of 100mg/kg/day produced a relative anti-tumor growth inhibitory effect but no actual tumor regression, and tumor growth resumed after cessation of therapy (figure 6A,B). However treatment with 800mg/kg/day in an intermittent schedule produced an immediate tumor regression and a much more long-lasting anti-tumor effect (figure 6A,B). Treatment at the high dose was associated with transient weight loss that is entirely regained during the off-cycle period (supplementary materials figure 3) and better target inactivation (figure 6C). The higher 800 mg/kg/day dose of lapatinib in these mice led to a proportionally higher steady-state plasma lapatinib concentration than the 100 mg/kg/day (figure 6D).

Discussion

Small molecule TKI treatment of cancer is based on a mechanistic understanding and has a proven track record in several disease types. TKIs that target the Bcr-abl oncoprotein produce responses in 95% of patients with Bcr-abl driven chronic myelogenous leukemia, including complete cytogenetic responses, suggesting that it eradicates the disease (29). TKIs that target the EGFR oncoprotein produce response rates of 60-94% in patients with lung cancers driven by mutationally activated EGFR, and disease recurrence is typically associated with mutational events that render the EGFR oncoprotein resistant to TKIs (30). Since HER2-amplified breast cancers are driven by and highly dependent on the HER2

tyrosine kinase, targeting of HER2 with TKIs would be expected to be similarly effective in the treatment of these cancers. However, highly potent HER2 TKIs only show a modest clinical anti-tumor activity and do not eradicate disease in patients with advanced cancer.

Our studies and other evidence converge to reveal previously unknown complexity in HER2 oncogenic signaling that underlies its resiliency to treatment with TKIs. A key role of the HER2 partner HER3 has been revealed by knockdown studies showing that HER3 is essential in HER2-driven tumorigenesis (18,19) and from TKI treatment studies showing that HER3 signaling persists despite an apparent inhibition of HER2 autophosphorylation (20). The relationship between HER2 and HER3 is more complex than that of a kinase and substrate. Kinase domain dimerization in the HER family occurs in an asymmetric configuration whereby one kinase functions as an allosteric activator of the other kinase, which contains all the catalytic activity of the dimer (31,32). The assignment of specialized stimulatory or catalytic functions to individual members in an asymmetric dimer explains the lack of catalytic activity in the HER3 kinase domain. This kinase domain is a specialized stimulatory kinase partner, as evidenced by its lack of measurable catalytic activity (14), while the HER2 kinase domain is an optimized catalytic kinase partner, evidenced by its robust catalytic function compared to other members (12). The HER3 kinase allosterically activates the HER2 kinase, while the HER3 c-terminal tail is the substrate of the HER2 kinase. As such, HER3 acts both upstream and downstream of HER2, and so the HER2-HER3 dimer is appropriately considered a single functional unit. Thus the functionally relevant tumorigenic driver of HER2-amplified tumors is the HER2-HER3 dimer. Indeed the HER3 signaling output is considerably more difficult to inhibit with TKIs than would be predicted from a simple kinase-substrate model. Here we have shown that this is due to signal buffering inherent in the HER2-HER3 pair that protects it against a nearly 2-log inhibition of HER2 catalytic function. This buffering can increase either the expression or the phosphorylation of HER3 and is driven by a downstream network topology that functions to preserve Akt signaling.

Next generation approaches in development include newer agents to specifically interfere with HER2-HER3 transactivation or with HER3 functions. Another approach to the inhibition of HER3 signaling is the simultaneous inhibition of PI3K, the immediate effector of phosphorylated HER3. Our data show that the success of this combination therapy may be undermined by the complexities in the downstream signaling circuitry. Inhibition of PI3K triggers negative feedback that desensitizes Akt to a PI3K inhibitor, necessitating higher concentrations of the PI3K inhibitor. Furthermore, inhibition of PI3K results in robust activation of MAPK, averting apoptosis, which requires inhibition of both pathways. The PI3K inhibitor produces both sensitizing and desensitizing effects, with the benefits dominating at higher concentrations. The clinical efficacy of this combination approach is difficult to predict from preclinical models as it depends on the therapeutic index of each drug in patients, and final determination must await appropriately designed clinical studies.

The ability of HER2-HER3 to signal despite TKI treatment requires residual HER2 kinase activity. If HER2 kinase is fully inactivated, HER2-HER3 signaling ultimately fails, despite maximal induction of HER3 expression, resulting in persistent inactivation of downstream Akt and MAPK signaling and consequent tumor apoptosis. This process accounts for the upper limit of the signal buffering capacity. The complete inactivation of HER2 requires much higher doses in patients. Although toxicity of the HER2 inhibiting drugs prevent their administration on continuous schedules, we have shown here in mice the feasibility of an alternative strategy that utilizes an intermittent dosing. An intermittent schedule delivers higher doses and produces more effective inactivation of HER2-HER3 in tumors, inducing tumor apoptosis and revealing an anti-tumor efficacy not seen with continuous schedules. Plasma lapatinib concentrations in the control mice given continuous daily dosing at the

MTD of the drug are similar to those observed at the MTD in continuous dosing in phase I studies in patients with cancer (33,34). The plasma concentrations of lapatinib in the mice given the higher dose on an intermittent schedule provide a concentration range that, at least in this preclinical model, achieves better anti-tumor efficacy than does continuous dosing at MTD and provides a pharmacologic reference point to guide clinical studies of this dosing strategy.

The full inactivation of tumor HER2 kinase in patients may not be feasible with promiscuous TKIs or irreversible TKIs. Despite potent effects *in vitro*, the maximal possible doses of such agents may be limited by their off-target effects, restricting their potential for dose escalation and complete inactivation of tumor HER2 kinase *in vivo*. Therapeutic index is a critical consideration in clinical experiments designed to fully inactivate tumor HER2 in patients.

HER2-HER3 signaling is thought to be critical for HER2-amplified cancer cells, generating optimism for the development of treatments that can inactivate it. The true scope and potential of this treatment approach will ultimately have to be determined in clinical studies using next generation treatments designed to inactivate the HER2-HER3 tumorigenic driver. Some clinical studies have suggested that breast cancers with hyperactivity of PI3K signaling due to loss of PTEN or mutational activation of PI3K may be resistant to HER2-targeting approaches (35,36). However these correlations, seen predominantly with the HER2-targeting antibody trastuzumab, do not appear to apply to HER2-targeting TKIs, which have a different mechanism of action. In *in vitro* and clinical studies, the lack of PTEN expression does not seem to confer resistance to lapatinib in HER2-amplified breast cancers (37,38). Indeed here we show that overcoming HER2-HER3 signal buffering capacity with higher intermittent dosing was effective in the HER2-amplified tumor model HCC1569. These tumor cells lack PTEN expression, and their sensitivity to HER2-HER3 inhibition supports the hypothesis that this treatment approach may be broadly effective in HER2-amplified cancers.

Methods

SKBr3 cells were obtained from ATCC and maintained at 37°C and 5%CO2 in DMEM:Ham's F12 1:1 media supplemented with 10% heat-inactivated Fetal Bovine Serum, 100 units/ml penicillin, 100 ug/ml streptomycin, and 4mM L-glutamine. LY29004, wortmannin, and 4-OH-tamoxifen were from EMD/Calbiochem. All compounds were reconstituted in DMSO, and 4-hydroxy-tamoxifen was reconstituted in ethanol. BEZ235 was obtained from Novartis. Lapatinib and erlotinib were purchased as tablets and the active ingredient purified by organic extraction as described in the supplementary materials section. In all TKI treatment experiments extending longer than 24 hours, the media was replaced with fresh media containing freshly mixed TKI every 24 hours.

Cell growth was assayed by seeding 20,000 cells per well in a 24-well cluster plate. After 24 hours, cells were treated with 200nM lapatinib or vehicle only. Media with fresh drugs was changed every 24 hours, and daily cell counts from triplicate wells were obtained for 7 days with a hematocytometer. Cell growth is reported as a percentage of the cell count obtained at time point “0”.

Total cellular lysates were obtained by harvesting cells in modified radioimmunoprecipitation assay (RIPA) buffer supplemented with leupeptin, aprotinin, phenylmethylsulphonyl fluoride, sodium vanadate and phosphatase inhibitor cocktail (Roche). Western blotting was performed by separating 50 ug of lysates on an SDS-PAGE, transferring to polyvinylidene fluoride membrane, and immunoblotting with the indicated

antibodies followed by enhanced chemoluminescence visualization. Immunoprecipitation of HER2 was performed with antibody to HER2 (SC-284) (Santa Cruz Biotechnology). Antibodies used for western blot analysis were against p-Y1248-HER2, pS473-AKT, pT202/Y204-MAPK, MAPK, AKT, p-S235/236-S6, S6 ribosomal protein (Cell Signaling), β-actin, HER2 SC-284, HER3 5A12, p-Tyr PY99 (Santa Cruz Biotechnology) and p-T246-PRAS40 (Biomol). Polyclonal antibodies against pY1289-HER3 were generated in rabbits with a phosphopeptide spanning Y1289, and antisera were affinity purified over a phosphopeptide column and counter-purified over a non-phosphopeptide column. The phosphospecificity of the antibody was verified in cells treated with phosphorylated or non-phosphorylated HER3 and treated with heregulin or lapatinib, or with overexpression of HER3.

Phospho-RTK profiling was done with the R&D systems Proteome Profiler Phospho-RTK Antibody Array according to manufacturer's instructions. Briefly, the array membranes were blocked in blocking buffer and incubated with diluted cell lysates overnight at 4C. After washing, the array membranes were incubated with the detection antibody, washed again, and developed with standard chemiluminescent reagents.

Apoptosis was assayed by fluorescence-activated cell sorting (FACS) analysis of nuclear degradation as described (39). In brief, cell nuclei were prepared and stained with ethidium bromide, and DNA content analyzed on FACS CaliburII with Modfit software. Apoptotic cells were identified by their Sub-G1 DNA content as analyzed by Modfit, and data averaged over triplicate experiments.

Total cellular RNA was isolated using the RNeasy miniprep kit with on-column DNAse treatment as per the manufacturer's protocols (Qiagen). Reverse transcription and real-time PCR amplification was perfomed as described using the IQ SybrGreen Supermix on a MyIQ I-cycler (BioRad) (40). Normalization was performed against GAPDH or β-microglobulin and relative expression was obtained using Pfaffl ratios. Data represents average from triplicates. The primers used were 5’CCCTGCCATGAGAACTGCAC and 5’TCACTGTCAAAGCCATTGTCAGAT for HER3, 5’AACTGCACCCACTCCTGTGT and 5’TGATGAGGATCCCAAAGACC for HER2, 5’GGTCTCCTCTGACTTCAACA and 5’AGCCAAATTCGTTGTCATAC for GAPDH, 5’TGCTGTCTCCATGTTTGATGTATCT and 5’TCTCTGCTCCCCACCTCTAAGT.

SkBr3 cells were engineered to express an inducible form of Akt. This construct was generated previously by fusing a myristylated Akt to a mutated ligand binding domain of the estrogen receptor (myrAktΔER) (41,42). Myristylated Akt is constitutively active, however the myrAktΔER fusion construct is auto-inhibited by the ER fragment. Treatment with the ER ligand 4-hydroxy tamoxifen (4HT) relieves the auto-inhibition leading to activation of myrAkt. Negative control is provided by the identical construct containing an inactivating mutation within the myristylation sequence (myr*AktΔER). SkBr3 cells were infected with retroviral particles generated with the pWZLneo-myrAkt-ΔER or the pWZLneo-myr*Akt-ΔER vectors and selected in neomycin.

Where indicated, statistical analysis was performed with t-tests based on two-tailed distribution and unequal variance; the calculated p values are stated in the figure legends.

Mouse experiments were done under an institutional IACUC approved protocol. Briefly 2×10^6 HCC1569 cells were orthotopically implanted into the mammary fat pad of 7-9 week old female nu/nu mice and allowed to grow into tumors. When tumors reached approximately $100mm^3$, mice were randomized and treated according to the experimental arms. Lapatinib was administered as a suspension in 0.5% hydroxypropylmethylcellulose,

0.2% tween-80 by oral gavage in two daily doses. Tumor sizes were measured once or twice weekly with calipers.

For biochemical and plasma analysis, some mice were sacrificed 4 hours after the morning dose of the 5^{th} day, the tumors rapidly dissected and flash frozen, and the plasma collected and frozen. Tumor lysates were prepared in RIPA buffer and western blotting performed as described. Plasma lapatinib concentrations were analyzed using a previously described method (43) with a sensitivity of 1 ng/ml and a precision and accuracy within 15%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and Notes

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987;235:177. [PubMed: 3798106]
2. Ursini-Siegel J, Schade B, Cardiff RD, Muller WJ. Insights from transgenic mouse models of ERBB2-induced breast cancer. Nat Rev Cancer May;2007 7:389. [PubMed: 17446858]
3. Moasser MM. The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis. Oncogene 2007;26:6469. [PubMed: 17471238]
4. Moody SE, Sarkisian CJ, Hahn KT, Gunther EJ, Pickup S, Dugan KD, Innocent N, Cardiff RD, Schnall MD, Chodosh LA. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. Cancer Cell Dec;2002 2:451. [PubMed: 12498714]
5. Blackwell KL, Pegram MD, Tan-Chiu E, Schwartzberg LS, Arbushites MC, Maltzman JD, Forster JK, Rubin SD, Stein SH, Burstein HJ. Single-agent lapatinib for HER2-overexpressing advanced or metastatic breast cancer that progressed on first- or second-line trastuzumab-containing regimens. Ann Oncol Jun;2009 20:1026. [PubMed: 19179558]
6. Burstein HJ, Storniolo AM, Franco S, Forster J, Stein S, Rubin S, Salazar VM, Blackwell KL. A phase II study of lapatinib monotherapy in chemotherapy-refractory HER2-positive and HER2-negative advanced or metastatic breast cancer. Ann Oncol Jun;2008 19:1068. [PubMed: 18283035]
7. Kaufman B, Trudeau M, Awada A, Blackwell K, Bachelot T, Salazar V, DeSilvio M, Westlund R, Zaks T, Spector N, Johnston S. Lapatinib monotherapy in patients with HER2-overexpressing relapsed or refractory inflammatory breast cancer: final results and survival of the expanded HER2+ cohort in EGF103009, a phase II study. Lancet Oncol Jun;2009 10:581. [PubMed: 19394894]
8. Iwata, H.; Toi, M.; Fujiwara, Y.; Ito, Y.; Fujii, H.; Nakamura, S.; Aogi, K.; Zaks, T.; Sasaki, Y.; Takashima, S. Phase II clinical study of lapatinib (GW572016) in patients with advanced or metastatic breast cancer.. San Antonio Breast Cancer Symposium. 2006. #1091
9. Gomez HL, Doval DC, Chavez MA, Ang PC, Aziz Z, Nag S, Ng C, Franco SX, Chow LW, Arbushites MC, Casey MA, Berger MS, Stein SH, Sledge GW. Efficacy and safety of lapatinib as first-line therapy for ErbB2-amplified locally advanced or metastatic breast cancer. J Clin Oncol Jun 20;2008 26:2999. [PubMed: 18458039]
10. Burstein, HJ.; Sun, Y.; Tan, AR.; Dirix, L.; Vermette, JJ.; Powell, C.; Zacharchuk, C.; Badwe, RA. Neratinib (HKI-272), an irreversible pan erbB receptor tyrosine kinase inhibitor: phase 2 results in patients with advanced HER2+ breast cancer.. San Antonio Breast Cancer Symposium. 2008. Abstr #37

11. Swaby R, Blackwell K, Jiang Z, Sun Y, Dieras V, Zaman K, Zacharchuk C, Powell C, Abbas R, Thakuria M. Neratinib in combination with trastuzumab for the treatment of advanced breast cancer: A phase I/II study. J Clin Oncol 2009;27:15s. Abst 1004.
12. Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, Ratzkin BJ, Yarden Y. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. Mol Cell Biol Oct;1996 16:5276. [PubMed: 8816440]
13. Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Kofler M, Jorissen RN, Nice EC, Burgess AW, Ward CW. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. Mol Cell Feb;2003 11:495. [PubMed: 12620236]
14. Sierke SL, Cheng K, Kim HH, Koland JG. Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein. Biochemical Journal Mar 15;1997 322:757. [PubMed: 9148746]
15. Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway KL 3rd. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc Natl Acad Sci U S A Aug 16;1994 91:8132. [PubMed: 8058768]
16. Alimandi M, Romano A, Curia MC, Muraro R, Fedi P, Aaronson SA, Di Fiore PP, Kraus MH. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene May 4;1995 10:1813. [PubMed: 7538656]
17. Zhang K, Sun J, Liu N, Wen D, Chang D, Thomason A, Yoshinaga SK. Transformation of NIH 3T3 cells by HER3 or HER4 receptors requires the presence of HER1 or HER2. J Biol Chem Feb 16;1996 271:3884. [PubMed: 8632008]
18. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A Jul 22;2003 100:8933. [PubMed: 12853564]
19. Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, Sliwkowski MX, Stern HM. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Res Jul 15;2008 68:5878. [PubMed: 18632642]
20. Sergina NV, Rausch M, Wang D, Blair J, Hann B, Shokat KM, Moasser MM. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature Jan 25;2007 445:437. [PubMed: 17206155]
21. Rusnak DW, Lackey K, Affleck K, Wood ER, Alligood KJ, Rhodes N, Keith BR, Murray DM, Knight WB, Mullin RJ, Gilmer TM. The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. Mol Cancer Ther Dec;2001 1:85. [PubMed: 12467226]
22. Fabian MA, Biggs WH 3rd, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, Carter TA, Ciceri P, Edeen PT, Floyd M, Ford JM, Galvin M, Gerlach JL, Grotzfeld RM, Herrgard S, Insko DE, Insko MA, Lai AG, Lelias JM, Mehta SA, Milanov ZV, Velasco AM, Wodicka LM, Patel HK, Zarrinkar PP, Lockhart DJ. A small molecule-kinase interaction map for clinical kinase inhibitors. Nat Biotechnol Mar;2005 23:329. [PubMed: 15711537]
23. Rusnak DW, Affleck K, Cockerill SG, Stubberfield C, Harris R, Page M, Smith KJ, Guntrip SB, Carter MC, Shaw RJ, Jowett A, Stables J, Topley P, Wood ER, Brignola PS, Kadwell SH, Reep BR, Mullin RJ, Alligood KJ, Keith BR, Crosby RM, Murray DM, Knight WB, Gilmer TM, Lackey K. The characterization of novel, dual ErbB-2/EGFR, tyrosine kinase inhibitors: potential therapy for cancer. Cancer Res Oct 1;2001 61:7196. [PubMed: 11585755]
24. Jani JP, Finn RS, Campbell M, Coleman KG, Connell RD, Currier N, Emerson EO, Floyd E, Harriman S, Kath JC, Morris J, Moyer JD, Pustilnik LR, Rafidi K, Ralston S, Rossi AM, Steyn SJ, Wagner L, Winter SM, Bhattacharya SK. Discovery and pharmacologic characterization of CP-724,714, a selective ErbB2 tyrosine kinase inhibitor. Cancer Res Oct 15;2007 67:9887. [PubMed: 17942920]
25. Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, Smith DJ, Landolfi S, Ramon y Cajal S, Arribas J, Baselga J. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene 2008;28:803. [PubMed: 19060928]

26. de Azambuja E, Bedard PL, Suter T, Piccart-Gebhart M. Cardiac toxicity with anti-HER-2 therapies: what have we learned so far? Target Oncol Apr;2009 4:77. [PubMed: 19418111]
27. Lacouture ME. Mechanisms of cutaneous toxicities to EGFR inhibitors. Nat Rev Cancer Oct;2006 6:803. [PubMed: 16990857]
28. Rhee J, Oishi K, Garey J, Kim E. Management of rash and other toxicities in patients treated with epidermal growth factor receptor-targeted agents. Clin Colorectal Cancer Nov;2005 5(Suppl 2):S101. [PubMed: 16336749]
29. Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, Niederwieser D, Resta D, Capdeville R, Zoellner U, Talpaz M, Druker B, Goldman J, O'Brien SG, Russell N, Fischer T, Ottmann O, Cony-Makhoul P, Facon T, Stone R, Miller C, Tallman M, Brown R, Schuster M, Loughran T, Gratwohl A, Mandelli F, Saglio G, Lazzarino M, Russo D, Baccarani M, Morra E. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med Feb 28;2002 346:645. [PubMed: 11870241]
30. Janne PA, Johnson BE. Effect of Epidermal Growth Factor Receptor Tyrosine Kinase Domain Mutations on the Outcome of Patients with Non-Small Cell Lung Cancer Treated with Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors. Clin Cancer Res July 15;2006 12:4416s. 2006. [PubMed: 16857820]
31. Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. Cell Jun 16;2006 125:1137. [PubMed: 16777603]
32. Qiu C, Tarrant MK, Choi SH, Sathyamurthy A, Bose R, Banjade S, Pal A, Bornmann WG, Lemmon MA, Cole PA, Leahy DJ. Mechanism of activation and inhibition of the HER4/ErbB4 kinase. Structure Mar;2008 16:460. [PubMed: 18334220]
33. Burris HA 3rd, Hurwitz HI, Dees EC, Dowlati A, Blackwell KL, O'Neil B, Marcom PK, Ellis MJ, Overmoyer B, Jones SF, Harris JL, Smith DA, Koch KM, Stead A, Mangum S, Spector NL. Phase I safety, pharmacokinetics, and clinical activity study of lapatinib (GW572016), a reversible dual inhibitor of epidermal growth factor receptor tyrosine kinases, in heavily pretreated patients with metastatic carcinomas. J Clin Oncol Aug 10;2005 23:5305. [PubMed: 15955900]
34. Burris HA 3rd, Taylor CW, Jones SF, Koch KM, Versola MJ, Arya N, Fleming RA, Smith DA, Pandite L, Spector N, Wilding G. A phase I and pharmacokinetic study of oral lapatinib administered once or twice daily in patients with solid malignancies. Clin Cancer Res Nov 1;2009 15:6702. [PubMed: 19825948]
35. Fujita T, Doihara H, Kawasaki K, Takabatake D, Takahashi H, Washio K, Tsukuda K, Ogasawara Y, Shimizu N. PTEN activity could be a predictive marker of trastuzumab efficacy in the treatment of ErbB2-overexpressing breast cancer. Br J Cancer Jan 30;2006 94:247. [PubMed: 16404430]
36. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, Klos KS, Li P, Monia BP, Nguyen NT, Hortobagyi GN, Hung MC, Yu D. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell Aug;2004 6:117. [PubMed: 15324695]
37. Johnston S, Trudeau M, Kaufman B, Boussen H, Blackwell K, LoRusso P, Lombardi DP, Ben Ahmed S, Citrin DL, DeSilvio ML, Harris J, Westlund RE, Salazar V, Zaks TZ, Spector NL. Phase II study of predictive biomarker profiles for response targeting human epidermal growth factor receptor 2 (HER-2) in advanced inflammatory breast cancer with lapatinib monotherapy. J Clin Oncol Mar 1;2008 26:1066. [PubMed: 18212337]
38. Xia W, Husain I, Liu L, Bacus S, Saini S, Spohn J, Pry K, Westlund R, Stein SH, Spector NL. Lapatinib antitumor activity is not dependent upon phosphatase and tensin homologue deleted on chromosome 10 in ErbB2-overexpressing breast cancers. Cancer Res Feb 1;2007 67:1170. [PubMed: 17283152]
39. Moasser MM, Srethapakdi M, Sachar KS, Kraker AJ, Rosen N. Inhibition of Src Kinases by a Selective Tyrosine Kinase Inhibitor Causes Mitotic Arrest. Cancer Res December 1;1999 59:6145. 1999. [PubMed: 10626805]
40. Amin DN, Hida K, Bielenberg DR, Klagsburn M. Tumor endothelial cells express epidermal growth factor receptor (EGFR) but not ErbB3 and are responsive to EGF and to EGFR kinase inhibitors. Cancer Res 2006;66:2173. [PubMed: 16489018]

41. Kohn AD, Barthel A, Kovacina KS, Boge A, Wallach B, Summers SA, Birnbaum MJ, Scott PH, Lawrence JC Jr. Roth RA. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. J Biol Chem May 8;1998 273:11937. [PubMed: 9565622]

42. Mirza AM, Kohn AD, Roth RA, McMahon M. Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. Cell Growth Differ Jun;2000 11:279. [PubMed: 10910095]

43. Hsieh S, Tobien T, Koch K, Dunn J. Increasing throughput of parallel on-line extraction liquid chromatography/electrospray ionization tandem mass spectrometry system for GLP quantitative bioanalysis in drug development. Rapid Commun Mass Spectrom 2004;18:285. [PubMed: 14755613]

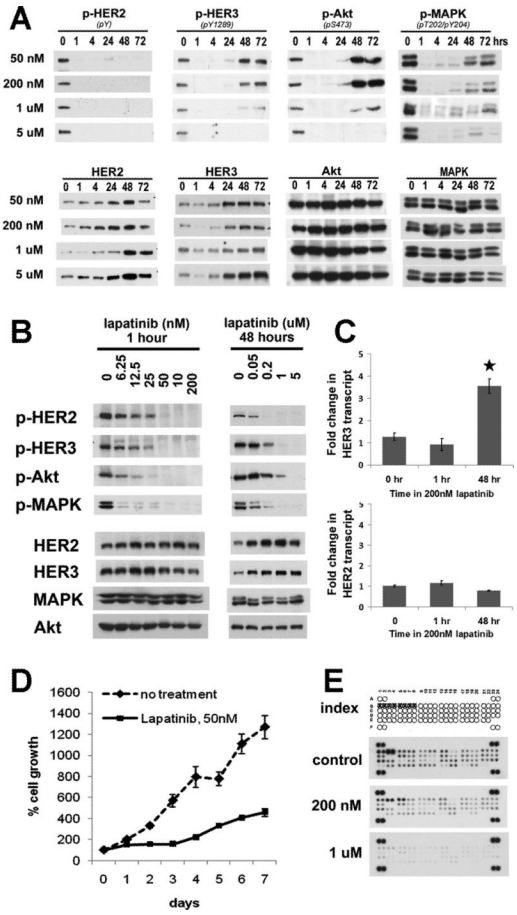

Fig. 1. The durability and dose-dependency of lapatinib effects on HER signaling

A) SkBr3 cells were treated with the indicated concentrations of lapatinib for the indicated lengths of time (in hours). For treatments longer than 24 hours, the media was replaced with fresh media containing fresh drug every 24 hours. Total cell lysates were immunoblotted as indicated. B) SkBr3 cells were treated with the indicated concentrations of lapatinib for one hour or for 48 hours. Cell lysates were immunoblotted as indicated. C) SkBr3 cells were treated for the indicated times with 200nM lapatinib, and the relative expression of HER2 and HER3 mRNA was evaluated by real-time RT-PCR analysis of total cellular RNA with HER2 and HER3-specific primers and normalization with β-microglobulin. Results are expressed as fold change and are the average of triplicates. Error bars represent SEM. *, significant induction compared with 0 hours ($p=0.004$). D) SkBr3 cells were placed in media containing 50nM lapatinib or vehicle and the number of cells counted daily. The media was replaced every 24 hours. The data are shown as a percentage of the starting cell count. Results are the average of triplicates and error bars represent SEM E) SkBr3 cells were treated with 200nM or 1uM lapatinib for 48 hours. Cell lysates were assayed with a phospho-RTK antibody array identifying activated receptor tyrosine kinases. Each RTK is spotted in duplicate on a membrane, and the location of the duplicate EGFR, HER2, HER3, and HER4 spots, in this order, are indicated by X in the index. The exposures have been normalized such that the positive control spots in the four corners have identical intensities.

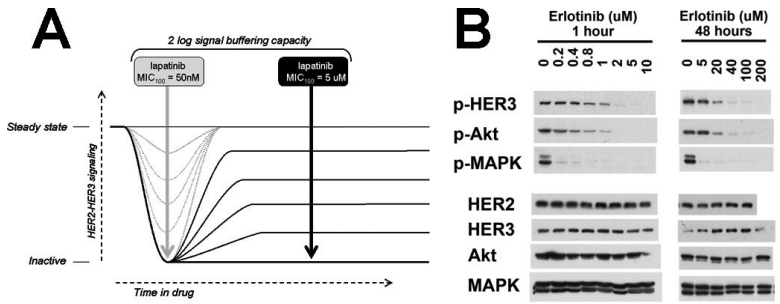

Fig. 2. Signal buffering capacity in the HER2-HER3 dimer

A) Schematic diagram depicting how the transient inhibition of HER2-HER3 signaling and its sustained inhibition exhibit different sensitivities to lapatinib. The two-log difference in the minimal inhibitory concentration required for full inactivation (MIC_{100}) reflects a two-log signal buffering capacity in HER2-HER3 signaling. B) SkBr3 cells were treated with the indicated concentrations of erlotinib for one hour or for 48 hours. Cell lysates were immunoblotted as indicated.

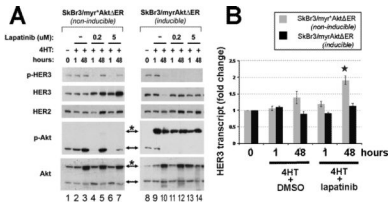

Fig. 3. Akt underlies HER3 signal buffering and protection against lapatinib

A) SkBr3/ myrAktΔER and SkBr3/ myr*AktΔER cells were treated with 4-hydroxy tamoxifen (4HT) to induce the activation of the engineered Akt construct and lapatinib at 0.2 or 5 μM for the indicated times. Cell lysates were immunoblotted as indicated. Arrows, endogenous cellular Akt; starred arrows, the larger engineered Akt-ΔER fusion proteins. B) SkBr3/ myr*AktΔER and SkBr3/ myrAktΔER cells were treated with 4HT to induce the activation of the engineered Akt construct and 200nM lapatinib for the indicated times (in hours). Cellular RNA was extracted and the relative expression of HER3 mRNA was assayed by real-time quantitative PCR with HER3 specific primers. GAPDH was used as normalization control. The results shown are the average of triplicates, and error bars represent SEM. Data are relative expression with values from untreated cells set as 1. * indicates significantly different from the adjacent induced data point (p=0.005), and significantly different from the 0 timepoint (p=0.01).

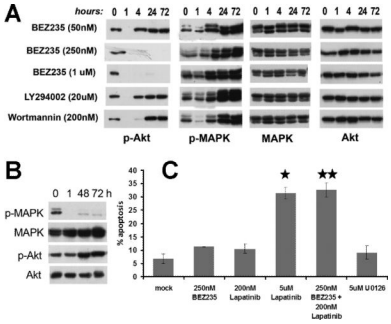

Fig. 4. Feedback and cross-talk in the MAPK and Akt pathways

A) SkBr3 cells were treated with the indicated concentrations of the specified PI3K inhibitors for the indicated times. Cell lysates were immunoblotted as indicated. B) SkBr3 cells were treated with 5 uM U0126 for the indicated durations of time. Cell lysates were immunoblotted as indicated. C) SkBr3 cells were treated with the indicated drugs for 72 hours, and the fraction of apoptotic cells quantified by FACS analysis of DNA degradation. * (p=0.0003) and ** (p=0.0006) indicate the significant induction of apoptosis compared with the mock treatment arm. BE, BEZ235; LY, LY294002; W, Wortmannin

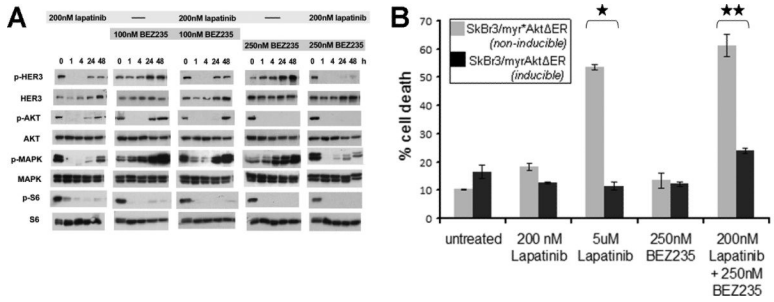

Fig. 5. Effects of lapatinib and BEZ235 alone and in combination on signaling

A) SkBr3 cells were treated with 200nM lapatinib or two concentrations of BEZ235 as single agents or in combination for the indicated durations of time. Cell lysates were immunoblotted as indicated. B) SkBr3/myrAktΔER and SkBr3/ myr*AktΔER cells were pre-treated with 4-hydroxy tamoxifen followed by treatment with the indicated concentrations of lapatinib or BEZ235 as single agents or in combination for 72 hours, and the fraction of apoptotic cells quantified by FACS analysis of DNA degradation. Significant protection from apoptosis by Akt induction is shown by * (p=0.00005) and ** (p=0.05). BE, BEZ235; LY, LY294002.

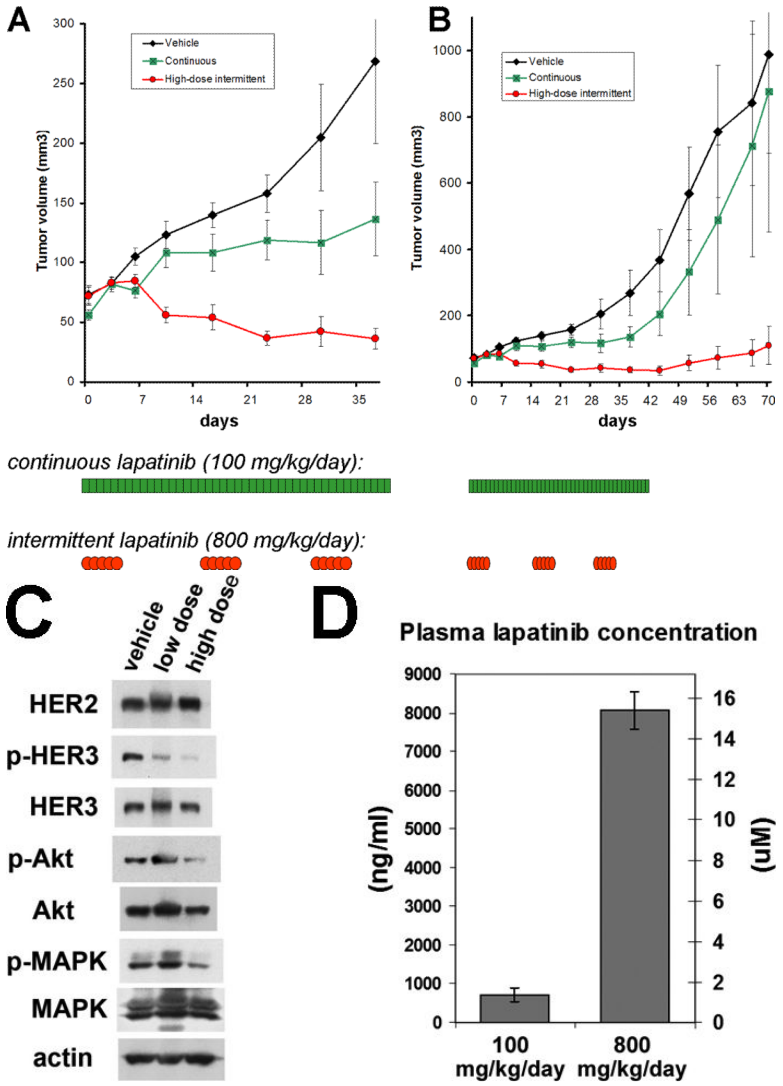

Fig. 6. Inactivation of HER2-HER3 signaling by high dose lapatinib

A) HCC1569 cells were orthotopically grown in the mammary fat pad of nude mice. When the average tumor sizes reached approximately 100 mm^3, mice were randomized to three groups of 10 mice each. One group was treated with lapatinib at 100mg/kg/day every day for 42 days. This is the maximum tolerated dose of lapatinib in continuous dosing. A second group was treated with lapatinib at 800mg/kg/day for a 5-day cycle followed by a 9-day off-treatment period for a total of three cycles. This is the maximum tolerated dose of lapatinib in this intermittent schedule. All treatments were administered by oral gavage in divided twice-daily dosing. Mice in the control arm were treated daily with vehicle. Tumor measurements were taken once or twice weekly. The treatment days for the continuous and high-dose intermittent arms are indicated below the graphs. Results shown are averages with the error bars indicating SEM. B) After completion of the 42-day treatment period, these mice were followed for another 30 days and tumor re-growth was measured and followed. Results shown are averages with the error bars indicating SEM. C) HCC1569 tumors from mice treated with the vehicle, 100mg/kg/day (low dose), or 800mg/kg/day (high dose) were resected 4 hours after the morning dose of the 5^{th} day of treatment and lysates prepared from frozen tissues. The lysates were subjected to immunoblotting as indicated. D) An additional seven mice bearing HCC1569 tumors were treated with vehicle or lapatinib at 100 mg/kg/

day or 800 mg/kg/day and plasma lapatinib concentrations were assessed 4 hours after the morning dose of the 5th day. Results represent average of replicates.