AKT Antagonist AZD5363 Influences Estrogen Receptor Function in Endocrine-Resistant Breast Cancer and Synergizes with Fulvestrant (ICI182780) In Vivo

Ricardo Ribas¹, Sunil Pancholi¹, Stephanie K. Guest¹, Elisabetta Marangoni², Qiong Gao¹, Aurélie Thuleau³, Nikiana Simigdala¹, Urszula M. Polanska³, Hayley Campbell³, Aradhana Rani¹, Gianmaria Liccardi¹, Stephen Johnston⁴, Barry R. Davies³, Mitch Dowsett¹,⁴ and Lesley-Ann Martin¹

Abstract

PI3K/AKT/mTOR signaling plays an important role in breast cancer. Its interaction with estrogen receptor (ER) signaling becomes more complex and interdependent with acquired endocrine resistance. Targeting mTOR combined with endocrine therapy has shown clinical utility; however, a negative feedback loop exists downstream of PI3K/AKT/mTOR. Direct blockade of AKT together with endocrine therapy may improve breast cancer treatment. AZD5363, a novel pan-AKT kinase catalytic inhibitor, was examined in a panel of ER-expressing breast cancer cell lines (MCF7, HCC1428, T47D, ZR75.1) adapted to long-term estrogen deprivation (LTED) or tamoxifen (TamR). AZD5363 caused a dose-dependent decrease in proliferation in all cell lines tested (GI₅₀ < 500 nmol/L) except HCC1428 and HCC1428-LTED. T47D-LTED and ZR75-LTED were the most sensitive of the lines (GI₅₀ ~100 nmol/L). AZD5363 reestablished TamR cells to tamoxifen and acted synergistically with fulvestrant. AZD5363 decreased p-AKT/mTOR targets leading to a reduction in ERα-mediated transcription in a context-specific manner and concomitant decrease in recruitment of ER and CREB-binding protein (CBP) to estrogen response elements located on the TFF1, PGR, and GREB1 promoters. Furthermore, AZD5363 reduced expression of cell-cycle-regulatory proteins. Global gene expression highlighted ERBB2-ERBB3, ERK5, and IGF1 signaling pathways driven by MYC as potential feedback-loops. Combined treatment with AZD5363 and fulvestrant showed synergy in an ER⁺ patient-derived xenograft and delayed tumor progression after cessation of therapy. These data support the combination of AZD5363 with fulvestrant as a potential therapy for breast cancer that is sensitive or resistant to E-deprivation or tamoxifen and that activated AKT is a determinant of response, supporting the need for clinical evaluation.

Introduction

Around 80% of breast cancers express estrogen receptor α (ER) and depend on estrogen (E) to grow. Strategies to target ER activity include depriving the hormone-dependent tumor cells of E by the use of aromatase inhibitors (1) or the use of antiestrogens such as tamoxifen or fulvestrant, both of which compete with E for the ER. Despite the efficacy of these agents, many tumors exhibit de novo or develop acquired mechanisms of resistance.

Deregulated signaling through the PI3K/AKT/mTOR pathway is a feature of most types of cancer cells (2) and has been linked to endocrine-resistant breast cancer (3–5). Activation of PI3K/AKT occurs via gain-of-function mutations in the catalytic domain of PI3K (PIK3CA) and the regulatory subunit p85α (PIK3R1), amplification of PIK3CA, PIK3CB, and PIK1 or by reduced expression of PTEN, an endogenous inhibitor of the PI3K/AKT pathway (6). Furthermore, mutation and amplification of AKT 1, 2, and 3 together with ERBB2, FGFR1, MET, and IGFIR have also been associated with activation of this signaling axis.

It is clear that the ER can become involved with the PI3K/AKT/mTOR pathway in breast cancer cells, with evidence for both genomic and nongenomic cross-talk between this signaling pathway and ER accounting for endocrine resistance (7–9). Growth factor–mediated activation of AKT can potentiate ER classical transcriptional activity via phosphorylation at serine 167 in the AF1 domain leading to ligand-independent transactivation (10–12). Similarly, elevated levels of AKT have been shown to change the genome-wide binding pattern of ER, effectively altering the ER program (13). These bidirectional interactions between hormonal and kinase signaling pathways create self-reinforcing synergistic loops that potentiate prosurvival signals and may allow breast cancer cells to escape normal endocrine responsiveness.
Targeting the PI3K/AKT/mTOR pathway in association with endocrine therapy has seemed a sensible strategy to explore. However, blockade of a single protein in a complex signaling cascade is unlikely to provide total or prolonged growth inhibition. An important negative feedback loop exists downstream in the PI3K/AKT/mTOR pathway that may limit the effectiveness of mTOR inhibitors in breast cancer. The mTOR-activated kinase, S6K1, phosphorylates and destabilizes the IRS1 and IRS2 proteins in IGF-responsive cells (14). In these cells, mTORC1 inhibition can lead to a reduction in S6K1 activity, which in turn allows IRS1/2 expression to be increased with associated enhanced activation of IGFI-dependent AKT activity. Concern has been raised that activation of this negative feedback loop may overcome the antitumor activity of mTORC1 blockade and limit the effectiveness of rapamycin analogs (14, 15).

In recent years, it has been suggested that direct blockade of AKT in combination with estrogen therapy, may provide a better rationale for treatment of endocrine-resistant breast cancer, affecting both cell survival/apoptosis and ER ligand-independent signaling. A novel pan-AKT kinase catalytic inhibitor (AZD5363) has been developed and shown to inhibit the growth of a range of human tumor xenografts, either as mono-therapy or in combination with HER2 inhibitors or with docetaxel (16). A recent study has also reported that combinations of AKT and IGFI/R/inR inhibitors can be effective in the treatment of hormone-independent ER⁺ breast cancer (17). On the basis of these data, AZD5363 is currently being investigated in phase I clinical trials.

Further investigations on intelligent combinations of signal transduction inhibitors are urgently required both in preclinical models and in clinical trials. In this article, we describe for the first time the effectiveness and molecular consequence of combining AZD5363 with the four most widely used approaches to endocrine therapy in the clinic, namely E-deprivation, tamoxifen, fulvestrant, and AIs in preclinical models of endocrine-sensitive and -resistant ER⁺ breast cancer, as well as in a patient-derived xenograft (PDX) model of ER⁺ breast cancer resistant to E-deprivation.

Materials and Methods

Antibodies and reagents

Primary antibodies against phospho-AKTSer473, phospho-AKTThr308, total-AKT, phospho-p70S6KThr389, total-p70S6K, phospho-S6 ribosomal protein (RPS6)Ser235/236, total-S6RP, phospho-EGFRY1068, total-EGFR, phospho-ERBB2Y1248, phospho-ERBB3Y1222, phospho-ERBB4Y1284, phospho-ERαSer167, phospho-ERβ1Thr17/46, total-4EBP1, phospho-RbSer807/811, total-Rb, phospho-RAFSer259, phospho-RAFSer338, total-RAF, phospho-PRAS40Thr246, total-PRAS40, Cyclin D1, Cyclin D3, total-ERK1/2, 14-3-3 were purchased from Cell Signaling Technology; IRS-1 and total-ERBB2 (Millipore); IRS-2 (Upstate) and cleaved-PARP, total-ERα, and total-ERBB3 were purchased from Santa Cruz Biotechnology; phospho-RAF (Novocastro or Dako); IRS-1 and total-ERBB2 (Millipore); IRS-2 (Upstate) and finally total-ERBB4 (Neomarkers). Secondary antibodies (anti-mouse or anti-rabbit horseradish peroxidase) were obtained from Dako or Cell Signaling Technology. Antibodies used for chromatin immunoprecipitation were purchased from Santa Cruz Biotechnology: ERα clone HC20 and CBP clone A22. 17β-estradiol and 4-hydroxytamoxifen (4-OHT) were purchased from Sigma Aldrich; fulvestrant (ICI182780), letrozole, and exemestane from Tocris Bioscience; AZD5363 was synthesized and supplied by AstraZeneca UK.

Tissue culture

Human breast cancer cell lines MCF7, T47D, ZR75.1, and HCC1428 were obtained from the ATCC between 2000 and 2012 and were cultured in phenol red-free RPMI1640 medium supplemented with 10% FBS and 1 nmol/L estradiol (E2). All cell lines were banked in multiple aliquots to reduce the risk of phenotypic drift and identity confirmed using iPLEX-pro (Agena Bioscience). LTED cells modeling resistance to an AI were derived from all four parental cell lines by long-term culture in the presence of RPMI1640 medium containing 10% dextran charcoal stripped serum (DCC), as described previously (18). The 1% MCF7 and TamR cell lines were cultured in DMEM/F-12 medium, as previously stated (19). MCF7-2A and BT474-A3 cells stably expressing CYP19 (AROM) were derived from parental MCF7 and BT474 cells, respectively, and were maintained in phenol red–free RPMI1640 medium containing 10% FBS supplemented with 1 nmol/L E2 and 1 mg/mL G-418 (Sigma-Aldrich; ref. 20). All cell lines were stripped of steroids for 48 to 72 hours prior to the start of experiments.

Proliferation and colony formation assays

Cells were seeded in 10% DCC into 96-well tissue culture plates and allowed to acclimatize overnight. Monolayers were then treated with RPMI1640 + 10% DCC containing increasing concentrations of AZD5363, with or without the presence of 0.01 nmol/L of E2. To assess the effects of AZD5363 in combination with endocrine agents, cells were maintained in the presence of GI50 values of AZD5363 along with increasing concentrations of estrogen agent 4-OHT, fulvestrant, or anastrozole. The medium was replaced after 3 days and cells were cultured for a total of 6 days. Each experiment was performed at least three times with 8 replicates per treatment. Cell viability was determined using the CellTitre-Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer’s protocol. Values were expressed as % viability relative to the untreated control.

For the colony formation assays, cells were seeded into 6-well plates and allowed to acclimatize for 24 hours. Subsequently, cells were treated with the drug combinations indicated, every 3 to 4 days, for the duration of 14 days until colonies were evident. Cells were then fixed using ice-cold methanol and stained using 0.5% w/v crystal violet in 90% H2O/10% methanol.

Drug interaction analysis

To determine the nature of the interaction between AZD5363 and endocrine agents, combination studies were carried out using Chou and Talalay constant ratio combination model (21). Cells were treated with increasing doses of AZD5363 alone, endocrine agent alone, or an equipotent combination of AZD5363 and endocrine agent for 6 days. The GI50 for each drug alone was used to define the ratio of equipotent doses and serial dilutions of the combinations used. Interactions were calculated using Calcu- synth software (BIOSOFT), based on the combination index (CI) equation of Chou and Talalay. The CIs for a growth inhibition of 50%, 75%, and 90% were obtained using mutually nonexclusive Monte Carlo CI simulations. Drug interaction was scored using the CI so that: CI = 1 is additive, CI < 1 is synergistic, CI > 1 is antagonistic.
Transcriptional assays

Cell lines were seeded in 24-well plates in 10% DCC medium and left to acclimatize for 24 hours, following transfection with Fugene 6 at a ratio of 6:1 (Promega) with 0.1 µg of estrogen response element linked luciferase (EREIlkluc) and 0.1 µg of β-galactosidase (pCH110) reporter constructs. The following day, cells were treated with the drugs combinations specified and left for 24 hours. Luciferase (Promega) and β-galactosidase (GALactoStar, Applied Biosystems) activity were measured using a luminometer. Each experiment was performed 3 times with 3 to 4 replicates per treatment.

Real-time quantitative PCR

mRNA from treated cells was extracted with RNeasy Mini Kit (Qiagen), as per the manufacturer’s instructions, and quantification performed using the Agilent 2100 Bioanalyzer (Expert Software version B.02.03) with RNA Nano LabChip Kits (Agilent Technologies). Total RNA was reverse transcribed using SuperScript III (Invitrogen) and random primers, in accordance with the manufacturer’s instructions. cDNA was subjected to quantitative PCR using 10 ng of cDNA in triplicate using the ABI Perkin-Elmer Prism 7900HT Sequence detection system (Applied Biosystems). TaqMan gene expression assays (Applied Biosystems) were used to quantify TFF1 (Hs00907239_m1), PGR (Hs01155670_m1), CDK7 (Hs00361486_m1); EIF2A (Hs00230684_m1); FOXO3 (Hs00818121_m1); IGFR (Hs00609566_m1); IR52 (Hs00275843_s1); MAP2K5 (Hs00177134); MYC (Hs00153408); NRIP1 (Hs00942766_s1); NRF2 (Hs00819630_m1); PIK3R1 (Hs00381459_m1); PTEN (Hs02621230_s1), and GREB1 (Hs00536409_m1), together with FKBP15 (Hs00391480_m1) as housekeeping genes, to normalize the data. The relative quantity was determined using ΔΔCq, according to the manufacturer’s instructions (Applied Biosystems).

Chromatin immunoprecipitation

MCF7-LTED cells were crosslinked in 1% formaldehyde at room temperature for 10 minutes and then quenched with 125 mmol/L glycine. Samples were then lysed, sonicated, and left to acclimatize for 24 hours, following transfection with the drug combinations indicated for 48 hours. Cells were fixed 90% ethanol and stained with propidium iodide. Cell-cycle analysis was carried out using fluorescence-activated cell sorting (FACS).

Gene expression data and microarray analysis

Gene expression analysis was performed in triplicate using RNA derived from MCF7 and their LTED derivatives after treatment with or without AZD5363 for 24 hours. mRNA extraction and quantification was performed, as previously stated. The 12 RNA samples were amplified, labeled, and hybridized on HumanHT-12_V4 Expression BeadChip, according to the manufacturer’s instructions (Illumina). Raw expression data were extracted using GenomeStudio (www.illumina.com) software; the data were then transformed and normalized using variance-stabilizing transformation and robust spline normalization method in the Lumi package in R (http://www.biocductor.org; ref. accession number: GSE69893). Probes for which expression levels were not reliably detected in one of the samples (detection P > 0.01) were filtered, the remaining differentially expressed genes were identified by using the Class Comparison (http://linus.nci.nih.gov/BRB-ArrayTools.html) using the thresholds of EDR < 5%. The significantly expressed gene lists were subject to further pathway analysis using Ingenuity Pathway Analysis (IPA) to identify altered pathways due to the AZD5363 treatment and the altered pathways with P < 0.05 were considered as significant.

Human tumor xenografts

In vivo efficacy studies were performed in 8- to 12-week-old female Swiss nude mice implanted with the PDX HBCx22OvaR, as previously described (22), in accordance with the French Ethical Committee. First, a pharmacodynamic study was performed for 4 days to assess biomarker changes with samples removed 2 and 4 hours after treatment. Between 5 and 7 mice were included in each group. Second, a long-term study to assess changes in tumor volume and progression over 90 days was initiated. To address the ability of the combination to delay tumor progression, mice were followed for a further 40 days after drug withdrawal. The treatment groups received AZD5363 solubilized in a 10% DMSO 25% w/v Kleptose HPB (Roquette) buffer by oral gavage, or fulvestrant suspended in corn oil by subcutaneous injection into the flank. For the combination groups, fulvestrant was dosed 2 hours before AZD5363. The control groups received both vehicles. In the combination treatment, treatments were started when tumors reached a volume of 40 to 65 mm3. Each group included between 11 and 13 xenografts. Tumor volumes (measured by caliper), animal body weight, and tumor condition were recorded twice weekly for the duration of the study. Tumor volumes were calculated as V = a x b²/2, where “a” is the largest diameter and “b” is the smallest. Growth inhibition from the start of treatment was assessed by comparison of the differences in tumor volume between control and treated groups. Because the variance in mean tumor volume data increases proportionally with volume (and is therefore disproportionate between groups), data were log-transformed to remove any size dependency before statistical evaluation. Tumor volumes were
reported to the initial volume as relative tumor volume (RTV). Percent of tumor growth inhibition (TGI) was calculated at the end of treatments (day 88) as follow: (1-RTVtreated/RTVcontrol) × 100. Statistical significance was evaluated using a one-tailed, two-sample t test.

Fludigim analysis

Tumor samples were homogenized and RNA was extracted as described above. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). ER-dependent genes listed (AQP3 (Hs01105469_G1); AREG (Hs00950669_m1); BMF (Hs00372937_m1); C3 (Hs00163811_m1); CASP9 (Hs02009467_m1); CCND1 (Hs00765553_m1); CCNG2 (Hs00159357_m1); C3 (Hs00950669_m1); ZEB1 (Hs00232783_m1)) were selected and analyzed by using the Fluidigm Biomark System, as per manufacturer's instructions (Fluidigm). Data were analyzed using a real-time PCR analysis software (version 3.1.3) and ΔΔCt calculated. All genes were normalized to the housekeeper PPIA (Hs04194521_s1). Linear fold changes were calculated on the basis of the relative difference in mRNA expression compared with vehicle control. Statistical analysis was subsequently performed using JMP 1.1.0 to determine significant changes with a P < 0.05.

Results

Effect of AZD5363 on cell growth of endocrine-sensitive and -resistant breast cancer cell lines

A panel of endocrine-sensitive and -resistant breast cancer cell lines were assessed for their sensitivity to the AKT inhibitor AZD5363, a pyrrolopyrimidine-derived compound (Fig. 1A). MCF7, ZR75, T47D, HCC1428 cell lines and their LTED derivatives were assessed both in the presence and absence of E2. TamR and their parental cell line (1%MCF7) were assessed in 1% FBS using JMP 11.0 to determine significance. Subsequently, we conducted formal assessment of the interaction between 4-OHT, fulvestrant, or anastrozole with AZD5363, using CalcuSyn software (Supplementary Fig. S2). T47D-LTED and ZR75-LTED were precluded from this analysis, as they lose the expression of ER. Furthermore, as the TamR cell line is resistant to 4-OHT formal analyses could not be assessed in response to the combination of AZD5363 with 4-OHT.

The combination of AZD5363 and 4-OHT was synergistic in MCF7, MCF7-LTED, and 1% MCF7, with CI's < 1, at 50% and 75% growth inhibition. In contrast, AZD5363 plus 4-OHT showed added benefit only in T47D at 50% and 75% growth inhibition. In contrast, ZR75 at 75% and 90% growth inhibition showed no added benefit (CI > 1; Supplementary Fig. S2A).

Combination of fulvestrant with AZD5363 was predominantly synergistic in MCF7, MCF7-LTED, 1% MCF7 and TamR. In contrast, the combination of fulvestrant and AZD5363 provided no further benefit in T47D and ZR75 cells (Supplementary Fig. S2B). The MCF7-2A and BT474-A3 cell lines showed synergy with almost all doses of anastrozole when combined with AZD5363 (Supplementary Fig. S2C).

In summary, these results suggest that the combination of AZD5363 with endocrine therapy is superior to monotherapy.

Analysis of the differential effects of AZD5363 ± endocrine therapy on PI3K/mTOR/AKT, ER signaling and cell-cycle arrest

To investigate the effect of AZD5363 in combination with endocrine agents on cellular signal transduction pathways (Fig. 3A), MCF7 and MCF7-LTED cells were treated with AZD5363 for 1, 8, 24, or 48 hours ± E2, 4-OHT, or fulvestrant, to establish the most suitable time points for further investigation. As few early changes were evident between 1 and 8 hours after treatment, the 1-hour timepoint was selected to depict early changes. Furthermore, as 24 and 48 hours also showed similar alteration, the 48-hour timepoint was selected to show later events associated with AKT blockade, in particular changes associated with feedback loops discussed later in this study (Supplementary Figs. S3A and S3B, 1-, 8-, and 24-hour timepoint) and Fig. 3B (48-hour timepoint). Addition of AZD5363 resulted...
in increased phosphorylation of AKT (S473 and T308) as early as 1-hour after treatment and was maintained even after 48 hours. Total AKT was suppressed in those cell lines showing the highest degree of sensitivity to AZD5363, most notably T47D, T47D-LTED, ZR75-LTED, and TamR. Treatment with AZD5363 caused a marked reduction, or even complete loss, of pPRAS40 and S6 kinase. Of particular note, total PRAS40 was suppressed in T47D and their LTED. Phosphorylation of 4EBP1, a downstream marker of the PI3K/AKT/mTOR pathway, was reduced in T47D, T47D-LTED, and ZR75-LTED in response to AZD5363 either alone or in combination with endocrine therapy (Fig. 3B). AZD5363 also reduced expression of pRAF (S259), most noticeably in MCF7, T47D, and their LTED derivatives; in contrast, the TamR cell line showed an increase in pRAF (S338). We have previously shown that inhibition of AKT can increase ERK signaling (19), therefore, we assessed the effect of AZD5363 on pERK1/2 and showed differential responses. For instance, AZD5363 suppressed phosphorylation of ERK1/2 in T47D but caused an increase in ZR75-LTED and TamR cells after 48 hours (Fig. 3B). As pAKT, pS6, and pERK1/2 are known to phosphorylate and activate ER, we investigated the effect of AZD5363 ± endocrine therapy on ER phosphorylation together with expression of the progesterone receptor (PGR), an endogenous E-regulated gene. Assessment of ER phosphorylation indicated AZD5363 had the clearest impact on reducing phosphorylation of ERser167 in the MCF7 and MCF7-LTED and this associated with a slight but noticeable reduction in PGR expression in the MCF7 and MCF7-LTED, particularly in the presence of 4-OHT. Of note, while T47D-LTED
Figure 2. Antiproliferative effect of AZD5363 in combination with escalating doses of various endocrine agents. 4-OHT (A), fulvestrant (B), and anastrozole (C), in endocrine-sensitive and -resistant breast cancer cell lines. Cell lines were treated with vehicle or GI50 concentrations of AZD5363 and increasing amounts of 4-OHT, fulvestrant, and anastrozole. After 6 days of treatment, cell viability was analyzed using Cell Titre-Glo and data expressed as fold change relative to vehicle-treated control. Error bars, ±SEM. D, colony-forming assay of MCF7 and MCF7-LTED, following treatment with AZD5363 in the presence or absence of endocrine agents. Cells were left for 14 days with treatment changes every 3 to 4 days.
Figure 3. Effect of the combination of AZD5363 with E-deprivation (DCC), 4-OHT, or fulvestrant on PI3K/mTOR/AKT, ER and cell-cycle signaling. A, schematic representation of the AKT signaling pathway and cross-talk with RTKs and ER. Endocrine-resistant and -sensitive breast cancer cell lines were treated for 48 hours with the drug combinations indicated to assess effect on PI3K/AKT/mTOR and ER pathways (B) and cell cycle (C). Cyclin D3 was assessed for MCF7-derived cell lines, while Cyclin D1 was tested in the remaining cell lines.
showed undetectable levels of ER, AZD5363 caused a marked suppression of PGR in this cell line, suggesting some residual ER activity may still be present.

To assess the effect of AZD5363 on cell-cycle arrest and apoptosis, we investigated changes in protein expression of cyclin D1/D3, Rb, and induction of PARP cleavage (Fig. 3C).

The combination of AZD5363 + endocrine therapy caused a decrease in expression of phosphorylated Rb and cyclin D in the majority of cell lines tested, with a higher effect being observed in combination with fulvestrant. Furthermore, cell-cycle analysis showed a significant reduction in S-phase in response to AZD5363 in MCF7 and MCF7-LTED (Supplementary Fig. S3C). Subtle increases in PARP cleavage were evident in MCF7 although this did not appear specific to treatment. In contrast, T47D-LTED showed increases in PARP cleavage specifically in response to AZD5363 addition (Fig. 3C).

In summary, these data show AZD5363 impedes downstream AKT signaling and reduces ER phosphorylation in a context-specific manner, leading to an increase in cell-cycle arrest.

The effect of AZD5363 alone or in combination with endocrine therapy on receptor tyrosine kinase activity and ER transactivation

We further assessed the impact of AZD5363 on the expression and phosphorylation of receptor tyrosine kinases (RTK) in the target cell lines (Fig. 4A). Inhibition of AKT with AZD5363 resulted in upregulation and activation of RTKs, including EGFR, ERBB2, but also IRS2, which was cell line specific (Fig. 4A). MCF7-LTED, ZR75, and most notably ZR75-LTED showed an increase in pERBB2 and IRS2; TamR revealed increases in pERBB3.

As cross-talk between ER and RTK pathways is well documented (12), we assessed alterations in ER-mediated transactivation after perturbation with AZD5363 alone or in combination with endocrine therapy. MCF7, MCF7-LTED, T47D, ZR75, and TamR cells were transiently transfected with an ER-E禄linked luciferase reporter construct and treated with E2, 4-OHT, or fulvestrant ± AZD5363 (Fig. 4B). AZD5363 showed a significant effect on ER transactivation of MCF7, MCF7-LTED, and T47D when in combination with all endocrine agents. In contrast, in ZR75 the effect on ER transactivation was minimal, apart from when combined with fulvestrant. TamR cells similarly showed minimal impact on ER-mediated transactivation. Further analyses of the expression of a panel of endogenous ER-regulated genes (TFF1, PGR, and GREB1), showed that AZD5363 had a similar impact in keeping with the ER禄linked luciferase assay with the exception of TFF1, which showed an increase in the expression of TFF1 when treated with AZD5363 but a decrease in PGR and GREB1 (Supplementary Fig. S4).

To address this further, chromatin immunoprecipitation was performed in MCF7-LTED cells. AZD5363 caused a reduction in recruitment of ER, AIB1, and CBP to the EREs on the TFF1, GREB1, and PGR promoters (Fig. 4C), supporting the impact of AZD5363 on ER-mediated transactivation.

Gene expression analysis of MCF7 and MCF7-LTED identified MYC as a potential regulator of resistance

Comparison of gene expression between MCF7 cells treated with or without AZD5363 in the absence of E2 showed a total of 1,695 genes differentially downregulated and 1,751 genes differentially upregulated. MCF7-LTED cells showed fewer genes altered in response to AZD5363 (983 genes differentially downregulated vs. 842 genes differentially upregulated). The downregulated genes in both cell lines were mainly associated with metabolic and cell-cycle pathways (Table 1).

The upregulated genes were associated with EIF2, mTOR, and IGF1 signaling. Of note, ERBB2–ERBB3 signaling and big MAPK1 (BMK1/ERK5) were significantly increased in both cell lines in response to AZD5363, together with ILK1 and integrin signaling. Furthermore, RAR pathway activation was also significantly upregulated in both MCF7-LTED and MCF7 cells in response to AZD5363. To interrogate these findings further, expression of selected genes representing activation of the various pathways was tested using qRT-PCR and was shown to be significantly overexpressed (Supplementary Fig. S5). Of particular note, interrogation of genes within these pathways identified MYC, PIK3R1, PTEF, and FOXO3 as common elements (Supplementary Table S1; Supplementary Fig. S5). Further analysis revealed that MYC is upregulated over 2-fold (P < 0.001) in the MCF7-LTED and over 1.2-fold (P < 0.05) in the MCF7 cell lines.

Combination of AZD5363 and fulvestrant in the luminal breast cancer xenograft HBCx22OvaR

A high degree of redundancy in the kinase networks and crosstalk with ER is well documented (23), suggesting destruction of ER with fulvestrant may be a better strategy than antagonism with tamoxifen or blockade of estrogen biosynthesis with an AI. Therefore, we assessed the combination of fulvestrant and AZD5363 compared with monotherapy in a patient-derived luminal breast cancer xenograft HBCx22OvaR modeling acquired resistance to E-deprivation (ref. 22; Fig. 5). Treatments were well tolerated. Fulvestrant monotherapy treatment caused a modest but nonsignificant reduction in tumor growth compared with the vehicle control group (30% inhibition, P = 0.11). Continuous dosing of AZD5363 at 50 mg/kg twice daily resulted in a significant reduction in tumor growth (57% inhibition, P = 5.0E–03), compared with vehicle control. Combination of fulvestrant with AZD5363 resulted in almost complete inhibition of tumor growth (80% inhibition P < 1E–05) compared with vehicle control. The combination dosing was significantly more active than AZD5363 and fulvestrant monotherapy groups (P = 3.75E–03 and 6.3E–04, respectively; Fig. 5A). After 90 days of treatment, the therapies were withdrawn and tumor volume assessed to establish the efficacy of the combination in delaying tumor progression.

Removal of AZD5363 showed a dramatic rise in tumor volume as early as 10 days after withdrawal (Fig. 5B), while the combination of AZD5363 plus fulvestrant showed sustained antitumor effect even after 50 days after cessation of treatment.

To assess dynamic changes in the PI3K/AKT/mTOR, ER, apoptotic, and cell-cycle pathways, a second xenograft experiment was carried out to study tumor pharmacodynamics. HBCx22OvaR were treated for 4 days with the combinations indicated and samples resected 2 and 4 hours after dosing of AZD5363. As expected, Western blot analysis showed significant increases in pAKT (S473) and pAKT (T308) and decreases in pPRAS40 in response to AZD5363. Two hours after treatment with AZD5363 showed a significant rise in PGR expression compared with vehicle control and was negated by the addition of fulvestrant (Fig. 5C).

To assess the effect of AZD5363 on ER-mediated transcription, we used Fluidigm analysis of several ER-dependent target genes (Fig. 5D). Fulvestrant caused a significant reduction in expression of the majority of the genes selected while AZD5363 showed a similar impact in keeping with the ERE-directed transcription. Further analysis of several ER-dependent target genes showed 983 genes differentially downregulated and 842 genes differentially upregulated. The downregulated genes in both cell lines were mainly associated with metabolic and cell-cycle pathways (Table 1). The upregulated genes were associated with EIF2, mTOR, and IGF1 signaling. Of note, ERBB2–ERBB3 signaling and big MAPK1 (BMK1/ERK5) were significantly increased in both cell lines in response to AZD5363, together with ILK1 and integrin signaling. Furthermore, RAR pathway activation was also significantly upregulated in both MCF7-LTED and MCF7 cells in response to AZD5363. To interrogate these findings further, expression of selected genes representing activation of the various pathways was tested using qRT-PCR and was shown to be significantly overexpressed (Supplementary Fig. S5). Of particular note, interrogation of genes within these pathways identified MYC, PIK3R1, PTEF, and FOXO3 as common elements (Supplementary Table S1; Supplementary Fig. S5). Further analysis revealed that MYC is upregulated over 2-fold (P < 0.001) in the MCF7-LTED and over 1.2-fold (P < 0.05) in the MCF7 cell lines.
Effect of AZD5363 in Endocrine-Resistant Breast Cancer

Figure 4.

Effect of the combination of AZD5363 with E-deprivation (DCC), 4-OHT, or fulvestrant on RTK expression (A), ER-mediated transactivation (B), and recruitment of the ER-transcriptional machinery to the TFF1, GREB1, and PGR promoters in response to AZD5363 in the absence of exogenous E2 (C). A, endocrine-resistant and -sensitive breast cancer cell lines were treated for 48 hours with the drug combinations indicated. Whole-cell extracts were assessed for expression on RTK markers by immunoblotting. B, cell lines were cotransfected with EREIItkLuc and pCH110, and treated for 24 hours with the drug combinations indicated. Luciferase activity was normalized by β-galactosidase from triplicate wells and fold changes expressed relative to the DCC control. C, MCF7-LTED cells were treated for 24 hours in DCC in the presence or absence of AZD5363 and ER/CBP binding to TFF1, GREB1, and Pgr assessed via chromatin immunoprecipitation studies. Error bars, ±SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Table 1. Top upregulated and downregulated canonical pathways after treatment with AZD5363 in MCF7-LTED and MCF7 cell lines in the absence of E2

| A. MCF7-LTED | 
|----------------|------------------|
| **MCF7-LTED-upregulated pathways** | **P** | **MCF7-LTED-downregulated pathways** | **P** |
| EIF2 signaling | 0.90E-07 | Mitotic roles of Polo-like kinase | 4.90E-06 |
| IGFl signaling | 3.26E-04 | Cell cycle: G2-M DNA damage checkpoint regulation | 3.55E-06 |
| RAR activation | 0.75E-03 | Remodeling of epithelial adherens junctions | 7.01E-04 |
| mTOR signaling | 0.95E-03 | ATM signaling | 7.46E-09 |
| Diphosphatase biosynthesis | 0.95E-03 | Hereditary breast cancer signaling | 8.50E-09 |
| Endometrial cancer signaling | 1.48E-02 | Role of CHK kinases in cell-cycle checkpoint control | 1.95E-08 |
| PTEN signaling | 1.55E-02 | Protein ubiquitination pathway | 3.00E-08 |
| ErbB2-ErbB3 signaling | 2.09E-02 | GADD45 signaling | 6.00E-08 |
| Insulin receptor signaling | 2.57E-02 | DNA damage-induced 14-3-3 signaling | 6.03E-08 |
| Clathrin-mediated endocytosis signaling | 2.63E-02 | 14-3-3-mediated signaling | 8.13E-08 |
| Mouse embryonic stem cell pluripotency | 3.09E-02 | Cyclins and cell-cycle regulation | 4.07E-07 |
| ERK5 signaling | 3.24E-02 | Role of BRCA1 in DNA damage response | 4.79E-07 |
| Aryl hydrocarbon receptor signaling | 3.39E-02 | Sertoli cell-Sertoli cell junction signaling | 1.70E-06 |
| Ketolysin | 3.63E-02 | Cell cycle control of chromosomal replication | 2.10E-06 |
| HGF signaling | 3.80E-02 | Germ-cell-Sertoli cell junction signaling | 2.75E-06 |
| DNA methylation and transcriptional repression signaling | 4.07E-02 | Mismatch repair in Eukaryotes | 3.72E-06 |
| Amyloid processing | 4.37E-02 | Estrogen-mediated S-phase entry | 8.13E-06 |
| Calcium transporters | 4.47E-02 | Breast cancer regulation by Stat5 | 2.69E-05 |
| Growth hormone signaling | 4.79E-02 | Epithelial adherens junction signaling | 4.07E-05 |
| ILK signaling | 4.90E-02 | Glycylase I | 4.27E-05 |

| B. MCF7 | 
|----------------|------------------|
| **MCF7-upregulated pathways** | **P** | **MCF7-downregulated pathways** | **P** |
| Hypoxia signaling in the cardiovascular system | 1.26E-06 | Mitotic roles of Polo-like kinase | 2.88E-07 |
| Protein ubiquitination pathway | 4.90E-06 | Cell cycle: G2-M DNA damage checkpoint regulation | 7.94E-07 |
| EIF2 signaling | 3.39E-05 | Cell-cycle control of chromosomal replication | 4.07E-06 |
| Aryl hydrocarbon receptor signaling | 1.29E-04 | Role of CHK kinases in cell-cycle checkpoint control | 5.25E-06 |
| IGFl signaling | 5.37E-03 | GADD45 signaling | 7.08E-06 |
| ERK5 signaling | 1.05E-03 | Estrogen-mediated S-phase entry | 8.95E-06 |
| ILK signaling | 1.20E-03 | Mismatch repair in Eukaryotes | 1.41E-05 |
| Molecular mechanisms of cancer | 1.35E-03 | ATP signaling | 1.41E-05 |
| Integrin signaling | 1.51E-03 | Remodeling of epithelial adherens junctions | 1.62E-05 |
| NGF signaling | 1.78E-03 | Role of BRCA1 in DNA damage response | 1.78E-05 |
| p53 signaling | 1.86E-03 | Sertoli cell-Sertoli cell junction signaling | 6.92E-05 |
| Regulation of eIF4 and p70S6K signaling | 2.00E-03 | Hereditary breast cancer signaling | 2.69E-04 |
| Neurotrophin/TRK signaling | 2.19E-03 | Cholesterol biosynthesis I | 3.09E-04 |
| Estrogen receptor signaling | 2.34E-03 | Cholesterol biosynthesis II (via 24, 25-dihydroxanossterol) | 3.09E-04 |
| PIGF signaling | 2.88E-03 | Cholesterol biosynthesis II (via desmosterol) | 3.09E-04 |
| Renal cell carcinoma signaling | 2.88E-03 | Breast cancer regulation by Stat5 | 3.80E-04 |
| Colonic acid building blocks biosynthesis | 3.55E-03 | Cyclins and cell-cycle regulation | 4.37E-04 |
| ErbB2-ErbB3 signaling | 4.27E-03 | DNA damage-induced 14-3-3 signaling | 5.15E-04 |
| VEGF signaling | 4.37E-03 | Germ-cell-Sertoli cell junction signaling | 7.41E-04 |
| RAR activation | 5.25E-03 | Superpathway of cholesterol biosynthesis | 1.07E-03 |

NOTE: MCF7-LTED and MCF7 cells were treated in the presence or absence of AZD5363 for 24 hours, and RNA was submitted to microarray analysis.

The combination of AZD5363 with endocrine therapy showed the greatest efficacy in both ER+ and ER− breast cancer cells, most notably those containing activating mutations in PIK3CA and/or loss of PTEN function (Fig. 1). In contrast, HCC1428, which are wild-type (wt) for both genes, were resistant to AKT inhibition (GI50 > 10000 nmol/L). The combination of AZD5363 with fulvestrant provided the greatest combination effect, which was later confirmed in a PDX model. Nonetheless, the combination of AZD5363 with an AI was also highly effective. Moreover, ZR75-LTED and T47D-LTED, which have suppressed interaction with ER (8, 10). Hence, AKT being a central mediator within this pathway poses a rational therapeutic target in endocrine resistant breast cancer. In support of this notion, we have shown that suppression of AKT activity with the catalytic AKT inhibitor AZD5363 inhibited the growth of ER+ human breast cancer cells modeling acquired resistance to E deprivation and tamoxifen and prevented the emergence of hormone-independent cells in vivo.

Discussion

Activation of PI3K/AKT/mTOR pathway has been shown to be involved in endocrine resistance in breast cancer, most notably via interaction with ER (8, 10). Hence, AKT being a central mediator within this pathway poses a rational therapeutic target in endocrine resistant breast cancer. In support of this notion, we have shown that suppression of AKT activity with the catalytic AKT inhibitor AZD5363 inhibited the growth of ER+ human breast cancer cells modeling acquired resistance to E deprivation and tamoxifen and prevented the emergence of hormone-independent cells in vivo.

The combination of AZD5363 with endocrine therapy showed the greatest efficacy in both ER+ and ER− breast cancer cells, most notably those containing activating mutations in PIK3CA and/or loss of PTEN function (Fig. 1). In contrast, HCC1428, which are wild-type (wt) for both genes, were resistant to AKT inhibition (GI50 > 10000 nmol/L). The combination of AZD5363 with fulvestrant provided the greatest combination effect, which was later confirmed in a PDX model. Nonetheless, the combination of AZD5363 with an AI was also highly effective. Moreover, ZR75-LTED and T47D-LTED, which have suppressed
Effect of AZD5363 alone or in combination with fulvestrant on biomarker changes and tumor progression in HBCx22OvaR. Long-term study assessing changes in tumor volume over 90 days of treatment (A) and assessment of tumor volume after drug withdrawal (B). Pharmacodynamic study performed for 4 days was conducted to assess biomarker changes in protein expression of pertinent targets (C) within the PI3K/AKT/mTOR pathway and ER signaling via measurement of PgR expression by semiquantitative Western blotting and mRNA analysis of ER-regulated, cell cycle, and apoptosis target genes using Fluidigm heatmap shows Log2 expression of samples extracted 2 and 4 hours after final administration of AZD5363 (D). Bars, ±SEM. *, P < 0.05; **, P < 0.01; *** P < 0.001.
expression of ER compared with their parental cells, were the most sensitive to the antiproliferative effects of single-agent AZD5363, with GI50 values less than 100 nmol/L. It is noteworthy, that PIK3CA mutation may not be the only governing feature of sensitivity to AKT inhibition. For instance, both MCF7 and T47D cells and their derivatives express mutant PIK3CA and wt-PTEN, yet T47D were at least 2-fold more sensitive than MCF7, and this is most pronounced when comparing T47D-LTED with MCF7-LTED, where there is a 5-fold difference in sensitivity. Furthermore, ZR75, which are wt-PIK3CA and PTEN null, are 5-fold less sensitive than ZR75-LTED, suggesting that while mutation within the pathway may delineate sensitivity in some circumstances, cell context remains a defining feature.

Treatment with AZD5363 reduced phosphorylation of 4EBP1, a downstream mediator of the mTOR pathway, and increased phosphorylation of AKT itself. The latter has been shown to occur with several ATP-competitive inhibitors of AKT, as a result of the protein being maintained in a hyperphosphorylated but catalytically inactive form (26). In all cell lines tested, AZD5363 caused cell-cycle arrest, most notably via suppression of phosphorylation of Rb and reduction in Cyclin D and in several cell lines this was associated with elevated apoptosis, most notably in the T47D-LTED. Furthermore, fluidigm analysis of our HBCx22OvaR PDX showed increased expression of CASP9 and BMF in response to AZD5363. These data are in keeping with previous studies in which AKT-dependent phosphorylation of BAD suppresses apoptosis (27).

Previous studies have shown that AKT can phosphorylate ER at serine 167 in a ligand-independent manner and is associated with resistance to endocrine therapy (10, 19); furthermore, elevated AKT has been associated with an altered ER transcriptional program (13). In this current study, we provided further support for these observations and showed that inhibition of AKT in MCF7-LTED reduced recruitment of ER, AIB1, and CBP to ERES. Furthermore, the combination of endocrine therapy with AZD5363 suppressed ER-mediated transcription to a greater extent than either agent alone. In contrast, however, although AZD5363 suppressed the proliferation of the TamR cell line and reconstituted it to the antiproliferative effects of tamoxifen, little impact on ER-mediated transcription in the presence of 4-OHT was evident compared with 4-OHT alone. One explanation for this finding may be due to the high degree of redundancy in the signal transduction pathways within the TamR cell line. We have previously shown that phosphorylation of ERSet167 can occur via pAKT or pERK1/2/pp90rsk. This was confirmed in the current study where AZD5363 had no impact on ERSet167 phosphorylation. It could be postulated, that continued signaling via the pERK1/2 pathway may, in the long-term, negate the initial anti-proliferative effect of AZD5363 in this setting. Previous studies have shown that targeting a single protein in a complex pathway can cause feedback loops resulting in increased ER-mediated transcription (28). Here, we assessed expression of several ER-mediated target genes both in our cell line models and in our PDX in response to AZD5363. Increased expression of ER-target genes appeared context specific and time dependent. One explanation for these contrasting observations is the nature of the cross-talk between ER and AKT. For instance, increased AKT signaling may lead to ligand-independent ER activity in which the cell still relies on ER function for proliferation. Alternatively, increased AKT can suppress ER expression circumventing the need for ER-driven transcription. In the latter setting, perturbation of AKT would therefore increase ER-mediated transcription. Taken together, these data indicate that the combination of AZD5363 with a selective ER downregulator, such as fulvestrant, may alleviate these potential ER-mediated feedback loops.

Studies have suggested that inhibition of kinases within the PI3K/mTOR/AKT pathway can lead to upregulation of several RTKs impacting on their potential clinical utility (17, 29–31). To address this, we assessed the impact of AZD5363 on expression and phosphorylation of type I/II growth factor receptors. AZD5363 caused a cell-type-specific upregulation of various RTKs, including IGFIR, EGFIR, ERBB2, and ERBB3. It has been suggested that altered expression of these RTKs may impede the effectiveness of AZD5363 (17). To assess this further, we used a global gene expression approach to identify networks of genes that were associated with response and resistance to AZD5363. The significantly downregulated canonical pathways, following treatment with AZD5363 were associated with cell-cycle progression and metabolism, confirming our observation in both proliferation and protein assays. One of the most significantly upregulated pathways was EIF2 which was evident in both MCF7 and their LTED derivative. The PERK–eIF2αP pathway mediates survival and facilitates adaptation to the deleterious effects of the inactivation of PI3K or AKT (32). The IGF1 signaling pathway, which encompasses IGF1R, IRS2, and FOXO3 was also significantly upregulated. Previous studies have shown that upregulation of IGF1R maintains PI3K and PIP3 formation, to counteract the inhibition of AKT which could potentially reduce the potency of AZD5363, via a FOXO-dependent transcriptional mechanism (17).

RAR signaling was also associated with inhibition of AKT activity. RAR transactivation is suppressed by phosphorylation via AKT (33). Of note, RAR-r is required for efficient ER-mediated transcription and cell proliferation (34). Assessment of the effect of AZD5363 on ER-mediated transactivation using an artificial reporter construct showed suppression of transactivation. However, assessment of transcript levels of several endogenous E2-regulated genes particularly in the T47D and TamR cell line as well as in our PDX model showed increases in TFF1 and GREB1 expression, in keeping with previous studies with a dual mTOR/PI3K inhibitor (35). Of note, assessment of genes within the RAR signaling pathways showed increases in expression of the IGF1R, IRS2, and FOXO3 was also significantly upregulated. Furthermore, high intratumoral RARs protein levels correlate with reduced relapse-free survival in ER+ patients treated with neoadjuvant tamoxifen (38). Taken together, activation of RAR as a result of AKT inhibition may potentiate ER activity in certain settings.

ERK5 signaling pathway was significantly upregulated in response to AZD5363. ERK5, similar to ERK1/2, regulates EGF-induced cell proliferation, and is known to cross-talk with ERBB2 and PDGFR. Both pathways were also elevated in response to AZD5363. ERK5 is also capable of phosphorylating the ERK1/2 substrates MYC and API1 (39). Moreover, ILK, HIF1, and VEGF pathways were upregulated in response to AKT inhibition. Previous studies have shown that β-parvin, which is lost in metastatic breast cancer (40), leads to an upregulation of ILK/AKT–mediated signaling resulting in increased HIF1α and VEGF (41). Both HIF1α and VEGF contain internal ribosome entry sites (IRES), located in their 5’ UTRs, allowing cap-independent translation, bypassing the requirement for mTOR/4EBP1 signaling (42). This
may provide a survival mechanism in response to chronic treat-
ment with AZD5363. Of particular interest, MYC expression was
identified as a potential driver in six of the upregulated pathways
in response to AZD5363. MYC activity has been shown to abro-
gate response to mTOR inhibition in prostate cancer via its
interaction and upregulation of 4EBP1 (43). Notably, 4EBP1
expression did not change in response to AZD5363 in our PDX
model.

Overall, the transcriptional profiling revealed multiple
mechanisms of compensation to AKT inhibition, highlighting
the complex interplay between ER and signal transduction path-
ways. This global gene expression analysis did, however, use only
one cell line model and its LTED counterpart.

In conclusion, the results provide mechanistic evidence for the
combination of AZD5363 and endocrine therapy to delay the
onset of resistance, as well as resensitize endocrine-resistant
tumors to the antiproliferative effects of endocrine therapy. Most
notably the combination of AZD5363 with fulvestrant, suppres-
sing both the ER and AKT signaling axes appeared superior to
either agent alone. The current study also highlights potential
routes of escape via RTK, RAR, ERK5, MYC, and PERK–eIF2εP
signaling, that merit investigation for further improvements in
treatment efficacy.

Disclosure of Potential Conflicts of Interest

S. Johnston is a consultant/advisory board member for AstraZeneca. B.R.
Davies has ownership interest as a shareholder at AstraZeneca. M. Dowsett
reports receiving a commercial research grant from AstraZeneca and has received speakers
bureau honoraria from AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Authors’ Contributions

Conception and design: S. Johnston, B.R. Davies, M. Dowsett, L.-A. Martin
Development of methodology: R. Ribas, S. Pancholi, S.K. Guest, E. Marangoni,
N. Simigdala, L.-A. Martin
Acquisition of data (provided animals, acquired and managed patients,
provided facilities, etc.): J. S. Pancholi, S.K. Guest, E. Marangoni, A. Thuleau,
U.M. Polanska, H. Campbell, G. Liccardi, L.-A. Martin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics,
computational analysis): R. Ribas, S. Pancholi, S.K. Guest, E. Marangoni,
Q. Gao, A. Thuleau, N. Simigdala, U.M. Polanska, H. Campbell, A. Rani,
G. Liccardi, B.R. Davies, M. Dowsett, L.-A. Martin
Writing, review, and/or revision of the manuscript: R. Ribas, S. Pancholi,
S.K. Guest, E. Marangoni, Q. Gao, U.M. Polanska, H. Campbell, G. Liccardi,
S. Johnston, B.R. Davies, M. Dowsett, L.-A. Martin
Administrative, technical, or material support (i.e., reporting or organizing
data, constructing databases): R. Ribas, S. Pancholi, S.K. Guest, A. Thuleau,
A. Rani, L.-A. Martin
Study supervision: M. Dowsett, L.-A. Martin

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