Kinome-wide Functional Screen Identifies Role of PLK1 in Hormone-Independent, ER-Positive Breast Cancer

Neil E. Bhola1, Valerie M. Jansen1, Sangeeta Bafna1, Jennifer M. Giltinan2,3, Justin M. Balko1,3, Mónica V. Estrada3, Ingrid Meszoely3,4, Ingrid Mayer1,3, Vandana Abramson1,3, Fei Ye5, Melinda Sanders2,3, Teresa C. Dougger1, Eliezer V. Allen6, and Carlos L. Arteaga1,3,7

Abstract

Estrogen receptor (ER) α–positive breast cancers initially respond to antiestrogens but eventually become estrogen independent and recur. ER+ breast cancer cells resistant to long-term estrogen deprivation (LTED) exhibit hormone-independent ER transcriptional activity and growth. A kinome-wide siRNA screen using a library targeting 720 kinases identified Polo-like kinase 1 (PLK1) as one of the top genes whose downregulation resulted in inhibition of estrogen-independent ER transcriptional activity and growth of LTED cells. High PLK1 mRNA and protein correlated with a high Ki67 score in primary ER+ breast cancers after treatment with the aromatase inhibitor letrozole. RNAi-mediated knockdown of PLK1 inhibited ER expression, estrogen-independent growth, and ER transcription in MCF7 and HCC1428 LTED cells. Pharmacologic inhibition of PLK1 with volasertib, a small-molecule ATP-competitive PLK1 inhibitor, decreased LTED cell growth, ER transcriptional activity, and ER expression. Volasertib in combination with the ER antagonist, fulvestrant, decreased MCF7 xenograft growth in ovariectomized mice more potently than each drug alone. JUNB, a component of the AP-1 complex, was expressed 16-fold higher in MCF7/LTED compared with parental MCF7 cells. Furthermore, JUNB and BCL2L1 (which encodes anti-apoptotic BCL-xl) mRNA levels were markedly reduced upon volasertib treatment in MCF7/LTED cells, while they were increased in parental MCF7 cells. Finally, JUNB knockdown decreased ER expression and transcriptional activity in MCF7/LTED cells, suggesting that PLK1 drives ER expression and estrogen-independent growth via JUNB. These data support a critical role of PLK1 in acquired hormone-independent growth of ER+ human breast cancer and is therefore a promising target in tumors that have escaped estrogen deprivation therapy. Cancer Res; 75(2); 1–10. ©2014 AACR.

Introduction

Most breast cancer express estrogen receptor α (ERα) and are driven by estrogen (1–3). Targeting the function of ER with receptor antagonists such as tamoxifen, fulvestrant, and aromatase inhibitors (AI), such as anastrozole and letrozole, are effective treatments for patients with this subtype of breast cancer (4–7). However, a significant number of patients with ER+ tumors, particularly those with advanced disease, exhibit primary and acquired resistance to antiestrogens (8–11). Most mechanisms that mediate this resistance are not yet fully understood. Therefore, identifying those mechanisms and strategies to interfere with them is needed to prevent or delay acquired endocrine resistance and improve patient outcome. We and others have modeled secondary resistance to treatment with AIs, the equivalent of estrogen deprivation in postmenopausal patients, by generating long-term estrogen-deprived (LTED) cells, whereby ER+ breast cancer cells have adapted to estrogen deprivation and acquired robust growth under hormone-free conditions (12–14). In this study, we used ER+ MCF7/LTED and HCC1428/LTED breast cancer cells, which overexpress ER and retain estrogen-independent ER binding to DNA and ER transcriptional activity (15).

To identify targetable molecules that drive hormone-independent growth and ER transcription in LTED cells, we performed a screen using a library with siRNA pools targeting 720 kinases. We used two metrics in this assay: (i) luciferase activity as assessed by using an estrogen-response-element (ERE)-regulated reporter and (ii) cell viability measured by the Alamar Blue assay. In this screen, we identified Polo-like kinase 1 (PLK1) as the top hit whose downregulation decreased both ER transcriptional activity and cell viability. PLK1 is a serine/threonine kinase that is highly expressed during the G2 phase of the cell cycle where it controls the metaphase-to-anaphase transition and mitotic exit (16–18). PLK1 is one of three isoforms of PLKs
(PLK2 and PLK3; refs. 19, 20) and has been shown to be overexpressed in several human cancers (21–25). Currently, PLK1-specific small-molecule inhibitors are in clinical trials in patients with advanced cancer (26, 27). Herein, we discovered that genetic and pharmacologic inhibition of PLK1 decreased ER expression, estrogen-independent ER-mediated transcriptional activity, and growth of ER+ xenografts alone and in combination with fulvestrant. These results provide a basis for combinations of PLK1 inhibitors with ER downregulators in patients with hormone-independent ER+ breast cancer.

Materials and Methods

Cell lines and reagents

Parental cancer cell lines were from ATCC and maintained in improved minimum essential medium (IMEM)/10% FBS (Gibco) as previously described (28). LTED cells were generated in and maintained in phenol red-free IMEM with 10% dextran/charcoal-treated FBS (DCC-FBS) as previously described (28). B12536 and volasertib were obtained from Selleckchem. Fulvestrant was obtained from the Vanderbilt Hospital Outpatient Pharmacy (Nashville, TN). FOXM1-E construct was a gift from Junjie Chen (Yale University; New Haven, CT; ref. 29). The myristoylated-PLK1 construct (Addgene plasmid 20859) was a gift from William Hahn (Dana Farber Cancer Institute; Boston, MA; ref. 30).

RNAi screen

MCF-7/LTED cells were transfected with the Dharmacon RTF Protein Kinase siRNA library (14) as described in Supplementary Methods. Secondary validation was performed with four independent siRNAs against GSG2, RPS6KA2, and PLK1 (Qiagen).

qRT-PCR

Cells maintained in 10% DCC-FBS were transfected with control or PLK1 siRNA oligonucleotides (Thermo Scientific) with Lipofectamine RNAiMAX for 48 to 72 hours. Cells were harvested and their RNA extracted using the RNeasy Mini Kit (Qiagen). Using the iScript CDNA Synthesis Kit (Bio-Rad), 1 μg of RNA was reverse transcribed to cDNA and real-time PCR reactions were conducted in 96-well plates using the iCycler iQ (Bio-Rad) and primers obtained from SA Biosciences (Qiagen). RNA was extracted from MCF7 and MCF7/LTED cells that had been treated with volasertib or transfected with PLK1 siRNA; cDNA was applied to the Estrogen Receptor Signaling PCR Array (SA Biosciences). Fold changes in gene expression were determined using the web-based software from SA Biosciences (http://www.qiagen.com/us/products/genes%20and%20pathways/data-analysis-center-overview-page/). Genes identified in the array were verified by qRT-PCR.

Cell proliferation assays

Cells seeded in triplicate in 12-well plates (2.5 × 10^5 cells/well for MCF-7/LTED and 4 × 10^4 cells/well for other lines) were treated in 10% DCC-FBS. Media and inhibitors were replenished every 3 days; after 5 to 10 days, adherent cells were trypsinized and counted using a Coulter Counter or fixed/stained with crystal violet. Crystal violet-stained plates were scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences) and the staining intensity was quantified using the manufacturer’s analysis software. For the combination studies of volasertib and fulvestrant, MCF7 and MCF7/LTED cells were seeded in 96-well black plates followed by the Alamar Blue Assay after 72 hours as described in the Supplementary Methods.

Immunoblot analysis

Cells were lysed with NP40 lysis buffer (150 mmol/L Tris, pH 7.4, 100 mmol/L NaF, 120 mmol/L NaCl, 0.5% NP-40, 100 μmol/L sodium vanadate, and 1× protease inhibitor cocktail; Roche). Lysates (40 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes; these were first incubated with primary antibodies at 4°C overnight or at room temperature for 2 hours, followed by incubation with horseradish peroxidase–conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Thermo Scientific). All primary antibodies were from Cell Signaling Technology except the ERα antibody (Santa Cruz Biotechnology).

Transcriptional reporter assays

Cells were plated as above and transfected with pGLB-MERE (encodes estrogen response element-regulated Firefly luciferase), pGL4.23 vectors (Peak2 or Peak5 Firefly luciferase; ref. 28) and pTK-Renilla (encodes TK-driven Renilla luciferase; Promega) plasmids. Cells were then treated as above; luciferase activity was measured 16 to 20 hours later using the Dual Luciferase Kit (Promega) according to the manufacturer’s instructions utilizing a Moonlight 3010 Luminometer (Analytical Luminescence Laboratory). The same procedure was used for the pCAGA (provided by J-M. Gautier, Laboratoire GlaxoSmithKline), pGL2-E-cadherin (31), and pGL-ErbB3 (32) Firefly luciferase reporters.

Xenograft studies

Animal experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee. Female ovariectomized athymic nude mice (Foxn1nu/nu 4–5 weeks old; Harlan–Sprague-Dawley) were implanted subcutaneously with a 14-day release, 0.17-mg, 17β-estradiol pellet (Innovative Research of America). Twenty-four hours later, 5 × 10^6 MCF7 cells suspended in IMEM and Matrigel (BD Biosciences) at 1:1 ratio were injected subcutaneously into the right flank of each mouse. Approximately 4 weeks later, mice bearing tumors measuring ≥150 mm^3 were randomized to treatment with vehicle (control), volasertib (10 mg/kg/day via orogastric gavage), fulvestrant (5 mg/week s.c.), or both drugs. Animal weight and tumor diameters (with calipers) were measured twice weekly and tumor volume was calculated with the formula: volume = width^2 × length/2. After 6 weeks, tumors were harvested and snap-frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin followed by embedding in paraffin for IHC analysis.

Results

PLK1 siRNA oligonucleotides inhibit ER transcriptional activity and cell growth

Initially, we transfected cells with ERE firefly-luciferase and Renilla-luciferase constructs. Transfection with ERα siRNA decreased ERE-firefly luciferase activity. Importantly, the Renilla reading was markedly decreased (93%), resulting in a greater firefly/Renilla ratio compared with control siRNA-transfected cells (Supplementary Table S1). In the Alamar Blue assay, ER siRNA...
decreased cell viability only by 62% (Supplementary Fig. S1B). These results suggested that RNAi oligonucleotides reducing ER expression had a nonspecific effect on Renilla expression in MCF7/LTED cells, thus skewing the results. For this reason, we could not use Renilla expression as a control in cells transfected with the siRNA pools. We next assessed whether LTED cell viability (Alamar Blue) and ERE luciferase activity can be measured consecutively. Firefly luciferase activity was similar in cells transfected with MERE-luc in the presence or absence of Alamar Blue dye (Supplementary Fig. S1A and S1C). Therefore, MCF7/LTED cells were next transfected with an ERE-luciferase construct and with siRNA pools targeting 720 kinases (schema in Supplementary Fig. S1A). Both cell viability (Alamar Blue) and ER reporter activity for each siRNA relative to nonsilencing controls (siCTL) were transformed to a Z-score; the data are presented as median Z-score across three independent experiments (Fig. 1A). Knockdown of 48 and 26 kinases significantly decreased MCF7/LTED cell proliferation and ER transcription, respectively. Knockdown of 10 kinases was found to decrease both cell viability and ER transcription. C. MCF7/LTED and HCC1428/LTED cells transfected with RNAi-targeting PLK1 and cultured for 5 days. Cells were trypsinized and their number determined in a Coulter Counter (*, P < 0.002). D. MCF7/LTED and HCC1428/LTED cells were transfected with control (CTL) or PLK1 siRNA and reseeded in 12-well plates in full growth media. Media were replenished every 3 days and the cells were stained with crystal violet after 7 days (*, P < 0.0002). E. RNAi-mediated PLK1 downmodulation was validated by qRT-PCR in MCF7/LTED and HCC1428/LTED cells (*, P < 0.01). F. MCF7/LTED cells were transfected with CTL or PLK1 siRNA for 72 hours. Cell lysates were prepared and subjected to immunoblot analysis for ER, PLK1, and actin.

PLK1 Drives Hormone-Independent ER⁺ Breast Cancer

Figure 1. RNAi screen identified PLK1 is required for hormone-independent ER transcriptional activity and growth. A, MCF-7/LTED cells were screened with a siRNA library targeting 720 kinases. Ligand-independent cell growth and ER transcriptional activities were sequentially measured 72 hours later using a high-throughput Alamar Blue and luciferase reporter assays, respectively. Both cell growth and ER transcriptional activity for each kinase siRNA relative to nonsilencing controls (siCTL) were transformed to a Z-score; the data are presented as median Z-score across three independent experiments. B, Knockdown of 48 and 26 kinases significantly decreased MCF7/LTED cell proliferation and ER transcription, respectively. Knockdown of 10 kinases was found to decrease both cell viability and ER transcription. C, MCF7/LTED and HCC1428/LTED cells transfected with RNAi-targeting PLK1 and cultured for 5 days. Cells were trypsinized and their number determined in a Coulter Counter (*, P < 0.002). D, MCF7/LTED and HCC1428/LTED cells were transfected with control (CTL) or PLK1 siRNA and reseeded in 12-well plates in full growth media. Media were replenished every 3 days and the cells were stained with crystal violet after 7 days (*, P < 0.0002). E, RNAi-mediated PLK1 downmodulation was validated by qRT-PCR in MCF7/LTED and HCC1428/LTED cells (*, P < 0.01). F. MCF7/LTED cells were transfected with CTL or PLK1 siRNA for 72 hours. Cell lysates were prepared and subjected to immunoblot analysis for ER, PLK1, and actin.
hormone independence because ER+ breast cancers that retain a high Ki-67 score after therapy are considered resistant to estrogen deprivation (with letrozole) and vice versa; tumors with a low posttreatment Ki-67 are considered highly hormone dependent (28). Notably, the effect of PLK1 siRNA on the ligand-independent ER transcription was similar to that of ER siRNA oligonucleotides (Fig. 3C). In addition, MCF7/LTED cells were transfected with both Peak5 firefly luciferase and Remila luciferase plasmids 2 days after transfection with two independent PLK1siRNAs to avoid the effects of RNAi on Remila activity. We observed that both PLK1 siRNA oligonucleotides decreased estrogen-independent ER reporter activity (Supplementary Fig. S3) when measured as a ratio of firefly to Renilla luciferase. Conversely, overexpression of PLK1 in MCF7/LTED cells increased >4-fold Peak5-Luc reporter activity (Fig. 3D). To show that the effect of PLK1 downregulation on ER transcription was specific to ER and not secondary to an overall effect on chromatin segregation during mitosis, we examined other transcriptional reporters. PLK1 and ER siRNA did not affect the activity of the transcriptional reporters driven by SMAD, E-cadherin, and ERBB3 promoter binding regions in MCF7/LTED cells (Figs. 3E–G).

Furthermore, both MCF7 and HCC1428 LTED cells displayed increased PLK1 expression compared with their parental cell lines (Fig. 2D). These results suggest that high PLK1 expression associates with and could be used as a marker of hormone-independent ER+ breast cancer.

PLK1 downmodulation decreases hormone-independent ER transcriptional activity

In MCF7/LTED cells, PLK1 siRNA decreased MERE-Luciferase activity (Fig. 3A) and expression of the ER-responsive genes progesterone receptor (PR), Trefoil factor 1 (TFF1), and growth regulation by estrogen in breast cancer 1 (GREB1; Fig. 3B). A more modest reduction in PR and TFF1 was observed in HCC1428/LTED cells transfected with PLK1 siRNA. To confirm that the effects of PLK1 siRNA were on ligand-independent ER transcription, we generated two luciferase reporters (Peak2 and Peak5) with ER-binding regions proximal to genes reported to be downregulated by ER siRNA or fulvestrant in the absence of estrogen (28). Notably, the effect of PLK1 siRNA on the ligand-independent reporters was similar to that of ER siRNA oligonucleotides (Fig. 3C). In addition, MCF7/LTED cells were transfected with both Peak5 firefly luciferase and Remila luciferase plasmids 2 days after transfection with two independent PLK1siRNAs to avoid the effects of RNAi on Remila activity. We observed that both PLK1 siRNA oligonucleotides decreased estrogen-independent ER reporter activity (Supplementary Fig. S3) when measured as a ratio of firefly to Renilla luciferase.

PLK1 inhibitor decreases hormone-independent growth and ER transcriptional activity in LTED cells

We next used BI2536 and volasertib, small-molecule inhibitors derived from the dihydropteridinone class of compounds that target the ATP-binding pocket in the PLK1 kinase domain (34). Growth of MCF7/LTED and HCC1428/LTED cells was inhibited by low nmol/L concentrations of both BI2536 and volasertib in dose-dependent fashion (Figs. 4A and B), whereas parental MCF7 cells were only inhibited by concentrations in excess of 10 nmol/L (Supplementary Fig. S4A). In both LTED cell lines, treatment with volasertib abrogated hormone-independent ER reporter activity (Fig. 4C) and reduced ER protein levels (Fig. 4D and Supplementary Fig. S4B). Furthermore, volasertib increased expression of cleaved PARP, an apoptotic marker, and phosphorylated Histone H2AX, a marker of DNA damage and fragmentation (Fig. 4E and Supplementary Fig. S4B).

Volasertib enhances the antitumor effect of fulvestrant in vivo

We next investigated whether combined inhibition of PLK1 and ER with volasertib and the ER antagonist fulvestrant, respectively, would have a synergistic antitumor effect. Volasertib enhanced the antiproliferative effect of fulvestrant in MCF7 and MCF7/LTED cells (Fig. 5A and B and Supplementary Fig. S4C). The combination of fulvestrant and volasertib also increased

**Table 1.** Ranking of the top 10 hits on cell proliferation and ER transcription in the RNAi screen of MCF7/LTED cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell proliferation rank</th>
<th>ER transcription rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTN1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CSNK1A1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PLK1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>MKI67</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>PRKCB1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>RPS6KA2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>TTK</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>PRKCB1</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>TTK</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

**Figure 2.** High PLK1 expression correlates with antiestrogen resistance in primary ER+ breast cancers. A, correlation PLK1 protein levels measured by RPPA with the Ki-67 score in 10 post-letrozole breast tumor biopsies (n = 10; P = 0.007). B, correlation of PLK1 and MKI67 expression from RNA-seq analysis of RNA extracted from post-letrozole biopsies of primary ER+ breast cancers (n = 47; P < 0.001). C, PLK1 expression was analyzed in patients' tumors that were categorized as drug-sensitive or -resistant based on the post-letrozole Ki-67 score in 10 post-letrozole breast tumor biopsies (n = 10; P = 0.002).
expression of cleaved PARP and phospho-Histone H2AX, whereas each drug alone did not (Fig. 5C). To examine the effect of the combination in vivo, we established MCF7 xenografts in ovariectomized nude mice. Mice with tumors measuring $\geq 150$ mm$^3$ were treated with vehicle, volasertib, fulvestrant, or the combination of both inhibitors. After 6 weeks of treatment, the combination induced a statistically superior antitumor effect compared with fulvestrant or volasertib alone (Fig. 5D). During this period, the mice in all 4 treatment groups displayed stable body weight (data not shown) and no signs of toxicity. Treatment with volasertib reduced ER levels but the combination of volasertib and fulvestrant more potently reduced both ER and PR levels as measured by IHC of tumor sections (Figs. 5E–G).

PLK1 inhibition decreases BCL-xL and JUNB expression in LTED cells

It has been shown that PLK1 may have a tumor-suppressive role in cancer, including MCF7 breast cancer cells (21, 35, 36). On the basis of the findings from the RNAi screen, we sought to elucidate mechanistic differences between parental MCF7 and estrogen-independent MCF7/LTEDs in response to PLK1 loss. Using an Estrogen Receptor Signaling PCR Array with 84 ER-associated genes, a 24-hour treatment with each volasertib and PLK1 siRNA oligonucleotides altered (>1.4-fold) the expression of seven genes, including ER$\alpha$, in MCF7 and MCF7/LTED cells (Supplementary Fig. S5A). Expression of BCL2L1, the gene encoding BCL-xL, and JUNB was also increased in hormone-dependent parental MCF7 cells following treatment with each volasertib and PLK1 siRNA. In MCF7/LTED cells, however, volasertib and PLK1 siRNA decreased the expression of both BCL-xL and JUNB (Fig. 6A and B). HCC1428/LTED cells treated with volasertib also displayed a modest reduction in BCL-xL and JUNB, whereas PLK1 siRNA markedly decreased BCL-xL and JUNB transcript levels (Fig. 6C). BCL-xL and JUNB expression were approximately 5-fold and 17-fold higher, respectively, in MCF7/LTED cells compared with the parental MCF7 cells (Fig. 6D). We next confirmed that treatment with volasertib and downmodulation of PLK1 with two independent siRNAs decreased protein levels of ER, BCL-xL, and JUNB (Fig. 6E). mRNA and protein levels of BCAR1 (breast cancer antiestrogen resistance 1), which has been associated with poor prognosis and resistance to tamoxifen (37), were also decreased upon treatment with volasertib (Supplementary Fig. S5A and S5B). JUNB is a component of the AP-1 transcriptional complex that...
drives the expression of genes involved in cellular growth and development (11). In addition, estrogen-activated ER is known to bind to AP-1 promoter sites in ER$^+$ breast cancer cells (38). Therefore, we examined whether PLK1-mediated regulation of JUNB affected ER expression and transcriptional activity. MCF7/LTED cells transfected with JUNB siRNA exhibited reduced hormone-independent ER transcriptional activity (Fig. 6F and Supplementary Fig. S5C) and ER expression (Fig. 6F). Conversely, JUNB siRNA increased ER expression in the parental MCF7 cells (Fig. 6G). These observations suggest that PLK1 regulates ER via transcriptional regulation of JUNB.

PLK1 transcription is driven by FOXM1, a member of the forkhead family of transcription factors (39). PLK1 and FOXM1 have an inter-regulatory relationship as part of a kinase-driven positive feedback loop that leads to the PLK1-mediated phosphorylation of FOXM1 and potentiation of its activity (29, 40). Consistent with this positive interaction, transfection of a vector encoding FOXM1 constitutively phosphorylated at the PLK1 site (FOXM1-EE) (29) increased ER expression and ER transcriptional activity (Supplementary Fig. S6A). Treatment with volasertib still reduced constitutive ER transcriptional reporter activity in the FOXM1-EE--expressing cells, suggesting that PLK1 mediates ER activity downstream of FOXM1 in LTED cells. Supporting an interaction between FOXM1 and PLK1 in hormone-independent primary tumors, FOXM1 transcript levels correlated positively with both Ki-67 and PLK1 mRNA levels in patients with ER$^+$ breast cancer treated with letrozole (Supplementary Fig. S6B and S6C). Finally, we determined the effect of PLK1 downmodulation on the expression of other nuclear receptors involved in breast cancer pathogenesis, androgen receptor (AR), and ER$\beta$. In ER$^+$ breast cancers, AR expression is associated with lower risk of recurrence and increased overall survival (41, 42). ER$\beta$ has been shown to antagonize ER$\alpha$ by decreasing growth and ER$\alpha$-mediated transcriptional activity (43). Furthermore, it was recently shown that ER$\beta$ can be regulated by activated AR to decrease ER$^+$ breast cancer cell growth (44). We observed that PLK1 downmodulation augmented the expression of both AR and ER$\beta$ while decreasing the expression of the ER$\alpha$ target gene PR (Supplementary Fig. S6D).

These results suggest that, in breast cancer cells that adapt to estrogen deprivation, PLK1 positively regulates antiapoptotic genes (BCL-xL) and components of the AP-1 (JUNB)
transcription complex (Fig. 6H). In turn, this promotes ERα expression and transcriptional activity and is therapeutically targetable with PLK1 inhibitors in hormone-independent, ER-driven breast cancers.

**Discussion**

In this study, we identified PLK1 as a potential therapeutic target in hormone-independent ER+ breast cancer cells. Using a high-throughput RNAi screen approach to identify kinases that contribute to hormone-independent transcriptional activity and tumor cell viability, PLK1 was the top candidate gene associated with both of these phenotypes. In addition to ER transcriptional activity, PLK1 downmodulation decreased ER expression. Consistent with these results, treatment with the PLK1 inhibitor volasertib reduced LTED cell viability, ER levels, and ER transcriptional activity. Furthermore, volasertib enhanced the antitumor action of fulvestrant in MCF7 xenografts via enhanced ER downmodulation. Using an Estrogen Receptor Signaling PCR Array, we compared the effect of PLK1 inhibition on gene expression in parental and LTED MCF7 cells. Inhibition of PLK1 with volasertib and PLKsiRNA resulted in a reduction of BCL-xL and JUNB expression in LTED cells, while these genes were upregulated in parental MCF7 cells. Taken together, these findings suggest that PLK1 inhibition may provide therapeutic benefit to patients with endocrine-resistant ER+ breast cancer.

RNAi kinome screens have previously identified PLK1 as a gene causally associated with growth of basal-like breast cancer and tumor-initiating cells (35, 45). This finding may reflect a crucial role of PLK1 in mitosis of rapidly proliferating cancer cells. PLK1 was also recently shown to cooperate with estrogen-dependent...
ER signaling (21). In this study, Wierer and colleagues showed that PLK1 phosphorylates the transcriptional regulator MLL2, which forms a complex with ER to drive transcription of tumor-suppressive genes. Those findings suggest that PLK1 drives proliferation via its role in interphase but it decreases growth via its interaction with ER. The findings in our report herein suggest that PLK1 has lost this negative cell cycle and transcriptional regulatory function in estrogen-independent breast cancer cells that still depend on unliganded ER function.

PLKs exist in three different isoforms, PLK1, PLK2, and PLK3. Although PLK1 was shown to significantly affect both viability and ER transcription, PLK3 siRNA only decreased ER transcriptional activity in the siRNA screen (Supplementary Table S2). PLK3 is not aberrantly expressed in breast cancer and maintains...
steady state levels during cell division (46). Volasertib has a high affinity for PLK1 (0.87 nmol/L), PLK2 (5 nmol/L), and PLK3 (56 nmol/L) (ref. 34). Therefore, treatment of antiestrogen-resistant ER+ breast cancer cells with volasertib may have a very potent effect on growth and ER transcriptional activity by inhibition of both PLK1 and PLK3.

Blockade of PLK1 with volasertib or siRNA decreased ER transcriptional activity and ER expression. JUNB, a component of the AP-1 transcription complex was also decreased upon inhibition of PLK1 in LTED cell lines and knockdown of JUNB decreased ER transcriptional activity and expression. It has been shown that ER can cooperate with AP-1 complexes at AP-I sites to decrease ER transcriptional activity and expression. It has been shown that inhibition of PLK1 in LTED cell lines and knockdown of JUNB mRNA and protein expressions have been reported as poor prognostic biomarkers in patients with ER+ breast cancer (48). We previously reported that an E2F transcriptional signature correlates with a resistance to estrogen deprivation in ER+ breast cancer cells (28). Treatment with volasertib also reduced expression of BCAR1 (breast cancer antiestrogen resistance), which encodes p130Cas (Crk-associated substrate; Supplementary Fig. S5A; ref. 49). BCAR1 overexpression has also been shown to correlate with poor prognosis and resistance to tamoxifen in patients with ER+ breast cancer (37). p130Cas complexes with ER and drives nongenomic signaling such as activation of c-Src and p85 PI3K (50). We have reported that hyperactivation of PI3K is causally associated with hormone-independent growth of ER+ breast cancer cells (15). Moreover, p130Cas has E2F and c-Jun–binding elements in its promoter. Taken together, these data suggest that inhibition of PLK1 may interfere with E2F transcription.

Finally, the combination of volasertib and fulvestrant exhibited synergistic antitumor efficacy and abrogated ER expression levels in vivo. Volasertib has currently completed phase I clinical trials where it showed a favorable pharmacokinetic profile with minor hematologic toxicities (34). It is under active investigation in phase II studies addressing efficacy (www.clinicaltrials.gov). Therefore, based on the two-pronged effect of PLK1 inhibition on endocrine-resistant ER+ breast cancer cell viability and ER transcriptional activity, we propose that PLK1 inhibitors like volasertib should be investigated in patients with ER+ breast cancer that have escaped estrogen deprivation therapy.

Disclosure of Potential Conflicts of Interest
E.V. Allen has ownership interest (including patents) in Syapse. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: N.E. Bhola, S. Rafna, J.M. Balko, C.L. Arteaga
Development of methodology: S. Bafna, J.M. Balko, M. Sanders, E.V. Allen, C.L. Arteaga
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.E. Bhola, V.M. Jansen, S. Bafna, J.M. Gilnane, I. Meszoely, I. Mayer, V. Abramson, M. Sanders, C.L. Arteaga
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.E. Bhola, V.M. Jansen, S. Bafna, J.M. Gilnane, J.M. Balko, I. Mayer, F. Ye, E.V. Allen, C.L. Arteaga
Writing, review, and/or revision of the manuscript: N.E. Bhola, V.M. Jansen, S. Bafna, J.M. Gilnane, J.M. Balko, I. Mayer, V. Abramson, F. Ye, M. Sanders, C.L. Arteaga
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.E. Bhola, S. Bafna, M. Sanders, T.C. Dugger, C.L. Arteaga
Study supervision: C.L. Arteaga
Other (pathology): M.V. Estrada

Acknowledgments
The authors acknowledge Genoptix Medical Laboratory for performing the quantification of Ki-67 nuclei staining by the AQUA methodology.

Grant Support
This work was supported by the NIH Breast Cancer Specialized Program of Research Excellence (SPORE) grant P50 CA98131, Vanderbilt-Ingram Cancer Center Support Grant P30 CA68485, Susan G. Komen for the Cure Foundation grants SAC100013 (C.L. Arteaga), a grant from the Breast Cancer Research Foundation, a Komen Post-Doctoral award PDF 1227859 (N.E. Bhola), and a NHGRI grant U54HG003067 (E.V. Allen).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 25, 2014; revised October 30, 2014; accepted November 11, 2014; published OnlineFirst December 5, 2014.

www.aacrjournals.org Cancer Res; 75(2) January 15, 2015 OF9

References


Kinome-wide Functional Screen Identifies Role of PLK1 in Hormone-Independent, ER-Positive Breast Cancer


Cancer Res  Published OnlineFirst December 5, 2014.

Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-2475

Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/12/06/0008-5472.CAN-14-2475.DC1

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.