Received: 3 August 2021 | Revised: 26 October 2021 | Accepted: 14 November 2021

DOI: 10.1111/nan.12780

ORIGINAL ARTICLE

Neuropathology and Applied Neurobiology
JOURNAL OF THE BRITISH NEUROPATHOLOGICAL SOCIETY

WILEY

AKT1^{E17K}-mutated meningioma cell lines respond to treatment with the AKT inhibitor AZD5363

Peter John[1] | **Natalie Waldt**[1] | **Josephine Liebich**[1] | **Christoph Kesseler**[1] | **Stefan Schnabel**[2] | **Frank Angenstein**[3] | **I. Erol Sandalcioglu**[4] | **Cordula Scherlach**[5] | **Felix Sahm**[6] | **Elmar Kirches**[1] | **Christian Mawrin**[1]

[1]Department of Neuropathology, Otto von Guericke University, Magdeburg, Germany

[2]Department of Neurosurgery, Paracelsus Hospital Zwickau, Zwickau, Germany

[3]Functional Imaging Group, DZNE, Magdeburg, Germany

[4]Department of Neurosurgery, Otto von Guericke University, Magdeburg, Germany

[5]Department of Neuroradiology, University Hospital Leipzig, Heidelberg, Germany

[6]Department of Neuropathology, University Hospital Heidelberg, Heidelberg, Germany

Correspondence
Christian Mawrin, Department of Neuropathology, Otto von Guericke University Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany.
Email: christian.mawrin@med.ovgu.de

Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: MA2530/8-1; Wilhelm Sander-Stiftung, Grant/Award Number: 2014.092.1; German Cancer Aid, Grant/Award Number: 70112956

Abstract

Aims: Meningiomas are the most frequent primary brain tumours. Recently, knowledge about the molecular drivers underlying aggressive meningiomas has been expanded. A hotspot mutation in the *AKT1* gene (*AKT1*^{E17K}), which is found in meningiomas at the convexity and especially at the skull base, has been associated with earlier tumour recurrence.

Methods: Here, we analysed the effects of the *AKT1*^{E17K} mutation and treatment response to the Akt inhibitor AZD5363 in transgenic meningioma cell clones and mouse xenografts modelling convexity or skull base meningiomas.

Results: We show that the *AKT*^{E17K} mutation significantly enhances meningioma cell proliferation and colony size in vitro, resulting in significantly shortened survival times of mice carrying convexity or skull base *AKT1*^{E17K} xenografts. Treatment of mutant cells or xenografts (150 mg/kg/d) with AZD5363 revealed a significant decrease in cell proliferation and colony size and a prolongation of mouse survival. Western blots revealed activation of AKT1 kinase (phosphorylation at Ser273 and Thr308) by the E17K mutation in human meningioma samples and in our in vitro and in vivo models.

Conclusions: Our data suggest that *AKT1*^{E17K} mutated meningiomas are a promising selective target for AZD5363.

KEYWORDS
AKT1, meningioma, targeted therapy

INTRODUCTION

Meningiomas are the most frequent intracranial tumours in adults, which can grow at intraspinal or different intracranial locations.[1] The majority of meningiomas occur intracranially, where meningiomas at the convexity or skull base can be observed.

Approximately 80% of meningiomas are benign WHO grade I tumours,[2] but a fraction (~15–20%) show aggressive biology with brain infiltration, reduced progression-free survival and impaired overall survival.[3,4] These meningiomas, classified as atypical meningiomas WHO grade II or the rarer anaplastic meningiomas WHO grade III (1–4%), can pose a treatment challenge.

Peter John and Natalie Waldt have equal contribution.

While about 50% of sporadic meningiomas are characterised by alterations in the tumour suppressor gene *NF2*,[5,6] the molecular basis of non-*NF2* meningiomas has been characterised recently in detail (reviewed in[7]). Among several recurrent mutations seen in non-*NF2* meningiomas are mutations in the genes *SMO* and *AKT1* and are found in meningiomas with preferential growth at the skull base.[8,9] Up to 28% of skull base meningiomas harbour hotspot mutations in the *SMO* gene,[10] while the hotspot mutation in the *AKT1* gene (*AKT1*^{E17K}) is found in 9–30% of convexity or skull base meningiomas, respectively.[10–13]

In many cancers, the phosphatidylinositol 3-kinase/protein kinase B (PI3K-AKT/PKB) signalling pathway is frequently activated by somatic mutations.[14] The PKB isoform AKT1 is a serine/threonine protein-kinase and a major downstream target of growth factor receptors and PI3K. A point mutation (G → A) at nucleotide 49 of the *AKT1* sequence results in a substitution of glutamic acid by lysine at codon 17 (E17K). This amino acid substitution leads to a conformational change in the Pleckstrin homology domain (PHD) of the protein, altering its localisation form the cytoplasm to the plasma membrane. This results in a constitutive activation of the AKT1 kinase and therefore the activation of mTOR and ERK1/2 signalling pathways.[15]

The *AKT1*^{E17K} hotspot mutation is most frequently detected in meningiomas, followed by breast, endometrium and skin cancer.[16,17] In meningioma, the mutation has been associated with earlier tumour recurrence on the one hand[10,11] but also more favourable prognosis than *NF2*-altered meningiomas.[18] However, given the fact that chemotherapy or targeted therapy of meningiomas has been unsuccessful so far,[19] there is a need to develop additional treatment options for patients suffering from aggressive or recurrent meningioma.[7]

Recently, a case report has described successful treatment of a metastatic *AKT1*^{E17K}-mutated meningioma by using AZD5363.[20] AZD5363 (Capivasertib) specifically targets the AKT signalling pathway, predominantly tumours harbouring the *AKT1*^{E17K} mutation.[21,22] Recent clinical studies (phase I and II) have suggested promising results with AZD5363 as monotherapy or combination therapy in breast cancer.[23] We had previously shown that *AKT1*^{E17K} mutant meningiomas show activation of the PI3K/mTOR signalling pathway.[11] Thus, *AKT1*^{E17K} mutant meningiomas seem to represent an attractive target for AZD5363.

Here, we show that *AKT1*^{E17K} meningioma cells are more aggressive than *AKT*^{WT} meningioma cells. Furthermore, we present data that cells harbouring the mutation are more susceptible to AZD5363 treatment in vitro and in vivo, providing a preclinical basis for future clinical studies to treat aggressive meningioma.

Key Points

- Meningioma cells with overexpression of the *AKT1*^{E17K} mutation have increased proliferation potential compared to meningioma cells overexpressing *AKT1*^{wt}.
- Mice with orthotopic xenografts of *AKT1*^{E17K} mutated meningioma cells have reduced overall survival compared with mice carrying *AKT1*^{wt}.
- The AKT inhibitor AZD5363 shows superior effects on *AKT1*^{E17K} meningioma cells compared to *AKT1*^{wt} cells in vitro and in vivo.

MATERIALS AND METHODS

Patient meningioma samples

Human meningioma tumour samples ($N = 6$) were obtained from patients after surgery at the Otto von Guericke University, Magdeburg.

Tissue sampling was approved by the local institutional review board (IRB). Tumour tissues were immediately frozen in liquid nitrogen and stored at −80°C until protein extraction for western blot analysis. Tumour samples were classified according to the 2016 WHO classification of brain tumours.[24] Mutational status of all six tumours was assessed by targeted Sanger sequencing as reported previously.[11] The three wild-type *AKT1* tumours were WHO grade I meningiomas (two transitional, one meningothelial), matching with the three *AKT1*^{E17K} tumours (two transitional, one meningothelial WHO grade I tumours).

Cell lines and cultivation conditions

The malignant meningioma cell line IOMM-Lee was obtained as described before (Pachow et al, 2013). The cells had originally been derived from the intraosseous part of a malignant meningioma WHO grade III of a 61-year-old male, were able to grow as xenografts in mice, to metastasise and expressed the mesenchymal tumour marker vimentin.[25] They possess a complex karyotype and were later found to contain a rare deletion of the tumour suppressor *CDKN2A*, which we confirmed by next generation sequencing (NGS) to be a homozygous loss (own unpublished data).[26] Normal expression of merlin was observed and no *NF2* gene losses or mutations detected, as well as no 'non-*NF2* mutations' in recently discussed meningeal oncogenes (including *AKT1*), except for the rare *TERT* C228T promoter mutation. All these features were in accordance with our own Western blot and NGS characterisation of IOMM-Lee, suggesting that *CDKN2A* loss and *TERT* promoter mutation may be relevant drivers already present in genetically unmodified IOMM-Lee cells. The cell line CH157-MN-LucNeo was kindly provided by Rachael E. Vaubel, Mayo Clinic, Rochester, MN, USA. The cells had been originally derived from the meningioma of 41-year-old female, without further classification or histologic description of the original meningioma.[27] Although a complex karyotype, the high number of CNVs and the presence of the C228T *TERT* promoter mutation in these cells are strong hints towards a malignant or at least higher grade origin, the precise meningioma type of origin must still be regarded as unknown. The cells

exhibited chromosome 22 losses, covering the *NF2* region and likely represent a high grade meningioma line, in which this tumour suppressor is affected.[26] SF4068 cells were provided by Frank Böhmer (University Jena, Germany).[28] They had been originally obtained in the group of Anita Lal (San Francisco) from a benign meningioma (WHO grade I), which had not been further characterised in the report.[29] To yield a stable cell culture from a low grade meningioma, the primary cells had been retrovirally transfected with human telomerase and the papilloma oncogenes E6/E7.

Cell lines were maintained in high glucose Dulbecco's modified Eagle (DMEM) medium (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal bovine serum (FBS; PAN Biotech, Aidenbach, Germany) and 100 U/ml penicillin/100 μg/ml streptomycin (PAN Biotech, Aidenbach, Germany) at 37°C, 5% CO_2.

Generation of transgenic cells

HEK293T cells (DSMZ, Braunschweig) were transfected using FuGene® HD transfection reagent (Promega, Mannheim, Germany) with pLV[Exp]-Bsd-EF1A > hAKT1[NM_005163.2] and pLV[Exp]-Bsd-EF1A > [hAKT1[NM_005163.2]*(E17K) (VectorBuilder) in combination with lentiviral packaging plasmid mix pC-Pack 2 (Cellecta) as reported.[30] Both plasmids contain the same lentiviral vector backbone. Supernatants were harvested after 24 h and 48 h, filtered and used for infection of IOMM-Lee cells. Infected cells were finally selected with Blasticidin (Corning).

Western blotting

Human meningioma tissues or cells were lysed in lysis buffer containing 10-mM Tris–HCl, 150-mM NaCl, 50-mM NaF, 1-mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, supplemented with sodium vanadate, dithiothreitol (DTT) and protease inhibitor cocktail. The protein samples were separated on SDS polyacrylamide gel and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Germany). After blocking, the membranes were incubated with following primary antibodies: AKT1 (Cell Signaling, Frankfurt am Main, Germany), Phospho-AKT1 (T308 and S473; Santa Cruz, Dallas, TX, USA) and β-actin (Sigma-Aldrich, St. Louis, MO, USA) as a loading control at 4°C overnight. Upon washing the membranes, protein expression was visualised using horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling, Frankfurt am Main, Germany) and the chemiluminescent substrate (Millipore, Billerica, MA, USA). For quantification of human samples, the intensity of detected bands was calculated using Kodak 1D3.6 software.

Trypan blue staining

To measure the survival of different tumour cells following drug treatment, we seeded 8000 cells/well in a standard 48-well plate and allowed the cells to settle down for 24 h. Next steps the media were removed, and the corresponding drug added. After 3 days incubation, the cells were washed, trypsinised and counted in a Neubauer cell chamber (Superior Marienfeld, Germany) with a depth of 0.1 mm and 0.0025 mm^2 squares. Drug-treated cells were normalised to solvent (DMSO) untreated ones. Cells were diluted 1:1 with a solution (0.4%) of trypan blue to exclude dead cells and counted with the help of a Carl Zeiss Axiovert 40 CFL microscope.

5-Bromo-2′-deoxyuridine (BrdU) labelling and detection

To assess cell proliferation, the Cell Proliferation ELISA (BrdU) from Roche Applied Sciences (Mannheim, Germany) was used. Two thousand cells were seeded per well of a 96-well plate (Sarstedt, Nümbrecht, Germany) and incubated at 37°C, 5% CO_2. After 24 h, the cells were stained according to manufacturer's instructions, using an incubation time with BrdU of 16 h for DNA labelling. Colour development was analysed using a Tecan-Reader (Infinite 200).

Colony forming assay and in vitro drug treatment

To analyse colony formation, 10,000 cells were seeded in 10-ml culture medium onto 10 cm culture dishes (TPP, Switzerland). After 5 days of cultivation, the cells were washed with phosphate buffered saline (PBS; PAN Biotech, Aidenbach, Germany) and stained with 0.5% crystal violet solution containing 20% methanol for 15 min. The colony sizes (colony area) were determined with the help of Carl Zeiss Axiovert 40 CFL microscope, Carl Zeiss AxioVision Rel. 4.8 software and ImageJ software.

For drug treatment, the added cells were allowed to attach for 24 h. After removing the media, an appropriate drug treatment was performed. The sizes of treated colonies were normalised to those of an untreated reference.

AZD5363 treatment

For in vitro assays, we used a final concentration of 2μM AZD5363 (Capivasertib; Selleck Chemicals, Houston, TX, USA) dissolved in DMSO. For in vivo mouse experiments, AZD5365 was kindly provided by AstraZeneca, London, UK. Three days after tumour inoculation mice were treated daily with 150 mg AZD5363 (dissolved in 30% Captisol) per kg mouse weight dispensed by gavage in two separate doses (every 12 h). The control mice received only 30% Captisol.

Subcutaneous mouse model

Animal experiments in all models were done in accordance with the regulations of animal protection. Animal experiments had been

approved by the responsible authority (Landesverwaltungsamt Sachsen-Anhalt, permission # 42502–2-1459 Uni MD). Fifteen 8- to 10-week-old nude mice (Swiss Nude, Charles River) were injected subcutaneously on both sides with 3×10^6 IOMM-Lee cells in 100-µl PBS/Matrigel (1:1). The tumour volume (V) was estimated by measurement (calliper rule) of two perpendicular axes (a, b) according to the formula $V = \pi/6 \times a \times b^2$ (a>b).[31] The tumour growth was monitored until the tumour reached a size of 1.5 cm in one of the perpendicular axes. This was the time point when animals had to be killed according to criteria of animal protection. After killing the mice, tumours and lungs were resected and embedded in paraffin for immunohistochemistry.

Convexity mouse model

Swiss Nude (Charles River, France) mice (at least 8 weeks old) were taken for intracranial injection of IOMM-Lee cells.[31] Mice were anaesthetised intraperitoneally with Rompun (Bayer Vital GmbH Leverkusen, Germany)/Ketamine (Bremer Pharma GmbH, Warburg, Germany) mixture and fixed in the stereotactic head frame. The head skin was cut longitudinally. Two holes were drilled 2 mm anterior of the bregma and 1.5 mm left and right from the sagittal suture. Using a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland), 2.5×10^5 cells in 2.5-µl phosphate-buffered saline (PBS; PAN Biotech, Aidenbach, Germany) were applied 1 mm deep in each hole. The skin was finally sealed with Histoacryl (B Braun Surgical, S.A., Rubi, Spain).

Skull base mouse model

Swiss Nude were taken for intracranial injection of IOMM-Lee cells. Mice were anaesthetised intraperitoneally with Rompun (Bayer Vital GmbH Leverkusen, Germany)/Ketamine (Bremer Pharma GmbH, Warburg, Germany) mixture and fixed in the stereotactic head frame. The head skin was cut longitudinally. A single hole was drilled 1.5 mm anterior of the bregma and 2 mm on the right side from the sagittal suture and 0.5×10^5 transfected IOMM-Lee cells (*AKT1*^{wt} or *AKT1*^{E17K}) in 0.5-µl PBS were deposited above the skull base bone (about 7.5 mm in depth).[30]

Mouse MRI

MRI was performed on a Bruker BioSpec 94/20 USR scanner at 9.4 T (free bore of 20 cm) equipped with a B-GA12S (440 mT/m) gradient system. A 1H MRI CryoProbeTM 2 element array kit for mice (Bruker BioSpin MRI GmbH, Ettlingen, Germany) was used for signal reception. Heating was provided from the ventral side and heart rate, and breathing rate was monitored during the entire experiment. High-resolution T2-weighted anatomical images were acquired using a RARE sequence (Hennig et al, 1986) with the following parameters: TR: 4185 ms; TEeff: 33 ms; RARE factor: 8; field of view: 20×20 mm; matrix: 256×256 (results in an in plane resolution of 78.1×78.1 µm); slice thickness 500 µm; averages: 2; total scanning time 4 min 28 s.

Statistics

Statistical analysis was performed using GraphPad Prism 7.03 software. Statistical significance was determined using Mann–Whitney *U* test in all in vitro experiments. For the analysis of pulmonary metastases in the subcutaneous mouse model, we used Fisher's exact test. Kaplan–Meier survival curves were analysed via log-rank (Mantel–Cox) test. Significance was assumed for $p \leq 0.05$. Error bars represent standard deviation (SD).

RESULTS

AZD5363 inhibits meningioma cell growth and clonogenicity

The effects of AZD5363 on meningioma cells have not been studied so far. Therefore, we analysed three meningioma cell lines (IOMM-Lee and CHI157 derived from malignant meningiomas,[26] SF4068 derived from a grade I meningioma[32]) following AZD5363 treatment. As shown in Figure 1A, all tested cell lines exhibited a similar (~50%) and highly significant reduction in viable cells, grown during a fixed time interval, as compared with the corresponding solvent (DMSO) controls. In addition, the sizes of colonies from cells seeded in low density decreased moderately but highly significantly in malignant and benign meningioma cells, if treated with AZD5363 (Figure 1B).

Meningioma cells overexpressing AKT1^{E17K} show a higher proliferation rate in vitro and respond to AZD5363 treatment

For the analysis of *AKT1*^{E17K}-related biological features in meningioma cells, we generated pairs of cell clones with overexpression of either *AKT1*^{wt} or *AKT1*^{E17K} as previously reported.[30] Figure 2A shows comparable expression levels of AKT1 in two independent, Blasticidin (Bsd) selected pairs of IOMM-Lee cell clones, expressing the wt or mutant variant. Overexpression of a control vector, expressing solely a short non-coding RNA from an identical promoter, did not affect AKT1 levels compared with naïve IOMM cells (not shown). Cells harbouring the *AKT1*^{E17K} mutation were characterised by increased cell proliferation (BrdU assay, Figure 2B) and formation of larger colonies (Figure 2C). Moreover, if cells are grown under serum restriction, the proliferative advantage of mutant cells as compared with wild type becomes more pronounced. This is in line with previous findings that the mutation causes a constitutive activation of AKT1, which remains active even in the absence of serum.[15,33]

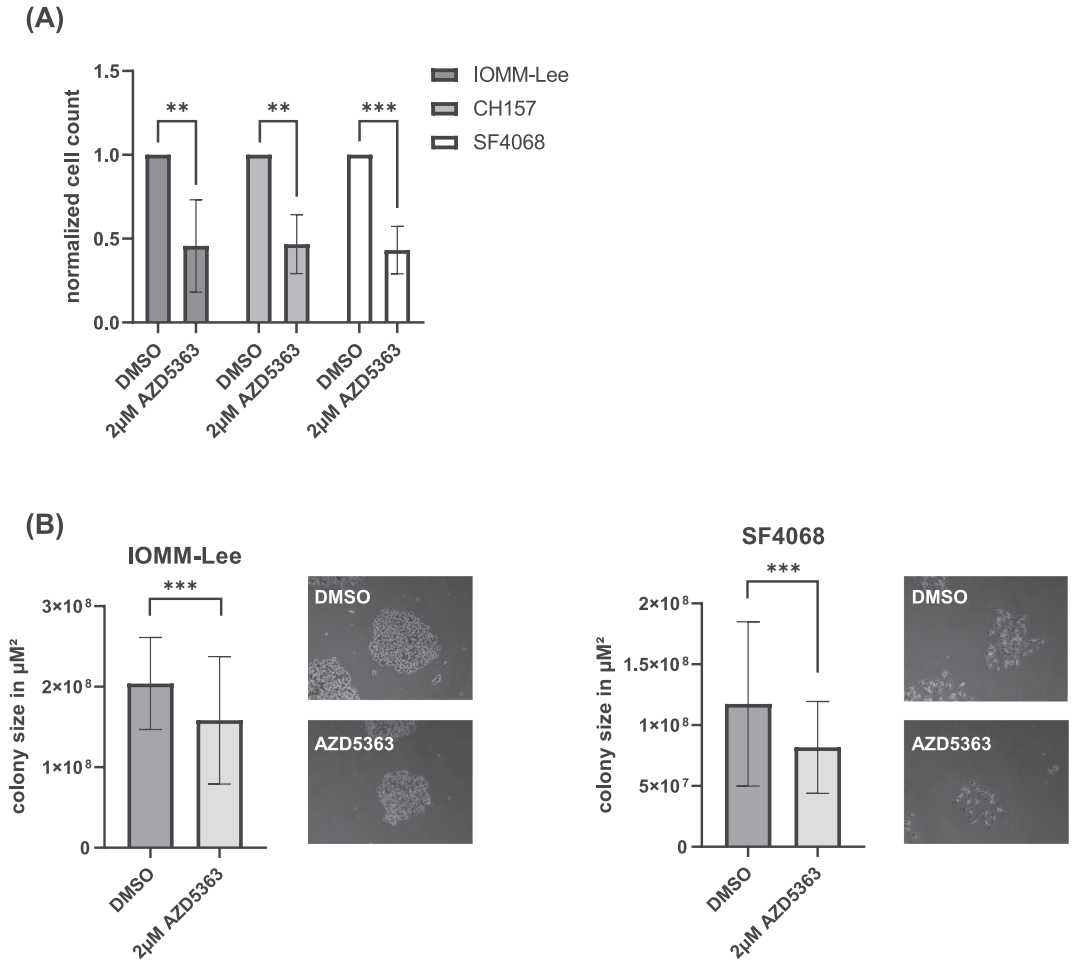

FIGURE 1 Meningioma cell line response to AZD5363 treatment. (A) Different naïve meningioma cells (malignant lines: IOMM-Lee and CHI157; benign meningioma cell line SF4068) display significant reduction of viable cells after treatment with the AKT1 inhibitor AZD5363 (**$p < 0.01$; ***$p < 0.001$). (B) Anchorage-independent growth of IOMM-Lee and SF4068 cells is impaired by AZD5363 treatment (***$p < 0.001$)

Next, we treated pairs of IOMM-Lee cell clones with AZD5363 and found that cell growth over a given time interval (Figure 2D) was strongly reduced by the drug, similarly in both wild-type and mutant IOMM-Lee cells, when compared with DMSO controls. The efficacy of the drug was also reflected in lower mean colony sizes after treatment (Figure 2E), but this did not reach significance.

Mice with transplanted IOMM-Lee AKT1^{E17K} cells show a decreased survival period compared with mice transplanted with IOMM-Lee AKT1^{WT} cells

In the first mouse model, we transplanted *AKT1*^{wt} or *AKT1*^{E17K} cells into the flank region of nude mice and measured the development of tumour size and lung metastases. As shown in Figure 3A, after 21 days, mice harbouring *AKT1*^{E17K} had bigger tumours than mice with *AKT1*^{wt} cells ($p \leq 0.05$), and the frequency of lung metastases (Figure 3B) was increased (not significant), suggesting a more aggressive in vivo phenotype of *AKT1*^{E17K} meningioma cells, despite the usage of an already aggressive malignant parental meningioma line for construction of the model.

Next, we used an orthotopic convexity meningioma model[31] to monitor intracranial growth of engineered meningioma cells. Figure 3C shows that mice harbouring *AKT1*^{E17K} cells had a shortened survival time (a). Moreover, treatment with AZD5363 had no effect in mice with the *AKT1*^{wt} tumours (b) but lead to a significant prolongation of survival ($p \leq 0.01$) in mice harbouring *AKT1*^{E17K} tumours (c).

Because *AKT1*^{E17K}-mutated meningiomas are preferentially found at the skull base,[8,9] we wondered whether AZD5363 could be effective in this tumour location. Using our recently developed orthotopic skull base meningioma model,[30] we treated mice with the same regime as for the convexity model (Figure 3D) and observed a more pronounced negative effect of the *AKT1*^{E17K} mutation on overall mouse survival ($p \leq 0.05$) (a), in accordance with the results obtained from the convexity model. A partial accordance between both models was also observed with respect to AZD5363 efficacy, which was seen only for mutant skull base tumours, although it reached no statistical significance (c). Interestingly, we observed that AZD5363 seemed to have even a negative impact on *AKT*^{wt} tumours grown at the skull base ($p \leq 0.01$).

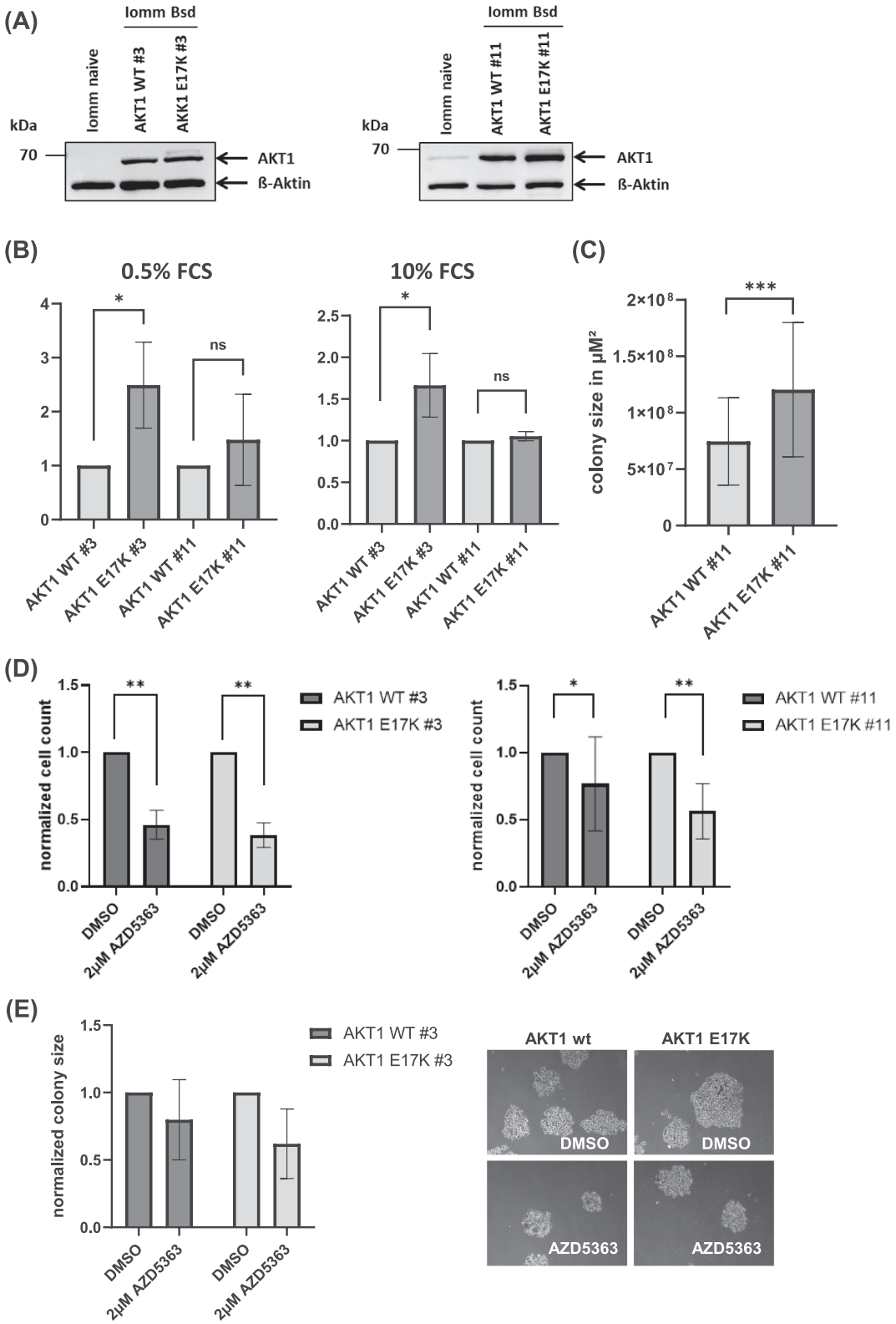

FIGURE 2 Generation and in vitro characterisation of IOMM-Lee cells overexpressing either wild-type *AKT1* or AKT1^{E17K}. (A) We isolated several transfected cell clones, which exhibited enhanced expression of the kinase AKT1 as compared with naïve (non-transfected) IOMM-Lee cells. In order to measure specifically effects of the mutation, we took care to select pairs of them without (wt) or with the E17K mutation, but expressing AKT1 protein at comparable levels (designated #3 and #11). They were used in subsequent studies. (B, C) Both proliferation and colony size are increased in *AKT1*^{E17K} cells (*$p < 0.05$; ***$p < 0.001$). (D) Both cell pairs of IOMM-Lee cells are characterised by significant reduction in viable cells after AZD5363 treatment (*$p < 0.05$; **$p < 0.01$). (E) Both AKT1^{wt} and *AKT1*^{E17} cells are susceptible to AZD5363 treatment in the colony formation assay (not significant)

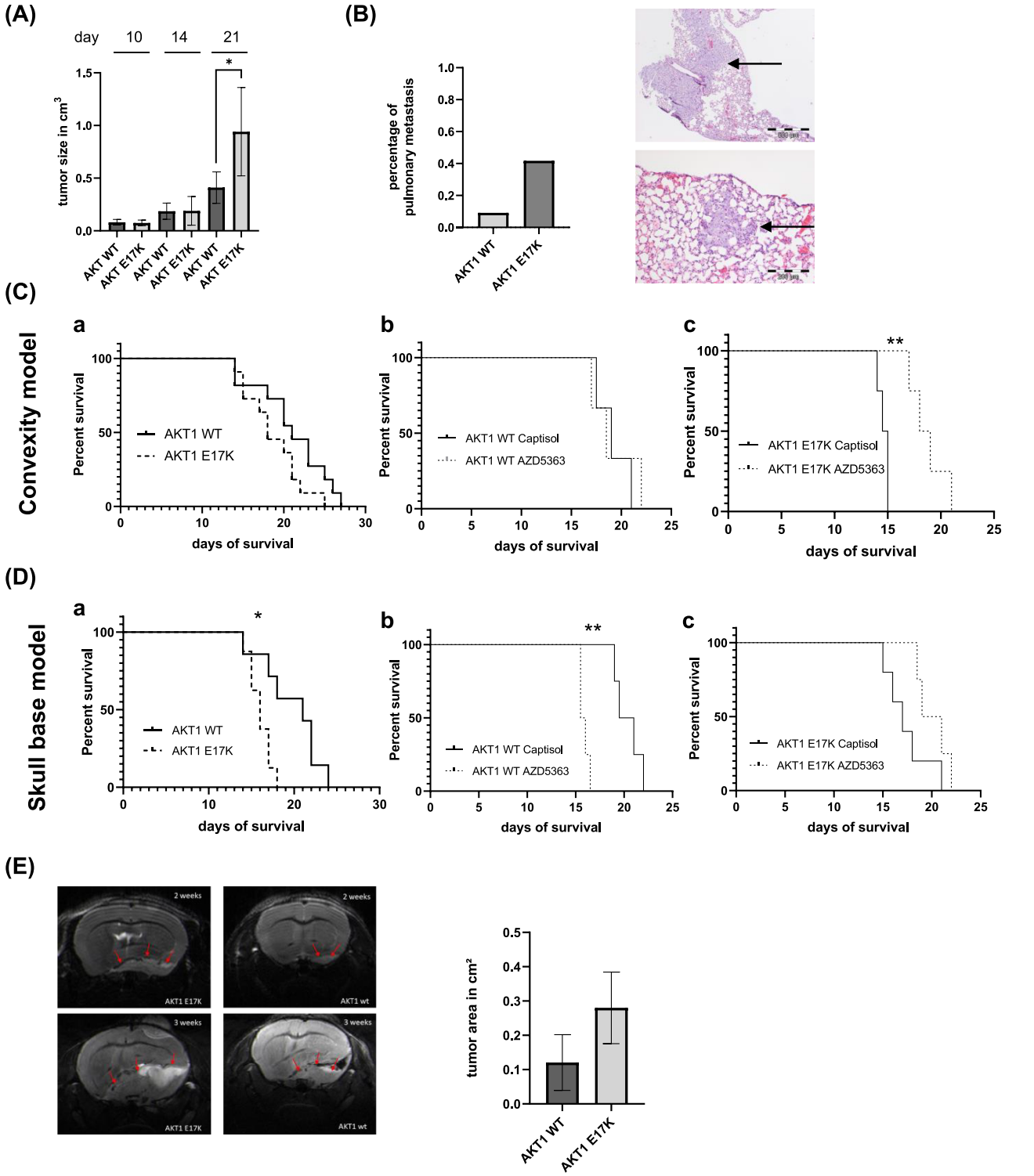

FIGURE 3 In vivo characterisation of *AKT1*wt or *AKT1*^{E17K} IOMM-Lee meningioma cells. (A) Subcutaneous tumour cell growth in Swiss-nude mice shows greater tumour size with *AKT1*^{E17K} cells ($p < 0.05$) and a higher frequency of lung metastases (B) (not significant). (C) Orthotopic xenograft growth in the convexity meningioma model shows impaired survival ($p = 0.105$) in mice with *AKT1*^{E17K} cells (a). Treatment with AZD5363 did not show any effect in mice with *AKT1*^{wt} tumours (b) but significantly ($p = 0.009$) increased survival in mice harbouring *AKT1*^{E17K} xenografts and AZD5363 treatment (c). (D) Orthotopic meningioma xenografts at the skull base are characterised by significant reduction ($p = 0.038$) of survival for *AKT1*^{E17K} xenografts (a) and a tendency for a prolonged survival with AZD5363 treatment (c) ($p = 0.09$). Surprisingly, treatment of mice harbouring *AKT1*^{wt} xenografts with AZD5363 reduced the overall survival (b) ($p = 0.006$). (E) Analysis of tumour size by small animal MRI reveals larger skull base meningiomas with *AKT1*^{E17K} compared with *AKT1*^{wt} tumours (not significant)

The mutation *AKT1*^{E17K} mediates enhanced phosphorylation of T308 and S473 in human meningioma samples and meningioma cells

The autonomous growth-factor-independent activity of the kinase AKT1 carrying the hotspot missense mutation can be expected to result in an enhanced phosphorylation of ATK1 at residues S473 and T308.[34] Enhanced phosphorylation at both sites ($p \leq 0.05$) was indeed observed by densitometric analysis in *AKT1*^{E17K} mutant human meningioma tissue samples of WHO grade I as compared with *AKT1*^{wt} tumours (Figure 4A).

Interestingly, despite the malignant character of the parental IOMM-Lee cell line used for the construction of our transgenic cell clones, the same upregulation of pAkt at S473 and T308 was seen in cells overexpressing *AKT1*^{E17K} (Figure 4B). Moreover, it appeared that treatment of meningioma cells itself results in enhanced phosphorylation at both sites independent of the mutation.

By analysing xenograft tumours grown in mice, we could confirm that immunoexpression of phosphorylated Akt at S473 and T308 was stronger in *AKT1*^{E17K} xenograft tumours and following AZD5363 treatment (Figure 4C,D).

DISCUSSION

Constitutive activation of mutant AKT1 causes enhanced meningioma cell aggressiveness

Treatment of aggressive meningiomas where standard treatment options such as surgery and radiotherapy have been exhausted, is still challenging. There is an urgent need to identify new treatment options based on recurrent somatic mutations in meningiomas.[35] Recent molecular characterisation has revealed a group of meningiomas with preferential growth at the skull base and a hotspot somatic mutation in the *AKT1* gene (*AKT1*^{E17K}). Here, we show that the mutation increases cell growth of meningioma cells in vitro, but also increases tumour aggressiveness in mouse xenograft models of convexity and skull base meningioma, leading to moderately decreased survival times. This was even more remarkable, when considering the circumstance that a malignant meningioma cell line had to be used for creation of the various models, because only the malignant cell line enabled sufficiently fast growing xenografts. AKT1 mutations, like most other currently known recurrent genetic alterations in meningiomas, are thought to be only mild drivers of tumourigenesis and occur mostly in tumours of WHO grade I, which constitute by far the largest fraction of human meningiomas.

Previous data had already suggested that meningioma patients with tumours harbouring the *AKT1*^{E17K} mutation are prone to earlier tumour recurrence.[10,11] Our results in meningioma cells, which strongly suggest a higher aggressiveness mediated by the E17K mutation, are well in accordance with the reported enhancement of proliferation, cell survival and accelerated cellular invasion.[36–40] Increased aggressiveness of meningioma cells in the present study is likely to be a result of enhanced AKT1 signalling, which was accompanied in our model cells and in analysed human meningioma tissues by an increased phosphorylation of Ser273 and Thr308. Phosphorylation at these sites had been described earlier to lead to a constitutive activation of AKT1, for example, in carcinoma cells, which persists after growth factor withdrawal.[33] We had observed by immunohistochemical staining that *AKT1*^{E17K}-mutated meningiomas have increased activity of the mTOR-S6K axis,[11] providing a plausible downstream target for the enhanced AKT1 activity due to the mutation.

The mutant AKT1 had already been associated earlier with enhanced migratory, invasive and metastatic properties of epithelial cells,[40] which may further be in accordance with the increase in lung metastases by the E17K mutation of meningioma cells observed in our subcutaneous xenograft model. While lung metastasis are exceptionally rare in meningiomas, a case report of an *AKT1*^{E17K}-mutated meningioma with lung metastases suggested that treatment with the inhibitor AZD5363 was able to control the disease.[20]

Treatment options with AZD5363

Due to the lack of established chemotherapy options for high-grade or recurrent meningiomas, the development of new strategies of pharmaceutical intervention is required.[19,41] New approaches should be mainly targeted therapies, directed towards known receptors, signalling routes or enzymes, playing a role in meningiomas. Recent sequencing data may partially illuminate the path to that goal.

Somatic mutations in the PI3K/AKT pathway provide reasonable targets in various cancers.[14] In meningiomas, the PI3K/AKT signalling is activated, mainly by PDGF receptor tyrosine kinase (RTK) activation.[42,43] Especially high-grade meningiomas show immunohistochemical evidence for activation of this signalling pathway.[44] However, the treatment of recurrent or aggressive meningiomas with RTK inhibitors such as Sunitinib or Erlotinib has delivered only modest results.[45,46] Downstream targets, such as mTORC1, which are activated in meningiomas,[47–49] can be effectively treated in mouse models,[31] but clinical studies have been unsuccessful.[50,51] Therefore, it seems to be more reasonable to target PI3K signalling components upstream of mTORC1.

In light of the frequent activation of the PDGFR/PI3K/AKT/mTORC1 pathway by the AKT1 mutation E17K in meningiomas particularly those located at the skull base,[11] and the positive effects reported for a single case,[20] it was reasonable to explore AZD5363 as a kinase inhibitor for *AKT1*^{E17K}-mutated meningiomas. In the present study, the drug exhibited efficacy at all levels in vitro and at a dosage of 150 mg/kg/d in a mouse xenograft model in vivo. Indeed, a phase I study of the drug in advanced cancers[52] (NCT01226316) has already delivered encouraging data in humans. Our preclinical models may point towards therapeutic efficacy in human meningiomas. Moreover, because meningiomas at the skull base can sometimes pose a challenge for the neurosurgeon,[53] AZD5363 might add an additional treatment option, especially in light of the positive effect seen in mice with E17K-mutated tumour xenografts located at the skull base.

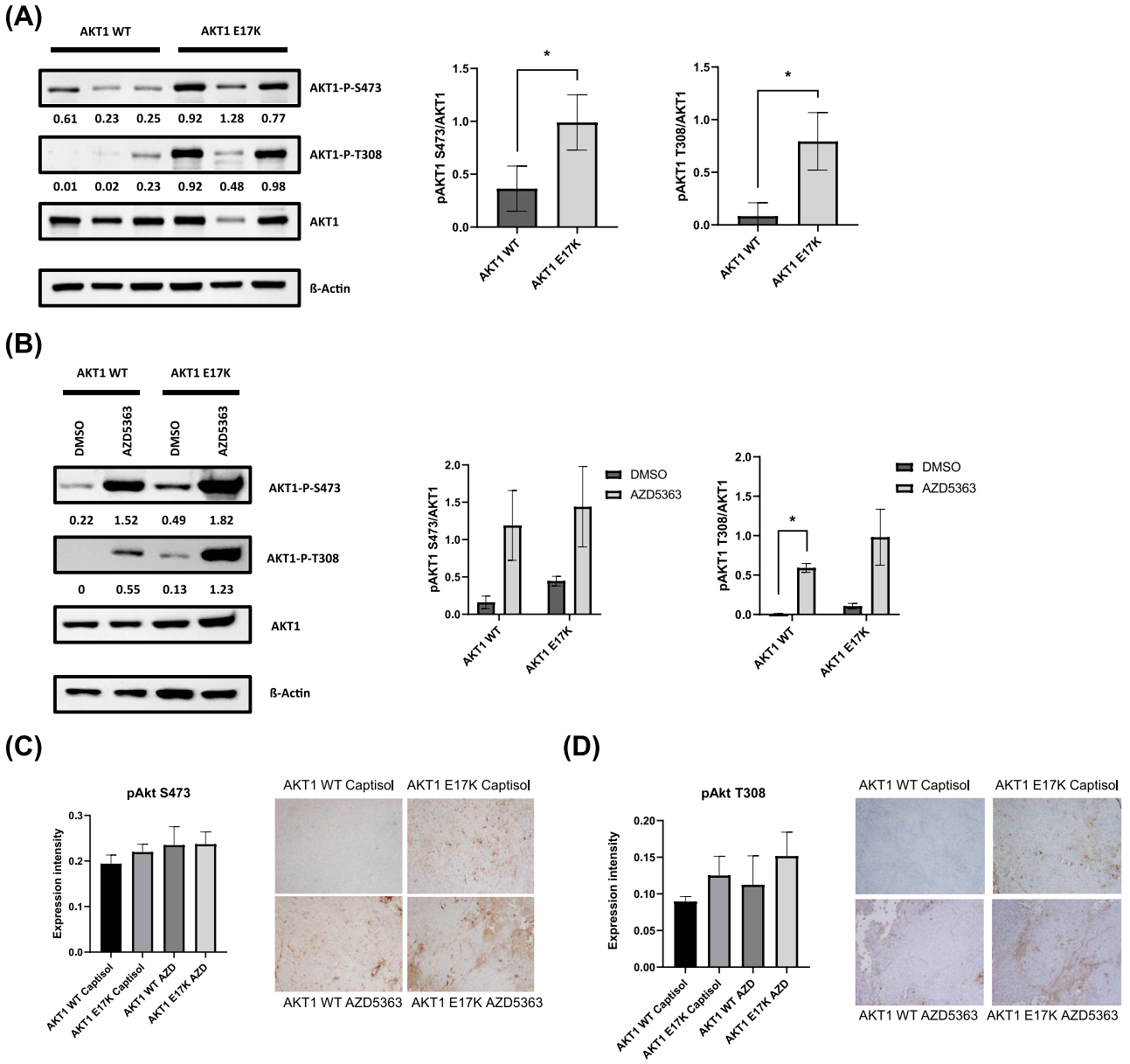

FIGURE 4 *AKT1* ^{E17K}-mutated meningiomas and meningioma cells are characterised by increased AKT phosphorylation at Ser473 and Thr308 sites. (A) Human WHO grade I meningioma samples with *AKT1*^{E17K} mutation have significantly increased S473 and T308 phosphorylation compared with wild-type tumours. (B) Phosphorylation of both sites is enhanced by treatment with AZD5363 in cells with the *AKT1*^{E17K} mutation. This indicates that even in the mutated kinase, the inhibitor can lead to a hyperphosphorylated, but inactive protein, as suggested as a general concept for ATP competitive inhibitors in non-mutated AKT isoforms. (C, D) According to visual inspection of stained tissue slices, explanted tumours grown in mice (skull base) are characterised by enhanced immunoexpression of both S473 and T308 phosphorylated forms, if carrying the E17K mutation. The difference between genotypes, as well as the moderate effect of AZD5363 treatment of mice (as compared with the solvent Captisol) did not reach statistical significance, if corresponding signal intensities were quantified

Although not necessarily expected, one might have argued for a potential difference in the responsiveness of convexity and skull base meningiomas, due to the differential embryologic origin of these two meningeal areas.[54]

In our in vitro experiments, the drug exhibited an effect not only in IOMM cells with mutant *AKT1*, but also towards non-mutated *AKT1*, while the effect towards the latter cells was not strongly supported by an increased survival of animals. The in vitro observations were not surprising given the fact that the inhibitor also effectively targets all (non-mutated) isoforms of the kinase, that is, AKT1, AKT2 and AKT3. At first sight, the enhanced phosphorylation of both regulatory residues of AKT1 (S473 and T308) under AZD5363 (Figure 4B) may appear astonishing. However, the phenomenon is known since long, for example, from various carcinoma cells.[21] It can

be explained by the circumstance that AZD5363, like all ATP competitive AKT inhibitors, binds to the APT site, thereby generating a hyperphosphorylated, but inactive kinase.[55] Despite our encouraging experiments with meningioma cells, expanded xenograft experiments, eventually with genetically edited cells expressing the normal intrinsic levels of the kinase, will be required prior to the inclusion of meningiomas without this specific activating mutation in clinical trials.

There may be some concerns regarding established protocols for radiotherapy, for example, in case of high-grade meningiomas, because the E17K mutation of AKT1 is suspected to generate a partial radio-resistance, based on cell culture data, by increasing the repair rate of double strand breaks in the DNA.[56,57] Therefore, AZD5363 may be of special interest in this setting, because AKT kinase inhibition partially restored radio-sensitivity. This may be an aspect worth of future analysis, for example, by a clonogenic assays similar to our colony formation assay or by agarose embedding.

Taken together, our in vitro and in vivo data provide evidence that treatment with AZD5363 might be an additional option for patients suffering from recurrent or otherwise aggressive *AKT1*^{E17K}-mutated meningiomas at the convexity and skull base, which should be explored in future clinical studies.

ACKNOWLEDGEMENTS

This study was supported by a generous grant from the Deutsche Forschungsgemeinschaft [DFG] (MA2530/8-1, to C.M.), the Wilhelm Sander-Stiftung (grant 2014.092.1, C.M.) and the German Cancer Aid (Deutsche Krebshilfe), Translational Oncology Project "*Aggressive Meningiomas,*" *70112956* (to C.M. and F.S.).

CONFLICT OF INTEREST

The authors declare to have no conflict of interest.

ETHICS STATEMENT

The research has been given ethical approval, including the use of human tumour tissue and all animal experiments.

AUTHOR CONTRIBUTIONS

PJ, NW, CK, JL and SS were responsible for the experiments. CM and EK were responsible for the planning. CS and FA were responsible for the imaging. CM, FS, ES and EK were responsible for the writing.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/nan.12780.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analysed in this study.

ORCID

Felix Sahm https://orcid.org/0000-0001-5441-1962
Christian Mawrin https://orcid.org/0000-0002-6677-3124

REFERENCES

1. Ostrom QT, Gittleman H, Truitt G, Boscia A, Kruchko C, Barnholtz-Sloan JS. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2011-2015. *Neuro Oncol*. 2018;20(suppl_4):iv1-iv86.
2. Mawrin C, Perry A. Pathological classification and molecular genetics of meningiomas. *J Neurooncol*. 2010;99(3):379-391.
3. Perry A, Scheithauer BW, Stafford SL, Lohse CM, Wollan PC. "Malignancy" in meningiomas: a clinicopathologic study of 116 patients, with grading implications. *Cancer*. 1999;85(9):2046-2056.
4. Mawrin C, Chung C, Preusser M. Biology and clinical management challenges in meningioma. *Am Soc Clin Oncol Educ Book/ASCO Am Soc Clin Oncol Meet*. 2015;(35):e106-e115.
5. Ruttledge MH, Xie YG, Han FY, et al. Deletions on chromosome 22 in sporadic meningioma. *Genes Chromosomes Cancer*. 1994;10(2):122-130.
6. Ruttledge MH, Sarrazin J, Rangaratnam S, et al. Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. *Nat Genet*. 1994;6(2):180-184.
7. Preusser M, Brastianos PK, Mawrin C. Advances in meningioma genetics: novel therapeutic opportunities. *Nat Rev Neurol*. 2018;14(2):106-115.
8. Clark VE, Erson-Omay EZ, Serin A, et al. Genomic analysis of non-NF2 meningiomas reveals mutations in TRAF7, KLF4, AKT1, and SMO. *Science*. 2013;339(6123):1077-1080.
9. Brastianos PK, Horowitz PM, Santagata S, et al. Genomic sequencing of meningiomas identifies oncogenic SMO and AKT1 mutations. *Nat Genet*. 2013;45(3):285-289.
10. Boetto J, Bielle F, Sanson M, Peyre M, Kalamarides M. SMO mutation status defines a distinct and frequent molecular subgroup in olfactory groove meningiomas. *Neuro Oncol*. 2017;19(3):345-351.
11. Yesiloz U, Kirches E, Hartmann C, et al. Frequent AKT1E17K mutations in skull base meningiomas are associated with mTOR and ERK1/2 activation and reduced time to tumor recurrence. *Neuro Oncol*. 2017;19(8):1088-1096.
12. Youngblood MW, Miyagishima DF, Jin L, et al. Associations of meningioma molecular subgroup and tumor recurrence. *Neuro Oncol*. 2020;22(Supplement_2):ii172-ii173.
13. Strickland MR, Gill CM, Nayyar N, et al. Targeted sequencing of SMO and AKT1 in anterior skull base meningiomas. *J Neurosurg*. 2016;127(2):1-7.
14. Brugge J, Hung MC, Mills GB. A new mutational AKTivation in the PI3K pathway. *Cancer Cell*. 2007;12(2):104-107.
15. Carpten JD, Faber AL, Horn C, et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature*. 2007;448(7152):439-444.
16. Bleeker FE, Felicioni L, Buttitta F, et al. AKT1(E17K) in human solid tumours. *Oncogene*. 2008;27(42):5648-5650.
17. de Bruin EC, Whiteley JL, Corcoran C, et al. Accurate detection of low prevalence AKT1 E17K mutation in tissue or plasma from advanced cancer patients. *PLoS One*. 2017;12(5):e0175779.
18. Yuzawa S, Nishihara H, Yamaguchi S, et al. Clinical impact of targeted amplicon sequencing for meningioma as a practical clinical-sequencing system. *Mod Path: An Off J US Can Acad Path Inc*. 2016;29(7):708-716.
19. Wen P, Quant E, Drappatz J, Beroukhim R, Norden A. Medical therapies for meningiomas. *J Neurooncol*. 2010;99(3):365-378.
20. Weller M, Roth P, Sahm F, et al. Durable control of metastatic AKT1-mutant WHO grade 1 meningothelial meningioma by the AKT inhibitor, AZD5363. *J Natl Cancer Inst*. 2017;109(3):1-4.
21. Davies BR, Greenwood H, Dudley P, et al. Preclinical pharmacology of AZD5363, an inhibitor of AKT: pharmacodynamics, antitumor activity, and correlation of monotherapy activity with genetic background. *Mol Cancer Ther*. 2012;11(4):873-887.

22. Davies BR, Guan N, Logie A, et al. Tumors with AKT1E17K mutations are rational targets for single agent or combination therapy with AKT inhibitors. *Mol Cancer Ther*. 2015;14(11):2441-2451.
23. Smyth LM, Zhou Q, Nguyen B, et al. Characteristics and outcome of AKT1 (E17K)-mutant breast cancer defined through AACR project GENIE, a clinicogenomic registry. *Cancer Discov*. 2020;10(4):526-535.
24. Louis DN, Perry A, Reifenberger G, et al. The 2016 World Health Organization classification of tumors of the central nervous system: a summary. *Acta Neuropathol*. 2016;131(6):803-820.
25. Lee W-H. Characterization of a newly established malignant meningioma cell line of the human brain: IOMM-Lee. *Neurosurgery*. 1990;27(3):389-396.
26. Mei Y, Bi WL, Greenwald NF, et al. Genomic profile of human meningioma cell lines. *PLoS One*. 2017;12(5):e0178322.
27. Tsai JC, Goldman CK, Gillespie GY. Vascular endothelial growth factor in human glioma cell lines: induced secretion by EGF, PDGF-BB, and bFGF. *J Neurosurg*. 1995;82(5):864-873.
28. Wilisch-Neumann A, Pachow D, Wallesch M, et al. Re-evaluation of cytostatic therapies for meningiomas in vitro. *J Cancer Res Clin Oncol*. 2014;140(8):1343-1352.
29. Baia GS, Slocum AL, Hyer JD, et al. A genetic strategy to overcome the senescence of primary meningioma cell cultures. *J Neurooncol*. 2006;78(2):113-121.
30. von Spreckelsen N, Waldt N, Poetschke R, et al. KLF4(K409Q)-mutated meningiomas show enhanced hypoxia signaling and respond to mTORC1 inhibitor treatment. *Acta Neuropathol Commun*. 2020;8(1):41.
31. Pachow D, Andrae N, Kliese N, et al. mTORC1 inhibitors suppress meningioma growth in mouse models. *Clin Cancer Res*. 2013;19(5):1180-1189.
32. Petermann A, Stampnik Y, Cui Y, et al. Deficiency of the protein-tyrosine phosphatase DEP-1/PTPRJ promotes matrix metalloproteinase-9 expression in meningioma cells. *J Neurooncol*. 2015;122(3):451-459.
33. Askham JM, Platt F, Chambers PA, Snowden H, Taylor CF, Knowles MA. AKT1 mutations in bladder cancer: identification of a novel oncogenic mutation that can co-operate with E17K. *Oncogene*. 2010;29(1):150-155.
34. Balasuriya N, Davey NE, Johnson JL, et al. Phosphorylation-dependent substrate selectivity of protein kinase B (AKT1). *J Biol Chem*. 2020;295(24):8120-8134.
35. Goldbrunner R, Stavrinou P, Jenkinson MD, et al. EANO guideline on the diagnosis and management of meningiomas. *Neuro Oncol*. 2021;23(11):1821-1834.
36. Keniry M, Parsons R. The role of PTEN signaling perturbations in cancer and in targeted therapy. *Oncogene*. 2008;27(41):5477-5485.
37. Guo G, Qiu X, Wang S, et al. Oncogenic E17K mutation in the pleckstrin homology domain of AKT1 promotes v-Abl-mediated pre-B-cell transformation and survival of Pim-deficient cells. *Oncogene*. 2010;29(26):3845-3853.
38. Lauring J, Cosgrove DP, Fontana S, et al. Knock in of the AKT1 E17K mutation in human breast epithelial cells does not recapitulate oncogenic PIK3CA mutations. *Oncogene*. 2010;29(16):2337-2345.
39. Mancini ML, Lien EC, Toker A. Oncogenic AKT1(E17K) mutation induces mammary hyperplasia but prevents HER2-driven tumorigenesis. *Oncotarget*. 2016;7(14):17301-17313.
40. De Marco C, Laudanna C, Rinaldo N, et al. Specific gene expression signatures induced by the multiple oncogenic alterations that occur within the PTEN/PI3K/AKT pathway in lung cancer. *PLOS One*. 2017;12(6):e0178865.
41. Rogers L, Barani I, Chamberlain M, et al. Meningiomas: knowledge base, treatment outcomes, and uncertainties. *A RANO Rev J Neurosurg*. 2015;122(1):4-23.
42. Figarella-Branger D, Vagner-Capodano AM, Bouillot P, et al. Platelet–derived growth factor (PDGF) and receptor (PDGFR) expression in human meningiomas: correlations with clinicopathological features and cytogenetic analysis. *Neuropathol Appl Neurobiol*. 1994;20(5):439-447.
43. Hilton DA, Shivane A, Kirk L, Bassiri K, Enki DG, Hanemann CO. Activation of multiple growth factor signalling pathways is frequent in meningiomas. *Neuropathol Off J Japanese Soc Neuropath*. 2016;36(3):250-261.
44. Mawrin C, Sasse T, Kirches E, et al. Different activation of mitogen-activated protein kinase and Akt signaling is associated with aggressive phenotype of human meningiomas. *ClinCancer Res*. 2005;11(11):4074-4082.
45. Kaley TJ, Wen P, Schiff D, et al. Phase II trial of sunitinib for recurrent and progressive atypical and anaplastic meningioma. *Neuro Oncol*. 2015;17(1):116-121.
46. Norden AD, Raizer JJ, Abrey LE, et al. Phase II trials of erlotinib or gefitinib in patients with recurrent meningioma. *J Neurooncol*. 2009;96(2):211-217.
47. Barresi V, Lionti S, La Rocca L, Caliri S, Caffo M. High p-mTOR expression is associated with recurrence and shorter disease-free survival in atypical meningiomas. *Neuropath Off J Japanese Soc Neuropath*. 2019;39(1):22-29.
48. James MF, Han S, Polizzano C, et al. NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth. *Mol Cell Biol*. 2009;29(15):4250-4261.
49. Johnson MD, O'Connell M, Vito F, Bakos RS. Increased STAT-3 and synchronous activation of Raf-1-MEK-1-MAPK, and phosphatidylinositol 3-kinase-Akt-mTOR pathways in atypical and anaplastic meningiomas. *JNeurooncol*. 2008;92(2):129-136.
50. Shih KC, Chowdhary S, Rosenblatt P, et al. A phase II trial of bevacizumab and everolimus as treatment for patients with refractory, progressive intracranial meningioma. *J Neurooncol*. 2016;129(2):1-8.
51. Mathew Thomas V, Bindal P, Vredenburgh JJ. Everolimus and bevacizumab in the management of recurrent, progressive intracranial NF2 mutated meningioma. *Case Rep Oncol*. 2019;12(1):126-130.
52. Hyman DM, Smyth LM, Donoghue MTA, et al. AKT inhibition in solid tumors with AKT1 mutations. *J Clin Oncol*. 2017;35(20):2251-2259.
53. Cornelius JF, Slotty PJ, Steiger HJ, Hänggi D, Polivka M, George B. Malignant potential of skull base versus non-skull base meningiomas: clinical series of 1,663 cases. *Acta Neurochir*. 2013;155(3):407-413.
54. Boetto J, Peyre M, Kalamarides M. Meningiomas from a developmental perspective: exploring the crossroads between meningeal embryology and tumorigenesis. *Acta Neurochir*. 2021;163(1):57-66.
55. Okuzumi T, Fiedler D, Zhang C, et al. Inhibitor hijacking of Akt activation. *Nat Chem Biol*. 2009;5(7):484-493.
56. Uematsu N, Weterings E, Yano K, et al. Autophosphorylation of DNA-PKCS regulates its dynamics at DNA double-strand breaks. *J Cell Biol*. 2007;177(2):219-229.
57. Fraser M, Harding SM, Zhao H, Coackley C, Durocher D, Bristow RG. MRE11 promotes AKT phosphorylation in direct response to DNA double-strand breaks. *Cell Cycle*. 2011;10(13):2218-2232.

How to cite this article: John P, Waldt N, Liebich J, et al. *AKT1* ^{E17K}-mutated meningioma cell lines respond to treatment with the AKT inhibitor AZD5363. *Neuropathol Appl Neurobiol*. 2022;48(2):e12780. doi:10.1111/nan.12780