

Targeted inhibition of dominant PI3-kinase catalytic isoforms increase expression of stem cell genes in glioblastoma cancer stem cell models

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Abstract. Cancer stem cells (CSC) exhibit therapy resistance and drive self-renewal of the tumour, making cancer stem cells an important target for therapy. The PI3K signalling pathway has been the focus of considerable research effort, including in glioblastoma (GBM), a cancer that is notoriously resistant to conventional therapy. Different isoforms of the catalytic subunit have been associated with proliferation, migration and differentiation in stem cells and cancer stem cells. Blocking these processes in CSC would improve patient outcome. We examined the effect of isoform specific PI3K inhibitors in two models of GBM CSC, an established GBM stem cell line 08/04 and a neurosphere formation model. We identified the dominant catalytic PI3K isoform for each model, and inhibition of the dominant isoform blocked AKT phosphorylation, as did pan-PI3K/mTOR inhibition. Analysis of *SOX2*, *OCT4* and *MSII* expression revealed that inhibition of the dominant p110 subunit increased expression of cancer stem cell genes, while pan-PI3K/mTOR inhibition caused a similar, though not identical, increase in cancer stem cell gene expression. This suggested that PI3K inhibition enhanced, rather than blocked, CSC activity. Careful analysis of the response to specific isoform inhibition will be necessary before specific subunit inhibitors can be successfully deployed against GBM CSC.

Introduction

Glioblastoma (glioblastoma multiforme, GBM) is a grade IV astrocytic tumour, the most aggressive form of astrocytic malignancy and the most common brain tumour in adults (1).

The disease can arise *de novo*, which accounts for 90% of cases, or as a secondary GBM, which is more common in younger people and progresses from a lower grade glial tumour. GBM is notoriously resistant to therapy, surgery cannot target the diffuse margins of the tumour, while cells have limited susceptibility to conventional radiation and temozolomide chemotherapy. With standard therapy, the median survival is 19 months post diagnosis (2). This dismal prognosis is in part due to the presence of cancer stem cells (CSC) in GBM. While the definition and characteristics of GBM CSC are controversial (3-6), these cells can generally considered to be intrinsically resistant to therapy (7), have self-renewal activity so can re-establish tumours after treatment (8) and are highly migratory and invasive (9), meaning they are likely to be present in the infiltrating edges of the tumour left behind after surgery. Therapies that target CSC and the characteristics of survival and self-renewal should dramatically improve the outcome for people with GBM.

Normal cellular processes of proliferation and survival are tightly regulated through a number of signalling pathways, including through the phosphatoinositide 3-kinase (PI3K) family proteins. The genes *PIK3CA*, *PIK3CB*, *PIK3CD* encode the catalytic subunit of class I_A kinases p110 α , p110 β and p110 δ , respectively, while the gene *PIK3CG* codes for a separate subunit class I_B kinase p110 γ . Mutation and dysregulation of the PI3K/AKT/mTOR signalling axis is a major contributor to tumorigenic behaviour in cells, with key roles in proliferation, migration and epigenetic silencing of developmental pathways. Hyper-phosphorylation of AKT is frequent in cancers, particularly where PI3K activity is decoupled from EGF signal transduction through the loss of PTEN function (10). This increases cancer proliferation and migration of cells (11,12) particularly in glioblastoma, where 85% of tumours have activating mutations in the RTK/PI3K/AKT pathway (13). This has made the PI3K pathway a potential pharmacological target in glioblastoma treatment (14). Inhibition of various kinases within these pathways is an effective treatment for GBM *in vitro*, however, clinical translation of these findings remains to be clarified. This is due in part to cross-talk and plasticity between cell signalling pathways (15) and to the

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intrinsic resistance of GBM cells to apoptosis (16,17). To overcome the adaptive response to PI3K/AKT/mTOR signalling, a complete understanding of the role of each individual effector is required.

The PI3K enzyme family mediate signal transduction via the phosphorylation of the 3'-OH of phosphatidylinositols localised to the internal surface of the cell membrane. Once activated, these messengers are responsible for downstream transduction through phosphorylation of AKT and the associated cellular responses. While class-I PI3K p110 isoforms share obvious structural similarities, a growing body of evidence describes discrete physiological roles for each isoform (18-24), with marked alterations in dominant isoform across malignancies (25), especially in PTEN deficient solid tumours (26). Activating point mutations are reported in the class I_A PI3K p110 α isoform in glioblastoma (27,28), along with gene copy amplifications of both PI3K p110 α and PI3K p110 δ (29,30). Isoform specific inhibition may provide significant benefits if used in the appropriate genetic background (31). A number of strategies using isoform specific PI3K inhibitors, alone or in combination with additional compounds are currently in clinical trial (26,32).

Most investigation has focused on PI3K activity in the proliferative and migratory phenotypes of differentiated glioblastoma cells (14,25, 33-36), however, little is known regarding the activity of PI3K isoforms in GBM CSC. In the present study, we examined expression and signalling of class I_A PI3K isoforms in two models of GBM CSC. The cell line 08/04 has high expression of embryonic and neural stem progenitor genes including SOX2, OCT4 and MSI1 and recapitulates a GBM phenotype following intra-cranial implantation (37). These cells were selected to model the effect of PI3K inhibition in maintenance of an established cancer stem cell phenotype. To determine how PI3K inhibition affects the acquisition of stem-like properties, an LN18 neurosphere model was utilised (37). PI3K isoforms p110 α , p110 β and p110 δ were selectively inhibited and effects on proliferation and migration assessed. This revealed a different dominant isoform in each model. Furthermore, changes in gene expression were evaluated following inhibition of the dominant isoform, to explore effects on stem-like and differentiation phenotypes. Inhibition increased transcription of cancer stem cell genes in both models, suggesting de-differentiation in response to blockade of the PI3K/AKT/mTOR signalling axis.

Materials and methods

Cell lines and tissue culture. The human GBM cell line LN18 was obtained from the American Type Culture Collection and was maintained in RPMI-1640 growth medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Auckland, New Zealand). Cells were discarded within 20 passages of initial receipt and replaced with cryopreserved stock cultures. Primary human glioblastoma stem cells 08/04 (37) were maintained as an adherent monolayer in serum-free stem cell medium (SCM) supplemented with heparin, hEGF and bFGF (NeuroCult NS-A proliferation kit; Stem Cell Technologies, Tullamarine, VIC, Australia) as recommended by the manufacturer. All cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere and subject to regular screening for myco-

plasma contamination (e-Myco™ Mycoplasma PCR detection kit; Intron Biotechnology, Sangdaewon-dong, Korea).

PI3K inhibitors. A66 (38), TGX221 (39), IC87114 (40), BEZ235 (41), targeting p110 α , p110 β , p110 δ and pan-specific PI3K, respectively, were dissolved in sterile DMSO. The starting dose for each inhibitor was chosen based on the IC₅₀ in a cell-free kinase assay, then titrated down from 100x IC₅₀ to find the maximal tolerated dose. A66 was used at 537.5 nM (12.5x IC₅₀), TGX221 at 850 nM (100x IC₅₀), IC87114 at 6 μ M (100x IC₅₀) and BEZ235 at 112.5 nM (1.5x IC₅₀).

RT-qPCR. RNA was extracted using the ZR Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA, USA) in accordance with the manufacturer's protocol. cDNA was synthesised from 250 ng of total RNA via the iScript cDNA synthesis kit (Bio-Rad Laboratories, Auckland, New Zealand). The reverse transcription was run with a Bio-Rad iCycle PCR machine with the following parameters: 25°C 5 min, 42°C 30 min, 85°C 5 min and the sample was then held at 4°C. QuantiTect Primer Assays (Qiagen, Melbourne, VIC, Australia) targeted to 18S (QT00199367), HPRT1 (QT00059066), SOX-2 (QT00237601), MSI-1 (QT00025389), OCT-4 (QT00210840), GFAP (QT00081151), PIK3CA (QT00014861), PIK3CB (QT01668821) and PIK3CD (QT00091840) were used with SensiMix SYBR Low-ROX kit (Bioline, London, UK). The qPCR was run on the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Auckland, New Zealand) with the following parameters: Stage 1: 94°C 15 min, Stage 2 (40 repeats): 94°C 15 sec, 55°C 30 sec, 72°C 35 sec. Each QuantiTect Primer Assay is pre-validated to have equivalent amplification efficiency, thus, direct normalisation to HPRT and 18s was carried out via the $\Delta\Delta$ Ct method. Ct (cycle threshold) values were exported from the 7500 System software into an Excel file (Microsoft, Redmond, WA, USA) and all following calculations were done using Excel software. The triplicate Ct values for each test primer pair were averaged and then normalized to the average Ct value of 18S primer pair for each sample to give the Δ Ct value. Then the difference between the Δ Ct value for the control and Δ Ct value for the treated cells was calculated to give a $\Delta\Delta$ Ct value. The fold change in gene expression was calculated using $2^{-\Delta\Delta$ Ct}.

Growth factor signalling. Two million cells were plated in SCM (08/04) or complete RPMI-1640 (LN18) and allowed to proliferate for 2 days. Media were replaced with NeuroCult NS-A Medium with proliferation supplement and heparin, but without EGF or FGF (08/04), or RPMI-1640 without serum (LN18). After 16-h growth factor withdrawal, cells were treated with PI3K inhibitor or vehicle control for 1 h before addition of 20 ng/ml hEGF and 10 ng/ml hFGF- β (08/04) or 10% FBS v/v (LN18) to re-stimulate PI3K activity. Cells were incubated for 15 min then harvested for analysis by western blot analysis.

Western blotting. Cells were lysed in 70 mM NaCl, 20 mM Tris, 0.1% Tween with 1X protease inhibitor (Complete ULTRA EDTA free; Roche, Auckland, New Zealand) and 1X phosphatase inhibitor (PhosStop; Roche) and maintained on ice for all subsequent steps. Soluble material was retained by centrifuga-

tion of lysates and total protein quantified. Protein (50 μg) was electrophoresed with Amersham GE Precast gels, transferred to PVDF membrane (Bio-Rad Laboratories) and blocked in 5% BSA (ICP Bio, Auckland, New Zealand) in PBS. The primary antibodies used were: polyclonal rabbit anti-human pAKT Ser473, monoclonal rabbit anti-human pAKT Thr308 (C31E5E), polyclonal rabbit anti-human AKT (Cell Signaling Technology, Inc., Danvers, MA, USA) and monoclonal, mouse anti-human α -tubulin (Sigma-Aldrich, Auckland, New Zealand). Primary antibodies were sequentially blotted at a concentration of 1:1,000 overnight followed by the appropriate anti-mouse or anti-rabbit horseradish peroxidase antibody at a concentration of 1:7,000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected by enhanced chemiluminescence (SuperSignal Pico; Pierce, Auckland, New Zealand). Chemiluminescent images were captured by the Carestream Gel Logic 4000 Pro using Carestream MI NE software (Carestream, Rochester, NY, USA). Membranes were stripped (Restore Western Blot stripping buffer; Thermo Fisher Scientific, Auckland, New Zealand) and re-imaged between antibodies to confirm complete signal ablation.

MTS assay. 08/04 or LN18 cells were seeded at 25,000 cell/well on a 96-well plate in the appropriate complete growth media. Cells were allowed to establish for 24 h before addition of PI3K inhibitors, which were refreshed every 48 h. Five days following treatment, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS) reagent per well (CellTiter 96 Aqueous One Solution; Promega, Madison, WI, USA) was added and incubated at 37°C, 5% CO₂ for 4 h before absorbance at 490 nm was measured. Data were normalised to the media only control.

Migration assay. 08/04 cells were seeded at 100,000 cells/well on a 24-well plate in SCM and allowed to become confluent (48-72 h), then pre-treated with PI3K inhibitor for 1 h. A scratch injury was formed in the monolayer and photographed using the x10 objective lens every 2 h for a total of 16 h using the Olympus IX51 inverted microscope with ColorView III 5 MP camera and Cell A software (Olympus, Auckland, New Zealand). Cell migration was assessed at each time-point by area measurements generated in ImageJ software (33).

Differentiation assay. 08/04 cells were seeded in SCM at 96,000 cells/well on a 6-well plate and allowed to establish for 2 days before addition of PI3K inhibitor, vehicle control, media only control or 10% FBS v/v to induce differentiation. Cells were incubated for 5 days with inhibitors renewed at day 3, then harvested for RT-qPCR analysis of embryonic and neural stem cell genes.

Immunofluorescence of GFAP. 08/04 cells were seeded at 96,000 cells/well on coverslips in 6-well plates in SCM and treated for 5 days with PI3K inhibitor, vehicle control, media only control or 10% FBS v/v to induce differentiation. Cells were fixed with 95% ethanol and 5% acetic acid v/v before permeabilisation with PBS/0.5% Tween. Cells were blocked in 2% BSA in PBS with 0.1% Tween, probed with monoclonal mouse anti-GFAP (GA5; Cell Signaling Technology, Auckland, New Zealand) at 4°C overnight. Slides were washed

Table I. The PI3K catalytic subunits expressed in 08/04 cells.

	α subunit	β subunit	δ subunit
Cycle threshold (Ct)	27.04	28.86	34.04
ΔCt (18s)	12.86	14.69	19.86

Expression of the PI3K catalytic subunits as shown by Cycle threshold (Ct) and ΔCt (relative to 18s) values in 08/04. Data are average of triplicate experiments.

then incubated with Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Auckland, New Zealand) at room temperature for 1 h. Slides were washed, DAPI stained (UltraCruz mounting media; Santa Cruz Biotechnology) then imaged by the Olympus BX51TF fluorescent compound microscope (Olympus). Representative pictures of each condition were taken in brightfield, DAPI and UV channels and combined using Pixelmator software for Mac (Pixelmator, Vilnius, Lithuania).

Neurosphere induction. LN18 cells were pre-treated with inhibitor for 5 days in complete RPMI-1640, with drug refreshed at day 3. Cells were then seeded into 24-well plate format at 500 cells/well in SCM containing PI3K inhibitor. After 7 days in culture, developed neurospheres were harvested and prepared for analysis of embryonic and neural stem cell genes by RT-qPCR.

Statistical analysis of in vitro experiments. All statistical analyses were performed using GraphPad Prism version 4.00 (Graphpad Software, Inc., La Jolla, CA, USA). Student's unpaired, two-tailed t-tests were performed in sequence to assess statistical significance between two groups, with P-values of <0.05 considered as statistically significant.

Results

PI3K p110 α , p110 β and p110 δ are expressed but differentially active in 08/04 cells. To determine which isoforms of p110 were expressed in the GBM stem cell model 08/04, expression of *PIK3CA*, *PIK3CB* and *PIK3CD* was measured by quantitative RT-PCR. This confirmed that all isoforms were expressed, although *PIK3CD* transcript was present at a lower level than the other two (Table I).

To determine which isoform/s contributed to growth factor-driven signal transduction in the 0904-SC models, cells were pre-treated with a specific subunit inhibitor, either A66 (p110 α), TGX221 (p110 β) or IC87114 (p110 δ) and the effect on growth factor-driven signal transduction measured by phosphorylation of AKT (Fig. 1). The initial dose chosen was 100x the IC₅₀ of each isoform in a cell-free kinase assay. For each inhibitor this was 10-20x the IC₅₀ in cell-based assays, and each was well within the specific range (38-41). The pan-PI3K/mTOR inhibitor BEZ235 was used as a positive control for lost AKT phosphorylation, and to compare specific isoform inhibition to broad-spectrum inhibition. The BEZ235 dose was titrated from 100x to 1.5x IC₅₀. At this concentration,

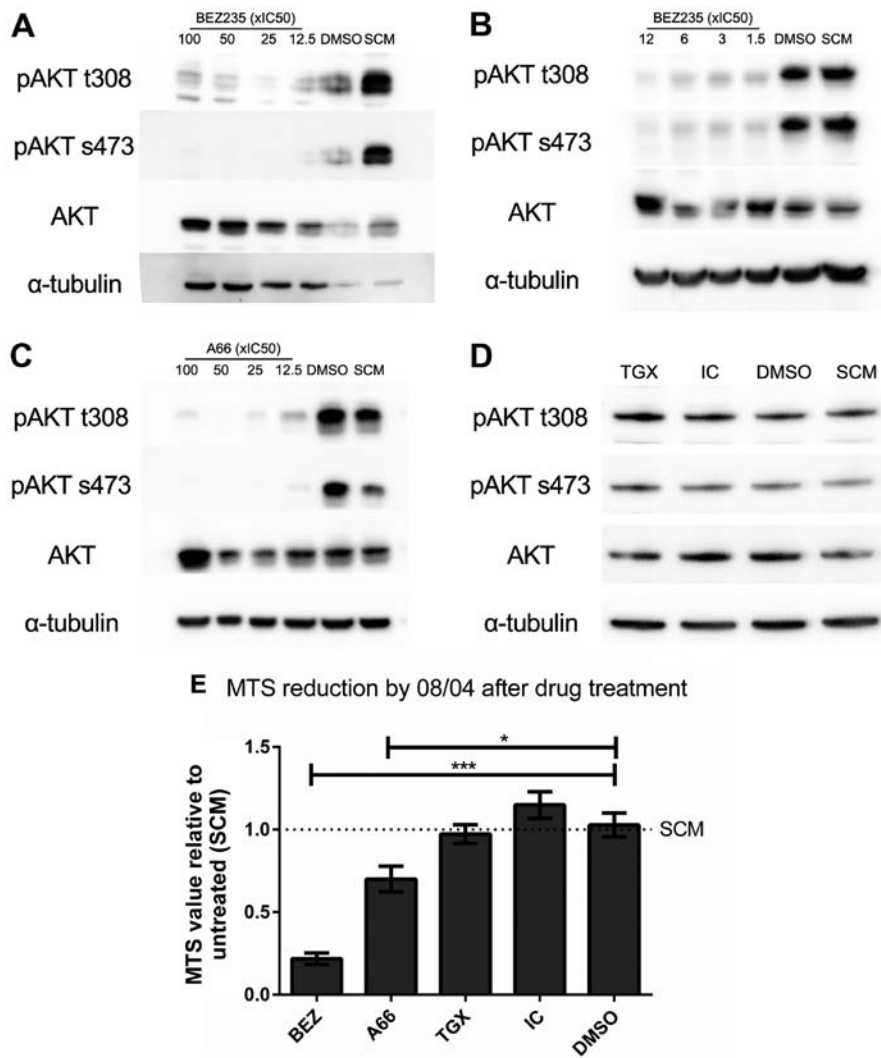


Figure 1. PI3K catalytic subunits are active in 08/04. Western blots showing a dose titration of (A) BEZ235 at 100-12.5x IC₅₀, (B) BEZ235 at 12-1.5x IC₅₀ (C) A66 at 100-12.5x IC₅₀ showing inhibition of pAKT at both t308 and s473 residues in 08/04, alongside DMSO and SCM (stem cell media) controls, representative of at least 3 independent experiments. (C) Western blot analysis of 08/04 treated with TGX221 (TGX) and IC87114 (IC) at 100x IC₅₀ showing no inhibition of pAKT, alongside DMSO and SCM controls. Data are representative of at least 3 independent experiments. (D) Graph showing reduction of MTS reagent by 08/04 treated with inhibitors, relative to untreated controls. Columns represent mean values ± SEM of triplicate experiments. *P<0.05, ***P<0.001 Student's t-test.

substantial inhibition of phospho-AKT was observed (Fig. 1A and B), but cellular viability and processes were sufficiently conserved to purify intact RNA from treated cells. The dose of 112.5 nM was used throughout. A similar titration of A66 was performed, and a dose of 12.5x IC₅₀ (537.5 nM) was identified. Inhibition of p110α by A66 produced a significant reduction in AKT phosphorylation at all doses tested, with no detectable signal for serine 473 and a marked reduction of threonine 308 phosphorylation (Fig. 1C). In contrast, inhibition by TGX221 and IC87114 did not change phosphorylation at either residue at the highest starting dose (Fig. 1D) and had no observable impact on viability, indicating that PI3K isoforms p110β and p110δ did not significantly contribute to signalling through AKT in the 08/04 model.

Inhibition of p110α, but not p110β or p110δ, inhibits proliferation in 08/04 cells. To examine the functional consequence of selective PI3K p110 inhibition, cytoplasmic reduction of NAD(P)H was measured using MTS reduction (42,43)

following 5 days treatment with each PI3K inhibitor. Based on the dose titration, treatment was not accompanied by widespread cell death so the MTS value directly correlated with cell number, and changes in value reflected cellular proliferation. Consistent with the reduction of pAKT, inhibition of p110α by A66, and PI3K/mTOR inhibition by BEZ235 significantly reduced final cell number, and hence proliferation, for 08/04 in contrast to untreated and vehicle treated cells (Fig. 1E). Neither TGX221 nor IC87114 affected final cell number, again consistent with the observation that p110β and p110δ did not contribute to signalling through AKT in this model. These data were indicative of a cytostatic, rather than toxic, effect of inhibition, as observed previously in inhibition of the PI3K/AKT/mTOR axis (15,44).

PI3K signalling does not drive migration in 08/04 cells. Given the potential for CSC to contribute to the invasive nature of glioblastoma *in vivo*, the effect of isoform selective PI3K inhibition on migration was assessed with the scratch assay. All

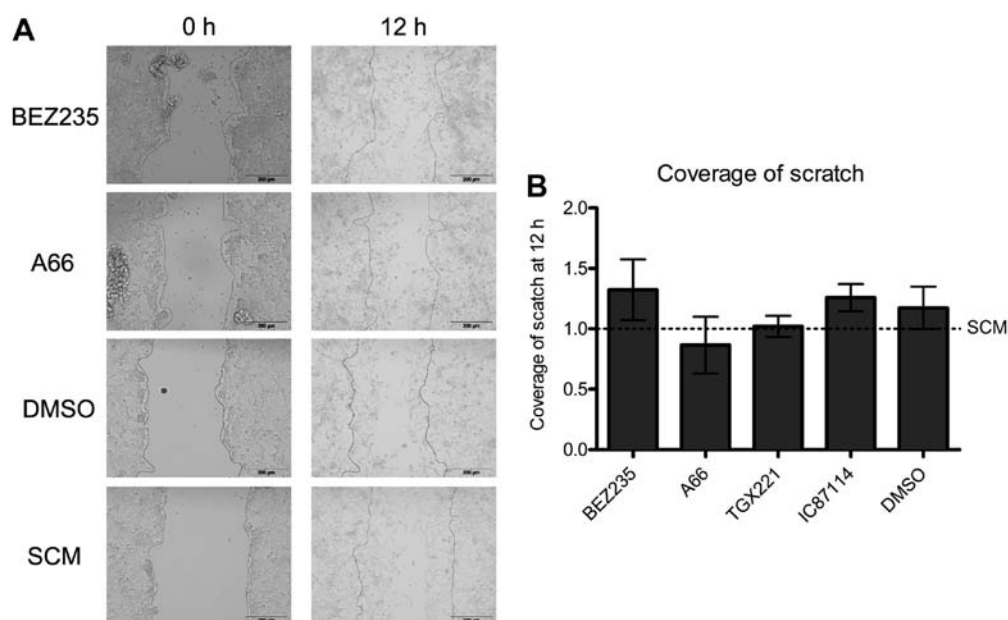


Figure 2. Migration of 08/04 cells was unchanged by inhibition with PI3K inhibitors. (A) Representative photos of scratch assay showing the migration of 08/04 pre-treated with PI3K inhibitors. (B) Percent coverage of scratch at 12 h for all treatment groups. Bars represent mean \pm SEM of triplicate experiments.

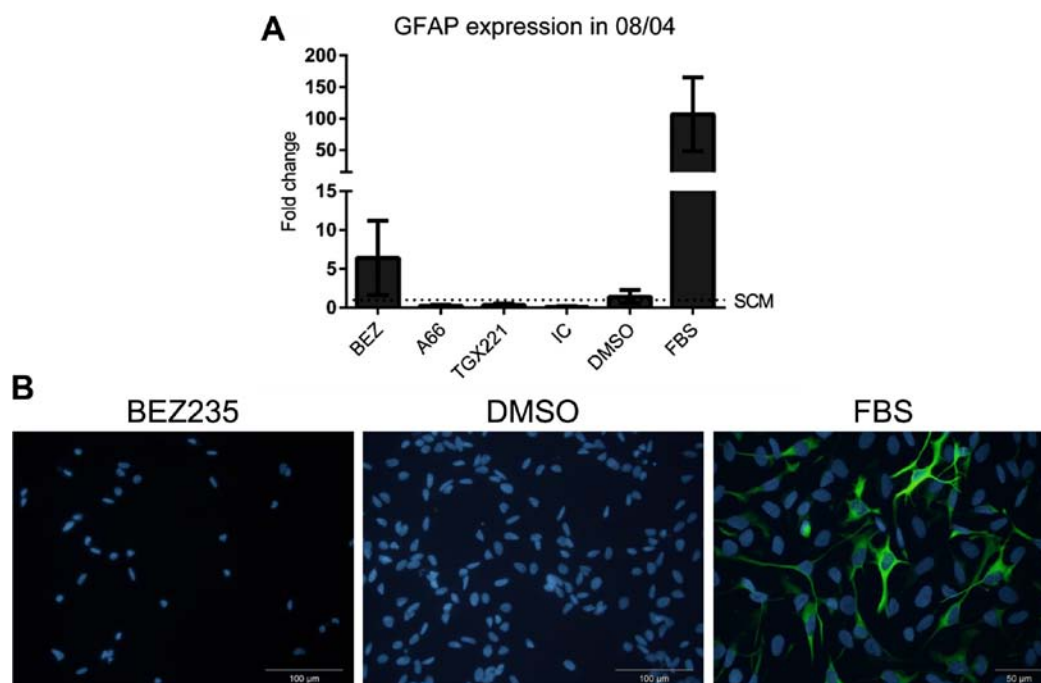


Figure 3. 08/04 exposed to PI3K inhibitors did not differentiate. (A) Expression of GFAP in 08/04 after exposure to PI3K inhibitors, DMSO control, SCM control and FBS containing media (FBS). Columns indicate mean \pm SEM of triplicate experiments. (B) Immunofluorescence of 08/04 indicating GFAP in green (Alexa Fluor-488) and nuclei in blue (DAPI) in BEZ235 treated cells, DMSO control and FBS. Data are representative of three independent experiments.

cells treated with PI3K inhibitors remained highly migratory, similar to vehicle treated cells (Fig. 2) and despite reduced AKT phosphorylation in the presence of A66 or BEZ235. No difference in migration was observed at any of multiple time-points, with all scratches filled in completely by 16 h. Migration data from 12 h is shown.

PI3K inhibition does not induce differentiation of 08/04 cells. Inhibition of either p110 α or the PI3K/mTOR pathway

suppressed proliferation of 08/04 cells through a cytostatic mechanism, as no cell death was observed. We next determined the effect of isoform selective PI3K inhibition on differentiation of cells. GFAP is an intermediate filament protein of lineage committed glial cells, and expression indicates differentiation down the glial lineage. Using GFAP as a marker for differentiation, 08/04 cells exposed to 10% FBS had 100-fold upregulation of GFAP mRNA (Fig. 3A) and increased protein expression (Fig. 3B). However, isoform selective inhibition did not upregu-

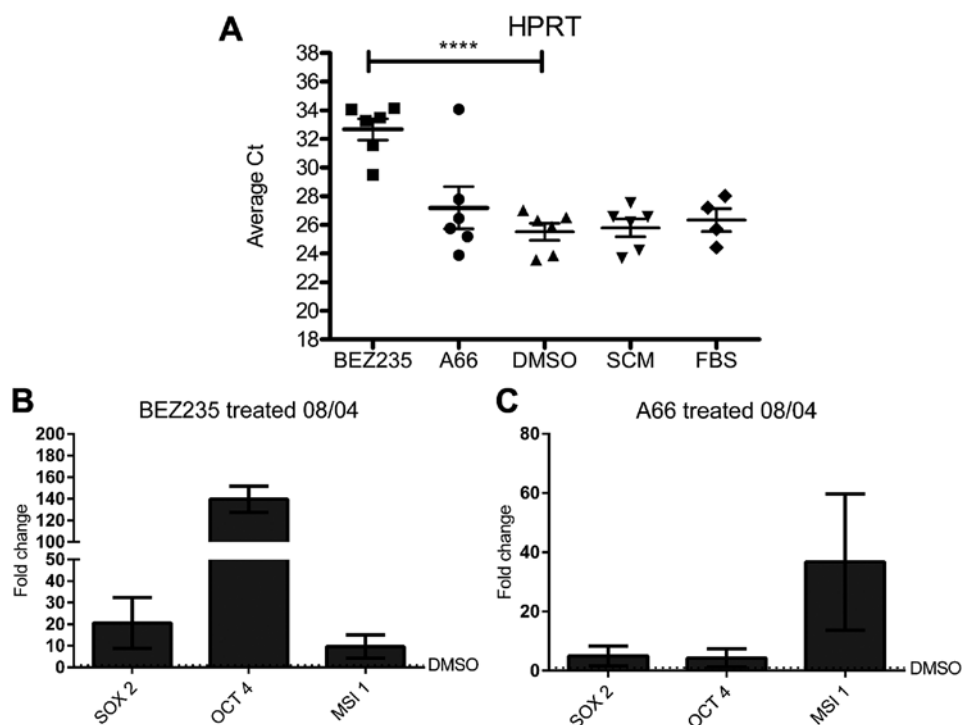


Figure 4. Treatment with PI3K inhibitors induced the expression of pluripotency and neural stem cell markers. (A) Cycle threshold (Ct) values for the expression of the housekeeping gene HPRT in 08/04 exposed to PI3K inhibitors BEZ235 (1.5x IC₅₀) and A66 (12.5x IC₅₀), DMSO, SCM and FBS. Expression of pluripotency markers SOX 2 and OCT 4 and neural stem cell marker MSI 1 in 08/04 treated with: (B) BEZ235; and (C) A66. Columns represent mean \pm SEM of triplicate experiments. ****P<0.0001 paired Student's t-test.

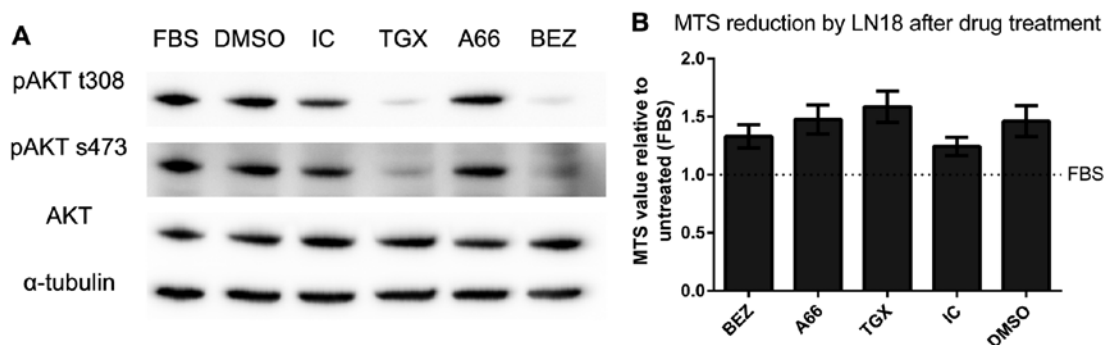


Figure 5. PI3K catalytic subunits were active in LN18. (A) Western blot analysis showing inhibition of pAKT with BEZ235 (1.5x IC₅₀) and TGX221 (100x IC₅₀) at both t308 and s473 residues in LN18 alongside LN18 treated with A66 (12.5x IC₅₀), IC87114 (100x IC₅₀), DMSO and FBS (RPMI containing 10% FBS) controls. Data are representative of at least 3 independent experiments. (B) Graph showing reduction of MTS reagent by LN18 treated with inhibitors, relative to untreated controls. Columns represent mean values \pm SEM of triplicate experiments.

late the GFAP transcript (Fig. 3A) and neither BEZ235 nor A66 induced expression of GFAP protein (Fig. 3B). This suggested that PI3K inhibition was not associated with increased differentiation of glioblastoma cancer stem cells.

Surprisingly, inhibition of PI3K by BEZ235 reproducibly appeared to upregulate GFAP mRNA, in contrast to the lack of GFAP protein detected by immunofluorescence. On further investigation, treatment by BEZ235 was found to decrease the relative transcript abundance of the gene used for normalisation of gene expression, HPRT (Fig. 4A) as well as a number of other genes commonly used for normalisation (18s rRNA, TBP, β -actin; data not shown). This led to the apparent increase in GFAP transcription. Importantly, the effect was restricted to BEZ235 treatment (Fig. 4A).

This prevented direct comparison of gene expression between BEZ235 treated cells and other inhibitor treatments. However, within a treatment group, gene expression data were directly comparable, and this approach was used for all further qRT-PCR analysis.

PI3K inhibition increased expression of CSC genes in 08/04 cells. We next examined expression of stem cell genes in our GBM CSC model. Cells were treated with inhibitor for 5 days and expression of the embryonic and neural stem cell transcription factors SOX2, OCT4 and MSI1 was measured. A surprising but consistent pattern of upregulation of CSC gene expression was observed. When compared to vehicle treated cells, A66 treatment led to an average 5-fold increase in SOX2

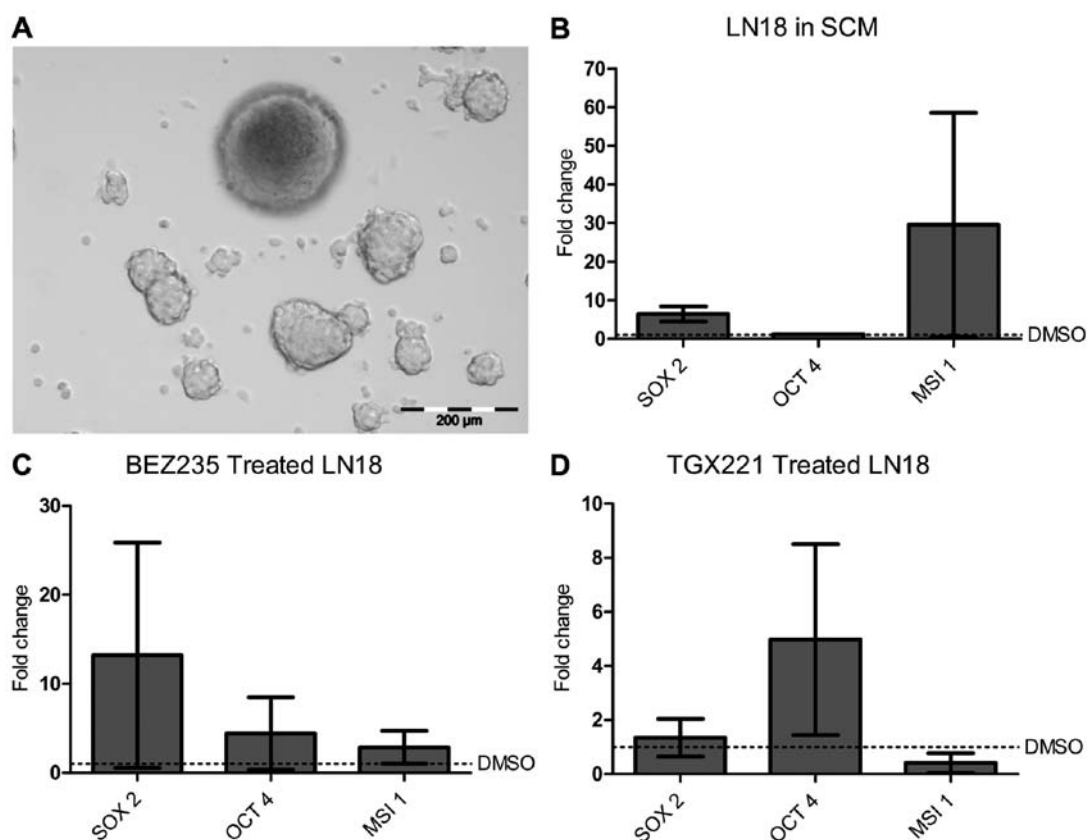


Figure 6. Induction of stem cell gene expression is changed by PI3K inhibition. (A) Representative picture of spheres formed when LN18 cells were grown in SCM. Expression of self-renewal marker SOX2, pluripotency marker OCT4 and neural stem cell marker MSI1 in LN18 cells in: (B) untreated cells grown in SCM; (C) cell treated with BEZ235, then grown in SCM; (D) cells treated with TGX221, then grown in SCM. Columns indicate mean \pm SEM of duplicate experiments.

Table II. The PI3K catalytic subunits expressed in LN18.

	α subunit	β subunit	δ subunit
Cycle threshold (Ct)	27.98	29.65	29.55
Δ Ct (HPRT)	1.81	3.91	4.53

Expression of the PI3K catalytic subunits as shown by cycle threshold (Ct) and Δ Ct (relative to HPRT) values in LN18. Data are average of triplicate experiments.

and OCT4, and a 30-fold average increase in *MSI1* mRNA (Fig. 4C). Treatment with BEZ235 also increased expression of *SOX2* and *MSI1*, with a notable increase in *OCT4* expression (Fig. 4B).

PI3K p110 α , p110 β and p110 δ are expressed but differentially active in LN18. The 08/04 cells effectively model maintenance of an established cancer stem cell phenotype. In order to determine the effect of PI3K p110 inhibition on the acquisition of CSC characteristics, a neurosphere formation model was used. LN18 cells proliferate as an adherent monolayer when cultured in 10% FBS, but form neurospheres on transfer to neural stem cell media at low cell density (37). This acquisition of a stem cell phenotype, or de-differentiation, is a model of cancer stem cell initiation.

Basal expression of PI3K p110 α , p110 β and p110 δ in serum-grown GBM cell line LN18 was established by qRT-PCR and the isoforms found to be of equal abundance (Table II). Analysis of signalling through the PI3K/AKT/mTOR axis revealed a difference in the dominant isoform between LN18 and 08/04. In contrast to 08/04, inhibition of catalytic subunit p110 α by A66 had no effect on pAKT relative to vehicle control (Fig. 5A). Instead, inhibition of p110 β by TGX221 suppressed phosphorylation at both sites. Inhibition of p110 δ by IC87114 had no significant effect on the phosphorylation of AKT at either residue, similar to 08/04. As expected, inhibition of PI3K/mTOR activity by BEZ235 suppressed phosphorylation of AKT at both threonine 308 and serine 473. Despite suppressed AKT phosphorylation by BEZ235 and TGX221, there was no significant difference in final number of LN18 cells (Fig. 5B) following PI3K inhibition, implying no loss of proliferation or increase in cell death.

PI3K inhibition prior to neurosphere formation enhances expression of neural and embryonic transcription factors. Formation of LN18 neurospheres was previously characterised by upregulation of embryonic and neural stem cell gene transcription (37). LN18 cells were pre-treated with TGX221, BEZ235 or DMSO, then transferred to serum-free stem cell media to induce sphere formation (Fig. 6A). The effect of sphere formation on expression of the CSC gene panel was compared between DMSO and inhibitor treated cells, either

BEZ235 (Fig. 6C) or TGX221 (Fig. 6D). There was no significant change in the rate of sphere formation (data not shown), but in each case, expression of at least 1 stem cell gene increased with PI3K inhibition, with a notable increase in *OCT4* expression compared to untreated cells, similar to the 08/04 data (Fig. 4B). Also similar to PI3K inhibition of 08/04, increased gene expression was more pronounced with pan-PI3K/mTOR inhibition (Fig. 6C), again suggesting that multiple kinases regulated CSC gene expression.

Discussion

Activation of the PI3K/AKT/mTOR signalling pathway reportedly enriches for highly tumorigenic stem-like cancer cells (45,46) and PI3K inhibition promotes differentiation, potentially reducing the self-renewal potential of cancer stem cells within tumours. The different isoforms of the PI3K catalytic subunit, p110 α , β and δ , have been suggested to have differential roles in pathway activation, and differential effects on proliferation, migration and differentiation. Based on the de-differentiated, self-renewing nature of glioblastoma cancer stem cells, we hypothesised that isoform-specific PI3K inhibition might specifically target components of cancer stem cell activity as has been found for embryonic stem cells (47). We looked at activity of the PI3K catalytic subunits in two different GBM cancer stem cell models, using specific inhibitors to look first at any role in cancer stem cell proliferation, and secondly in the acquisition of a stem-like phenotype. The origin of the GBM CSC is generally thought to be the acquisition of key stem cell characteristics by a lineage-committed cell (48). The LN18 sphere formation assay was used to look specifically at PI3K activity in the acquisition of stem cell gene expression, as a marker of de-differentiation of a lineage-committed cell.

The specificity and selectivity of the PI3K inhibitors was critical to these experiments. The dose titrations started at 100x the IC₅₀ for a cell-free kinase assay. At this dose, there was no effect of p110 δ inhibition in either cell line with IC87114, which we interpreted as no activity. This was equivalent to 12x the cellular IC₅₀. Similarly, there was no effect of TGX221 on 08/04, at 17x the cellular IC₅₀. While we have not formally excluded that either inhibitor would not have been effective at higher concentration this seems unlikely. There is also the possibility of non-specific activity if an inhibitor is used at a high dose. There was no non-specific activity of TGX221 and IC87114, but this could potentially have been observed with the p110 α inhibitor. However, selectivity of A66 is very high. At 537.5 nM, the dose used in this study, there is >40-fold higher concentration needed to inhibit the δ isoform, and >400-fold for inhibition of β (38). Hence, we were well within the selective range.

While activation of the overall PI3K pathway led to constitutive AKT phosphorylation in both GBM CSC models, the activity of the p110 catalytic sub-units were different in each model. A single dominant isoform was identified in each model, which differed between the two models, p110 α was active in 08/04, and p110 β in the LN18 neurosphere model. Inhibition of PI3K, whether specific to the dominant p110 isoform, or pan-PI3K/mTOR inhibition, had similar effects on proliferation in each model. PI3K inhibition blocked AKT phosphorylation and reduced proliferation in 08/04. However,

neither inhibitor had any effect on LN18 proliferation despite blocking AKT phosphorylation. The two GBM cell lines used are very similar in appearance and behaviour (37), but these data indicate that there are fundamental differences underlying their PI3K biology. This could include differences in the mutational status of the PI3K pathway, such as PTEN loss of function or activating PI3K mutations. In this regard, LN18 cells are known to have wild-type PTEN (49), but the genetic background of the 08/04 line is completely unknown. As differential expression of targets obviously leads to differential efficacy of targeted therapies, these data highlight the importance of further study into the PI3K pathway in GBM before any successful implementation of PI3K inhibition for cancer stem cell targeting.

A recently proposed competition model for p110 association with activated receptor tyrosine kinases (RTKs) (18) highlights the differential role of the isoforms in the regulation of PI3K activity. Differences in both lipid- and protein-kinase activities have been described for PI3K p110 isoforms (50,51), which allow precise control over downstream transduction in response to activating signals. In addition, rebound signalling in other pathways following inhibition of dominant signalling isoforms has been identified in various cell lines (52,53), demonstrating the adaptive nature of cancer cell signalling pathways and consequent cell survival.

Inhibition of either PI3K/mTOR or the dominant isoform altered expression of embryonic and neural stem cell genes in both CSC models, with overall increased stem cell gene expression. The precise effect of PI3K inhibition on stem cell gene expression varied between inhibitors. Whether this difference between pan-PI3K/mTOR and dominant isoform inhibition indicates a link between a given isoform and a corresponding specific CSC gene is not clear. Any contributory role of minor p110 isoforms, or of other signalling pathways to CSC gene expression requires further analysis.

Given that PI3K/AKT activation was previously reported to increase cancer stem cell gene expression (45,46), the increased stem cell gene expression with PI3K inhibition was unexpected and inconsistent with those previous reports. Instead, it reflected data on PI3K inhibition in embryonic stem cells (47). It is possible that in our models, PI3K inhibition directly increased cancer stem cell gene expression, and simply reflected de-differentiation and acquisition of a more stem-like phenotype. Alternatively, the increase we observed could result from a more complex disruption to the stem cell phenotype. We speculate that while PI3K inhibition attempted to force cells to differentiate, another signalling pathway intervened to push stem gene expression back and restore the CSC phenotype, limiting the effect. In this circumstance, the increased stem cell gene expression we observed could be an 'over-shoot' phenomenon. Signal transduction in the PI3K/AKT/mTOR axis is partially mediated through a cross-inhibitory relationship with the MEK/ERK pathway in GBM (54). MEK/ERK signalling is highly integrated with the downstream transcriptional activation of embryonic and neural stem cell genes (55) and it is plausible that PI3K/MEK/ERK cross-talk plays a role in cancer stem cell gene expression and activity (56,57).

Regardless of the mechanism, increased expression of genes that drive a cancer stem cell phenotype would make

PI3K inhibition counter-productive for cancer stem cell targeting, and for treatment of GBM. These data illustrate the complexity of PI3K biology in the cancer stem cell phenotype. A careful analysis of the role of other signalling pathways in the response to specific isoform inhibition will be necessary before these inhibitors can be successfully deployed against GBM cancer stem cells, or other tumours with a cancer stem cell component.

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