Altered Steroid Milieu in AI-Resistant Breast Cancer Facilitates AR Mediated Gene-Expression Associated with Poor Response to Therapy

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Abstract

Divergent roles for androgen receptor (AR) in breast cancer have been reported. Following aromatase inhibitor (AI) treatment, the conversion of circulating androgens into estrogens can be diminished by >99%. We wished to establish whether the steroid environment can dictate the role of AR and the implications of this for subsequent therapy. This study utilizes models of AI resistance to explore responsiveness to PI3K/mTOR and anti-AR therapy when cells are exposed to unconverted weak androgens. Transcriptomic alterations driven by androstenedione (4AD) were assessed by RNA-sequencing. AR and estrogen receptor (ER) recruitment to target gene promoters was evaluated using ChIP, and relevance to patient profiles was performed using publicly available data sets. Although BEZ235 showed decreased viability across AI-sensitive and -resistant cell lines, anti-AR treatment elicited a decrease in cell viability only in the AI-resistant model. Serum and glucocorticoid-regulated kinase 3 (SGK3) and cAMP-dependent protein kinase inhibitor β (PKIB) were confirmed to be regulated by 4AD and shown to be mediated by AR; crucially, reexposure to estradiol suppressed expression of these genes. Meta-analysis of transcript levels showed high expression of SGK3 and PKIB to be associated with poor response to endocrine therapy (HR = 2.551, P = 0.003). Furthermore, this study found levels of SGK3 to be sustained in patients who do not respond to AI therapy. This study highlights the importance of the tumor steroid environment. SGK3 and PKIB are associated with poor response to endocrine therapy and could have utility in tailoring therapeutic approaches.

Introduction

Estrogen receptor (ER)-positive tumors account for approximately 75% of all breast cancer diagnoses (1). The emergence of selective ER modulators in the 1970s proved to be a rubicon in the fight against breast cancer, with all first-line therapies thereafter focusing on estrogen-driven ER activity (2). Since the mid-2000s, aromatase inhibitors (AI) have been recommended as first-line therapy for hormone receptor-positive, postmenopausal breast cancers (3). However, the development of resistance to these drugs is a perennial problem with disease recurrence in ~30% of patients (4). Mechanisms of resistance to antiestrogen therapy are multifaceted and include alterations in coactivator recruitment (5), dominance of growth factor pathways (6), upregulation of androgens (7), ER mutations (8), and alterations in steroid handling in breast tumor epithelial cells (9).

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and androgens have been demonstrated to activate intracellular signalling pathways (19). Here, we show that AR protein levels are elevated in cell line models of letrozole resistance. Significantly, due to the mechanism of action of AI therapy, the intracellular environment becomes saturated with androgens, and will become largely estrogen depleted. The risk that this alteration in the steroid environment may facilitate resistance is supported by clinical evidence, which has shown that serum levels of the direct precursor steroid androstenedione (4AD) are elevated in patients progressing on AI therapy (20, 21). Understanding individual tumor intracrinology will be critical to evaluating this clinically as it is known that elevated levels of androgens (4AD and DHEA) are associated with breast cancer risk >2 years prior to cancer detection (22). This suggests that hormone levels effect risk rather than hormone levels being altered by localized steroid production. 4AD is known to bind AR and can induce AR nuclear translocation in vitro, albeit with a lower affinity than 5-Dihydrotestosterone (DHT; ref. 23). Herein, we report that this chronically altered androgenic steroid environment enhances expression of SGK3 and PKIB transcripts, which are both identified as AR/ER regulated genes. SGK3 has previously been identified as a downstream target of both PI3K and AR in prostate cancer (24), highlighting it as a potential central mediator for both signaling pathways in AI-resistant breast cancer (25, 26). This current study highlights the impact of steroid levels on transcriptional regulation and identified SGK3, in particular as a potential indicator of poor response to AI therapy. As SGK proteins can be targeted pharmacologically, this pinpoints SGK3 as a potential therapeutic target for ER-positive breast cancer that may not respond to conventional endocrine treatment.

Materials and Methods

Cell culture
Cell lines were cultured as follows: endocrine-sensitive MCF7 were grown in DMEM (low glucose) with 10% of fetal bovine serum and 100 U penicillin/0.1 mg/mL streptomycin (Pen-strep) plus 10−8 mol/L 17-β-estradiol (Sigma E8875). MCF7-derived AI-sensitive cells (MCF7-Aro) were developed in-house and cultured in phenol red free MEM (Sigma-Aldrich), 10% charcoal dextran stripped FCS, 1% Pen-Strep, 1% L-glutamine, and 200 μg/mL G418 (Geneticin). MCF7-Aro-derived letrozole-resistant cells (MCF7-Aro-LetR) were created by long-term treatment of MCF7-Aro with letrozole and 4AD >3 months (Novartis) in charcoal dextran-stripped FCS, 1% Pen-Strep, 1% L-glutamine, 200 μg/mL G418, 2.5−8 mol/L 4AD, and 10−6 mol/L letrozole; ref. 27). An obvious morphologic change was noted in the transition of MCF7 cells to AI-resistant MCF7aro-LetR, with cells displaying a large increase in cell-surface area. To further assess the impact of the steroid environment on these cells, they were then maintained in medium supplemented with estradiol (10−8 mol/L for 15 weeks) and designated as MCF7aro-LetR-Est. After 15 weeks, MCF7aro-LetR-Est cells had reverted to smaller cell sizes with cobblestone morphology, as is observed in MCF7 cells grown in estradiol.

ZR75.1 were included as an additional luminal A model that express high AR. They are known to have a PTEN mutation and were cultured in MEM, 10% FCS, 1% Pen-Strep, 1% L-glutamine (28). ZR75.1-derived AI-sensitive cells (ZR-Aro) were developed in-house by lentiviral transduction of ZR75.1. ZR-Aro-derived letrozole-resistant cells (ZR-Aro-LetR) were then generated by long-term treatment of ZR-Aro with 4AD and letrozole >3 months using the same culture medium as above and are fully characterized (Supplementary Fig. S2A–S2C). All cells were maintained in the steroid-depleted medium for 72 hours before treatment with steroids or drugs. All cells were incubated at 37°C under 5% CO2 in a humidified incubator. In-house cells were authenticated and are routinely verified as endocrine resistant, Mycoplasma free, and all cells are utilized within 10 passages for triplicate experiments. The Aro cell lines from which the AI-resistant cells are derived are generally not used as comparators in these studies as it is well established that CYP19 amplification is a hallmark of endocrine resistance (29) and if cultured in the absence of AI and steroid these cells are even less endocrine sensitive (30–32).

Western blotting
Cells were harvested, lysed, electrophoresed, and immunoblotted with specific primary antibodies and corresponding horseradish peroxidase-conjugated secondary antibody (Dako). See Supplementary Materials and Methods for antibody detail.

MTS assay
For drug treatments, MCF7 and MCF7-Aro-LetR cells were steroid depleted for 72 hours. MCF7 cells were plated in steroid-depleted media supplemented with estradiol 10−8 mol/L to enable cell growth, and MCF7-Aro-LetR were seeded in steroid-depleted media prior to the addition of BEZ335 (Selleck Chemicals) as per optimal dose concentration, enzalutamide (10 μmol/L; Selleck chemicals), or DMSO vehicle (Sigma-Aldrich). Treatments were replenished every 3 days. Colorimetric outputs were analyzed by measuring the absorbance at 490 nm using a spectrophotometer (PerkinElmer). Prior to transfection, MCF7-Aro-LetR and ZR-Aro-LetR cells were steroid depleted for 72 hours. Cells were transfected with siRNA against SGK3 (L-004162-00, Dharmacon) or siRNA-negative control (d-001206-13-05) and seeded in a 96-well plate prior to the addition of 4AD (10−7 mol/L). Two-way ANOVA with Bonferroni posttest was used for statistical analysis of results for treatment 1 to 3 days. Student t test (two-tailed) was used to compare means for siRNA experiments.

Colony-forming assay
Assays were performed as previously described (14).

RNA extraction, library preparation, and RNA-sequencing
To assess the transcriptional effects of 4AD in endocrine-resistant breast cancer cells, RNA-sequencing was performed using MCF7-Aro-LetR cells, which were steroid depleted for 72 hours and treated with either 4AD (10−7 mol/L) or vehicle in the presence of letrozole 10−8 mol/L in triplicate. RNA was extracted using an RNeasy Kit (Qiagen). Sequencing was performed on an Illumina HiSeq technology (minimum 10 million clean reads of the RNA-quantification/sample). Three independent biological libraries were prepared for each sample to facilitate the expression detection and variance estimation.

Transcriptomic analysis (RNA-seq analysis and microarray analysis)
RNA-seq preprocessing: see Supplementary Material and Methods.
Microarray preprocessing: see Supplementary Material and Methods.

For both transcriptomic experiments, differential expression was determined using the eBayes function of the R package Limma (33). In each case, a binary comparison was performed between 4AD-treated and controls. In the case of the microarray experiment, the following samples were included: GSM1016474, GSM1016475, and GSM1016476 (controls) and GSM1016486, GSM1016487, and GSM1016488 (treated). A P value of less than 0.05 and a fold change greater than 1.4-fold were considered significant. The package Limma was chosen here for differential expression analysis as it is particularly robust when dealing with small sample sizes in both microarray and RNA-seq experiments (34). These two lists of differentially regulated genes were overlapped with each other and a publicly available list of genes associated with acquired endocrine therapy resistance in breast tumors expressing ESR1 but not ESRB2 (35). The original authors referred to this as their "group 4 set" of genes which exhibited strong hormone responsiveness. A detailed description of how it was generated can be found in the original manuscript. These data were used to generate a candidate gene list for further investigation. All calculations were carried out in the R statistical environment (https://cran.r-project.org/).

RNA-seq validation

A panel of breast cancer cell lines (MCF7, MCF7-Aro-LetR, MCF7-Aro-LetR-Est, ZR75.1, and ZRaro-LetR) was steroid depleted for 72 hours followed by treatment with 4AD (10⁻⁷ mol/L) and ethanol vehicle (0.0001% (v/v)) for 24 hours. Letrozole 10⁻⁶ mol/L was also added to AI-resistant cell lines. RNA was extracted using an RNeasy Kit (Qiagen). cDNA synthesis was performed using Superscript III First-Strand Synthesis System (Life Sciences). See Supplementary Material and Methods for primer details.

MassArray analysis

See Supplementary Material and Methods.

siRNA transfection

AR, ESR1, and SGK3 were silenced by transient transfection using experimentally verified pools of siRNA. AR was silenced using siRNA pools SMARTpool (catalog number L-003400-00; 30 nmol/L), SGK3 SMARTpool (catalog number L-004162-00; 30 nmol/L), and ESR1 SMARTpool (catalog number L-003401-00-0005; 30 nmol/L), all purchased from Dharmacon. All transfections were carried out using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions (Invitrogen) and a nontargeting siRNA pool-negative control (Dharmacon) was used as a control for all siRNA experiments. Confirmation of mRNA knockdown was performed using primers for AR (F: 5'-AGCGACCTCACGGACACCC-3'; R: 5'-CAGTCTCCAAA CGGTAGTCCCG-3'), ESR1 (F: 5'-TGACCTGCAACAGCAG CATACC-3'; R: 5'-TCCTCAGTGATGCGCACCC-3') 24 hours after transfection. siRNA efficacy was also confirmed at protein level (Supplementary Fig. S2D and S2E).

Chromatin immunoprecipitation

MCF7-Aro-LetR cells were treated with 4AD 10⁻⁷ mol/L (30 minutes), estradiol 10⁻⁸ mol/L (45 minutes) or ethanol vehicle. ChIP was performed as previously described (27). Rabbit anti-AR (3 μg; sc-816, SCBT), anti-ESR1 (6 μg; SC-543, SCBT), or mouse IgG (6 μg)/rabbit (3 μg; Dako) was added to the supernatant fraction and incubated overnight at 4°C with rotation. Proteins were un-crosslinked. DNA extracted, and specific primers were used to amplify the DNA of the SGK3 proximal promoter. SGK3 proximal promoter primers: Forward 5'-GTCTCAGTCTATATCT-3' and reverse 5'-CAAGTCTAATCTGACCCCTCATATCT-3' (24).

Meta-analysis of SGK3 mRNA expression and breast cancer patient survival

BreastMark (36) is an algorithm that enables the identification of subsets of gene transcript/miRNAs that are associated with disease progression and survival in breast cancer and its subtypes. Using this resource, the association of expression levels of SGK3 mRNA and survival was evaluated in endocrine-treated/untreated data sets.

Evaluation of SGK3 in clinical cohorts and responsiveness to endocrine therapy

Series GSE59515 (Accurate Prediction and Validation of Response to Endocrine Therapy in Breast Cancer; ref. 37) was used to assess mRNA expression levels of SGK3 and AR pre and 3 months after AI treatment in a cohort of postmenopausal breast cancer patients (n = 25). The USCS Xena browser (https://xenabrowser.net/heatmap/) was used to interrogate TCGA breast cancer data sets filtered to focus analysis on the study's postmenopausal, endocrine therapy–treated patients (n = 415). Copy-number variation for SGK3 was evaluated, and cohorts were stratified by masked copy-number repeat to eliminate sex chromosome and germline artifacts. Kaplan–Meier graphs (quartiles) were generated from these data to ascertain association of copy-number amplification and overall survival.

RNA extraction from formalin-fixed, paraffin-embedded (FFPE) tissues

Breast cancer patients (n = 6) who were responsive (n = 3) or nonresponsive (n = 3) to AI therapy were selected. Informed written consent was received from all patients, and the study was approved by institutional review board Royal College of Surgeons in Ireland (CTI09/07). Hematoxylin and eosin–stained sections of FFPE tumor tissues were analyzed by a pathologist for histologic and tumor cellularity classifications. Tumor content was annotated on sections, and RNA was extracted from these areas using the Qiagen AllPrep DNA/RNA FFPE kit according to the manufacturer’s instructions.

Statistical analysis

A two-tailed Student t test was used to compare means. Two-way ANOVA with Bonferroni posttest was used to compare replicate means. Dose response curves were normalized to 100% and 0% viability based on the lowest and highest drug concentrations (GraphPad Prism v8).

Data accession code

RNA-seq data are available at the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/), provisional accession code SRP148035.

Androstenedione Altered Transcriptome in AI Resistance

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Results

Al-sensitive MCF7 and Al-resistant MCF7-Aro-LetR cells exhibit decreased cell viability when treated with BEZ235; however, only MCF7-Aro-LetR cells display decreased cell viability when treated with anti-AR therapy

 Estradiol-dependent parental MCF7 cells were found to harbor a PIK3CA E545K missense mutation [Fig. 1A and B (i)], which is a known hotspot mutation in breast cancer [38]. Analysis of the letrozole-resistant MCF7 derivative cell line MCF7-Aro-LetR confirmed they retained the same mutation [Fig. 1A and B (ii)]. The cell lines exhibited varied levels of AR protein expression with low amounts in the MCF7 and higher levels in the MCF7-Aro-LetR [Fig. 1C; Supplementary Fig. S1A (i)]. This was mirrored in the ZR75.1 letrozole-resistant derivative cell line ZR75aro-LetR when compared with their parental cell line; ER protein levels did not vary across the panel of cell lines [Fig. 1C; Supplementary Fig. S1A (ii)]. Dose response analysis was used to establish sensitivity to PI3K/mTOR inhibition by the pan class inhibitor BEZ235 (Selleckchem) and to a δ9 PI3K inhibitor, pictilisib [Selleckchem; Supplementary Fig. S1B (i–iv) and S1C (i–ii)].

Endocrine-sensitive (MCF7) and Al-resistant cells (MCF7-Aro-LetR) were treated with BEZ235 or enzalutamide (anti-AR) as single or combination treatment followed by an evaluation of cell viability. BEZ235 treatment significantly decreased cell viability in all cell lines tested compared with vehicle (Fig. 1D and E). Of note, the Al-resistant MCF7-Aro-LetR cells showed a significant decrease in levels of cell viability following enzalutamide treatment (Fig. 1E); this was in contrast to the parental MCF7 cells, in which viability was sustained when AR was targeted compared with vehicle (Fig. 1D). Combined BEZ235 and enzalutamide treatment resulted in a significant decrease in viability across all cell lines; however, this effect was most likely carried by BEZ235 (Fig. 1D and E). This result was mirrored by colony-forming assays suggesting that it is cell proliferation that is being affected [Fig. 1F (i–ii)].

4AD upregulates genes associated with steroid and PI3K signaling

Transcriptomic analysis enabled the identification of genes differentially regulated when Al-resistant cells are exposed to 4AD in the presence of letrozole to recapitulate the steroid environment under Al therapy (Supplementary Table S1, RNA-seq data). This in-house–generated gene list was further supported by comparison with genes identified in a publicly available data set of MCF7-Aro cell treated with 4AD without Al. This helped define gene-expression changes attributed solely to the effects of 4AD and provided evidence of putative 4AD-specific regulation in Al resistance. Subsequent comparison with the “Group 4” genes associated with ER”, HER2-endocrine resistance (GSE1480; ref. 35) yielded a subset of 8 genes (Fig. 2A). As using data from independently published data sets introduces confounding factors the genes identified were validated in-house in two genetically disparate Al-resistant models.

Chronic adaptation to the steroid environment induces a unique transcriptome that can be reverted by reintroduction of estradiol

Validation of the transcriptomic analysis was performed by treating endocrine-sensitive MCF7 and ZR75.1 cells and their endocrine-resistant derivatives (MCF7-Aro-LetR and ZR-Aro-LetR cells) with 4AD and investigating the impact on the transcript levels of the top 4 genes in our list: growth regulation by estrogen in breast cancer 1 (GREB1), serum/glucocorticoid-regulated kinase family member 3 (SGK3), cAMP-dependent kinase inhibitor β (PKIB), and MYB proto-oncogene like 1 (MYBL1). Each of these genes validated in both Al-resistant cell lines, MCF7-Aro-LetR and ZR-Aro-LetR, with significant increases in GREB1, SGK3, PKIB, and MYBL1 in response to 4AD treatment [Fig. 2B (i–viii)]. GREB1, SGK3, PKIB, and MYBL1 did not increase in response to 4AD treatment in endocrine-sensitive MCF7 cells and no change was observed for 3 out of 4 of these genes in endocrine-sensitive ZR75.1 cells with the exception of PKIB, which demonstrated a very modest increase [Fig. 2C (i–viii)]. Overexpression of the aromatase gene was used to generate both models of Al resistance and has also been a feature observed in the development of Al resistance in the clinic. In order to address whether CYP19A overexpression was a driving factor in the increased expression of SGK3, PKIB, MYBL1, and GREB1 a series of experiments using MCF7 cells overexpressing aromatase and cultured in the presence of 4AD were performed. Data from these experiments confirmed that aromatase overexpression alone did not induce increased levels of these genes [Supplementary Fig. S3A (i–iv)]. Further evidence that aromatase overexpression is inconsequential for these genes is provided via analysis of GSE 10911 (39) and from TCGA cohorts (Supplementary Figs. S3B and S6). The former study focused on MCF7-Aro cells cultured in the absence of steroid, with testosterone (T) or with T plus Al. SGK3 levels were increased in the MCF7aro plus T compared with steroid unstimulated cells; most interestingly, there was a further significant increase in SGK3 mRNA in cells cultured with T and AI. Reversion of the MCF7-Aro-LetR transcriptional response to 4AD could be achieved by culturing MCF7-Aro-LetR in estradiol for 15 weeks. Cell growth initially decreased with notable alteration in cell morphology to the cobbledstone appearance of estradiol-dependent MCF7 and smaller cell size (see Fig. 6A for illustrative images). Treatment of these cells with 4AD (24 hours) produced very minimal effects on gene expression in comparison with those observed in the MCF7-Aro-LetR from which they were derived [Figs. 3A (i–iv), 3B and 6A].

Androstenedione-stimulated induction of target gene mRNA is reduced post-siRNA knockdown of either AR or ER; both AR and ER are recruited to a common region on each gene promoter

As it has been established that many transcripts are under cooperative AR:ER regulation (13), we then wished to establish whether the genes identified in this current study were regulated by ER and/or AR. To determine if AR and/or ER are integral to the 4AD-mediated upregulation of GREB1, SGK3, PKIB, and MYBL1, siRNA inhibition of AR and ER was performed, and siRNA efficacy was confirmed [Supplementary Fig. S2D (i–ii) and S2E (i–ii)]. The impact of knockdown of nuclear receptors (NR) on target gene expression in MCF7-Aro-LetR cells was evaluated. siRNA knockdown of AR combined with 4AD treatment resulted in a significant decrease in SGK3 and PKIB transcript levels but had no significant impact on GREB1 or MYBL1 [Fig. 4A (i–iv)], siRNA knockdown of ER combined with 4AD treatment resulted in significantly reduced levels of all four targets [Fig. 4B (i–iv)]. Regulation of SGK3 protein by AR and ER was then confirmed at the level of protein expression [Supplementary Fig. S3C (i–ii)]. AR and ER recruitment to these target genes was then evaluated in a
publicly available data set (GSE104399; ref. 40). Although primarily a male breast cancer data set, it contains ChIP-seq data for both AR and ER recruitment in a female breast cancer patient (patient 8: ER⁺, PR⁺, HER2⁻, postmenopausal; Fig. 4C, top). Using the ChIP-seq peak information published by the original authors, we were able to confirm the binding of ER to the proximal promoter of SGK3, GREB1, MYBL, and PKIB and AR to GREB1 and PKIB in this patient sample (see Supplementary Table S2 and Supplementary Fig. S4 for additional details for binding locations). Enhanced AR recruitment and ER occupancy of the SGK3 promoter were confirmed using ChIP in 4AD-stimulated MCF7-Aro-LetR (Fig. 4C and D). Although this analysis was limited to one patient, it does suggest that AR and ER bind at regions enriched with ESR1 motifs. ER ChIP-seq data for the MCF7-Aro-LetR confirmed ER recruitment to the proximal promoters of all 4 genes in the presence of 4AD plus letrozole (Fig. 4C, bottom; Supplementary Table S2 and Supplementary Fig. S4).

Knockdown of SGK3 inhibits AI-resistant cell viability in the presence of androstenedione or estradiol

SGK3 has previously been documented as a downstream target of AR and ER (α and β isoforms) in prostate and ERα in breast cancer (24, 25) and is also associated with stabilization of endoplasmic reticulum stress. Here, we have shown that SGK3 mRNA and protein levels are significantly increased in MCF7-Aro-LetR cultured in the presence of 4AD [Fig. 5A (i–ii)]. Treatment with BEZ235 or a combination of the anti-AR therapy, bicalutamide, with BEZ235 decreased SGK3 mRNA expression (Fig. 5B). We found that when SGK3 levels are abrogated by siRNA targeting SGK3 [Fig. 5C (i–iii)] in the absence of steroid, there was no change in cell viability [Fig. 5D (i)]; in contrast, when SGK3 was targeted for degradation in the presence of 4AD, there was a significant decrease in cell viability [Fig. 5D (ii)]. This result was also reflected in cell counts of MCF7-Aro-LetR cells exposed to siRNA targeting SGK3 [Fig. 5D (iii)]. Further validation was carried out in
ZR-Aro-LetR cells [Supplementary Fig. S5A (i–iii)]. Follow-up siSGK3 experiments using a variety of steroids demonstrated that decreased viability is only evident when cells are exposed to 4AD or estradiol, but there is no alteration observed when cells are cultured in the potent androgen, R1881, or the control, cholesterol (Supplementary Fig. S5B).

SGK3 and PKIB are associated with poor recurrence-free survival (RFS) in the postmenopausal, endocrine-treated breast cancer patient population (ER\(^+\) PR\(^-\)).

In vitro data presented have shown the impact of the steroid microenvironment upon target gene expression identified by RNA-seq (schematic overview: Fig. 6A). In postmenopausal women, 100% of sex hormones are synthesized in peripheral tissues from circulating adrenal and ovarian precursor steroids. Clinical data have shown levels of 4AD to be elevated in patients whose disease progresses on AI therapy (19, 20), and more recent data have shown 4AD dominates breast tumor intracrinology (41). In contrast, serum and tissue levels of estradiol are markedly reduced in patients treated with an AI (3). Our study identified SGK3 and PKIB as 4AD regulated transcripts mediated in part by AR in collaboration with ER. This supports the hypothesis that transcriptional reprogramming as a result of the steroid environment can facilitate resistance to AI therapy (summarized: Fig. 6B). The BreastMark meta-analysis resource was used to evaluate the impact of SGK3 and PKIB transcript levels on endocrine-treated breast cancer patient outcome (36). The high expression group in each plot (blue) accounts for the upper quartile of expression levels for a particular transcript and the low expression group (red) the remaining 75%. This was applied to each of the individual data sets, and the information was then combined to perform a global pooled survival analysis. Meta-analysis of the AR-mediated genes SGK3 and PKIB showed that these genes have no impact on RFS in treatment-na\(\bar{v}\)e, postmenopausal ER-\(\bar{v}\)positive breast cancer; however, when we evaluate their impact in an endocrine-treated population, it is clear that patients with high levels of these transcripts do not benefit from endocrine therapy [Fig. 6C (i–ii)].

Figure 2.
4AD upregulates genes associated with steroid and PI3K signaling in two models of AI resistance but not in the parental cell lines. A, RNA-sequencing was performed following androstenedione (4AD \(10^{-7}\) mol/L) treatment plus letrozole for 24 hours in Al-resistant MCF7-Aro-LetR cells versus vehicle. These data were then compared with array data for MCF7aro-treated with 4AD versus vehicle to identify 4AD-specific transcripts. These genes were then integrated with the ‘Group 4’ gene set of endocrine-resistant genes associated with ER\(^+\) HER2\(^-\) disease yielding a list of 8 genes. B, (i–viii) qRT-PCR validated differentially expressed genes (GREB1, SGK3, PKIB, and MYBL1) in Al-resistant cell models MCF7-Aro-LetR and ZR-Aro-LetR cells. C, (i–viii) qRT-PCR showed expression of these genes in parental MCF7 and ZR75.1 cells on exposure to 4AD. Graphs representative of \(n = 3\). Error bars are representative of mean ± SEM. Student paired, two-tailed \(t\) test established significance: *, \(P < 0.05\); **, \(P < 0.001\); \(n\), ref. (32); $, ref. (36).
SGK3 levels are sustained in breast cancer patients who do not respond to AI therapy

To evaluate these observations further, SGK3 levels were assessed in a clinical cohort of breast cancer patients defined as responsive or nonresponsive to AI therapy. Data Series GSE59515 (37) was used to assess mRNA expression levels of SGK3 and AR before and 3 months after AI treatment in a cohort of postmenopausal breast cancer patients (n = 25). SGK3 levels are lower in responsive patients posttherapy (drop in mean expression: 16.75 ± 0.563, one-tailed t test: P = 0.0015; Fig. 7A(i)). Conversely, SGK3 mRNA in nonresponders is somewhat sustained (drop in mean expression 16.64 ± 6.44, one-tailed t test: not significant; Fig. 7A(ii)). This was mirrored by levels of AR mRNA with a significant drop in responders (one-tailed t test: P = 0.0196) compared with sustained levels in nonresponders to AI therapy (one-tailed t test: not significant; Fig. 7A(ii)). These data were validated in a second cohort of AI responders and nonresponders (n = 6). SGK3 mRNA was detectable only in postmenopausal nonresponders [Fig. 7A(iii)], a result that is mirrored by the levels of AR mRNA [Fig. 7B(iii)]. Of note, the only patient nonresponsive to AI with no detectable SGK3 or AR transcript was premenopausal, highlighting the postmenopausal hormone state as being crucial to the expression of these target genes. SGK3 has previously been reported to be amplified in breast cancers (42), and thus whilst exploring the expression of SGK3 transcript in clinical cohorts, it was noted that there is a large percentage (16%) of breast cancer patients with an alteration in the SGK3 gene. The impact of SGK3 copy-number amplification was then evaluated in postmenopausal, breast cancer patients treated with endocrine therapy (n = 132). Kaplan–Meier survival curves showed that copy-number amplification of SGK3 significantly associates with poor survival in the postmenopausal patients (P = 0.016). Analysis of the premenopausal patient cohort yielded no association; however, it should be noted that numbers in this cohort were <50 (Fig. 7C).

Discussion

PI3K signaling as a driver of endocrine resistance in ER-positive breast cancer has been validated in many previous studies and clinical trials (17, 43). Given that the combination of the α6-PI3Kinase inhibitor pictilisib plus fulvestrant does not improve survival in AI-resistant breast cancer suggests that the PI3K pathway alone or in combination with ER is not the sole driver of AI resistance (44). Our cell line models of AI-resistant breast cancer are cultured in letrozole and 4AD, which results in elevation of AR protein levels in contrast to parental cell lines. In light of the abundance of data from the field of prostate cancer, which have elucidated mechanisms by which AR can act as a mediator of second messenger signaling, a potentiating role for AR in our model of AI resistance was evaluated (45). In this study, we have utilized transcriptomic analysis to elucidate gene-expression changes associated with resistance to AI therapy.

AI therapy creates a unique androgenic environment that permits exploration of steroid drivers of resistance that may not be wholly dependent upon a functional ER; this is exemplified...
clinically by the similar overall survival achieved when AI or selective estrogen receptor degrader is used as second-line therapy after recurrence on AI (46). AR is normally associated with good patient outcome (11, 16) and the prosurvival response observed in the enzalutamide-treated MCF7 cells in this study would fully support this role. However, the strongly opposing action of the same drug in the isogenic MCF7-Aro-LetR cells highlights the importance of the steroid microenvironment in directing the action of AR. As we have observed contrasting impact on cell growth with anti-AR therapy, we may presume the response is heavily influenced by the steroid driver (estradiol in MCF7 and androstenedione in MCF7-Aro-LetR). Our study is not without limitations as the cell line models used are engineered to over-express CYP19 (amplification of which is known to be associated with AI resistance; ref. 29); however, control experiments clearly show that aromatase overexpression in this system does not increase expression of the 4AD target genes. Furthermore, analyses of clinical TCGA data sets show no association of CYP19 amplification and SGK3 expression, suggesting that this is a disparate mechanism of resistance. It is therefore plausible that estrogen depletion as a consequence of AI therapy may be permissive of AR-mediated transcriptomic alterations.

Elevated levels of sex steroids and their prohormones have long been associated with increased breast cancer risk primarily via epidemiologic and clinical observation (20, 47). In this study, we have focused on the effects of 4AD arising from the adrenal gland and ovarial interstitial cells, which acts as the direct sex-steroid precursor for both androgens and estrogens (48, 49). Disappointingly, clinical trials of the CYP17 inhibitor AA (abiraterone), alone or in combination with AI, have shown no difference in progression-free survival between modalities, results that are perhaps confounded by an associated rise in progesterone and the potential of AA to act as an ER agonist in breast cancer (50, 51). It is also plausible that 4AD will be metabolized to other steroids such as 5α-androstenedione or 3β-androstanediol, themselves known to act as drivers of breast cancer proliferation (52). Interestingly, a proliferative role of androstene-3β,17β-diol has recently been suggested for...
ER-positive breast cancer with low intratumoral estradiol levels (53). While weaker sex steroids are incapable of inducing the conformational change required for classic NR genomic action, the and/or their metabolites may play a role in driving nongenomic activity. Indeed, the sheer overabundance of weaker steroids and the formation of transient NR associations may well be able to initiate second messenger signaling, which is not dependent upon low dissociation constants (19). It has often been reported that male and female sex steroids are associated with both ER-positive and ER-negative breast cancers, highlighting the importance of the steroid drivers which may be acting independently of the classic ER action in some tumors (47).

As the majority of breast cancers express AR, it is of interest to understand how the estradiol depleted steroid environment, which accompanies AI therapy, modifies AR action. Many studies have focused on the transcriptomic role of AR in this setting with much of the data indicating a cooperative AR:ER dynamic (13, 40). Indeed from the RNA-sequencing data in our current study, the main network of genes altered in response to 4AD treatment was also found to be transcriptionally regulated by ER alpha. Of note, SGK3 and PKIB were also confirmed to be regulated, in part, by AR. Recruitment of both NRs to regions harboring classic EREs in the proximal promoter region was further confirmed in ChIP sequencing data from a female breast cancer patient in the Severson study (40). Importantly, we have established that SGK3

Figure 5.
BEZ235 or combination of BEZ235 with the anti-AR bicalutamide decreases SGK3 mRNA expression. Knockdown of SGK3 inhibits AI-resistant cell proliferation only in the presence of androstenedione. A. (i) SGK3 protein expression is increased in the presence of 10^{-7} mol/L 4AD in MCF7-Aro-LetR cells. (ii) Densitometry analysis of 3 independent Western blots confirmed significant increase in SGK3 protein expression with 4AD treatment. B, qRT-PCR evaluation of SGK3 mRNA expression with BEZ235 ± anti-AR therapy bicalutamide in MCF7-Aro-LetR cells. C, (i) Western blot of SGK3 protein levels following siSGK3 in MCF7-Aro-LetR. (ii) Densitometry analysis shows SGK3 protein levels following siSGK3. (iii) qRT-PCR of SGK3 mRNA following siSGK3 in MCF7-Aro-LetR. D, MTS of MCF7-Aro-LetR cell viability following knockdown of SGK3 in the absence of steroid. (i) MTS assay of MCF7-Aro-LetR cell viability following knockdown of SGK3 combined with 4AD treatment. (ii) Cell counts confirmed decreased cell viability in MCF7-Aro-LetR cells following siSGK3. Graphs representative of n = 3. Error bars are representative of mean ± SEM. Student two-tailed t test established significance for A, B, and D. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
is indeed a steroid regulated, AR target gene and that its expression is enhanced when estrogen synthesis is inhibited in models of AI resistance.

The most interesting observation made in this study was the shift in gene expression induced by 4AD which was shown, not only in AI-resistant cells derived from MCF7, but also those generated from ZR75.1. This was in stark contrast to the unre- sponsive parental cell lines, suggesting that chronic alterations in steroid bioavailability can induce transcriptomic reprogramming. This was further supported by data from the long-term estradiol-treated MCF7-Aro-LetR, whose gene-expression response to 4AD was completely reverted to reflect that of estradiol-dependent MCF7. SGK3, in particular, has recently been associated with poor response to AI therapy in breast cancer and has been demonstrated to play a role in stabilization of the endoplasmic reticulum during cell stress (25). SGK3 has a high degree of similarity to AKT with both kinases targeting many of the same substrates. The SGK family has been implicated in breast cancer resistance to PI3K inhibition in numerous in vitro studies and in a clinical trial (26); of note, SGK3 can be activated independently of class I PI3K (54). In our study, reduction in SGK3 transcript resulted in a loss of cell viability but only in the presence of 4AD or estradiol. This mirrors the finding reported by Wang and colleagues (25), wherein they observed complete loss in viability when SGK3 is depleted; the more pronounced effect reported was likely due to the increased efficacy of knockdown achieved and also because of the different steroid environments (testosterone vs. 4AD).

Figure 6.
SGK3, PKIB, and GREB1 are 4AD regulated transcripts mediated in part by AR in collaboration with ER. Associated outcome in clinical cohorts highlights a significant impact on therapeutic response to endocrine therapy. A, Schematic overview of key study findings highlights the morphologic and transcriptional changes driven by chronic exposure of MCF7 to 4AD in the presence of letrozole when stably overexpressing CYP19. These same cells, when maintained in estradiol, revert to their original morphology and no longer exhibit alterations in 4AD-mediated gene expression. B, Study summary: 1. In postmenopausal women, 100% of sex hormones are synthesized in peripheral tissues from circulating adrenal and ovarian precursors steroids. Clinical data have shown levels of 4AD to be elevated in patients whose disease progresses on AI therapy, and more recent data have shown 4AD dominates breast tumor intracrinology. Cyp 19 image: DOI: 10.2210/pdb4GL7/pdb. 2. In contrast, serum and tissue levels of estradiol are markedly reduced in patients treated with an aromatase inhibitor. C, (i) Meta-analysis of 4AD-regulated genes SGK3 and PKIB showed that there is no impact on RFS in the endocrine-untreated population [ER⁺ PR⁺, n = 379, HR = 1.23 (0.76–2.08), log-rank P = 0.37]. (ii) Meta-analysis of 4AD-regulated genes SGK3 and PKIB showed that SGK3 and PKIB is associated with poor RFS in the endocrine-treated population [ER⁺ PR⁺, n = 231, HR = 2.55 (1.34–4.85), log-rank P = 0.003].
To understand the clinical relevance of these findings, a meta-analysis of the AR-mediated genes SGK3 and PKIB was performed. This analysis showed that these genes have no impact on RFS in treatment-naïve, postmenopausal, ER-positive breast cancer; however, when we evaluate their impact in an endocrine-treated population, it is clear that patients with high levels of these transcripts do not benefit from endocrine therapy. When this was evaluated in AI-responsive versus nonresponsive patients, it was clear that levels of SGK3 and AR mRNA are sustained in patients failing on therapy. As previously reported (Gasser et al., 2014), it was also noted that many breast cancer patients have elevation of SGK3 copy number; whether this is as a consequence of an altered steroid microenvironment is an intriguing possibility that is yet to be determined. We would purport, from these observations, that AI-treated breast cancers adapt to utilize bioavailable steroids such as those of adrenal origin. As highlighted in this study, evaluating the implications of steroid alterations in the clinical management of disease is warranted.

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

Availability of Data
The data sets generated and/or analyzed during the current study are available in the Gene-Expression Omnibus repository (accession code: SRP148035).

Ethics approval and informed written consent to participate/consent for publication. CTI 09/07.

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References
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Molecular Cancer Therapeutics

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