Coamplification of miR-4728 protects HER2-amplified breast cancers from targeted therapy

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HER2 (ERBB2) amplification is a driving oncogenic event in breast cancer. Clinical trials have consistently shown the benefit of HER2 inhibitors (HER2i) in treating patients with both local and advanced HER2+ breast cancer. Despite this benefit, their efficacy as single agents is limited, unlike the robust responses to other receptor tyrosine kinase inhibitors like EGFR inhibitors in EGFR-mutant lung cancer. Interestingly, the lack of HER2 efficacy occurs despite sufficient intracellular signaling shutdown following HER2i treatment. Exploring possible intrinsic causes for this lack of response, we uncovered remarkably depressed levels of NOXA, an endogenous inhibitor of the antiapoptotic MCL-1, in HER2-amplified breast cancer. Upon investigation of the mechanism leading to low NOXA, we identified a micro-RNA encoded in an intron of HER2, termed miR-4728, that targets the mRNA of the Estrogen Receptor α (ESR1). Reduced ESR1 expression in turn prevents ERα-mediated transcription of NOXA, mitigating apoptosis following treatment with the HER2i lapatinib. Importantly, resistance can be overcome with pharmacological inhibition of MCL-1. More generally, while many cancers like EGFR-mutant lung cancer are driven by activated kinases that when drugged lead to down-regulating the pro-apoptotic NOXA. Importantly, coinhibition of MCL-1 inhibitors overcomes this resistance.

NOXA | apoptosis | HER2 amplification | targeted therapies | MCL-1 inhibitor

Genetic amplification of the receptor tyrosine kinase (RTK) HER2 (ERBB2) is found in breast cancers with amplification of the 17q12-21 locus, which occurs in 20–25% of invasive breast cancers. It has been demonstrated that trastuzumab, a humanized monoclonal antibody targeting HER2, when combined with chemotherapy, significantly improves progression-free survival (PFS) (7.4 vs. 4.6 mo) and overall survival (OS) (25.1 vs. 20.3 mo) (1) in patients with HER2-amplified breast cancer. A number of studies have since demonstrated that adjuvant use of HER2 kinase inhibitors, such as lapatinib, improves outcomes in HER2-amplified breast cancers, including reduced recurrence rates and increased OS (2). Interestingly, despite these clear benefits, HER2 kinase inhibitors used as monotherapy have demonstrated only minor benefit, with objective response rates (ORRs) below 20% (3). In contrast, other RTK-driven cancers like EGFR-mutant lung cancers (4) and ALK-translocated lung cancers (5) have ORRs approaching 60–70%.

Laboratory studies have indicated that EGFR, ALK, and HER2 inhibitors have comparable ability to block the critical PI3K/mTOR and MEK/ERK pathways (6, 7). This suggests that the poorer response rates of HER2 inhibitor monotherapy relative to those of EGFR and ALK inhibitors are not due to decreased inhibition of key intracellular signaling. Further downstream, these pathways converge on the BCL-2 family of proteins, which govern the ability of the cell to undergo apoptosis (8). The degree of apoptosis induced by targeted therapies is critical and directly influences targeted therapy responses (9–15). For instance, in response to MEK/ERK pathway inhibition, all three of these RTK-driven cancer subtypes up-regulate the expression of the proapoptotic, BH3-only BCL-2 family member, BIM. Loss of BIM expression universally protects these cancers from kinase inhibitor-induced apoptosis (6, 9, 14).

Interestingly, it is now appreciated that cancers with lower basal expression of functional BIM, either due to genetic polymorphisms (12) or other less defined causes (9, 11), are deficient in HER2-amplified breast cancers, HER2 inhibitors have been very successful as adjuvant therapy but not as monotherapy. Here, we demonstrate that coamplification of a HER2 intrinsic microRNA causes intrinsic resistance to HER2 inhibitors by indirectly down-regulating the pro-apoptotic NOXA. Importantly, coinhibition with MCL-1 inhibitors overcomes this resistance.


Conflict of interest statement: J.D.L. and A.J.S. are employees and shareholders of AbbVie Inc. A.C.F. analyzed data; and K.V.F. and A.C.F. wrote the paper.

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in an apoptotic response following kinase inhibitor treatment, even in the presence of MEK/ERK (and PI3K/mTOR) signal shutdown. Clinical studies have now been designed to overcome these BIM-centric deficiencies, by adding apoptosis-inducing agents to sensitize cancers to kinase inhibitors (16, 17).

In our investigations, we made the unexpected discovery that levels of NOXA (encoded by PMAIP1) were markedly low in breast cancers. We subsequently found that this phenomenon is strongly enriched in HER2-amplified breast cancers. As NOXA acts primarily as an endogenous inhibitor of the antiapoptotic BCL-2 family member MCL-1, we hypothesized that NOXA deficiency serves as a universal apoptotic block in HER2-amplified breast cancers, which could explain the lack of clinical HER2 inhibitor monotherapy activity. In this work, we tested this hypothesis and investigated the cellular mechanism(s) underlying this phenomenon.

**Results**

**NOXA Is Uniformly Suppressed in HER2-Amplified Breast Cancer.** Using gene expression datasets of cancer cell lines to understand the landscape of BCL-2 family member expression in breast cancer, we found that, within the cancer cell line encyclopedia (CCLE), levels of mRNA encoding the proapoptotic protein NOXA (PMAIP1) were markedly reduced in breast cancer cells compared with other cancer cell subtypes (Fig. S1) (18). Parsing multiple datasets (19–25) in the Oncomine database (26) and the R2 genomics application (r2.amc.nl), we found that NOXA mRNA levels were remarkably and specifically depressed in the HER2-amplified subset of breast cancer (Fig. I4), while mRNA levels of other major proapoptotic BCL-2 family members were not depressed (Fig. S24). We also found an inverse correlation

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**Fig. 1.** NOXA (encoded by PMAIP1) is uniformly suppressed in HER2-amplified breast cancer. (A) Scatter plots comparing PMAIP1 mRNA levels in HER2-amplified versus non-HER2-amplified breast cancers from six tumor databases of breast cancers obtained from Oncomine (https://www.oncomine.com/resource/login.html, May 2017, Thermo Fisher Scientific) (TCGA dataset: HER2−, n = 194; HER2+, n = 67; Bonnefoi dataset: HER2−, n = 83; HER2+, n = 29; Kao dataset: HER2−, n = 252; HER2+, n = 75; Lu dataset: HER2−, n = 69; HER2+, n = 26; Richardson dataset: HER2−, n = 29; HER2+, n = 8; Chin dataset: HER2−, n = 71; HER2+, n = 8). Red lines represent means. Error bars indicate ± SEM. (B) Scatter plots of PMAIP1 mRNA expression against HER2 mRNA expression from three datasets obtained from the R2 genomics application (r2.amc.nl) (Black dataset: n = 107; Sotiriou dataset: n = 120; Iglehart dataset: n = 123). (C) HER2 (ERBB2) and NOXA (PMAIP1) mRNA expression correlation from 1,102 breast cancer tumors from TCGA. Red box indicates NOXA levels of high HER2 cancers. Yellow box indicates HER2 mRNA levels of tumors that have higher NOXA mRNA levels. P value was calculated using the Mann–Whitney U test (with P values < 0.05 equaling significance). (D) Untreated cells from a panel of HER2-amplified and TNBC cell lines were lysed and separated by SDS/PAGE, subjected to immunoblotting, and probed for HER2 or NOXA. GAPDH was used as a loading control.
between HER2 and NOXA mRNAs in breast cancer (Fig. 1B). Consistently, from 1,102 breast cancer specimens deposited in The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov/), we found a striking relationship between the highest HER2-expressing tumors and low NOXA expression (Fig. 1C). On the other hand, our analysis did not indicate a statistically significant relationship between MCL1 and HER2 mRNA (Fig. S2D). Furthermore, breast cancers determined to be HER2-positive (HER2+) by protein overexpression as measured by immunohistochemistry had significantly lower NOXA levels (Fig. S2B).

We next performed laboratory-based experiments to confirm the low levels of NOXA in HER2-amplified breast cancer and to understand the biological consequences of this deficiency. To investigate the expression levels of NOXA protein in HER2-amplified breast cancers, we interrogated whole-cell lysates from a panel of HER2-amplified and triple-negative breast cancer (TNBC) cell lines that were otherwise randomly selected. Consistently, the HER2-amplified breast cancer cell lines had markedly lower NOXA levels compared with the TNBC cell lines (Fig. 1D). Interestingly, BT-474 and MDA-MB-361 (HER2+)/ER+ cell lines (27), which have elevated ERα protein levels among the HER2+ cell lines (Fig. S2C), demonstrated higher NOXA levels than the (HER2+)/ER− lines but lower levels than the TNBC cell lines. In contrast to NOXA, the key antiapoptotic proteins MCL-1 and BCL-xL did not exhibit expression patterns associated with HER2 status (Fig. S2C). Of note, while MCL-1 and BCL-xL were both expressed similarly in HER2-amplified and TNBC cell lines, the antiapoptotic protein BCL-2 was lower in most HER2-amplified cell lines (Fig. S2C), consistent with the mRNA data (Fig. S2A).

**ER Status Affects NOXA Levels.** To investigate whether ER status also impacted NOXA levels, we interrogated TCGA breast tumors in the Oncomine database (26). The lowest levels of NOXA mRNA expression were observed in the ER−/HER2+ subset of tumors, implying an association between both HER2 and NOXA and ER and NOXA (Fig. S2A). Consistently, within HER2-amplified breast cancers, ER− breast cancers had substantially lower levels of NOXA than ER+ breast cancers (Fig. 2A). These data indicate that both HER2 status and ER status correlate with NOXA levels. ER has been reported to directly up-regulate NOXA mRNA levels (28). In a different set of ER+ breast cancers (29), ER+ breast cancers demonstrated markedly higher NOXA levels compared with ER− tumors (Fig. 2B). In addition, when 22 HER2+ and 88 luminal A subtype breast cancers (29) [the latter are typically ER+/HER2− (30)] were compared, there was a striking difference in NOXA expression (Fig. 2C) (29). To determine whether there is also functional evidence to support these findings, we analyzed gene expression data from a clinical trial of presurgical treatment with the aromatase inhibitor letrozole, which suppresses estrogen biosynthesis to inhibit ER (31). Strikingly, NOXA was among the most significantly altered genes, markedly down-regulated after 14 d of letrozole treatment compared with baseline among 58 primary ER+ breast tumors.
Coamplification of miR-4728 in HER2-Amplified Breast Cancer Leads to ER-Mediated Down-Regulation of NOXA. Our previous data showed a striking inverse relationship between HER2 and NOXA (Figs. 1 and 2 A and C). To gain insights into the mechanism that leads to NOXA deficiency in HER2-amplified breast cancers, we introduced siRNA targeting HER2 in three HER2-amplified breast cancer cell lines. Surprisingly, silencing HER2 did not affect NOXA mRNA levels (Fig. S3A). To further investigate a potential causative link between HER2 and NOXA, we analyzed the protein levels of NOXA in isogenic breast cancer cell lines with low (endogenous) vs. high (exogenous cDNA) HER2 (MCF7-GFP vs. MCF7-HER2) (32). Consistent with the siRNA analysis, we did not find that HER2 overexpression altered NOXA levels (Fig. S3B). We conclude that the expression of NOXA is independent of HER2 signaling. However, evidence in Figs. 1 and 2 suggested that NOXA expression is dependent on HER2 amplification and HER2 expression; thus, there seems to be a contradiction.

We then investigated what would explain this seeming contradiction. Another potential mechanism by which NOXA may be down-regulated is through expression of micro-RNAs (miRNAs) that target the gene. Analyzing TCGA data from 964 tumors, we found that the top correlating miRNA with HER2 mRNA, out of 1,626 miRNAs, is miR-4728, with a correlation coefficient of 0.7465 (Fig. 3A). These data led us to focus on miR-4728, which interestingly is located within intron 23 of the HER2 gene and, as such, could be processed from the same primary transcript (33). Two mature miRNAs are formed from the precursor miR-4728—miR-4728-5p and miR-4728-3p—with miR-4728-3p being more prominently expressed as a mature miRNA (34). As previously mentioned, HER2 drives breast cancer formation as a focal amplicon manifesting as 17q12-21 amplification (35). An example of the amplicon in a HER2-amplified breast cancer from TCGA is depicted in Fig. S4A, illustrating the coamplification of miR-4728. Consistently, we found that the expression levels of miR-4728-3p in our panel of HER2-amplified breast cancers are much higher compared with the TNBC cell lines (Fig. 3B).

Importantly, it has recently been reported that miR-4728 targets ERα (ESR1) mRNA (36, 37) and that ERα can function as a transcriptional factor for NOXA (28). Indeed, selective inhibition of ERα in BT-474 and MDA-MB-361 cells resulted in down-regulation of NOXA (Fig. S4B). Furthermore, our analyses indicated an inverse correlation between ER mRNA and miR-4728, which became apparent when we separated breast tumors by miR-4728 expression: The highest miR-4728 expressions had markedly low ER mRNA (Fig. 3C) as well as NOX4 mRNA (Fig. 3D). We therefore hypothesized that when HER2 is amplified in breast cancer, coamplification of miR-4728 leads to down-regulation of NOXA expression in breast cancer.

Fig. 3. miR-4728 is coamplified and strongly correlated with HER2, while NOXA (PMAIP1) and ERα (ESR1) are inversely correlated with miR-4728-3p in breast cancer. (A) HER2 (ERBB2) and miR-4728 (hsa-miR-4728) correlation over 964 breast cancer tumors from TCGA. The Spearman correlation coefficient (r) and the P value (using Mann–Whitney U test) were calculated. (B) Total RNA enriched with small RNAs was isolated from a panel of HER2-amplified and TNBC cell lines. MiRNAs were reverse-transcribed using a TaqMan miRNA assay and finally analyzed by qPCR. miR-26a-5p was used as an endogenous control, and the data were expressed as a relative value to the cell line expressing the highest levels of miR-4728-3p (UACC-893); n = 3; error bars are ±SD. (C) Scatter plots comparing the ESR1 mRNA levels in the top 5% miR-4728-3p expressors versus the next 5% miR-4728-3p expressors versus the lowest 90% miR-4728-3p expressors from data analyses of 964 breast tumor samples. For (C) and (D), the red lines are means and the error bars are ± SEM.

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of ER and NOXA, resulting in a mitigated response to HER2 inhibitors through the MCL-1 function.

We inhibited miR-4728-3p after transfection of MD-MB-361 and BT-474 cells with a specific pLV-hsa-miR-4728-3p locker plasmid or a scrambled vector control (38). As demonstrated in Fig. 4A, we observed a concomitant increase of estrogen receptor α (ERα), the dominant ER isoform in breast cancer (39), and of NOXA. Of note, the phosphorylation status of ERK served as a positive control for miR-4728 inhibition (38). In contrast, overexpression of miR-4728-3p in T47D (HER2+/ER+) and BT-474 (HER2+/ER+) cells was sufficient to decrease both ERα and NOXA (Fig. 4B). The increased expression levels of miR-4728-3p, in our overexpression models, were verified by qPCR analysis (Fig. S4C). Moreover, inhibition of ERα with a selective antagonist (fulvestrant) (40) mitigated the effect of pLV-hsa-miR-4728-3p locker plasmid on NOXA induction in MDA-MB-361 cells (Fig. 4C). We then tested whether inhibition of miR-4728 sensitizes HER2-amplified breast cancer cells to the HER2 inhibitor, lapatinib. Indeed, MDA-MB-361 as well as BT-474 cells transfected with the pLV-hsa-miR-4728-3p locker plasmid demonstrated markedly elevated cleaved PARP compared with the control
cells (Fig. 4 D and E), while overexpression of miR-4728 in BT-474 cells rescued lapatinib-treated cells from apoptosis (Fig. 4E). Altogether, these data demonstrate that miR-4728 amplification results in ER silencing, which in turn prevents expression of NOXA (28). Our model is depicted in Fig. 4F.

**Pharmacological Inhibition of MCL-1 Sensitizes HER2-Amplified Breast Cancer Cells to Lapatinib.** Altogether, our data indicate that amplification of miR-4728 plays a heretofore undiscovered role in miR-4728 amplified breast cancer and that deficient expression of the intrinsic MCL-1 inhibitor NOXA may lead to an underlying apoptotic block in HER2-amplified breast cancers. To determine whether NOXA expression confers sensitivity to HER2 inhibition, we first artificially increased the levels of NOXA by transducing viral particles containing a NOXA-expressing plasmid into HER2-amplified breast cancer cells, creating the cells with a time course of lapatinib (Fig. 5A). Consistent with our hypothesis, NOXA-expressing cells were markedly sensitized to lapatinib, compared with control cells, as evidenced by increased PARP cleavage in HCC-1419 and MDA-MB-453 cells (Fig. 5A), as well as in EFM-192A and...
MDA-MB-361 cells (Fig. S5A). Immunoprecipitation analysis of these lysates revealed that MCL-1:BIM and MCL-1:BAK complexes were reduced by NOXA overexpression, consistent with the understanding of NOXA as an endogenous MCL-1 inhibitor (41) (Fig. S5 B and C). To further confirm this effect of NOXA expression, we reduced MCL-1 expression with a siRNA targeting MCL-1 (15) or a siRNA with a scrambled sequence and treated HER2-amplified HCC-1419 and MDA-MB-453 breast cancer cells with lapatinib. As expected, we found that the cells treated with siRNA targeting MCL-1 were markedly sensitized to lapatinib compared with the scramble siRNA-treated cells (Fig. S5D). These data indicate that NOXA overexpression confers sensitivity to HER2 inhibitors in HER2-amplified breast cancers.

Due to the emerging role of MCL-1 in cancer development, progression, and cell survival (42), MCL-1 inhibitors are being heavily pursued as anticancer agents. A1210477 is a BH3 mimetic that has been described to bind with high affinity to MCL-1 and selectively disrupts the interaction between proapoptotic BIM and MCL-1 (43). We therefore tested A1210477 in combination with lapatinib. Our data revealed that the addition of A1210477 effectively reduced the apoptotic threshold and markedly sensitized HER2-amplified breast cancer cells to lapatinib, as evidenced by cleaved PARP induction (Fig. S6A). The increased amount of apoptosis from the combination of lapatinib and A1210477 translated to enhanced sensitivity in 72-h cell viability assays (Fig. S6B). To further confirm the mode of activity of A1210477, we treated MDA-MB-361 breast cancer cell lines with lapatinib, A1210477, or the combination and evaluated the status of the major intracellular signaling pathways downstream of HER2 (6, 44). As expected, A1210477 did not alter the PI3K/mTOR or MEK/ERK pathways (Fig. S7), indicating that its sensitizing effects were specific to MCL-1 inhibition.

To better understand the mechanism linking sensitization of the HER2-amplified breast cancer cells to A1210477, we immunoprecipitated MCL-1 complexes in HCC-1419 and MDA-MB-453 cells. As expected, addition of A1210477 disrupted the interaction between MCL-1 and BIM (45) (Fig. S8A), releasing BIM to activate the effector molecules BAX and BAK and induce apoptosis (46–50). Furthermore, it is worth noting that lapatinib and A1210477 increased BIM and MCL-1 levels, respectively, consistent with previous reports (43, 51). The consequence of these increases was, at least in part, the accumulation of MCL-1:BAK complexes (Fig. S8B). To further study the roles of BIM and BAK in lapatinib/A1210477-mediated apoptosis, we stably knocked down BAK (shB4AK) in the same HER2-amplified breast cancer cell lines (HCC-1419 and MDA-MB-453) and silenced BIM with siRNA. In both cell lines, there was a clear requirement for both BIM and BAK in lapatinib/A1210477-mediated cell death, with the HCC-1419 cells particularly reliant on BAK (Fig. S8B).

The MCL-1 Inhibitor S63845 Synergizes with Lapatinib by Disrupting both MCL-1:BAK and MCL-1:BIM Complexes in HER2-Amplified Breast Cancers. The addition of A1210477 to lapatinib appeared effective because low NOXA caused the increase in MCL-1:BIM complexes, and A1210477 disrupted these complexes, therefore increasing the amount of liberated BIM. This is a critical step in apoptosis caused by kinase inhibitors and BCL-2 family inhibitors (8, 9, 15, 52–55). However, A1210477 did not robustly disrupt MCL-1:BAK complex formation (Fig. S8A). The new selective MCL-1 inhibitor, S63845, reportedly disrupts both BIM:MCL-1 and BAK:MCL-1 complexes (45), which may be a result of higher affinity for binding to the MCL-1 hydrophobic pocket than A1210477 (56). We first combined lapatinib with S63845 and found that the combination potently induced apoptosis as evidenced by cleaved PARP (Fig. 5B) and FACS analysis of annexin V-stained cells (Fig. S9A). Additionally, the combined treatment reduced viable cell numbers in HER2-amplified breast cancer cells (Fig. S9B). Intriguingly, in some of the cell lines (BT-474 and UACC-893), the combination of the two drugs displayed remarkable synergy, leading to robust cell death in less than 24 h (Fig. 5B and Fig. S9B). To ensure that S63845 functions exclusively through MCL-1 inhibition, we immunoprecipitated BIM complexes in both BT-474 and UACC-893 cells and found that addition of S63845 disrupts only the BIM:MCL-1 and not the BIM:BCL-xL or the BIM:BCL-2 complex (Fig. S10A). As in the case of A1210477, S63845 leaves PI3K/mTOR and MEK/ERK signaling unperturbed (Fig. S10B), confirming previously published data (57).

We next examined the requirement for BIM and BAK in lapatinib-, S63845-, and S63845/lapatinib-mediated toxicity in the HER2-amplified HCC-1419 and MDA-MB-453 cells. Similar to the data with A1210477, knockdown of both BIM and BAK substantially mitigated drug-induced toxicity in the cells, with knockdown of BAK alone markedly protecting HCC1419 cells (Fig. 5C). Consistently, knockdown of BAK and BIM translated to significantly increased cell viability following treatment compared with controls (Fig. S11A). We next investigated any involvement of BAX in drug-induced toxicity. Knockdown of BAX expression with shBAX also protected cells from MCL-1/- lapatinib-induced toxicity, albeit to a lesser degree than BAK (Fig. S11B). Interestingly, the protective effects of BAX and BIM appear redundant, as knockdown of BIM in the presence of knockdown of BAX did not markedly increase the amount of protection in either cell line (Fig. S11B). Using specific antibodies that recognize the active conformation of BAX or BAX (clone AB-1 for BAX and clone 6A7 for BAX, respectively) (58, 59), we immunoprecipitated lysates from HCC-1419 and MDA-MB-453 cells treated overnight and individually with lapatinib, A1210477, and S63845 or the combinations of lapatinib/A1210477 and lapatinib/S63845, with the AB-1 and 6A7 antibodies. Consistently, we detected levels of both active BAK and BAX following drug exposure that were generally much more pronounced in the combination treatments compared with single agents (Fig. S5D and Fig. S11C).

To verify that both MCL-1:BIM and MCL-1:BAK complexes were disrupted by S63845, we immunoprecipitated MCL-1 complexes in lysates of the HER2-amplified breast cancer cells, HCC-1419 and MDA-MB-453, following treatment with lapatinib, S63845 (at two concentrations of 300 nM and 1 μM), or their combination (Fig. 5E). Immunoprecipitation complex investigation confirmed that both MCL-1:BIM and MCL-1:BAK complexes were disrupted following treatment with 1 μM of S63845. These data imply that S63845-induced apoptosis involves disruption of BIM:MCL-1 and BAK:MCL-1 complexes in HER2-amplified breast cancer cells.

We next analyzed lysates from these HER2-amplified breast cancer cells to assess dependence upon the three main antiapoptotic BCL-2 family proteins for survival, following HER2 inhibition. Treatment with the combination of lapatinib/S63845 led to a marked increase in PARP cleavage, compared with lapatinib/A1331852 (a BCL-xL inhibitor) or lapatinib/venetoclax (a BCL-2 inhibitor) (Fig. S12A). However, knocking down BAK in HCC-1419 and MDA-MB-453 cells conferred resistance to MCL-1 inhibition (Fig. S12B).

Combination Treatment with Lapatinib and S63845 Consistently Induces Tumor Regression. To expand and corroborate our findings in vivo, BT-474 xenografts were established in NOD SCID gamma (NSG) female mice, and mice were treated with 100 mg/kg of lapatinib, 25 mg/kg of S63845, or their combination, for 5 consecutive days as previously described (60). Both drugs showed modest efficacy as single agents and variably slowed tumor growth or induced modest regression. However, the combination led to marked regression of 10/10 tumors (Fig. 6A and Fig. S13A). Cleaved PARP was up-regulated following combination drug treatment (Fig. 6B), indicating cell death. Furthermore, we assessed the drug treatments in a HER2-/-ER– breast cancer patient-derived xenograft (PDX) model via injection into NSG mice following the same dosing schedule. Again, the single agents displayed modest activity, inhibiting tumor growth, whereas the combination shrank most of the tumors.
Combination treatment with lapatinib and S63845 leads to antitumor activity in vivo. Values were calculated using the Student t test. BT-474 cells were injected orthotopically into each NSG mouse (both sides) and monitored for subsequent growth. After tumors reached a size of ∼150 mm³, mice were treated with 25 mg/kg S63845, 100 mg/kg lapatinib, or the combination for 5 consecutive days. Tumor measurements were performed every day by calipers, and the percentage (%) of changes in volume for each tumor is shown by a waterfall plot (control, 6 tumors; lapatinib, 6 tumors; S63845, 9 tumors; combination, 10 tumors). P values were calculated using the Student t test. (B) Tumors were harvested from BT-474 tumor-bearing mice ∼2 h after lapatinib administration, and tumor lysates were subjected to Western blot analyses and probed for cleaved PARP and GAPDH (*No Rx*: No drug). (C) Approximately 1.5 × 10⁶ cells derived from a PDX breast cancer model were injected orthotopically into each NSG mouse (both sides) and monitored for subsequent growth. After tumors reached a size of 150–200 mm³, mice were treated with 25 mg/kg S63845, 100 mg/kg lapatinib, or the combination for 5 consecutive days. Tumor measurements were performed every day by calipers, and the percentage (%) of changes in volume for each tumor is shown by a waterfall plot (control, 8 tumors; lapatinib, 7 tumors; S63845, 9 tumors; combination, 11 tumors). P values were calculated using the Student t test.

Immunohistochemistry detection of cleaved caspase-3 confirmed induction of apoptosis in a PDX tumor treated with S63845, with more pronounced apoptosis seen with the combination (Fig. S13B), consistent with the in vitro data (Fig. 5). Altogether, these data indicate that the miR-4728–ERα–NOXA pathway can be successfully targeted by cotreatment of HER2 inhibitors together with MCL-1 inhibitors.

**Discussion**

Breast cancers with amplification of 17q12-21 are driven by, and addicted to, HER2 (60). In genetically engineered mice, HER2 expression driven by the MMTV promoter is sufficient to cause the development of multiple invasive mammary carcinomas, and tumor regressions follow inactivation of MMTV-HER2 in these mice (61). Follow-up studies in these mouse models focused on the proapoptotic BH3-only protein BIM, the abundance of which was increased upon HER2 inhibition. When HER2 was inactivated in MMTV-HER2 mice crossed with BIM−/− knockout mice, tumors did not regress upon MMTV-HER2 inactivation (14). These studies elegantly demonstrated the importance of a BCL-2 family member-mediated apoptotic response upon HER2 inhibition in HER2-amplified breast cancer.

Clinically, despite their clear benefit in the adjuvant setting (62), HER2 inhibitors have shown only modest activity as single agents (63). This is somewhat surprising given that RTK-addicted cancers in other solid tumor types are effectively treated with RTK inhibitor monotherapies (4, 64, 65). Given the demonstrated role of apoptosis in targeted therapy efficacy (reviewed in ref. 8), we reasoned that there may be a widespread deficiency in the HER2 inhibitor-induced apoptotic response.

Surprisingly, we uncovered that coamplification of the HER2 intronic miRNA, miR-4728, is responsible for depressed NOXA expression. We demonstrated that while blocking miR-4728 increases phosphorylation and activation of ERK, as previously demonstrated (38), it also leads to up-regulation of ER and NOXA. Thus, our data point to a dual role for miR-4728: as a tumor antagonistic gene through down-regulation of pERK (38) and as an antagonist of HER2 inhibitor therapy by suppression of NOXA. Importantly, since HER2 inhibitors like lapatinib block ERK signaling in HER2-amplified breast cancers (Figs. 4 D and E and 5 and Figs. S6A, S8B, S11B, and S12, and ref. 9), it is likely that miR-4728 only has an antagonistic effect in the presence of HER2 inhibitors, while miR-4728 likely promotes tumorigenesis in the absence of HER2 inhibition. Indeed, we provide evidence that coamplification of miR-4728 plays a role...
in the lack of responses to HER2 inhibitors (Fig. 4). Moreover, the lack of single agent efficacy of HER2-targeting therapeutic antibodies, like trastuzumab (Herceptin) (66), might also be at least partly explained by down-regulation of NOXA expression. However, since trastuzumab-mediated cytotoxicity includes function outside of HER2 pathway inhibition (67, 68), the relationship between deficient NOXA and trastuzumab efficacy may differ. Importantly, however, Merino et al. (57) demonstrated robust activity of trastuzumab and S63845 in a HER2-amplified PDX model.

More globally, these data reveal that for kinases activated by focal amplification and treated with therapies targeting that kinase, a thorough understanding of coamplified genes may inform treatment strategies. In this case, amplification of mir-4728 forms that HER2-amplified breast cancers should be treated with a HER2 inhibitor and a MCL-1 inhibitor. This may be particularly relevant for patients with high HER2 copy number as well as HER2+/ER− patients that have the lowest levels of NOXA (Fig. 2A).

Direct MCL-1 inhibitors are now being developed, reflecting the growing understanding of the importance of MCL-1 in cancer. A1210477 was the first widely tested, specific MCL-1 inhibitor. Underlying the emerging importance of MCL-1 in breast cancer, Xia et al. (53) demonstrated that A1210477 has single-agent activity in a subset of breast cancers, including HER2-amplified breast cancer cell lines. Similar to our findings, the disruption of MCL-1:BIM complexes was reported as a major mechanism of A1210477 efficacy, and BIM reduction by siRNA markedly mitigated the efficacy of the agent. They also noted that MCL-1 increased in whole-cell lysates of SKBR3 cells following A1210477 treatment, similar to our findings (Fig. S8A). Moreover, they observed an increase in MCL-1:BAX complexes at lower concentrations (2.5 μM) of A1210477. Since it is already known that MCL-1 has a preference for binding to BAX over BAX (69), the disruption of MCL-1:BAX complexes is of significant importance for the induction of apoptosis, which we achieved with S63845 (Fig. 5E) and could explain why we saw a larger amount of BAX activation after treatment with S63845 vs. A1210477 (Fig. S5).

Both selective MCL-1 inhibitors used in our study function as derepressors by displacing proapoptotic BCL-2 family proteins from MCL-1. The increased binding of BAX (Fig. S11B) may be partly explained by mediating the interaction of MCL-1 with proteins such as NOXA that facilitate proteasomal degradation (43, 56). Over the last 10 y, several models have been developed to investigate the dependence of different types of cancer on the proapoptotic BCL-2 family proteins for survival (70). Our data support that the observed sensitivity of HER2-amplified breast cancer cell lines to MCL-1 inhibition in the presence of lapatinib was BIM−, BAX−, but also BAX-dependent, since silencing these three proapoptotic BCL-2 family members significantly rescued the cells from apoptosis. It should be noted that BIM knockdown and BAX knockdown seemed redundant (Fig. S11B), consistent with BIM preferentially activating BAX to kill (49, 71). Our data, therefore, highlight the importance of both displacement of BIM from the prosurvival BCL-2 family proteins (to activate BAX) and displacement of BAX from MCL-1.

While this manuscript was in preparation, Merino et al. (57) demonstrated that S63845 and the HER2-directed antibody trastuzumab combined to induce marked antitumor activity in HER2-amplified breast cancer. Their treatment schedule differed from ours: Merino et al. treated mice once a week with S63845, demonstrating that long-term use of the combination (60 d) is both tolerable and efficacious. Our data also support the use of this combination in vivo (Fig. 6 and Fig. S13), and the sum of our work provides a mechanistic rationale for its implementation. Furthermore, as we found that NOXA (PMAIP1) mRNA levels are lowest in the HER2-amplified/ER− subgroup of human breast tumors (Fig. 2A), this subgroup of patients may benefit from combined targeting of HER2 and MCL-1. These findings may have further implications, as we also report NOXA was among the most highly down-regulated genes following treatment of ER+ breast cancers with the anti-erbB receptor trastuzumab (Fig. 2D), which we recapitulated in vitro (Fig. S4B). These data suggest these breast cancers may also acquire a reliance on MCL-1 as treatment progresses with antiestrogen drugs, and our future work will focus on delineating this precise point.

Overall, our study uncovers an important role for an amplified miRNA within the 17q12-21 ampiclon, driving resistance to HER2 inhibitors. This study represents an example of a coamplified gene that mitigates response to a kinase inhibitor within the same ampiclon that created the kinase addiction in the first place. It also highlights difficulties that may arise when targeting an oncogene within a cancer-addicted ampiclon with a targeted therapeutic—difficulties that do not arise when the addictive oncogene is mutated or translocated, as in the case of EGFR-mutant and ALK-translocated lung cancers. This fundamental difference would appear to contribute to the differential efficacy of these drugs compared with HER2 inhibitors, and analogous situations may arise in other cancers addicted to oncogenes that are activated by amplification.

Materials and Methods

Cell Lines. The cell lines in this study were from the Massachusetts General Hospital. SKBR3, BT-474, MDA-MB-231, MDA-MB-468, and BT-2094 cells were grown in DMEM/F12 (HyClone Laboratories, Inc.) with 10% FBS in the presence of 1 μg/mL penicillin and streptomycin. MDA-MB-361, MDA-MB-453, HCC-1419, UACC-893, HCC-1395, HCC-1937, and HCC-38 cells were cultured in RPMI with 10% FBS in the presence of 1 μg/mL penicillin and streptomycin. UACC-812, CAL-120, MCF-7, and 293T cells were cultured in DMEM with 10% FBS in 1% FBS in the presence of antibiotics. EFM-192A cells were grown in RPMI with 20% FBS in the presence of 1 μg/mL penicillin and streptomycin.

Reagents and Antibodies. The following drugs were kindly provided by Abbvie: venetoclax, A1210477, and A1331852. The following drugs were purchased: S63845 for in vitro and in vivo studies (S-63845; Chemietek), Lapatinib Ditosylate (Tykerb) for in vitro and in vivo studies (M1802; Abmole), and Fulvestrant (S1191; Selleckchem).

The antibodies used in this study (clone/cat. no.) were as follows: Anti-Bak (Ab-1 clone for IP) (AM003; EMD Millipore), anti-BAX (6A7 clone for IP) (sc-23959; Santa Cruz), anti-BAX (N-20 clone) (sc-493; Santa Cruz), anti-Bak (38145; Cell Signaling), anti-Bim (BiMC45S; 29335S; Cell Signaling), anti-Noxa (BD Biosciences, 646803), anti-BCL-2 (D55G8) (Human Specific) (4223S; Cell Signaling), anti-Cleaved PARP (Asp214) (D464E10) (56255S; Cell Signaling), anti-GAPDH (6C5) (sc-23223; Santa Cruz), anti-HER2/ErbB2 (2908) (21656S; Cell Signaling), anti-MCL-1 (S-19) (sc-819; Santa Cruz), anti-BCL-xl (S464) (27645; Cell Signaling), 4E-BP1 (53H11) (9644S; Cell Signaling), phospho-4E-BP1 (Thr37/46) (9258S; Cell Signaling), anti-Phospho-56 Ribosomal Protein S6 (Ser223/244) (28178S; Cell Signaling), anti-Phospho-Akt (Thr308) (4060S; Cell Signaling), anti-Erk (DB8) (8644S; Cell Signaling), anti-Phospho-HER2/ErbB2 (Tyr1248) (2247S; Cell Signaling), anti-Phospho-ALK (Tyr1278) (9254S; Cell Signaling), anti-Phospho-PI3K (Ser473) (4223S; Cell Signaling), anti-Phospho-EGFR (Tyr1068) (4265S; Cell Signaling), anti-Phospho-ERK (Thr202/Tyr204) (4370S; Cell Signaling), anti-Phospho-ERK (Thr560) (4376S; Cell Signaling), and anti-Phospho-MAPK (Thr185/Tyr187) (4380S; Cell Signaling).

All mouse experiments were approved and performed in accordance with the Institutional Animal Care and Use Committee at Virginia Commonwealth University (VCU).

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