AZD9496: An Oral Estrogen Receptor Inhibitor That Blocks the Growth of ER-Positive and ESR1-Mutant Breast Tumors in Preclinical Models

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Abstract

Fulvestrant is an estrogen receptor (ER) antagonist administered to breast cancer patients by monthly intramuscular injection. Given its present limitations of dosing and route of administration, a more flexible orally available compound has been sought to pursue the potential benefits of this drug in patients with advanced metastatic disease. Here we report the identification and characterization of AZD9496, a nonsteroidal small-molecule inhibitor of ERα, which is a potent and selective antagonist and downregulator of ERα in vitro and in vivo in ER-positive models of breast cancer. Significant tumor growth inhibition was observed as low as 0.5 mg/kg dose in the estrogen-dependent MCF-7 xenograft model, where this effect was accompanied by a dose-dependent decrease in PR protein levels, demonstrating potent antagonist activity. Combining AZD9496 with PI3K pathway and CDK4/6 inhibitors led to further growth-inhibitory effects compared with monotherapy alone. Tumor regressions were also seen in a long-term estrogen-deprived breast model, where significant downregulation of ERα protein was observed. AZD9496 bound and downregulated clinically relevant ESR1 mutants in vitro and inhibited tumor growth in an ESR1-mutant patient-derived xenograft model that included a D538G mutation. Collectively, the pharmacologic evidence showed that AZD9496 is an oral, nonsteroidal, selective estrogen receptor antagonist and downregulator in ERα breast tumors that could provide meaningful benefit to ERα breast cancer patients. AZD9496 is currently being evaluated in a phase I clinical trial. Cancer Res; 76(11); 3307–18. ©2016 AACR.

Introduction

With over 70% of breast cancers expressing the estrogen receptor alpha (ERα), treatment with antihormonal therapies that directly antagonize ER function (e.g., tamoxifen) or therapies that block the production of the ligand, estrogen (e.g., aromatase inhibitors) are key to management of the disease both in adjuvant and metastatic settings (1, 2). Despite initial efficacy seen with endocrine therapies, the development of acquired resistance ultimately limits the use of these agents although the majority of tumors continue to require ERα for growth. Through the use of preclinical models and cell lines, changes in growth factor receptors have been implicated in driving resistance in cells, for example, receptor tyrosine kinases (HER2), EGFR, and their downstream signaling pathways, Ras/Raf/MAPK and PI3K/protein kinase B (AKT) signaling (3–5). In some instances, signaling from different growth factor receptor kinases leads to phosphorylation of various proteins in the ER pathway, including ER itself, leading to ligand-independent activation of the ER pathway (6).

In the metastatic setting, 500 mg fulvestrant, given as 2 × 250 mg intramuscular injections, has been shown to offer additional benefit over the 250 mg dose with improved median overall survival (7, 8). Recent analysis of presurgical data from a number of trials showed a dose-dependent effect on key biomarkers such as ER, progesterone receptor (PR), and Ki67 labeling index (9). Although fulvestrant is clearly effective in this later treatment setting, there are still perceived limitations in its overall clinical benefit due to very low bioavailability, the time taken to achieve plasma steady-state of 3–6 months and ultimately the level of ER inhibition seen in clinical samples (10, 11). It is widely believed that a potent, orally bioavailable SERD that could achieve higher steady-state free drug levels more rapidly would have the potential for increased receptor knockdown and lead to quicker clinical responses, reducing the possibility of early relapses (12). Moreover, the recent discovery of ESR1 mutations in metastatic breast cancer patients that had received prior endocrine treatments, where the mutated receptor is active in the absence of estrogen,
highlights an important potential resistance mechanism. Preclinical in vitro and in vivo studies have shown a reduction in signaling from ESR1 mutants by tamoxifen and fulvestrant, but at higher doses than required to inhibit the wild-type receptor, which implies that more potent SERDs may be required to treat these patients or to use in the adjuvant setting to limit the emergence of mutations (13–15).

Achieving oral bioavailability has remained a key challenge in the design of ER downregulators and until now no oral estrogen receptor downregulators, other than GDC-0810 and RAD1901, have progressed into clinical trials, despite encouraging reports from others of preclinical activity in mouse xenograft models (16–21). Here, we describe a chemically novel, nonsteroidal ER antagonist and downregulator, AZD9496, that can be administered orally and links increased tumor growth inhibition to enhanced biomarker modulation compared with fulvestrant in a preclinical in vivo model of breast cancer. Moreover, we show that AZD9496 is a potent inhibitor of ESR1-mutant receptors and can drive tumor growth inhibition in a patient-derived ESR1 mutant in vivo model (PDX). Hence, AZD9496 is anticipated to yield improved bioavailability and clinical benefit through enhanced ER pathway modulation.

Materials and Methods

Cell lines and culture

All AZ cell lines were tested and authenticated by short-tandem repeat (STR) analysis. MCF-7 was obtained from DSMZ and last STR tested in February 2013. MDA-MB-361 (STR tested in October 2011), MDA-MB-134 (STR tested in October 2010), T47D (STR tested in June 2012), CAMA-1 (STR tested in March 2012), HCC1428 (STR tested in December 2011), HCC70 (STR tested in October 2013), LnCAP (STR tested in August 2011), MDA-MB-468 (STR tested in March 2014), BT474 (STR tested in July 2013), PC3 (STR tested in October 2012), HCT116 (STR tested in August 2011) were all obtained from the ATCC. MDA-MB-134 (STR tested in October 2010), T47D (STR tested in February 2013. MDA-MB-361 (STR tested in October 2010), T47D (STR tested in February 2013). PC3 (STR tested in October 2012), HCT116 (STR tested in August 2011) were all obtained from the ATCC. MDA-MB-134 (STR tested in October 2010), T47D (STR tested in February 2013. MDA-MB-361 (STR tested in October 2010), T47D (STR tested in February 2013). MCF-7 was obtained from DSMZ and last STR tested in September 2012. Other cell lines in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility. The rat uterine weight assay for the measurement of estrogenic activity was performed as described previously (31). Details of individual models, protein analysis of tumor fragments, and pharmacokinetic analysis of plasma samples are included in Supplementary Methods.

Results

AZD9496 is a selective ER antagonist, downregulator, and inhibitor of ER+ tumor cell growth

AZD9496 ((E)-3-(3,5-difluoro-4-((1R,3R)-2-(2-fluoro-2-methyl-propyl)-3-methyl-2,3,4,9-tetrahydropyrido[3,4-b]indol-1-yl)phenyl)acrylic acid; Fig. 1A) was identified through structure-based design and iterative medicinal chemistry structure–activity relationship (SAR) studies from a novel ER-binding motif (32). A directed ER-binding screen was established to identify novel binding motifs that could then be optimized for both cellular phenotype and potency while maintaining good pharmacokinetic properties (23). Unlike fulvestrant, and other well-known historical modulators of the ER receptor, including hydroxytamoxifen (Fig. 1B), AZD9496 lacks a phenolic moiety in which the phenol mimics the A-ring phenol group of the endogenous ligand estradiol but gains its potency through a different series of novel interactions with the ER protein (Fig. 1C). The indole NH picks up an interaction by forming a hydrogen bond to the carbonyl of Leu-346, with additional lipophilic interactions achieved by both the chiral methyl substituent, adjacent to the ring nitrogen, and the N-substituted isopropyl fluoro side chain occupying a “lipophilic hole” in the ER protein. The acrylic acid side chain picks up an unusual acid–acid colocalization with Asp-351 in the helix-12 region of the ER that has been proposed to be crucial for achieving a downregulator–antagonist profile (33). AZD9496 showed high oral bioavailability across three species (% 63, 91, and 74, rat, mouse, and dog, respectively) with generally low volume and clearance across species, albeit a higher clearance in mouse. The percent free levels in human plasma of 0.15% were 5-fold higher than those measured for fulvestrant.

The key characteristics of a SERD is the ability to both antagonize and downregulate ER protein without inducing any partial agonist effects in breast cells. A series of in vitro cell
assays were developed specifically to measure ERα downregulation, agonism, and antagonism through simultaneous measurement of ER and PR protein levels in cells after treatment with AZD9496 (23). PR expression is known to be regulated by ER transcription and was measured as a marker of ER activation in cells (34). The potency of AZD9496 approached that observed with fulvestrant in achieving pmol/L IC50 in ERα binding, downregulation, and antagonism and significant cell growth inhibition in hormone-depleted growth conditions. Adding 250 nmol/L tamoxifen significantly lowered the IC50 value, as expected, if AZD9496 and tamoxifen are competing for binding to the ligand-binding domain (LBD; Table 1). The potent downregulation of ERα was also verified by immunoblotting techniques (Supplementary Fig. S1).

To understand the binding kinetics of AZD9496 to human ERα LBD, binding studies were performed in the presence of increasing concentrations of AZD9496 and association (kass) and dissociation (kdiss) rate constants were calculated. AZD9496 was calculated as having an apparent dissociation rate constant (kdiss) for ERα LBD of 0.00043/sec, at 25°C equating to a half-life (t1/2) of 27 ± 2 minutes and the affinity derived from the ratio of the rate constants gave a Kd of 0.33 nmol/L (Supplementary Table S2). This is in good agreement with the binding IC50 of 0.82 nmol/L and correlates well with previously published data on estrogen and tamoxifen binding to ER LBD (35). AZD9496 showed pmol/L equipotent binding to both ERα and ERβ isoforms, as expected, given their high sequence homology and similarity in tertiary structure in their LBDs (36), and highly selective binding compared with progesterone (~650-fold), glucocorticoid (~11,223-fold), and androgen (~36,375-fold) receptor LBDs (Supplementary Table S3). Selectivity for ER was also evident by measuring cell growth inhibition in a panel of ER+ and ER− cell lines (Supplementary Table S4). No significant growth inhibition was seen in any ER− cell lines (EC50 > 10 μmol/L) in contrast to the low nanomolar activity seen in a range of ER+ cell lines.

![Crystal structure of the LBD of ERα in complex with AZD9496. A, two-dimensional chemical representation of AZD9496. B, two-dimensional chemical representation of 4-hydroxy tamoxifen. C, three-dimensional structure of the complex between AZD9496 and the LBD of ERα.](cancerres.aacrjournals.org)
AZD9496 directly targets ERα for downregulation in vitro

To measure the rate at which AZD9496 downregulated ERα, SILAC experiments were performed in MCF-7 cells using mass spectroscopy analysis to detect ERα protein levels after treatment with compounds. Addition of AZD9496 or fulvestrant increased the rate of degradation of the isotope-labeled ERα compared with DMSO control. Levels of newly synthesized ERα peptide were also reduced following AZD9496 and fulvestrant treatment, presumably due to ongoing degradation of newly synthesized ERα protein by compound present, whereas ERα protein levels continued to increase over time in the presence of tamoxifen or DMSO (Fig. 2). The 11/12 of ERα was decreased from 3 hours, in the presence of DMSO, to 0.75 hours with 100 nmol/L AZD9496 and 0.6 hours with 100 nmol/L fulvestrant (Supplementary Fig. S2). Downregulation of ER occurs through the 26S proteosomal pathway as no decrease in ERα protein level in MCF-7 cells was seen in the presence of the proteosome inhibitor MG132 (Supplementary Fig. S3). In addition, ERα downregulation was shown to be reversible in compound wash-out experiments, where ERα levels increased in a time-dependent manner back to basal levels over a 48-hour period following compound removal (Supplementary Fig. S4).

Agonism of ERα in human breast and uterine cells

In contrast to an AstraZeneca reference compound (AZL0557841), which has known ERα agonist activity in breast cells, AZD9496 was shown to cause no increase in PR protein levels but rather a slight decrease below the detected basal levels (Fig. 3A). Some endocrine therapies, such as tamoxifen, can act as partial ER agonists in certain tissues, for example, endometrium, due to conformational changes that take place at the ER and the resulting effect on cofactor recruitment and transcriptional gene activation from the tamoxifen-bound receptor (37). To test whether AZD9496 could act as a partial agonist in other tissues, agonist effects were measured in vivo in a previously validated immature female rat model designed to detect agonistic properties of test compounds by measuring increases in uterine weight (31). AZD9496, given once daily orally at 5 and 25 mg/kg produced statistically significant increases in uterine weight compared with the fulvestrant control (P < 0.001) but significantly lower than tamoxifen (P = 0.001; Fig. 3B). Histologic staining of uterine tissue samples also showed that the lengthening of the endometrial cells in the rat uteri appeared to have decreased compared with tamoxifen (Fig. 3C). In further studies, ERα protein levels were reduced with fulvestrant and AZD9496, but not tamoxifen, compared with relevant vehicle controls, whereas PR levels were significantly increased with tamoxifen alone (Fig. 3D).

AZD9496 is a potent, oral inhibitor of breast tumor growth in vivo

The effect of chronic, oral dosing of AZD9496 was explored in MCF-7 human breast xenografts, as a representative ER+/PR-/HER2+ breast cancer model. Good bioavailability and high clearance gave a terminal t1/2 of 5–6 hours after oral dosing in the mouse and resulted in significant dose-dependent tumor growth inhibition with 96% inhibition at 50 mg/kg and no toxicity or weight loss relative to the vehicle control group (Fig. 4A). To confirm that AZD9496 was targeting the ER pathway, PR protein levels were measured in tumor samples taken at the end of the study and a significant reduction in PR was seen, which correlated with tumor growth inhibition. A >90% reduction in PR was seen with both 10 and 50 mg/kg doses and a 75% decrease even with the 0.5 mg/kg dose, demonstrating that AZD9496 can clearly antagonize the ER pathway (Fig. 4B). Dosing 5 mg/kg of AZD9496, the minimal dose required to see significant tumor inhibition, gave greater tumor growth inhibition compared with 5 mg/mouse fulvestrant given 3 times weekly and tamoxifen given 10 mg/kg orally, daily (Fig. 4C). A series of pharmacodynamic in vivo studies were conducted to measure time taken to reach maximal inhibition of PR levels and time taken to recover back to basal levels. Three days of dosing with 5 mg/kg of AZD9496 gave 98% reduction of PR protein and continued to suppress protein levels at 48 hours (Fig. 4D), with full recovery by 72 hours (data not shown), which indicates a long pharmacologic half-life in vivo. Fulvestrant, given as 3 × 5 mg/mouse doses over one week, gave a 60% reduction in PR protein over a prolonged period with measured plasma levels approximately 8-fold higher than those achieved clinically, at steady state, with 500 mg fulvestrant (Fig. 4E). As estrogen itself is known to downregulate ER protein (38, 39), we were unable to detect further decreases in ERα protein compared with control animals with AZD9496 or fulvestrant in the MCF-7 model presumably due to the high circulating plasma levels of estrogen from implanted pellets at the time tumor samples were taken (Supplementary Fig. S5). A mouse-specific metabolite of AZD9496 was detected in circulating plasma at similar levels to AZD9496 and showed a similar pharmacokinetic profile. Testing this metabolite in the in vitro MCF-7 assays resulted in approximately 5-fold lower ERα antagonism activity and 7-fold lower ERα downregulation activity than AZD9496 (data not shown). Using a pharmacokinetic/pharmacodynamic model based on PR inhibition data, at the 5 mg/kg dose in vivo, which gives 98% inhibition of PR, the inhibitory activity that could be attributed to the parent compound alone was 85% when the activity of the metabolite was discounted.

To explore mechanistic effects on ER pathway regulation further, a gene expression analysis study was performed using tumor samples dosed at 10 mg/kg for 3 days with AZD9496 or tamoxifen and fulvestrant given as a single dose 5 mg dose subcutaneously. Tumors were taken 24 hours after the last dose of AZD9496 and tamoxifen and mRNA levels measured using a Human Transcriptome Array (HTA 2.0). A number of known ER-regulated genes were examined to see whether AZD9496 could antagonize these genes in a similar manner to fulvestrant and tamoxifen (40–42). The heatmap shows that AZD9496 could indeed downregulate...
the mRNA levels of estrogen-responsive genes (Supplementary Fig. S6A). As differences in the levels of mRNA downregulation in vivo could be due to different pharmacokinetic profiles of the molecules, the effects of compounds on the mRNA levels of a subset of estrogen-regulated genes were also measured in MCF-7 cells that had been treated with or without estrogen (Supplementary Fig. S6B–S6E). For two of these genes, TFF1 and AREG, tamoxifen was shown to increase mRNA levels in the absence of estrogen but not with either fulvestrant or AZD9496 (Supplementary Fig. S6D and S6E). Where transcript levels were increased to varying levels in the presence of estrogen, fulvestrant and AZD9496 inhibited transcript levels in a dose-dependent fashion compared with tamoxifen. No significant effects were seen in ESR1 mRNA levels in vitro, indicating that the protein downregulation described previously is due to downregulation of the protein and not a decrease in transcript levels per se (Supplementary Fig. S6F).

AZD9496 causes tumor regressions in combination with PI3K pathway and CDK4/6 inhibitors and in an estrogen-deprived ER+ model of resistance

Acquired resistance to aromatase inhibitors and antiestrogens is driven through a variety of mechanisms, which include effects on the ER pathway itself, i.e., downregulation of ER expression and altered expression of ER coregulators, as well as activation of alternative prosurvival or proliferative pathways and cross-talk between growth factor receptors and ER pathways. This has led to a number of clinical trials with PI3K, mTOR, AKT inhibitors, as well as CDK4/6 inhibitors in combination with aromatase inhibitors or fulvestrant and early results have shown increased progression-free survival (PFS) in many cases (43). Combinations of AZD9496 with AZD2014 (dual mTORC1/2), AZD8835 (PIK3CA/8) and palbociclib (CDK4/6) inhibitors were tested in the MCF-7 in vivo model and in all cases tumor regressions seen with the combination arms alone compared with tumor stasis with monotherapy (Fig. 5A–C). AZD9496 was also tested in a long-term estrogen-deprived model (LTED), using the HCC-1428 LTED cell line that was previously adapted to grow in the absence of estrogen and as such represents a model of aromatase inhibitor resistance (22). Tumor regressions were seen with both AZD9496 and fulvestrant (Fig. 5D) and both compounds completed ablated ER protein levels in the end-of-study tumors (Supplementary Fig. S7).

AZD9496 antagonizes and downregulates mutant ER in vitro and in vivo

The recent identification of ESR1 mutations in patients that had received one or more endocrine therapy treatments has led to the discovery that these mutations can drive cell proliferation in the absence of estrogen and may constitute a resistance mechanism to some endocrine agents. In vitro binding studies were performed using wt, D538G, and Y537S LBDs and both AZD9496 and fulvestrant were able to bind to mutant LBD with nmol/L potency although 2- to 3-fold reduced compared with wt (Table 2). The different IC50 values obtained for binding of AZD9496 and fulvestrant to wt ERα compared with the value in Table 1 is

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<th>Table 2. In vitro binding to ERα mutant LBDs</th>
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<td>ERα LBD binding IC50 nmol/L</td>
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<tr>
<td>AZD9496 nmol/L       Fulvestrant nmol/L</td>
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<tr>
<td>wt                      0.2                   0.5</td>
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<tr>
<td>D538G                  0.5                   3.3</td>
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<tr>
<td>Y537S                  0.6                   3.8</td>
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NOTE: IC50 for binding of AZD9496 and fulvestrant to ERα wt and mutant LBDs; data shown are representative of the mean IC50 from three independent experiments.
Figure 3.

ERα-mediated agonism in MCF-7 cells and immature female rat endometrial tissue. A, MCF-7 cells were treated with AZD9496 or a known agonist compound AZ10557841 and PR levels (response) measured using in-cell immunofluorescence quantification using an Acumen Ex3 platform. Data was normalized for cell number by analyzing a ratio of fluorescence resulting from stimulation of two emission wavelengths and used to perform curve fitting analysis. A concentration response was plotted using nonlinear regression analysis. B and C, immature female rats were dosed orally with AZD9496 or tamoxifen or subcutaneously with fulvestrant for 3 days. PEG/Captisol, polysorbate, and peanut oil were used as vehicle controls for AZD9496, tamoxifen, and fulvestrant, respectively. Uterine tissue was removed 24 hours after the final dose and weighed before tissues were formalin-fixed and paraffin embedded to allow histologic staining of the endometrial tissue. Significant differences in mean uterine weight are shown: ***, P < 0.001; **, P < 0.01. D, tissue was subsequently analyzed by Western blot analysis for levels of ERα, PR, and vinculin. Protein levels were measured by chemiluminescent and quantified using Syngene software. ER and PR protein levels were normalized to vinculin as a loading control and plotted as shown. Statistically significant differences in PR levels are shown. ***, P < 0.001; **, P < 0.01; and *, P < 0.05.
Figure 4.

In vivo efficacy of AZD9496 in MCF-7 xenograft model. A, MCF-7 xenografts, grown in male SCID mice, were dosed daily with either PEG/captisol (vehicle) or AZD9496 at doses shown. Tumor growth was measured by caliper at regular intervals and mean tumor volumes plotted for each dosed group. B, tumors from treated mice collected at the end of study were analyzed by Western blot analysis for levels of PR and vinculin. Protein levels were measured by chemiluminescent and quantified using Syngene software. PR protein levels were normalized to vinculin as a loading control and plotted as shown with standard error bars. *, P < 0.05; **, P < 0.001. C, MCF-7 xenografts were also dosed daily with 5 mg/kg AZD9496, 10 mg/kg tamoxifen, or 5 mg/mouse three times weekly with fulvestrant subcutaneously. D and E, for acute dosing studies, MCF-7 xenografts were dosed for 5 days with doses shown of AZD9496 and tumors collected at 24 or 48 hours after the last dose (D) or dosed with three doses of 5 mg/mouse of fulvestrant over 5 days and tumors collected at 24, 48, and 96 hours after the last dose (E). PR and vinculin protein levels in the tumors were analyzed and plotted as described above. p.o., orally; q.d., once daily.
likely due to different sources of protein used in the Lanthascreen kit assays versus the laboratory-derived wt and ESR1-mutant proteins. Downregulation of ER-mutant proteins was measured using MCF-7 doxycycline-inducible stable cell lines in vitro. ER protein levels increased on induction with 1 μg/mL doxycycline and led to increased levels of PR in the absence of estrogen. AZD9496 and fulvestrant were able to downregulate all mutant ER proteins and decreased PR levels, whereas tamoxifen appeared to stabilize ER protein levels and reduced PR levels to a lesser extent (Fig. 6A). To explore activity against an ESR1 mutant in vivo, tumor growth inhibition studies were performed in a PDX model, CTC-174, which harbors a D538G mutation in the LBD and constituted 31% of the transcripts variants identified by RNA-seq analysis. Tumors were grown from implanted tumor fragments either with or without estrogen and in both cases viable tumors grew. Given published data that higher doses of SERMs/SERDs may be needed to inhibit ESR1 mutants (14, 15), 25 mg/kg of AZD9496 was used alongside 5 mg/mouse of fulvestrant, which was known to achieve higher pharmacokinetic levels in mouse than the current clinical dose. AZD9496 and fulvestrant inhibited tumor growth by 66% and 59% respectively, compared with tamoxifen, which gave 28% inhibition. Efficacy correlated with antagonism of the pathway with AZD9496 giving a 94% decrease in PR levels compared with 63% with fulvestrant (Fig. 6B and C). In the absence of estrogen, and given the significant PR changes seen with 25 mg/kg, the dose of AZD9496 was lowered but a 5 mg/kg dose still achieved growth inhibition of approximately 70% and led to a significant decrease of 73% in ERE protein levels at the end of the study (Fig. 6D and E). Given the similarities in growth inhibition either with or without estrogen, residual tumor growth is likely to be due to either additional mutations (e.g., PIKCA N345K) and/or growth factor signaling pathways involved in driving tumor growth in addition to the ER pathway.

Figure 5.
Tumor regressions with AZD9496 in combination with mTOR, PIKC, and CDK4/6 inhibitors in MCF-7 xenograft model and in HCC1428 LTED model. A–C, MCF-7 xenografts, grown in male SCID mice, were dosed daily with AZD9496 at 5 mg/kg with or without AZD2014 dosed at 20 mg/kg b.i.d. 2 days out of 7 (A), with or without AZD8835 dosed at 50 mg/kg twice daily on day 1 and 4 (B), and with or without palbociclib dosed daily at 50 mg/kg (C). D, HCC1428LTED engrafts were grown in ovariectomized NSG mice and were dosed daily with either AZD9496 or once weekly with fulvestrant at doses shown. Tumor growth was measured by caliper at regular intervals and mean tumor volumes plotted for each dosed group. p.o., orally; q.d., once daily.
Figure 6. AZD9496 is efficacious against ESR1 mutants in vitro and in vivo. A, stable MCF-7 cell lines containing either pTRIPZ vector alone or vector containing ESR1 mutants were induced to express mutant protein with 1 µg/mL doxycycline for 24 hours before treating with either 0.1% DMSO (vehicle) or 100 nmol/L of fulvestrant, AZD9496, or tamoxifen for 24 hours. Immunoblotting was performed to detect expression of ERα, PR, and GAPDH levels. B, CTC-174 tumor fragments were grown in female NSG mice implanted with estrogen pellets and dosed daily with either PEG/captisol (vehicle) or AZD9496, tamoxifen, and fulvestrant at doses shown. Tumor growth was measured by caliper at regular intervals and mean tumor volumes plotted for each dosed group. C, tumors from treated mice collected at the end of study were analyzed by Western blot analysis for levels of PR and vinculin. Protein levels were measured by chemiluminescent and quantified using Syngene software. PR protein levels were normalized to vinculin as a loading control and plotted as shown. *** P < 0.001. D, CTC-174 tumor fragments were grown in female NSG mice without estrogen pellets to enable measurement of ER levels and dosed daily with either PEG/captisol (vehicle) or AZD9496 at doses shown. E, tumors from treated mice collected at the end of study were analyzed by Western blot analysis for levels of ERα and vinculin and measured as described above. *, P < 0.05. p.o., orally; q.d., once daily.
Discussion

Despite the enhanced clinical benefits of directly antagonizing and downregulating ER and thereby targeting any ligand-independent ER-driven pathways, the pharmacokinetic and IHC biomarker data for ER, PR, and Ki67 from clinical trials with fulvestrant would suggest that a plateau of effect has not been reached, and there is scope to further improve on the 50% reduction in ER levels seen after 6 months of treatment (8, 12). As one of the potential drawbacks associated with fulvestrant is the very low bioavailability, which is seen both in patients and in preclinical models, there is scope to further improve on the 50% reduction in ER protein, and signiﬁcant decreases in ER protein levels but at signiﬁcantly higher potency for the receptor between AZD9496 and fulvestrant, increased efficacy is likely due to the higher free drug levels of AZD9496 versus fulvestrant in this model. It would therefore be interesting to test the activity of potent oral SERDs, such as AZD9496, in the premenopausal setting where there is no enhanced risk of endometrial cancer observed for tamoxifen.

We were unable to detect a reduction in ER protein levels in the MCF-7 in vivo model with AZD9496 at any dose tested (data not shown). Having shown in vitro that estradiol can efficiently downregulate ER protein, we postulate that downregulation of ER in AZD9496 is colocalized with the Asp 351 residue in the helix-12 region of ER in an unusual acid–acid interaction, and it is therefore proposed that AZD9496 also repositions residues associated with the helix-12 via specific contacts with the N-terminus region, resulting in a mixed antagonist and downregulator proﬁle (33, 44). Evidence that AZD9496 was directly binding and downregulating the receptor at the same site as other antagonists was seen by the reduced potency observed on prior addition of tamoxifen to the downregulation assay (Table 1). Mechanistically, we have shown that AZD9496 has very similar effects on antagonism, ER downregulation, and agonism to fulvestrant. Even where ER is overexpressed in MCF-7 cells, unlike tamoxifen, AZD9496 was able to downregulate and antagonize the overexpressed receptor (Fig. 6A). Our transcriptional studies in both MCF-7 cells and the in vivo model also demonstrated antagonism of the transcriptional activity of the receptor as seen by decreased mRNA levels of ER-activated genes in a dose-dependent manner (Supplementary Fig. S6).

Unlike fulvestrant, AZD9496 did show some degree of agonism in endometrial tissue (Fig. 3). This effect was dose independent and would indicate that there are subtle differences in how AZD9496 interacts with ER compared with tamoxifen and fulvestrant and therefore the extent to which tissue specific coactivators can interact with the receptor. Given the widespread use of tamoxifen in the adjuvant setting for 5–10 years, the low level of agonism seen with AZD9496 is not viewed as a deterrent for clinical development. A number of studies have looked at the incidence of endometrial cancer in patients taking tamoxifen over a number of years and despite the small increase in the incidence of endometrial cancer, the benefits of tamoxifen greatly outweigh any risks of developing uterine malignancy in postmenopausal patients (45, 46).

A pharmacokinetic/pharmacodynamic efﬁcacy model was established, integrating diverse endpoints such as drug exposure, PR modulation, and inhibition of tumor growth (data not shown). The model reﬂected relative contributions of AZD9496 and its active mouse metabolite to the overall biomarker modulation and efﬁcacy. Given the half-life of the parent drug and metabolite are around 5 hours, to explain the prolonged pharmacodynamic effect observed with AZD9496 at 48 hours, an indirect response model for PR was developed, which estimated a degradation rate constant of around 23 hours for PR. This modulation in PR was linked directly to tumor growth inhibition by a proportional decrease of the tumor growth constant, and correlated well with the wide range of measured efﬁcacies.

We consistently saw enhanced tumor growth inhibition of AZD9496 compared with fulvestrant in the MCF-7 in vivo model (Fig. 4). This model contains high circulating levels of plasma estradiol over the dosing period of the study as a result of the estrogen pellets used to drive growth of the MCF-7 cells as a xenograph. With plasma levels between 750 and 1,000 pg/mL over day 21–35, this model is more representative of the premenopausal setting where estrogen levels can vary between 20 and 400 pg/mL (47). As the receptor binding data does not suggest a signiﬁcant difference in afﬁnity for the receptor between AZD9496 and fulvestrant, increased efﬁcacy is likely due to the higher free drug levels of AZD9496 versus fulvestrant in this model. It would therefore be interesting to test the activity of potent oral SERDs, such as AZD9496, in the premenopausal setting where there is no enhanced risk of endometrial cancer observed for tamoxifen.

Achieving increased serum levels of a biologically active SERD could be particularly beneﬁcial in the recently identiﬁed ESR1-mutant patient setting. Preclinical in vivo studies using PDX models derived from ESR1-mutant patients grown in the absence of estrogen supplementation have shown that high doses of fulvestrant (>500 mg/kg) can lead to tumor regression and signiﬁcant decreases in ER protein levels but at signiﬁcantly higher doses than could be achieved clinically (48). Our preclinical data would indicate that both AZD9496 and fulvestrant can potently bind and downregulate D538G and Y537C/N/S ERα proteins in vitro and signiﬁcant tumor growth inhibition rather than regression seen in vivo. This is in contrast to published work showing relatively little downregulation of Y537N mutant protein in vitro with increasing doses of fulvestrant (15) but could be attributed to different expression systems and cell lines used for the analysis. It is notable that the doxycycline-inducible mutant receptors do not
appear to be constitutively downregulated in a similar manner to wt ER exposed to estradiol (Supplementary Fig. S1). This may be due to the level of overexpression of the receptor in each stable cell line and it will be interesting to compare these findings from engineered expression systems to those from genetically altered mutant cell lines where expression is under control of the native promoter. Although very high doses of both compounds were not tested in the PDX CTC-174 ERα-mutant model, the lack of any significant dose response might indicate the need to inhibit other growth factor–activated pathways in addition to the ER pathway to achieve significant antitumor growth. As trials commence to explore treatment of ERα-mutant patients with new oral SERDs, data will hopefully emerge that will indicate whether lack of response is due to inability to fully inhibit mutated ER receptors or whether, in these advanced metastatic patients, other mutations and pathways are or become dominant in driving tumor growth. In metastatic breast cancer, the median survival time is still around two years as a result of acquired endocrine resistance, highlighting the need for additional therapies (43). This has led to a number of clinical trials using PI3K–Akt–mTOR pathway inhibitors in combination with AIs as well as cyclin-dependent kinase inhibitors such as palbociclib, which was recently approved for combination treatment with letrozole in the metastatic breast cancer. Results from the phase III BOLERO-2 trial also demonstrated the PFS benefits of a steroidal AI plus everolimus in patients that had progressed on a nonsteroidal AI (49). More recently, further trials have been initiated using fulvestrant as the combination endocrine partner, which will allow analysis and understanding of any additional benefit from combination with a SERD versus AIs in this setting. Our data clearly demonstrated enhanced combination effects on tumor growth of an oral SERD agent with either dual mTORC1/2, PI3KCA/6 or CDK4/6 inhibitors and furthermore opens up the possibility of using a combined endocrine and targeted inhibitor approach with longer term dosing in the adjuvant setting, assuming emerging data from ongoing metastatic trials supports the testing of combinations in early breast disease. The identification of a potent, orally bioavailable compound such as AZD9496 is a step forward in the next generation of SERD agents to undergo clinical evaluation as a future monotherapy or combination partner of choice.

Disclosure of Potential Conflicts of Interest
J.O. Curwen is an associate principal scientist at AstraZeneca. G. Davies has ownership interest (including patents) in AstraZeneca as a shareholder. S. Powell is a team leader at AstraZeneca. G. Richmond has ownership interest (including patents) in AstraZeneca. All authors are current or former AstraZeneca employees and shareholders.

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Acknowledgments
The authors thank Elaine Hurt, Hai Feng Bao, Sanjoo Jalla for access to the CTC-174 in vivo model developed at MedImmune. The authors also thank Paul Hemsley and Emma Linney for providing ER-mutant binding assay data. Helen Gould for supporting the crystallography work and Zara Ghazoui and Henry Brown for the transcript data and analysis at AstraZeneca. The authors also thank Carlos Arteaga for use of the HCC1428-LTED cell line.

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Received August 28, 2015; revised February 1, 2016; accepted March 16, 2016; published OnlineFirst March 28, 2016.

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