Seribantumab, an Anti-ERBB3 Antibody, Delays the Onset of Resistance and Restores Sensitivity to Letrozole in an Estrogen Receptor–Positive Breast Cancer Model

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Abstract

Heregulin-driven ERBB3 signaling has been implicated as a mechanism of resistance to cytotoxic and antiendocrine therapies in preclinical breast cancer models. In this study, we evaluated the effects of seribantumab (MM-121), a heregulin-blocking anti-ERBB3 monoclonal antibody, alone and in combination with the aromatase inhibitor letrozole, on cell signaling and tumor growth in a preclinical model of postmenopausal breast cancer. In vitro, heregulin treatment induced estrogen receptor–positive (ER⁺) breast cancer. In vitro, heregulin treatment induced estrogen receptor phosphorylation in MCF-7CA cells, and long-term letrozole-treated (LTLT-Ca) cells had increased expression and activation levels of EGFR, HER2, and ERBB3. Treatment with seribantumab, but not letrozole, inhibited basal and heregulin-mediated ERBB receptor phosphorylation and downstream effector activation in letrozole-sensitive (MCF-7Ca) and -refractory (LTLT-Ca) cells. Notably, in MCF-7Ca–derived xenograft tumors, cotreatment with seribantumab and letrozole had increased antitumor activity compared with letrozole alone, which was accompanied by downregulated PI3K/MTOR signaling both prior to and after the development of resistance to letrozole. Moreover, the addition of an MTOR inhibitor to this treatment regimen did not improve antitumor activity and was not well tolerated. Our results demonstrate that heregulin-driven ERBB3 signaling mediates resistance to letrozole in a preclinical model of ER⁺ breast cancer, suggesting that heregulin-expressing ER⁺ breast cancer patients may benefit from the addition of seribantumab to antiendocrine therapy. Mol Cancer Ther; 14(11): 2642–52. ©2015 AACR.

Introduction

Hormone receptor–positive tumors, comprising both estrogen and progesterone receptor–positive (ER/PR⁺) tumors, represent the predominant subtype of breast cancer, accounting for approximately 70% of breast cancer diagnoses (1, 2). The estrogen receptor mediates the effects of estrogen on normal and cancerous breast tissue. Binding of estrogen (17β-estradiol) to the ER induces nuclear localization of this complex. Recruitment to specific DNA-binding sites and subsequent activation of transcription culminates in increased cell proliferation and survival (3). In the context of ER⁺ breast cancer, ER activation drives tumor growth and hence disrupting estrogen activity represents the primary approach to therapy. In postmenopausal women, estrogen biosynthesis occurs predominantly in peripheral tissues under the control of the aromatase enzyme. Aromatase inhibitors (AI), such as letrozole, anastrozole, or exemestane, that block estrogen-mediated ER activation by suppressing estrogen production, have demonstrated clinically meaningful activity and have emerged as the standard therapy for postmenopausal ER⁺ breast cancer (4). Despite the effectiveness of these agents, however, some patients are intrinsically insensitive to AIs and most patients eventually develop resistance to these treatments.

Dysregulated expression of receptor tyrosine kinases (RTK) and their growth factors (ligands) has been implicated in the development of resistance to therapy in breast cancer (5, 6). When mutated or overexpressed, RTKs can act as primary oncogenic drivers of cancer growth, as is the case for HER2 amplification in breast cancer (7, 8). In addition, paracrine-, autocrine-, or juxta-crine-derived growth factors in the tumor microenvironment bind to RTKs, activating a variety of downstream signaling pathways, including the PI3K/AKT/MTOR prosurvival pathway (9). In this way, cancer cells may become desensitized to treatment by exposure to growth factors that activate compensatory signaling pathways (10).

Bidirectional signaling crosstalk has been shown to exist between the ERBB family of RTKs and estrogen receptors (11, 12). The ERBB family of receptors comprises four related transmembrane proteins, EGFR, HER2, ERBB3, and ERBB4, that form homo- or heterodimers with distinct binding affinities for...
specific growth factors (13). Growth factor–induced receptor dimerization stimulates intrinsic receptor kinase activity, resulting in activation of intracellular signaling cascades that regulate and promote a variety of cellular functions, including cell survival and proliferation (14, 15). Although no direct ligand for HER2 has been identified (16), and although ERBB3 has very weak catalytic activity (17–19), the HER2:ERBB3 heterodimer represents the most potent driver of PI3K/AKT signaling and cell proliferation (20, 21). The growth factor heregulin (HRG), which binds directly to ERBB3, stimulates signaling through the HER2:ERBB3 heterodimer and can induce phosphorylation of the estrogen receptor independent of estrogen, leading to ER activation (22). Moreover, HRG expression in breast cancer cells can contribute to cancer progression by activating HER2:ERBB3 signaling through autocrine mechanisms (23, 24). Thus, if HRG-driven HER2:ERBB3 signaling is active in ER+ breast cancer, blocking both estrogen-dependent and HRG-mediated, estrogen-independent signaling may be necessary to fully block tumor progression and provide the maximum benefit to patients.

Computational modeling and sensitivity analysis of the ERBB signaling network previously identified ERBB3 as the key node driving AKT activation following growth factor stimulation (25). On the basis of these findings, seribantumab (MM-121) was developed as a fully human IgG2 antibody that recognizes ERBB3 and blocks HRG binding to the receptor. In addition to preventing HRG-mediated ERBB3 activation, seribantumab also prevents heterodimerization of ERBB3 with other receptors in the ERBB family and induces ERBB3 receptor internalization and degradation.

HER2 and ERBB3 signaling have previously been implicated in the development of resistance to antiestrogens such as tamoxifen, and inhibition of ERBB3 signaling was shown to overcome HER2-mediated tamoxifen resistance in ER+ breast cancer cells (26). More recently, studies using an in vitro mouse model of postmenopausal ER+ breast cancer indicated that the development of resistance to the nonsteroidal AI letrozole was associated with upregulation of HER2 expression in xenograft tumors (27). Here, we used this same model of postmenopausal ER+ breast cancer to determine the effect of blocking HRG-mediated ERBB3 signaling and/or estrogen-mediated ER activation on tumor growth. We found that a combination of seribantumab and letrozole had increased antitumor activity in vivo compared with either agent alone, both before and after the development of resistance to letrozole. This effect was accompanied by decreased signaling through the AKT/MTOR/S6 pathway, suggesting a mechanistic explanation for these observations. Overall, our results suggest that targeting ERBB3 signaling in ER+ breast cancer tumors that express HRG may extend the activity of AIs in postmenopausal breast cancer patients.

Materials and Methods

Materials

Letrozole and everolimus (RAD001) were purchased from Selleck Chemicals, LLC. Androstenedione (Δ4A) was obtained from Sigma Aldrich. Matrigel Basement Membrane Matrix was obtained from BD Biosciences. All antibodies were purchased from Cell Signaling Technology, except for total ERBB3 (Abcam) and pER (EMD Millipore).

Cell culture

MCF-7Ca (human breast cancer cells, which have been stably transfected with human aromatase gene) were provided by Dr. S Chen (City of Hope, Duarte, CA) in 2012. These cells were maintained in DMEM base media (Life Technologies) supplemented with 5% charcoal–dextran–treated FBS (CDT-FBS), 1% penicillin/streptomycin (P/S; Life Technologies) and 700 μg/mL G418 (Life Technologies). Long-term letrozole-treated (LITT-Ca) cells were developed from MCF-7Ca xenograft tumors that had been treated with letrozole (10 μg/day, i.p. injection) for 56 weeks in mice (28). These cells were routinely cultured in phenol red-free Modified IMEM base media (Life Technologies) supplemented with 5% CDT-FBS, 1% P/S, 750 μg/mL G418, and 1 μmol/L letrozole. Both cell lines were maintained in a humidified atmosphere of 5% CO2, at 37°C. All cell lines used in this study were authenticated with short tandem repeat (STR) profile analysis in August 2013 using CellCheck service provided by Idexx Radil. The cell lines were found to be identical to the genetic profile reported for the MCF-7 cell line (ATCC# HTB-22). Frozen aliquots of cell lines were used within 6 months of culturing.

For in vitro assays, cells were seeded in phenol-red free IMEM containing 4% CDT-FBS, 1% P/S and 75 μg/mL G418. Twenty-four hours before treatment initiation, cells were washed in PBS and media was replaced with low serum media (phenol-red free IMEM containing 0.5% CDT-FBS, 1% P/S). Treatments were administered as detailed in the Figure legends.

In vivo studies

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland, Baltimore, MD. Female ovariectomized BALB/c athymic nude mice (4–6 weeks of age) were obtained from the NCI-Frederick Cancer Research and Development Center (Frederick, MD). Mice were housed in a pathogen-free environment under controlled conditions and received food and water ad libitum.

Tumor xenografts were established by subcutaneous injection of 100 μL of a cell suspension consisting of 2.5 × 10⁶ MCF-7Ca cells, diluted 1:1 in Matrigel, into single sites on both flanks of each mouse. Mice were injected daily with Δ4A (100 μg/mouse), a precursor for estrogen synthesis and substrate for aromatase.

Figure 1.

HRG activates ER phosphorylation in MCF-7Ca cells. Serum-starved MCF-7Ca cells were pretreated with seribantumab (Seri; 1 μmol/L) for 1 hour, followed by treatment with 10 nmol/L HRG for 10 minutes, where indicated (+). Cell lysates were analyzed by immunoblotting with antibodies for p-ERBB3 (Y1289), p-AKT (S473), and p-ER (S305) and p-ER (S167). Anti-β-actin antibody was used as a loading control.
Tumor formation was monitored weekly and tumor volumes were calculated following caliper measurement according to the formula \( \frac{4}{3} \pi \left( \frac{\text{radius 1} \times \text{radius 1} \times \text{radius 2}}{2} \right) \), where radius 1 < radius 2. Once the average measured tumor volume had reached approximately 300 mm\(^3\), mice were randomized into groups and treatment was administered. Overall, the average starting tumor volume per group was equivalent across all groups.

For injection, letrozole and \( \Delta^4 \) were prepared in 0.3% hydroxypropylcellulose (HPC) in 0.9% NaCl solution, seribantumab was diluted in 0.9% NaCl solution, and everolimus was diluted in 5% glucose. Drug treatments were administered as indicated in the Figure legends. Following the development of resistance to letrozole, defined as the time point at which the average tumor volume in the letrozole group had doubled from the average starting tumor volume, mice in the letrozole-treated group were rerandomized to various treatment groups, maintaining an equivalent average tumor volume per group. Mouse body weights were measured weekly over the course of treatment and mice were euthanized if their total body weight loss.

**Figure 2.**
Seribantumab, but not letrozole, inhibits basal and HRG-induced activation of ERBB receptors and downstream effectors in MCF-7Ca and LTLT-Ca cells. MCF-7Ca and LTLT-Ca cells were serum-starved (0.5% serum) for 24 hours before treating with seribantumab (1 \( \mu \)mol/L), letrozole (1 \( \mu \)mol/L) or HRG (10 nmol/L) for 2 hours, alone and in combination, as indicated. Cell lysates were analyzed by immunoblotting with antibodies for phosphorylated or total EGFR, HER2, and ERBB3 (A), and phosphorylated or total AKT, ERK, MTOR, S6K1, and ER (B). Anti-\( \beta \)-actin antibody was used as a loading control. The blots show a single representative of two independent experiments.
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surpassed 15%. Tumors were flash-frozen in liquid nitrogen following extraction for pharmacodynamic analyses, where indicated.

Immunoblotting
For in vitro studies, cells were treated as indicated for each experiment, then media was removed, dishes were placed on ice, and cells were washed with ice-cold PBS. To generate cell lysates, M-PER Mammalian Protein Extraction Buffer (Thermo Scientific) supplemented with protease and proteinase inhibitors (Roche) was added and cells were scraped and pipetted into fresh tubes. Cell debris was removed by centrifugation (14,000 rpm for 10 minutes). To generate tumor lysates, flash-frozen tumors were pulverized in a CryoPrep pulverizer (Covaris) and resuspended in T-PER Tissue Protein Extraction Buffer (Thermo Scientific), supplemented with protease and proteinase inhibitors (Roche). Following a 30-minute incubation on ice, cell debris was removed by centrifugation.

Total protein was measured using a BCA assay (Thermo Scientific). Thirty micrograms of protein samples were analyzed by SDS-PAGE and bands were visualized using the Odyssey detection system (LI-COR). Quantitative analyses were performed using ImageStudio (LI-COR), and measurements were corrected for loading control.

HRG ELISA
For detection of HRG1-beta 1 (HRG1-β1) in tumor tissue lysates, Reacti-Bind plates (Thermo Scientific) were coated overnight with recombinant histidine-tagged ERBB3 capture antibody (29), then blocked with 1% BSA (w/v) in PBS before adding cell and tumor lysates. HRG1-β1 was detected with biotinylated goat anti-human NRG1-β (R&D Systems; BAF377) for 2 hours at room temperature followed by streptavidin HRP (Jackson ImmunoResearch) for 30 minutes at room temperature. HRG1-β1 levels were quantified using purified recombinant HRG1-β1 protein (R&D Systems, 377-HB) as a standard. ELISA signal was visualized using One-step Ultra TMB solution (Thermo Scientific) and absorbance was measured at 450 nm.

For detection of HRG1-β1 in media derived from cells in culture, the human NRG1-beta 1/HRG1-beta 1 DuoSet ELISA kit was used (R&D Systems, DY377). Signal was visualized as described for cell and tumor lysates.

Statistical analyses
Statistical analyses were used to evaluate potential differences in tumor growth rates between the various groups of mice treated with different drugs and drug combinations. For in vitro models, tumor growth rates \( G_i \) of a lesion \( i \) were estimated from the measured volume \( V_{obs}^{i} \) of each tumor lesion \( i \) at time \( t \) using the following nonlinear mixed effect model:

\[
V_{it} = V_0 e^{G_i t} + \varepsilon_i
\]

where \( G_{TRT} \) indicates the population average growth rates of each treatment TRT, \( G_i \) indicates the individual growth rates for

Figure 3.
Seribantumab and letrozole cotreatment delays the onset of resistance and restores sensitivity to letrozole in MCF-7Ca xenografts. MCF-7Ca xenograft tumors were generated in female, ovariectomized nude mice, which were randomized to receive vehicle [Control; 0.3% HPC in 0.3% NaCl, twice weekly (Q2W), i.p.; 15 mice/group], seribantumab (750 µg/mouse, Q2W, i.p.; 15 mice/group), letrozole (10 µg/mouse/day × 5 days/week (QD × 5), subcutaneous injection (SQ); 60 mice/group), or letrozole in combination with seribantumab, dosed as indicated for the monotherapies (15 mice/group). Changes in mean tumor volume (±SEM) were determined weekly by caliper measurement. Following the development of resistance to letrozole (week 14), mice in the letrozole-only group were rerandomized into 15 mice/group to receive: letrozole alone, seribantumab alone, or a combination of letrozole and seribantumab. All agents were administered as described above. Mice included for pharmacodynamic analyses were taken from each group at specified time points, and were not included in efficacy study calculations.
Seribantumab treatment blocks ERBB3 activation and downregulates MTOR pathway activation in combination with letrozole. A, MCF-7Ca xenograft tumors (n = 4/treatment group) were harvested 24 hours after one dose of vehicle, letrozole, seribantumab, or letrozole + seribantumab, administered as described in Fig. 3. Tumor lysates were generated and analyzed by immunoblotting with antibodies for phosphorylated or total ERBB3. Anti-\(\beta\)-actin antibody was used as a loading control. Quantified expression, normalized to \(\beta\)-actin, is shown in the dot plots. B, dot plots showing quantified expression (normalized to \(\beta\)-actin) of tumor-derived proteins analyzed by immunoblotting following 24 hour treatment as indicated. Antibodies were for t-MTOR, p-S6K1 (T389), p-S6 (S235/S236) and p-S6 (S240/S244). *P < 0.05 vs. control and vs. letrozole; †P < 0.05 vs. control; ‡P < 0.05 vs. letrozole and vs. seribantumab; §P < 0.05 vs. all other groups.
each tumor lesion (each mouse’s left and right flank of tumor lesions). \( V_{t} \) indicates the tumor volume at time \( t \), and \( V_{0}^{obs} \) indicates the observed tumor volume at time \( t \). Tumor volumes below the limit of detection were treated as censored observations. The resulting \( G \) estimates were compared across treatments and lines of therapy using ANOVA, with the letrozole alone treatment group used as the baseline comparator. The nonlinear mixed-effect modeling approach was implemented in NONMEM version 7.3 and the ANOVA was implemented in JMP version 10.0.2. All \( P \) values < 0.05 were considered statistically significant.

**Results**

**HRG stimulation of ERBB3 promotes ER phosphorylation**

To explore the effect of inhibiting HRG-mediated ERBB3 signaling on ER phosphorylation, we stimulated MCF-7Ca cells in vitro with HRG, in the presence and absence of seribantumab, and measured phosphorylated protein levels by immunoblotting. Treatment with HRG potently stimulated ERBB3 and AKT phosphorylation, as well as ER phosphorylation at serine 167 and serine 305 (Fig. 1), indicating direct cross-talk between the ERBB3 and ER signaling pathways in this cell line. Treatment with seribantumab reduced HRG-stimulated phosphorylation of ERBB3, AKT, and ER in MCF-7Ca cells.

**Seribantumab inhibits basal and HRG-induced receptor activation in MCF-7Ca and LTLT-Ca cells**

Upregulation of HER2 has been implicated as an adaptive mechanism of resistance to letrozole treatment in breast cancer cells (27). To further explore network adaptation to long-term treatment with letrozole leading to resistance, we compared the expression of ERBB receptors and downstream effector proteins in MCF-7Ca and LTLT-Ca cells following treatment with HRG, letrozole and seribantumab. The parental MCF-7Ca cell line displayed low levels and activation states of EGFR, HER2, and ERBB3 receptors. In addition to the previously reported upregulation of HER2 in LTLT-Ca cells (27), we found that total and phosphorylated levels of EGFR and ERBB3 were also increased (Fig. 2A and Supplementary Table S1), potentially implicating these receptors in contributing to letrozole resistance. Treatment with seribantumab alone decreased p-EGFR (Y1068), p-HER2 (Y1196), p-ERBB3 (Y1289), and total ERBB3 (t-ERBB3) levels in MCF-7Ca and LTLT-Ca cell lines in the absence of exogenously added HRG, indicating that HRG-mediated ERBB3 signaling persists in these cells. As the only source of HRG in vitro is autocrine-derived, we measured HRG levels in cell lysates and conditioned media by ELISA and found that HRG was present in both cell lines at low levels (Supplementary Fig. S1). Both cell lines were highly responsive to exogenously added HRG, as indicated by increased levels of p-EGFR, p-HER2, and p-ERBB3. Whereas letrozole had no effect on HRG-induced ERBB3 receptor activation, seribantumab blocked HRG-induced HER2 and ERBB3 receptor phosphorylation.

Given the increase in ERBB receptor activation following the development of resistance to letrozole, we analyzed effector activation downstream of these receptors. LTLT-Ca cells displayed increased levels of p-AKT (S473), p-ERK (T202/Y204), p-MTOR (S2448), p-S6K1 (T389), and p-ER (S167 and S305),
Figure 6.
Full inhibition of tumor cell proliferation and survival in HRG-expressing, ER⁺ tumors requires dual inhibition of HRG/ERBB3 and estrogen/ER signaling. A, ER-driven tumor growth can arise by estrogen-dependent and HRG-dependent, estrogen-independent mechanisms. Estrogen binding to the ER or phosphorylation and activation of ER via non-estrogen-mediated RTK signaling can induce gene transcription and ultimately promote tumor cell proliferation, growth, and survival. (Continued on the following page.)
and reduced levels of t-ERK and t-ER compared with parental MCF-7Ca cells (Fig. 2B and Supplementary Table S1). Addition of exogenous HRG resulted in increased levels of p-AKT, p-ERK, p-S6K1, and p-ER, which were substantially reduced by cotreatment with seribantumab. As before, cotreatment with letrozole did not counteract HRG-mediated activation of these survival signaling-associated proteins. We conclude that, in the presence of HRG, blocking the action of estrogen alone is insufficient to block ER activation and survival signaling.

Seribantumab delays the onset of resistance to letrozole and restores sensitivity to letrozole in letrozole-resistant tumors

The in vitro studies detailed above revealed that MCF-7Ca and LITF-Ca cell lines are both responsive to HRG. We therefore sought to determine whether HRG-induced signaling is active in tumors generated from these cell lines in vivo, and if blocking this signaling pathway affects tumor growth. To do so, we generated MCF-7Ca xenograft tumors in ovariectomized mice and tested the effects of treatment with seribantumab and/or letrozole on tumor growth in vivo. As previously shown, the MCF-7Ca–derived xenograft tumors initially responded to letrozole, but started to develop resistance after approximately 7 weeks of treatment (Fig. 3). When mice were cotreated with letrozole and seribantumab, however, tumor growth was inhibited and resistance to letrozole substantially delayed (Fig. 3 and Supplementary Fig. S2A). This suggests that HRG/ERBB3 signaling was either present at the start of the study or developed relatively quickly in response to letrozole treatment. Once resistance to letrozole was clearly established (week 14), mice in the letrozole-treated group were randomized to one of three cohorts: (i) continued letrozole monotherapy; (ii) seribantumab monotherapy; or (iii) seribantumab in combination with letrozole (Fig. 3). Notably, the letrozole-resistant tumors displayed significantly decreased tumor growth when cotreated with letrozole and seribantumab compared with treatment with either drug alone (Fig. 3 and Supplementary Fig. S2B). This is consistent with the hypothesis that blocking both estrogen/ER- and HRG/ERBB3-driven signaling provides greater antitumor activity than blocking either pathway alone.

Xenograft tumors harvested 24 hours after initiating treatment with letrozole and/or seribantumab were analyzed to determine the effects of treatment on cellular signaling. Treatment with seribantumab, alone or in combination with letrozole, resulted in a substantial decrease in total and phosphorylated levels of ERBB3, consistent with the known mechanism of action of seribantumab (Fig. 4A). Cotreatment with seribantumab and letrozole also led to decreased levels of t-MTOR, p-S6K1, and p-S6 levels compared with the other treatment groups (Fig. 4B), indicating an enhanced inhibitory effect of combination treatment on this well-established survival signaling pathway. HRG was detected in all tumor samples and no significant differences were observed between the treatment groups harvested at various time points after treatment initiation (Supplementary Fig. S3A). In addition, pERBB3 expression was elevated at the end of the study outlined in Fig. 3 following letrozole treatment (Supplementary Fig. S3B). The elevated pERBB3 levels are decreased when seribantumab treatment is provided. On the basis of these results, we attribute the response to seribantumab to an increased sensitivity to HRG rather than an increase in HRG expression.

The antitumor activity of seribantumab and letrozole is not enhanced by an MTOR inhibitor

As cotreatment with seribantumab and letrozole substantially affects MTOR signaling, we sought to determine (i) whether blocking this resistance pathway at a downstream node (MTOR) would prove more or less effective than blocking it at the receptor level; and (ii) if dual pathway blockade at HRG/ERBB3 and MTOR would prove even more effective at restoring sensitivity to letrozole. Everolimus (RAD001), a specific inhibitor of MTOR, was selected for these studies as it is currently approved by the Food and Drug Administration for the treatment of postmenopausal women with advanced hormone receptor positive, HER2-negative breast cancer in combination with the aromatase inhibitor, exemestane based on a randomized Phase III clinical trial (30). MCF-7Ca tumor cells were inoculated into ovariectomized nude mice and, following the development of tumors, the mice were randomized to receive letrozole, alone or in combination with seribantumab. As before, cotreatment with letrozole and seribantumab significantly inhibited tumor growth compared with letrozole alone (P < 0.05; Fig. 5 and Supplementary Fig. S4), confirming our previous findings. Following the development of resistance to letrozole (week 16), mice in the letrozole-treated group were randomized to receive one of four treatment options: (1) letrozole alone; (2) letrozole in combination with seribantumab; (3) letrozole in combination with everolimus; and (4) letrozole in combination with both seribantumab and everolimus. Interestingly, treatment with either seribantumab or everolimus in combination with letrozole led to tumor regression. Seribantumab exhibited slightly more activity in combination with letrozole than everolimus, but this effect was not significant (P > 0.05). Notably, cotreatment with letrozole and everolimus induced increased tumor activation of ERBB3 (Supplementary Fig. S5), suggesting that ERBB3 signaling may act as an escape route to MTOR inhibition in the context of antidiocrine therapy. In addition, adding everolimus to the combination of letrozole and seribantumab did not increase antitumor activity and was more toxic, as evidenced by significantly reduced mouse body weights at 4 weeks after treatment (P < 0.05; Supplementary Fig. S6).

(Continued)
In this study, we used a preclinical model of postmenopausal breast cancer to investigate the role of ERBB3 signaling in mediating resistance to the AI letrozole. Long-term exposure of MCF-7Ca cells to letrozole induced resistance to letrozole and was accompanied by an increase in the total and phosphorylated levels of EGFR, HER2, and ERBB3 (Fig. 2A). This is consistent with a broad range of studies associating ERBB receptor activation with acquired drug resistance (5, 31–34), and with clinical data showing that expression levels of EGFR, HER2, and ERBB3 are associated with impaired overall survival in breast cancer patients (35, 36). Both parental and letrozole-resistant MCF-7Ca cells are responsive to HRG in vitro, as evidenced by phosphorylation of EGFR, HER2, ERBB3, and downstream proteins in the MAPK and AKT signaling pathways (Fig. 2B). In addition, the HRG induces activation of ER in an estrogen-independent manner (Fig. 1), and letrozole alone is unable to offset these effects (Fig. 2B). In contrast, the HRG-blocking anti-ERBB3 antibody seribantumab effectively blocks HRG-induced pathway activation. When implanted in ovariectomized mice, MCF-7Ca-derived xenografts acquire resistance to letrozole after 7 to 14 weeks. Resistance is delayed, however, when mice are cotreated with seribantumab, and resistance is reversed when seribantumab is coadministered with letrozole after tumors acquire resistance to letrozole (Fig. 3). Consistent with our in vitro findings and with those of Linn and colleagues (9), molecular analyses of in vivo–treated tumors implicate downregulation of the MTOR/S6 signaling axis as the key mechanism underlying tumor growth inhibition following dual inhibitor treatment (Fig. 4). Although seribantumab inhibited both AKT and ERK signaling in vitro, either alone or in combination with letrozole, the same inhibitory effects were not observed in our in vivo experiment. This discrepancy likely arises from a difference in timing; tumors were acquired 24 hours after dosing mice in our in vivo experiment.

Consistent with the observed downregulation of the MTOR/S6 signaling axis by seribantumab in vivo, everolimus was also able to reverse resistance to letrozole in MCF-7Ca–derived xenografts (Fig. 5). The combination of seribantumab and letrozole, however, appeared to have higher antitumor activity than the combination of everolimus and letrozole. The dose of everolimus used in this study was the highest dose that displayed antitumor activity in MCF-7Ca xenografts without inducing weight loss when given as a monotherapy, based on a dose finding study in female ovariectomized BALB/c athymic nude mice. Although the difference between seribantumab and everolimus was not statistically significant, the observed trend suggests that blocking HRG-mediated ERBB3 signaling at the receptor level may result in more potent inhibition of resistance than targeting MTOR directly. Notably, targeting both nodes in combination with letrozole (triple inhibition) was toxic in mice (Supplementary Fig. S6).

In our initial in vivo experiment, we observed increased tumor growth near the very end of the studies following long-term coadministration of seribantumab and letrozole (Fig. 3). This suggests that alternative molecular pathways of escape may also arise that drive tumor growth independent of HRG and ER inhibition. This is consistent with the signaling redundancy that exists among RTKs and that allows for compensatory signaling upon targeted inhibition (10, 37, 38). Identifying these compensatory signaling mechanisms remains the focus of future studies.

Our study, and those of others (39), implicate HRG-driven ERBB3 signaling as an important mechanism of resistance to conventional endocrine therapy. The overall model that emerges is summarized in Fig. 6. In ER$^+$ breast cancers in which HRG is expressed, ER stimulates tumor cell proliferation in both an estrogen-dependent and HRG-dependent, estrogen-independent fashion (Fig. 6A). In addition, HRG stimulates prosurvival signaling through the PI3K/AKT/MTOR pathway. Inhibition of estrogen-dependent ER signaling through the action of AIs like letrozole is insufficient to block HRG-mediated compensatory signaling (Fig. 6B). Dual blockade of these pathways can be achieved through coadministration of seribantumab and letrozole (Fig. 6C). This suggests that patients with ER$^+$ breast cancer whose tumors also express HRG could potentially benefit from the addition of seribantumab to their standard endocrine therapy. This hypothesis was recently tested in a global, double-blinded, placebo-controlled Phase II study of postmenopausal women with locally advanced or metastatic ER/PR$^+$, HER-2-negative breast cancer (ClinicalTrials.gov identifier: NCT01151046; ref. 40). Patients ($N = 115$) were randomized to receive either seribantumab or placebo in combination with the steroidal AI exemestane. Although the addition of seribantumab to exemestane did not significantly prolong progression-free survival (PFS) in the unselected population, a subset of patients (45%) with high levels of tumor HRG mRNA was identified who benefited from the combination of seribantumab and exemestane relative to placebo and exemestane (hazard ratio of 0.26, $P = 0.003$; ref. 41).

These data are also in agreement with Phase II clinical trials in platinum-resistant ovarian cancer (ClinicalTrials.gov identifier: NCT01447706) and EGFR wild-type non–small cell lung cancer (ClinicalTrials.gov identifier: NCT00994123), where tumor HRG mRNA levels were found to be the principal biomarker for seribantumab activity (42, 43).

In conclusion, these data provide further evidence that HRG-driven ERBB3 signaling mediates resistance to AI-based therapy in postmenopausal ER$^+$ breast cancer. Our in vivo data show that seribantumab can delay the onset of resistance and restore sensitivity to an aromatase inhibitor in an AI-sensitive model of ER$^+$ breast cancer. These and other results provide the molecular and biologic basis for the increased PFS observed in patients with high HRG mRNA levels receiving seribantumab in combination with exemestane compared with those receiving exemestane alone. We submit that continued efforts to identify mechanisms of resistance to therapy, along with their biomarkers, will extend the effectiveness of existing therapies and provide an armamentarium of treatment options for patients with breast cancer.
Disclosure of Potential Conflicts of Interest

G. MacBeath has ownership interest (including patents) in Merrimack Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: M.D. Curley, G.J. Sabnis, L. Wille, G. Garcia, V. Moyo, A. Brodie, G. MacBeath

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Grant Support

This research was funded by Merrimack Pharmaceuticals. A. Brodie received research support from Merrimack Pharmaceuticals.

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Received February 25, 2015; revised August 17, 2015; accepted August 20, 2015; published OnlineFirst August 26, 2015.

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Seribantumab, an Anti-ERBB3 Antibody, Delays the Onset of Resistance and Restores Sensitivity to Letrozole in an Estrogen Receptor–Positive Breast Cancer Model


Mol Cancer Ther 2015;14:2642-2652. Published OnlineFirst August 26, 2015.

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