Synthesis of a novel Pyrano[2,3-c]pyrazole enabling PKB β /AKT2 inhibitory and *in vitro* anti-glioma activity

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Abbreviations: PKB, Protein Kinase B; GBM, Glioblastoma multiforme; NMR, nuclear magnetic resonance; HRMS, High-resolution mass spectrometry; UVM, Uveal Melanoma; LIHC, Liver hepatocellular carcinoma; KICH, Kidney Chromophobe; ESCA, Esophageal carcinoma; CHOL, Cholangiocarcinoma; UCS, Uterine Carcinosarcoma; KIRP, Kidney renal papillary cell carcinoma; BRCA, Breast invasive carcinoma; READ, Rectum adenocarcinoma; HNSC, Head and Neck squamous cell carcinoma; MESO, Mesothelioma; PAAD, Pancreatic adenocarcinoma; PRAD, Prostate adenocarcinoma; SARC, Sarcoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; LUAD, Lung adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; SKCM, Skin Cutaneous Melanoma; OV, Ovarian serous cystadenocarcinoma; THCA, Thyroid carcinoma; LUSC, Lung squamous cell carcinoma; KIRC, Kidney renal clear cell carcinoma; ACC, Adrenocortical carcinoma; UCEC, Uterine Corpus Endometrial Carcinoma; LGG, Brain Lower Grade Glioma; TCGA, The Cancer Genome Atlas; EGF, Epidermal growth factor; FGF, Fibroblast growth factor; IDH, isocitrate dehydrogenase.

Abstract

A series of novel pyrano[2,3-c]pyrazoles were synthesized and screened for their potential to inhibit kinases and exhibit anti-cancer activity against primary patient derived glioblastoma 2D cells and 3D neurospheres. A collection of 10 compounds were evaluated against glioma cell lines, with compound 4j exhibiting promising glioma growth inhibitory properties. Compound 4j was screened against 139 purified kinases and exhibited low micromolar activity against kinase AKT2/PKB β . AKT signalling is one of the main oncogenic pathways in glioma and is often targeted for novel therapeutics. Indeed AKT2 levels correlated with glioma malignancy and poorer patient survival. Compound 4j inhibited the 3D neurosphere formation in primary patient derived glioma stem cells and exhibited potent EC₅₀ against glioblastoma cell lines. Herein we establish a novel biochemical kinase inhibitory function for a pyrano[2,3-c]pyrazole derivative and further report its anti-glioma activity *in vitro* for the first time.

1. Introduction

Protein kinases have diverse roles in cellular physiology¹. Over 520 protein kinases have been included in the kinome tree² with more being identified as research progress³. The oncogenic roles of protein kinases have long been targeted for therapeutic benefits^{4,5} while a few kinases exhibit tumour

suppressor properties as well ^{4, 6}. Although over 70 kinase inhibitors have been approved for clinical use ⁵, very few options are available for patients with grade IV glioma or glioblastoma multiforme where median survival remains at 12-18 months from initial diagnosis⁷. Gliomas are the most common central nervous system cancer, arising from glial cells within the brain. Histologically subclassifying the tumour into IDH1 mutation and chromosome 1p/19q co-deletion status can improve the patients therapeutic options^{9, 10}. Although IDH1 mutant and 1p/19q co-deleted glioma improves the outcome of most patients¹⁰, many often develop resistance and refractory tumour development. Chromosome 19q, in fact, has a pro-tumorigenic resident kinase called AKT2/PKB β ¹¹. AKT is an established oncogenic kinase with at least three mammalian isoforms AKT1/PKB α , AKT2/PKB β , and AKT3/PKB γ of which AKT2 plays major pro-oncogenic roles in cancers including glioma¹¹.

Pyrano[2,3-c]pyrazoles are worthy candidates in the field of medicinal chemistry. They possess important biological properties such as anti-inflammatory activity,¹² antimicrobial activity,¹³ antioxidant activity,^{14, 15} anticancer activity¹⁶ and antifungal activity.¹⁴ Furthermore, they show inhibitory activity against various enzymes such as yeast α -glucosidase,¹⁷ and cyclooxygenase ¹². Although having structural and often conformational resemblances to adenine-based ATP-competitive kinase inhibitors, no biochemical activity toward kinases have been reported till date for pyrano[2,3-c]pyrazoles beyond *in silico* virtual screens¹⁸. Here, we report the synthesis of a series of pyrano[2,3-c]pyrazoles with 4-chlorophenyl substitution at N-1 and report compound 4j as an inhibitor of AKT2/PKB β kinase. Using the multicomponent synthesis strategy as reported previously,¹⁹⁻²⁵ 6-amino-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitriles were synthesised via a three-component reaction of aldehyde, 5-pyrazolone and malononitrile. This reaction has previously been catalysed by basic catalysts ^{8, 26-29}, ionic liquids³⁰ or metal catalysts³¹. However, in this work, pyrano[2,3-c]pyrazoles were synthesised using 1,4-diazabicyclo[2.2.2]octane (DABCO), which is a highly reactive, inexpensive, eco-friendly, and non-toxic catalyst for various organic transformations. From the multiple derivatives synthesized, compound 4j inhibited AKT2/PKB β specifically among 139 purified kinases tested and induced cell death in primary patient derived glioma 2D cells and 3D neurospheres. In addition, the ADME pharmacokinetic properties of 4j was investigated to gain preliminary information regarding the potential drug-like profile. Herein we report the first pyrano[2,3-c]pyrazole derivative with kinase inhibitory and anti-glioma potency.

2. Results and Discussions

2.1. Chemistry

As depicted in scheme 1, we prepared 6-amino-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitriles **4(a-j)** by three-component reaction of aldehyde **1**, 1-(4-chlorophenyl)-3-methyl-5-pyrazolone **2** and malononitrile **3**. The reaction was efficiently catalysed by DABCO with ethanol as a reaction medium and gave moderate to excellent yield in 30 minutes. The synthesised compounds were characterised by ^1H -NMR, $^{13}\text{C}\{^1\text{H}\}$ -NMR and HRMS analysis.

In ^1H -NMR, the singlet peak observed at δ 1.78-1.84 ppm confirmed the presence of a methyl group of pyrazole ring. Chiral proton present at the fourth position of pyran ring confirmed by singlet peak observed at δ 4.62-4.93 ppm. Signals observed in the region δ 1.78-1.84 ppm due to the presence of eight aromatic protons. In this region, the singlet peak of the amine group substituted in the pyran ring also appears at δ 7.18-7.53 ppm.

$^{13}\text{C}\{^1\text{H}\}$ -NMR of **4a** show a total of 16 equivalent carbons. A signal appears at δ 12.56 ppm due to methyl carbon substituting at the C-3 position. In comparison, C-3 carbon gave a signal at δ 143.95 ppm. The signal that appeared at δ 36.70 ppm confirmed the presence of chiral carbon C-4. Other pyran carbons appear at δ 58.16 (C-5), 159.34 (C-6), 145.74 (C-8), 98.89 (C-9) ppm. The signal of nitrile carbon appears at δ 119.93 ppm. Eight signals that appear in range δ 121.37-143.48 ppm confirmed the presence of aromatic carbons. The same pattern of signals was observed in $^{13}\text{C}\{^1\text{H}\}$ -NMR of other synthesised product. Some deviation in the position of signals observed due to the effect of substituted function group at the fourth position of phenyl ring substituted in pyran ring.

The molecular ion peak observed in HRMS of each synthesised compound match with its calculated value. Hence, all synthesised pyrano[2,3-c]pyrazoles are characterised by spectral analysis; we proceed to their biological evaluation.

2.2. Biology

2.2.1 Compound **4j** had the most potent antiproliferative activity in GL261 cells

To determine the biological relevance of these molecules in vitro, EC₅₀ values were conducted on all synthesized compounds in triplicates against murine glioblastoma cell line GL261. Out of the ten molecules tested, **4j** had the lowest EC₅₀ concentration at 20 μM (Table 1).

I.D.	~R	GL261 EC ₅₀
4a	~H	30
4b	~CH ₃	30
4c	~NO ₂	>100
4d	~Cl	>100
4e	~Br	>100

4f	~F	>100
4g	~OH	>100
4h	~OMe	>100
4i	~OEt	>30
4j	~Ph	20

Table 1: EC₅₀ (μM) values of compounds 4a-4j in GL261 cells *in vitro*. The IC₅₀ values for antiproliferation activity were determined by a dose-response inhibition curve. All assays were performed in triplicate.

2.2.2 Compound 4j specifically inhibits AKT2/PKBβ *in vitro*

To identify any potential kinase inhibitory activity of 4j, 5 μM 4j was screened over 139 purified kinases at the International Centre for Protein Kinase Profiling, University of Dundee, UK. Interestingly, 4j significantly inhibited PKBβ/AKT2 with high degree of specificity (**Fig 1A**). To ascertain whether 4j inhibited other AKT isoforms, biochemical IC₅₀ analyses were conducted on both purified AKT1/PKBα and AKT2. 4j exhibited IC₅₀s of 12 μM and 14 μM respectively AKT2 (**Fig 2B**) and AKT1 (**Fig 2C**). After obtaining promising inhibitory activity of 4j against AKT2/PKBβ, the molecular docking has been carried out to determine the binding modes of 4j with

AKT2 (**Fig 2D**). As 4j contain one chiral carbon C-4, both R and S stereoisomers exist in a racemic mixture. So, we analysed both stereoisomers and found that they were docked into the active site of AKT2 kinase domain (**Fig 2D & Supplementary Fig S1**). The docking score is listed in **Supplementary Table S1**. 4j was docked into the active site of AKT2/PKB β where molecule G93 co-crystallised in AKT2 (PDB IDP: 3d0e). The binding mode of 4j in AKT2 is shown

(**Supplementary Figure S1**) in two-dimensional and three-dimensional patterns. In R-isomer, two conventional hydrogen bonds of 1.8 Å bond length with GLU 236 and GLU 279 formed by amino group of pyran ring. They stabilised the binding of 4j in the active site. In addition, carbon hydrogen bond, of bond length 3.06 Å, formed between nitrile group of pyran ring of R-isomer and Gly-159. Whereas 4-biphenyl ring of S-isomer formed π -donor hydrogen bond, of bond length 2.76 Å, with Asp-293. Hydrophobic contacts involving different amino residues can also be observed (**Supplementary Figure S1**). Furthermore, Van der Waals interaction enhanced the stability of docked isomer in binding site. Thus, The binding mode of 4j in AKT2 provide supportive information to kinase binding and inhibition of AKT2.

Figure 3 AKT2 is overexpressed in glioma (A) High expression of AKT2 mRNA correlates with poorer survival in LGG patients. The individual values are sign-corrected log10 p-values of correlation. (B) AKT2 expression is highest in the tumour cell population of IDH1 WT GBM single-cell RNAseq dataset. (C) AKT2 expression is highest in the tumour cell population of IDH1 mutant astrocytoma single-cell RNAseq dataset.

2.2.3 AKT2 is overexpressed in low-grade glioma and primarily expressed in malignant cells

The mRNA expression of AKT2 was correlated with overall survival which showed that patients with low grade glioma (LGG) have the highest AKT2 mRNA expression along with the lowest overall survival (**Fig 3A**). To understand the expression levels of AKT2 within the glioma microenvironment and cell states, we queried the expression level

AKT2 levels in two widely used single-cell RNA sequencing (scRNAseq) datasets. In the scRNAseq dataset for adult and pediatric IDH1 wild-type glioblastoma³⁴, AKT2 expression was maximally identified in the malignant cell population with minimal expression observed in the tumour associated

macrophages (TAM), oligodendrocytes (ODCs), and T-cells (**Fig 3B**). Similar expression profile of AKT2 was also observed in the scRNAseq dataset of IDH1 mutant astrocytoma³⁵ (**Fig 3C**). This suggests that AKT2 is a potential driver of gliomagenesis since the highest expression is observed in malignant cells. This further suggests that targeting AKT2 is a good therapeutic strategy for gliomas.

2.2.4 Effects of 4j on glioma cells and neurospheres

In order to evaluate the biological activity of 4j, we utilized immortalized murine glioblastoma cell line GL261 along with primary patient-derived xenograft cells (GBM6, GBM22) and a glioblastoma stem 3D neurosphere (GBM76). The cell lines and accompanying de-identified histopathological informations were established at the Mayo Clinic Brain Tumor PDX National Resource, USA. All three patient derived primary glioma cells were IDH1 wild-type with *in vivo* bilateral invasive structures observed via neuro-pathology. The effect of 4j on cell viability was measured against GL261, GBM6, and GBM22 cell lines. After 72h incubation, cell viability was measured and the EC₅₀ values for 4j in GL261 is approximately 10-30 μ M, and 30 μ M in GBM6 and GBM22 (**Fig 4A**). To further determine whether 4j treatment could impact a 3D glioma stem cell culture system, we treated GBM76 pre-formed neurospheres with 20 μ M 4j for 7 days. 4j treated neurospheres were significantly smaller in size as observed under bright-field (**Fig 4B**). The cumulative diameter of 4j treated neurospheres were quantified and found to be significantly smaller than control DMSO treated neurospheres (**Fig 4C**).

3. Conclusion

A series of pyrano[2,3-c]pyrazole derivatives were synthesized (**Fig 1**) and compound 4j was determined to be kinase inhibitors with anti-glioma activity. Upon kinase screening and IC₅₀ analyses, compound 4j showed inhibitory activity towards AKT2/PKB β (**Fig 2**). Abberent AKT2 signalling is common in many cancers, such as glioma. We confirmed this by bioinformatic analysis of AKT2 mRNA in many cancers where we found AKT2 levels to be highest along with correlating to poor patient survival in low grade glioma (**Fig 3A**). Furthermore in our analysis of scRNA-seq data of glioma tumours, we found that AKT2 expression is highest in the malignant tumour cells themselves when compared to the immune and stromal populations (**Fig 3B&C**). Upon treating murine and patient derived glioma cell lines with 4j, EC₅₀ values are approximated to 20-30 μ M (**Fig 4A**) Treating patient derived glioma stem 3D neurospheres with 4j also significantly reduced neurosphere size (**Fig 4B&C**) thus suggesting potent anti-glioma activity in organoid models.

Gliomas being one of the most common brain cancers, there is always a need for more therapeutic strategies. Here we have established a novel pyrano[2,3-c]pyrazole compound 4j enabling kinase inhibitory and anti-glioma activity for the first time.

Figure 4 Compound 4j induces anti-glioma activity in 2D and 3D glioblastoma cell cultures. (A) Glioblastoma cells were treated with the indicated concentrations of 4j for 72 hours, and fold viability was measured using MTS assay. (B) GBM76 neurospheres were allowed to form for 14 days, then were treated with either DMSO or 20 μ M 4j for 7 days. A representative image of the neurospheres is shown, and (C) the diameter of the neurospheres were quantified using ImageJ. The significance of the differences were measured using the Mann-Whitney test and calculated p value = 0.0250.

4. Experimental protocols

4.1. Chemistry

4.1.1. General

We purchased 1,4 Diazabicyclo[2.2.2]octane (DABCO) from Alfa Aesar Chemical Company. 4-Ethoxy benzaldehyde, 4-phenyl benzaldehyde and 1-(4-chlorophenyl)-3-methyl-5-pyrazalone were purchased from TCI Chemicals (India) Pvt. Ltd. Benzaldehyde, p-tolualdehyde, 4-nitro benzaldehyde, 4-chloro benzaldehyde and 4-hydroxy benzaldehyde were purchased from Sisco Research Laboratories Pvt. Ltd. 4-Bromo benzaldehyde and 4-fluoro benzaldehyde were purchased from Spectrochem Pvt. Ltd. ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were recorded on a Bruker AV-400 or Bruker AV-600 device. Chemical shifts are expressed in ppm relative to TMS in DMSO- d_6 . High-resolution mass spectra were obtained on a Bruker Compass Data Analysis 4.0 spectrometer. For NMR spectra and HRMS details, please refer to **Supplementary Information Fig S2-S32**.

4.1.2. General procedure for the synthesis of pyrano[2,3-c]pyrazoles

In a round bottom flask, 1 mmol aldehyde, 1 mmol 1-(4-chlorophenyl)-3-methyl-5-pyrazolone, 1.1 mmol malononitrile and 0.1 mmol DABCO were mixed in 5 ml ethanol. The reaction mixture was refluxed and stirred at 78 °C for 30 minutes in a water bath. The water bath temperature should be below 80 °C; otherwise, it caused the reaction mixture to more brownish with Increasing the effort in work up process. If the reaction mixture solidified in some cases, then extra 3 ml ethanol were added. The progress of the reaction was monitored by TLC (hexane/ethyl acetate = 1/1). After completing the reaction, the reaction mixture was cooled to room temperature filtered to separate the product. Product washed with warm water three times to remove colour impurity.

4.1.2.1. 6-amino-1-(4-chlorophenyl)-3-methyl-4-phenyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4a**)

White solid (0.277 g, 77%), mp: 196-198 °C; ^1H NMR (400 MHz, DMSO- d_6) δ : 1.78 (s, 3H), 4.68 (s, 1H), 7.26-7.28 (m, 5H), 7.35(t, J = 7.4 Hz, 2H), 7.54 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, DMSO- d_6) δ : 12.6, 36.7, 58.2, 98.9, 119.9, 121.4, 127.1, 127.8, 128.5, 129.2, 130.2, 136.4, 143.5, 144.0, 145.7, 159.3; HRMS (ESI-TOF) m/z calcd. for $\text{C}_{20}\text{H}_{15}\text{ClN}_4\text{O}$ ($\text{M}+\text{H}$) $^+$: 363.10126, found: 363.10024.

4.1.2.2. 6-amino-1-(4-chlorophenyl)-3-methyl-4-p-tolyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4b**)

White solid (0.298 g, 79%), mp: 210-212 °C; ^1H NMR (400 MHz, DMSO- d_6) δ : 1.78 (s, 3H), 2.29 (s, 3H), 4.62 (s, 1H), 7.14 (s, 3H), 7.23 (s, 2H), 7.53 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, DMSO- d_6) δ : 12.6, 20.7, 36.3, 58.4, 99.0, 119.9, 121.3, 127.7, 129.1, 129.2, 130.1, 136.1, 136.4, 140.5, 143.9, 145.8, 159.2; HRMS (ESI-TOF) m/z calcd. for $\text{C}_{21}\text{H}_{17}\text{ClN}_4\text{O}$ ($\text{M}+\text{H}$) $^+$: 377.11691, found: 377.11585.

4.1.2.3. 6-amino-1-(4-chlorophenyl)-3-methyl-4-(4-nitrophenyl)-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4c**)

Light yellow solid (0.341 g, 84%), mp: 228-230 °C; ¹H NMR (400 MHz, DMSO-d₆) δ: 1.79 (s, 3H), 4.93 (s, 1H), 7.42 (s, 2H), 7.57 (dd, J = 8.8 Hz, 17.6 Hz, 4H), 7.84 (d, J = 8.8 Hz, 2H), 8.23 (d, J = 8.8 Hz, 2H); ¹³C{¹H} NMR (100 MHz, DMSO-d₆) δ: 12.6, 36.3, 56.9, 97.8, 119.7, 121.5, 123.9, 129.2, 130.4, 136.3, 144.1, 145.6, 146.6, 151.1, 159.6; HRMS (ESI-TOF) m/z calcd. for C₂₀H₁₄ClN₅O₃ (M+H)⁺: 408.08634, found: 408.08520.

4.1.2.4. 6-amino-1,4-bis(4-chlorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4d**)

White solid (0.299 g, 75 %), mp: 218-220 °C; ¹H NMR (400 MHz, DMSO-d₆) δ: 1.79 (s, 3H), 4.73 (s, 1H), 7.30-7.32 (m, 4H), 7.41 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H); ¹³C{¹H} NMR (100 MHz, DMSO-d₆) δ: 12.6, 36.0, 57.7, 98.4, 119.8, 121.4, 128.5, 129.2, 129.7, 130.3, 131.6, 136.3, 142.6, 144.0, 145.7, 159.4; HRMS (ESI-TOF) m/z calcd. for C₂₀H₁₄Cl₂N₄O (M+H)⁺: 397.06229, found: 397.06122.

4.1.2.5. 6-amino-4-(4-bromophenyl)-1-(4-chlorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4e**)

White solid (0.344 g, 78%), mp: 224-226 °C; ¹H NMR (400 MHz, DMSO-d₆) δ: 1.79 (s, 3H), 4.71 (s, 1H), 7.25 (d, J = 8.4 Hz, 2H), 7.31 (s, 2H), 7.52-7.56 (m, 4H), 7.83 (d, J = 8.8 Hz, 2H); ¹³C{¹H} NMR (100 MHz, DMSO-d₆) δ: 12.6, 36.1, 57.6, 98.4, 119.8, 120.2, 121.4, 129.2, 130.1, 130.3, 131.5, 136.3, 143.0, 144.0, 145.7, 159.4; HRMS (ESI-TOF) m/z calcd. for C₂₀H₁₄BrClN₄O (M+H)⁺: 441.01178, found: 441.01042.

4.1.2.6. 6-amino-1-(4-chlorophenyl)-4-(4-fluorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4f**)

White solid (0.305 g, 80%), mp: 192-194 °C; ¹H NMR (400 MHz, DMSO-d₆) δ: 1.78 (s, 3H), 4.72 (s, 1H), 7.17 (t, J = 8.4 Hz, 2H), 7.28 (s, 2H), 7.30-7.33 (m, 2H), 7.54 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H); ¹³C{¹H} NMR (100 MHz, DMSO-d₆) δ: 12.6, 35.9, 58.0, 98.7, 115.3 (d, J = 22 Hz), 119.9, 121.4, 129.2, 129.7 (d, J = 8 Hz), 130.2, 136.4, 139.7 (d, J = 3 Hz), 144.0, 145.7, 159.3, 161.2 (d, J = 241 Hz); HRMS (ESI-TOF) m/z calcd. for C₂₀H₁₄ClFN₄O (M+H)⁺: 381.09184, found: 381.09077.

4.1.2.7. 6-amino-1-(4-chlorophenyl)-4-(4-hydroxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4g**)

White solid (0.310 g, 82%), mp: 212-214 °C; ¹H NMR (400 MHz, DMSO-d₆) δ: 1.79 (s, 3H), 4.56 (s, 1H), 6.72 (d, J = 8.4 Hz, 2H), 7.04 (d, J = 8.4 Hz, 2H), 7.18 (s, 2H), 7.53 (d, J = 8.8 Hz, 2H), 7.82 (d, J = 8.8 Hz, 2H), 9.36 (s, 1H); ¹³C{¹H} NMR (100 MHz, DMSO-d₆) δ: 12.6, 36.0, 58.8, 99.3, 115.2, 120.0, 121.3, 128.8, 129.2, 130.1, 133.8, 136.4, 143.8, 145.8, 156.3, 159.1; HRMS (ESI-TOF) m/z calcd. for C₂₀H₁₅ClN₄O₂ (M+H)⁺: 379.09618, found: 379.09505.

4.1.2.8. 6-amino-1-(4-chlorophenyl)-4-(4-methoxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4h**)

White solid (0.343 g, 87%), mp: 218-220 °C; ¹H NMR (400 MHz, DMSO-d₆) δ: 1.78 (s, 3H), 3.75 (s, 3H), 4.63 (s, 1H), 6.90 (d, J = 8.4 Hz, 2H), 7.17 (d, J = 8.4 Hz, 2H), 7.21 (s, 2H), 7.53 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H); ¹³C{¹H} NMR (100 MHz, DMSO-d₆) δ: 12.6, 35.9, 55.0, 58.5, 99.1, 113.8, 120.0, 121.3, 128.9, 129.2, 130.1, 135.5, 136.4, 143.9, 145.8, 158.2, 159.2; HRMS (ESI-TOF) m/z calcd. for C₂₁H₁₇ClN₄O₂ (M+H)⁺: 393.111829, found: 393.11077.

4.1.2.9. 6-amino-1-(4-chlorophenyl)-4-(4-ethoxyphenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4i**)

White solid (0.322 g, 79%), mp: 190-192 °C; ¹H NMR (400 MHz, DMSO-d₆) δ: 1.32 (t, J = 6.8 Hz, 3H), 1.78 (s, 3H), 4.00 (q, J = 6.8 Hz, 2H), 4.62 (s, 1H), 6.88 (d, J = 8.4 Hz, 2H), 7.15 (d, J = 8.8 Hz, 2H), 7.21 (s, 2H), 7.53 (d, J = 8.8 Hz, 2H), 7.83 (d, J = 8.8 Hz, 2H); ¹³C{¹H} NMR (100 MHz, DMSO-d₆) δ: 12.6, 14.7, 35.9, 58.6, 62.9, 99.1, 114.3, 120.0, 121.3, 128.8, 129.2, 130.1, 135.4, 136.4, 143.9, 145.8, 157.5, 159.2; HRMS (ESI-TOF) m/z calcd. for C₂₂H₁₉ClN₄O₂ (M+H)⁺: 407.12748, found: 407.12642.

4.1.2.10. 6-amino-4-(biphenyl-4-yl)-1-(4-chlorophenyl)-3-methyl-1,4-dihydropyrano[2,3-c]pyrazole-5-carbonitrile (**4j**)

White solid (0.390 g, 89%), mp: 204-206 °C; ¹H NMR (400 MHz, DMSO-d₆) δ: 1.84 (s, 3H), 4.75 (s, 1H), 7.29 (s, 2H), 7.35-7.38 (m, 3H), 7.47 (t, J = 7.6 Hz, 2H), 7.55 (d, J = 8.8 Hz, 2H), 7.69 (t, J = 7.4 Hz, 4H), 7.85 (d, J = 8.8 Hz, 2H); ¹³C{¹H} NMR (100 MHz, DMSO-d₆) δ: 12.6, 36.3, 58.0, 98.8, 120.0, 121.4, 126.6, 126.9, 127.4, 128.4, 128.9, 129.2, 130.2, 136.4, 138.9, 139.7, 142.7, 144.0, 145.8, 159.4; HRMS (ESI-TOF) m/z calcd. for C₂₆H₁₉ClN₄O (M+H)⁺: 439.13256, found: 439.13138.

4.2. Biochemical analysis

4.2.1 Kinase screen analysis of **4j**

Kinase inhibitor specificity profiling assays were carried out at The International Centre for Protein Kinase Profiling (<http://www.kinase-screen.mrc.ac.uk/>). **4j** biochemical kinase inhibitory property was determined against a panel of 139 protein kinases as described previously ^{32,33}. The assay mixes and ³³P-γ-ATP were added by Multidrop 384 (Thermo). Results are presented as a percentage of kinase activity in DMSO control reactions. Protein kinases were assayed *in vitro* with 5 μM final concentration of **4j** and the results are presented as an average of triplicate reactions in the form of comparative histograms using Adobe Illustrator.

4.2.2 IC₅₀ analyses

IC₅₀ analyses were carried out at The International Centre for Protein Kinase Profiling (<http://www.kinase-screen.mrc.ac.uk/>). **4j** IC₅₀ measurements were carried out against the kinases with final concentrations between 0.003 to 100 μM *in vitro* (**4j** was added to the kinase reaction prior to ATP master mix). PKBβ (ΔPH-PKBβ-S474D) and PKBα (ΔPH-PKBα-S473D) were purified using a baculoviral expression system as stated previously ³². PKBβ or PKBα (5-20 mU diluted in 50 mM Tris pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 1 mg/ml bovine serum albumin) were assayed against a modified Crosstide peptide (GRPRTSSFAEGKK) in a final volume of 25.5 μl containing 50 mM Tris pH 7.5, 0.05% β-mercaptoethanol, 30 μM substrate peptide, 10 mM magnesium acetate and 0.05 mM [³³P-γ-ATP] (50-1000 cpm/pmole) and incubated for 30 min at room temperature. Assays were stopped by addition of 5 μl of 0.5 M (3%) orthophosphoric acid and then harvested onto P81 Unifilter plates with a wash buffer of 50 mM orthophosphoric acid. IC₅₀ curves were developed as % of DMSO control and IC₅₀ values were calculated using GraphPad Prism software.

4.2.3 Molecular docking studies

All calculations were performed using autodock 4.2.6 software installed on 2.5GHz intel® Core™ i5-7200U CPU. AutoDock Tools (ADT) version 1.5.6 was used for structure pre-processing. Discovery Studio Visualizer, PyMOL and Chimera 1.14 were used to analyse bound conformations and different interactions. Crystal structure AKT2 (PDB ID: 3d0e) was obtained from the Protein Data Bank (<http://www.rcsb.org/>). With help of ADT, the co-crystallised ligand and crystal water were removed,

then the polar hydrogens were added. Finally, Gasteiger charges were added to each atom, and merged the non-polar hydrogen atoms to the protein structure. The structure was saved in PDBQT file format for further studies in ADT. 2D structure of 4j were drawn using ChemBioDraw Ultra 11.0 (ChemBioOffice 2008). ChemBio3D Ultra 11.0 was used to convert the 2D structure into 3D. Minimise energy to minimum RMS gradient of 0.100 was set in each iteration. All structures were saved as SYBYL2 (.mol2) file format for input to ADT. All ligand structures were then saved in PDBQT file format, to carry out docking in ADT. A grid box with a dimension of $50 \times 50 \times 50 \text{ \AA}^3$ with $0.397 \text{ \AA}$ spacing and centred on 21.768, -18.472, -7.540 was created surrounding co-crystallized ligand G93 using ADT. Then, grid energy calculations were carried out. For the AutoDock docking calculation, default docking parameters were used and 50 docked conformations were generated for each compound. The energy calculations were done using genetic algorithms.

4.2.4 Bioinformatic analysis

4.2.4.1 Expression correlation with overall survival for tumor indication in TCGA

The AKT2 expression correlation with overall survival for tumor indication is based upon data generated through the Lumin Bioinformatics Software of Champions Oncology, Inc. This signature reflects correlation of AKT2 mRNA expression with the overall survival in each of the TCGA cancer indications described. The individual values are sign-corrected log₁₀ p-values of correlation. For example, a value of -2, (representing a p-value of 0.01), for AKT2 indicates that when AKT2 expression is high patient outcome is better with longer survival, whereas a positive 2 value, (representing a p-value of 0.01), indicates the opposite: high expression of AKT2 predicts worse outcome or poorer survival.

4.2.4.2 AKT2 expression analysis from single cell RNA sequencing datasets

To understand expression across various cell states of glioblastoma, we queried AKT2 levels in two previously published single cell RNA sequencing datasets available on the Single Cell portal of Broad Institute, MIT and Harvard, USA (https://singlecell.broadinstitute.org/single_cell). We explored the ‘Single cell RNA-seq of adult and pediatric glioblastoma’ dataset consisting of 24,131 single cell sequences from patients with IDH1 wild-type glioblastoma³⁴ and the ‘single cell RNA-seq analysis of astrocytoma’ dataset consisting of 6,341 single cell sequences from 10 patients with IDH1 mutant astrocytoma³⁵. Data represented with t-distributed stochastic neighbor embedding (t-SNE) clustering and sub-sampling of ‘All Cells’.

4.3. Cell based assays

4.3.1 Cell Culture

Mammalian cells were all grown in a humidified incubator with 5% CO₂ at 37°C. GL261 cells (gift from Prof Kun-Liang Guan, University of California San Diego, USA) were cultured in Dulbecco’s Modified Eagle Media (DMEM, Gibco) supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin and streptomycin. GBM6 and GBM22 cells were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin and streptomycin, 10 µg/ml insulin, and 20 ng/ml hEGF. GBM76 cells were cultured in KnockOut DMEM/F-12 Basal Media supplemented with StemPro NSC SFM Supplement, 10 µg FGF, 10 µg EGF, L-glutamine (Corning #25005CI) 10 ml of 200 mM solution, and 1% penicillin and streptomycin. GBM6, GBM22, and GBM76 cells were acquired from the Brain Tumour PDX National Resource, Mayo Clinic, USA.

4.3.2 Cell Viability Assay

Cell viability assays were carried out as stated previously^{36, 37}. To measure cell viability, actively proliferating cells were seeded at with equal number of cells per well. Cell viability was ascertained as fold proliferation using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit following manufacturer's instructions. Cell viability assays were carried out with or without 72 hr treatment of the respective inhibitor using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit following manufacturer's instructions and data was represented as %viability compared to DMSO treated control.

4.3.3 Neurosphere Formation Assay

GBM76 cells were plated at 4,000 cells per well and incubated for 14 days for neurospheres to form. Once neurospheres were formed, neurospheres were treated with either DMSO, or 20 µM 4j in triplicates for 7 days. Three brightfield images were taken per week using the digital camera on the Zeiss Axiovert Live microscope. Diameters of the neurospheres were quantified using ImageJ software and graphs were plotted on GraphPad Prism.

4.4 Statistical analysis

Details of all statistical tests and multiple comparisons used to derive p value has been detailed in Figure Legends. All experiments were repeated 2-3 times with multiple technical replicates to be eligible for the indicated statistical analyses, and representative image has been shown. All results are presented as mean ± SD unless otherwise mentioned. Data were analysed using Graphpad Prism statistical package unless otherwise mentioned. TCGA data mining and corresponding statistical analyses were performed by Lumin Bioinformatics webtool as stated previously.

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Conflicts of interests

The authors declare that there are no conflicts of interests.

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Supporting Information

Synthesis of a novel Pyrano[2,3-c]pyrazole enabling PKB β /AKT2 inhibitory and *in vitro* anti-glioma activity

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Table of Contents

1	Table and Figure of Molecular docking study	S1
2	Copies of ¹ H NMR and ¹³ C{ ¹ H} NMR spectra of 4a-4j	S2-S22
3	Copies of HRMS of 4a-4j	S23-S32

Table and figure of Molecular docking study

Table S1. Docking score of 4j with PKB β /AKT2		
Protein kinase B	PKB β /AKT2	
Stereoisomer of 4j	R	S
Estimated Free Energy of Binding (kcal/mol)	-8.53	-9.54
Estimated Inhibition Constant, K_i (nM)	560.47	102.12
Final Intermolecular Energy (kcal/mol)	-9.72	-10.43
Final Total Internal Energy (kcal/mol)	-1.32	-1.22
Torsional Free Energy (kcal/mol)	+1.19	+0.89
Unbound System's Energy (kcal/mol)	-1.32	-1.22

Table S1

Figure S1 Binding mode of **4j** in the active pocket of AKT2 (PDB ID: 3d0e) in 2D-ligand interaction diagram (left) and 3D-ligand interaction diagram (right).

Copies of ^1H NMR and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra of **4a-4j**

Figure S2. ^1H NMR spectrum of compound **4a** at 400 MHz in DMSO-d_6

Figure S3. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4a** at 100 MHz in DMSO- d_6

Figure S4. ¹H NMR spectrum of compound **4b** at 400 MHz in DMSO-d₆

Figure S5. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4b** at 100 MHz in DMSO- d_6

Figure S6. ¹H NMR spectrum of compound **4c** at 400 MHz in DMSO-d₆

Figure S7. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4c** at 100 MHz in DMSO- d_6

Figure S8. ¹H NMR spectrum of compound **4d** at 400 MHz in DMSO-d₆

Figure S9. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4d** at 100 MHz in DMSO-d₆

Figure S10. ^1H NMR spectrum of compound **4e** at 400 MHz in DMSO-d_6

Figure S11. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4e** at 100 MHz in DMSO- d_6

Figure S12. ¹H NMR spectrum of compound **4f** at 400 MHz in DMSO-d₆

Figure S13. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4f** at 100 MHz in DMSO- d_6

Figure S14. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4f** at 100 MHz in DMSO- d_6

Figure S15. ^1H NMR spectrum of compound **4g** at 400 MHz in DMSO- d_6

Figure S16. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4g** at 100 MHz in DMSO- d_6

Figure S17. ¹H NMR spectrum of compound **4h** at 400 MHz in DMSO-d₆

Figure S18. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4h** at 100 MHz in DMSO- d_6

Figure S19. ¹H NMR spectrum of compound **4i** at 400 MHz in DMSO-d₆

Figure S20. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4i** at 100 MHz in DMSO- d_6

Figure S22. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of compound **4j** at 100 MHz in DMSO- d_6

Copies of HRMS of **4a-4j**

Generic Display Report (all)

Figure S23. HRMS of compound **4a**

Generic Display Report (all)

Figure S24. HRMS of compound **4b**

Generic Display Report (all)

Figure S25. HRMS of compound **4c**

Generic Display Report (all)

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Page 1 of 1

Figure S26. HRMS of compound **4d**

Generic Display Report (all)

Figure S27. HRMS of compound **4e**

Generic Display Report (all)

Figure S28. HRMS of compound **4f**

Generic Display Report (all)

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Page 1 of 1

Figure S29. HRMS of compound **4g**

Generic Display Report (all)

Figure S30. HRMS of compound **4h**

Generic Display Report (all)

Figure S31. HRMS of compound **4i**

Generic Display Report (all)

Figure S32. HRMS of compound **4j**