Tumors with AKT1E17K Mutations Are Rational Targets for Single Agent or Combination Therapy with AKT Inhibitors

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Abstract

AKT1E17K 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 AKT1E17K mutation is not known. Expression of exogenous copies of AKT1E17K 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 AKT1E17K mutation. Furthermore, in a phase I clinical study, the catalytic Akt inhibitor AZD5363 induced partial responses in patients with breast and ovarian cancer with tumors containing AKT1E17K mutations. In MGH-U3 bladder cancer xenografts, which contain both AKT1E17K and FGFR3Y373C mutations, AZD5363 monotherapy did not significantly reduce tumor growth, but tumor regression was observed in combination with the FGFR inhibitor AZD4547. The data show that tumors with AKT1E17K 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, whereas mutation of PTEN is most common in endometrial cancer, prostate cancer, and glioblastomas (3). AKT proteins are less frequently mutated; the most frequently mutated AKT isoform being AKT1, which is mutated in 2% to 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, which 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 leukemia in mice (15). Although knock-in of AKT1E17K into isogenic MCF-7 breast cancer cells depleted of endogenous PIK3CA E545K restored proliferation in vitro and tumor growth in vivo (16), knock-in of E17K into the AKT1 gene of nontransformed 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 plekstrin homology 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 antitumor activity in xenograft models with PI3CA and PTEN mutations (19). We now report that both allosteric and catalytic inhibitors of AKT can inhibit downstream signaling, reverse the human EGF receptor (HER2) signal transduction of mammary epithelial cells induced by expression of AKT1E17K, and inhibit the growth of breast cancer explant models with homozygous copies of AKT1E17K. Most importantly, AZD5363 monotherapy induced partial responses in breast and ovarian cancer patients with tumors containing AKT1E17K 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 AKT1E17K 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 (November 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. M.A. Knowles (University of Leeds, 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-AKT1E17K and MCF10A-AKT1WT cell lines were generated by infecting MCF10A cells with lentivirus expressing AKT1E17K, or wild-type AKT1 under a tet-inducible promoter (pTRIPZ; Thermo Scientific), followed by selection in 0.5 μg/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-AKT1WT was subcloned into pTRIPZ as an AgeI/XbaI fragment. The AKT1E17K fragment was cloned as above after AKT1 was mutated via site directed mutagenesis (QuikChangeII XL; Stratagene). MCF10A-RFP and MCF10A-HER2V659E cell lines were similarly generated and selected in puromycin following infection with pTRIPZ-derived lentivirus expressing RFP or HER2V659E under tet-inducible promoters. Cell lines were maintained at 37°C/5% CO2 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"). MCF10A cells were acquired directly from ATCC (November 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. M.A. Knowles (University of Leeds, 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-AKT1E17K and MCF10A-AKT1WT cell lines were generated by infecting MCF10A cells with lentivirus expressing AKT1E17K, or wild-type AKT1 under a tet-inducible promoter (pTRIPZ; Thermo Scientific), followed by selection in 0.5 μg/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-AKT1WT was subcloned into pTRIPZ as an AgeI/XbaI fragment. The AKT1E17K fragment was cloned as above after AKT1 was mutated via site directed mutagenesis (QuikChangeII XL; Stratagene). MCF10A-RFP and MCF10A-HER2V659E cell lines were similarly generated and selected in puromycin following infection with pTRIPZ-derived lentivirus expressing RFP or HER2V659E under tet-inducible promoters. Cell lines were maintained at 37°C/5% CO2 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").

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 1 μg/mL doxycycline and incubated at 37°C/5% CO2 for 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. After which colonies were counted using a Gel-Count 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):

\[ \text{No. of cells/colony} = 2.40 \times (\text{colony diameter})^{0.577} (\text{cell diameter})^{0.804} \]

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

Soft agar colony formation assays

To evaluate growth in soft agar, 1 ml 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°C was added to each well of a 12-well plate and allowed to solidify (base agar). One milliliter of 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 1 mL RPMI medium containing 1 μg/mL doxycycline) and incubated for 14 days at 37°C. Medium was changed twice a week. Colonies were then stained with P-iodininotetrazolium violet dye (Sigma), which was added for 16 hours at 37°C, after which colonies were counted using a Gel-Count instrument (Oxford Optronix). Counts from three replicate wells were averaged and the standard deviation calculated.
SDS-polyacrylamide premade gels (Bio-Rad) and transferred to nitrocellulose membranes. Membranes were then incubated with primary antibodies (see "reagents" above) and subsequently with HRP-conjugated anti-mouse or anti-rabbit IgG diluted in 5% milk in phosphate-buffered saline (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 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;Alp/C) were bred at AstraZeneca 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 PBS. Only single-cell suspensions of greater than 90% viability, as determined by trypan blue exclusion, were used for injection. A total of 2 × 106 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 passed down from donor tumors when they reached volumes of 1 to 2 cm3. Tumors were cut into fragments of approximately 20 mm3 and transplanted immediately. Recipient animals were anesthetized with ketamine/xylazine, the skin aseptically with chlorohexidine solution, and then incised in the intercapsular region prior to a tumor fragment being placed in the subcutaneous tissue. The skin was closed with clips. When mean tumor sizes reached 0.1 to 0.2 cm3, 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: \( V = \pi/6 \times length \times width^2 \); ref. 21). Growth inhibition from the start of treatment was assessed by comparison of the differences in tumor volume between control and treated groups. As 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-AKT1E17K or MCF10A-AKT1WT cells were grown using standard techniques with 0.5 μg/mL puromycin. In groups treated with doxycycline (Dox), cells were pretreated with 1 μg/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 (un supplemented)/50% Matrigel (Coming) at 5 × 106/50 μL. A total of 5 × 105 cells were injected into the third mammary fat pad of NSG (NOD.Cg-Pkdcsid1I22gptm1Wjl/SzJ) mice (Jackson Labs) using an insulin syringe. Dox groups were pretreated with Dox chow (625 mg/kg; Harlan-Teklad, cat. no. 2018) for 48 hours 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 histologic examination, tumors were collected, fixed in 10% neutral buffered formalin and processed for hematoxylin/eosin staining according to standard techniques.

Phase I study of AZD5363 in Japanese patients

A phase I, open-label, multicentre, dose-escalation study was carried out in Japanese patients with advanced solid tumors (NCT01353781). Full results from this study will be published separately. Briefly, three schedules were assessed in 21-day cycles: continuous (twice a day, every day), intermittent-1 (twice a day, 4 days on/3 days off), and intermittent-2 (twice a day, 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 antitumor 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, and 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 tumor tissue was carried out using the National Cancer Centre (NCC) Oncopanel based on an Agilent SureSelect system and Illumina MiSeq sequencer. The target regions to be captured were the exons of 90 druggable or actionable genes and 10 protein kinase fusion genes. AKT1E17K mutations were validated by next-generation sequencing and with the commercial Foundation Medicine platform.
Results

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

To determine whether the AKT1E17K mutation was oncogenic, we established MCF10A cell lines that express either AKT1 wild type (AKT1WT) or AKT1E17K 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 (Fig. 1A). The effect of AKT1E17K expression on downstream signaling was examined, both in basal media and in full growth media, containing the added growth factors, cytokines, and supplements. Cells expressing AKT1E17K 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 (Fig. 1A). This is in contrast to cells expressing AKT1WT where maximum pathway activation only occurs in full growth media. Phospho S6 levels were similar in cells expressing AKT1WT or AKT1E17K but were markedly increased when cells were stimulated with full growth medium.

Ectopic expression of AKT1E17K in MCF10A cells did not affect the expression of P13Kα or mTOR (Supplementary Fig. S1), suggesting that increased phosphorylation of AKT and its substrates is because of expression of AKT1E17K rather than increased expression of these upstream activating proteins. To further investigate whether the AKT1E17K mutation was oncogenic, we assessed anchorage independent growth in soft agar. Colony formation was increased following doxycycline addition in cells expressing AKT1E17K compared with AKT1WT or RFP control protein (Fig. 1B and C), although it was notable that the mean colony size was smaller than that observed for cells expressing HER2V659E, a constitutively active form of HER2, a known oncogene (Supplementary Fig. S2). The mean colony diameter produced by MCF10A-AKT1E17K cells in the presence of doxycycline was 226 mmol/L, 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 Fig. S3).

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, whereas MCF10A-HER2V659E cells produced rapidly growing tumors in all the mice that reached a mean size of 672 $\pm$ 115 mm$^3$ at 13 days after implantation. MCF10A-AKT1E17K cells produced small tumors in 6 of 10 mice, which reached 5.0 $\pm$ 1.8 mm$^3$ at 13 days after implantation (Fig. 2A and B). At 54 days after implantation, the mean tumor size reached 6.7 $\pm$ 2.7 mm$^3$. One animal was killed at this point and the tumor was examined histologically. The lesion appeared to consist of well-differentiated, noninvasive 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 (Fig. 2C and 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. First, we confirmed that two independent inhibitors of AKT, AZD5363 and MK-2206, were able to inhibit AKT and subsequent downstream signaling. 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 (Fig. 3A). Further analysis of a panel of downstream signaling molecules in MCF10A-AKT1E17K cells demonstrated that PRAS40, GSK3b, and S6 phosphorylation were all inhibited by AZD5363 and MK2206 at concentrations of 0.3 mmol/L and above, with pS6 and pGSK3b being inhibited by greater than 80% at 3 mmol/L. These AKT inhibitors also inhibited signaling in MCF10A cells with ectopic expression of AKT1WT (Fig. 3B). Having demonstrated that AKT pathway signaling was inhibited by AZD5363 and MK2206 at concentrations of 0.3 μmol/L and above, with pS6 and pGSK3b being inhibited by greater than 80% at 3 μmol/L, we went on to determine whether colony formation could also be inhibited in the presence of the two inhibitors. Concentrations of 0.1 μmol/L of either drug were sufficient to reduce colony formation by >80% compared with DMSO controls (Fig. 3C and D).
Figure 3. AKT inhibitors abrogate activation of AKT signaling and colony formation in MCF10A cells expressing AKT1E17K. MCF10A cells were infected with lentivirus expressing AKT1E17K under a tet-inducible promoter followed by selection in 0.6 μg/mL puromycin and cultured in growth media in the presence or absence of 1 μg/mL doxycyclin, 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 AKT1E17K mutations. A and B, triple negative, AKT1E17K-mutant breast cancer explant models HBC-x2 (A) and HBC-x31 (B) (Xentech) were chronically dosed with vehicle, AZD5363, or MK-2206 and tumor growth was monitored using calipers; b.i.d., twice daily. C, pathway biomarkers were measured in lysates from HBC-x2 tumors at the end of the antitumor study, by Western blotting or ELISA. D and E, nuclear FOXO3a and Ki67 in sections from HBC-x2 tumors at the end of the antitumor study were measured by immunohistochemistry.
Forty-one patients were enrolled into a phase I study. Conformal partial responses (PR) 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 AKT1E17K mutation in archival primary tumor (Table 1). No other mutations were found in 100 other druggable genes. Another patient, who had primary ovarian cancer of endometrioid histology with metastatic disease in lung, maintained the PR for more than 2 years (Fig. 6A) and remains in response at the time of writing. Another patient

49, indicative of production of AKT1E17K protein (Supplementary Fig. S4). 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 tumor volume (T/C = 42%; P < 0.01), and AZD5363, when dosed at 150 mg/kg twice a day, gave 76% inhibition (T/C = 24%; P < 0.001; Fig. 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 (Fig. 4B).

Pharmacodynamics were studied in the residual tumor tissue at the end of the HBC-x2 experiment, after 32 days of treatment. MK-2206, with a GI50 of 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- to 3-fold hyperphosphorylation of AKT at both 2 and 8 hours after the final dose. AZD5363 inhibited phosphorylation of the AKT substrate PRAS40 by approximately 70% at 2 hours, and this recovered to 30% at 8 hours, whereas MK-2206 inhibited PRAS40 phosphorylation by approximately 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 (Fig. 4C). Both AZD5363 and MK-2206 reduced nuclear Ki67 expression and increased localization 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 relocalized to the cytoplasm (Fig. 4D and 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 GI50 of 2.4 and 1.6 μmol/L, respectively, and concentrations of approximately 1 μmol/L of both compounds were also required to inhibit AKT substrate phosphorylation by >50% (Supplementary Fig. S5). However, this cell line also contains an activating mutation in FGFR3. Another bladder cancer cell line with AKT1E17K and NRAS mutation, K1-19-19, was even more resistant to AZD5363 and MK-2206, with a GI50 of >10 μmol/L for both compounds (data not shown).

We hypothesized that combination with an FGFR inhibitor would mask resistance to AZD5363 in MGH-U3 xenografts. Treatment of these xenografts with AZD5363 at 150 mg/kg twice a day caused a small but nonsignificant inhibition of tumor growth (T/C = 80%; NS), whereas 12.5 mg/kg twice a day 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 with vehicle controls); this was significantly superior to AZD4547 monotherapy (P < 0.0001; Fig. 5).

**Patients with AKT1E17K mutations show RECIST responses to AZD5363**

Forty-one patients were enrolled into a phase 1 study. Confirmed partial responses (PR) 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 AKT1E17K mutation in archival primary tumor (Table 1). No other mutations were found in 100 other druggable genes. Another patient, who had primary ovarian cancer of endometrioid histology with metastatic disease in lung, maintained the PR for more than 2 years (Fig. 6A) and remains in response at the time of writing. Another patient...
Response of AKTIE17K-Mutant Tumors to AKT Inhibitors

Figure 6.
Tumor response by AZ5363 in two patents with somatic AKTIE17K 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 to 8.1 mm (S1) and from 43.4 to 22.3 mm (S2). B, metastases of mediastinal lymph node and liver in patient with ERþ, HER2-negative, papillary carcinoma breast cancer decreased in size at day 126 in comparison with baseline, from 19.5 to 4.8 mm (S3) and from 17.2 to 14.8 mm (S4). Each arrow shows the location of the metastasis.

had primary estrogen receptor–positive, HER2-negative, papillary carcinoma breast cancer with metastatic disease in mediastinal lymph node and liver (Fig. 6B) and also remains in response at time of writing.

Discussion

The mutation AKTIE17K has been shown to transform rat fibroblasts and induce leukemia in mice (15). However, the oncogenic potential of this mutated protein in epithelial cell lines is less clear. Moreover, the effects of pharmacologic inhibition of the protein in models of breast and urothelial carcinoma with endogenous AKTIE17K mutation have not been reported. Lauring and colleagues reported that AKTIE17K had minimal phenotypic consequences and failed to induce oncogenic transformation of MCF10 breast epithelial cells, whereas PIK3CAE545K and PIK3CAH1047R mutations were transforming in the same cells, leading them to suggest that these mutations may not be functionally equivalent, and that cooperating genetic changes are required for AKTIE17K 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 cooperative with AKT1 for oncogenic transformation of breast epithelial cells, or indeed progression of breast tumors to malignancy. In our experimental system using the same recipient cell line, AKTIE17K 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 because of AKTIE17K 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 and colleagues is unclear, but it may be because of 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 nontumorigenic in immunodeficient mice, and were not transformed by AKT1WT. Whatever the explanation, AKTIE17K clearly has the potential to confer two properties of oncogenic transformation on recipient breast epithelial cells: the ability to grow anchorage independently in semisolid medium and the ability to form mammary lesions in immunodeficient rodents. Moreover, recent data have shown that knock-in of AKTIE17K into isogenic MCF-7 cells that have previously been depleted of their endogenous PIK3CAE545K mutation restored proliferation and tumor growth (16).

Both catalytic and allosteric inhibitors of AKT can reverse pathway activation by AKTIE17K 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 semisolid 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 AKTIE17K mutations, and AZD5363 monotherapy was sufficient to induce partial responses in two patients with tumors containing AKTIE17K mutation. These data provide strong evidence that tumors with AKTIE17K 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 AKTIE17K mutations tend to be mutually exclusive.
with mutations in components of the MAPK pathway, receptor tyrosine kinases, and other proteins in the PI3K signaling 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 a FGFR inhibitor is necessary to unmask resistance and achieve tumor regression. Collectively, these data suggest that tumors with AKT1E17K 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 AKT1E17K-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 plekstrin homology and kinase domains, and prevents translocation of AKT to the membrane and activation. AKT1E17K 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 with wild-type AKT1. The allosteric inhibitor VIII has previously been reported to be less effective than several different catalytic inhibitors at inhibiting purified AKT1E17K protein and proliferation of AKT1E17K 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 in vitro and inhibit tumor growth in vivo.

In conclusion, our data demonstrate that tumors with AKT1E17K 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.

Disclosure of Potential Conflicts of Interest
B. Davies, M. Zinda, and E.L. Jenkins have ownership interest (including patents) in AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
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). The authors thank Xentech for carrying out the live phase of the HBC-x2 and HBC-x31 explant model studies.

Received March 19, 2015; revised August 14, 2015; accepted August 20, 2015; published OnlineFirst September 8, 2015.

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Molecular Cancer Therapeutics

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Mol Cancer Ther 2015;14:2441-2451. Published OnlineFirst September 8, 2015.

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