AZD2014, an inhibitor of mTORC1 and mTORC2, is highly effective in ER+ breast cancer when administered using intermittent or continuous schedules.
Sylvie M. Guichard1*, Jon Curwen2*, Teeru Bihani1, Celina M. D'Cruz1, James W.T. Yates5, Michael Grondine1, Zoe Howard3, Barry Davies2, Graham Bigley2, Teresa Klinowska2, Kurt G. Pike4, Martin Pass4, Christine M. Chresta2, Urszula Polanska2, Robert McEwen2, Oona Delpuech5, Stephen Green2, and Sabina C. Cosulich5
*these authors contributed equally to the work

1AstraZeneca Oncology, Gatehouse Park, 35 Gatehouse Drive, Waltham, MA02451, USA;
2AstraZeneca Oncology, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK;
3Roche, Hochstrasse 16, 4070, Basel, Switzerland;
4AstraZeneca Oncology, Building 310 Cambridge Science Park, 319 Milton Rd, Cambridge, CB4 0WG, UK
5AstraZeneca Oncology, CRUK Cambridge Institute, Robinson Way, Cambridge, CB2 0RE, UK.

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Abbreviations: PTEN: phosphatase and tensin homolog; AKT: PKB (Protein kinase B); PI3K: Phosphoinositide 3 Kinase; TSC: tuberous sclerosis; LKB: liver kinase B; PIK3CA (phosphoinositide 3-kinase alpha); mLST8: mTOR associated LST8 protein; SREBP: sterol regulatory binding protein; PRAS40: proline-rich AKT substrate of 40 kDa; SGK: serum and glucocorticoid-regulated kinase.

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Corresponding author:
Sabina Cosulich, CRUK Cambridge Institute, Li Ka Shing Centre, Robinson Way, Cambridge CB2 0RE.
sabina.cosulich@astrazeneca.com
Tel: +44 7818523830
ABSTRACT

The mammalian target of rapamycin (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 macrolide rapamycin (rapalogues). Encouraging effects have been observed with rapalogues 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 which inhibits both mTORC1 and mTORC2 complexes and has a greater inhibitory function against mTORC1 than the clinically approved rapalogues. Here we demonstrate that AZD2014 has broad anti-proliferative effects across multiple cell lines, including ER+ breast models with acquired resistance to hormonal therapy and cell lines with acquired resistance to rapalogues. In vivo, AZD2014 induces dose-dependent tumour growth inhibition in several xenograft and primary explant models. The anti-tumour 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 tumour 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, make this compound an ideal candidate for combining with endocrine therapies in the clinic.

AZD2014 is currently in phase II clinical trials.
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 tumour types \((1,2)\). Mutations or loss of function of upstream regulators such as TSC1/2, LKB1, or components of the phosphatidylinositol-3 kinase (PI3K) pathway such as PIK3CA, AKT or PTEN have been reported in most types of human tumours \((3,4)\).

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, sub-cellular 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, \(6\)) 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 which modulate autophagy and lysosome biogenesis \((9)\). mTORC2 has been reported to play a role in the cellular response to extra-cellular growth factors through largely unknown mechanisms. Its activation requires association with ribosomes and results in the phosphorylation 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 tumours \((11)\). The inhibition of mTORC1 by rapamycin and its related compounds (rapalogues) 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 hyper-activation of mTORC2 and AKT phosphorylation \((12)\). In addition, rapalogues 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 rapalogues suggest 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 anti-estrogen modalities, such as aromatase inhibitors (anastrozole, letrozole and exemestane) \((14)\), 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 US Food and Drug Administration (19). Previous reports on ATP competitive mTORC1 and 2 inhibitors such as torin1 (20) or AZD8055 (21) have described inhibition of rapamycin-resistant mTOR signaling, which translated into a greater biological activity in ER+ breast cancer models compared to rapalogues (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 which are resistant to endocrine therapy and/or to the rapalogue 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 mM stock solution in DMSO and stored under nitrogen.

Antibodies
Antibodies were obtained from the following sources: pPRAS40 T246 (Biosource); p-AKT S473 and T308 (Cell Signaling Technology, CST), p-P70S6K T389 (CST), p-NDRG1 T346 (CST), p-S6RP S235/236 and S240/244 (CST), p-4EBP1 T37/46 (CST), yH2AX S139 (Millipore), 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 + 2mM 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 (24). MCF7-T52, MCF7-100-16 and MCF7-40-6 (fulvestrant resistant) were obtained from T. Shioda and cultured in the presence of 1 μM of tamoxifen or 1 μM fulvestrant as described (25). The HCC1428 LTED-eveR line was generated and cultured in phenol red-free medium + 10% charcoal dextran-treated fetal bovine serum (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 et al (27). All cell lines were authenticated at AstraZeneca cell banking using DNA fingerprinting short-tandem repeat (STR) assays (Supplementary materials and methods).

**Colony formation assays**

Cells were plated at 5000 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 prior to scanning.

**Three-dimensional Matrigel assays**

Cells were plated in phenol-red free growth factor reduced Matrigel (BD). After plating, cells were incubated for four days before adding the indicated concentrations of compound. Phase-contrast photographs were taken using a 10X 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.
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 MDA-MB-468 cells, using an Acumen laser scanning cytometer (TTP Labtech, UK) to analyse 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% Tris-HCl 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 (Cell Signaling) with phosphatase/protease inhibitor cocktail (Cell Signaling).

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 US and France) and a third facility (UK) operating under the UK Home Office regulatory framework. MCF7 experiments: 5 x 10^6 MCF7 cells were injected subcutaneously in a volume of 0.1 mL in male SCID mice and were randomized into control and treatment groups when tumour size reached 0.2 cm^3. AZD2014 was dissolved in captisol (Cydex, Lenexa, KS), 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. Tumour volumes (measured by calliper), animal body weight and condition were recorded twice weekly for the duration of the study. The tumour volume was calculated (taking length to be the longest diameter across the animal and width to be the corresponding perpendicular diameter) using the formula: (length x width) x √((length x width) x (π/6)). Because the variance in mean tumour 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.

BID (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 x 362 D x 185 H, Allentown, USA) with sterilized and dust-free bedding cobs, access to sterilized food and water ad libitum, under a light-dark cycle (14-hour circadian cycle of artificial light) and controlled room temperature and humidity. Tumour fragments were obtained by collecting a tumour from a donor mouse and cutting it into 50mm³ pieces. Fragments were implanted orthotopically adjacent to mammary fat pad. 0.18mg 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. 9mm staples were used to close all incision sites and were removed one week post surgery. Mice were randomized into control and treatment groups once tumour sizes reached approximately 150mm³.

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

HCC1428 experiments used the same female NSG mice (Jackson Laboratories) but s were induced by inoculation of a cell suspension rather than fragments. Animals were anesthetized and 10×10⁶ cells were implanted in a total of 50uL (of 50% phenol-red free Matrigel (BD): 50% RPMI media) transdermally in the third mammary fat pad. Tumours were measured with vernier calipers, and volumes were calculated using the formula (L*W²)*0.52. When the s reached an average of 100mm³, the mice were randomized into treatment groups by volume. Animals were treated with vehicle control, everolimus (5mg/kg p.o., q.d.) or AZD2014 (20mg/kg p.o. 2 days on/5 days off) for 7 weeks. Tumour volumes are displayed as fold change relative to the average volume on the first day of treatment.

HBCx3 experiments: HBCx3 tumour fragments of around 40 mm³ were implanted into male nude mice (athymic strain Foxn1nu, supplied by Harlan Laboratories) under general anaesthesia (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 tumour growth, mice were given drinking water containing 8.5 mg/L ß-oestradiol rather than an implanted pellet as in the other models above.
Tumours were homogenised using a Fastprep instrument and all samples were sonicated prior to lysing and immunoblotting. The numerical data for each biomarker was determined using Genetools software and normalised 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:

\[
\frac{dGUT}{dt} = -ka \ GUT \\
\frac{dCEN}{dt} = -\frac{(Q + CL)}{V_1} \ CEN + \frac{Q}{V_2} \ PER + ka_{\text{FAST}} \ GUT_{\text{FAST}} + ka_{\text{SLOW}} \ GUT_{\text{SLOW}} \\
\frac{dPER}{dt} = Q \left( \frac{CEN}{V_1} - \frac{PER}{V_2} \right)
\]

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:

\[
\text{Effect} = \text{Baseline} \left( \frac{1}{1 + \frac{C_p}{IC50}} \right)
\]

**Immunohistochemical analysis of xenograft tissue**

Tumours were excised from animals and placed into 10% Buffered Formalin. Tissues were fixed for 24-48 hours before being processed and embedded into paraffin wax. Sections were cut and allowed to dry overnight in an oven at 40°C. Immunohistochemistry was performed on the LabVision immunostainer platform. Slides were scanned using the Aperio
Scanscope and analysed using a modified version of the Colour Deconvolution algorithm. This algorithm separates the image into three channels, corresponding to the actual colours 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 manufacturer's instructions. Targeted gene profiling was performed using the Fluidigm platform and cDNA was pre-amplified (14 cycles) using a pool of TaqMan primers (Life Technologies), following manufacturer's instructions. Sample and assay preparation of the 96.96 Fluidigm Dynamic arrays was carried out according to the manufacturer's instructions. Data was collected and analyzed using the Fluidigm Real-Time PCR Analysis 2.1.1 software. Gene expression values were calculated after normalisation to the average of the house keeping genes (PPIA, IPO8, YWHAZ): dCt; negative ddCt was calculated by subtracting the average dCT value of the vehicle group to each animal (negative ddCt = AVG dCt-vehicle group – dCt), and fold change after log2 transformation ($2^{\text{negddCt}}$). The mean and standard error 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 rapalogues.**

Benzamide 3-[2,4-bis[(3S)-3-methyl-4-morpholinyl]pyrido[2,3-d]pyrimidin-7-yl]-N-methyl-, (Figure 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 (IC$_{50}$ of 2.81 nM) as well as in cellular assays measuring both mTORC1 and mTORC2 activities (Supplementary table 1). In MDAMB468 cells, AZD2014 decreased the phosphorylation of the mTORC1 substrate ribosomal protein S6 (Ser235/236) with a mean IC50 of 210 nM and the mTORC2 substrate AKT (Ser473) with a mean IC50 of 78 nM (Supplementary table 1). Everolimus tested in the same conditions showed a greater inhibitory effect against S6 phosphorylation (mean IC50 of 0.15 nM) but had no inhibitory effect against phosphorylation of AKT (mean IC50 of 20.6 μM). The activity of AZD2014 against a number of mTOR substrates was also assessed in MCF7 cells (Figure 1B). AZD2014 inhibited both mTORC1 and mTORC2 substrates, whereas everolimus only inhibited mTORC1 substrates and caused an up-regulation of phosphorylation of AKT, as
previously reported (12). AZD2014 was tested against a number of PI3K family enzymes and showed more than a 1000-fold selectivity against all PI3K isoforms (Supplementary table 1).

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 μM (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 μM (Supplementary table 2).

AZD2014 and everolimus were tested in vitro against a panel of cell lines from multiple tumour types (27). Two parameters were derived from the data: GI50, (the concentration decreasing cell number by 50% compared to untreated cells) and total growth inhibition (the concentration at which there is no net growth). AZD2014 showed a good relationship between GI50 and total growth inhibition values, consistent with a complete growth inhibition in the vast majority of cell lines (Figure 1C). As previously reported for rapamycin and its analogues, everolimus treatment resulted in a ‘disconnect’ between GI50 and total growth inhibition values, indicating a partial growth inhibitory effect in many cell lines (Figure 1D). Breast cancer cell lines were particularly sensitive to either everolimus or AZD2014, compared to cell lines from other lineages.

In order to evaluate the biological 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. Long term estrogen deprived (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 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 nM and total growth inhibition values of <500 nM 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 μM in 6 out of the 8 cell lines tested (Supplementary table 3).

In order to test the activity of AZD2014 in models of acquired resistance to rapalogues, we generated everolimus resistance in ER+ breast cancer cell lines and long-term estrogen deprived (LTED) cell lines. The effects of AZD2014 on cellular proliferation were measured in HCC1428 parental cells and compared with everolimus-resistant (eveR), long-term
estrogen deprived (LTED), and long-term estrogen deprived/everolimus-resistant (LTED-everR) cells (Figure 2A). AZD2014 blocked proliferation in all cell lines, including everolimus resistant cells and everolimus resistant-long term estrogen deprived cells. In addition, AZD2014 effectively inhibited signaling to S6, PRAS40 as well as feedback phosphorylation of AKT in these cells (Figure 2B). The effects of AZD2014 were confirmed using colony formation assays and three dimensional matrigel assays (Supplementary figures 1A and B).

**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 dose-dependent increase in Cmax and AUC was observed following single dose and repeat dosing of AZD2014: Cmax ranged from 1 to 16 µM and AUC ranged from 220 to 5042 µM.h across this dose range (Supplementary figure 2A). 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 µM total, 53% standard error, Supplementary figure 2B and estimated p-S6 IC50 0.392 µM, 28.8% standard error, Supplementary figure 2C).

To evaluate the biological effect associated with specific modulation of the biomarkers over time, the tumour 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 (Figure 3A) and HCC1500 (Figure 3B), as well as three patient derived primary explant models BR0555, CTC174, and HBCx3 (Figure 3C,D,E). Tumour growth inhibition (TGI) values ranged from 61% at 15mg/kg in the HCC1500 model, to complete stasis or regression in the MCF7 model at 15 mg/kg (Figure 3 and Supplementary table 3). Interestingly, AZD2014 delivered anti-tumour activity in the CTC174 model (84% TGI, Figure 3D and Supplementary table 4), which carries a mutation in the estrogen receptor (ESR1 D538G) indicating that AZD2014 may have activity in this patient population.

The anti-tumour activity of AZD2014 administered in combination with fulvestrant was also assessed in a number of ER+ breast cancer models (Figure 3 and Supplementary table 4). Both agents were administered at a well tolerated dose and the combination did not alter the tolerance (based on body weight measurements) observed with each agent alone. The effect of fulvestrant on tumour growth was visible from the second week of treatment.
onwards 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 (Figure 3 and Supplementary table 4). Furthermore, in the HBCx3 model, which is relatively insensitive to the ER antagonist tamoxifen, the combination of AZD2014 and tamoxifen had a similar anti-effect to AZD2014 alone (Figure 3E).

In order to study the mechanisms of tumour growth inhibition delivered by the combination of AZD2014 and fulvestrant on the ER and mTOR pathways in more details, we analysed the modulation of downstream pathway markers. Consistent with the overall anti-tumour effects of AZD2014, a significant modulation of both mTORC1 (p-P70S6K, p-S6, p-4EBP1) and mTORC2 (p-NDRG1, p-AKT) biomarkers was confirmed using western blotting (Figure 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 to once daily dosing: Cmax reached ~15 µM with an AUC on day 2 of 4320uM.hrs and concentrations were still ~1µM 48h later. A dose of 20 mg/kg BID for 2 days achieves exposures greater than the IC50s for both biomarkers (p-AKT and p-S6) for 48 hours continuously (Supplementary figure 3).

Using this intermittent dosing regimen (2 days on, 5 days off), AZD2014 induced a rapid tumour regression observed during the treatment period followed by re-growth during the drug holiday (Figure 4A). Consistent with these findings, immunohistochemical analysis of samples treated with a high dose of AZD2014 (20mg/kg) compared with 15mg/kg, showed increased cleaved caspase and γ-H2AX staining (Figure 4B) indicative of apoptosis and DNA damage and suggesting the occurrence of cell death during the growth regression period. Administration of AZD2014 using a day1/day4 intermittent dosing schedule was also effective, but did not display the same pattern of growth inhibition, followed by recovery (Figure 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 long-term estrogen deprived/everolimus-resistant (LTED-eveR) xenografts (Figure 4C, D).
In order to investigate whether intermittent dosing schedules of AZD2014 were able to deliver anti-tumour activity in combination with fulvestrant, we treated MCF7 xenografts with 20mg/kg AZD2014 (BID, 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 tumour regressions the MCF7 model (Figure 5A). Whilst 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 tumour regression phase (Figure 5B).

To study the distinct responses observed with different dosing schedules, we carried out gene expression analysis from tumour samples dosed continuously vs. samples from tumours dosed intermittently. More profound changes in the expression of some genes (e.g. IRS2, FBXO32, PDK4) were observed in samples from the tumours which had received a higher dose of AZD2014, consistent with the increased regressions observed at the time of sampling (Figure 6A). A subset of genes showed enhanced modulation (statistically significant values) in the combination group compared to AZD2014 or fulvestrant alone (e.g. AREG, C3, PDZK1, SEPP1), including some apoptosis genes (e.g. BIK, BMF) (Figure 6B). A larger number of genes were observed to be significantly modulated compared to the vehicle group, including classic ER target genes (Supplementary figure 4).

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

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 >1000 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 to everolimus in a number of cell lines. ER positive 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 tumour 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 tumour 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 to their parental counterparts, suggesting that inhibition of mTORC1 and 2 could be beneficial in patients which have become resistant to endocrine therapy (Supplementary table 2). Consistent with these findings, Jordan et al. recently reported that AZD8055 modulated phosphorylation of estrogen receptor in both tamoxifen and LTED models of breast cancer. In vivo, AZD2014 induces tumour 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 (Figure 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 long term estrogen deprived 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 rapalogues.

Mutations in the estrogen receptor are rapidly emerging as a mechanism of resistance to aromatase inhibitors (31). AZD2014 delivers anti-tumour activity in the CTC174 explant model, which has a mutation in the estrogen receptor (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 which could be targeted by AZD2014. In vivo, the combination of AZD2014 with fulvestrant has greater anti-tumour 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 this combination could offer an alternative for therapy in patients that have relapsed on aromatase inhibitors and/or rapalogues. Moreover, the data in this study suggest that AZD2014 delivers efficacy in a wide range of ER+ pre-clinical 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 tumour growth inhibition compared to 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. Re-growth 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/re-growth following high intermittent dosing may be partly due to the growth rate of the tumour model. Increases in the levels of cleaved caspase and phosphorylation of γ-H2AX (Figure 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 Cmax and AUC, causing a succession of regression/re-growth which reflects exposure to compound. This suggests that careful optimisation of dose and schedule may maximise therapeutic benefit while minimising toxicities. This concept has now been tested clinically with very encouraging initial results suggesting a lower incidence of macula-papular 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 (Figure 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 to the single treatment samples. Whilst the biological 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/re-growth.

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 anti-hormonal agents.

ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

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 tumour types are represented in the main graph, whereas breast cancer cell lines are represented in the insert. GI50 (the concentration decreasing cell number by 50% compared to untreated cells) and total growth inhibition (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 colours and cell lines which harbour a mutation in the PI3K pathway are indicated with a cross.

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), everolimus-resistant (eveR), long-term estrogen deprived (LTED), and long-term estrogen deprived/everolimus-resistant (LTED-eveR) cells. Five days post-treatment of the indicated compounds cell density was measured by CellTiterGlo assay. (B) Immunoblotting analysis of the indicated HCC1428 derivatives, six hours post-treatment with AZD2014.

Figure 3: Activity of AZD2014 in a number of in vivo models.
(A-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, Foxn1nu). (F) Ex-vivo analysis of the activity
of AZD2014 in MCF7 xenografts. AZD2014 was administered at 15mg/kg. Samples were collected 4 hours after the last dose, following 3 days of treatment, prior to immunoblotting.

**Figure 4: AZD2014 delivers efficacy when administered using an intermittent dosing schedule.**

(A) *In vivo* tumour xenografts of MCF7 human breast cancer cells. Tumours were randomised 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 day 1 and 4 of a weekly schedule as indicated.  
(B) Higher doses of AZD2014 cause significant increases in cleaved caspase 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* tumour xenografts of HCC1428-LTED or (D) HCC1428-LTED-eveR cells. Tumours were treated with everolimus (5mpk, q.d.), AZD2014 (20mpk, 2 days on, 5 days off) or a vehicle control as indicated.

**Figure 5: AZD2014 delivers efficacy when administered using an intermittent dosing schedule in combination with fulvestrant.**

(A) *In vivo* tumour xenografts of MCF7 human breast cancer cells treated as indicated.  
(B) The combination of AZD2014 (20mg/kg B.I.D., 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 AZD2014 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 analysed by immunoblotting.

**Figure 6: Analysis of gene expression changes by different AZD2014 schedules and combinations in vivo.**

(A) Example genes which display a statistically significant increase in the intermittent dosing schedule samples (AZD2014 20 mg/kg twice daily for 2 days), compared to 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.
Figure 1

A

B

4EBP1 pT37/46
4EBP1 pS65
4EBP1
S6 pS240/244
S6 pS235/236
S6
p70S6K pT389
p70S6K
AKT pS473
AKT pT308
AKT
VINCULIN

C

D

Cell line lineage
- Haems
- Bladder
- Breast
- Colon
- Gastric
- Liver
- Lung
- Pancreas
- Prostate
- PI3K pathway WT
- PI3K pathway mutant
Figure 2

A

![Bar charts showing net growth](chart_a.png)

B

<table>
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<tr>
<th>Par</th>
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<th>LTED</th>
<th>LTED-eveR</th>
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AZD2014 (500nM)

- pAkt (S473)
- pAkt (T308)
- pPRAS40 (T246)
- pS6 (S240/244)
- Vinculin
Figure 3

(A) Vehicle (polysorbate) p.o. q.d.
Vehicle (peanut oil) s.c. x3 weekly
Fulvestrant 5 mg/mouse s.c. x3 weekly
AZD2014 15 mg/kg p.o. q.d.
Fulvestrant + AZD2014

(B) Vehicle (polysorbate) p.o. q.d.
Fulvestrant 5 mg/mouse s.c. x3 weekly
AZD2014 15 mg/kg p.o. q.d.
Fulvestrant + AZD2014

(C) Vehicle (polysorbate) p.o. q.d.
AZD2014 15 mg/kg p.o. q.d.

(D) Vehicle (polysorbate) p.o. q.d.
Fulvestrant 5 mg/mouse s.c. x3 weekly
Tamoxifen 4 mg/kg p.o. 5 days on/2 off
AZD2014 15 mg/kg p.o. q.d.
AZD2014 + Fulvestrant

(E) Vehicle (polysorbate) p.o. q.d.
Tamoxifen 4 mg/kg p.o. q.d.
AZD2014 15 mg/kg p.o. q.d.
AZD2014 + Tamoxifen

(F) Protein expression (% control)

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Figure 4

A. Mean Tumour Volume (cm$^3$) vs Days of Dosing

- Black square: Vehicle (polysorbate) p.o. q.d.
- Green triangle: AZD2014 15 mg/kg p.o. q.d.
- Orange triangle: AZD2014 20 mg/kg p.o. b.i.d. Day 1 and 2
- Blue triangle: AZD2014 20 mg/kg p.o. b.i.d. Day 1 and 4

B. CC Positive Cell (% of total)

- Controls
- AZD2014 15 mg/kg
- AZD2014 20 mg/kg

P-values: P=0.04, P=0.07, P=0.03, P=0.08

C. Tumor Volume (mm$^3$) vs Days of Dosing

- Black square: Vehicle 1% polysorbate p.o. q.d.
- Blue triangle: Everolimus 5 mg/kg p.o. q.d.
- Red triangle: AZD2014 20 mg/kg p.o. b.i.d. 2 days on/5 off

D. Tumor Volume (mm$^3$) vs Days of Dosing

- Black square: Vehicle 1% polysorbate p.o. q.d.
- Blue triangle: Everolimus 5 mg/kg p.o. q.d.
- Red triangle: AZD2014 20 mg/kg p.o. b.i.d. 2 days on/5 off
AZD2014, an inhibitor of mTORC1 and mTORC2, is highly effective in ER+ breast cancer when administered using intermittent or continuous schedules.

Sylvie M. Guichard, Jon Curwen, Teeru Bihani, et al.

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