Molecular Cancer Therapeutics

AZD2014, an Inhibitor of mTORC1 and mTORC2, Is Highly Effective in ER⁺ Breast Cancer When Administered Using Intermittent or Continuous Schedules ⊠

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Abstract

mTOR is an atypical serine threonine kinase involved in regulating major cellular functions, such as nutrients sensing, growth, and proliferation. mTOR is part of the multiprotein complexes mTORC1 and mTORC2, which have been shown to play critical yet functionally distinct roles in the regulation of cellular processes. Current clinical mTOR inhibitors only inhibit the mTORC1 complex and are derivatives of the macro-lide rapamycin (rapalogs). Encouraging effects have been observed with rapalogs in estrogen receptor–positive (ER⁺) breast cancer patients in combination with endocrine therapy, such as aromatase inhibitors. AZD2014 is a small-molecule ATP competitive inhibitor of mTOR that inhibits both mTORC1 and mTORC2 complexes and has a greater inhibitory function against mTORC1 than the clinically approved rapalogs. Here, we demonstrate that AZD2014 has broad antipro-

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Introduction

The mTOR serine threonine kinase plays a critical role in regulating cellular energy sensing, growth and metabolism. Deregulation of mTOR signaling is observed in many tumor types (1, 2). Mutations or loss of function of upstream regulators such as TSC1/2, LKB1, or components of the PI3K pathway such as

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doi: 10.1158/1535-7163.MCT-15-0365

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liferative effects across multiple cell lines, including ER⁺ breast models with acquired resistance to hormonal therapy and cell lines with acquired resistance to rapalogs. *In vivo*, AZD2014 induces dose-dependent tumor growth inhibition in several xenograft and primary explant models. The antitumor activity of AZD2014 is associated with modulation of both mTORC1 and mTORC2 substrates, consistent with its mechanism of action. In combination with fulvestrant, AZD2014 induces tumor regressions when dosed continuously or using intermittent dosing schedules. The ability to dose AZD2014 intermittently, together with its ability to block signaling from both mTORC1 and mTORC2 complexes, makes this compound an ideal candidate for combining with endocrine therapies in the clinic. AZD2014 is currently in phase II clinical trials. *Mol Cancer Ther;* 14(11); 2508–18. ©2015 AACR.

PIK3CA, AKT, or PTEN have been reported in most types of human tumors (3, 4).

mTOR kinase forms two distinct multiprotein complexes called mTORC1 and mTORC2. Both complexes share common subunits, including mTOR and mLST8, but the distinct cellular functions and localizations of the two complexes are regulated by the presence of a number of different subunits. The mTORC1 complex contains the cofactor RAPTOR, whereas the mTORC2 complex includes the proteins RICTOR and mSIN1. The different cofactors define the assembly, subcellular localization, substrate binding, and unique functions of the two complexes (2, 5). Additional regulators such as PRAS40, PROTOR, and DEPTOR also regulate the cellular functions of the different complexes.

The mTORC1 complex plays a key role in coupling nutrient sensing with the regulation of protein translation and cellular metabolism processes. It directly phosphorylates proteins such as P70S6K (S6K; ref. 6) and 4E-BP1 (7), which are involved in controlling cellular growth and proliferation, as well as SREBP (8), a key modulator of metabolism and lipid synthesis. mTORC1 also phosphorylates a number of substrates that modulate autophagy and lysosome biogenesis (9). mTORC2 has been reported to play a role in the cellular response to extracellular growth factors through largely unknown mechanisms. Its activation requires association with ribosomes and results in the phosphorylation



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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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of downstream targets such as the AGC family of protein kinases, which includes AKT, SGK, and PKC (10).

Rapamycin and its analogues temsirolimus and everolimus have been approved for the treatment of certain types of tumors (11). The inhibition of mTORC1 by rapamycin and its related compounds (rapalogs) is through an indirect mechanism, which does not involve mTOR kinase and results in the release of the negative feedback loop between S6K and IRS1, leading to the hyperactivation of mTORC2 and AKT phosphorylation (12). In addition, rapalogs have been reported to be partial inhibitors of mTORC1 signaling, limiting the inhibitory effect of 4E-BP1 in the initiation of protein translation (13). The resulting AKT activation and the lack of complete inhibition of 4E-BP1 phosphorylation in response to rapalogs suggest that targeting both mTORC1 and mTORC2 would be a more efficacious and beneficial approach.

Approximately three quarters of patients diagnosed with advanced breast cancer have hormone receptor-positive (HR⁺) disease. Patients with HR⁺ advanced breast cancer typically respond well to endocrine therapies and antiestrogen modalities, such as aromatase inhibitors (anastrazole, letrozole, and exemestane; ref. 14), tamoxifen (15) or fulvestrant (16). Although aromatase inhibitors have become standard of care in postmenopausal women and endocrine therapy is generally very effective, disease progression remains a major challenge in this setting. Recent advances in elucidating the molecular mechanisms of pathway "cross-talk" between the estrogen receptor (ER) and intracellular signaling pathways, including the PI3K-mTOR pathway (17), have provided the rationale for combining endocrine therapies with a targeted agent against a compensatory pathway (18). In the BOLERO-2 trial, the inhibitor everolimus, in combination with exemestane, improved progression-free survival of patients with advanced breast cancer previously treated with aromatase inhibitors, leading to its approval by the FDA (19). Previous reports on ATP-competitive mTORC1 and two inhibitors such as torin1 (20) or AZD8055 (21) have described inhibition of rapamycin-resistant mTOR signaling, which translated into a greater biologic activity in ER⁺ breast cancer models compared with rapalogs (22).

In this study, we describe AZD2014, an ATP-competitive inhibitor of mTOR kinase and a close analogue of AZD8055, with improved pharmacokinetic properties. We demonstrate the activity of AZD2014 in ER⁺ breast cancer models that are resistant to endocrine therapy and/or to the rapalog everolimus. Furthermore, we demonstrate that intermittent dosing schedules of AZD2014 deliver efficacy in a number of *in vivo* models of ER⁺ breast cancer.

Materials and Methods

Chemicals

Benzamide, 3-[2,4-bis](3S)-3-methyl-4-morpholinyl]pyrido[2,3-d]pyrimidin-7-yl]-N-methyl-(AZD2014, Fig. 1A) was identified following an extensive optimization campaign around an initial pyridopyrimidine hit uncovered by a screening approach (23).

AZD2014 was prepared as 10 mmol/L stock solution in DMSO and stored under nitrogen.

Antibodies

Antibodies were obtained from the following sources: pPRAS40 T246 (Biosource); pAKT S473 and T308 (Cell Signaling

Technology, CST), pP70S6K T389 (CST), pNDRG1 T346 (CST), p-S6RP S235/236 and S240/244 (CST), p-4EBP1 T37/46 (CST), yH2AX S139 (Millipore), and Vinculin (Sigma). NDRG1 (total) and pNDRG1 antibodies were obtained from Dario R. Alessi (University of Dundee).

Cell lines and cell culture

Cells lines were grown in RPMI-1640 (Gibco) + 10% FCS + 2 mmol/L glutamine at 37°C, 5% carbon dioxide, unless otherwise indicated. HCC1428/LTED and ZR75-1/LTED were obtained from C. Arteaga and cultured in charcoal-stripped serum complemented medium as described previously (24). MCF7-T52, MCF7-100-16, and MCF7-40-6 (fulvestrant resistant) were obtained from T. Shioda and cultured in the presence of 1 µmol/L of tamoxifen or 1 µmol/L fulvestrant as described (25). The HCC1428 long-term estrogen-deprived everolimus-resistant (LTED-eveR) line was generated and cultured in phenol red-free medium + 10% charcoal dextran-treated FBS (Hyclone), 1% L-glutamine and antibiotics. Everolimus resistance was generated as previously described (26). To perform compound treatment assays, everolimus was removed during plating and cells were treated the following day with indicated compounds.

Cell panel screen details and associated cell line identification procedures are summarized in Davies and colleagues (27). All cell lines were authenticated at AstraZeneca cell banking using DNA fingerprinting short-tandem repeat assays (Supplementary Materials and Methods).

Colony formation assays

Cells were plated at 5,000 cells per well in 6-well dishes. Colonies were allowed to form for approximately 3 weeks or until control colonies were sufficient to be visualized by eye. Plates were then rinsed in PBS, fixed with 4% paraformaldehyde and stained with 0.1% crystal violet before scanning.

Three-dimensional Matrigel assays

Cells were plated in phenol-red free growth factor reduced Matrigel (BD Biosciences). After plating, cells were incubated for 4 days before adding the indicated concentrations of compound. Phase-contrast photographs were taken using a $10 \times$ objective using an Olympus DP71 microscope and images were captured using Olympus DP-BSW software.

Cell growth assays

Cells were plated in 96-well plates for the indicated time. For CellTiterGlo assays: CellTiterGlo (Promega) was mixed with the cells as per manufacturer's instructions. Cells were normalized to day 0 control and net growth was determined using the following formula: ((x - y)/(z - y)) = net growth, where x = reading of treated sample at end of study, y = average reading on day 0, and z = reading of DMSO-treated sample at end of study. The concentration of DMSO did not exceed 0.03% for any experiment. For MTS assays: adherent cell lines were grown in 96-well plates, as described above. MTS reagent (Promega) was added on day 0 and on day 3 post-compound addition.

Suspension lines were assayed using the Alamar Blue reagent (Promega) according to manufacturer's instructions, 72 hours after compound addition.



Figure 1.

Structure, biochemical, and cellular activity of AZD2014. A, chemical structure of AZD2014. B, immunoblotting analysis of AZD2014 activity against mTOR substrates in MCF7 cells. C and D, cell proliferation activity in a panel of cell lines. Multiple tumor types are represented in the main graph, whereas breast cancer cell lines are represented in the insert. GI_{50} (the concentration decreasing cell number by 50% compared with untreated cells) and TGI (the concentration at which there is no net growth) are plotted on the *x* and *y* axis, respectively. Different cell lineages are represented by colors and cell lines that harbor a mutation in the PI3K pathway are indicated with a cross.

Enzyme assays

Recombinant truncated FLAG-tagged mTOR (aa1362-2549) expressed in HEK 293 cells was used in biochemical assays, together with a biotinylated p70S6K peptide substrate. Streptavidin donor and protein A acceptor beads were used to assemble the capture complex for generation of the assay signal. The activity of the lipid kinases, PI3 kinase alpha, beta, delta, and gamma were measured using recombinant proteins and the lipid PIP2 as substrate. Assays for ATM and DNA-PK activity were performed as described previously (21, 23). The selectivity of AZD2014 was tested against kinase panels from Dundee, Millipore and Ambit. mTOR cellular activity was measured in MDAMB468 cells, using an Acumen laser scanning cytometer (TTP Labtech) to analyze the levels of phosphorylation of S6 (Ser235/236) and AKT (Ser473).

Immunoblotting

Expression levels of total and phosphorylated protein were assessed using standard Western blotting techniques (NuPAGE Novex 4%–12% Bis-Tris 15-well gels, or Criterion 4%–20% TrisHCl 26-well gels). Antibodies were diluted in 5% BSA/PBS-tween and signal detected using SuperSignal West Pico, Dura or Femto HRP substrates. Cells were harvested and lysed in cell lysis buffer (CST) with phosphatase/protease inhibitor cocktail (CST).

In vivo studies

All mice were used between the ages of 8 and 12 weeks in accordance with institutional guidelines and all procedures were performed in accordance with federal, state and institutional guidelines in AAALAC-accredited facilities (in the United States and France) and a third facility (United Kingdom) operating under the UK Home Office regulatory framework.

MCF7 experiments: 5×10^{6} MCF7 cells were injected s.c. in a volume of 0.1 mL in male SCID mice and were randomized into control and treatment groups when tumor size reached 0.2 cm³. AZD2014 was dissolved in captisol (Cydex), and diluted to a final captisol concentration of 30% (w/v). AZD2014 was administered by oral gavage (0.1 mL/10 g body weight). The control group received vehicle only. Tumor volumes (measured by calliper), animal body weight and condition were recorded twice weekly for

the duration of the study. The tumor volume was calculated (taking length to be the longest diameter across and width to be the corresponding perpendicular diameter) using the formula: (length × width) × $\sqrt{(\text{length} \times \text{width})} \times (\pi/6)$. 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. Statistical significance was evaluated using a one-tailed, two-sample *t* test. Twice daily dosing was carried out every 12 hours.

HCC1500 s were implanted into female SCID Beige mice (CB17.B6-PrkdcscidLystbg/Crl) supplied by Charles River Laboratories. Mice were housed in pathogen-free housing in individually ventilated cages (IVC) of Polysulfone (PSU) plastic (mm 213 W \times 362 D \times 185 H) with sterilized and dust-free bedding cobs, access to sterilized food and water ad libitum, under a lightdark cycle (14-hour circadian cycle of artificial light) and controlled room temperature and humidity. Tumor fragments were obtained by collecting a tumor from a donor mouse and cutting it into 50 mm³ pieces. Fragments were implanted orthotopically adjacent to mammary fat pad. The 0.18 mg 90 day 17β estradiol pellets obtained from Innovative Research of America and were implanted at the same time into dorsal scapular region. Anesthesia was maintained with isoflurane during the surgical procedures. Staples (9 mm) were used to close all incision sites and were removed 1 week after surgery. Mice were randomized into control and treatment groups once tumor sizes reached approximately 150 mm^3 .

CTC174 studies were also conducted following implantation of a tumor fragment in a similar manner to the HCC1500 described above. Surgical methodology was the same, but female NSG mice obtained from The Jackson Laboratory were used.

HCC1428 experiments used the same female NSG mice (The Jackson Laboratory) but were induced by inoculation of a cell suspension rather than fragments. Animals were anesthetized, and 10×10^6 cells were implanted in a total of 50 µL [of 50% phenol-red free Matrigel (BD Biosciences): 50% RPMI media] transdermally in the third mammary fat pad. Tumors were measured with vernier calipers, and volumes were calculated using the formula (L × W²) × 0.52. When the s reached an average of 100 mm³, the mice were randomized into treatment groups by volume. Animals were treated with vehicle control, everolimus (5 mg/kg p.o., daily) or AZD2014 (20 mg/kg p.o. 2 days on/5 days off) for 7 weeks. Tumor volumes are displayed as fold change relative to the average volume on the first day of treatment.

HBCx3 experiments: HBCx3 tumor fragments of around 40 mm³ were implanted into male nude mice (athymic strain Foxn1^{nu}, supplied by Harlan Laboratories) under general anesthesia (ketamine/xylazine). Fragments were placed in a subcutaneous site on the left flank to enable simple caliper measurements and to avoid impairing animal movement. Fragments were produced from donor mice of the same strain. To support tumor growth, mice were given drinking water containing 8.5 mg/L β -oestradiol rather than an implanted pellet as in the other models above.

Tumors were homogenized using a Fastprep instrument and all samples were sonicated before lysing and immunoblotting. The numerical data for each biomarker were determined using Genetools software and normalized to vinculin control. A two sided t test was performed on data assuming unequal variance.

Plasma pharmacokinetic analysis

Blood samples were taken from mice following administration of AZD2014 and plasma prepared by centrifugation. The concentration of AZD2014 in the plasma samples was determined using a protein precipitation extraction procedure, followed by LC/MS-MS detection using Masslynx and processed using Quanlynx.

Pharmacokinetic-pharmacodynamic modeling

The pharmacokinetics of AZD2014 in the mouse were modeled using a linear two compartment model with first-order absorption:

$$\frac{dGUT}{dt} = -ka \ GUT$$
$$\frac{dCEN}{dt} = -\frac{(Q+CL)}{V_1} CEN + \frac{Q}{V_2} PER + ka.GUT$$
$$\frac{dPER}{dt} = Q\left(\frac{CEN}{V_1} - \frac{PER}{V_2}\right)$$

where predicted plasma concentration of AZD2014 is described by:

 $C_p = \frac{CEN}{V_1}$

The model, therefore, characterizes the oral clearance, CL and volume of distribution, V1 + V2.

The pAKT and pS6 pharmacodynamics were linked to the simulated plasma concentrations using a direct sigmoid relationship:

Effect = Baseline
$$\left(\frac{1}{1 + \frac{Cp}{IC_{50}}}\right)$$

Immunohistochemical analysis of xenograft tissue

Tumors were excised from animals and placed into 10% buffered formalin. Tissues were fixed for 24 to 48 hours before being processed and embedded into paraffin wax. Sections were cut and allowed to dry overnight in an oven at 40°C. Immuno-histochemistry was performed on the LabVision immunostainer platform. Slides were scanned using the Aperio Scanscope and analyzed using a modified version of the Color Deconvolution algorithm. This algorithm separates the image into three channels, corresponding to the actual colors of the stains used (single staining/dual staining and the Counterstain).

Gene-expression analysis

Tissue was excised from snap-frozen xenografts and RNA was isolated using the RNeasy MiniKit (Qiagen-RLT Buffer), with an additional DNAse treatment step, following the manufacturer's protocol. Reverse transcription was performed using 50 ng of RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), following the manufacturer's instructions. Targeted gene profiling was performed using the Fluidigm platform and cDNA was preamplified (14 cycles) using a pool of TaqMan primers (Life Technologies), following the manufacturer's instructions. Sample and assay preparation of the 96.96 Fluidigm Dynamic arrays was carried out according to the manufacturer's instructions. Data were collected and analyzed using the Fluidigm Real-Time PCR Analysis 2.1.1 software. Gene-expression values were calculated after normalization to the average of the house keeping genes (PPIA, IPO8, and YWHAZ): ΔC_t , negative $\Delta \Delta C_t$ was

calculated by subtracting the average ΔC_t value of the vehicle group to each animal (negative $\Delta \Delta C_t = \text{AVG } \Delta C_t$ vehicle group— ΔC_t), and fold change after log₂ transformation (2^{neg $\Delta \Delta C_t$}). The mean and SE of each group and statistical analysis was performed using jmp-11.

Results

AZD2014 is a potent and selective mTORC1/2 inhibitor, with distinct properties from rapalogs

Benzamide 3-[2,4-bis[(3S)-3-methyl-4-morpholinyl]pyrido[2,3d]pyrimidin-7-yl]-N-methyl- (Fig. 1A), AZD2014, is a close analogue of the mTORC1/2 inhibitor AZD8055 (21, 23). The inhibitory effects of AZD2014 were measured against isolated recombinant mTOR enzyme (IC₅₀ of 2.81 nmol/L) as well as in cellular assays measuring both mTORC1 and mTORC2 activities (Supplementary Table S1). In MDAMB468 cells, AZD2014 decreased the phosphorylation of the mTORC1 substrate ribosomal protein S6 (Ser235/236) with a mean IC_{50} value of 210 nmol/L and the mTORC2 substrate AKT (Ser473) with a mean IC₅₀ value of 78 nmol/L (Supplementary Table S1). Everolimus tested in the same conditions showed a greater inhibitory effect against S6 phosphorylation (mean IC₅₀ of 0.15 nmol/L) but had no inhibitory effect against phosphorylation of AKT (mean IC₅₀ of 20.6 µmol/L). The activity of AZD2014 against a number of mTOR substrates was also assessed in MCF7 cells (Fig. 1B). AZD2014 inhibited both mTORC1 and mTORC2 substrates, whereas everolimus only inhibited mTORC1 substrates and caused an upregulation of phosphorylation of AKT, as previously reported (12). AZD2014 was tested against a number of PI3K family enzymes and showed more than a 1,000-fold selectivity against all PI3K isoforms (Supplementary Table S1).

The selectivity of AZD2014 was also tested in two distinct, commercially available panels to assess broad spectrum kinase activity. In a panel of 220 biochemical (kinase) assays, only one hit showed >50% inhibition at 10 μ mol/L (Wnk2); in a further panel of 393 kinases/catalytic domains assessed by competition binding assays, AZD2014 showed no or weak binding to the majority of kinases when tested at 1 μ mol/L (Supplementary Table S2).

AZD2014 and everolimus were tested *in vitro* against a panel of cell lines from multiple tumor types (27). Two parameters were derived from the data: GI_{50} (the concentration decreasing cell number by 50% compared with untreated cells) and total growth inhibition (TGI; the concentration at which there is no net growth). AZD2014 showed a good relationship between GI_{50} and TGI values, consistent with a complete growth inhibition in the vast majority of cell lines (Fig. 1C). As previously reported for rapamycin and its analogues, everolimus treatment resulted in a "disconnect" between GI_{50} and TGI values, indicating a partial growth inhibitory effect in many cell lines (Fig. 1D). Breast cancer cell lines were particularly sensitive to either everolimus or AZD2014, compared with cell lines from other lineages.

To evaluate the biologic effect of AZD2014 in more clinically relevant models, the impact on cell growth was determined in a series of parental and endocrine therapy–resistant cell lines. LTED cell lines have been widely accepted as *in vitro* models mimicking the hormonal environment experienced by postmenopausal women and/or patients treated with primary endocrine therapy, in particular aromatase inhibitors (24, 28). Furthermore, LTED lines demonstrate activation of the PI3K–AKT–mTOR pathway

(24, 28), a phenomenon observed in postmenopausal patients relapsed on endocrine therapy, underscoring the clinical relevance of studying mTOR inhibition in the LTED setting.

AZD2014 caused growth inhibition with GI₅₀ values of <200 nmol/L and TGI values of <500 nmol/L across a panel of LTED cell lines, indicating complete growth inhibition and a cytotoxic effect at concentrations consistent with inhibition of biomarkers downstream of mTOR. In addition, AZD2014 showed similar activity in lines resistant to tamoxifen or fulvestrant. As previously reported and in contrast, everolimus induced only a partial growth inhibition with TGI values >10 μ mol/L in 6 of the 8 cell lines tested (Supplementary Table S3). To test the activity of AZD2014 in models of acquired resistance to rapalogs, we generated everolimus resistance in ER⁺ breast cancer cell lines and LTED cell lines. The effects of AZD2014 on cellular proliferation were measured in HCC1428 parental cells and compared with eveR. LTED, and LTED-eveR cells (Fig. 2A). AZD2014 blocked proliferation in all cell lines, including everolimus-resistant cells and everolimus-resistant LTED cells. In addition, AZD2014 effectively inhibited signaling to S6, PRAS40 as well as feedback phosphorylation of AKT in these cells (Fig. 2B). The effects of AZD2014 were confirmed using colony formation assays and three-dimensional Matrigel assays (Supplementary Fig. S1A and S1B).

AZD2014 delivers efficacy in *in vivo* models of ER⁺ breast cancer alone or in combination with fulvestrant

The pharmacokinetics of AZD2014 in mice was tested upon administration of doses between 7.5 and 15 mg/kg. A dosedependent increase in C_{max} and AUC was observed following single dose and repeat dosing of AZD2014: C_{max} ranged from 1 to 16 µmol/L and AUC ranged from 220 to 5,042 µmol/L·h across this dose range (Supplementary Fig. S2A). The pharmacodynamic effect of AZD2014 against an mTORC1 biomarker (phosphorylation of S6) and an mTORC2 biomarker (phosphorylation of AKT) was assessed in SCID mice bearing MCF7 xenografts following administration of 3.75, 7.5, and 15 mg/kg AZD2014. There was a good relationship between the drug plasma concentrations and biomarker levels (estimated p-AKT IC50 of 0.119 µmol/L total, 53% SE, Supplementary Fig. S2B and estimated p-S6 IC50 0.392 µmol/L, 28.8% SE, Supplementary Fig. S2C).

To evaluate the biologic effect associated with specific modulation of the biomarkers over time, the tumor growth inhibitory effect of AZD2014 was tested in a number of ER⁺ breast xenograft models. AZD2014 induced significant dose-dependent growth inhibition in several hormone therapy–sensitive ER⁺ breast cancer xenograft models, such as MCF7 (Fig. 3A) and HCC1500 (Fig. 3B), as well as three patient-derived primary explant models BR0555, CTC174, and HBCx3 (Fig. 3C–E). Tumor growth inhibition (TGI) values ranged from 61% at 15 mg/kg in the HCC1500 model, to complete stasis or regression in the MCF7 model at 15 mg/kg (Fig. 3 and Supplementary Table S3). Interestingly, AZD2014 delivered antitumor activity in the CTC174 model (84% TGI, Fig. 3D and Supplementary Table S4), which carries a mutation in the ER (ESR1 D538G), indicating that AZD2014 may have activity in this patient population.

The antitumor activity of AZD2014 administered in combination with fulvestrant was also assessed in a number of ER⁺ breast cancer models (Fig. 3 and Supplementary Table S4). Both agents were administered at a well-tolerated dose and the combination did not alter the tolerance (based on body weight measurements)



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Figure 2.

HCC1428-eveR and HCC1428-LTED-eveR cell lines resistant to everolimus remain sensitive to mTORC1/2 inhibitor AZD2014. A, proliferation of HCC1428 parental (Parental), eveR, LTED, and LTED-eveR cells. Five days after treatment of the indicated compounds, cell density was measured by CellTiterGlo assay. B, immunoblotting analysis of the indicated HCC1428 derivatives, 6 hours after treatment with AZD2014.

observed with each agent alone. The effect of fulvestrant on tumor growth was visible from the second week of treatment onward, but induced only partial growth inhibition in most models. The combination of continuous dosing of AZD2014 with fulvestrant was generally more effective than either agent alone (Fig. 3 and Supplementary Table S4). Furthermore, in the HBCx3 model, which is relatively insensitive to the ER antagonist tamoxifen, the combination of AZD2014 and tamoxifen had a similar antitumor effect to AZD2014 alone (Fig. 3E).

To study the mechanisms of tumor growth inhibition delivered by the combination of AZD2014 and fulvestrant on the ER and mTOR pathways in more details, we analyzed the modulation of downstream pathway markers. Consistent with the overall antitumor effects of AZD2014, a significant modulation of both mTORC1 (p-P70S6K, p-S6, and p-4EBP1) and mTORC2 (p-NDRG1 and p-AKT) biomarkers was confirmed using Western blotting (Fig. 3F).

AZD2014 causes significant growth inhibition when dosed continuously or using an intermittent dosing schedule

"High-dose pulsatile" administration has been proposed as a means to switch a phenotypic output from growth inhibition to cell death by inducing a transient but complete abrogation of a signaling pathway (29, 30). The administration of AZD2014 20 mg/kg twice daily for 2 days significantly increased the plasma exposure during the dosing days and into the third day compared with once daily dosing: $C_{\rm max}$ reached approximately 15 µmol/L with an AUC on day 2 of 4,320 µmol/L h and concentrations were still approximately 1 µmol/L 48 hours later. A dose of 20 mg/kg twice daily for 2 days achieves exposures greater than the IC₅₀s for both biomarkers (p-AKT and p-S6) for 48 hours continuously (Supplementary Fig. S3).

Using this intermittent dosing regimen (2 days on, 5 days off), AZD2014 induced a rapid tumor regression observed during the treatment period followed by regrowth during the drug holiday



Figure 3.

Activity of AZD2014 in a number of *in vivo* models. A to E, *in vivo* activity of AZD2014 alone or in combination with endocrine therapy. Animals were treated as indicated. A, MCF7 xenografts (SCID mice); B, HCC1500 xenografts (SCID-beige mice); C, BR0555 xenografts (NSG mice); D, CTC174 xenografts (NSG mice); E, HBCx3 xenografts (Male nude mice, Foxn1^{nu}). F, *ex vivo* analysis of the activity of AZD2014 in MCF7 xenografts. AZD2014 was administered at 15 mg/kg. Samples were collected 4 hours after the last dose, following 3 days of treatment, before immunoblotting.

(Fig. 4A). Consistent with these findings, immunohistochemical analysis of samples treated with a high dose of AZD2014 (20 mg/kg) compared with 15 mg/kg, showed increased cleaved caspase and γ -H2AX staining (Fig. 4B) indicative of apoptosis and DNA damage and suggesting the occurrence of cell death during the growth regression period. Administration of AZD2014 using a day 1/day 4 intermittent dosing schedule was also effective, but did not display the same pattern of growth inhibition, followed by recovery (Fig. 4A).

To confirm the effects of the AZD2014 intermittent dosing schedule in models of endocrine and everolimus resistance *in vivo*, we tested AZD2014 in the HCC1428 LTED and LTED-eveR derivatives using an intermittent dosing schedule. AZD2014 was efficacious when administered intermittently in HCC1428 (LTED) xenografts and LTED-eveR xenografts (Fig. 4C and D).

To investigate whether intermittent dosing schedules of AZD2014 were able to deliver antitumor activity in combination with fulvestrant, we treated MCF7 xenografts with 20 mg/kg AZD2014 (twice daily, 2 days on/5 days off) in combination with fulvestrant (5 mg/kg s.c. three times weekly, estimated to deliver concentrations consistent with clinical exposure). This combination caused significant tumor regressions the MCF7 model (Fig. 5A). Although the combination treatment did not cause additional effects on mTORC1 or mTORC2 biomarkers, we observed a significant increase in γ -H2AX phosphorylation during the first 3 days of dosing, again indicating potential induction of apoptosis, during the tumor regression phase (Fig. 5B).

To study the distinct responses observed with different dosing schedules, we carried out gene-expression analysis from tumor samples dosed continuously versus samples from tumors dosed intermittently. More profound changes in the expression of some genes (e.g., *IRS2*, *FBXO32*, and *PDK4*) were observed in samples from the tumors that had received a higher dose of AZD2014, consistent with the increased regressions observed at the time of sampling (Fig. 6A). A subset of genes showed enhanced modulation (statistically significant values) in the combination group compared with AZD2014 or fulvestrant alone (e.g., *AREG*, *C3*, *PDZK1*, and *SEPP1*), including some apoptosis genes (e.g., *BIK* and *BMF*; Fig. 6B). A larger number of genes were observed to be significantly modulated compared with the vehicle group, including classic ER target genes (Supplementary Fig. S4).

These results suggest that mechanistically distinct effects may come into play when AZD2014 is dosed using an intermittent schedule versus a continuous schedule, and that an intermittent dosing schedule could be used clinically to deliver improved antitumor activity.



Figure 4.

AZD2014 delivers efficacy when administered using an intermittent dosing schedule. A, *in vivo* tumor xenografts of MCF7 human breast cancer cells. Tumors were randomized into 4 groups and treated with vehicle or AZD2014 in three schedules: 15 mg/kg once daily, 20 mg/kg twice daily on days 1 and 2 of a weekly schedule or 20 mg/kg twice daily on days 1 and 4 of a weekly schedule as indicated. B, higher doses of AZD2014 cause significant increases in cleaved caspase (CC) and γ -H2AX. Animals were dosed with vehicle or AZD2014 for 2 days as indicated. Samples were collected 4 hours after the last dose for immunohistochemistry analysis. C, *in vivo* tumor xenografts of HCC1428-LTED or D, HCC1428-LTED-eveR cells. Tumors were treated with everolimus (5 mg/kg, daily), AZD2014 (20 mg/kg, 2 days on, 5 days off), or a vehicle control as indicated.

Discussion

This study demonstrates the activity of AZD2014, a potent and selective mTORC1/mTORC2 kinase inhibitor in ER⁺ breast cancer models. AZD2014 is a close analogue of AZD8055, a previously reported mTORC1 and 2 inhibitor, with similar protein kinase

selectivity profile (21) and a >1,000 selectivity window versus class I PI3 kinases. AZD2014 shows a clear differentiation from rapamycin analogues, from a mechanistic and a phenotypic perspective. AZD2014 induces a more complete growth inhibition and cell death *in vitro* compared with everolimus in a number

Figure 5.

AZD2014 delivers efficacy when administered using an intermittent dosing schedule in combination with fulvestrant. A, in vivo tumor xenografts of MCF7 human breast cancer cells treated as indicated B the combination of AZD2014 (20 mg/kg twice daily, 2 days on, 5 off) and fulvestrant (5 mg/mouse s.c. three times weekly) has no additional effect on TORC1 or TORC2 substrates over A7D2014 alone but causes a significant increase in γ -H2AX phosphorylation. Samples were collected following 3 days of treatment, 4 hours after the last dose and analyzed by immunoblotting





Figure 6.

Analysis of gene-expression changes by different AZD2014 schedules and combinations *in vivo*. A, example genes that display a statistically significant increase in the intermittent dosing schedule samples (AZD2014 20 mg/kg twice daily for 2 days), compared with samples derived from the continuous dosing schedule (AZD2014 15 mg/kg once daily) as indicated. B, expression of a subset of genes modulated (increased or decreased) in the AZD2014 + fulvestrant combination group, versus either single agents fulvestrant or AZD2014.

of cell lines. ER^+ cell lines are amongst the most sensitive to AZD2014, consistent with the PI3K–mTOR pathway being important in this setting (17, 18). *In vivo*, AZD2014 causes significant tumor growth inhibition in a number of xenograft and patient derived models. AZD2014 is efficacious when dosed using a continuous or intermittent dosing schedules, and in combination with fulvestrant, delivers complete tumor growth inhibition or regressions in ER^+ breast cancer models.

AZD2014 has a broad spectrum of growth inhibition in cell lines in vitro but induces cell death more prominently in ER⁺ breast cancer models. We therefore extended these observations to more clinically relevant ER⁺ breast cancer models. AZD2014 has equal or greater activity against a variety of cell lines mimicking resistance to a number of different hormone therapies (tamoxifen, fulvestrant, and long-term estrogen deprivation) compared with their parental counterparts, suggesting that inhibition of mTORC1 and 2 could be beneficial in patients who have become resistant to endocrine therapy (Supplementary Table S2). Consistent with these findings, Jordan and colleagues recently reported that AZD8055 modulated phosphorylation of ER in both tamoxifen and LTED models of breast cancer. In vivo, AZD2014 induces tumor stasis or regressions in a number of models of ER⁺ breast cancer. In a primary explant model refractory to tamoxifen and expressing low levels of ER (HBCx3), AZD2014 causes significant inhibition of growth (Fig. 3). Around 10% of ER⁺ breast cancers show a decrease or loss of ER expression at relapse. The ZR75-1/LTED model, which has very low ER, is also sensitive to AZD2014. Interestingly, this cell line is also sensitive to everolimus.

The mTORC1 and 2 activity of AZD2014 is further demonstrated in models of everolimus resistance (eveR) and in models of everolimus resistance in a LTED background (LTED-eveR) *in vitro* and *in vivo*, suggesting that inhibition of mTORC1 and 2 could be effective in patients which have become resistant to rapalogs.

Mutations in the ER are rapidly emerging as a mechanism of resistance to aromatase inhibitors (31). AZD2014 delivers antitumor activity in the CTC174 explant model, which has a mutation in the ER (ESR1 D538G), indicating that TORC1/2 inhibition could also be effective in patients with ER mutations. Furthermore, as this patient-derived model is grown in the absence of estrogen supplementation, we believe it closely represents the clinical population that could be targeted by AZD2014. In vivo, the combination of AZD2014 with fulvestrant has greater antitumor activity than either agent alone in ER⁺ breast cancer models sensitive to fulvestrant. Encouragingly, the combination of fulvestrant and AZD2014 in vivo does not alter the tolerance profile of the agents, suggesting that this combination could offer an alternative for therapy in patients that have relapsed on aromatase inhibitors and/or rapalogs. Moreover, the data in this study suggest that AZD2014 delivers efficacy in a wide range of ER⁺ preclinical models representing different patient populations and stages of disease, including LTED (endocrine resistance), tamoxifen resistance, fulvestrant resistance, ESR mutants, and everolimus resistance settings. Therefore, AZD2014 treatment alone or in combination with endocrine therapy could provide superior efficacy in a number of distinct breast cancer disease segments.

Acute and complete abrogation of the mTOR pathway using AZD8055 administered as a pulsatile dosing (75 mg/kg twice a week) has been shown previously to enhance tumor growth inhibition compared with chronic daily dosing (12). This concept was applied to AZD2014 in ER⁺ breast cancer models. Intermittent dosing of AZD2014 (2 days on/5 off) clearly shows regression induced during the drug exposure period. Regrowth observed during the drug holiday was partly alleviated by the presence of fulvestrant. At the end of the study, chronic daily dosing and high intermittent dosing achieved similar growth inhibitory effects. These cycles of regression/

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regrowth following high intermittent dosing may be partly due to the growth rate of the tumor model. Increases in the levels of cleaved caspase and phosphorylation of γ -H2AX (Fig. 5B) may also indicate the occurrence of cell death during the regression periods. The high-dose intermittent schedule altered the pharmacokinetics of AZD2014, both in terms of C_{max} and AUC, causing a succession of regression/regrowth that reflects exposure to compound. This suggests that careful optimization of dose and schedule may maximize therapeutic benefit while minimizing toxicities. This concept has now been tested clinically with very encouraging initial results suggesting a lower incidence of maculopapular rash and stomatitis in patients treated with an intermittent dosing schedule (32).

Gene-expression profiles of the intermittent dosing schedule versus the continuous dosing schedule indicated differences in the response to exposure to higher doses of compound (Fig. 6). A number of genes such as *AREG*, *C3*, *PDZK1*, *SEPP1*, *BIK*, and *BMF* were differentially modulated in a significant manner in the combination samples compared with the single treatment samples. Although the biologic significance of such changes would require further investigation, the pattern indicates that a more profound effect on cell signaling and physiology is being observed in the combination groups and that differences in gene expression may also reflect the distinct profiles observed in the growth curves, which suggest a succession of regression/ regrowth.

AZD2014 is currently in phase II study clinical development. The preclinical studies reported here support the evaluation of AZD2014 in combination with fulvestrant in a number of breast cancer disease settings and in patients with ER^+ breast cancer who have progressed on antihormonal agents.

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Disclosure of Potential Conflicts of Interest

B. Davies has ownership interest as a shareholder in AstraZeneca. M. Pass has ownership interest (including patents) in AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Michael Dymond for his work on data statistical analysis. T. Bihani is a current employee of Radius Health. Z. Howard is a current employee of Roche.

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Received May 6, 2015; revised August 20, 2015; accepted August 30, 2015; published OnlineFirst September 10, 2015.

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