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Mutant *PIK3CA* controls DUSP1-dependent ERK 1/2 activity to confer response to AKT target therapy

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Background: Alterations in the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signalling pathway are frequent in urothelial bladder cancer (BLCA) and thus provide a potential target for novel therapeutic strategies. We investigated the efficacy of the AKT inhibitor MK-2206 in BLCA and the molecular determinants that predict therapy response.

Methods: Biochemical and functional effects of the AKT inhibitor MK-2206 were analysed on a panel of 11 BLCA cell lines possessing different genetic alterations. Cell viability (CellTiter-Blue, cell counts), apoptosis (caspase 3/7 activity) and cell cycle progression (EdU incorporation) were analysed to determine effects on cell growth and proliferation. cDNA or siRNA transfections were used to manipulate the expression of specific proteins such as wild-type or mutant PIK3CA, DUSP1 or CREB. For *in vivo* analysis, the chicken chorioallantoic membrane model was utilised and tumours were characterised by weight and biochemically for the expression of Ki-67 and AKT phosphorylation.

Results: Treatment with MK-2206 suppressed AKT and S6K1 but not 4E-BP1 phosphorylation in all cell lines. Functionally, only cell lines bearing mutations in the hotspot helical domain of *PIK3CA* were sensitive to the drug, independent of other genetic alterations in the PI3K or MAPK signalling pathway. Following MK-2206 treatment, the presence of mutant *PIK3CA* resulted in an increase in DUSP1 expression that induced a decrease in ERK 1/2 phosphorylation. Manipulating the expression of mutant or wild-type PIK3CA or DUSP1 confirmed that this mechanism is responsible for the induction of apoptosis and the inhibition of tumour proliferation *in vitro* and *in vivo*, to sensitise cells to AKT target therapy.

Conclusion or interpretation: *PIK3CA* mutations confer sensitivity to AKT target therapy in BLCA by regulating DUSP1 expression and subsequent ERK1/2 dephosphorylation and can potentially serve as a stratifying biomarker for treatment.

The phosphoinositide 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signalling pathway has a fundamental role in tumourigenesis and tumour progression. Molecular alterations resulting in the hyperactivation of this pathway have been observed frequently in human cancer making it particularly suited for targeted therapy (Yuan and Cantley, 2008). Chemical inhibitors targeting different molecules in this pathway have been developed, but their clinical implementation has had limited success (Rodon *et al*, 2013). This might be explained by the underlying tumour genetics as well as the complex network of feedback loops in this signalling pathway that rescues their inhibitory effects (Weigelt and Downward, 2012; Klempner *et al*, 2013).

Activated PI3K regulates PDK1- and mTORC2-mediated phosphorylation of AKT, which is an oncogenic serine/threonine kinase with the three isoforms AKT 1, 2 and 3, a mechanism that can be reversed by PTEN (Manning and Cantley, 2007). AKT can induce different cellular responses such as proliferation and growth, cell cycle progression, apoptosis, metabolism, metastasis, invasion and angiogenesis. One of its

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downstream targets is the mTORC1 protein complex that is involved in controlling protein translation by regulating the proteins S6K1 and 4E-BP1. Upon treatment of cells with mTOR inhibitors, AKT is reactivated via molecular feedback loops that might protect the cell from therapeutic effects (Sun *et al*, 2005; O'Reilly *et al*, 2006; Nawroth *et al*, 2011). AKT has emerged as a potential target molecule in cancer therapy. Several pharmacologically distinct inhibitors possessing varying affinities towards the different AKT isoforms have been developed (Mattmann *et al*, 2011) and are currently being tested in more than 150 registered clinical trials in different tumour entities (U.S. National Library of Medicine Clinical Trials, 2014a).

One such well-characterised inhibitor in early-phase clinical trials is MK-2206, an allosteric AKT inhibitor that targets all three isoforms (Yap *et al*, 2011). Preclinical data in various tumour entities suggest that it is effective only in a subset of tumours possessing specific molecular alterations in PI3K or MAPK signalling. However, these alterations that are associated with sensitivity are not uniform across various tumour types suggesting that distinct molecular patterns, specific for a particular tumour entity might predict response to therapy (Meng *et al*, 2010; Liu *et al*, 2011; Sangai *et al*, 2012). Thus, the complexity behind the signalling events that contribute to the selective efficacy of AKT inhibition in the presence of specific genetic alterations remains to be understood. MK-2206 is now being tested in phase II trials in which patients are pre-stratified for *PIK3CA*, *AKT* or *PTEN* mutations in breast and lung cancer or thymic malignancies, those with *KRAS* wild type (WT) and *PIK3CA* mutations in metastatic colorectal cancer, or *PIK3CA* mutations in advanced endometrial cancer (U.S. National Library of Medicine Clinical Trials, 2014b, c, d, e).

In this preclinical study, we have focused on the characterisation of AKT target therapy in urothelial bladder cancer (BLCA). BLCA is the ninth most common cancer worldwide with more than 330 000 new patients each year, of which around 30% are clinically advanced with either muscle invasive or metastatic disease (Witjes *et al*, 2013). The current recommended treatment for patients with non-organ-confined muscle-invasive disease is radical cystectomy and lymphadenectomy, combined with neoadjuvant chemotherapy, which provides a 5-year survival of 50–55%. However, around 50% of these patients relapse, and of these, about 70% present with distant metastases. Advanced BLCA has a dismal prognosis with first-line chemotherapy regimens of methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) or gemcitabine plus cisplatin (GC) providing a modest survival of around 14 months (von der Maase *et al*, 2000). The only approved second-line agent vinflunine provides just a 2-month survival advantage (Bellmunt *et al*, 2013). These treatment strategies and overall prognosis have remained largely unchanged over the last 25 years.

About 50–70% of BLCA tumours possess molecular alterations that affect PI3K/AKT/mTOR signalling, including activating *PIK3CA* mutations in 20–25%, inactivating *TSC1* mutations in 8–16% and inactivating *PTEN* mutations or loss of expression in 13–16% (Knowles *et al*, 2009; Ciriello *et al*, 2013; Cancer Genome Atlas Research Network, 2014). These tumours might stand to benefit from target therapy against the PI3K signalling pathway. Different clinical trials for patients with advanced BLCA were initiated with mTORC1 inhibitors (rapalogues) in recent years. However, the results from these trials have been disappointing with only up to 20–30% of patients showing a clinical benefit (Gerullis *et al*, 2011; Seront *et al*, 2012; Milowsky *et al*, 2013). A potential association between the presence of *TSC1* mutations and response to rapalogues in BLCA has been reported and demonstrates that a thorough understanding of the signalling events initiated by the PI3K/AKT/mTOR pathway is required to maximise the potential benefit of available inhibitors in patients (Iyer *et al*, 2012).

It has been previously demonstrated, that the use of rapalogues and also dual PI3K/mTOR inhibitors in BLCA triggers feedback loops that hyperphosphorylate AKT, suggesting that AKT might be a key molecule in regulating tumour growth in BLCA (Nawroth *et al*, 2011). Consequently, we set out to determine the *in vitro* and *in vivo* response of BLCA tumour models to AKT inhibition and the molecular determinants and mechanisms involved in mediating and predicting responsiveness to AKT target therapy.

MATERIALS AND METHODS

Cell lines and cell culture. Cell lines HT1197, HT1376, RT4, UMUC3, J82 and T24 were obtained from the American type culture collection (Manassas, VA, USA), RT112 and 647V from the Leibniz institute German collection of microorganisms and cell cultures (Braunschweig, Germany), 253J were a kind gift from Professor Dr G. Unteregger (University of Saarland, Homburg/Saar, Germany) and 639 V and VmCUB1 were kindly provided by Professor Dr WA Schulz (Heinrich-Heine-University, Düsseldorf, Germany). Cells were maintained as early passages of subconfluent cultures in RPMI or DMEM (Biochrom AG, Berlin, Germany) at 5% or 10% CO2, respectively, supplemented with 10% FBS (Biochrom AG) and 1% NEAA (Biochrom AG). A total of 1×10^6, 1×10^5 or 2×10^5, and 1000 or 8000 cells were seeded in 10 cm, 6-well and 96-well formats, respectively.

Small molecule inhibitor and treatment. MK-2206 (Active Biochem, Bonn, Germany) was stored as a 10 mM stock solution in DMSO and working concentrations were freshly prepared in medium. As a control, DMSO was used corresponding to the DMSO in the highest MK-2206 concentration.

siRNA oligonucleotides and transfection. Ten nanomolar of stealth RNAi oligonucleotides (Life Technologies, Darmstadt, Germany) against AKT1 (UGCAGCAUCGCUUCUUUGCCGGUAU, GACGUGGCUAUUGUGAAGGAGGGUU), AKT2 (GGCACGGGCTAAAGTGACCATGAAT, CCUUGGCAAGGGAACCUUUGGCAAA), AKT3 (GGCACACACUCUAACUGAAAGCAGA, ACCTCAAGATGTGGATTTACCTTAT), PIK3CA (AAGAGCCCCGAGCGUUUCUGCUUUU targeting the 5'UTR), DUSP1 (GCCAUUGACUUCAUAGACUCCAUCA), negative control Hi GC duplex #2, and Silencer siRNA (Life Technologies) for CREB (GCUGGCUAACAAUGGUACCtt) were used for transfecting cells with Lipofectamine RNAimax (Life Technologies) according to the manufacturer's instructions. All assays were conducted 48 or 72 h after transfection.

Plasmid construction and transfection. The HA PIK3CA E545K fragment from pBABE puro HA PIK3CA E545K (Addgene, Cambridge, MA, USA) (plasmid number 12525) (Zhao *et al*, 2005) was cloned into pcDNA3.1/V5-His-TOPO (Life Technologies) and verified by sequencing. QiaQuick Gel extraction kit and Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany) were used as per the manufacturer's protocol. pCMV2-Tag 2A PIK3CA-WT was purchased from Addgene (plasmid number 16643) (Samuels *et al*, 2004) and verified by sequencing. pcDNA3.1/V5-his-TOPO was used as a control. Cells were transfected using FuGENE HD (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. siRNA and DNA co-transfection was performed by using Lipofectamine 2000 (Life Technologies) as per the manufacturer's protocol. All assays were conducted 48 or 72 h after transfection.

Cell viability assay. CellTiter-Blue cell viability assay (Promega Corporation) was performed in triplicates according to the manufacturer's protocol. Fluorescence was measured using VictorX3 Multilabel plate reader (PerkinElmer, Waltham, MA, USA) and the absolute IC50 was determined using the 4-parameter logistic model (GraphPad Software Inc., La Jolla, CA, USA) (Sebaugh, 2011).

Cell counts. Cells were harvested and stained with 0.5% Trypan Blue (Biochrom AG). Non-stained living cells were counted using a Neubauer chamber in triplicates.

Apoptosis assay. Caspase-Glo 3/7 assay (Promega Corporation) was performed in triplicates according to the manufacturer's protocol. Luminescence was measured using VictorX3 Multilabel plate reader (PerkinElmer) and normalised to the number of living cells.

Cell cycle analysis. Click-it EdU Alexa Fluor 488 flow cytometry assay kit (Life Technologies) was used as per the manufacturer's protocol with 10 uM EdU incorporation for 2 h in triplicate samples. Total DNA was stained with 7-AAD (Life Technologies). Flow cytometry was conducted on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed with FlowJo software (FlowJo LLC, Ashland, OR, USA).

Immunoblotting. Immunoblotting was performed as described previously (Nawroth *et al*, 2011). Antibodies against following antigens were used: Akt1, Akt2, Akt3, pAkt Thr 308, pAkt Ser 473, pan Akt, pS6K1Thr 389,S6K1, p4EBP1 Thr 37/46, p4EBP1 Ser 65, p4EBP1 Thr 70, 4EBP1, pGSK3ß Ser 9, GSK3ß, pERK 1/2 Thr 202/ Tyr 204, ERK 1/2, PI3 kinase p110alpha, pc-RAF Ser 338, pc-RAF 259, c-RAF, GAPDH, CREB (all from Cell Signaling Technology, Beverly, MA, USA), Actin (Sigma-Aldrich Chemie GmbH, Munich, Germany), DUSP1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and secondary antibodies were purchased from Dianova (Hamburg, Germany). Amersham ECL Prime Western blotting reagent (GE Healthcare Europe GmbH, Freiburg, Germany) was used for detection.

Chicken chorioallantoic membrane (CAM) assay. The CAM assay was performed as described previously with some modifications (Kunzi-Rapp *et al*, 2001). Fertilised white Leghorn chicken eggs (Brueterei Sued, Regenstauf, Germany) were incubated at 37°C. On day 4 of incubation, the eggs were opened at the convex end. On day 9, sterilised lens paper was used to irritate a region of the CAM until bleeding was observed and a silicone ring was placed in this area. One million cells suspended in 10 ul medium and 10 ul phenol red free Matrigel (BD Biosciences) were incubated at 37°C to form a viscous mixture and then pipetted within the ring. On days 11 and 14, 500 nM of MK-2206 was used for topical treatment of the tumours. Concentration was calculated for the total blood volume of the developing embryo (Kind, 1975). Tumours were harvested on day 15, trimmed under a stereomicroscope (Stemi DV4, Carl Zeiss, Oberkochen, Germany) and weighed on a fine balance.

Immunohistochemistry. Immunohistochemistry was performed on 2.5 uM sections from formalin-fixed, paraffin-embedded tissues with antigen retrieval by heating in 0.01 M citrate buffer, pH 6.0, blocking in 6% hydrogen peroxide for 5 min and 5% FCS-TBS for 2 h. Ki-67 (Dako Deutschland GmbH, Hamburg, Germany) or pAkt Thr 308 (Bioworld Technology Inc., St Louis Park, MN, USA) antibodies were incubated for 2 h at room temperature. Detection was performed using the Dako REAL detection system (Dako Deutschland GmbH) according to the manufacturer's protocol and sections were counterstained with haematoxylin. Images were obtained using Zen Lite 2012 software (Carl Zeiss) and analysed manually for the presence of staining in at least three fields from three independent specimens.

RESULTS

AKT isoforms contribute differentially to AKT phosphorylation and cell viability. AKT inhibitors have varying affinities towards the three AKT isoforms. Hence, we analysed the expression of AKT isoforms and their contribution to downstream signalling in BLCA to determine prerequisites of potential AKT inhibitors. We could demonstrate expression of all three AKT isoforms in a panel of 11 different BLCA-derived cell lines (Figure 1A). As these isoforms have distinct roles in regulating cell growth (Gonzalez and McGraw, 2009), we determined their activation status in two representative cell lines by silencing their expression using two different isoform-specific siRNA oligonucleotides and analysed their expression and phosphorylation by immunoblotting (Figure 1B). The phosphorylation pattern of the individual isoforms differed in both cell lines. While only isoforms 2 and 3 were phosphorylated in 253J cells, all three isoforms were phosphorylated in HT1197 cells. When characterising the effect of AKT silencing on cell viability, each of the isoforms contributed to a decrease in cell viability. The greatest effect was seen after silencing the expression of all three isoforms (Figure 1C), indicating heterogeneous contribution of AKT isoforms to the regulation of cell growth and viability.

MK-2206 inhibits AKT/S6K1 phosphorylation and causes a cell-line-dependent decrease in viability via induction of apoptosis. In order to effectively inhibit all three AKT isoforms, we used MK-2206, a pan-AKT allosteric inhibitor. When treating cells with this compound, we first analysed the biochemical response of AKT downstream targets to increasing doses of MK-2206 (Figure 2A). All 11 BLCA cell lines showed a dose-dependent decrease in AKT phosphorylation in a range of 50–100 nM corresponding to the described activity of MK-2206 (Yap *et al*, 2011) that was accompanied by a decrease in S6K1 phosphorylation. Although RT4 cells had no detectable phosphorylated AKT, they responded with a similar dephosphorylation of S6K1 upon MK-2206 treatment. Interestingly, none of the cell lines showed an effect on 4E-BP-1 phosphorylation of the amino acid residues Thr 37/46, Ser 65 and Thr 70.

Although the biochemical data did not differ among the various cell lines, only three cell lines, 253J, HT1197 and VmCUB1, responded with a decrease of 40–60% in viability after MK-2206 treatment (Figure 2B) with an absolute IC50 of 674, 242 and 1469 nM respectively (Figure 2C). We confirmed this effect by directly assessing the number of living cells in six representative cell lines. 253 J, HT1197 and VmCUB1 cells showed a decrease of 37–48% after MK-2206 treatment but no such decrease was observed in 647V, RT112 and T24 cells (Supplementary Figure 1A).

In order to further characterise the effect of MK-2206 on cell growth, we performed apoptosis assays and cell cycle analysis in the sensitive and representative resistant cell lines. The MK-2206-sensitive 253J, HT1197 and VmCUB1 cells showed an increase in caspase 3/7 activity of 97%, 95% and 23%, respectively, after treatment. No effect was apparent in the resistant 647V, RT112 or T24 cells (Supplementary Figure 1B). Not only all three sensitive cell lines but also the resistant T24 cells showed a modest but significant decrease in S phase after treatment (Supplementary Figure 1C).

DUSP1 controls ERK 1/2 phosphorylation and sensitivity to MK-2206. Apart from its regulation of mTOR signalling, AKT is an upstream component of several signalling cascades. Thus, we determined whether the biochemical basis for the differential response to MK-2206 treatment could be explained by its effects on GSK3β, a regulator of cell proliferation (Rayasam *et al*, 2009), or responses in MAPK signalling that interacts at multiple levels with PI3K/AKT signalling and controls BLCA proliferation (Nawroth *et al*, 2011). GSK3β phosphorylation decreased upon AKT inhibition in six representative cell lines (Figure 3). Phosphorylation of RAF-1 at serine 338 increased after treatment, indicating activation of MAPK signalling. Serine 259 phosphorylation of RAF-1 that has been described to be negatively regulated by AKT

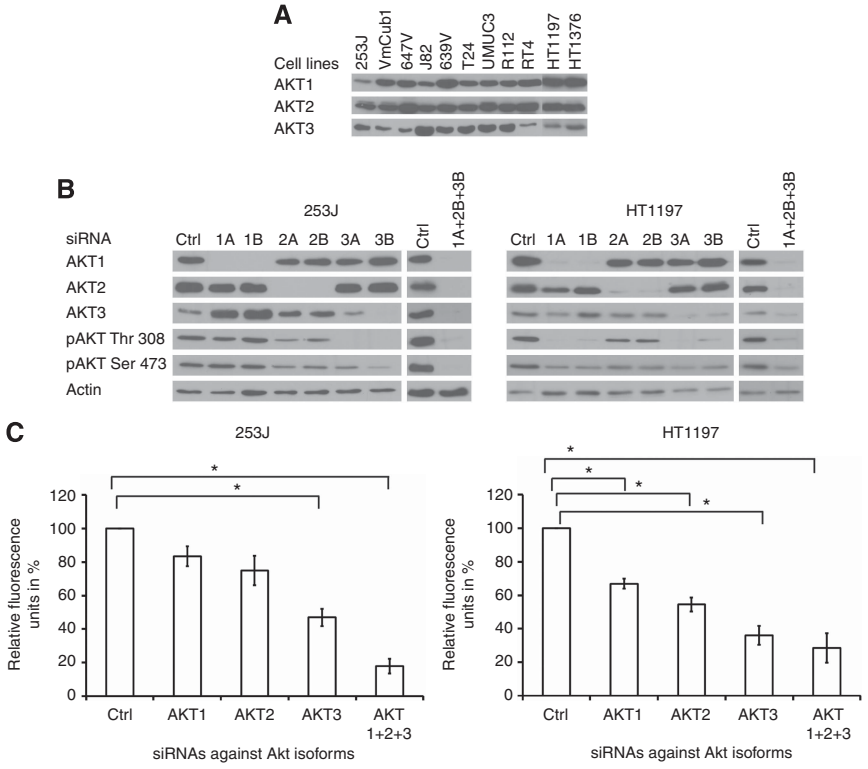

Figure 1. All three AKT isoforms are expressed in BLCA but contribute heterogeneously to active phosphorylated AKT and maintenance of cell viability. (**A**) Protein expression of the three AKT isoforms was analysed in a panel of cell lines by immunoblotting with the respective antibodies. (**B**) Representative cell lines were transfected with two different siRNA oligonucleotides against each AKT isoform (A or B) or control (ctrl), and protein expression was analysed by immunoblotting with the respective antibodies. (**C**) Cell viability of transfected cells was analysed at 72 h. Values indicate mean +/− s.e. expressed as percentage of control from three independent experiments. Statistical comparison was performed using the student *t*-test (* indicates $P<0.05$).

phosphorylation remained unaffected in HT1197 and VmCUB1 cells but showed an increase in 253J cells (Zimmermann, 1999). However, MK-2206-sensitive and -resistant cell lines showed opposite responses in the level of phosphorylated ERK 1/2. While the sensitive 253J, HT1197 and VmCUB1 cells showed a reduction in phosphorylated ERK 1/2, the resistant 647V, RT112 and T24 cells showed an increase indicating that ERK 1/2 phosphorylation is regulated differently in responsive cells by a RAF-1-independent mechanism.

A potential candidate for regulating ERK 1/2 could be DUSP1 (also known as MKP-1), a phosphatase that negatively regulates ERK 1/2 by dephosphorylation (Keyse, 2008). We observed an inverse relationship between DUSP1 protein expression and ERK 1/2 phosphorylation in all the examined cell lines (Figure 3), with sensitive cells showing an increase and resistant cells showing a decrease in DUSP1 expression after treatment.

In order to rigorously characterise this correlation, we used siRNA oligonucleotides to suppress DUSP1 expression in all three sensitive cell lines and examined the effects on ERK phosphorylation and cell viability. MK-2206 treatment in the absence of DUSP1 failed to produce a decrease in ERK 1/2 phosphorylation in all three responsive cell lines (Figure 4A). Additionally, upon DUSP1 silencing, previously sensitive cells became resistant to MK-2206 treatment (Figure 4B). We conclude that the regulation of DUSP1 expression upon treatment and the subsequent dephosphorylation of ERK 1/2 determine the responsiveness to MK-2206.

A possible link between AKT and DUSP1 regulation is CREB, an AKT downstream target and transcriptional regulator of DUSP1 (Du and Montminy, 1998; Xu *et al*, 2007). Thus, we suppressed the expression of CREB in HT1197 cells using siRNA oligonucleotides and treated them with MK-2206 (Supplementary Figure 2). Despite successful CREB silencing, an increase in DUSP1 expression after AKT inhibition was observed as noted previously, demonstrating that CREB does not regulate DUSP1 in these cells.

Sensitivity to MK-2206 is associated with the presence of *PIK3CA* mutations. Using public databases, we analysed the panel of cell lines used in this study for correlation of alterations in *PTEN*, *PIK3CA*, *TSC1*, *RAS* and *FGFR3* with the response to MK-2206 in order to explain the differential sensitivity to AKT inhibition (Platt *et al*, 2009; Forbes *et al*, 2011; Lamont *et al*, 2011; Wellcome Trust Sanger Institute website, 2014) (Table 1). All three cell lines sensitive to MK-2206 possess activating hotspot mutations in the helical domain (HD) of the *PIK3CA* gene that encodes for the p110 alpha subunit of PI3K. In contrast, mutations in other *PIK3CA* domains, *TSC1*, *RAS*, or alterations in *PTEN* or *FGFR3*, did not correlate with sensitivity to MK-2206.

Mutant *PIK3CA* regulates ERK 1/2 phosphorylation and sensitivity to MK-2206. In order to examine the correlation between mutations in the HD of *PIK3CA* and response to MK-2206 at a molecular level, we used two different strategies of genetic manipulation. First, we expressed a *PIK3CA* E545K mutation in 647V, RT112 and T24 cells that are WT for *PIK3CA* and examined the biochemical and functional response after MK-2206 treatment (Figure 5A). Expression of the activating mutation led to an increase in phosphorylated AKT and ERK 1/2 as compared with WT cells (comparing lane 1 and 3), demonstrating that the presence of mutant *PIK3CA* results not only in hyperphosphorylation of AKT but also in the activation of ERK signalling. After MK-2206 treatment in the presence of the *PIK3CA* mutation, levels of phosphorylated ERK remained

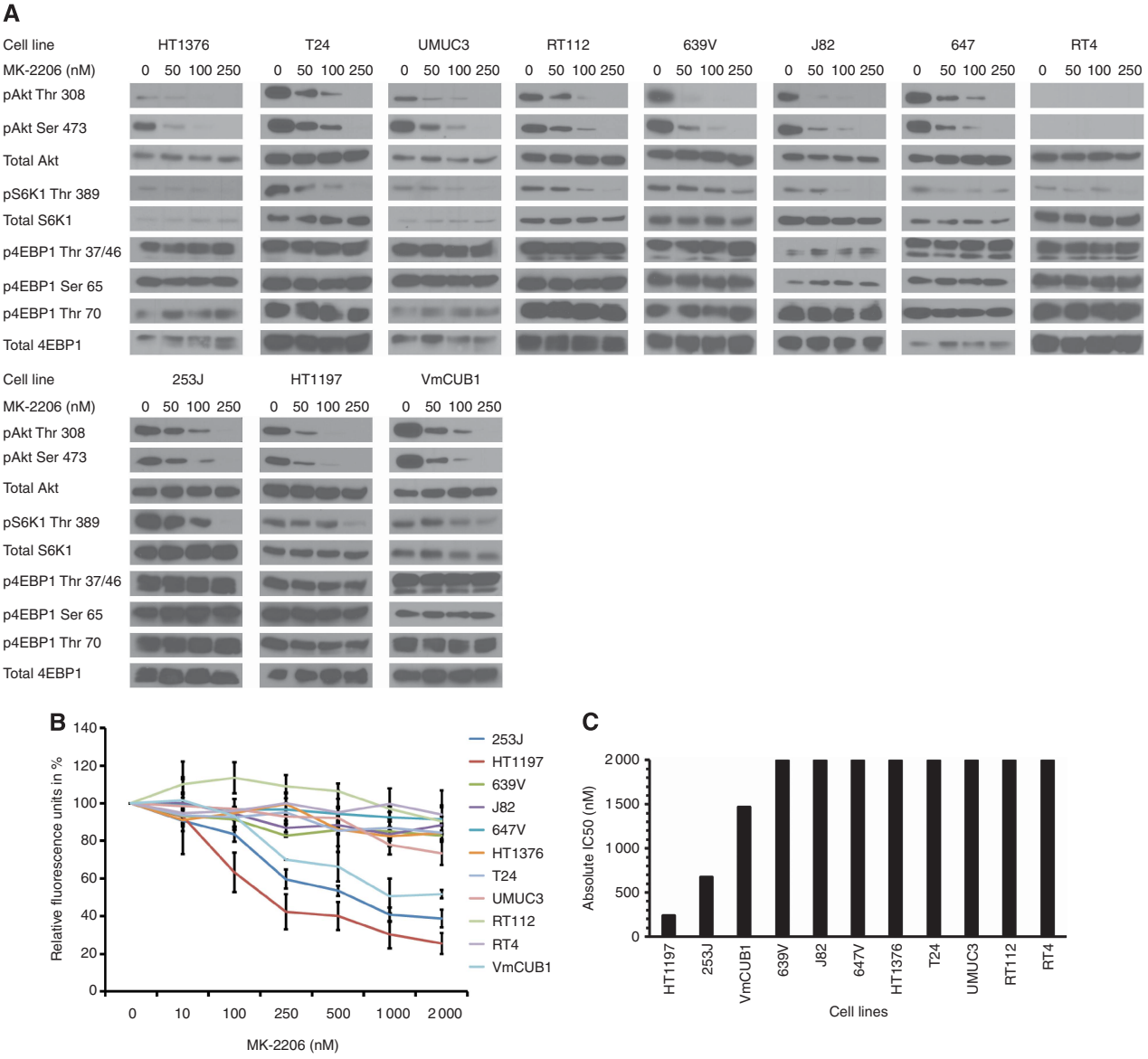

Figure 2. MK-2206 decreases AKT/mTORC1 signalling in BLCA cell lines but selectively affects BLCA viability. (**A**) Respective cell lines were treated for 1 h with indicated concentrations of MK-2206 and protein expression and phosphorylation was analysed by immunoblotting. (**B**) Cell viability was assessed 72 h after the addition of respective MK-2206 concentrations and (**C**) absolute inhibitory concentration (IC50) was determined. Graphs represent mean +/− s.e. expressed as a percentage of control from at least three independent experiments.

unchanged in 647V cells, whereas RT112 and T24 cells showed a decrease, resembling the observed biochemical response in MK-2206-sensitive *PIK3CA* mutant cells (as shown in Figure 3). Furthermore, the *PIK3CA*-transfected cells responded to MK-2206 treatment with a 37–68% decrease in cell viability (Figure 5B). This was accompanied by a 30–70% decrease in cell number (Supplementary Figure 3A) and a 200–300% increase in caspase 3/7 activity (Supplementary Figure 3B).

As a second strategy, we silenced the expression of *PIK3CA* in 253J and HT1197 cells with an siRNA oligonucleotide directed against the 5' untranslated region of the gene (Figure 6A). We then reconstituted them with WT *PIK3CA* and treated them with MK-2206 (Figure 6B). Replacement of mutant *PI3KCA* to WT resulted in a decrease in AKT phosphorylation and also phosphorylated ERK 1/2 (comparing lanes 1–3). Upon MK-2206 treatment, cells negative for the *PIK3CA* mutation no longer showed dephosphorylation of ERK 1/2. Additionally, previously sensitive cells became resistant to treatment in the absence of the mutant *PIK3CA* (Figure 6C).

These observations demonstrate that sensitivity to MK-2206 requires the presence of mutant *PIK3CA* that regulates a biochemical response mechanism involving ERK 1/2 phosphorylation.

MK-2206 reduces proliferation of BLCA cells in a three-dimensional *in vivo* model in the presence of mutant *PIK3CA*. We used a three-dimensional, *in vivo* xenograft model to extend our *in vitro* findings. The chicken CAM assay serves as an excellent substitute for animal experiments (Flecknell, 2002) and represents an *in vivo* immunodeficient model where cells can form three-dimensional tumours with a complex vascular network (Kunzi-Rapp *et al*, 2001). We generated tumour xenografts of the representative cell lines HT1197 and RT112 on the developing CAM (Supplementary Figure 4A). Tumours were treated with MK-2206 and analysed for weight, AKT phosphorylation and proliferation as determined by Ki-67 expression. Upon treatment, tumour weights were significantly reduced by about 50% in HT1197 cells, whereas RT112 tumours showed no such decrease (Figure 7A). MK-2206 induced a decrease in AKT phosphorylation

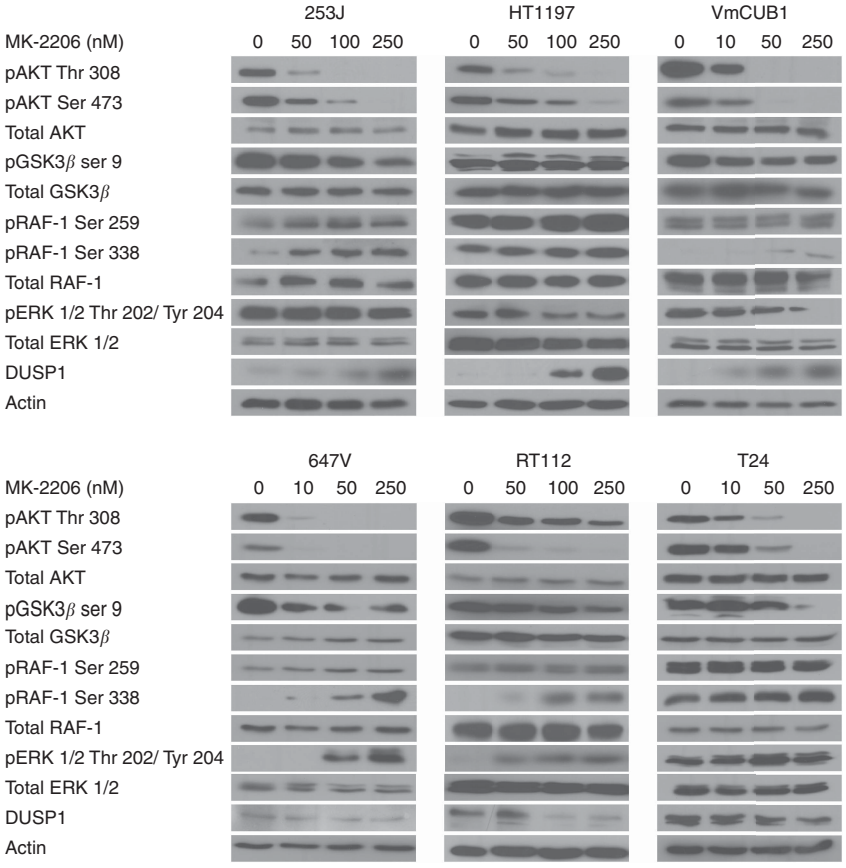

Figure 3. MK-2206-sensitive cells show a reduction in ERK 1/2 phosphorylation and an increase in DUSP1 expression after treatment. Cell lines were incubated with indicated MK-2206 concentrations for 1 h and protein expression and phosphorylation pattern was analysed by immunoblotting.

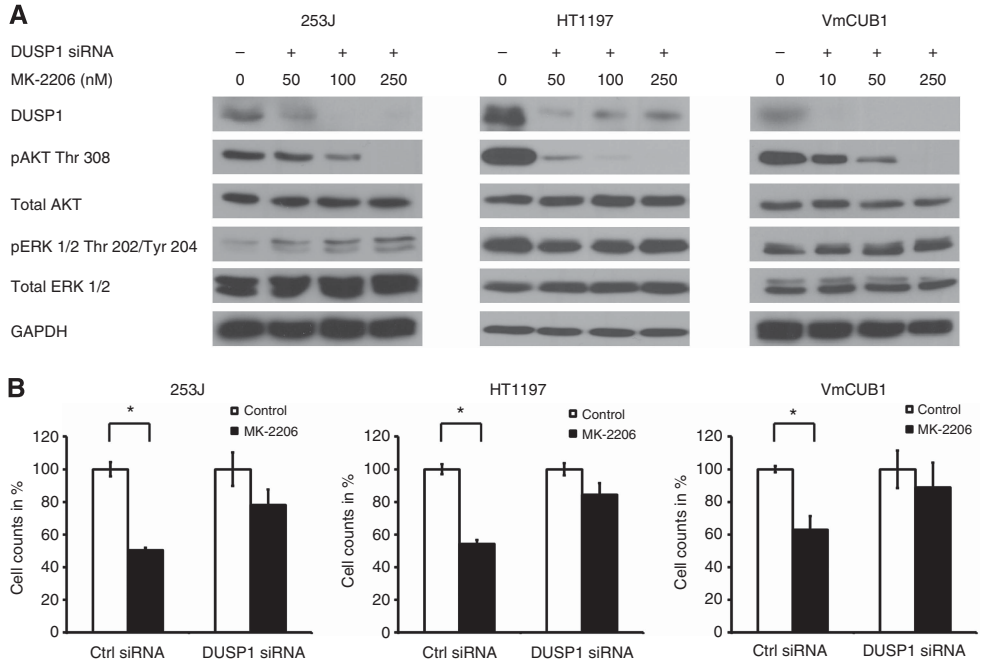

Figure 4. DUSP1-mediated regulation of ERK 1/2 phosphorylation regulates sensitivity to MK-2206. (**A**) Cells transfected with control (ctrl) or DUSP1-directed siRNA oligonucleotides were treated with indicated concentrations of MK-2206 for 1 h in the respective cell lines. '+' indicates present and '−' indicates absent. (**B**) Transfected cells were treated with 1000 nM of MK-2206 for a further 72 h and the number of viable cells was assessed. Graphs indicate mean viable cell counts +/− s.e. expressed as a percentage of control and represent at least three independent experiments. Statistical comparison was performed using the student *t*-test (* indicates $P<0.05$).

Table 1. Genetic background of cell lines and their response to MK-2206

Cell line	*PIK3CA*	*PTEN* status	*TSC1*	*RAS*	*FGFR3* status/ expression	Response to MK2206
RT112	WT	WT	WT	WT	WT overexpressed	Resistant
RT4	WT	WT, reduced copy number	Mutant	WT	WT overexpressed	Resistant
647V	WT	WT, reduced copy number	WT	WT	WT	Resistant
HT1376	WT	WT	WT	WT	WT	Resistant
T24	WT	Mutant	WT	*HRAS* Mutant, *KRAS* WT, *NRAS* WT	WT	Resistant
UMUC3	WT	Homozygous deletion	WT	*KRAS* Mutant, *NRAS* WT, *HRAS* WT	WT	Resistant
J82	P124L rare mutant	Homozygous deletion	WT	WT	Mutant	Resistant
639V	A1066V rare mutant	Mutant	Mutant	*KRAS* Mutant, *NRAS* Mutant, *HRAS* WT	WT	Resistant
253J	E545G hotspot helical domain mutant	WT	WT	WT	WT	Sensitive
HT1197	E545K hotspot helical domain mutant	WT, reduced copy number	WT	*NRAS* Mutant, *HRAS* WT, *KRAS* WT	WT	Sensitive
VM-CUB1	E542K hotspot helical domain and E674Q helical domain mutant	WT	WT	WT	WT	Sensitive

Abbreviation: WT = wild type. Sensitivity to MK-2206 correlates with the presence of hotspot HD PIK3CA mutations. List of described molecular alterations in *PIK3CA*, *PTEN*, *TSC1*, *RAS* and *FGFR3* in relation to the response to MK-2206.

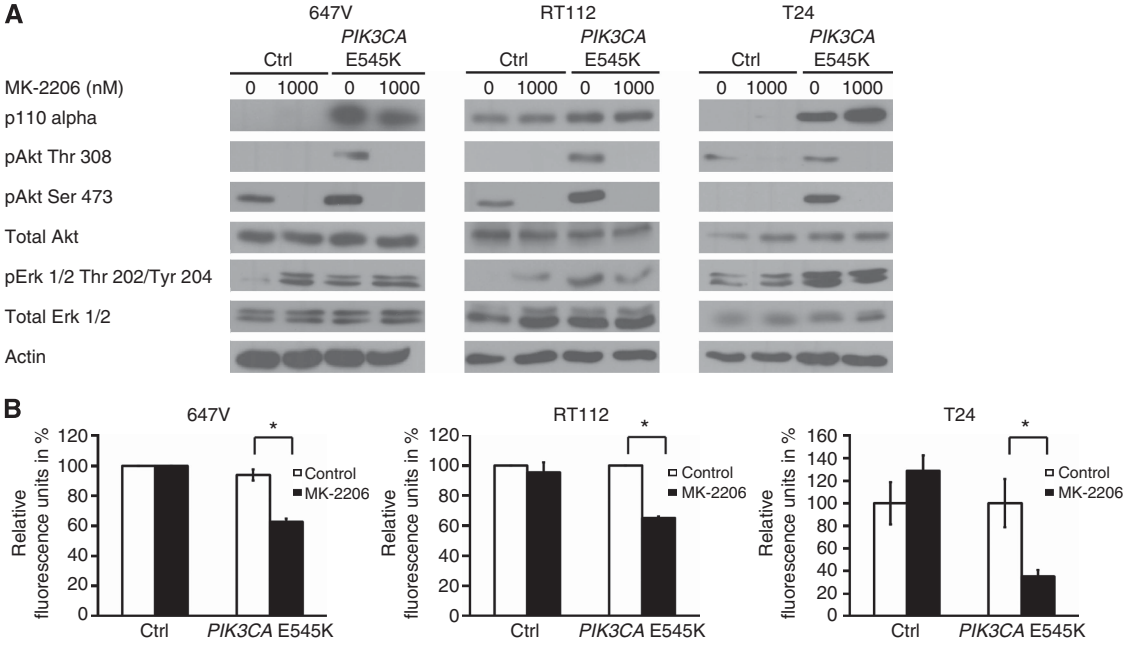

Figure 5. Resistant cells are sensitive to MK-2206 in the presence of mutant *PIK3CA* and show a decrease in ERK 1/2 phosphorylation. (**A**) Cells transfected with either control vector (ctrl) or mutant *PIK3CA* E545K were treated for 1 h with 1000 nM MK-2206 for immunoblotting. (**B**) Transfected cells were treated for a further 72 h to assess cell viability. Graphs indicate mean +/− s.e. expressed as a percentage of control and are representative of at least three independent experiments. Statistical comparison was performed using the student *t*-test (* indicates $P<0.05$).

of around 50–70% in both and a reduction of Ki-67 expression by 54% in HT1197 tumours, with no significant change in RT112 tumours (Figure 7B, Supplementary Figure 4B). We then grafted RT112 cells transfected with a control vector or with *PIK3CA* E545K and treated the tumours with MK-2206. While tumours containing the control vector did not respond to MK-2206, cells expressing the mutant *PIK3CA* showed a 56% decrease in weight after treatment (Figure 7C). While MK-2206 induced a decrease in AKT phosphorylation of around 80% in both, Ki-67 expression was significantly reduced by 55% only in cells containing *PIK3CA* E545K (Supplementary Figure 4C).

DISCUSSION

In this study, we describe a molecular mechanism that regulates the response to AKT therapy in BLCA.

We first demonstrated in a panel of 11 cell lines with characterised genomic alterations that all AKT isoforms are expressed and that they contribute heterogeneously to active phosphorylated AKT. Although diverse roles of these isoforms have been described in cell cycle regulation and cell migration (Gonzalez and McGraw, 2009), their functional role in BLCA has

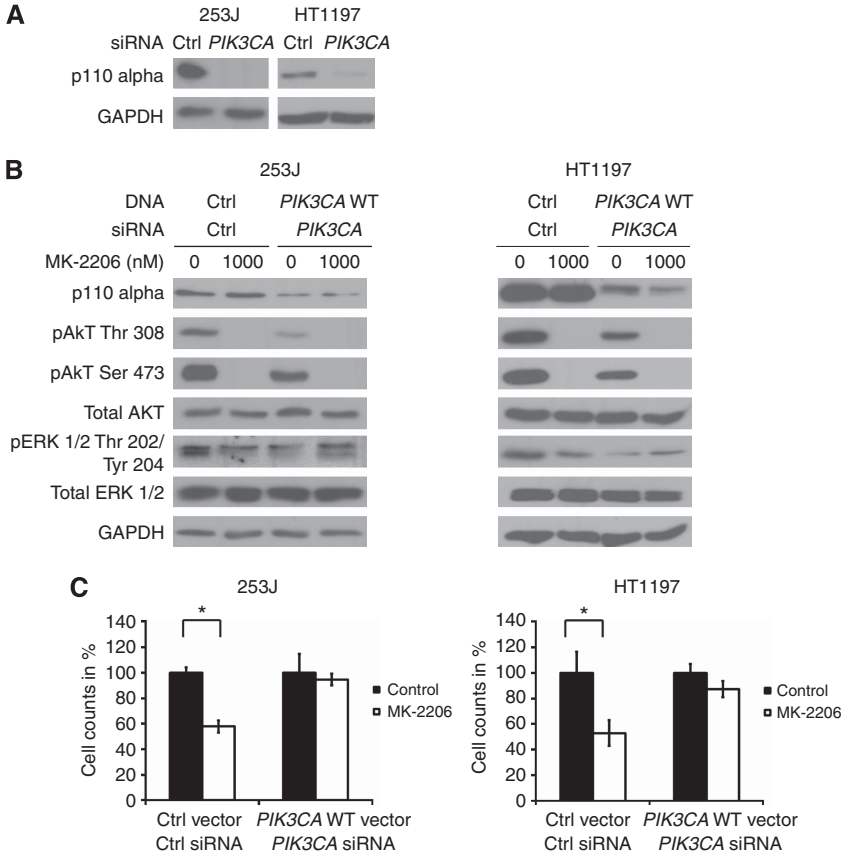

Figure 6. Sensitive cells become resistant to MK-2206 upon loss of mutant *PIK3CA* and show an increase in ERK 1/2 phosphorylation. (**A**) Cells were transfected with control (ctrl) or siRNA oligonucleotides against *PIK3CA*. (**B**) siRNA-transfected cells were then transfected with control (ctrl) or WT *PIK3CA* containing vector and treated with 1000 nM MK-2206 for 1 h for immunoblotting or (**C**) incubated for another 72 h to assess cell viability. Graphs indicate mean viable cell counts +/− s.e. expressed as a percentage of control and represent at least three independent experiments. Statistical comparison was performed using the student *t*-test (* indicates $P<0.05$).

not been previously examined. AKT inhibitors differ in their affinity towards AKT isoforms and several selectively target only AKT1 and AKT2 (Mattmann *et al*, 2011). Our results demonstrate that inhibitors used in BLCA should target all three AKT isoforms.

For characterising the therapeutic effect of AKT target therapy in BLCA, we examined the biochemical and functional response of cell lines to the pan-AKT inhibitor MK-2206. Upon treatment with MK-2206, decreased phosphorylation of AKT and its downstream mTOR substrate S6K1 could be observed. No dephosphorylation was observed in different amino acid residues of 4E-BP1, a second important downstream target of mTORC1. We have previously shown that rapalogues (RAD001, CCI-779) or shRNA-mediated silencing of mTOR affects only the phosphorylation of S6K1 and not 4E-BP1 in BLCA. However, dual PI3K inhibitors such as NVP-BEZ235 did result in dephosphorylation of both S6K1 and 4E-BP1 (Nawroth *et al*, 2011). These data extend our previous results that 4E-BP1 phosphorylation is not regulated by the AKT/mTOR axis of the pathway but that there might be another link between PI3K inhibition and 4E-BP1 regulation that has not been revealed to date. Interestingly, the same response was observed in RT4 cells that had no detectable AKT phosphorylation. This might indicate that either MK-2206 is not as specific as described or that RT4 cells possess phosphorylation levels that are non-detectable by western blotting but are sufficient to regulate further downstream signalling events.

On a functional level, despite successful downregulation of AKT phosphorylation, MK-2206 treatment inhibited cell viability only in cell lines possessing mutations in the HD of *PIK3CA* that result in constitutive activation of PI3K. Mutations found in non-hotspot regions of the HD result in AKT activation to a lesser degree (Ross *et al*, 2013) and were not associated with sensitivity to MK-2206 in our panel of cell lines (Table 1). Bladder cancer possesses a different spectrum of *PIK3CA* mutations compared with other tumour entities, with about 80% occurring in hotspot regions of the HD (Platt *et al*, 2009). Around 15–20% of BLCA patients are predicted to bear these mutations (Platt *et al*, 2009; Cancer Genome Atlas Research Network, 2014) and thus have the potential to benefit from AKT target therapy.

Representative sensitive and resistant cell lines showed a modest decrease in cell cycle progression after MK-2206 treatment, as described previously for breast cancer and T-cell leukaemia (She *et al*, 2008; Sangai *et al*, 2012; Simioni *et al*, 2012). Only sensitive cells demonstrated an increase in caspase 3/7 activity suggesting that the response to MK-2206-induced inhibition of tumour growth is mediated by an increase in apoptosis.

The association between sensitivity to AKT inhibition and specific *PIK3CA* mutations has also been observed in thyroid and breast cancer. However, in these studies, sensitivity also correlated with *PTEN* and in thyroid cancer also with *RAS* mutations (Liu *et al*, 2011; Sangai *et al*, 2012). No correlations with *PIK3CA*, *PTEN* or *RAS* mutations and AKT target therapy have been observed in non-small cell lung cancer (Meng *et al*, 2010). This suggests that the predictive character of the genetic determinants of drug sensitivity differs between tumour entities. Also in our study, cell lines displaying similar alterations in *PTEN* or *RAS* were not responsive.

An ATP-competitive AKT inhibitor, AZ7328, has been tested on a panel of BLCA-derived cell lines before (Dickstein *et al*, 2012). In this study, no clear predictable pattern of associations between

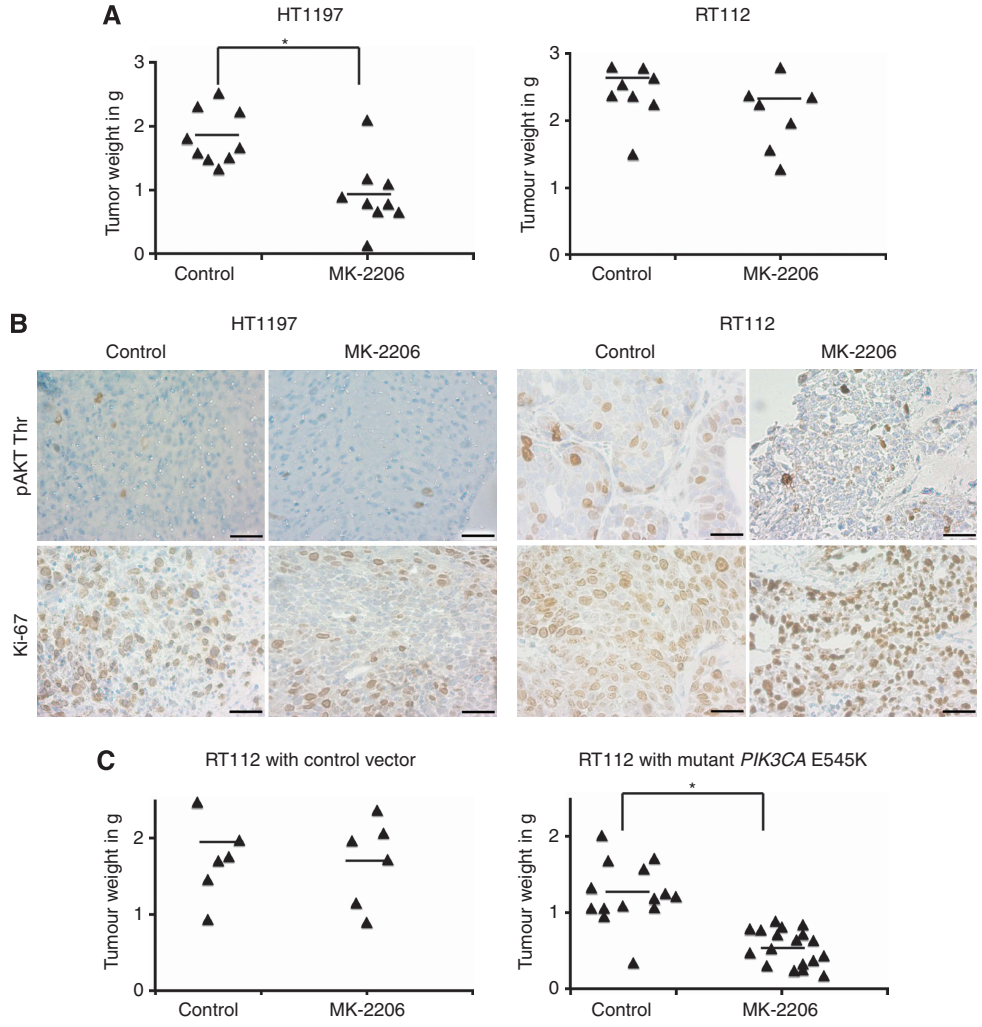

Figure 7. HD mutant *PIK3CA* confers sensitivity to MK-2206 *in vivo*. (**A**) Tumours from indicated cell lines were harvested and weighed after MK-2206 or control treatment. (**B**) Representative images from tumours, which were analysed with IHC using the indicated antibodies. Scale bar = 20 μM. (**C**) RT112 tumours transfected with either control vector or mutant *PIK3CA* E545K were harvested after control or MK-2206 treatment and weighed. For all graphs, each dot indicates a data point and line indicates the mean. Statistical comparison was performed using the student *t*-test (* indicates $P<0.05$). All results are representative of at least two independent experiments.

the presence of alterations in *PIK3CA* and the sensitivity to AKT inhibition was observed, although cells possessing mutant *PIK3CA* did respond with a lower IC50 for growth inhibition. This suggests, that the mode of action of different inhibitors used might largely influence therapy response (Dickstein *et al*, 2012; Haibe-Kains *et al*, 2013). MK-2206 was also previously examined in a panel of BLCA cell lines. Mutations in *PIK3CA* or *AKT1* correlated with sensitivity to the drug, whereas *PTEN* and *TSC1* alterations did not (Iyer *et al*, 2013). However, the authors did not provide a direct molecular characterisation of specific mutations and therapy response. We have extended these data to provide a mechanistic explanation by rigorously examining the contribution of a single mutation in *PIK3CA* to drug sensitivity. Our results clearly demonstrate that the presence of only a *PIK3CA* mutation is sufficient to confer sensitivity to MK-2206 both *in vitro* and *in vivo*.

Upon treatment with MK-2206, activation of the MAPK signalling pathway by an increase in Raf-1 phosphorylation was observed in both WT and mutant *PIK3CA* expressing cells. This observation is in agreement with studies that have demonstrated an upregulation of tyrosine kinases after AKT inhibition via feedback loops (Chandarlapaty *et al*, 2011; Cen *et al*, 2013). Also, introduction of the mutant *PIK3CA* E545K resulted in an increase in ERK phosphorylation confirming a recent study that demonstrated crosstalk between PI3K and MAPK signalling (Will *et al*, 2014). However, we show here that expression of *PIK3CA* E545K has a regulatory role in DUSP1 expression. Inhibition of AKT in this situation results in a downregulation of ERK 1/2 and subsequently induces apoptosis. This represents a biochemical signature of response and has not been described previously. The reduction in ERK 1/2 activity promotes apoptosis and decreases tumour growth, which is in line with reports that this decrease after PI3K inhibition is essential to initiate apoptosis and sensitise cells to the inhibitor (Will *et al*, 2014).

It remains to be examined whether AKT inhibition in the presence of mutant *PIK3CA* influences DUSP1 expression due to hyperactivation of the PI3K or MAPK signalling pathway or due to other properties of the mutant such as protein complex formation. We have verified that this mechanism is not regulated via CREB, which has been described as an AKT substrate (Du and Montminy, 1998). DUSP1 is expressed at higher levels in early stages of bladder carcinogenesis (Loda *et al*, 1996). Given its impact on the regulation of cell proliferation and response to treatment, it warrants further investigation as a potential therapeutic target in BLCA.

The interplay between mutant *PIK3CA*, DUSP1, ERK phosphorylation and AKT inhibition demonstrates the complexity in cell signalling involving a non-linear signalling cascade and

crosstalk between the PI3K and MAPK pathways, which ultimately influences therapy response. These interactions make it likely that the application of AKT target therapy will have a complicated transition to the clinic. We demonstrate that this therapy is likely to benefit a subset of patients whose tumour genetics determine the responsiveness to therapy. In BLCA, a disease for which target therapy is still in early stages of development, our data suggest that AKT therapy can benefit patients possessing hotspot HD *PIK3CA* mutations. In addition, this mutation might be used as a stratifying biomarker to personalise treatment in patients with advanced BLCA.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

AS and RN conceived and designed the study. AS, FG, MVC and RN developed the methodology. AS and FG acquired the data. AS and RN analysed and interpreted it. The manuscript was written and reviewed by AS, JEG, MR and RN; administrative and material support was provided by MMH and MT.

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