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Extended adjuvant therapy with neratinib plus fulvestrant blocks ER/HER2 crosstalk and maintains complete responses of ER+/HER2+ breast cancers: Implications to the ExteNET trial

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Abstract

Purpose: The phase III ExteNET trial showed improved invasive disease-free survival in patients with HER2+ breast cancer treated with neratinib vs. placebo after trastuzumab-based adjuvant therapy. The benefit from neratinib appeared to be greater in patients with ER+/HER2+ tumors. We thus sought to discover mechanisms that may explain the benefit from extended adjuvant therapy with neratinib.

Experimental Design: Mice with established ER+/HER2+ MDA-MB-361 tumors were treated with paclitaxel plus trastuzumab ± pertuzumab for 4 weeks, and then randomized to fulvestrant ± neratinib treatment. The benefit from neratinib was evaluated by performing gene expression analysis for 196 ER targets, ER transcriptional reporter assays, and cell cycle analyses.

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Results: Mice receiving ‘extended adjuvant’ therapy with fulvestrant/neratinib maintained a complete response whereas those treated with fulvestrant relapsed rapidly. In three ER+/HER2+ cell lines (MDA-MB-361, BT-474, UACC-893) but not in ER+/HER2– MCF7 cells, treatment with neratinib induced ER reporter transcriptional activity whereas treatment with fulvestrant resulted in increased HER2 and EGFR phosphorylation, suggesting compensatory reciprocal crosstalk between the ER and ERBB RTK pathways. ER transcriptional reporter assays, gene expression and immunoblot analyses showed that treatment with neratinib/fulvestrant but not fulvestrant potently inhibited growth and downregulated ER reporter activity, P-AKT, P-ERK, and cyclin D1 levels. Finally, similar to neratinib, genetic and pharmacological inactivation of cyclin D1 enhanced fulvestrant action against ER+/HER2+ breast cancer cells.

Conclusions: These data suggest that ER blockade leads to re-activation of ERBB RTKs and thus extended ERBB blockade is necessary to achieve durable clinical outcomes in patients with ER+/HER2+ breast cancer.

Keywords

HER2; ER; neratinib; fulvestrant; breast cancer

INTRODUCTION

HER2 gene amplification and/or overexpression occur in ~20 % of patients with operable breast cancer and used to be strong predictors of early disease relapse and mortality (1,2). With the advent of HER2 targeted therapies, the outcome of patients with HER2-overexpressing (HER2+) breast cancer has vastly improved (3–5). The current standard of care for early stage operable HER2+ breast cancer includes one year of trastuzumab based adjuvant therapy. However, a fraction of patients relapse with metastatic disease (6). The HERA trial tested 24 months of adjuvant trastuzumab. Results from this study showed that 2 years of adjuvant trastuzumab had an unfavorable benefit-to-risk ratio compared to 1 year of trastuzumab (6). Conversely, the phase III ExteNET trial reported that extended adjuvant therapy with 12 months of treatment with neratinib, an irreversible pan-ERBB tyrosine kinase inhibitor (TKI), resulted in a significant improvement in invasive disease-free survival compared to placebo following trastuzumab based adjuvant therapy (7–9). Interestingly, the benefit was greater in patients with hormone receptor positive (HR+) breast cancer compared to those with HR-negative disease. Of note, patients with HR+ cancer remained on antiestrogen therapy during extended adjuvant neratinib. On this basis, neratinib was recently approved by the FDA for use in patients with HER2+ breast cancer following completion of adjuvant trastuzumab (10). Analysis of long term outcomes of patients enrolled in the GeparQuinto trial revealed similar survival benefit in patients with HR+ tumors receiving prolonged anti-HER2 treatment with neoadjuvant lapatinib followed by adjuvant trastuzumab (11).

To study how extended adjuvant neratinib achieved a better clinical outcome in patients with ER+/HER2+ breast cancer, we developed a human-in-mouse breast cancer model. We found that ER+/HER2+ MDA-MB-361 tumors rapidly evade ER blockade through ERBB pathway hyper-activation. Conversely, inhibition of ERBB tyrosine kinase activity with neratinib stoked up ER activity. These compensatory bypass mechanisms have been documented by

previous studies (12,13). However, the molecular underpinnings of resistance to endocrine therapies in HER2+ setting remain incompletely understood. We further observed that resistance to fulvestrant treatment in ER+/HER2+ breast cancer models was mediated, at least in part, through maintenance of cyclin D1 expression and cell cycle progression. The addition of neratinib led to a complete loss of cyclin D1 expression and tumor progression, thereby, supporting simultaneous blockade of both axes to achieve durable remissions in patients with ER+/HER2+ breast cancer.

MATERIALS AND METHODS

Cell culture:

MCF7 (ATCC® HTB-22™), BT-474 (ATCC® HTB-20™), MDA-MB-361 (ATCC® HTB-27™) and UACC-893 (ATCC® CRL-1902™) human breast cancer cell lines were purchased from American Type Culture Collection (ATCC) within the past 10 years. All cell lines were maintained in ATCC recommended media supplemented with 10% FBS (Gibco) at 37°C in a humidified atmosphere of 5% CO₂ in air. All cell lines were tested for mycoplasma contamination and authenticated by ATCC using short tandem repeat (STR) profiling method in January 2017. Prior to performing any *in vitro* experiments, cells were rinsed with PBS, and maintained in phenol red free media supplemented with 10% dextran-coated charcoal treated FBS (DCC-FBS) for 72 h.

Xenograft studies:

All animal experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee (IACUC protocol M/14/028). MDA-MB-361 cells suspended in serum-free IMEM were injected subcutaneously (s.c.) into the right flank of 4–6 week old, ovariectomized athymic nu/nu mice. When the average tumor volume reached ~200 mm³, the mice were treated with trastuzumab (20 mg/kg i.p. twice/week), paclitaxel (15 mg/kg i.p. twice/week; Sigma) ± pertuzumab (20 mg/kg i.p. twice a week) for 4 weeks and then randomized to fulvestrant (5 mg/week s.c.; from AstraZeneca) ± neratinib (20 mg/kg p.o. daily; from Puma Biotechnology). In our previous studies, we have found neratinib to cause modest mouse weight loss due to lack of appetite. This weight loss could be averted by dietary supplementation with flavor-enhanced DietGel 76A (Clear H₂O). Therefore all mice were prophylactically supplemented with DietGel in addition to regular chow. Animal weights and tumor dimensions were measured twice weekly using calipers. Tumor volume was calculated using the formula: volume = width² x length/2. Tumors were harvested 24 h and 6 h after the last dose of fulvestrant and neratinib, respectively, fixed in 10% neutral buffered formalin, dehydrated and paraffin embedded. Tumors were sliced into 5-µm sections and stained for P-HER2 (Cell Signaling #2249), ERα (Santa Cruz Biotech #8002), and Ki67 (Dako #M7240). Sections were scored by an expert pathologist (P.G.E.) blinded to the treatment arm. Staining intensities were determined using a semiquantitative weighted histoscore system that takes both intensity and percentage positivity into account. H-score formula: 3*[% of 3+ cells] + 2*[% of 2+ cells] + 1*[% of 1+ cells] (14,15).

Fluorescent in-situ hybridization (FISH):

FISH was performed using *CCND1/CEN11* Dual Color Probe (ZytoVision, catalog# ZTV-Z-2071). Images were captured at 100X magnification and analysed using Cytovision software by an expert pathologist (P.G.E). *CCND1* amplification was defined following HER2 guidelines.

Immunoblot analysis:

Flash-frozen tumor fragments were homogenized using a TissueLyser (Qiagen) and lysed in RIPA buffer (Sigma) supplemented with 1X protease inhibitor (Roche) and phosphatase inhibitor (Roche) cocktails. Cells were washed with ice-cold PBS twice and lysed in RIPA buffer as described above. Lysates were gently rocked for 30 min at 4°C and centrifuged at 13,000 rpm for 15 min. Protein concentrations in supernatants were measured with the BCA protein assay (Pierce); 20 µg of total protein were fractionated by SDS-PAGE and transferred to nitrocellulose membranes (BioRad). Membranes were blocked with 5% non-fat dry milk and then incubated at 4°C overnight with the following primary antibodies: [from Cell Signalling Technologies: P-HER2 (#2249 1:1000), HER2 (#2242; 1:5000), P-HER3 (#4791; 1:1000), HER3 (#4754; 1:500), HER4 (#4795; 1:500), P-HER4 (#4757; 1:500), P-EGFR (#2237; 1:1000), EGFR (#2646; 1:5000), AKT (#9272; 1:10000), P-AKT_{S473} (#9271; 1:500), P-ERK1/2 (#9101; 1:10000), ERK (#9102; 1:10000), pRB (#9308; 1:1000), and Calnexin (#2679; 1:10000)]; [from Santa Cruz Biotechnology: ERα (sc-8002; 1:1000) and cyclin D1 (sc-718; 1:200)]. Nitrocellulose membranes were then incubated with HRP conjugated anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature and immunoreactive bands were detected by enhanced chemiluminescence (Perkin Elmer).

Cell viability assays:

To determine cell viability in presence of drugs, cells were seeded in 12-well plates in estrogen-free media; 24 h later, they were treated with DMSO, neratinib (200 nM), fulvestrant (1 µM), or fulvestrant/neratinib. At experiment endpoint, plates were fixed, stained with crystal violet, and scanned using a Nikon flat-bed scanner. Staining intensities were then quantified using a LICOR Odessey infra-red plate reader.

ERα transcriptional reporter assay:

Cells were seeded in 96-well plates in estrogen-free media and co-transfected with pGLB-MERE (encoding firefly luciferase flanked by estrogen response elements) and pCMV-Renilla (encoding CMV driven Renilla luciferase) plasmids; 16 h later, cells were treated with DMSO, fulvestrant (1 µM), neratinib (200 nM) or fulvestrant/neratinib. Luciferase activities in drug treated cells were determined 24 h later using Dual-Luciferase® reporter assay system (Promega) as per manufacturer's instructions.

Quantitative PCR and nanoString analysis:

Cells were seeded in 6-well dishes in estrogen depleted media; 72 h later, cells were treated with DMSO, fulvestrant (1 µM), neratinib (200 nM) or fulvestrant/neratinib for 4–6 h. Cells were then lysed and RNA was isolated using Maxwell® LEV simplyRNA cell kit (Promega)

as per manufacturer's instructions. Total RNA content was quantified using a Nanodrop spectrophotometer and reverse transcribed using the iScript cDNA synthesis kit (BioRad). cDNAs of interest were amplified using RT2 qPCR primer assays for human *PGR*, *GREB1*, *CCND1* and *GAPDH* (Qiagen). Relative gene expression was determined by performing quantitative PCR using the CFX-96 thermocycler (BioRad). NanoString analysis was performed on human xenograft RNA using nanoString nCounter Human Breast Cancer ER panel as previously described (16). RNA was extracted from MDA-MB-361 tumors using Maxwell® LEV simplyRNA tissue kit (Promega) as per manufacturer's instructions; 50 ng of total RNA was used for input into nCounter hybridizations. Quality-control measures and normalization of data were performed using the nSolver analysis package and R. Data were normalized in nSolver (version 3.0) by using the geometric mean of the positive control probes to compute the normalization factor as well as the geometric mean of the housekeeping genes (*CLTC*, *GAPDH*, *GUSB*, *HPRT1*, *PGK1*, *TUBB*). Data were then Log₂ transformed to establish normal distribution and a one-way ANOVA was performed with a Benjamini and Hochberg false discovery rate correct to examine the difference between treatment groups. The FDR cut-off for statistical significance was set to 10%. Significant genes were then averaged for each treatment group and z-scores were visualized using a heatmap.

Flow cytometry:

Cells were plated in 60-mm dishes in estrogen depleted media and 3 days later treated with DMSO, fulvestrant (1 μ M), neratinib (200 nM) or fulvestrant/neratinib for 24 h. The cells were then harvested using phenol-red-free TrpLE Xpress dissociation medium (Gibco), rinsed with PBS, and fixed with 70% ethanol at 4°C for 30 min followed by 2 washes with PBS and incubation with 0.1 mg/ml RNase A (Qiagen) and 40 μ g/ml propidium iodide (Sigma) for 10 min at room temperature. Cell cycle distribution was assessed using a 3 laser LSRII bioanalyzer.

Statistical analysis:

Paired and unpaired *t* tests were used to determine statistically significant differences in cell proliferation assays, *in vivo* tumor growth assays, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays, and immunohistochemistry (IHC) H-scores. A *p* value of less than .05 was considered statistically significant, and all statistical tests were two-sided. Bar graphs show mean $\pm$ S.E.M., unless otherwise stated in the figure legend.

RESULTS

Adjuvant therapy with fulvestrant/neratinib maintains complete responses of ER+/HER2+ tumors.

We first established a human-in-mouse model that simulates the clinical outcomes seen in the ExteNET trial. Mice with established ER+/HER2-amplified MDA-MB-361 tumors were treated with trastuzumab (tz) + paclitaxel (pac) for 4 weeks, before receiving 'extended adjuvant' therapy with fulvestrant $\pm$ neratinib for 4 weeks (Fig. 1A). All MDA-MB-361 tumors exhibited a prompt and marked reduction in volume after tz/pac treatment with some mice exhibiting a complete response (CR) and others a partial response (PR). Within the CR

cohort, mice receiving fulvestrant/neratinib remained in complete remission during treatment. After treatment discontinuation only 2/5 tumors recurred during the next 6 weeks; these xenografts responded to retreatment with fulvestrant/neratinib. However, mice treated with fulvestrant alone relapsed rapidly ($p < 0.05$ at week 8). Even within the PR cohort, fulvestrant/neratinib was able to significantly suppress tumor growth compared to single agent fulvestrant. We did not notice any signs of overt toxicities or considerable weight loss in mice receiving neratinib. We next evaluated ER and P-HER2 levels in fulvestrant-treated tumors on week 8 and fulvestrant/neratinib-treated tumors on week 18 (* in Fig. 1A). ER α levels were markedly downregulated in both fulvestrant and fulvestrant/neratinib treated tumors compared to untreated controls. HER2 phosphorylation was significantly higher in tumors treated with fulvestrant alone but not fulvestrant/neratinib, suggesting activation of the HER2 pathway as an adaptation mechanism upon ER downregulation (Fig. 1B,C).

Since ~50% mice had failed to achieve a tumor complete response prior to initiation of extended adjuvant therapy, we next repeated this experiment using double blockade of HER2 with pertuzumab and trastuzumab. Mice with established MDA-MB-361 xenografts were treated with pertuzumab/tz/pac for 4 weeks. Following a complete tumor response, mice were randomized to fulvestrant/neratinib vs. fulvestrant alone. Mice treated with the combination remained in complete remission for 6 months after treatment discontinuation and were ultimately euthanized. On the other hand, tumors in mice treated with fulvestrant monotherapy relapsed within a week, but the addition of neratinib to fulvestrant on week 8 resulted in marked tumor shrinkage (Fig. 1D). Tumors recurring on fulvestrant were harvested before and after the addition of neratinib (* in Fig. 1D). IHC analysis of tumor sections showed robust P-HER2 and undetectable ER levels before neratinib, and a significant reduction in P-HER2 staining following the addition of the pan-HER TKI (Fig. 1E,F). These data suggest that extended ERBB blockade with a pan-HER inhibitor may overcome activation of the HER2 pathway in ER+/HER2+ breast cancers treated with adjuvant antiestrogens alone.

Neratinib and fulvestrant block ER/HER2 crosstalk and potentially inhibit growth of ER+/HER2+ breast cancer cells.

We next examined the effect of fulvestrant, neratinib or both drugs against ER+/HER2- MCF7 cells and a panel of ER+/HER2+ cell lines, BT-474, MDA-MB-361, and UACC-893. Except for P-HER3 in MCF7 cells, treatment with 200 nM neratinib completely eliminated detectable P-HER2, P-EGFR, P-HER3 and P-HER4 levels in all cell lines. Neratinib also markedly downregulated P-AKT and P-ERK in all three HER2+ cell lines but not in MCF7 cells (Fig. 2A), suggesting that, in these cells, activation of PI3K and MEK is not entirely dependent on the ERBB pathway. Total EGFR and total HER2 levels were reduced in all four cell lines upon treatment with neratinib, with HER2 downregulation being more evident in MDA-MB-361 and UACC893 cells. Consistent with the *in vivo* findings shown in Fig. 1B,E, fulvestrant treatment resulted in increased HER2 phosphorylation in BT-474 and UACC-893 cells but not in ER+/HER2- MCF7 cells (Fig. 2A). While MDA-MB-361 did not recapitulate the increase in P-HER2 levels at 24 h, we noted a robust upregulation in HER2 phosphorylation in response to long term (2 week) fulvestrant exposure (supplementary fig. 1). Finally, clonogenic growth assays showed that HER2+ lines were generally resistant to

fulvestrant. However, in line with the effects on signal transduction, treatment with fulvestrant/neratinib resulted in complete growth inhibition of all three HER2+ cells whereas neratinib did not add to fulvestrant action against fulvestrant-sensitive MCF7 cells (Fig. 2B,C).

We next tested whether trastuzumab would achieve similar suppression of the adaptive responses induced by ER blockade. The growth of ER+/HER2+ cells was only marginally hampered by fulvestrant or fulvestrant/trastuzumab. On the other hand, addition of fulvestrant/neratinib completely ablated the growth of cells refractory to fulvestrant/trastuzumab (supplementary fig. 2A,B). We then tested the phosphorylation status of other ERBB receptors in MDA-MB-361 tumors that recurred on fulvestrant following complete regression on trastuzumab-based therapy (* in Fig. 1A and 1D). While P-HER3 levels remained unaltered, we noted a significant increase in P-EGFR in tumors maintained on fulvestrant but not fulvestrant/neratinib (supplementary fig. 2C-F). Consistent with these *in vivo* findings, long term (2 weeks) treatment of ER+/HER2+ UACC-893 cells with fulvestrant led to an increase in P-EGFR and P-HER4 in addition to P-HER2 upregulation (supplementary fig. 2G). While the addition of trastuzumab completely ablated HER2 phosphorylation, it did not revert P-EGFR and P-HER4 to basal levels. Consistently, we noted higher AKT phosphorylation in fulvestrant and fulvestrant/trastuzumab treated cells compared to untreated controls. Collectively these data suggest that ER+/HER2+ tumors evade ER blockade through concomitant activation of members of the ERBB family, which would be effectively overcome by a pan-HER TKI.

We next asked whether suppression of ERBB receptor signaling with neratinib resulted in a compensatory effect on estrogen receptor activity. In all three ER+/HER2+ cell lines but not in ER+/HER2- MCF7 cells, treatment with neratinib resulted in a significant increase in ER reporter transcriptional activity (MCF7, 0.2-fold; BT474, 12-fold; MDA-MB-361, 2-fold; UACC893, 8-fold), which was dampened by the addition of fulvestrant. Treatment with fulvestrant alone reduced ligand-independent ER reporter activity in MCF7 but not in any of the HER2+ cell lines (Fig. 3A). Whereas fulvestrant treatment downregulated ER protein levels in all cell lines, neratinib treatment resulted in a subtle and transient increase in ER levels in BT474 and UACC893 cells (Fig. 3B). To examine ER transcriptional activity further, we examined the gene expression status for progesterone receptor (*PGR*) and *GREB1*. In all three HER2+ cell lines, neratinib treatment induced variable increase in *PGR* and *GREB1* mRNA expression which, except for *GREB1* in UACC893 cells, was reduced by the addition of fulvestrant (Fig. 3C). Collectively, these data further suggest the need of dual targeting of ER and HER2 in order to block crosstalk and achieve durable growth inhibition of ER+/HER2+ breast cancer cells.

Combined treatment with neratinib plus fulvestrant targets cyclin D1.

To further investigate the effects of fulvestrant/neratinib on ER-HER2 crosstalk at a molecular level, we screened for ER regulated genes that are un-responsive to fulvestrant treatment but sensitive to the combination. MDA-MB-361 tumor-bearing mice were treated with fulvestrant, neratinib or fulvestrant/neratinib for 7 days and then harvested (Fig. 4A). IHC of tumor sections showed downregulation of ER α and P-HER2 levels in fulvestrant and

neratinib treated tumors, respectively, confirming drug target inhibition (Fig. 4B, C). Tumor RNA was extracted and subjected to gene expression analysis using a nanoString breast cancer ER panel consisting of 196 ER-regulated genes. Out of 196 ER-regulated genes tested, 42 were significantly altered by at least one of the treatments as shown in heatmap in Figure 4D. Single agent neratinib enhanced the expression of several ER target genes, consistent with the upregulation of ER transcriptional activity observed *in vitro* (Figure 3A). *CCND1* (cyclin D1) and *GABRP* (Gamma aminobutyric acid A receptor, Pi subunit) were the only genes unaffected by fulvestrant but that were ablated by the combination treatment (Fig. 4D). Notably, *CCND1* amplification is present in 26% of ER+/HER2+ breast cancers in the Cancer Genome Atlas (TCGA; Fig. 4E). Interestingly, all three ER+/HER2+ cell lines used herein, BT-474, MDA-MB-361 and UACC-893, also harbor *CCND1* gene amplification (Fig. 4F).

We next examined if downregulation of cyclin D1 was central to the efficacy of combined ER/HER2 targeting with fulvestrant/neratinib. Immunoblot analysis of MDA-MB-361 tumor lysates (shown in Fig. 4A), confirmed near complete loss of cyclin D1 expression upon treatment with fulvestrant/neratinib, but not in tumors treated with fulvestrant or neratinib alone (Fig. 5A). Consistent with these results, neratinib ± fulvestrant but not fulvestrant alone reduced cyclin D1 protein and P-Rb levels in all three ER+/HER2+ breast cancer cell lines (Fig. 5B). These results were corroborated at the mRNA level as we observed significant inhibition of *CCND1* mRNA in all three ER+/HER2+ breast cancer cell lines treated with neratinib ± fulvestrant (Fig. 5C). These observations were further supported by a significant reduction in Ki67-positive cells in fulvestrant/neratinib treated tumors compared to fulvestrant-treated and untreated tumors (Fig. 5D,E). There was no statistically significant difference in the number of apoptotic cells among all treatments as measured by TUNEL analysis. Cell cycle analysis of ER+/HER2+ breast cancer cell lines also showed a marked reduction in the number of cells in 'S-phase' upon treatment with fulvestrant/neratinib (Fig. 5F).

Cyclin D1 inactivation adds to fulvestrant action against ER+/HER2+ breast cancer cells.

In MCF7 cells, with low levels of HER2, but not in ER+/HER2 gene-amplified cells, treatment with fulvestrant resulted in downregulation of cyclin D1 mRNA and protein levels. Addition of neratinib to fulvestrant suppressed cyclin D1 expression in ER+/HER2+ cells (Fig. 5C), suggesting cyclin D1 transcription is co-regulated by ERα and PI3K/AKT and/or MEK/ERK, downstream of amplified HER2 (17–19). Phosphorylation of the tumor suppressor Rb by the cyclin D1-CDK4/6 complex uncouples Rb from E2F transcription factors. As a result, E2Fs induce transcription of genes necessary for the G1-to-S transition (20). Also, cyclin D1 has been shown to be necessary for ErbB2 (neu)-driven carcinogenesis (21,22). Thus, we next examined if genetic and pharmacological inactivation of cyclin D1 would resemble the growth inhibitory effect of neratinib ± fulvestrant against ER+/HER2+ cells. Treatment with the CDK4/6 antagonist abemaciclib (23) inhibited growth of BT-474, MDA-MB-361 and UACC893 cells. The combination of abemaciclib/fulvestrant was markedly more inhibitory than single agent fulvestrant (Fig. 6A). Similar results were observed with two independent cyclin D1 siRNAs (Fig. 6C). In all 3 ER+/HER2+ cell lines, cyclin D1 knockdown resulted in growth inhibition. The combination of cyclin D1 siRNA

and fulvestrant was generally more potent at inhibiting cell growth than each intervention alone (Fig. 6C). Due to the transient nature of siRNA mediated knockdown, growth modulating effects were assessed within 3 days of drug treatment. MDA-MB-361 cells have a PIK3CA E545K activating mutation which we speculate may dampen their responsiveness to a brief exposure to neratinib compared to longer term treatments (Fig. 2C). Collectively, these data suggest a central role of cyclin D1 in limiting the action of antiestrogens alone against ER+/HER2+ breast cancer cells. They also provide a plausible explanation for the synergistic effect of adjuvant fulvestrant/neratinib against ER+/HER2+ xenografts following treatment with chemotherapy and anti-HER2 therapy (Fig. 1), reminiscent of the results in the ExteNET trial.

DISCUSSION:

Patients with early stage ER+/HER2+ breast cancer receive at least 5 years of adjuvant antiestrogen therapy with one year of trastuzumab after completion of primary therapy. Since the advent of trastuzumab and other HER2 targeting agents, the outcome of patients with HER2+ breast cancer has vastly improved. However, ~15% patients still recur with metastatic disease (6). Neratinib has been recently approved as an extended adjuvant treatment for early stage HER2+ breast cancer patients who have completed trastuzumab based adjuvant therapy. The approval was based on the phase III ExteNET trial, which showed a significant improvement in invasive disease free survival in patients receiving 12 months of neratinib treatment after completion of adjuvant trastuzumab (7,8). In this study using experimental models of ER+/HER2+ breast cancer, we attempted to identify potential mechanisms that would support the results of the ExteNET trial. We found that ER+/HER2+ MDA-MB-361 tumors in mice maintained on fulvestrant alone, relapsed rapidly compared to mice receiving neratinib and fulvestrant (Fig. 1A, D). Tumor recurrences within the fulvestrant arm exhibited a marked increase in HER2 and EGFR phosphorylation suggesting that ER+/HER2+ cancers can adapt to ER blockade through hyperactivation of the ERBB RTK pathway (Fig. 1 and supplementary fig. 2). These observations are consistent with previous pre-clinical and clinical reports of HER2 overexpression as a mechanism of intrinsic or acquired resistance to endocrine therapy (12,24,25). Using HER2 overexpressing ER+ MCF7 cells, Massarweh et al. demonstrated that resistance to prolonged estrogen deprivation or fulvestrant treatment was achieved through HER2-reactivation (12). Similarly, retrospective analysis of the IMPACT neoadjuvant trial comparing the clinical efficacy of tamoxifen vs. aromatase inhibitors revealed a lower response rate among HER2+ tumors, irrespective of the antiestrogen arm (26). In line with HER2-mediated resistance to antiestrogens, we noted a prompt upregulation in P-HER2 levels upon fulvestrant treatment, in three ER+/HER2+ breast cancer cell lines (Fig. 2A). In addition, we observed a significant increase in P-EGFR in tumors recurring on fulvestrant (supplementary fig. 2C-F) as well as in cells exposed to fulvestrant for 2 weeks (supplementary fig. 2G). The addition of trastuzumab to fulvestrant did not overcome activation of ERBB receptors or AKT (supplementary fig. 2G). These findings are consistent with several pre-clinical and clinical reports that have associated EGFR activation with resistance to both endocrine therapy (27–30) and trastuzumab (31,32). Further, phase II randomized trials in ER+ metastatic breast cancer patients have shown an improvement in progression free survival with the addition of

the EGFR inhibitor gefitinib to tamoxifen or to anastrozole (33,34). Similarly, high EGFR expression has been associated with lesser benefit to adjuvant trastuzumab in the NCCTG N9831 (Alliance) trial (32). Of note, phase III GeparQuinto trial reported similar survival benefit in patients with ER+ tumors receiving prolonged HER2 blockade with 6 months of neoadjuvant lapatinib, followed by 1 year of adjuvant trastuzumab (11).

We acknowledge that our mouse model does not entirely recapitulate the design of the ExteNET trial. It is extremely challenging to power mouse studies to evaluate disease recurrence rates in response to sequential adjuvant treatments in a statistically meaningful manner. In order to overcome this inherent limitation of mouse models, we tested the efficacy of trastuzumab and neratinib based treatments in tumor bearing mice. Even though our model is closer to metastatic setting, we believe that the overall findings could be extended to adjuvant settings as well.

HER2 signaling has been previously shown to promote ligand independent activation of ER through various mechanisms including ER phosphorylation and modulation of co-regulators of ER transcription (35,36). We therefore tested the effect of HER2 inactivation with neratinib on ER activity. Counterintuitive to the above studies, we noted a significant upregulation in ER transcriptional activity upon neratinib treatment, thereby suggesting that effective ERBB inhibition leads to rapid restoration of ER function in HER2 gene amplified cells (Fig. 3). This is in agreement with the reported induction of ER activity in primary HER2+ tumors upon short term treatment with the HER2 TKI lapatinib (36). Further, a retrospective analysis of HER2+ primary tumors treated with neoadjuvant lapatinib showed a switch from ER-negative to ER+ status in about 20% of patients' cancers (37). Other pre-clinical studies have also reported ER activation as a mechanism of acquired resistance to HER2 targeting in experimental models of HER2+ breast cancer (37–39). Collectively, these findings suggest that ER upregulation might occur as a prompt response to HER2 inhibition and gradually gets hardwired as a mechanism of resistance to anti-HER2 therapy.

Although patients with ER– tumors did not gain benefit from extended adjuvant neratinib, there appeared to be a benefit while the patients remained on treatment (8). The discrepancy in treatment outcomes within ER+ versus ER– cohorts could be ascribed to several factors. The biology and natural history of ER+/HER2+ versus ER–/HER2+ breast cancers are very distinct. ER–/HER2+ tumors are at a higher risk of early recurrence (40). Retrospective subgroup analysis of patients receiving 1 year of adjuvant trastuzumab in the HERA trial revealed a trend toward inferior 3-year disease free survival in patients with ER– cancers compared to the ER+ cohort, likely due to their inherent higher risk of early relapse (41). On the other hand, ER+ tumors may recur late and, as such, may require more prolonged combined blockade of ER-HER2 signaling crosstalk. In line with this notion, the phase III TAnDEM and EGF30008 trials in patients with ER+/HER2+ metastatic breast cancer, showed an improved PFS with the addition of trastuzumab to anastrozole and of lapatinib to letrozole, respectively (42,43). Collectively, these pre-clinical and clinical observations suggest a plausible explanation to the benefit of combined anti-ER and anti-HER2 therapies in the ExteNET and GeparQuinto trials.

While the question of combined ER/HER2 targeting has been addressed to some extent by previous studies (42,44,45), the molecular underpinnings of the observed benefit remain less understood. Thus, to further our understanding of potential mechanisms to explain how addition of the HER2 inhibitor neratinib overcame fulvestrant resistance, we screened for ER regulated genes that are un-responsive to fulvestrant but remain sensitive to the combination. Gene expression analysis of 196 ER regulated genes revealed that cyclin D1 was one of the two main ER responsive genes that remained unaffected by fulvestrant but ablated by fulvestrant/neratinib. Cyclin D1 upregulation has been shown to drive resistance to both endocrine therapy and anti-HER2 agents. Cyclin D1 has also been shown to be a key mediator of the mitogenic effects of estrogen and thus purported as a potential driver of endocrine resistance (46). Similarly, robust cyclin D1 downregulation has been shown to be required for the antitumor action of HER2-targeted drugs (47). Goel et al. recently demonstrated that tumor recurrences in a genetically engineered mouse model of HER2+ breast cancer was primarily mediated by cyclin D1/Cdk4 upregulation and thus could be overcome by combined inhibition of HER2 and Cdk4/6 (48). Mouse mammary glands deficient in cyclin D1 are largely resistant to the tumor initiating effects of ErbB2 (21,22,49). The mitogenic effects of several distinct growth stimuli converge on cyclin D1 either via its transcriptional upregulation or through increased stabilization, and ERBB mediated activation of RAS/RAF/MEK/ERK signaling promotes cyclin D1 transcription through increased recruitment of E2F and SP1 transcription factors to *CCND1* promoter (17). Likewise, AKT, a major substrate of PI3K downstream of the HER2 receptor, post-translationally stabilizes intracellular cyclin D1 levels by inhibiting its proteasomal degradation (50). In the study reported herein, we show that fulvestrant monotherapy yields incomplete suppression of cyclin D1 levels in ER+/HER2+ cells and tumors, whereas addition of neratinib results in robust ablation of cyclin D1 levels and cell cycle progression.

In conclusion, we show herein that fulvestrant/neratinib but not fulvestrant monotherapy maintained complete responses of ER+/HER+ tumors following treatment with tz/pac or pertuzumab/tz/pac, reminiscent of the results in the phase III ExteNET trial. We found that ER+/HER2+ tumors rapidly evade ER blockade through ERBB pathway hyperactivation and, conversely, inhibition of ERBB tyrosine kinase activity with neratinib stoked up ER activity. Finally, treatment with neratinib/fulvestrant but not fulvestrant alone reduced cyclin D1 mRNA and protein levels, and induced cell cycle arrest, suggesting that simultaneous targeting of both ER and HER2 axes is required to overcome compensatory crosstalk between ER and amplified HER2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES:

1. Arteaga CL, Sliwkowski MX, Osborne CK, Perez EA, Puglisi F, Gianni L. Treatment of HER2-positive breast cancer: current status and future perspectives. *Nat Rev Clin Oncol* 2011;9(1):16–32 doi 10.1038/nrclinonc.2011.177. [PubMed: 22124364]
2. 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(4785):177–82. [PubMed: 3798106]
3. Swain SM, Baselga J, Kim SB, Ro J, Semiglazov V, Campone M, et al. Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer. *N Engl J Med* 2015;372(8):724–34 doi 10.1056/NEJMoa1413513. [PubMed: 25693012]
4. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 2002;20(3):719–26 doi 10.1200/JCO.2002.20.3.719. [PubMed: 11821453]
5. Marty M, Cognetti F, Maraninchi D, Snyder R, Mauriac L, Tubiana-Hulin M, et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. *J Clin Oncol* 2005;23(19):4265–74 doi 10.1200/JCO.2005.04.173. [PubMed: 15911866]
6. Goldhirsch A, Gelber RD, Piccart-Gebhart MJ, de Azambuja E, Procter M, Suter TM, et al. 2 years versus 1 year of adjuvant trastuzumab for HER2-positive breast cancer (HERA): an open-label, randomised controlled trial. *Lancet* 2013;382(9897):1021–8 doi 10.1016/S0140-6736(13)61094-6. [PubMed: 23871490]
7. Chan A, Delaloge S, Holmes FA, Moy B, Iwata H, Harvey VJ, et al. Neratinib after trastuzumab-based adjuvant therapy in patients with HER2-positive breast cancer (ExteNET): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2016;17(3):367–77 doi 10.1016/S1470-2045(15)00551-3. [PubMed: 26874901]
8. Martin M, Holmes FA, Ejlertsen B, Delaloge S, Moy B, Iwata H, et al. Neratinib after trastuzumab-based adjuvant therapy in HER2-positive breast cancer (ExteNET): 5-year analysis of a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* 2017;18(12):1688–700 doi 10.1016/S1470-2045(17)30717-9. [PubMed: 29146401]
9. Unni N, Sudhan DR, Arteaga CL. Neratinib: Inching up on the cure rate of HER2+ breast cancer? *Clin Cancer Res* 2018 doi 10.1158/1078-0432.CCR-18-1114.
10. Singh H, Walker AJ, Amiri-Kordestani L, Cheng J, Tang S, Balcazar P, et al. U.S. Food and drug administration approval: Neratinib for the extended adjuvant treatment of early stage HER2-positive breast cancer. *Clin Cancer Res* 2018 doi 10.1158/1078-0432.CCR-17-3628.
11. Untch M, von Minckwitz G, Gerber B, Schem C, Rezai M, Fasching PA, et al. Survival analysis after neoadjuvant chemotherapy with trastuzumab or lapatinib in patients with human epidermal growth factor receptor 2-positive breast cancer in the GeparQuinto (G5) study (GBG 44). *J Clin Oncol* 2018;JCO2017759175 doi 10.1200/JCO.2017.75.9175.
12. Massarweh S, Osborne CK, Jiang S, Wakeling AE, Rimawi M, Mohsin SK, et al. Mechanisms of tumor regression and resistance to estrogen deprivation and fulvestrant in a model of estrogen receptor-positive, HER-2/neu-positive breast cancer. *Cancer Res* 2006;66(16):8266–73 doi 10.1158/0008-5472.CAN-05-4045. [PubMed: 16912207]
13. Bender LM, Nahta R. Her2 cross talk and therapeutic resistance in breast cancer. *Front Biosci* 2008;13:3906–12. [PubMed: 18508484]
14. Kurebayashi J, Kanomata N, Yamashita T, Shimo T, Mizutoh A, Moriya T, et al. Prognostic value of phosphorylated HER2 in HER2-positive breast cancer patients treated with adjuvant trastuzumab. *Breast Cancer* 2015;22(3):292–9 doi 10.1007/s12282-013-0478-y. [PubMed: 23749689]
15. Spears M, Pederson HC, Lyttle N, Gray C, Quintayo MA, Brogan L, et al. Expression of activated type I receptor tyrosine kinases in early breast cancer. *Breast Cancer Res Treat* 2012;134(2):701–8 doi 10.1007/s10549-012-2076-7. [PubMed: 22562124]

16. Balko JM, Giltane JM, Wang K, Schwarz LJ, Young CD, Cook RS, et al. Molecular profiling of the residual disease of triple-negative breast cancers after neoadjuvant chemotherapy identifies actionable therapeutic targets. *Cancer Discov* 2014;4(2):232–45 doi 10.1158/2159-8290.CD-13-0286. [PubMed: 24356096]
17. Lee RJ, Albanese C, Fu M, D'Amico M, Lin B, Watanabe G, et al. Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol Cell Biol* 2000;20(2):672–83. [PubMed: 10611246]
18. Sabbah M, Courilleau D, Mester J, Redeuilh G. Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. *Proc Natl Acad Sci U S A* 1999;96(20):11217–22. [PubMed: 10500157]
19. Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL. Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. *Cancer Res* 2002;62(14):4132–41. [PubMed: 12124352]
20. Knudsen ES, Knudsen KE. Tailoring to RB: tumour suppressor status and therapeutic response. *Nat Rev Cancer* 2008;8(9):714–24 doi 10.1038/nrc2401. [PubMed: 19143056]
21. Yu Q, Geng Y, Sicinski P. Specific protection against breast cancers by cyclin D1 ablation. *Nature* 2001;411(6841):1017–21 doi 10.1038/35082500. [PubMed: 11429595]
22. Yu Q, Sicinska E, Geng Y, Ahnstrom M, Zagodzko A, Kong Y, et al. Requirement for CDK4 kinase function in breast cancer. *Cancer Cell* 2006;9(1):23–32 doi 10.1016/j.ccr.2005.12.012. [PubMed: 16413469]
23. Sledge GW, Jr., Toi M, Neven P, Sohn J, Inoue K, Pivot X, et al. MONARCH 2: Abemaciclib in combination with fulvestrant in women with HR+/HER2- advanced breast cancer who had progressed while receiving endocrine therapy. *J Clin Oncol* 2017;35(25):2875–84 doi 10.1200/JCO.2017.73.7585. [PubMed: 28580882]
24. Prat A, Baselga J. The role of hormonal therapy in the management of hormonal-receptor-positive breast cancer with co-expression of HER2. *Nat Clin Pract Oncol* 2008;5(9):531–42 doi 10.1038/ncponc1179. [PubMed: 18607391]
25. Lipton A, Ali SM, Leitzel K, Demers L, Chinchilli V, Engle L, et al. Elevated serum Her-2/neu level predicts decreased response to hormone therapy in metastatic breast cancer. *J Clin Oncol* 2002;20(6):1467–72 doi 10.1200/JCO.2002.20.6.1467. [PubMed: 11896093]
26. Dowsett M, Ebbs SR, Dixon JM, Skene A, Griffith C, Boeddinghaus I, et al. Biomarker changes during neoadjuvant anastrozole, tamoxifen, or the combination: influence of hormonal status and HER-2 in breast cancer—a study from the IMPACT trialists. *J Clin Oncol* 2005;23(11):2477–92 doi 10.1200/JCO.2005.07.559. [PubMed: 15767642]
27. Zhang X, Diaz MR, Yee D. Fulvestrant regulates epidermal growth factor (EGF) family ligands to activate EGF receptor (EGFR) signaling in breast cancer cells. *Breast Cancer Res Treat* 2013;139(2):351–60 doi 10.1007/s10549-013-2541-y. [PubMed: 23686416]
28. Frogne T, Benjaminsen RV, Sonne-Hansen K, Sorensen BS, Nexø E, Laenkholm AV, et al. Activation of ErbB3, EGFR and Erk is essential for growth of human breast cancer cell lines with acquired resistance to fulvestrant. *Breast Cancer Res Treat* 2009;114(2):263–75 doi 10.1007/s10549-008-0011-8. [PubMed: 18409071]
29. Okubo S, Kurebayashi J, Otsuki T, Yamamoto Y, Tanaka K, Sonoo H. Additive antitumour effect of the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib (Iressa, ZD1839) and the antioestrogen fulvestrant (Faslodex, ICI 182,780) in breast cancer cells. *Br J Cancer* 2004;90(1):236–44 doi 10.1038/sj.bjc.6601504. [PubMed: 14710235]
30. Massarweh S, Osborne CK, Creighton CJ, Qin L, Tsimelzon A, Huang S, et al. Tamoxifen resistance in breast tumors is driven by growth factor receptor signaling with repression of classic estrogen receptor genomic function. *Cancer Res* 2008;68(3):826–33 doi 10.1158/0008-5472.CAN-07-2707. [PubMed: 18245484]
31. Ritter CA, Perez-Torres M, Rinehart C, Guix M, Dugger T, Engelman JA, et al. Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. *Clin Cancer Res* 2007;13(16):4909–19 doi 10.1158/1078-0432.CCR-07-0701. [PubMed: 17699871]

32. Cheng H, Ballman K, Vassilakopoulou M, Dueck AC, Reinholz MM, Tenner K, et al. EGFR expression is associated with decreased benefit from trastuzumab in the NCCTG N9831 (Alliance) trial. *Br J Cancer* 2014;111(6):1065–71 doi 10.1038/bjc.2014.442. [PubMed: 25117817]
33. Cristofanilli M, Valero V, Mangalik A, Royce M, Rabinowitz I, Arena FP, et al. Phase II, randomized trial to compare anastrozole combined with gefitinib or placebo in postmenopausal women with hormone receptor-positive metastatic breast cancer. *Clin Cancer Res* 2010;16(6):1904–14 doi 10.1158/1078-0432.CCR-09-2282. [PubMed: 20215537]
34. Osborne CK, Neven P, Dirix LY, Mackey JR, Robert J, Underhill C, et al. Gefitinib or placebo in combination with tamoxifen in patients with hormone receptor-positive metastatic breast cancer: a randomized phase II study. *Clin Cancer Res* 2011;17(5):1147–59 doi 10.1158/1078-0432.CCR-10-1869. [PubMed: 21220480]
35. Osborne CK, Shou J, Massarweh S, Schiff R. Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. *Clin Cancer Res* 2005;11(2 Pt 2):865s–70s. [PubMed: 15701879]
36. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst* 2004;96(12):926–35. [PubMed: 15199112]
37. Giuliano M, Hu H, Wang YC, Fu X, Nardone A, Herrera S, et al. Upregulation of ER signaling as an adaptive mechanism of cell survival in HER2-positive breast tumors treated with anti-HER2 therapy. *Clin Cancer Res* 2015;21(17):3995–4003 doi 10.1158/1078-0432.CCR-14-2728. [PubMed: 26015514]
38. Xia W, Bacus S, Hegde P, Husain I, Strum J, Liu L, et al. A model of acquired autoresistance to a potent ErbB2 tyrosine kinase inhibitor and a therapeutic strategy to prevent its onset in breast cancer. *Proc Natl Acad Sci U S A* 2006;103(20):7795–800 doi 10.1073/pnas.0602468103. [PubMed: 16682622]
39. Sabnis G, Schayowitz A, Goloubeva O, Macedo L, Brodie A. Trastuzumab reverses letrozole resistance and amplifies the sensitivity of breast cancer cells to estrogen. *Cancer Res* 2009;69(4):1416–28 doi 10.1158/0008-5472.CAN-08-0857. [PubMed: 19190349]
40. Vaz-Luis I, Ottesen RA, Hughes ME, Marcom PK, Moy B, Rugo HS, et al. Impact of hormone receptor status on patterns of recurrence and clinical outcomes among patients with human epidermal growth factor-2-positive breast cancer in the National Comprehensive Cancer Network: a prospective cohort study. *Breast Cancer Res* 2012;14(5):R129 doi 10.1186/bcr3324. [PubMed: 23025714]
41. Untch M, Gelber RD, Jackisch C, Procter M, Baselga J, Bell R, et al. Estimating the magnitude of trastuzumab effects within patient subgroups in the HERA trial. *Ann Oncol* 2008;19(6):1090–6 doi 10.1093/annonc/mdn005. [PubMed: 18296421]
42. Kaufman B, Mackey JR, Clemens MR, Bapsy PP, Vaid A, Wardley A, et al. Trastuzumab plus anastrozole versus anastrozole alone for the treatment of postmenopausal women with human epidermal growth factor receptor 2-positive, hormone receptor-positive metastatic breast cancer: results from the randomized phase III TAnDEM study. *J Clin Oncol* 2009;27(33):5529–37 doi 10.1200/JCO.2008.20.6847. [PubMed: 19786670]
43. Bauerfeind I, Elling D, Heinemann V. Lapatinib in the treatment of hormone receptor-positive/ ErbB2-positive breast cancer. *Breast Care (Basel)* 2010;5(s1):13–5 doi 10.1159/000285775. [PubMed: 20847831]
44. Johnston S, Pippen J, Jr., Pivot X, Lichinitser M, Sadeghi S, Dieras V, et al. Lapatinib combined with letrozole versus letrozole and placebo as first-line therapy for postmenopausal hormone receptor-positive metastatic breast cancer. *J Clin Oncol* 2009;27(33):5538–46 doi 10.1200/JCO.2009.23.3734. [PubMed: 19786658]
45. Huober J, Fasching PA, Barsoum M, Petruzelka L, Wallwiener D, Thomssen C, et al. Higher efficacy of letrozole in combination with trastuzumab compared to letrozole monotherapy as first-line treatment in patients with HER2-positive, hormone-receptor-positive metastatic breast cancer - results of the eLEcTRA trial. *Breast* 2012;21(1):27–33 doi 10.1016/j.breast.2011.07.006. [PubMed: 21862331]

46. Butt AJ, McNeil CM, Musgrove EA, Sutherland RL. Downstream targets of growth factor and oestrogen signalling and endocrine resistance: the potential roles of c-Myc, cyclin D1 and cyclin E. *Endocr Relat Cancer* 2005;12 Suppl 1:S47–59 doi 10.1677/erc.1.00993. [PubMed: 16113099]
47. Lenferink AE, Busse D, Flanagan WM, Yakes FM, Arteaga CL. ErbB2/neu kinase modulates cellular p27(Kip1) and cyclin D1 through multiple signaling pathways. *Cancer Res* 2001;61(17): 6583–91. [PubMed: 11522658]
48. Goel S, Wang Q, Watt AC, Tolaney SM, Dillon DA, Li W, et al. Overcoming therapeutic resistance in HER2-positive breast cancers with CDK4/6 inhibitors. *Cancer Cell* 2016;29(3):255–69 doi 10.1016/j.ccell.2016.02.006. [PubMed: 26977878]
49. Landis MW, Pawlyk BS, Li T, Sicinski P, Hinds PW. Cyclin D1-dependent kinase activity in murine development and mammary tumorigenesis. *Cancer Cell* 2006;9(1):13–22 doi 10.1016/j.ccr.2005.12.019. [PubMed: 16413468]
50. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998;12(22):3499–511. [PubMed: 9832503]

Translational relevance:

A significant proportion of early stage ER+/HER2+ breast cancer patients relapse with metastatic disease following standard of care treatment with 1 year of trastuzumab and 5 years or longer of endocrine therapy. The phase III ExteNET trial reported improved invasive disease-free survival in patients with ER+/HER2+ breast cancer receiving 'extended adjuvant' treatment with neratinib. We found that in a ER+/HER2+ setting, endocrine therapy alone leads to rapid activation of cyclin D1 regulating survival pathways and thus, combined ER and ERBB blockade is essential to achieve durable cyclin D1 suppression. Our study provides a plausible explanation to the benefit of extended anti-HER2 therapy in treating ER+/HER2+ breast cancers.

Fig. 1. Extended adjuvant therapy with neratinib/fulvestrant prevents recurrence of ER+/HER2+ xenografts.

(A) Nude mice with established MDA-MB-361 xenografts were treated with trastuzumab (20 mg/kg i.p. twice/week) and paclitaxel (15 mg/kg i.p. twice/week) for 4 weeks and then randomized to fulvestrant (5 mg/week s.c.) ± neratinib (40 mg/kg p.o. daily). Number of mice per treatment are shown in parentheses. (B) Representative IHC staining for ERα and P-HER2 in 'complete response' tumors. Scale bars are 100 μm for ERα and P-HER2. (C) H-scores for ERα and P-HER2 (D) Nude mice with established MDA-MB-361 xenografts were treated with trastuzumab (20 mg/kg i.p. twice/week), pertuzumab (20 mg/kg i.p. twice a week) and paclitaxel (15 mg/kg i.p. twice/week) for 4 weeks and then randomized to fulvestrant (5 mg/week s.c.) ± neratinib (40 mg/kg p.o. daily). Number of mice per treatment are shown in parentheses. (E) Representative IHC staining for ERα and P-HER2 in recurrent

tumors from fulvestrant alone arm harvested before or after fulvestrant+neratinib retreatment. Scale bars are 100 μ m for ER α and P-HER2. (F) H-scores for ER α and P-HER2.

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Fig. 2. Combined ER and HER2 blockade potently inhibits proliferation of ER+/HER2+ breast cancer cells.

(A) Immunoblot analysis of cells treated with fulvestrant (1 μ M), neratinib (200 nM), or both under estrogen free conditions for 24 h. (B) Representative images of cells seeded in 24-well plates, treated every 2 days with fulvestrant (1 μ M), neratinib (200 nM), or both under estrogen free conditions. On day 7, monolayers were stained with crystal violet. (C) Quantification of viability on day 7 based on cell counting. Values are mean $\pm$ s.e.m from three independent experiments, Student's *t* test.

Fig. 3. HER2 inhibition results in upregulation of ER transcriptional activity.

(A) ERE reporter activity in cells co-transfected with an ERE-firefly luciferase reporter plasmid and Renilla luciferase plasmid as an internal control. Cells were treated with fulvestrant (1 μ M), neratinib (200 nM), or both for 24 h. Values represent mean $\pm$ s.e.m from three independent experiments, Student's *t* test. (B) Immunoblot analysis of cells treated with fulvestrant (1 μ M), neratinib (200 nM), or both for the indicated times. (C) Relative expression of ER target genes in cells treated with fulvestrant (1 μ M), neratinib (200 nM), or both for 6 h. Values represent mean $\pm$ s.e.m from three independent experiments.

Fig. 4. Combined treatment with neratinib and fulvestrant targets cyclin D1.

(A) Nude mice bearing MDA-MB-361 xenografts were treated for 7 days with fulvestrant (5 mg/week s.c.), or neratinib (40 mg/kg p.o. daily), or both. Number of mice per treatment are shown in parentheses. (B) Representative IHC staining for ERα and p-HER2 in FFPE sections of tumors shown in (A). Scale bars are 100 μm ERα and p-HER2. (C) H-scores for ERα and P-HER2. (D) Gene expression analysis of 196 ER-regulated genes. RNA extracted from tumors shown in (A) was normalized and ran on the nanoString Human Breast Cancer Estrogen Receptor Panel. Genes were compared across treatments using one-way ANOVA

and FDR corrected at 10%. Significantly altered genes plotted as row-standardized Z-scores are visualized with a heatmap. (E) Tile plot depicting *cyclin D1* amplification status in HER2+ breast cancers in TCGA (Cell 2015). Cases are categorized by ER status. (F) *CCND1:CEN11* ratio measured by FISH in the indicated xenografts as described in Methods.

Fig. 5. Combined HER2 and ER blockade is required to suppress cell cycle progression in ER+/HER2+ cells.

(A) Immunoblot analysis of MDA-MB-361 tumors treated with fulvestrant (5 mg/week s.c.), or neratinib (40 mg/kg p.o. daily), or both for 7 days (shown in Fig. 4A). (B) Immunoblot of cells treated with fulvestrant (1 μ M), neratinib (200 nM), or both under estrogen free conditions for 24 h. (C) Relative cyclin D1 mRNA levels in cells treated with fulvestrant (1 μ M), neratinib (200 nM), both, estradiol (1 nM), or neuregulin (10 ng/ml) under estrogen free conditions for 4h. Values represent mean $\pm$ s.e.m from three independent experiments. (D) Representative IHC staining for Ki67 in FFPE sections of tumors shown in Fig. 4A. (E)

H-scores for Ki67 staining (n = 4). (F) Cell cycle analysis of cells treated with fulvestrant (1 μ M), neratinib (200 nM), or both under estrogen free conditions for 24 h. Values represent mean $\pm$ s.e.m from three independent experiments.

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Fig. 6. Cyclin D1 inactivation adds to fulvestrant action against ER+/HER2+ breast cancer cells. (A) Growth assay of cells seeded in a 24 well plate and treated with fulvestrant (1 μM), neratinib (200 nM), palbociclib (1 μM), abemaciclib (500 nM), or indicated drug combinations, under estrogen free conditions. 3 days later, cells were stained with crystal violet and viability was quantified based on crystal violet staining intensity. Values are mean ± s.e.m from three independent experiments, Student's *t* test. (B) Immunoblot analysis of cyclin D1 knockdown efficiency. (C) Growth assay of cells treated with fulvestrant (1 μM), neratinib (200 nM) in the presence or absence of cyclin D1 ablation; After 3 days of treatment, cells were stained with crystal violet and viability was determined based on staining intensity of cell monolayers.