Activation of AR Sensitizes Breast Carcinomas to NVP-BEZ235's Therapeutic Effect Mediated by PTEN and KLLN Upregulation

Yu Wang1, Qi Yu1, Xin He1, Todd Romigh1, Jessica Altemus1, and Charis Eng1,2,3,4,5

Abstract

NVP-BEZ235 is a newly developed dual PI3K/mTOR inhibitor, being tested in multiple clinical trials, including breast cancer. NVP-BEZ235 selectively induces cell growth inhibition in a subset, but not all, breast cancer cell lines. However, it remains a challenge to distinguish between sensitive and resistant tumors, particularly in the pretreatment setting. Here, we used ten breast cancer cell lines to compare NVP-BEZ235 sensitivity and in the context of androgen receptor (AR) activation during NVP-BEZ235 treatment. We also used female SCID mice bearing breast tumor xenografts to investigate the beneficial effect of dihydrotestosterone/NVP-BEZ235 combination treatment compared with each alone. We found that AR-positive breast cancer cell lines are much more sensitive to NVP-BEZ235 compared with AR-negative cells, regardless of PTEN or PI3KCA status. Reintroducing AR expression in NVP-BEZ235 nonresponsive AR-negative cells restored the response. DHT/NVP-BEZ235 combination not only resulted in a more significant growth inhibition than either drug alone, but also achieved tumor regression and complete responses for AR+/ER+ tumors. This beneficial effect was mediated by dihydrotestosterone (DHT)-induced PTEN and KLLN expression. Furthermore, DHT could also reverse NVP-BEZ235–induced side effects such as skin rash and weight loss. Our data suggest that AR expression may be an independent predictive biomarker for response to NVP-BEZ235. AR induction could add benefit during NVP-BEZ235 treatment in patients, especially with AR+/ER+ breast carcinomas.

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Introduction

The PI3K/AKT/mTOR pathway is mainly involved in the regulation of cell growth and survival in tumorigenesis. The PI3K/AKT/mTOR inhibitors (PI3K/AKT/mTORi) have shown some promise in clinical trials for patients with cancer. However, long-term inhibition of the PI3K/AKT/mTOR signaling pathway could lead to drug resistance and apparent failure of the therapeutic trials (1–3). NVP-BEZ235 is a newly developed dual PI3K/mTOR inhibitor, currently being tested in multiple clinical trials for its antiproliferative effects in cancer. Enzymatic evidence shows that NVP-BEZ235 inhibits phosphoinositide 3-kinase (PI3K) pathway activity at doses as low as 100 nmol/L; however, a much higher dose was found to be required to suppress tumor cell growth (4, 5). It has also been shown that NVP-BEZ235 selectively inhibits proliferation and induces cell death in a subset, but not all, breast cancer cell lines (4, 6), fueling the urgency to find a predictive biomarker(s) of each patient’s response to this class of drug.

Unaromatizable androgens, such as dihydrotestosterone (DHT), inhibit tumor proliferation and induce apoptosis by activating androgen receptor (AR) in AR+/ER+ breast cancers (7–9). Among all female breast carcinomas, 60% to 70% are AR positive. Maintaining AR expression in patients with breast cancers has been reported to be significantly associated with increased survival and may be used as a marker of good prognosis (10, 11). The tumor suppressor gene PTEN (deleted on chromosome 10) is one of the most frequently mutated or deleted genes in inherited and sporadic human cancers, including breast carcinomas (12). We have previously shown that AR is positively correlated with PTEN expression in breast carcinomas, which is due to the direct activation of PTEN transcription, mediated by an androgen response element (ARE) in the PTEN promoter (9, 13).

Here, we sought to address the hypothesis that depending on the AR expression status, different subgroups of breast cancers differentially respond to NVP-BEZ235 treatment at low doses, whereby AR-positive tumors are much more sensitive than AR-negative tumors. We...
further hypothesize that combining DHT and NVP-BEZ235 could achieve maximum therapeutic effects in AR+/ER+ breast cancer while decreasing the side effects from this compound.

Materials and Methods

Cell culture and treatments
Breast cancer cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, MCF-7, BT-474, HCC1395, BT20, MDA-MB-436, SKBR3, and AU565 cells were purchased from and authenticated (between 2009 to 2012) by short tandem repeat (STR) analysis by the American Type Culture Collection (ATCC). The MDA-MB-231 cell line was reauthenticated by STR in 2013 (DCC Medical, Fairfield, OH). All cell lines were resuscitated from low passage and all the experiments were performed with cells that were passaged in the laboratory for less than 6 months after receipt or resuscitation. Cells were monitored by microscopy and confirmed to maintain their original morphology. Cell molecular profiles, including AR, estrogen receptor (ER), and PTEN expression, were tested by Western blot analyses and are consistent with those in the existing literature (6, 9). Cultures were cultivated in “complete medium” per ATCC recommendation, with 10% heat-inactivated FBS, 50 IU/mL penicillin, and 50 μg/mL streptomycin. DHT is from Steraloids. NVP-BEZ235 is a kind gift from Novartis. Control siRNA is a pool of four scrambled nonspecific siRNA (sc-37007, Santa Cruz Biotechnology). ON-TARGETplus Human AR siRNA (LU-003400-00-0005) is from Thermo Fisher Scientific.

MTT assay
Cell growth rates were estimated by MTT assay. Fifteen thousand cells were seeded in 24-well plates 24 hours before the assay. “Day 0” point was measured at the time of drug treatments. After adding drugs to the culture medium, MTT assay was conducted on days 1, 3, and 5. To measure cell growth rate, each well was incubated with 25 μL of 5 mg/mL MTT for 1 hour in a CO2 incubator at 37°C. The medium was aspirated and 0.5 mL dimethyl sulfoxide (DMSO) was added per well. After a 10-minute incubation, 200 μL per well were transferred to a 96-well plate and proliferation rates were measured by colorimetric assay of formazan intensity in a plate reader at 560 nm.

Clonogenic assay
Breast cancer cells were trypsinized and single-cell suspensions of 1,000 cells were seeded into 100 mm culture dishes. Cells were treated with vehicle (0.1% DMSO), DHT, NVP-BEZ235, or the DHT/NVP-BEZ235 combination. After 7 days, colonies were fixed in 100% methanol for 15 minutes at room temperature and stained with 10% Giemsa (Gibco). Dishes were washed with water and visible colonies containing approximately 50 or more cells were counted.

Western blotting
Western blotting was performed as described elsewhere (1). Mouse monoclonal anti-AI is from Santa Cruz Biotechnology. Mouse monoclonal anti-tubulin antibody is from Sigma-Aldrich. Mouse monoclonal anti-PTEN antibody is from Cascade Biosciences. Rabbit polyclonal anti-phospho-AKT (Ser473), total AKT, N-cadherin, E-cadherin, vimentin, and occludin antibodies are from Cell Signaling Technology.

Promoter activity assay
AR activity assay was measured by dual luciferase assay as described previously (1). AR transcriptional activity was measured by Cignal report assay kit (SA Bioscience) as per the manufacturer’s instructions.

Mouse xenograft assay
MCF-7 and MDA-MB-231 cells were injected (5 × 10⁶ cells in 50 μL Matrigel and 50 μL medium mix, BD Bioscience) subcutaneously into both flanks of 4- to 6-week-old female NOD/SCID mice (Jackson Laboratory). The take rates for MCF-7 and MDA-MB-231 were 90.4% and 88.0%, respectively. Tumors were measured twice a week and volume was calculated as 0.5 × (L × W²) mm³. Depending on the cell lines, the average tumor size reached 100 mm³ after 2 to 3 weeks, and mice carrying xenografts were randomly separated into control and DHT groups matched by body weight. Mice assigned to the DHT group were implanted with DHT pellets (10 mg/90 days; Innovative Research of America). We used sesame oil as the vehicle and delivered NVP-BEZ235 by gavage at doses of 1 mg/kg/d, 5 mg/kg/d, 10 mg/kg/d, 25 mg/kg/d, or 50 mg/kg/d. After 5 weeks of treatment, mice were sacrificed. Tumors are weighed, fixed, and dissected for immunohistochemistry and quantitative real-time PCR (qRT-PCR). The Cleveland Clinic Animal Care and Use Committee approved the protocol for animal experiments conducted at the Cleveland Clinic (Cleveland, OH).

Immunohistochemistry
Breast cancer xenograft samples were rehydrated in xylene and a series of graded ethanol. Antigen retrieval was performed with 0.01 mol/L citrate buffer at pH 6.0 for 20 minutes in a 121°C pressure chamber. Slides were allowed to cool for another 30 minutes, followed by sequential rinsing in phosphate-buffered saline (PBS)-T (0.01% Triton X). Endogenous peroxidase activity was quenched by incubation in PBS-T containing 5% hydrogen peroxide. Each incubation step was carried out at room temperature and was followed by three sequential washes in PBS-T. Sections were incubated in rabbit polyclonal anti-KLLN antibody (Abgent) diluted in PBS-T containing 5% goat serum albumin, followed by incubations with biotinylated secondary antibody for 1 hour, peroxidase-labeled streptavidin (Vectastain system, Vector Laboratories) for 1 hour, and diaminobenzidine substrate for peroxidase-based immunohistochemistry (Cardussian

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AR Sensitizes Breast Carcinomas to NVP-BEZ235

DAB Chromogen, Biocare Medical). Slides were counterstained with hematoxylin (Vector Laboratories) and mounted.

Results

AR-positive breast cancer cells are sensitive to NVP-BEZ235 treatment

Previous studies have shown that at high doses, NVP-BEZ235 inhibits tumor cell proliferation in a subset of breast cancer cell lines (5, 6, 14). The mechanism by which this series of cancer cells differentially responded to NVP-BEZ235 is still unclear. To elucidate the difference between NVP-BEZ235-sensitive cells and -resistant cells, we first treated ten different breast cancer cell lines with increasing doses of NVP-BEZ235. Four of the breast cancer cell lines (MCF-7, MDA-MB-453, SKBR3, and BT474) are sensitive to NVP-BEZ235 and responded to the minimum (1 nmol/L) dose (Fig. 1A). In this group, all four cell lines showed a reduction in cell number below the initial count with 25 nmol/L NVP-BEZ235 (Fig. 1A). In contrast, the remaining six cell lines were relatively insensitive to NVP-BEZ235 (Fig. 1B). It required a much higher dose to inhibit cell growth and NVP-BEZ235 did not induce cell death in these six lines (Fig. 1B). To characterize the molecular differences between these two groups of cell lines, we first listed the cell lines’ molecular status and subtypes (Table 1; refs. 15–18). A previous study suggested that NVP-BEZ235 induces cell death in breast cancer cells with PIK3CA mutations or HER2 amplification, whereas all of the PTEN deleted, mutated, or silenced cell lines are insensitive to NVP-BEZ235 (5). Here, we noticed that the sensitive MCF-7, MDA-MB-453, SKBR3, and BT474 cells are all AR positive, whereas the insensitive cell lines are AR negative (Table 1). These correlative observations led us to compare NVP-BEZ235’s growth inhibition of 50% (GI50) values in these ten breast cancer cell lines. All four AR-positive breast cancer cell lines have much lower GI50 (mean = 5.67 nmol/L) compared with AR-negative cells (mean = 34.32 nmol/L; P < 0.001; Fig. 1C). Our data suggest that maintaining AR expression might be important for NVP-BEZ235 response in breast cancer cells.

AR activation enhances NVP-BEZ235 response in AR-positive breast cancer cells

To examine whether AR indeed sensitizes breast cancer cells to NVP-BEZ235, we used DHT to activate AR and anti-AR siRNA to knock down AR in these lines. In AR+/ER/C0 breast cancer cells, DHT or NVP-BEZ235 alone can inhibit cell growth (Fig. 2A). Combining DHT during NVP-BEZ235 treatment not only resulted in a more significant suppressive effect to cell proliferation, but also reduced cell density below their starting levels (Fig. 2A). This combination effect is synergistic with the combination index less than 1 (Supplementary Fig. S1). In contrast, knocking down AR expression by siRNA promoted cell growth and desensitized the cells to NVP-BEZ235 treatment (Fig. 2A).

Next, we used a clonogenic assay to investigate whether DHT enhances NVP-BEZ235’s suppressive effect on tumor cell clonal formation. After 7 days of treatment, tumor cell clonogenic formation was decreased significantly by either DHT (50%) or NVP-BEZ235 (41%) alone in MCF cells, whereas the combination of DHT and NVP-BEZ235 completely abolished the breast cancer cells’ clonogenic ability (Fig. 2B). In addition, the presence of DHT sensitized cells to NVP-BEZ235 and significantly decreased GI50 in AR+/ER+/C0 (MCF-7 and BT474) cells (Fig. 2C). Interestingly, this beneficial effect was not observed in AR+/ER– (MDA-MB-453 and SKBR3) cells (Fig. 2C). MCF-7 cells grew in tightly packed cobblestone-like clusters, which is a typical characteristic of epithelial cells, whereas 25% serum promoted cell scattering and spindle-like transformation (Fig. 2D). DHT, but not NVP-BEZ235, reversed these morphologic changes in monoculture cultures, suggesting that AR might suppress epithelial-to-mesenchymal transitions (EMT) in MCF-7 cells (Fig. 2D). We also observed significant amount of cell death during DHT/NVP-BEZ235 combination treatment (Fig. 2D). We then evaluated EMT by examining the expression patterns of epithelial and mesenchymal markers. DHT decreased the mesenchymal markers vimentin and N-cadherin, and increased the epithelial markers E-cadherin and occludin (Supplementary Fig. S2). Collectively, these morphologic, biochemical, and cell-biologic observations suggest that activation of AR inhibits EMT and sensitizes response to NVP-BEZ235 in AR+/ER+ breast cancer cells.

Reexpression of AR enhanced NVP-BEZ235 response in AR-negative breast cancer cells

Our data above show that AR-positive breast cancer cells are sensitive to NVP-BEZ235 and activation of AR by DHT further sensitizes AR+/ER+ breast cancer cells to NVP-BEZ235-induced growth inhibition. Such DHT/BEZ235 combination, compared with the same concentrations of NVP-BEZ235 agent alone, did not provide additional beneficial effect on growth inhibition in AR+/ER+ and AR+/ER– breast cancer cell lines (Supplementary Fig. S3). Therefore, we hypothesized that activation of the AR pathway is a key factor in maintaining cell response to NVP-BEZ235. To test this hypothesis, we reintroduced AR into AR-negative breast cancer cell lines. In all six AR-negative cell lines that showed drug resistance to NVP-BEZ235 at 2 nmol/L dose, reexpression of AR inhibited cell growth, regardless of ER status (Fig. 3A). After AR reexpression, 2 nmol/L NVP-BEZ235 could further suppress cell growth, suggesting that presence of AR restored drug sensitivity in these cells (Fig. 3A). We also noticed that in MDA-MB-468, BT20, AU565, and MDA-MB-436 cells, NVP-BEZ235 treatment after AR reexpression decreased cell density below their starting levels (Fig. 3A). These observations not only indicated a restored drug response, but also suggested resultant cell death that was not achieved by AR expression alone or NVP-BEZ235 exposure alone. Reexpression of AR also
Figure 1. AR-positive breast cancer cells are sensitive to NVP-BEZ235 treatment compared with AR-negative cells. A, four AR-positive breast cancer cell lines were treated with control or NVP-BEZ235 for 5 days. Cell growth rates were measured by MTT assay. Each data point represents a mean value of three independent experiments and the error bars indicate the SD, unless otherwise indicated. B, the remaining six breast cancer cell lines that are AR negative were treated with control or NVP-BEZ235 for 5 days. Cell growth rates were measured by MTT assays. C, GI50 values of NVP-BEZ235 in breast cancer cell lines treated with 5 days of treatment.
significantly reduced the GI50 of NVP-BEZ235 in all six AR-negative cell lines (Fig. 3B). The reexpressed AR showed appropriate nuclear localization and inhibited cell growth, whereas combining additional NVP-BEZ235 resulted in significant cell death (Fig. 3C). Thus far, our data show that reexpression of AR in AR-negative breast cancer cells not only suppressed cell growth, but also restored cell responsiveness to NVP-BEZ235.

Table 1. Subtypes and molecular status of breast cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Subtype</th>
<th>Expression</th>
<th>Mutation</th>
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<tbody>
<tr>
<td>MCF-7</td>
<td>Luminal A</td>
<td>++ ++ ++</td>
<td>PTEN</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>Unclassified</td>
<td>++</td>
<td>HER2</td>
</tr>
<tr>
<td>SKBR3</td>
<td>HER2</td>
<td>++</td>
<td>PTEN</td>
</tr>
<tr>
<td>BT-474</td>
<td>Luminal B</td>
<td>++ ++ ++</td>
<td>PTEN</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>TN</td>
<td>++ ++ ++ +</td>
<td>PI3KCA</td>
</tr>
<tr>
<td>BT20</td>
<td>Basal</td>
<td>++ ++ ++</td>
<td>p53</td>
</tr>
<tr>
<td>MDA-MB-436</td>
<td>TN</td>
<td>++ ++ ++ +</td>
<td>PI3KCA</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>TN</td>
<td>++ ++ ++ +</td>
<td>PI3KCA</td>
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<tr>
<td>AU565</td>
<td>HER2</td>
<td>++ ++ ++</td>
<td>PI3KCA</td>
</tr>
<tr>
<td>HCC1395</td>
<td>Basal</td>
<td>++ ++ ++</td>
<td>PI3KCA</td>
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Abbreviation: TN, triple negative.

A) NVP-BEZ235 sensitive with cell death.

DHT/NVP-BEZ235 combination results in better therapeutic effect for breast cancer

To examine whether DHT benefits NVP-BEZ235 treatment in vivo, we generated MCF-7 (AR wt) and MDA-MB-231 (AR-) xenograft models in female nude mice. Mice carrying control or DHT capsules received NVP-BEZ235 of 0, 1, 5, 10, 25, or 50 mg/kg/d. After 4 weeks, DHT inhibited MCF-7 xenograft growth by 50% (Fig. 4A and D).
Supplementary Fig. S4). NVP-BEZ235 suppressed tumor growth at 5 mg/kg/d and higher doses (Fig. 4A and Supplementary Fig. S4). The combination of DHT and NVP-BEZ235 not only inhibited tumor growth more effectively than either drug alone, but also reduced tumor size to below that at their starting point (Fig. 4A and Supplementary Fig. S4). More importantly, 1 mg/kg/d NVP-BEZ235, without tumor-suppressive effects by itself, was able to induce tumor regression in the presence of DHT (Fig. 4A). In AR-negative MDA-MB-231 xenografts, it required 50 mg/kg/d NVP-BEZ235 to achieve 50% inhibition of tumor growth, and additional DHT did not benefit tumor suppression due to the lack of AR (Fig. 4B).

Although high doses of NVP-BEZ235 significantly suppressed tumor growth, this single drug alone could not achieve a complete response or a partial response (Table 2). After combined DHT and NVP-BEZ235 (50 mg/kg/d) in AR-positive xenografts, we observed responses in all tumors, including complete responses in 36% of tumors and partial response in 64% of tumors (Table 2). Even the combination of DHT and a “noneffective” dose of NVP-BEZ235 (1 mg/kg/d) can achieve complete responses in 13% of tumors and partial response in 63% of tumors (Table 2).

Next, we intend to elucidate the mechanism of DHT’s beneficial effects during NVP-BEZ235 treatment in AR-negative breast cancer cells. A reexpression of AR restored NVP-BEZ235 response in AR-negative breast cancer cells. A, six AR-negative cell lines were transfected with control or AR, and treated with 2 nmol/L NVP-BEZ235. Cell growth rates were assessed by MTT assay. B, GI50 values of NVP-BEZ235 for AR-negative breast cancer cell lines transfected with control or AR. C, morphologic changes of AU565 cells after DHT or NVP-BEZ235 treatment.

Figure 3. Reexpression of AR restored NVP-BEZ235 response in AR-negative breast cancer cells. A, six AR-negative cell lines were transfected with control or AR, and treated with 2 nmol/L NVP-BEZ235. Cell growth rates were assessed by MTT assay. B, GI50 values of NVP-BEZ235 for AR-negative breast cancer cell lines transfected with control or AR. C, morphologic changes of AU565 cells after DHT or NVP-BEZ235 treatment.
AR+/ER+ breast cancer. Earlier studies demonstrated that NVP-BEZ235 inhibits the activity of the PI3K/AKT/mTOR pathway at high doses (4, 5). Surprisingly, we found that NVP-BEZ235, at low doses, decreases AR activity and suppresses AR expression in AR+/ER+ breast cancer (Fig. 4C and E, second row). DHT successfully reversed NVP-BEZ235–induced AR suppression by increasing AR expression and promoting AR nuclear localization, representing reactivation of the AR pathway after NVP-BEZ235 treatment (Fig. 4E, second row). PTEN is a well-known tumor suppressor that plays roles in growth inhibition and apoptosis in breast cancer. KLLN is a newly identified tumor suppressor, inhibiting proliferation and inducing apoptosis (19–22). KLLN and PTEN share the same promoter region but are transcribed in opposite directions. Our previous studies have shown that AR induces the expression of both PTEN and KLLN in breast cancers by direct promoter activation (9, 22). NVP-BEZ235–induced AR suppression resulted in decreased expression of both PTEN and KLLN in AR+/ER+ breast cancer cells (Fig. 4D). Tumor tissues without functioning AR expressed very low levels of PTEN and
KLN (Fig. 4E, third and fourth row). DHT, but not NVP-BEZ235, significantly increased the expression of PTEN and KLN (Fig. 4E). NVP-BEZ235 or DHT decreased tumor cell density in AR-positive xenografts and the combination of DHT and NVP-BEZ235 almost completely eradicated all cancer cells (Fig. 4E, top). DHT or NVP-BEZ235 single compound treatment was associated with decreased Ki-67 expression, indicating the inhibitory effect on cancer cell proliferation (Fig. 4E, fifth row). The DHT/NVP-BEZ235 combination resulted in a further decrease of Ki-67 expression in the remaining tumor tissues (Fig. 4E). More importantly, DHT/BEZ235 combination, but not single agent treatment, induced a dramatic increase of apoptosis in AR⁺/ER⁺ tumor xenograft (Supplementary Fig. S5). We did not observe changes in mTOR or PI3K protein expression levels upon DHT, NVP-BEZ235, or combination treatment (Supplementary Fig. S5). Although NVP-BEZ235 suppresses AKT phosphorylation at higher concentrations, it actually increases the levels of AKT phosphorylation (Ser473) at low doses (Supplementary Fig. S6A). Similar adverse effects have been shown among other mTOR inhibitors, such as RAD001 and rapamycin (1, 3). Combining DHT with NVP-BEZ235 effectively prevents this elevation in AKT phosphorylation (Supplementary Fig. S6B). Taken together, our in vitro and in vivo data showed that combining DHT and NVP-BEZ235 achieves a better therapeutic effect in breast cancer than single drug exposure.

MYC is a well-known AR target gene, which is differentially regulated by AR in a tissue-specific manner. The transcription of MYC is promoted by AR in prostate cancers, whereas AR suppresses MYC expression in female breast carcinomas (23, 24). Recent studies show that long-term NVP-BEZ235 treatment upregulates MYC expression in breast cancer cells, leading to NVP-BEZ235 resistance (25, 26). Therefore, we postulated that the presence of DHT during NVP-BEZ235 treatment could effectively block NVP-BEZ235-induced MYC upregulation in breast cancers. In MCF-7 xenografts, NVP-BEZ235 suppressed tumor growth, however, any remaining tumor cells showed increased MYC expression and increased MYC nuclear localization (Fig. 4E, bottom row). This upregulation of MYC is at the transcriptional level, as MYC mRNA increased 3-fold after NVP-BEZ235 treatment (Supplementary Fig. S6C). Immunohistochemistry and qRT-PCR demonstrated that DHT not only decreases MYC expression, but also prevents NVP-BEZ235–induced MYC upregulation, resulting in further tumor regression in breast cancers (Fig. 4E). In AR-negative MDA-MB-231 xenografts, NVP-BEZ235 decreased tumor density by 23% and the beneficial effect from DHT was not observed because of the absence of AR expression (Fig. 4F). Thus far, we conclude that DHT effectively prevents NVP-BEZ235–induced drug resistance and also enhances NVP-BEZ235 treatment outcome in AR⁺/ER⁺ breast cancers mediated by several mechanisms.

DHT reduces side effects during NVP-BEZ235 treatment

Another mTOR inhibitor, rapamycin, has been shown to inhibit hair growth mediated by cyclosporin A and FK506 (27–30). During the 4-week NVP-BEZ235 treatment, we observed hair loss in the head, neck, and front torso areas in mice that received 10 mg/kg/d or higher doses, with the most severe side effects found in the 50 mg/kg/d group (Supplementary Fig. S7A). Surprisingly, combining DHT during the NVP-BEZ235 treatment prevented the hair loss (Supplementary Fig. S7A). Multiple studies have reported that high doses of NVP-BEZ235 resulted in weight loss in mouse models (31, 32). After 4 weeks of treatment, NVP-BEZ235 reduced mouse body weight in a dose-dependent manner (Supplementary Fig. S7B). We found that mice under NVP-BEZ235 treatment consumed less food compared with the control group (data not shown), which is consistent with previous reports that BEZ235 is associated with reduced food

<table>
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<th>Table 2. DHT/NVP-BEZ235 combination induces breast cancer response</th>
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<tr>
<td>NVP-BEZ235 (mg/kg/d)</td>
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<tr>
<td>Complete responsea</td>
</tr>
<tr>
<td>DHT</td>
</tr>
<tr>
<td>Partial responseb</td>
</tr>
<tr>
<td>DHT</td>
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<tr>
<td>Tumor growth (fold)c</td>
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<tr>
<td>Control</td>
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aDenominators represent (number of complete response)/(total number of tumors) in MCF-7 xenograft models. Mantel–Cox test, P = 0.0016.

bPartial response: at least 50% decrease in tumor volume after 5 weeks of treatment compared with initial measurement.

cThe tumor volume of remaining MCF-7 xenografts after 5 weeks of treatment compared with initial measurement.
consumption and severely impaired whole-body glucose metabolism in mice (33). In our study, DHT slightly increased body weight and completely reversed the NVP-BEZ235–induced weight loss (Supplementary Fig. S7B). Mice receiving 50 mg/kg/d NVP-BEZ235 lost 31% of body weight on average, whereas the addition of DHT prevented this side effect (Supplementary Fig. S7B). Our data here suggest that the presence of DHT during NVP-BEZ235 treatment can prevent the standard side effects of the latter.

Discussion

Because of the important role of the PI3K/AKT/mTOR pathway in tumor development and progression, numerous PI3K/AKT/mTORi have been developed for cancer treatment. Currently, several types of PI3K/AKT/mTORi have been in the U.S. Food and Drug Administration pipeline for breast cancer clinical trials. This category of small-molecule drugs includes PI3K inhibitors BGT226 and BKM120, AKT inhibitor KRX-0401 (perifosine), and mTOR inhibitor RAD-001 (everolimus), CCI-779 (temsirolimus), and rapamycin (sirolimus). NVP-BEZ235 seems to be a better candidate for cancer treatment as it inhibits both PI3K and mTOR activities simultaneously. However, it seems that breast cancers with different genetic backgrounds respond differently to NVP-BEZ235. Previous observations have suggested using PI3K, PTEN, or AKT as biomarkers to predict whether NVP-BEZ235 can induce cell death in certain breast cancer lines (5). In contrast, irrespective of the status of these biomarkers, we have shown that AR expression effectively distinguishes the drug-sensitive cell lines from drug-resistant ones. AR-positive tumors are sensitive to NVP-BEZ235, whereas AR-negative tumors are drug resistant. We show here that the presence of DHT adds benefit to NVP-BEZ235 treatment in AR+/ER+, but not AR+/ER−, tumors. In addition, by reintroducing AR back into drug-resistant AR-negative cells, we show reactivation of the AR pathway restored the NVP-BEZ235 response.

Using PI3K/AKT/mTOR inhibition has been shown to successfully inhibit tumor growth during the initial stages of treatment. However, the common reason for failure of PI3K/AKT/mTOR inhibitor trials seems to be development of drug resistance, which remains a challenge before this class of compounds can be effectively applied to the routine clinical armamentarium. Recent studies have demonstrated several potential mechanisms for NVP-BEZ235–induced drug resistance. For example, NVP-BEZ235 upregulates ER expression in ER-positive luminal B subtype of breast carcinomas (34). Long-term NVP-BEZ235 treatment also stimulates MYC expression and leads to drug resistance in breast cancers (25, 26).

In endocrine-related cancers in general, targeting both hormone receptor and the PI3K/AKT/mTOR pathways has shown promise. For example, combining the ER inhibitor tamoxifen and a PI3K inhibitor in breast cancers achieves a better therapeutic effect than either single drug alone (34). Studies have also shown that combination of mTOR inhibitors and AR antagonists could effectively treat advanced prostate cancer (1, 35).

Unaromatizable androgens are well known in inhibiting ER-mediated tumor proliferation, inducing apoptosis and cell-cycle arrest in AR-positive breast cancer cell lines, such as CAMA-1, ZR75-1, and T47D (9, 36–39). Two recent pieces of literature suggest that the androgenic effect on breast cancer cell growth may depend on tumor cell ER status, as DHT exerts antiproliferative effects in AR+/ER+ breast cancer cell lines while inducing proliferation in AR+/ER− cells (40, 41). However, Lehman and colleagues examined the effect of Casodex (AR antagonist) in 23 different breast cancer cell lines and found that Casodex does not have antiproliferative effects in some AR+/ER− cells (6). Interestingly, in the MCF-7 cell line, which is the most widely used AR+/ER+ breast cancer cell model, different groups also reported androgens’ antiproliferative (7, 8, 39, 42, 43) as well as proliferative effects (44, 45). These lines of controversial evidence most likely are due to the complexity of the AR pathway and lack of acknowledgment of broad mechanisms of androgenic effects in breast cancer, in vitro and in vivo. Most importantly, very few AR-regulated targets have been identified in breast cancer, making it extremely challenging to directly demonstrate the androgenic effect, especially in different subtypes of breast carcinomas.

Here, we provide in vitro and in vivo evidence to show that activation of AR can lend added benefits to PI3K/AKT/mTORi treatment in certain subgroups of breast cancers. This combinatorial benefit is also observed by using DHT and other PI3K/AKT/mTORi, such as rapamycin (data not shown). It seems that DHT’s beneficial effect in AR+/ER− breast cancers is mediated through multiple mechanisms, including MYC suppression and PTEN upregulation. Our observations here suggest that more clinical studies are needed to survey the correlation between patient tumor AR status and PI3K/AKT/mTOR inhibitors and perhaps other drug classes. It is also tempting to speculate that such drug resistance can be reversed by judicious reactivation of the AR signaling pathway depending on the tissue and on the hormone and molecular status of each malignancy. Currently, breast carcinomas are categorized on the basis of ER, PR, HER2, EGF receptor, and Ki-67 molecular status without consideration of AR. Our study suggests that routine screening for AR status in breast tumors may provide valuable information for cancer management and guidelines for chemotherapy planning.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y. Wang, C. Eng
Development of methodology: Y. Wang, C. Eng
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wang, X. He, C. Eng

Writing, review, and/or revision of the manuscript: Y. Wang, J. Altemus, C. Eng

Study supervision: C. Eng

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