UNIVERSITY OF CALIFORNIA SAN DIEGO

Small Molecule SGK Inhibitor Decreases Glioblastoma Cell Growth

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by

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The thesis of Ramie Kotadia is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

DEDICATION

To my parents and brother for their unwavering encouragement and support on this path. Their belief in my abilities and enthusiasm for my future success has been a blessing that has driven me throughout this journey, and I hope to make them proud as I take this next step in my career.

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
AGC	Containing PKA, PKG, and PKC
BBB	Blood-Brain Barrier
BBTB	Blood-Brain Tumor Barrier
BOHV-1	Bovine Herpes-1
CDK	Cyclin-dependent Kinase
CNS	Central Nervous System
DIPG	Diffuse Intrinsic Pontine Glioma
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
GBM	Glioblastoma
GSC	Glioblastoma Stem-like Cell
HDAC	Histone Deacetylase
HER2	Human Epidermal Growth Factor Receptor 2
HR	Homologous Recombination
HSV-1	Herpes Simplex Virus-1
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
NDRG1	N-Myc Downstream Regulated 1
NHEJ	Non-Homologous End Joining
p27	$p27^{kip1}$

pAKT	Phosphorylated Akt
pHGG	Pediatric High-Grade Glioma
PI3K	Phosphatidylinositol 3-Kinases
PI3Kα	PI3K alpha subunit
PDK1	3-Phosphoinositide-Dependent Kinase
SGK	Serum and Glucocorticoid-Regulated Kinase
TMZ	Temozolomide

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ABSTRACT OF THE THESIS

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Master of Science in Biology

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Glioblastoma is the most common of all tumors in the brain and central nervous system and has an average survival time of less than one year. The current standard of care treats these tumors with radiation and temozolomide, a DNA alkylating agent, but a vast majority of these tumors have begun to acquire temozolomide resistance, highlighting the need for new therapies. The heterogeneous nature of glioblastoma tumors as well as the difficulty of drugs to cross the blood-brain barrier leads to minimal treatment options. To test potential small molecule

inhibitors as treatment for glioblastoma, we exposed glioblastoma cells to four commercially available SGK (serum and glucocorticoid-regulated kinase) inhibitors, which target downstream effectors in the PI3K/Akt pathway regulating cell survival and proliferation. We found that one of the four SGK inhibitors, GSK650394, actively inhibits glioblastoma cell viability in U87MG and selectively target SGK at concentrations below 100 μM. This inhibitor could potentially be used to treat glioblastoma but must be further tested in heterogenous glioblastoma tumors and *in vivo* models, especially to study if these small molecule inhibitors can cross the blood-brain barrier, and the mechanism of action in glioblastoma must be further elucidated.

INTRODUCTION

Glioblastoma (GBM) is the most common of all malignant tumors of the brain and central nervous system (CNS).[1] The World Health Organization classifies GBM as a Grade IV glioma, as it is the most aggressive collection of tumors in the CNS generated from glial cells or glial precursors, which are non-neuronal cells of the nervous system.[1,2] The tumors' aggressiveness leads to an average survival time of less than one year with an average five-year GBM survival rate of 5.6%.[1,3] The survival time is limited because of the difficulty in treating these tumors, due in part to their molecular nature, as the tumor is heterogeneous and composed of multiple different cell types.[4–6] Limited treatment options have led the current standard of treatment to be more palliative than curative. Treatment consists of surgical resection of the tumor followed by radiotherapy and chemotherapy.[2] In 2005, concurrent and adjuvant chemotherapy with temozolomide (TMZ), a DNA alkylating agent, was added to the standard of care.[7] TMZ significantly increases the survival time of GBM patients from 12.1 months with radiotherapy alone to 14.6 months with TMZ added to radiotherapy.[7] However, recent studies have shown that TMZ eventually loses its efficacy, as tumors can acquire TMZ resistance through the altered expression levels of DNA alkylating proteins and DNA repair enzymes in addition to changes in cellular signaling.[8–10] Acquired TMZ resistance by tumor cells poses a complication in the treatment of GBM.

Another problem for GBM treatment is its inherent tumor heterogeneity.[4–6] Both inter-patient and intra-tumoral heterogeneity are a hallmark of GBM.[11] There are two main subtypes of glioblastoma: primary and secondary glioblastoma. Primary glioblastoma appears as a full-blown tumor without any lesser-grade tumors present beforehand, while secondary glioblastomas progress from lower-grade tumors, such as diffuse astrocytoma (WHO grade II) or anaplastic

astrocytoma (WHO grade III), which are tumors that develop in the astrocytes that support nerve cells.[12] Inter-patient heterogeneity has led to the classification of glioblastoma into four more subtypes based on transcriptional signatures: classical, mesenchymal, neural, or proneural.[11] Classification into these subtypes is mostly based on the genetic alterations that are most seen within that subtype, for example, classical is associated with *EGFR* amplification, mesenchymal with *Neurofibromin 1* loss, and proneural with *IDH1/2* mutation.[11] Typically primary glioblastomas are either classical, mesenchymal, or neural subtypes and secondary glioblastomas are proneural.[11] Intra-tumoral heterogeneity stems from the presence of multiple GBM subtypes within samples from the same tumor, adding to the difficulty in diagnosing the specific GBM subtype a patient may have.[13] Due to tumor heterogeneity and the various subclassifications of GBM, patients are given different prognoses and respond differently to therapy based on the genetic makeup of their tumors.

Patients also respond differently to therapy based on the presence of glioblastoma stem-like cells (GSCs) within their tumors. GSCs are a population of cells with unlimited proliferative potential, progressing tumor development and maintenance.[4,6] These GSCs are resistant to chemotherapeutic agents and have the ability to continue to recover and proliferate even after exposure to these agents.[6,14] The presence of these cells can cause TMZ resistance, contributing to the increased difficulty in treating GBM.[4,8,10] Due to the presence of multiple subtypes of GBM and the multiple cell types present within a tumor, this has brought the need for new therapies or small molecule drugs specifically targeting GSCs.

Small molecule drugs, however, can only be effective if they can pass the blood-brain barrier (BBB), which is a protective cellular barrier between the blood compartment and the brain that secures proper function of neurons.[15] This barrier prevents unwanted cells and

macromolecules, such as potential drugs, from entering the brain and protects the CNS from neurotoxic substances.[15] The BBB is composed of special endothelial cells that are packed together with intercellular tight junctions that prevent anything other than small or gaseous molecules, such as water or carbon dioxide, from crossing in, and ATP-binding cassette (ABC) efflux transporters that send out any unwanted compounds.[15] These junctions are regulated by multiple proteins based on their organization and interactions. The BBB can be affected by the presence of tumors or other pathological conditions. Lower-grade gliomas have a relatively normal functioning BBB but higher-grade gliomas, including glioblastoma, can cause a disrupted or "leaky" BBB which is still not enough for drug penetration for treatment in the brain.[15] This disrupted BBB can be referred to as the blood-brain tumor barrier (BBTB) which is formed from brain tumor capillaries and is distinct from the BBB.[15] In glioblastoma specifically, a subset of tumor cells at the BBTB express ABC efflux transporters and possibly confer drug resistance to these tumors, causing another complication in the treatment of glioblastoma.[15] Therefore for a potential small molecule drug to be effective in treating glioblastoma, a way of either crossing, disrupting, or avoiding the BBB or efflux transporters is needed.

To develop small molecule drugs that can effectively target the glioblastoma cells in the brain, current research has turned to understanding the multiple pathways involved in glioblastoma. Receptor tyrosine kinases are an attractive target as they are involved in multiple downstream pathways, including Ras/MAP/ERK and Ras/PI3K/Akt.[16] These receptors are involved in the cell cycle, differentiation, and survival as well as cancer hallmarks including angiogenesis and tumor invasiveness.[16,17] EGFR is a particularly promising target as it is commonly amplified and mutated in approximately 45-57% of GBM cases.[18–20] In addition to

EGFR, there are several receptor tyrosine kinases and downstream pathways that are potential drug targets for GBM.[21]

One of the downstream pathways regulated by receptor tyrosine kinase activity is the PI3K/Akt/mTOR pathway. The pathway begins with phosphatidylinositol 3-kinases (PI3K), which are part of a family of intracellular lipid kinases, and are mainly involved in cell growth and survival,[22] but also play a role in metabolism and intracellular trafficking.[23,24] PI3K consists of three classes: Class 1 (further split into 1A and 1B), Class 2, and Class 3.[25] Current PI3K inhibitors studied for GBM target class 1A PI3Ks, as they are activated by EGFR, which is amplified in the majority of GBM cases.[18–20,25] Once PI3K is activated it proceeds to activate Akt through its downstream targets. Akt, a serine/threonine kinase, is activated through phosphorylation by 3-phosphoinositide- dependent kinase (PDK1) and the mammalian target of rapamycin complex 2 (mTORC2) (Figure 1).[25] Akt then goes on to activate the mammalian target of rapamycin complex 1 (mTORC1) by phosphorylating TSC2[26,27] and PRAS40[28,29], which are both mTORC1 suppressors (Figure 1). Akt regulates a variety of cellular processes involving differentiation, proliferation, survival, metabolism, angiogenesis, and apoptosis.[25,30,31]

The PI3K/Akt/mTOR pathway is of great interest for GBM because 15% of GBM patients have shown amplification mutations of *PIK3CA* or *PIK3R1*, which encode subunits of PI3K.[25] Loss of function mutations of *PTEN*, a negative regulator of PI3K, are present in 40% of GBM patients and is correlated with poor survival.[32,33] Alterations in *EGFR, PTEN,* and/or *PIK3* genes are present in 63-86% of primary GBM and 31% of secondary GBM cases, highlighting the need for drugs targeting this pathway.[25] Fimepinostat (CUDC-907) and Alpelisib (BYL719) are two such PI3K inhibitors that are currently in clinical trials.

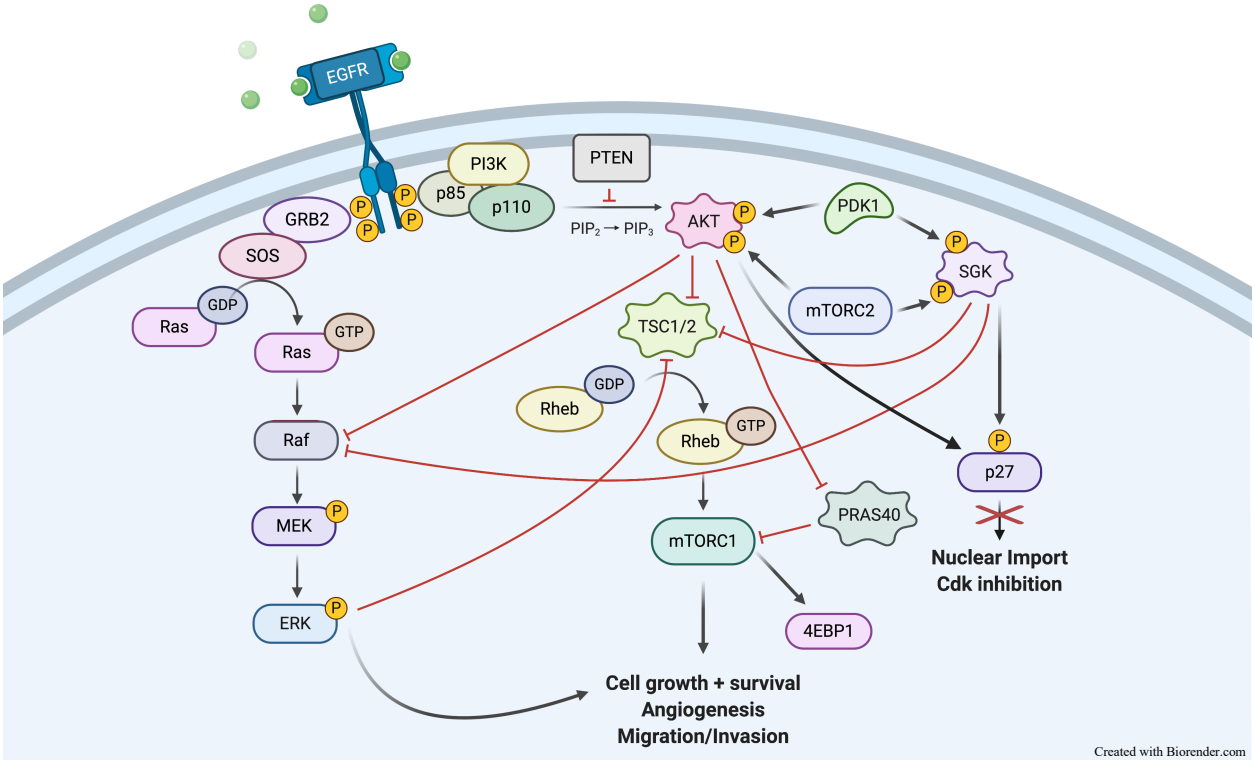


Figure 1. **Intracellular Signaling via PI3K/AKT pathway.** Akt and SGK are activated through phosphorylation by PDK1 and mTORC2 and downstream activate mTORC1 and play a role in a variety of cellular processes.

Fimepinostat (CUDC-907) is a dual histone deacetylase (HDAC) and PI3K inhibitor that is currently in phase one trials for children and young adults with glioblastoma and other recurrent gliomas[34] as well as for refractory lymphoma and multiple myeloma.[35,36] Pediatric high-grade glioma (pHGG) and diffuse intrinsic pontine glioma (DIPG) are two gliomas with very low survival rates in children and young adults.[37] The PI3K pathway is activated in these gliomas through either PTEN silencing or activating mutations in the PI3K pathway.[37] Fimepinostat is a potential radiosensitizer for these gliomas as HDAC and PI3K inhibitors disrupt DNA repair, with HDAC inhibition decreasing the expression of homologous recombination (HR) and non-homologous end joining (NHEJ) regulators, including BRCA1, and PI3K inhibition also downregulating BRCA1 in breast cancer.[37] The combination of the two inhibitors in Fimepinostat works synergistically with radiation both *in vitro* and *in vivo*, with

increased survival seen in the combinatory treatment samples compared to either treatment alone.[37] Fimepinostat induces G_1 cell cycle arrest while radiation induces G_2—M cell cycle arrest, allowing for DNA damage, while Fimepinostat further induces DNA damage and inhibits DNA repair by causing functional defects in NHEJ and HR in a dose-dependent manner.[37] Fimepinostat also causes apoptosis in higher amounts when in combination with radiation, while on its own Fimepinostat can induce low amounts and radiation even less.[37] Fimepinostat is thought to evade drug resistance based on studies done on activated AKT1-expressing melanoma cells, which became insensitive to treatment by PI3K inhibitor GDC-0941 but remained sensitive to Fimepinostat, most likely due to the presence of the HDAC inhibitory activity.[38] Fimepinostat is able to effectively inhibit tumor growth *in vivo* and *in vitro* through the suppression of multiple signaling pathways, including the RAF/MEK/MAPK pathway and SRC/STAT pathway which contains EGFR, as well as the induction of apoptosis and cell cycle arrest.[37,38]

Alpelisib (BYL719), marketed as Piqray, is a selective PI3K inhibitor against the PI3K alpha subunit (PI3Kα), which is encoded by the *PIK3CA* gene.[39] It has been developed by Novartis for the treatment of Hormone receptor-positive, human epidermal growth factor receptor-2 (HER2) negative breast cancer, in conjunction with fulvestrant, and was approved in the USA on May 24th 2019.[39] Alpelisib has also undergone phase I trials in Japan[40] and the USA[41] to determine the efficacy and safety in patients with advanced solid-state tumors, however, none of the patients enrolled had glioblastoma or other brain tumors. Tumors and cell lines with altered *PIK3CA* seemed to have more antitumor activity upon treatment compared to wild type *PIK3CA*,[41] however this is not the only defining factor to determine if a cell line will be sensitive to Alpelisib.[42] Alpelisib is able to prevent the phosphorylation and activation of Akt[42], but there is contrasting data regarding the ability of Alpelisib to inhibit the mammalian

target of rapamycin (mTOR) activity.[42,43] Studies have found that sensitivity to Alpelisib inhibition in breast cancer is associated with inhibition of mTORC1 signaling, and resistance to Alpelisib treatment is associated with constantly active mTORC1.[44] Inhibition of mTOR or Akt along with Alpelisib decreased breast cancer cell line growth.[43] In medulloblastoma cell lines, the combination of Alpelisib with mTOR inhibitor OSI-027 yielded synergistic inhibitory activity, with the combination increasing the rate of apoptosis more than either treatment alone.[45] This dual inhibition was also able to decrease the ability of stem-like cancer cells to form and grow neurospheres, as well as reduce tumor growth in xenograft mouse models.[45] These previous studies suggest that Alpelisib would be best suited to be used alongside mTOR inhibitors.

The PI3K/Akt/mTOR pathway can potentially be targeted by a protein parallel in function to Akt, the serum and glucocorticoid-regulated kinase (SGK) family of serine/threonine kinases which are activated in a PI3K-dependent manner.[46] These SGKs lie downstream of PI3K, at the same level as Akt, and phosphorylate many of the same targets as Akt, making it parallel to PI3K/Akt signaling (Figure 2).[46] The three SGK isoforms (SGK1, SGK2, and SGK3) share a similar domain structure and have multiple variants, and while the specific role of these multiple isoforms remains to be elucidated, SGK1 is of primary focus for its role in the mTOR pathway.[46] The SGK isoforms are activated downstream of PI3K, and their catalytic domain is similar to Akt, as SGK and Akt are both part of the Containing PKA, PKG, and PKC (AGC) kinase family of serine/threonine kinases.[46] Unlike Akt, SKG does not localize to the cell membrane for activation and can be phosphorylated by both mTORC1 and mTORC2.[46] SKG is activated similarly to Akt, with mTORC2 phosphorylating its hydrophobic motif while PDK1 phosphorylates its activation loop.[47] Its expression is transcriptionally regulated and degraded through ubiquitination.[46] SGK1 is involved in cell growth and proliferation as well as in cell

survival and migration, specifically anti-apoptotic signaling.[48–51] SGK1 expression is upregulated in multiple human cancers, including prostate cancer,[52] squamous subtype small cell lung cancer,[53] and in gastrointestinal tumors.[54] SKG1 interacts with the RAS/RAF/ERK pathway and is believed to repress the pathway's activity through a negative feedback loop (Figure 1).[55] SGK1 can also activate mTORC1 through phosphorylation of mTORC1 repressors,[56] especially TSC2.[57] PDK1 is able to maintain mTORC1 activity through SGK1, which is upregulated partly through epigenetic regulation in cells and tumors that are resistant to Alpelisib.[57] To prevent resistance to PI3Kα inhibition, dual inhibition by a PDK1 or SGK1 inhibitor and a PI3Kα inhibitor is necessary to prevent the downstream activities of both Akt and SGK1.[57]

Recently, Kulkarni and colleagues conducted an RNAi screen in GBM and found that *in vitro* knockdown and drug-based inhibition of SGK1 decreased GSC proliferation and survival, while inducing apoptosis that led to decreased growth.[58] *In vivo* studies of glioblastoma tumor growth in mice showed that SGK1 shRNA inhibition caused a significant increase in mouse survival.[58] These studies show SGK as a possible target for inhibition of tumor growth, which is especially maintained by GSCs.

There are currently five main drugs available that inhibit SGK1: GSK650394, EMD638683, SGK1-IN-1, SGK1-inh, and SI113. While these inhibitors have been tested in other disease models, there is little data from glioblastoma research.

GSK650394 is a competitive inhibitor for SGK1[52], and has been explored along with analogs in multiple other diseases[59–61] including prostate cancer,[52] where *in vitro* and *in vivo* studies exhibit a decrease in androgen-dependent cell viability when exposed to GSK650394, as well as bovine herpes-1 (BoHV-1) and herpes simplex virus-1 (HSV-1),[62] where exposure reduced viral replication in cells. Kulkarni and colleagues also found that GSK650394 decreased

cell viability and induced apoptosis in GSCs, but had a less pronounced cell viability decrease in normal neural progenitor lines.[58] In addition, differentiated GSCs were no longer sensitive to SGK1 inhibition by GSK650394, although SGK1 levels were only slightly decreased, suggesting that there is no need for SGK1 by differentiated GSCs.[58] In prostate cancer cells, GSK650394 is believed to induce G_2—M cell cycle arrest and activate autophagy-dependent and caspase-dependent apoptosis.[59]

EMD638683 was first developed as an SGK inhibitor for hypertension treatment,[63] as SGK can be activated by insulin acting on PDK1. In Type II diabetes and metabolic syndrome models, EMD638683 prevented high blood pressure in response to fructose or high salt.[63] EMD638683 has since then also been tested in human colon carcinoma cells in conjunction with radiotherapy and was found to *in vitro* increase cell shrinkage and sensitize the cells to the apoptotic effects of radiation while *in vivo* decrease tumor growth and survival.[64]

SGK1-IN-1 and SGK1-inh are N-[4-(1H-Pyrazolo[3,4-b]pyrazin-6-yl)-phenyl]-sulfonamides that have been developed based on 3D ligand-based virtual screens and further tested to determine activity and specificity.[65] SGK1-inh has previously been tested in two breast cancer lines, HC1954 and JIMT, where it selectively inhibited SGK1 activity as measured by the level of phosphorylated N-Myc Downstream Regulated 1(NDRG1), which is a downstream substrate that is phosphorylated by SGK1.[57,66] SGK-IN-1 has not been previously tested in any other disease models.

SI113 has been minimally tested *in vitro* in colorectal adenocarcinoma, breast cancer, and glioblastoma cell lines, with most research focusing on colorectal adenocarcinoma and glioblastoma.[67–69] SI113 consistently increased the amount of nonviable cells in multiple GBM cell lines and was more effective in combination with radiation than alone.[68] Furthermore, SI113

reduces mTORC1 and/or mTORC2 activity in a GBM cell line dependent manner, based on if the cell line is adherent or in suspension.[69] This decrease in mTORC1 and mTORC2 activity led to decreased phosphorylation of Akt and SGK1 and a reduction of apoptosis-related endpoints.[69] Overall, SI113 treatment led to a pro-survival autophagic response in neurospheres, which diverted from a proliferative state, and dual treatment with autophagy inhibitor quinacrine led to a synergistic decrease in glioblastoma cell viability.[69]

For this study GSK650394, EMD638683, SGK1-IN-1, and SGK1-inh were tested. While some of these inhibitors have been tested in other cancer models and show desirable results, there is still minimal research regarding most of these inhibitors in glioblastoma models. To test the effects of these inhibitors in GBM, *in vitro* cell proliferation assays were conducted with varying concentrations of these SGK1 inhibitors on U87MG, an adherent GBM cell line, to determine if the drugs are active inhibitors of glioblastoma cell growth. The SGK1 inhibitors were compared with PI3K inhibitors that have been proven to have activity in GBM and are currently undergoing phase 1 trials. The discovery of SGK inhibitors that show activity in glioblastoma could allow for the improvement of these drugs and subsequent clinical trials that could lead to SGK inhibitors as a treatment to manage glioblastoma.

MATERIALS AND METHODS

Reagents

GSK650394 (Catalog # HY-15192; CAS: 890842-28-1), EMD638683 (Catalog # HY-15193; CAS: 1181770-72-8), and SGK1-IN-1 (Catalog # HY-18607; CAS: 1279829-87-6) were procured from MedChemExpress, while SGK1-inh (ID # GS-9007; CAS: 1426214-51-8) was from Key Organics. Alpelisib (Catalog # 76203-088; CAS: 1217486-61-7) was acquired from VWR International and Fimepinostat (Catalog # 501872903; CAS: 1339928-25-4) from Fischer Scientific. All inhibitors were diluted to working concentrations in dimethyl sulfoxide (DMSO).

Cell Culture

U87MG cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated Fetal Bovine Serum and 1% Penstrep. The cells were disassociated with trypsin. All cells were maintained at 37 degrees with 5% CO_2.

Drug Administration

The U87MG cells were plated in a 96-well plate at a density of $1x10^4$ cells per well in 50 μL of media. 24 hours after plating, the cells were either treated with each of the SGK inhibitors (GSK650394, EMD638683, SGK1-IN-1, and SGK1-inh), the PI3K inhibitors (Alpelisib and Fimepinostat), or DMSO (at the same final concentration as other solutions). The SGK inhibitors were tested at various concentrations as indicated. The cells were treated for either 24, 48, or 72 hours and the total final volume for all time points was 100 μL. The 24-hour treatment had one drug dose administration, the 48-hour treatment had 2 (one at 24 hours after plating, and one 24 hours after the first dose), and the 72-hour treatment had 3 administrations (in a similar fashion to the 48 hour treatment with a third dose 24 hours after the second dose). Any drugs that did not

show a time or dose-dependent response up to 100 μM were claimed to not effectively inhibit cell growth.

MTS Assay

After the cells had been treated with their appropriate drug for either 24, 48, or 72 hours, their cell viability was measured using the CellTiter 96 Cell Proliferation Assay (Promega, Madison, WI), an assay that is an accurate measure of cell viability, especially after drug treatment of cancer cells.[70] 20 μL was added to each well and incubated for 4 hours, after which it was shaken for 45 seconds and the Optical Density (OD) was read at 490 nm on a Biotek Synergy 2 automated plate reader.

Data Analysis and Statistical Calculations

The cell viability percentage was calculated as:

$$Percent\ cell\ viability = OD\ test\ sample / OD\ DMSO\ control \times 100$$

The OD test sample was the mean of 5 inhibitor-treated wells while the OD DMSO control was the mean of 5 DMSO treated wells. All drugs were tested in triplicate and the results are one trial representative of all trials. The error bars are representative of the standard error of the mean (SEM). The SGK inhibitors were compared to the DMSO control using an unpaired t-test assuming Gaussian distribution with a confidence level of 95%. The statistical significance is as follows: 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), <0.0001 (****).

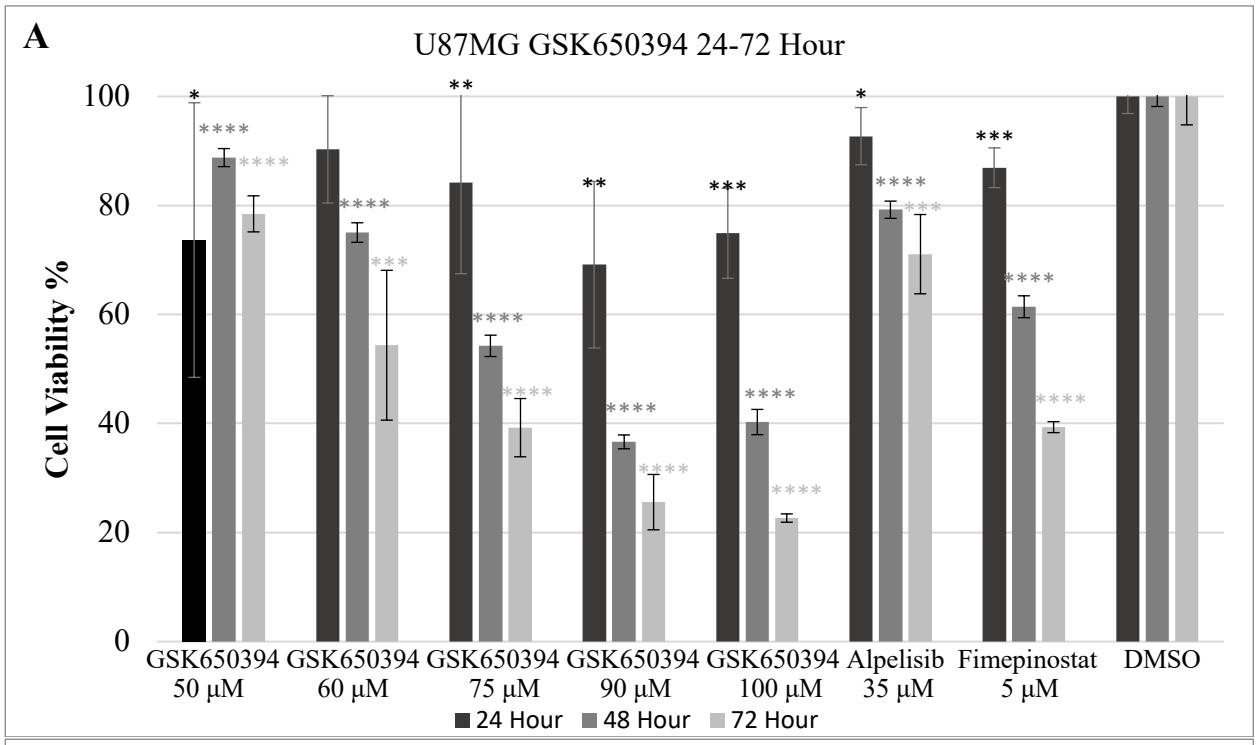
RESULTS

To evaluate the efficacy of various SGK inhibitors for glioblastoma, a color-based MTS assay was conducted on the glioblastoma cell line U87MG exposed to the SGK inhibitors GSK650394, EMD638683, SGK1-IN-1, and SGK1-inh. The cells were treated for either 24, 48, or 72 hours, after which an MTS assay was administered to measure percent absorbance at 490 nm to determine cell viability. The cells treated with SGK inhibitors were compared against DMSO as a negative control, while the PI3K inhibitors Alpelisib and Fimepinostat were used as positive controls (Figure 2).

Overall, GSK650394 was active in decreasing cell viability at concentrations between 50 and 100 μM when compared against DMSO, especially at 48 and 72 hours (Figure 2A). The inhibition of cell viability appeared to be both time and dose-dependent. The longer the cells were treated with a specific dose, the less cell viability was measured. Additionally, higher concentrations were able to inhibit cell viability to a greater extent than the lower concentrations.

The other three inhibitors including EMD638698, SGK1-IN-1, and SGK-inh were unable to effectively inhibit cell growth at concentrations under 100 μM in either a dose-dependent or time-dependent manner. Although there was some inhibition measured at 150 μM, they did not inhibit in a time dependent manner, as the same level of inhibition was measured regardless of 24, 48, or 72-hour treatment (Figure 2B). EMD638698 showed the least effect on cell viability, with the maximum inhibitory activity of approximately 70% cell viability at 72 hours at 150 μM (Figure 1B). SGK1-IN-1 and SGK-inh were able to bring the cell viabilities down to approximately 50% and 35% respectively, with SGK-inh showing inhibitory activity that is comparable to Fimepinostat at 48 hours (Figure 2B). However, the lack of a time-dependent response and such a high dosage is unfavorable to further studies.

Figure 2. **Cell viability analysis of SGK inhibitor-treated glioblastoma cell line.** A, B: Cell viability quantification based on MTS assay of glioblastoma cell line U87MG after treatment with various SGK inhibitors and PI3K inhibitors (positive controls) for 24 to 72 hours. Cell viability based on percentage absorbance at 490 nm compared to DMSO (negative control). Data are representative of triplicate experiments. Error bars indicate SEM. Statistical significance calculated via T-test against DMSO control (*≤ 0.0322; ** ≤ 0.0021; *** ≤0.0002; **** ≤ 0.0001).


A
U87MG GSK650394 24-72 Hour
Cell Viability %
100
80
60
40
20
0
GSK650394 50 µM
GSK650394 60 µM
GSK650394 75 µM
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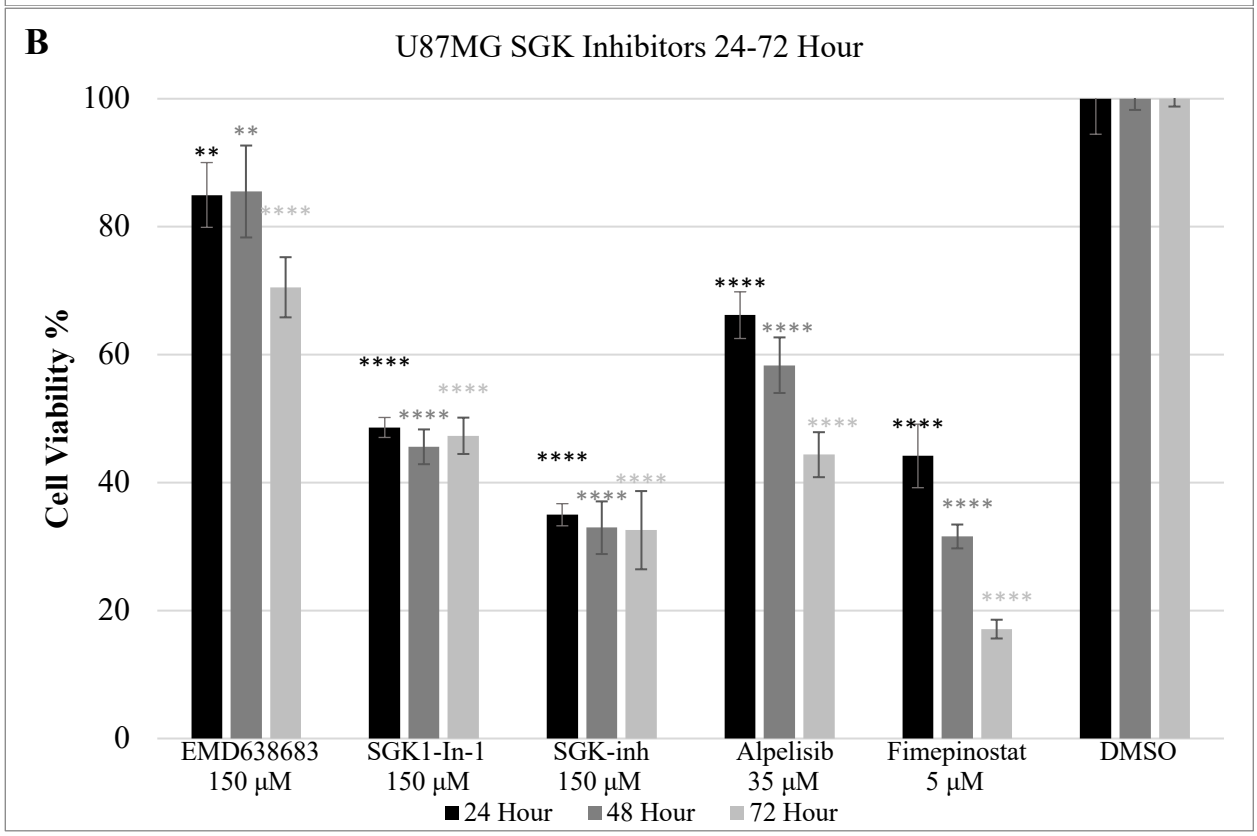
GSK650394 100 µM
Alpelisib 35 µM
Fimepinostat 5 µM
DMSO
24 Hour
48 Hour
72 Hour


B
U87MG SGK Inhibitors 24-72 Hour
Cell Viability %
100
80
60
40
20
0
EMD638683 150 µM
SGK1-In-1 150 µM
SGK-inh 150 µM
Alpelisib 35 µM
Fimepinostat 5 µM
DMSO
24 Hour
48 Hour
72 Hour

DISCUSSION

In this study, we found that GSK650394 is an SGK inhibitor that can potentially be used to inhibit SGK1 and glioblastoma cell growth. It is active within a wide range of concentrations, although not at such low concentrations as PI3K inhibitors Alpelisib and Fimepinostat (Figure 1A). Other SGK inhibitors including EMD638683, SGK1-IN-1, and SGK-inh did not efficiently inhibit SGK activity at concentrations below 100 μM, although some inhibitory activity was measured at 150 μM (Figure 1B).

Future *in vitro* studies should be done in other glioblastoma cell types with different traits compared to U87MG. U87MG has increased VEGFR expression due to EGFR activation, which is present in 50-60% of glioblastomas,[71] and this leads to increased PI3K activity and more downstream Akt phosphorylation, which is especially present in U87MG when compared to other glioblastoma cell lines.[72] U87MG also has known PTEN deficiency due to PTEN mutations, preventing PI3K from being properly regulated.[73] These inhibitors should be tested in other glioblastoma cell lines with different genetic profiles, such as TS576 and GBM6, to elucidate the cell lines showing the most drug sensitivity. TS576 has virtually no EGFR expression and lower levels of Akt,[74,75] while GBM6 has EGFR amplification and the EGFRvIII mutation, which constitutively activates EGFR.[76] Furthermore, while U87MG is an adherent cell line, TS576 and GBM6 form neurospheres in suspension, which provides a new microenvironment that has been proven to independently predict the outcome of patient gliomas.[77] This experiment could be done not only to determine if GSK650394 and other SGK inhibitors are functional in these other cell lines, but it may also allow us to determine the IC50 values of these inhibitors in different glioblastoma cell lines.

With GBM6 and TS576, neurosphere formation assays could also be done to study if the SGK inhibitors affect the ability to form neurospheres, as was seen by Matteoni and colleagues when treating GBM3-Luc and GBM-I neurosphere forming cells with the SGK1 inhibitor SI113.[69] TS576 and GBM6 cells could be treated for 48 hours with a specific concentration of GSK650394 based on its IC50, then dissociated and replated in 6 well plates at a low concentration, where the number of neurospheres could then be counted after approximately 1 week.

Future *in vitro* studies could also include dual inhibition with both a PI3K inhibitor and an SGK1 inhibitor, most likely GSK650394, as previous studies have suggested that the inhibition of mTOR by inhibiting both Akt and SGK activity would have the most beneficial effect.[57] Based on the activity of GSK650394 measured previously, a specific concentration of GSK650394 could be tested in combination with the most active concentrations of Alpelisib or Fimepinostat. This would show the effects of dual inhibition of Akt and SGK through PI3K inhibition (Alpelisib) in conjunction with SGK inhibition (GSK650394) compared with dual HDAC/PI3K inhibition (Fimepinostat) with SGK inhibition (GSK650394). In addition to dual inhibition, radiation can also be tested as a possible variable, as many previous studies have seen increased *in vitro* inhibitory effects when treated with an inhibitor and radiation.[37, 64, 68]

One of the next steps would be to study the mechanism of how GSK650394 and the other SGK inhibitors function in glioblastoma cells to try and glean some information on why they were unable to effectively inhibit glioblastoma cell growth. Perhaps the lack of sensitivity of the cells to the ineffective inhibitors was due to an epigenetic upregulation of Akt, similar to the epigenetic upregulation of SGK in cells insensitive to Alpelisib inhibition.[57] A western blot could be conducted looking at the effects of GSK650394 on protein expression. U87MG and TS576

cells could be treated with 60 μM GSK650394 for 48 hours along with other cells treated with Alpelisib, Fimepinostat, and DMSO as controls. The concentrations for Alpelisib and Fimepinostat should show approximately the same cell viability as the GSK650394 at 48 hours. Some proteins of interest would be p27, Akt, phosphorylated Akt (pAKT), and 4E-BP1.

$P27^{kip1}$ (p27) is a cyclin-dependent kinase (Cdk) inhibitor that functions as a tumor suppressor.[78] Its mRNA levels are constant throughout the cell cycle, regulated by translational controls and ubiquitin-mediated proteolysis, although p27 is most active at G0 and early G1 to inhibit G1 cyclin Cdks.[78] If p27 is unable to regulate Cdk nuclear activity due to displacement in the cytoplasm, this leads to loss of cell cycle inhibition and deregulation, one of the hallmarks of cancer.[79] Although p27 is not completely mutated or deleted, low levels of p27 are associated with many cancers and these are often linked with worse prognosis and increased proliferation, especially in glioblastoma.[78,79] Loss of p27 appears to be mediated by activation of Ras, MAPK, and PI3K although PI3K inhibitors wortmannin and LY294002 appear to nullify the Ras effects on p27 loss.[78,80–82] Akt, mediated by either PI3K or mTOR, and mTORC1- mediated SGK activation contribute to the phosphorylation of p27, delaying its nuclear import and Cdk inhibitory activity (Figure 1).[80,83] In glioblastoma, accumulation of cytoplasmic p27, especially in U87MG tumor cells, is associated with pro-oncogenic effects, promoting tumor metastasis and glioblastoma cell invasion.[84,85] Inhibition of SGK1 with GSK650394 in colorectal cancer was found to inhibit cancer cell proliferation *in vivo* and *in vitro* while simultaneously enhancing p27 protein levels and nuclear accumulation.[60] The increased expression of downstream p27 would show that SGK activity is being inhibited.

The levels of phosphorylated Akt (pAKT) and total Akt could also be measured. Since Akt is phosphorylated by mTORC2, if pAKT levels decrease in GSK650394 treated cells, that

would suggest lower activity levels of mTORC2, as is seen with SI113.[69] Based on lower measured protein levels of pAKT and phosphorylated SGK1, SI113 was determined to decrease mTORC2 activity, an effect only seen in neurosphere glioblastoma cells, not in adherent cells.[69] Conducting a western blot on both adherent and neurosphere-forming glioblastoma cell types would allow us to see if any effects are only specific to one cell type. If the inhibitors that showed a lack of activity below 100 μM indeed do cause an epigenetic upregulation of Akt, then we would expect to see increased levels of Akt and pAKT compared to cells with no treatment or even perhaps cells treated with GSK650394.

To measure mTORC1 activity, we could measure the levels of 4E-BP1, a downstream substrate activated by phosphorylation at multiple sites through mTORC1 (Figure 1).[86] Treatment with SI113 was found to decrease 4E-BP1 levels, and therefore it can be assumed that mTORC1 is also inhibited. However, this decrease was only measured in neurosphere but not adherent glioblastoma cell lines.[69] By measuring 4E-BP1 levels in U87MG and TS576 cell lines, we can study if GSK650394 has a similar mechanism to SI113. The inhibition of mTORC1 and mTORC2 by SI113 in only neurospheres opens an intriguing mechanistic avenue to explore if the same is seen with GSK650394 inhibition in the adherent U87MG cell line and the neurosphere forming TS576 cell line.

Overall, the finding that GSK650394 is active in U87MG glioblastoma provides the basis for future *in vitro* experiments that can further explore the inhibitory potential of SGK inhibitors, either on their own or in combination with other treatments such as PI3K inhibitors. Better understanding of the mechanisms behind the inactivity of EMD638683, SGK1-IN-1, and SGK-inh in U87MG, such as possible compensatory Akt upregulation, could allow an expansion in the range of possible glioblastoma cell types that could be targeted by these inhibitors. With the

variety of challenges faced by glioblastoma treatment, this study provides evidence that targeting SGK in glioblastoma and possibly other gliomas may be beneficial in preventing the spread and further growth, and future *in vivo* studies could potentially offer up SGK inhibition as a valid treatment for glioblastoma.

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