Tumors with AKT1<sup>E17K</sup> mutations are rational targets for single agent or combination therapy with AKT inhibitors

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Conflict of interest statement: Barry Davies, Nin Guan, Armelle Logie, Claire Crafter, Lyndsey Hanson, Vivien Jacobs, Neil James, Philippa Dudley, Kelly Jacques, Brendon Ladd, Celina D’Cruz, Michael Zinda, Justin Lindemann and Emma Jenkins are employees of AstraZeneca.
Abstract

AKT1<sup>E17K</sup> mutations occur at low frequency in a variety of solid tumors, including those of the breast and urinary bladder. Although this mutation has been shown to transform rodent cells in culture, it was found to be less oncogenic than PIK3CA mutations in breast epithelial cells. Moreover, the therapeutic potential of AKT inhibitors in human tumors with an endogenous AKT1<sup>E17K</sup> mutation is not known. Expression of exogenous copies of AKT1<sup>E17K</sup> in MCF10A breast epithelial cells increased phosphorylation of AKT and its substrates, induced colony formation in soft agar, and formation of lesions in the mammary fat pad of immunodeficient mice. These effects were inhibited by the allosteric and catalytic AKT inhibitors MK-2206 and AZD5363, respectively. Both AKT inhibitors caused highly significant growth inhibition of breast cancer explant models with AKT1<sup>E17K</sup> mutation. Furthermore, in a phase 1 clinical study, the catalytic Akt inhibitor AZD5363 induced partial responses in breast and ovarian cancer patients with tumors containing AKT1<sup>E17K</sup> mutations. In MGH-U3 bladder cancer xenografts, which contain both AKT1<sup>E17K</sup> and FGFR<sup>Y373C</sup> mutations, monotherapy AZD5363 did not significantly reduce tumor growth, but tumor regression was observed in combination with the FGFR inhibitor AZD4547. The data show that tumors with AKT1<sup>E17K</sup> mutations are rational therapeutic targets for AKT inhibitors, although combinations with other targeted agents may be required where activating oncogenic mutations of other proteins are present in the same tumor.

Introduction

The signaling network containing PI3K, AKT and mTOR is the most frequently mutated in human cancer. AKT is a central node in this signaling network, with a plethora of substrates controlling growth, apoptosis and metabolism (1,2). The most commonly mutated genes that encode for proteins in this signaling network are those that activate PIK3CA, the gene encoding the catalytic subunit of PI3K, which catalyses the phosphorylation of PI4,5-P2 to PI3,4,5-P3, and inactivating mutations or loss of the phosphatase PTEN, which catalyses
the reverse reaction. PIK3CA mutations are most common in breast and endometrial cancers, whilst mutation of PTEN is most common in endometrial cancer, prostate cancers and glioblastomas (3). AKT proteins are less frequently mutated; the most frequently mutated AKT isoform being AKT1, which is mutated in 2-3% of breast and urinary bladder tumors (4-7). Mutations in AKT1 have also been reported in other solid tumors (8), including endometrial (9,10), prostate (11) and lung (12) cancers. The E17K mutation, that results in a glutamic acid to lysine substitution at amino acid 17 in the lipid-binding pocket, is the most common mutation in AKT1, comprising 89% of the mutations found in this gene (13). Less common non-hotspot mutations have also been found, some of which are functionally transforming (14). The E17K mutation has been shown to constitutively activate AKT1 by increased localization to the plasma membrane, where it stimulates downstream signaling and can transform Rat1 fibroblasts and induce leukaemia in mice (15). Whilst knock in of AKT1E17K into isogenic MCF-7 breast cancer cells depleted of endogenous PIK3CAE545K restored proliferation in vitro and tumor growth in vivo (16), knock in of E17K into the AKT1 gene of non-transformed MCF10A mammary epithelial cells had minimal phenotypic consequences and did not recapitulate the transforming properties induced by somatic cell knock in of PIK3CA hotspot mutations (17). In addition, the consequences of directly inhibiting this target in endogenous models of breast and urinary bladder cancer have not been reported.

Two distinct types of relatively selective AKT inhibitor are being tested in the clinic. Allosteric inhibitors such as MK-2206 bind to the region that interacts with both the PH and kinase domains, and prevent translocation of AKT to the membrane and subsequent pathway activation (18). Classical ATP competitive kinase domain inhibitors, which prevent substrate phosphorylation by AKT, have also been developed. This latter group includes AZD5363, a potent pan AKT kinase inhibitor with pharmacodynamic properties consistent with mechanism in vivo, and anti-tumour activity in xenograft models with PIK3CA and PTEN mutations (19). We now report that both allosteric and
catalytic inhibitors of AKT can inhibit downstream signaling, reverse the transformation of mammary epithelial cells induced by expression of AKT$^{E17K}$, and inhibit the growth of breast cancer explant models with homozygous copies of AKT$^{E17K}$. Most importantly, monotherapy AZD5363 induced partial responses in breast and ovarian cancer patients with tumours containing AKT$^{E17K}$ mutations. However, in a urinary bladder cancer xenograft model with both AKT1 and FGFR3 mutations, inhibiting AKT alone was insufficient to inhibit tumor growth, but combination with a potent and selective inhibitor of FGFR1-3 tyrosine kinases (AZD4547) caused tumor regression. The data show that tumors with AKT$^{E17K}$ mutations are rational targets for AKT inhibitors, but may require combination therapy with other signaling inhibitors when complementary mutations are present.

Materials and Methods

Cell culture, antibodies and compounds

MCF10A cells were acquired directly from ATCC (Nov 2009) and stored frozen at early passage until use. ATCC uses short tandem repeat (STR) profiling for testing and authentication of cell lines. No additional authentication was performed. MGH-U3 cells were obtained from Dr MA Knowles (University of Leeds, UK) in November 2010, and tested by STR analysis in June 2011. All experiments using cell lines were performed within 6 months of resuscitation after cryopreservation. MCF10A-AKT$^{1E17K}$ and MCF10A-AKT$^{1WT}$ cell lines were generated by infecting MCF10A cells with lentivirus expressing AKT$^{1E17K}$, or wild type AKT1 under a tet-inducible promoter (pTRIPZ; Thermo Scientific), followed by selection in 0.5ug/ml puromycin 48 hours following infection (Sigma). AKT1 was cloned into pTRIPZ by PCR using Origene cDNA (SC116883, NM_005163.2) as a template. An N-terminal FLAG tag was added as part
of the PCR primer. The FLAG AKT1\textsuperscript{WT} was subcloned into pTRIPZ as an Age1/Xho1 fragment. The AKT1\textsuperscript{E17K} fragment was cloned as above after AKT1 was mutated via site directed mutagenesis (QuikChange II XL; Stratagene). MCF10A-RFP and MCF10A-HER2\textsuperscript{V659E} cell lines were similarly generated and selected in puromycin following infection with pTRIPZ-derived lentivirus expressing RFP or HER2\textsuperscript{V659E} under tet-inducible promoters. Cell lines were maintained at 37\textdegree C/5% CO\textsubscript{2} in Mammary Epithelial Cell Growth Medium (MEGM) containing no growth factors, cytokines or supplements (“Basal Medium”) or supplemented with all of the MEGM SingleQuot additions except GA-1000 (MEGM BulletKit, Lonza, #CC-3150), but with the addition of 100 ng/ml cholera toxin (Sigma) (“Growth medium”). Antibodies against FLAG (#8146), AKT1 (#2938), phospho-AKT S473 (#4060); PRAS40 (#2610), phospho-PRAS40 T246 (#2997), GSK-3\textbeta (#9315), phospho-GSK3\textbeta S9 (#9323), S6 (#2217), phospho-S6 S235/236 (#2211), FOXO3a (#2497), mTOR (#2972), phospho-mTOR S2448 (#2971), phospho- mTOR S2481 (#2974), GAPDH (#2118), \beta actin (#4970), horseradish peroxidase conjugated anti-mouse IgG (#7076) and anti-rabbit IgG (#7074) were obtained from Cell Signaling Technology. Antibody to Ki67 was obtained from Dako (#7240) and antibody to PI3K\textalpha was obtained from Thermo Scientific (#14870). AZD4547, AZD5363 and MK-2206 were synthesized by AstraZeneca. For in vitro studies, compounds were diluted in dimethylsulfoxide (DMSO) to a concentration of 10 mMol/L and subsequently diluted in the relevant assay media.

Soft agar colony formation assays

To evaluate growth in soft agar, 1ml of 0.8% agarose (Lonza, #50101) in RPMI supplemented with MEGM growth factors and human EGF, insulin, hydrocortisone and bovine pituitary extract (Lonza #CC4136) at 37\textdegree C was added to each well of a 12 well plate and allowed to solidify (base agar). 1ml cell suspension made up in 0.5% agarose
in RPMI (with the same supplements as above) containing DMSO or compound diluted at the desired assay concentration, was added on top of the base agar and allowed to solidify (top agar). Cells were seeded at a density of 50,000 cells/well (in 1ml RPMI medium containing 1μg/ml doxycycline) and incubated for 14 days at 37°C. Medium was changed twice a week. Colonies were then stained with P-iodonitrotetrazolium violet dye (Sigma) which was added for 16 hours at 37°C, after which colonies were counted using a GelCount instrument (Oxford Optronix). Counts from three replicate wells were averaged and the standard deviation calculated. Statistical analysis between groups was by one-way analysis of variance followed by the Bonferroni post hoc test. P values of less than 0.05 were considered statistically significant.

To determine the number of cells in a typical colony, we used the following formula (20):

\[
\text{No of cells/colony} = 2.40 \times (\text{colony diameter})^{2.379} / (\text{cell diameter})^{2.804}
\]

The average cell diameter was measured by Cellometer and the average colony diameter was calculated using a GelCount instrument.

**Immunoblotting and pharmacodynamic studies**

For *in vitro* studies, cells were seeded into 6-well plates at 75,000 cells/well in RPMI with supplements added as detailed above. After overnight incubation at 37°C/5%CO₂, cells were treated with or without 1μg/ml doxycycline and incubated at 37°C/5%CO₂ for a further 48 hours, after which the cells were washed twice in RPMI (without supplements) followed by overnight incubation prior to compound treatment. DMSO or serially diluted compounds were then added to the cells for 2 hours at 37°C/5%CO₂ prior to cell harvesting. Cells were subsequently lysed in buffer containing 100mM Tris-HCl buffer (pH7.5), 1% SDS and 10% Glycerol. Cell lysates were transferred into tubes, heated at 100°C for 5 minutes then centrifuged at 14,000rpm for 10 minutes at room temperature. Sample loading buffer and 10x Reducing Reagents (Invitrogen) were then added and
samples heated at 100°C for 5 minutes. An equal volume of cell lysates (15μg protein) was subjected to electrophoresis on NuPAGE 4-12% Bis-Tris 1.5mm 15-well Gels (Life Technologies) using MES SDS running buffer. Protein was then transferred onto nitrocellulose membranes and probed with primary antibodies raised against the protein of interest as indicated. After incubation with the appropriate secondary antibody, proteins were detected by Immobilon ECL western blotting detect reagent (Millipore). Pharmacodynamics were carried out on tumor samples at the end of the efficacy studies. Half of the tumor was snap-frozen in liquid nitrogen and stored at –80°C for pharmacodynamic analysis; the other half was fixed in 10% formalin buffer for 24 hours and then embedded in paraffin for immunohistochemical (IHC) staining. Frozen tumors were homogenized using Fastprep methodology lysis matrix A (MP Biomedicals) and lysates generated using adjusted Lysis buffer (1% Triton X 100). Equivalent amounts of protein (12 μg/lane) were resolved by 4–15% gradient SDS-polyacrylamide pre-made gels (Bio-Rad) and transferred to nitrocellulose membranes. Membranes were then incubated with primary antibodies (see “reagents” above) and subsequently with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG diluted in 5% marvel in PBS. Immunoreactive proteins were detected by enhanced chemiluminescence (Pierce) and bands quantified with a ChemiGenius (Syngene). Phosphorylated PRAS40 (T246) was measured using solid phase sandwich ELISA (Biosource #KHO0421). FOXO3a and Ki67 were detected by immunohistochemistry. Briefly following peroxidase blocking (H₂O₂) and incubation with serum-free block (Dako, X0909) for 20 minutes to block nonspecific binding, primary antibodies were applied for 1 hour at room temperature and binding detected using Rabbit Envision + System HRP–labeled Polymer for 30 minutes (Dako). Antibodies were detected by incubation with 3,3′-diaminobenzadine chromagen (Dako) for 10 minutes and then counterstained with Carazzi's hematoxylin. The nuclear signal was quantified using an algorithm developed for scoring percentage positive nuclei on
an ACIS II image analyzer (ChromaVision Medical Systems, Inc.) using standard threshold settings.

**Tumorigenicity studies**

For the MGH-U3 efficacy study, pathogen-free, male nude mice (nu/nu:Alpk) were bred at AstraZeneca (Alderley Park, UK) and housed in pathogen-free conditions. For the HBC-x2 and HBC-x31 explant model studies, pathogen-free female athymic nude mice (Foxn1nu) were obtained from Harlan Laboratories, Gannat, France. Animals were maintained in rooms under controlled conditions of temperature (19–23°C), humidity (55 ± 10%), photoperiod (12 hours light/12 hours dark) and air exchange. Animals were housed in standard cages, with food and water provided ad libitum. The facilities have been approved by the UK Home Office or Direction des Services Vétérinaires, Ministère de l'Agriculture et de la Pêche, France. For *in vivo* implants, MGH-U3 cells were harvested from T25 tissue culture flasks with 0.05% trypsin (Invitrogen) in EDTA solution followed by suspension in basic medium and three washes in phosphate buffered saline. Only single-cell suspensions of greater than 90% viability, as determined by trypan blue exclusion, were used for injection. $2 \times 10^6$ tumor cells were injected subcutaneously into the left flank of the animal in a volume of 0.1 mL PBS containing 50% Matrigel. The HBC-x2 and HBC-x31 primary breast cancer explant models were passaged from donor tumors when they reached volumes of 1 to 2 cm$^3$. Tumours were cut into fragments of approximately 20 mm$^3$ and transplanted immediately. Recipient animals were anaesthetised with ketamine/xyazine, the skin ascepticised with chlorhexidine solution, and then incised in the interscapular region prior to a tumour fragment being placed in the subcutaneous tissue. The skin was closed with clips. When mean tumor sizes reached 0.1 to 0.2 cm$^3$, the mice were randomized into control and treatment groups. Compounds were administered by oral gavage. AZD5363 was solubilized in a vehicle
containing 10% DMSO 25% w/v Kleptose HPB (Roquette Pharma), AZD4547 was suspended in 0.1% Polysorbate 80 and MK-2206 was suspended in 0.5% HPMC, 0.1% Tween 80. Tumor volumes (measured by caliper), animal body weight and tumor condition were recorded twice weekly for the duration of the study. Mice were sacrificed by CO2 euthanasia. The tumor volume was calculated (taking length to be the longest diameter across the tumor and width to be the corresponding perpendicular diameter using the formula: \( \pi/6 \times \text{length} \times \text{width}^2 \) (21). Growth inhibition from the start of treatment was assessed by comparison of the differences in tumor volume between control and treated groups. Because the variance in mean tumor volume data increases proportionally with volume (and is therefore disproportionate between groups), data were log-transformed to remove any size dependency before statistical evaluation. Statistical significance was evaluated using a one-tailed, two-sample t-test.

For xenograft assays, MCF10A-AKT1^{E17K} or MCF10A-AKT1^{WT} cells were grown using standard techniques (described above) with 0.5ug/ml puromycin. In groups treated with doxycycline (Dox), cells were pretreated with 1ug/μl Doxycycline 24 hours prior to implantation. At the time of implant, cells were harvested by trypsinization, counted, centrifuged (1,200 RPM for 5 minutes), and resuspended in 50% MEBM (unsupplemented)/50% Matrigel (Corning) at 5 x 10^6/50μl. 5X10^6 cells were injected into the 3rd mammary fat pad of NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice (Jackson Labs) using an insulin syringe. Dox groups were pretreated with Dox chow (625mg/kg, Harlan-Teklad, Cat# 2018) for 48hrs prior to implantation and maintained on this chow ad libitum. All procedures were performed in accordance with federal, state and Institutional guidelines in an AAALAC-accredited facility and were approved by the AstraZeneca Institutional Animal Care and Use Committee (IACUC). For histological examination, tumors were collected, fixed in 10% neutral buffered formalin and processed for hematoxylin/eosin staining according to standard techniques.
Phase 1 study of AZD5363 in Japanese Patients

A phase 1, open-label, multicentre, dose-escalation study was carried out in Japanese patients with advanced solid tumours (NCT01353781). Full results from this study will be published separately. Briefly, three schedules were assessed in 21-day cycle; Continuous (twice daily (bid), everyday), Intermittent-1 (bid, 4 days on/3 days off) and Intermittent-2 (bid, 2 days on/5 days off). The primary objective was to assess the safety and tolerability of AZD5363 in Japanese patients. Secondary objectives included defining the MTD and an assessment of preliminary anti-tumour activity. The study protocol was approved by the Institutional Review Board of each participating institution and all patients gave their written informed consent before study entry. The primary tumor locations were breast (n = 8), colorectal (n =2), liver (n = 2), lung (n = 5), ovary (n = 2), pleura (n = 3) uterus (n =5) and one tumor from each of the following: anterior mediastinum, cecum, duodenum, endometrium stroma, hypopharyngeal, pelvis, leiomyosarcoma, oesophagus, pancreas, rectal, stomach, thymus, urinary bladder, unknown. RECIST 1.1 guidelines were applied for tumor assessments. Baseline assessments (CT or MRI) were performed no more than 28 days before the start of treatment. Follow-up assessments were performed on day 1 of cycle 2 and cycle 3 (at weeks 3 and 6 ± 1 week), and then at approximately 6 week intervals until discontinuation of treatment or withdrawal of consent. Screening for mutations in archival primary tumour tissue was carried out using the NCC Oncopanel based on an Agilent SureSelect system and Illumina MiSeq sequencer. The target regions to be captured were the exons of ninety druggable or actionable genes and ten protein kinase fusion genes. AKT1E17K mutations were validated by next generation sequencing and with the commercial Foundation Medicine platform.
Results

AKT1E17K mutation activates AKT signalling and induces colony formation in MCF10A cells

To determine whether the AKT1\textsuperscript{E17K} mutation was oncogenic, we established MCF10A cell lines which express either AKT1 wild-type (AKT1\textsuperscript{WT}) or AKT1\textsuperscript{E17K} in the presence of doxycycline. Inducible expression of each protein was confirmed by western blot using an anti-FLAG antibody to detect the flag-tagged exogenous AKT1 (Figure 1A). The effect of AKT1\textsuperscript{E17K} expression on downstream signalling was examined, both in basal media and in full growth media, containing the added growth factors, cytokines and supplements. Cells expressing AKT1\textsuperscript{E17K} had high levels of phosphorylated AKT and PRAS40 in both basal and full growth media indicating that the pathway is active even in the absence of exogenous growth factors (Figure 1A). This is in contrast to cells expressing AKT1\textsuperscript{WT} where maximum pathway activation only occurs in full growth media. Phospho S6 levels were similar in cells expressing AKT1\textsuperscript{WT} or AKT1\textsuperscript{E17K} but were markedly increased when cells were stimulated with full growth medium.

Ectopic expression of AKT1\textsuperscript{E17K} in MCF10A cells did not affect the expression of PI3K\(\alpha\) or mTOR (Supplementary Figure 1), suggesting that increased phosphorylation of AKT and its substrates is due to expression of AKT1\textsuperscript{E17K} rather than increased expression of these upstream activating proteins. To further investigate whether the AKT\textsuperscript{E17K} mutation was oncogenic we assessed anchorage independent growth in soft agar. Colony formation was increased following doxycycline addition in cells expressing AKT1\textsuperscript{E17K} compared to AKT1\textsuperscript{WT} or RFP control protein (Figures 1B and C), although it was notable that the mean colony size was smaller than that observed for cells expressing HER2\textsuperscript{V659E}, a constitutively active form of HER2, a known oncogene (Supplementary Figure 2). The mean colony diameter produced by MCF10A-AKT1\textsuperscript{E17K} cells in the presence of doxycycline was 226 \(\mu\text{M}\), which corresponds to 479 cells per colony, whereas in the absence of doxycycline, a mean of only...
71 cells per colony was obtained. Moreover, MCF10A-AKT1WT and MCF10A-RFP cells produced an average of <70 cells per colony, in the presence and absence of doxycycline supplementation (Supplementary Figure 3). A xenograft study was carried out by injecting the various engineered MCF10A-derived cells into the mammary fat pad of NOD scid gamma mice. None of the cell lines formed tumors in the absence of doxycycline in the chow. In animals administered doxycycline, MCF10A-AKT1WT cells did not form tumors, whilst MCF10A-HER2V659E cells produced rapidly growing tumors in all the mice that reached a mean size of 672 ± 115 mm³ at 13 days after implantation. MCF10A-AKT1E17K cells produced small tumors in 6/10 mice, which reached 5.0 ± 1.8 mm³ at 13 days after implantation (Figure 2A, B). At 54 days after implantation, the mean tumor size reached 6.7 ± 2.7 mm³. One animal was killed at this point and the tumor was examined histologically. The lesion appeared to consist of well differentiated, non-invasive polygonal epithelial cells with round to oval nuclei and an open chromatin pattern. The lesion was surrounded by a fibrovascular capsule infiltrated with mixed inflammatory cells. Some clusters of cells around the periphery were distended by small lipid vacuoles. Mitotic figures and vessels did not appear to be present within the lesion (Figure 2 C,D). The formation of small lesions in the mammary fat pad of immunodeficient rodents is consistent with the colony formation data in showing that AKT1E17K can weakly transform MCF10A cells.

**AKT inhibitors inhibit colony formation by MCF10A-AKT1E17K cells**

Having established that the AKT1E17K mutation could drive anchorage-independent growth in MCF10A cells, we assessed whether this transformation could be blocked by inhibiting AKT. Firstly we confirmed that two independent inhibitors of AKT, AZD5363 and MK-2206, were able to inhibit AKT and subsequent downstream signalling. Increasing concentrations of AZD5363 resulted in AKT hyperphosphorylation, as expected from a catalytic AKT inhibitor, whereas increasing concentrations of the allosteric inhibitor, MK2206, potently decreased AKT phosphorylation (Figure 3A). Further analysis of a panel of downstream signalling molecules in MCF10A-AKT1E17K cells demonstrated that PRAS40, GSK3β and S6
phosphorylation were all inhibited by AZD5363 and MK2206 at concentrations of 0.3μM and above, with pS6 and pGSK3β being inhibited by greater than 80% at 3μM. These AKT inhibitors also inhibited signalling in MCF10A cells with ectopic expression of AKT1WT (Figure 3B). Having demonstrated that AKT pathway signalling was inhibited by AZD5363 and MK2206 in MCF10A cells expressing AKT1E17K, we went on to determine whether colony formation could also be inhibited in the presence of the two inhibitors. Concentrations of 0.1 μM of either drug were sufficient to reduce colony formation by >80% compared to DMSO controls (Figures 3C and D).

**Allosteric and catalytic inhibitors of AKT inhibit the growth of breast cancer explant models with AKT1E17K mutation**

We have identified two triple negative (ER, PR and HER2 negative) breast cancer explant models with G to A mutation at nucleotide 49, indicative of production of AKT1E17K protein (Supplementary Figure 4). The HBC-x2 explant model was established from an axillary lymph node metastasis of a patient with infiltrating ductal carcinoma of the breast, is unresponsive to docetaxel, and has high levels of PTEN and phosphorylated AKT expression (22). The HBC-x31 model was established from a primary basal ductal breast carcinoma. In HBC-x2 explants, MK-2206 dosed at 120 mg/kg every other day, gave a 58% inhibition of tumour volume (T/C = 42%; p < 0.01), and AZD5363, when dosed at 150 mg/kg bid, gave 76% inhibition (T/C = 24%; p < 0.001) (Figure 4A). In HBC-x31 explants, the same dosing schedules of MK-2206 and AZD5363 gave 56% inhibition (T/C = 44%; p < 0.05) and 89% inhibition (T/C = 11%; p < 0.01), respectively (Figure 4B). Pharmacodynamics were studied in the residual tumour tissue at the end of the HBC-x2 experiment, after 32 days of treatment. MK-2206 inhibited AKT phosphorylation by more than 98% at 2 hours but this recovered to a level that did not significantly differ from controls at 24 hours after the final dose, whereas AZD5363 treatment resulted in a 2-3 fold hyperphosphorylation of AKT at both 2 and 8 hours after the final dose. AZD5363 inhibited
phosphorylation of the AKT substrate PRAS40 by ~70% at 2 hours, and this recovered to 30% at 8 hours, whereas MK-2206 inhibited PRAS40 phosphorylation by ~50% at 2 hours, but this recovered to control levels by 24 hours. AZD5363 and MK-2206 both caused a more modest but time-dependent reduction in phosphorylation of S6 (Figure 4C). Both AZD5363 and MK-2206 reduced nuclear Ki67 expression and increased localisation of FOXO3a to the nucleus of the tumor cells at 2 hours after the final dose, whereas at 8 hours after the final dose of AZD5363 and 24 hours after the final dose of MK-2206, FOXO3a had re-localised to the cytoplasm (Figure 4D,E). Increased localization of FOXO3a to the nucleus is consistent with inhibition of FOXO3a phosphorylation by AKT; which enables FOXO3a to switch on the expression of genes such as p27, FasL and BIM, which collectively induce cell cycle arrest and/or apoptosis (1-2,19).

Combination of AZD5363 and AZD4547 induces tumor regression in MGH-U3 xenografts

Given the strong monotherapy activity of AKT inhibitors in two breast cancer explant models with AKT1E17K mutation, we subsequently investigated whether these compounds were also potent inhibitors of bladder cancer models with AKT1E17K mutation. Surprisingly, both AZD5363 and MK-2206 only modestly inhibited in vitro growth of MGH-U3 cells with GI50s of 2.4 and 1.6 μM respectively, and concentrations of ~ 1 μM of both compounds were also required to inhibit AKT substrate phosphorylation by >50% (Supplementary Figure 5). However, this cell line also contains an activating mutation in FGFR3. Another bladder cancer cell line with AKT1E17K and NRAS mutations, KU-19-19, was even more resistant to AZD5363 and MK-2206, with a GI50 of >10 μM for both compounds (data not shown). We hypothesised that combination with an FGFR inhibitor would unmask resistance to AZD5363 in MGH-U3 xenografts. Treatment of these xenografts with AZD5363 at 150 mg/kg bid caused a small but non-significant inhibition of tumour growth (T/C = 80%; NS), whereas 12.5 mg/kg bid AZD4547 resulted in significant tumor growth inhibition (T/C = 53%;
p = 0.004). The combination of AZD5363 and AZD4547 resulted in tumor regression (T/C = 10%, p < 0.0001 compared to vehicle controls); this was significantly superior to monotherapy AZD4547 (p < 0.0001) (Figure 5).

**Patients with AKT1\textsuperscript{E17K} mutations show RECIST responses to AZD5363**

Forty one patients were enrolled into a phase 1 study. Confirmed partial responses (PRs) in RECIST criteria were experienced by two patients, both receiving intermittent AZD5363 dosing (480 mg 4 days on/3 days off and 640 mg 2 days on/5 days off). Tumor samples from these two responders were subjected to mutation profiling by Next Generation Sequencing and each was found to have AKT1\textsuperscript{E17K} mutation in archival primary tumour (Table 1). No other mutations were found in 100 other druggable or actionable known cancer or protein kinase genes that constitute the NCC cancer gene panel. One of these patients, who had primary ovarian cancer of endometrioid histology with metastatic disease in lung, maintained the PR for more than 2 years (Figure 6A) and remains in response at the time of writing. Another patient had primary estrogen receptor positive, HER2 negative, papillary carcinoma breast cancer with metastatic disease in mediastinal lymph node and liver (Figure 6B) and also remains in response at time of writing.

**Discussion**

The mutation AKT1\textsuperscript{E17K} has been shown to transform rat fibroblasts and induce leukaemia in mice (15). However, the oncogenic potential of this mutated protein in epithelial cell lines is less clear. Moreover, the effects of pharmacological inhibition of the protein in models of breast and urothelial carcinoma with endogenous AKT1\textsuperscript{E17K} mutation have not been reported. Lauring et al. reported that AKT1\textsuperscript{E17K} had minimal phenotypic consequences and failed to induce ongogenic transformation of MCF10 breast epithelial cells, whereas PIK3CA\textsuperscript{E545K} and PIK3CA\textsuperscript{H1047R} mutations were transforming in the same cells, leading them to suggest that these mutations may not be functionally equivalent, and that co-operating
genetic changes are required for AKT1E17K to transform these cells (17). In breast cancer, PIK3CA and AKT1 mutations tend to be mutually exclusive, which suggests that either these mutations do have some redundancy of function, or that PIK3CA is not co-operative with AKT1 for oncogenic transformation of breast epithelial cells, or indeed progression of breast tumours to malignancy. In our experimental system using the same recipient cell line, AKT1E17K was sufficient to weakly transform MCF10A cells, as shown by an increase in colony number and size in soft agar, and the formation of mammary lesions in immunodeficient mice, although the colony and tumor sizes were notably smaller than that achieved by an oncogenic form of the HER2 oncogene. These colonies and mammary lesions are almost certainly due to AKT1E17K expression because (i) expression of wild type AKT1 from the same promoter failed to induce colony or tumor formation, (ii) colonies and tumors only formed when the medium or chow was supplemented with doxycycline and (iii) colony formation was reversed by the presence of two different types of small molecule AKT inhibitor. The reason for the discrepancy between our data and that of Lauring et al. is unclear, but it may be due to the different expression vector systems employed, or some variability in the phenotype of the recipient MCF10A cells, resulting in a lower threshold of transformation in our experiments. However, the MCF10A recipient cells in the present experiments remain non-tumorigenic in immunodeficient mice, and were not transformed by AKT1WT. Whatever the explanation, AKT1E17K clearly has the potential to confer two properties of oncogenic transformation on recipient breast epithelial cells: the ability to grow anchorage independently in semi-solid medium and the ability to form mammary lesions in immunodeficient rodents. Moreover, recent data has shown that knock in of AKT1E17K into isogenic MCF-7 cells that have previously been depleted of their endogenous PIK3CAEs45K mutation restored proliferation and tumour growth (16).

Both catalytic and allosteric inhibitors of AKT can reverse pathway activation by AKT1E17K and colony formation in soft agar. The concentration of drug required to inhibit colony formation appears to be lower than that required to inhibit AKT substrate phosphorylation by
the same extent, suggesting that it is only necessary to very modestly inhibit the target to prevent growth in semi-solid medium. More importantly, we show for the first time that both catalytic and allosteric inhibitors of AKT, dosed as monotherapy, are sufficient to cause highly significant growth delay in two breast cancer primary explant models with endogenous AKT1<sup>E17K</sup> mutations, and monotherapy AZD5363 was sufficient to induce partial responses in two patients with tumors containing AKT1<sup>E17K</sup> mutation. This data provides strong evidence that tumors with AKT1<sup>E17K</sup> mutations are rational targets for monotherapy with AKT inhibitors, at least in tumor types where other activating oncogenic mutations do not tend to co-occur. Breast cancers with AKT1 mutations may be particularly sensitive, as AKT1<sup>E17K</sup> mutations tend to be mutually exclusive with mutations in components of the MAPK pathway, receptor tyrosine kinases, and other proteins in the PI3K signalling network (3, 23). However, where other oncogenic mutations are present that activate the MAPK pathway, combinations may be required. The modest monotherapy activity of AZD5363 in the MGH-U3 bladder cancer model, which contains an activating mutation in FGFR3, supports this concept, where combination with an FGFR inhibitor is necessary to unmask resistance and achieve tumour regression. Collectively, this data suggests that tumours with AKT1<sup>E17K</sup> mutations are rational targets for therapy with AKT inhibitors, and that monotherapy may be sufficient in some, but not all, tumors with this genotype.

An illuminating aspect of our preclinical studies is the finding that the allosteric inhibitor MK-2206 can also effectively inhibit AKT1<sup>E17K</sup> mediated growth. MK-2206 was able to reverse colony formation and pathway activation in vitro, and cause growth delay in vivo, although it was less effective than AZD5363 at the maximum tolerated dosing schedules tested. MK-2206 binds to the region that interacts with both the PH and kinase domains, and prevents translocation of AKT to the membrane and activation. AKT1<sup>E17K</sup> mutation alters the electrostatic interactions of the lipid binding pocket and dramatically increases the affinity of the protein for the constitutive membrane lipid PI(4,5)P2 (24), which probably
explains increased membrane localization compared to wild type AKT1. The allosteric inhibitor VIII has previously been reported to be less effective than several different catalytic inhibitors at inhibiting purified AKT1^{E17K} protein and proliferation of AKT1^{E17K} transfected fibroblasts (15), but presumably because membrane translocation is still a necessary step and/or because it is structurally distinct from inhibitor VIII, MK-2206 is still able to inhibit colony formation \textit{in vitro} and inhibit tumour growth \textit{in vivo}.

In conclusion, our data demonstrate that tumours with AKT1^{E17K} mutation are rational targets for therapy with allosteric or catalytic AKT inhibitors. The proportion of tumors of various different types that harbor this mutation that can be successfully treated with monotherapy AKT inhibitors, relative to those that may require rational combination therapy, remains to be determined in the clinic. With the era of whole tumor genotyping now upon us, AKT inhibitors seem very likely to take their place in the arsenal of targeted cancer therapeutics.

**Acknowledgements**

AZD5363 was discovered by AstraZeneca subsequent to a collaboration with Astex Therapeutics (and their collaboration with the Institute of Cancer Research and Cancer Research Technology Limited). We thank Xentech (Evry, France) for carrying out the live phase of the HBC-x2 and HBC-x31 explant model studies.

**References**


20. Meyskens FL, Thomson SP and Moon TE. Quantitation of the Number of Cells within Tumor Colonies in Semisolid Medium and Their Growth as Oblate Spheroids. Cancer Res 1984; 44: 271-277


Table 1: Details of patients with partial responses

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<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Cancer Type</th>
<th>Histological subtype</th>
<th>Disease distribution</th>
<th>No of previous treatments</th>
<th>Mutational status</th>
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<tr>
<td>1</td>
<td>38</td>
<td>F</td>
<td>Ovarian</td>
<td>Endometrioid carcinoma</td>
<td>Multiple lung metastases</td>
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<td>AKT1^{E17K}</td>
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<tr>
<td>2</td>
<td>57</td>
<td>F</td>
<td>Breast</td>
<td>Invasive ductal carcinoma; ER (+), PgR (+), HER2 (-)</td>
<td>Right hilar LN, liver (S2) metastases</td>
<td>2 (chemotherapy) 6 (hormonal therapy)</td>
<td>AKT1^{E17K}</td>
</tr>
</tbody>
</table>
Figure 1: Doxycycline-inducible expression of AKT1\textsuperscript{E17K} in MCF10A cells activates the AKT signalling pathway and induces colony formation in soft agar. MCF10A cells were infected with lentivirus expressing AKT1\textsuperscript{E17K}, AKT1\textsuperscript{WT} or RFP under a tet-inducible promoter followed by selection in 0.6 \( \mu \text{g/mL} \) puromycin and cultured in the presence or absence of 1 \( \mu \text{g/mL} \) doxycycline. A: proteins were detected by western blotting; B: colonies growing in 0.5% agarose were counted after 14 days. Mean colony counts from three experimental replicates are shown. *A statistically significant increase in colony number was observed in the AKT1\textsuperscript{E17K} + doxycycline group compared to the RFP + doxycycline group, \( p < 0.0001 \). C: Representative images from 3 experimental replicates are shown.

Figure 2. AKT1\textsuperscript{E17K} overexpression promotes tumor formation in vivo. The indicated MCF10A cells were implanted in the mammary fat of mice +/- Doxycycline chow. A: Thirteen days after implanting cells, only mice supplemented with doxycycline chow with MCF10A-HER2\textsuperscript{V659E} or MCF10A-AKT1\textsuperscript{E17K} implanted grew measurable tumors. B: Growth kinetics of the MCF10A-HER2\textsuperscript{V659E} tumors. C and D: Representative low and high magnification images of an MCF10A-AKT1\textsuperscript{E17K} tumor 35 days after implantation. Magnification is 3X and 20X, respectively.

Figure 3: AKT inhibitors abrogate activation of AKT signalling and colony formation in MCF10A cells expressing AKT1\textsuperscript{E17K}. MCF10A cells were infected with lentivirus expressing AKT1\textsuperscript{E17K} under a tet-inducible promoter followed by selection in 0.6 \( \mu \text{g/mL} \) puromycin and cultured in growth media in the presence or absence of 1 \( \mu \text{g/mL} \) doxycycline, AZD5363 and MK-2206. A and B: proteins were detected by western blotting; C and D:
colonies growing in 0.5% agarose were counted after 14 days. Average colony counts from three experimental replicates are shown.

Figure 4: AKT inhibitors inhibit tumor growth and demonstrate pharmacodynamic activity in breast cancer explant models with AKT1<sup>E17K</sup> mutations. A and B: Triple negative, AKT1<sup>E17K</sup> mutant breast cancer explant models HBc-x2 (panel A) and HBc-x31 (panel B) (Xentech) were chronically dosed with vehicle, AZD5363 or MK-2206 and tumor growth was monitored using callipers. C: pathway biomarkers were measured in lysates from HBC-x2 tumors at the end of the anti-tumor study, by western blotting or ELISA. D and E: Nuclear FOXO3a and Ki67 in sections from HBC-x2 tumors at the end of the anti-tumor study were measured by immunohistochemistry.

Figure 5: Combination of AKT and FGFR inhibitors induces tumor regression in MGH-U3 (AKT1<sup>E17K</sup>, FGFR3<sup>S249C</sup>) bladder cancer xenografts. Nude mice were implanted subcutaneously with MGH-U3 bladder cancer cells to establish xenografts, which were dosed by oral gavage with compounds as indicated. Tumor measurements were monitored using callipers.

Figure 6: Tumor response by AZ5363 in two patients with somatic AKT1<sup>E17K</sup> mutation. A: Multiple metastases in a patient with endometrioid ovarian cancer have revealed definite tumor shrinkage at day 433 in comparison with start of the treatment (baseline) from 42.3 mm to 8.1 mm (S1) and from 43.4 mm to 22.3 mm (S2). B: Metastases of mediastinal lymph node and liver in patient with ER+, HER2- breast cancer decreased in size at day 126 in comparison with baseline, from 19.5 mm to 4.8 mm (S3) and from 17.2 mm to 14.8 mm (S4). Each arrow shows the location of the metastasis.
**Figure 1**

A) Western blot analysis of E17K and WT AKT1 under Basal medium and Growth medium conditions with and without Dox.

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<tr>
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<tr>
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<tr>
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B) Bar graph showing the number of colonies for AKT1E17K, AKT1WT, and RFP under -Dox and +Dox conditions.

C) Photographs of colony formation with and without Dox for AKT1E17K, AKT1WT, and RFP.
Figure 3

A

<table>
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B

MCF10A-AKT<sup>E17K</sup>

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MCF10A-AKT<sup>WT</sup>

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C

D

Relative colony formation

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**Figure 4**

(A) Tumour volume (mm$^3$) over Days of treatment for different treatments: Vehicle, AZD5363 150 mg/kg bid, and MK-2206 120 mg/kg 3 times weekly.

(B) Tumour volume (mm$^3$) over Days of treatment for different treatments: Vehicle, AZD5363 150 mg/kg bid, and MK-2206 120 mg/kg 3 times weekly.

(C) PD % of control +/- SEM for pPRAS40, pAKT, and ps6.

(D) Nuclear H Score +/- SEM for Ki67 and FOXO.

(E) Representative images of FOXO and Ki67 staining for Vehicle and AZD5363 treatments.
Figure 5

Tumour volume (cm$^3$) vs. Days of dosing

- Vehicle control
- AZD4547 12.5 mg/kg qd
- AZD5363 150 mg/kg bid
- AZD4547 12.5 mg/kg qd + AZD5363 150 mg/kg bid
Endometrioid ovarian cancer

Baseline Day 433

A

B

Breast cancer

Baseline Day 126
Molecular Cancer Therapeutics

Tumors with AKT1E17K mutations are rational targets for single agent or combination therapy with AKT inhibitors

Barry Davies, Nin Guan, Armelle Logie, et al.

*Mol Cancer Ther* Published OnlineFirst September 8, 2015.

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