

# Resiliency and Vulnerability in the HER2-HER3 Tumorigenic Driver

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About 25% of breast cancers harbor the amplified oncogene human epidermal growth factor receptor 2 (HER2) and are dependent on HER2 kinase function, identifying HER2 as a vulnerable target for therapy. However, HER2-HER3 signaling 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 have shown that the compensatory buffering prevents apoptotic tumor cell death from occurring as a result of the combined loss of mitogen-activated protein kinase (MAPK) and Akt signaling. To overcome the cancer cells' compensatory mechanisms, we coadministered a phosphoinositide 3-kinase-mammalian target of rapamycin inhibitor and a HER2 tyrosine kinase inhibitor (TKI). This treatment strategy proved equivocal because it induced both TKI-sensitizing and TKI-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 little toxicity. This strategy for inactivation of HER2-HER3 tumorigenic activity is proposed for clinical testing.

## INTRODUCTION

A subset of human cancers is characterized by amplification and overexpression of the human epidermal growth factor receptor 2 (HER2) oncogene. HER2 has been best studied in the context of breast cancers, where it accounts for ~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 metastatic 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 (RTKs) composed 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 C-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 principal 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. HER2 is the

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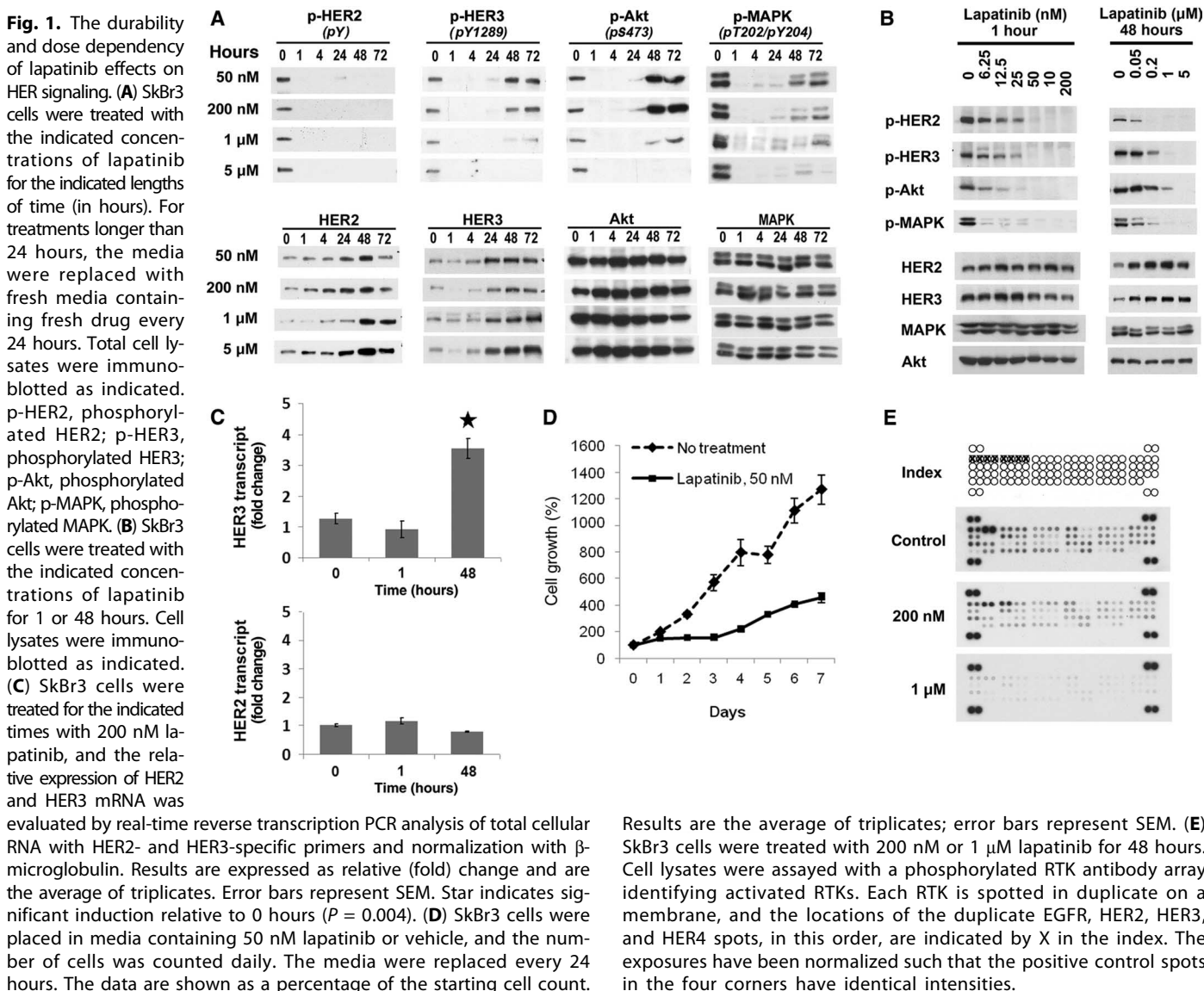
preferred dimerization partner for most other family members (12, 13). On the other hand, although HER3 can bind ligand, the HER3 kinase domain lacks catalytic activity, and HER3 depends on a heterodimeric partner for signaling (14, 15). The functions of HER2 and HER3 are complementary to one another, 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 cannot transform cells by itself, its coexpression synergistically enhances HER2 transformation (16, 17). 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 relation 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.

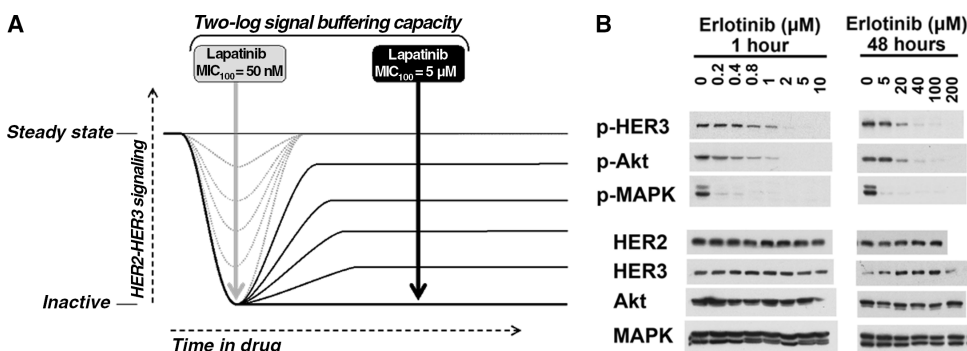
## RESULTS

Lapatinib is a reversible TKI that inhibits the EGFR and HER2 enzymes with in vitro  $IC_{50}$ s (median inhibitory concentrations) of

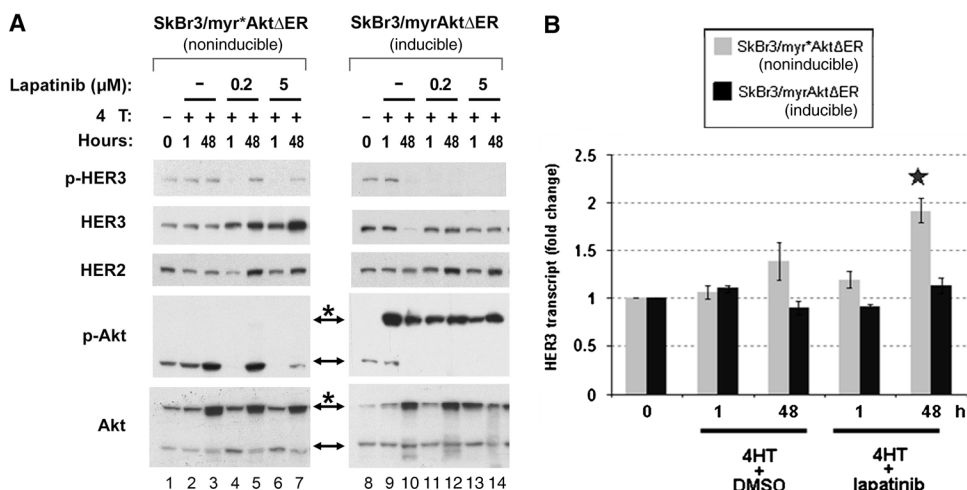


~10 nM (21). Lapatinib is selective for the HER family and has little interaction with the entire rest of the human kinome, making it an ideal agent to interrogate HER family signaling with minimal impact from off-target effects (22). Treatment of HER2-amplified human breast cancer cells with lapatinib initially induces a potent and rapid inactivation of HER2-HER3 and downstream Akt and mitogen-activated protein kinase (MAPK) signaling with inhibition complete at 50 nM (Fig. 1, A and B). HER2-HER3 ultimately escapes inhibition when the drug is at these nanomolar concentration ranges, although durable inhibition of HER2-HER3 signaling occurs at ~5 μM (Fig. 1, A

and B). When downstream phosphorylated Akt and MAPK pathway signaling recovers after ~48 hours at the lower dose of lapatinib, the amount of HER2 and HER3 protein is also increased (Fig. 1A). The up-regulation of HER3 is, at least in part, due to its transcriptional up-regulation (Fig. 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 (Fig. 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 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 (Fig. 1E).



**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 1 or 48 hours. Cell lysates were immunoblotted as indicated.



**Fig. 3.** Akt underlies HER3 signal buffering and protection against lapatinib. (A) SkBr3/myrAkt $\Delta$ ER and SkBr3/myr\*Akt $\Delta$ ER cells were treated with 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, larger engineered Akt $\Delta$ ER fusion proteins. (B) SkBr3/myr\*Akt $\Delta$ ER and SkBr3/myrAkt $\Delta$ ER cells were treated with 4HT to induce the activation of the engineered Akt construct and 200 nM 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. Star indicates significant difference from the adjacent induced data point ( $P = 0.005$ ) and from the 0 time point ( $P = 0.01$ ). DMSO, dimethyl sulfoxide.

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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 Fig. 2). This buffering capacity is not inherent in the HER2 tyrosine kinase enzyme because, 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 (Fig. 2B). HER2-HER3 signaling is similarly buffered in other HER2-amplified breast cancer cells (Fig. S1).

To test whether Akt was the driver of HER2-HER3 signal buffering, we engineered SkBr3 cells expressing myrAkt $\Delta$ ER, a 4-hydroxy tamoxifen (4HT)-inducible form of Akt, and the relevant noninducible control (myr\*Akt $\Delta$ ER, described in Materials and Methods). The myr\*Akt $\Delta$ ER fusion product (Fig. 3A, left panel) is not activated by 4HT because of the mutated myristylation domain, whereas the myrAkt $\Delta$ ER fusion product (Fig. 3A, right panel) is activated rapidly after 4HT treatment. 4HT-induced activation of Akt in SkBr3/myrAkt $\Delta$ ER cells leads to down-regulation of HER3 protein abundance, revealing that the steady-state HER3 signaling is under Akt control (Fig. 3A, lanes 8 to 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 (Fig. 3A, compare lanes

4 to 7 with lanes 11 to 14). The lapatinib induction of HER3 expression is prevented by activated Akt (lanes 12 and 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 up-regulation of HER2 is not mediated through Akt (Fig. 3A, compare lanes 4 to 7 with lanes 11 to 14). This up-regulation 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 posttranscriptional mechanisms. Although the suppression of steady-state HER3 protein concentrations with the 4HT-induced activation of Akt (Fig. 3A, lanes 1 to 3 and 8 to 10) is not associated with reduction of HER3 mRNA expression (Fig. 3B, 4HT/DMSO), the prevention of the lapatinib-induced increase in HER3 protein (Fig. 3A, lanes 4 to 8 and 11 to 14) is associated with the prevention of the lapatinib-induced increase in HER3 mRNA expression (Fig. 3B, 4HT/lapatinib).

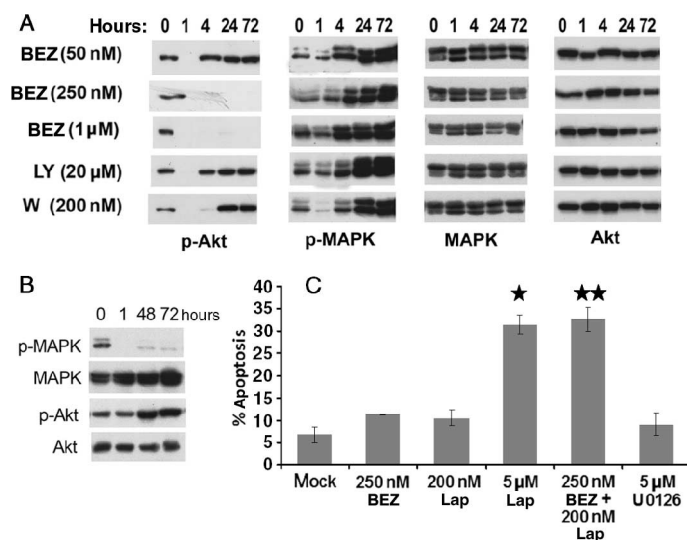
Because HER3 promotes downstream signaling predominantly through activation of phosphoinositide 3-kinase (PI3K), more effective inactivation of HER2-HER3 signaling could possibly be achieved by the addition of a PI3K or PI3K-mammalian target of rapamycin (mTOR) inhibitor to HER2 TKI treatment. Although 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 (Fig. 4A). This feedback signaling can be overcome at 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 MAPK kinase inhibitor U0126 similarly up-regulates Akt signaling (Fig. 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 200 nM lapatinib and 250 nM BEZ235 induces tumor cell apoptosis (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 5, A and 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 (Fig. 5A). This is also seen in BT474 cells treated with BEZ235 (Fig. S2). 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 (Fig. 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. Because 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  $\mu$ M and overcomes HER2-HER3 signal buffering, inducing a durable suppression of MAPK and PI3K-Akt pathways despite causing maximal up-regulation of HER3 (Fig. 1A) and apoptotic tumor cell death (Fig. 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 because of 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.



**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  $\mu$ M 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 was quantified by FACS analysis of DNA degradation. \* $P = 0.0003$ , \*\* $P = 0.0006$ , significant induction of apoptosis relative to the mock treatment arm. BEZ, BEZ235; LY, LY294002; W, wortmannin; Lap, lapatinib.

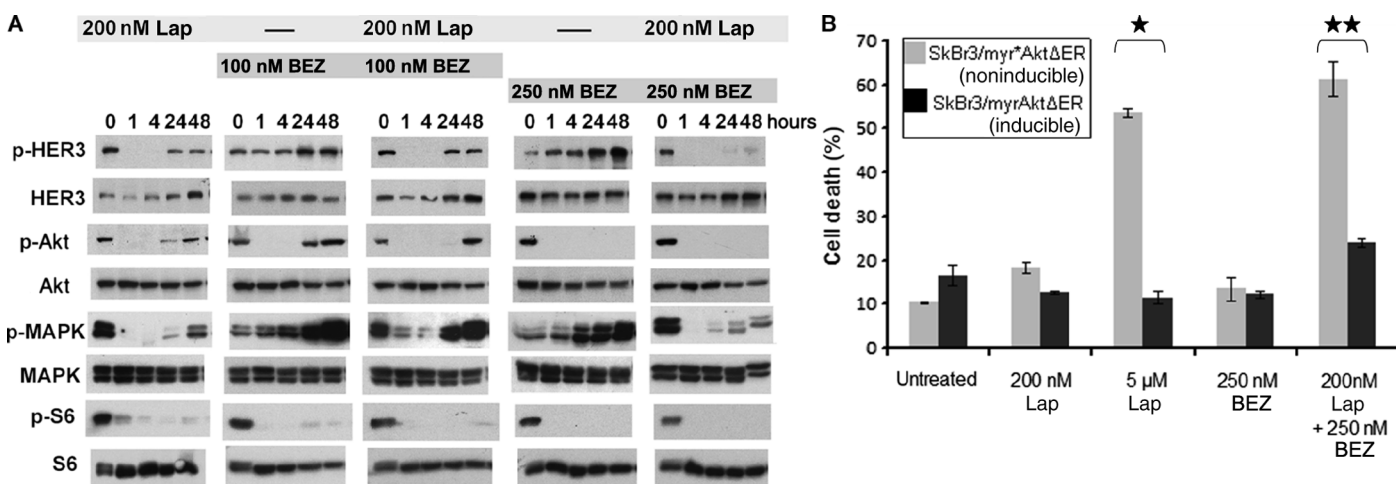
Because HER2-inactivating TKI therapy is expected to induce potent tumor apoptosis, the intermittent administration of TKI might be expected to induce significant antitumor effects when given in repeated cycles. To test this treatment hypothesis, we determined the maximum tolerated dose (MTD) of lapatinib given as a 5-day course repeated every 2 weeks in mice. The MTD was 800 mg/kg per day, which is higher (by a factor of 8) than the MTD of lapatinib administered continuously. In mice bearing HCC1569 HER2-amplified tumors, treatment with lapatinib at the continuous dosing MTD of 100 mg/kg per day produced a relative antitumor growth-inhibitory effect but no actual tumor regression, and tumor growth resumed after cessation of therapy (Fig. 6, A and B). However, treatment with 800 mg/kg per day in an intermittent schedule produced an immediate tumor regression and a much more long-lasting antitumor effect (Fig. 6, A and B). Treatment at the high dose was associated with transient weight loss that is entirely regained during the off-cycle period (fig. S3) and better target inactivation (Fig. 6C). The higher 800 mg/kg per day dose of lapatinib in these mice led to a proportionally higher steady-state plasma lapatinib concentration than the 100 mg/kg per day dose (Fig. 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 can eradicate the disease (29). TKIs that target the EGFR oncoprotein produce response rates of 60 to 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). Because 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 antitumor 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 relation 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), whereas the HER2 kinase domain is an optimized catalytic kinase partner, evidenced by its robust catalytic function relative to other members (12). The HER3 kinase allosterically activates the HER2 kinase, whereas 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 two-log inhibition of HER2 catalytic function. This buffering can increase either the expression or



**Fig. 5.** Effects of lapatinib and BEZ235 alone and in combination on signaling. **(A)** SkBr3 cells were treated with 200 nM 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/myrAkt $\Delta$ ER and SkBr3/myr\*Akt $\Delta$ ER cells were pretreated with

4HT 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 was quantified by FACS analysis of DNA degradation. \* $P = 0.00005$ , \*\* $P = 0.05$ , significant protection from apoptosis by Akt induction.

the phosphorylation of HER3 and is driven by a downstream network topology that functions to preserve Akt signaling.

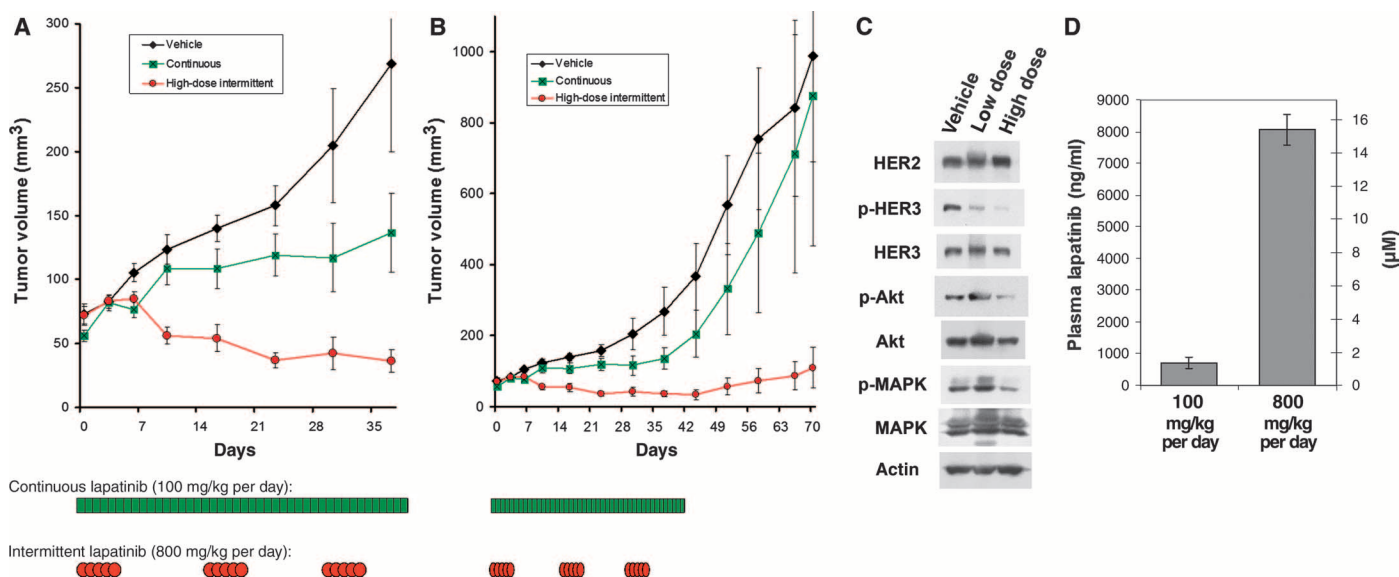
Next-generation approaches in development include newer agents to specifically interfere with HER2-HER3 transactivation or with HER3 functions. Another approach to the inhibition of HER3 signaling is the simultaneous inhibition of PI3K, the immediate effector of phosphorylated HER3. Our data show that 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 prevents their administration at inactivating doses on continuous schedules, we have shown here in mice the feasibility of an alternative strategy that uses 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 antitumor 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 antitumor 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 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 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



**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  $\sim 100$  mm<sup>3</sup>, mice were randomized to three groups of 10 mice each. One group was treated with lapatinib at 100 mg/kg per day every day for 42 days. This is the MTD of lapatinib in continuous dosing. A second group was treated with lapatinib at 800 mg/kg per day for a 5-day cycle followed by a 9-day off-treatment period for a total of three cycles. This is the MTD 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 regrowth was measured and followed. Results shown are averages with the error bars indicating SEM. (C) HCC1569 tumors from mice treated with the vehicle, 100 mg/kg per day (low dose), or 800 mg/kg per day (high dose) were resected 4 hours after the morning dose of the fifth day of treatment and lysates were 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 or 800 mg/kg per day, and plasma lapatinib concentrations were assessed 4 hours after the morning dose of the fifth day. Results represent average of replicates.

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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.

## MATERIALS AND METHODS

SkBr3 cells were obtained from the American Type Culture Collection and maintained at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium–Ham's F12 (1:1) media supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 4 mM L-glutamine. LY29004, wortmannin, and 4HT were from EMD-Calbiochem. All compounds were reconstituted in dimethyl sulfoxide, and 4HT was reconstituted in ethanol. BEZ235 was obtained from Novartis. Lapatinib and erlotinib were purchased as tablets, and the active ingredient was purified by organic extraction as described in the Supplementary Material. In all TKI treatment experiments extending longer than 24 hours, the media were 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 200 nM lapatinib or vehicle only. Media with fresh drugs were changed every 24 hours, and daily cell counts from triplicate wells were obtained for 7 days with a hemacytometer. 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, phenylmethylsulfonyl fluoride, sodium vanadate, and phosphatase inhibitor cocktail (Roche). Western blotting was performed by separating 50 µg of lysates on an SDS–polyacrylamide gel electrophoresis, transferring to polyvinylidene fluoride membrane, and immunoblotting with the indicated antibodies followed by enhanced chemiluminescence visualization. Immunoprecipitation of HER2 was performed with antibody to HER2 (Santa Cruz Biotechnology). Antibodies used for Western blot analysis were against pY1248-HER2, pS473-Akt, pT202/Y204-MAPK, MAPK, Akt, pS235/236-S6, S6 ribosomal protein (Cell Signaling), β-actin, HER2, HER3 5A12, pTyr PY99 (Santa Cruz Biotechnology), and pT246-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 counterpurified over a nonphosphopeptide column. The phosphospecificity of the antibody was verified in cells treated with phosphorylated or nonphosphorylated

HER3 and treated with heregulin or lapatinib, or with overexpression of HER3.

Phosphorylated RTK profiling was done with the Proteome Profiler Phospho-RTK Antibody Array (R&D Systems) according to the manufacturer's instructions. Briefly, the array membranes were blocked in blocking buffer and incubated with diluted cell lysates overnight at 4°C. 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 was analyzed on FACSCalibur II with ModFit software. Apoptotic cells were identified by their sub-G<sub>1</sub> DNA content as analyzed by ModFit, and data were averaged over triplicate experiments.

Total cellular RNA was isolated with the RNeasy Miniprep kit with on-column DNase treatment as per the manufacturer's protocols (Qiagen). Reverse transcription and real-time polymerase chain reaction (PCR) amplification was performed as described with the iQ SYBR Green Supermix on an MyiQ iCycler (Bio-Rad) (40). Normalization was performed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-microglobulin, and relative expression was obtained with Pfaffl ratios. Data represent average from triplicates. The primers used were 5'-CCCTGCCATGAGAACTGCAC and 5'-TCACTGTCAAAGCCATTGTCAGAT for HER3, 5'-AACTGCACC-CACTCCTGTGT and 5'-TGATGAGGATCCCAAAGACC for HER2, 5'-GGTCTCCTCTGACTTCAACA and 5'-AGCCAAATTCGTTGT-CATAC for GAPDH, and 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 autoinhibited by the ER fragment. Treatment with the ER ligand 4HT relieves the autoinhibition, 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-myrAktΔER or the pWZLneo-myr\*AktΔER vectors and selected in neomycin.

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

Mouse experiments were done under an Institutional Animal Care and Use Committee–approved protocol. Briefly, 2 × 10<sup>6</sup> HCC1569 cells were orthotopically implanted into the mammary fat pad of 7- to 9-week-old female *nu/nu* mice and allowed to grow into tumors. When tumors reached ~100 mm<sup>3</sup>, mice were randomized and treated according to the experimental arms. Lapatinib was administered as a suspension in 0.5% hydroxypropylmethylcellulose and 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 killed 4 hours after the morning dose of the fifth day, the tumors were rapidly dissected and flash-frozen, and the plasma was collected and frozen. Tumor lysates were prepared in RIPA buffer and Western blotting was performed as described. Plasma lapatinib concentrations were analyzed with a previously described method (43), with a sensitivity of 1 ng/ml and a precision and accuracy within 15%.

## SUPPLEMENTARY MATERIAL

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Fig. S1. The HER2-amplified HCC1569 and BT474 breast cancer cells were treated with 200 nM lapatinib for the indicated durations of time.

Fig. S2. The HER2-amplified BT474 cells were treated with BEZ235 for the indicated time points, and cell lysates were immunoblotted with the indicated antibodies.

Fig. S3. Body weight changes during lapatinib therapy in mice bearing orthotopic HCC1569 tumors.

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## Resiliency and Vulnerability in the HER2-HER3 Tumorigenic Driver

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### How to Outsmart Breast Cancer

Patients with breast cancer enrolled in recent clinical trials of a drug called lapatinib had reason to be optimistic. The growth and metastasis of many breast cancers depend critically on the target of this drug, the Erb receptor human epidermal growth factor 2 (HER2), and it made sense that its inhibition would hobble the cancer's ability to survive. But some of these patients were ultimately disappointed as only a fraction of cancers responded to the drug, and those responses tended to be partial and transient. New work by Amin *et al.* in human breast cancer cells tests alternative treatment strategies and suggests that one of these might outwit these cancers.

In certain breast tumors, the protein kinase activity of HER2, which is blocked by lapatinib, signals to downstream targets that cause cancer. One of these targets is another member of the same family, HER3, which can bind ligand but does not have catalytic activity of its own, and which in turn activates phosphoinositide 3-kinase (PI3K)–Akt signaling. In previous work, Amin and colleagues showed in human breast cancer cells that drug-induced altered regulation of HER3 through feedback from Akt is responsible for allowing cell to escape the lethal effects of lapatinib. Here, they probe this effect further and try to find a way to bypass the cells' compensatory mechanism.

The first approach was to try to inhibit PI3K at the same time as HER2 tyrosine kinase, but this proved ineffective as these cells were also able to up-regulate the growth signaling pathways and bypass inhibition by this combined treatment. Next, they used much higher doses of lapatinib, which were in fact able to completely and durably extinguish HER2 activity, but which have the disadvantage of being very toxic in vivo. They found a way around this problem by giving these high doses to mice with HER2-dependent tumors on an intermittent schedule, periodically driving blood concentrations high enough to generate a wave of apoptosis in the tumor and effectively preventing growth.

The success of these authors in this skirmish with breast cancer marks a reason for renewed optimism in patients with HER2-dependent breast cancer. These second-generation approaches will need to be tested in the clinic, but the HER2-HER3 tumorigenic driver still seems to be an opponent keeping in our sites.

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