Screen of Glioblastoma Cancer Stem Cells with a Kinase Inhibitor Library

A thesis submitted by

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Abstract

Glioblastoma Multiforme (GBM) is the most common and most aggressive form of brain cancer in adults. Despite aggressive treatment modalities, median survival post diagnosis has changed little since the 1980s and remains stagnant at 12-14 months. There are a host of novel, targeted therapies being developed such as immunotherapy but up until now, none of them has been able to extend progression-free survival in any significant way. Because of this, research into the underlying mechanisms that control cell proliferation and tumor invasion is critical to the development of more targeted therapies. GBM is a prime example of the cancer stem cell theory in which a subset of tumor cells with stem-like properties is responsible for the maintenance and recurrence of tumors. These GBM stem cells (GSCs) are resistant to chemo- and radiotherapy making their eradication the largest obstacle to treatment.

We obtained a commercially available kinase inhibitor (KI) library and used it to screen three of our GSC lines. After incubating the cells with KIs for 6-8 days, we performed the PrestoBlue cell viability assay to determine which compounds were growth inhibitory. Our results show that inhibition of the kinases in the RTK/Ras/PI3K signaling pathway, commonly altered in GBM, leads to significant suppression of GSC proliferation. Additionally, protein kinase C (PKC) inhibitors were among the most effective compounds in the entire library. We performed Western Blot analysis of the downstream targets of PKC in order to further understand this result. We show that

inhibition of PKC suppressed phosphorylation of Ribosomal Protein S6 but had no effect on the activation of extracellular-signal related kinases (ERK1/2) or Nuclear Factor Kappa-Light-Chain-Enhancer of B Cells (NF-κB) p65.

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List of Abbreviations

BIM-IX	Bisindolylmaleimide-IX (mesylate)
CNS	Central Nervous System
CSC	Cancer Stem Cell
EGF	Epidermal Growth Factor
EGFR	EGF-Receptor
ERK	Extracellular-Signal Related Kinase
FGF	Fibroblast Growth Factor
GBM	Glioblastoma Multiforme
GSC	Glioblastoma Stem Cell
KI	Kinase Inhibitor
MAPK	Mitogen-Activated Protein Kinase
MEK	MAPK kinase
mTOR	Mammalian Target of Rapamycin
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of B Cells
PDGFR	Platelet Derived Growth Factor Receptor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
RTK	Receptor Tyrosine Kinase
S6	Ribosomal Protein S6
SCM	Stem Cell Media
TBS	Tris-buffered Saline
TBST	TBS with 0.2% Tween-20
TMZ	Temozolomide
VEGFR	Vascular-Endothelial Growth Factor Receptor

1. Introduction

Cancer is one of the world's leading causes of mortality. In the United States, it is second only to heart disease and cancer deaths number over 600,000 in 2017 (Siegel, Miller, & Jemal, 2017). Characterized by uncontrolled growth and invasion of surrounding tissues, cancer is a disease that can affect virtually any part of the body. And while important advances have been made in the treatment of many cancers, there are some for which little to no progress has been made. Glioblastoma Multiforme (GBM) is among the deadliest types of cancer known to man. While uncommon relative to other types of cancer, the median survival with therapy is only 15 months, only 5% of patients survive more than 5 years and only 2.6% survive longer than 10 years (Ostrom et al., 2014). The latter statistic looks to be closer to and even less than 1% when looking at a larger population of cases as was done by Tykocki and Eltayeb (Tykocki & Eltayeb, 2018) It is the most common and most severe grade of primary brain tumor in humans and the progression free survival rate can best be described as abysmal. The adjective multiforme as used to describe glioblastoma has multiple meanings; this term can be used to describe the heterogenous histopathology that arise within a GBM tumor but also the multitude of genetic mutations that occur leading to a diverse population of cells.

1.1. Brain Cancers

Generally speaking, solid tumors are graded based on the TNM Classification of Malignant Tumors. In this staging system the 'T' refers to the tumor size measured in centimeters-cubed, the 'N' to the nodal status or the degree to which the tumor has spread to surrounding or distant lymph nodes, and 'M' refers to the presence or absence of metastasis. This system is not used, however, to describe brain cancers for multiple reasons. For one, tumor size in the case of brain tumors is not usually associated with severity. Additionally, there are no lymphatics in the Central Nervous System and therefore tumors of the CNS never have a positive nodal status. Lastly, it is rare that a patient lives long enough with a brain tumor for it to develop into metastatic disease (Board, 2002).

According to the World Health Organization, there are 4 histological grades of CNS malignancies. Grade I is the lowest grade and concerns tumors that are largely non-invasive and show very little evidence of proliferation. These are generally removed surgically without recurrence. Grades II and III are tumors that demonstrate low to medium proliferative characteristics (i.e., mitotic activity and atypical nuclei) and recur more frequently than Grade I tumors. Tumors of these grades have a higher chance of progressing into higher grades. Grade IV is the highest and most severe grade of brain tumor and describes lesions with high mitotic activity and are prone to necrosis (Board, 2002). GBM are not only the highest grade, but also the most common out of all types of malignant brain tumor (Holland, 2000).

1.2. Glioblastoma: Symptoms and Treatment

There are a number of factors to consider when discussing the high mortality rate of GBM. For one, there are a number of obstacles to the diagnosis of brain tumors. GBM presents with symptoms that can vary from headaches and migraines to mood changes and cognitive dysfunction such as aphasia or visual loss. For instance, about 20% of new cases presented with seizures as a symptom. The nature and severity of the symptoms depends largely on the location of tumor in question. The milder of these symptoms are easily ignored and without progression are usually without need for concern, leading to unchecked progression. The more severe of these symptoms can be indicative of a number of conditions including but not limited to hypoglycemia or aseptic meningitis. The median age of diagnosis for GBM is between 55 and 70 years of age, a time when symptoms from many age-related diseases, such as Alzheimer's or Parkinson's, would begin to present. All these factors together contribute to an estimated death toll of 16,700 patients in 2017 (Siegel et al., 2017).

The first-line standard of care treatment of GBM has not changed substantially in the past couple decades. In fact, temozolomide has been indicated by the FDA for the treatment of newly-diagnosed GBM or refractory anaplastic astrocytoma since its approval in 1999 (Mutter & Stupp, 2006), nearly twenty years ago. In 2005, Dr. Roger Stupp published a suggested protocol of treatment with TMZ and radiation therapy and it is the basis for most GBM therapy to this day (Stupp et al., 2005). Treatment commonly consists of maximum safe surgical resection followed by adjuvant

radiotherapy in addition to or instead of treatment with TMZ (Louis Burt Nabors et al., 2015). In recent years, these guidelines have been updated to include an emphasis on enrolling patients in clinical trials in addition to checking O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation (L. B. Nabors et al., 2018). MGMT is a DNA repair protein and is responsible for repairing the kind of DNA damage caused by TMZ. Methylation of the MGMT promoter is therefore a prognostic indicator of response to TMZ treatment. Nonetheless, TMZ is still part of the recommended therapy regimen for those patients without MGMT promoter methylation. In virtually every case these treatments are incapable of achieving long -term remission, and the tumors inevitably return, frequently with new mutations (Muscat et al., 2018).

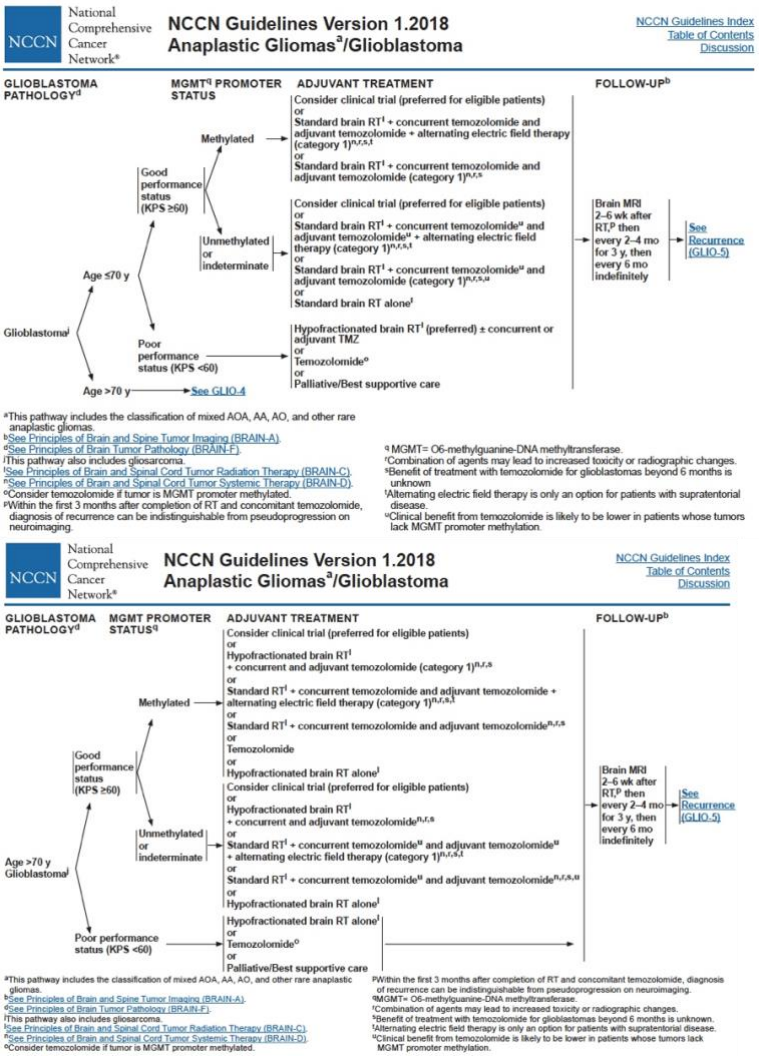


Figure 1.1: Standard treatment regiments for patients under and over age 70 with Glioblastoma as recommended by the National Comprehensive Cancer Network. Adapted by combining figures GLIO-3 and GLIO-4, with permission from the NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) for Central Nervous System Cancers V.1.2018. © 2018 National Comprehensive Cancer Network, Inc. All rights reserved. The NCCN Guidelines® and illustrations herein may not be reproduced in any form for any purpose without the express written permission of NCCN. To view the most recent and complete version of the NCCN Guidelines, go online to NCCN.org. The NCCN Guidelines are a work in progress that may be refined as often as new significant data becomes available. NCCN makes no warranties of any kind whatsoever regarding their content, use or application and disclaims any responsibility for their application or use in any way.

Research is being done to assess the effectiveness of newer therapies in the treatment of GBM. There is some evidence to suggest that Tumor Treating Fields Technology, the use of alternating electrical fields to disrupt mitotic spindle formation, when used concurrent to treatment with TMZ, can improve both PFS and OS in patients with newly diagnosed GBM (Benson, 2018). Medical advances such as immunotherapy and angiogenesis inhibitors also have the potential to be more effective in halting or reversing the disease (Coleman, Ameratunga, & Lopez, 2018; Lotshaw, 2016). Cutting edge techniques such as CRISPR/Cas9 gene editing also provide some hope of tackling the underlying genetic basis for resistance (Cai & Sughrue, 2018).

1.3. Cancer Stem Cells in GBM

There are two main models used to explain the heterogeneity of solid tumors in many types of cancer. The stochastic model, also referred to as clonal evolution, tells us that any stem or progenitor cell is capable of becoming tumorigenic if exposed to the right oncogenic triggers (Greaves & Maley, 2012). From there, each daughter cell has the potential to undergo further genetic mutation caused by either endogenous or exogenous factors. These alterations and the resulting selection occur in a manner consistent with Darwinian evolution. The Cancer Stem Cell (CSC) model, on the other hand, theorizes that intratumoral heterogeneity is the result of a cellular hierarchy (Fabian, Vereb, & Szollosi, 2013). CSC's are capable of generating an epigenetically diverse population of daughter progenitor cells which proliferate and differentiate into the cells that make up the majority tumor cells. Importantly, CSC's are the only cells

with the multipotency and self-renewal characteristics of stem cells. It is likely that the true origin of intratumoral heterogeneity is some combination of these two hypotheses, but the existence of CSC's in common solid tumors such as breast, lung, and brain cancers is well evidenced (Dawood, Austin, & Cristofanilli, 2014; Hong et al., 2018; Maiuthed, Chantarawong, & Chanvorachote, 2018). CSC's arise through a dysregulation of stem cell signaling pathways including WNT, Hedgehog, and Notch (Kaur, Sharma, Dogra, & Singh, 2018).

Therapies used to treat GBM and other solid tumors fail or are ineffective for any number of reasons. An important one is the inability to target and eliminate the CSC population within the tumor. Liu et all showed that CD133+, a stem cell marker in GBM, cancer cells were significantly resistant to treatment with TMZ, carboplatin, and other anticancer drugs with various targets, relative to cancer cells lacking the CD133 marker (Liu et al., 2006). Additionally, they found that cells from patients with recurrent GBM had higher levels of CD133 expression than patients with newly diagnosed disease. Together, these results indicate that not only are GSC responsible for the resistance to therapy, they are also to blame for recurrence and the increased resistance to therapy. Stem cell regulatory pathways are also able to confer resistance to radiotherapy in addition to chemotherapy. High activation of stem cell pathways such as Notch, Wnt, and Sox2 contribute to GSC stemness. Research by Lee et al demonstrates that inhibition of FoxM1 in combination with radiotherapy reduce stemness and proliferation

in GBM xenograft models, in addition to directly affecting Sox2 expression levels (Lee et al., 2015).

Stem-like behaviors are not the only way GSC's escape the effects of anticancer therapies. In addition to enhanced DNA repair and high drug efflux transporter activity, a large portion of these cells lie within hypoxic regions (Aderetti, Hira, Molenaar, & van Noorden, 2018). Many solid tumors have been characterized with substantial hypoxic regions. Furthermore, these regions have been found to confer added resistance to both radiation and chemotherapy to cells found within them. The lack of oxygen limits the mechanism of action of radiation killing and the reduced blood flow limits the penetration of chemotherapeutic agents (Khanal, Hiemstra, & Pappas, 2014; Li et al., 2013; Mitani et al., 2014; H. M. Wu, Jiang, Ding, Shao, & Liu, 2015). In addition, by up- or down-regulating various key pathways and proteins, hypoxia renders relative resistance to treatments that otherwise would actually be effective.

1.4. Important Pathways and Proteins in GBM

There are a number of commonly altered signaling pathways in GBM that contribute to cell growth and progression. The first of these, the p53 signaling pathway, plays a role in promoting cell-cycle arrest and apoptosis (Kastenhuber & Lowe, 2017). In GBM and many other types of cancer, signaling by p53 is inactivated either by direct mutation or deletion (35% of GBM cases), or by amplification of its negative regulators, Mouse double minute 2 and 4 (MDM2 14% and MDM4 7% of GBM cases) (Brennan et al., 2013). Another important upstream regulator of p53 signaling is $p14^{ARF}$. Arf

negatively regulates MDM2 and the gene that codes for it, CDKN2A, frequently undergoes homozygous deletion or methylation in GBM (Nakamura et al., 2001).

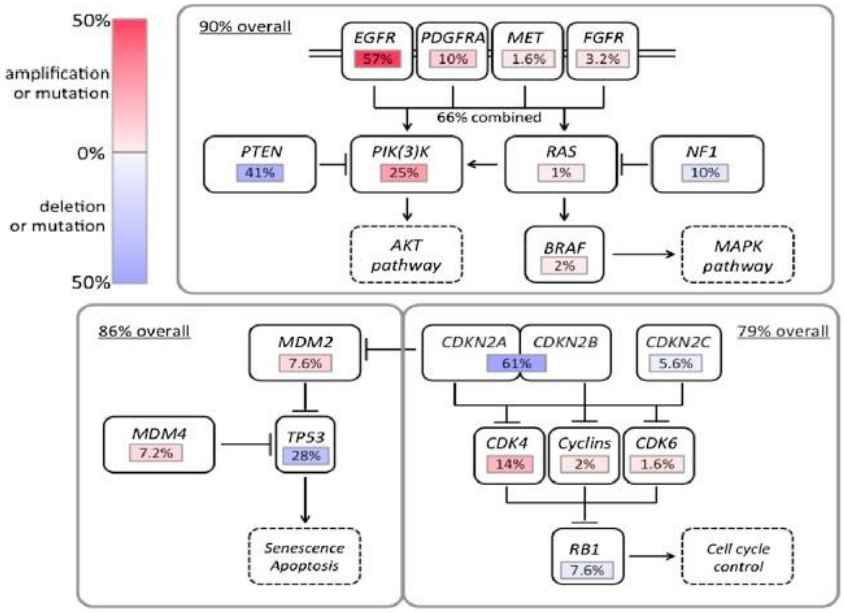


Figure 1.2: Commonly altered signaling pathways in GBM. Adapted and reproduced here with permission from the publisher (Brennan et al., 2013). Changes include cropping the figure to include only part A above.

Another significantly altered pathway is the Retinoblastoma (Rb) signaling pathway. Rb is a tumor suppressor and normally acts to inhibit cell cycle progression until the cell in question is ready to begin the DNA synthesis phase of mitosis. In primary GBMs, the RB gene is either mutated or deleted in 11% of cases and its promoter is methylated in 14% of cases (Crespo et al., 2015). In healthy cells, CDK4/cyclin D1 and CDK2/cyclin E are activated by degradation of bound CDK inhibitor p27. They then hyperphosphorylate Rb leading to entry into S phase by activating transcription of growth-promoting genes (Furnari et al., 2007). Additionally, $p16^{INK4A}$, which is generated by alternative splicing of the CDKN2A gene, is an endogenous inhibitor of CDK4 and 6

(LaPak & Burd, 2014). As has been previously mentioned, this gene is frequently mutated or deleted which leads not only to dysregulation of p53 signaling, but also Rb pathway signaling.

Yet another pathway that contributes considerably in the development and progression of GBMs is the Receptor Tyrosine Kinase (RTK)/Ras/Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt signaling pathway. RTKs such as Epidermal Growth Factor Receptor (EGFR) are activated by the binding of their ligands (EGF) or by activating mutations triggering a downstream signaling cascade regulating proliferation and survival. In GBM, EGFR is found to be amplified in approximately 60% of cases (Hurtt, Moossy, Donovan-Peluso, & Locker, 1992). Of these, about half are the EGFRvIII mutant variant of the receptor. This mutation is a deletion of exons 2 through 7 resulting in a ligand-independent, constitutively active RTK (Hatanpaa, Burma, Zhao, & Habib, 2010). While this is the most commonly deregulated RTK, there are also a significant portion of cases with amplification of the Platelet Derived Growth Factor Receptor (PDGFR), which may be to blame for the lack of tumor response to treatment with single RTK-inhibitors (Chakravarty et al., 2017).

The downstream effectors of this pathway are also frequently altered in GBM. Upon its activation, PI3K generates phosphatidylinositol-4,5-bisphosphate (PIP2) and -3,4,5-triphosphate (PIP3). These go on to recruit and bind Akt, activating it to regulate one of many cellular processes including but not limited to cell growth, proliferation, metabolism, and survival (Risso, Blaustein, Pozzi, Mammi, & Srebrow, 2015). In GBM,

mutations to PI3K occur around the binding domains on either the p85 regulatory subunit or the p110 catalytic subunit, limiting the ability for p85 to regulate p110 (The Cancer Genome Atlas Research, 2008).

The regulators of this signaling pathway are also commonly silenced. Phosphate and Tensin homolog (PTEN) negatively regulates PI3K signaling but it is mutated in deleted in 30% of GBM cases (Parsons et al., 2008). Neurofibromin 1 (NF1), which negatively regulates Ras signaling, has also been found to be mutated or deleted in 15% of GBMs. Together, these alterations contribute to maintaining high levels of cell proliferation in GBMs.

1.5. Kinase Inhibitors

Kinases are proteins that catalyze the transfer phosphate groups from donors (i.e., ATP) to substrate proteins. This process, referred to as phosphorylation, is used by cells to regulate many cellular processes. Kinases are integral to signal transduction and as we have already mentioned, they are almost always deregulated in cancer. Ever since the first Kinase Inhibitor (KI) was approved by the FDA in 1999, research into the field of kinase inhibition has exploded. From the approval of Sirolimus up to 2012, there was around one approval per year; since then, the rate has jumped to almost 4 per year with a total of 43 approved drugs as of 6/30/2018 (Roskoski, 2018). Of these, 38 (or 88%) are indicated for use in the treatment of various types of cancer.

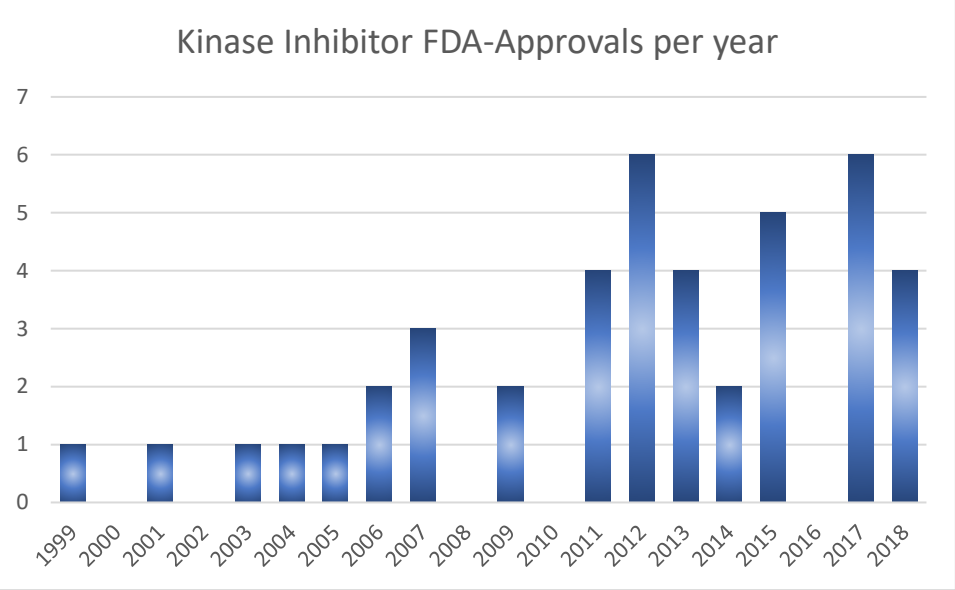


Figure 1.3: Number of KIs approved by the FDA per year since 1999. Adapted from public website (Roskoski, 2018). Data was summarized into the above bar-graph.

Aside from their obvious clinical use, KIs are excellent tools for research. Kinases have long been the subject of academic research, with well over 1 million publications (P. Wu, Nielsen, & Clausen, 2015). As our knowledge and understanding of the human kinome increases, we are better able to comprehend the inner workings of both healthy and diseased cells and thus better prepared to develop targeted treatments. By utilizing a KI library, we tested our GSC lines against a wide variety of inhibitors in order to determine which proteins and which signaling pathways are important to the survival of these cells.

The KI library that we used in this screen is made up of 152 specific and non-specific KIs covering 96 different targets. Many of the inhibitors target members of the important signaling pathways in GBM, notably the RTK/PI3K/Akt and the RTK/Ras/RAF pathways. Many of the other inhibitors included in this library target peripheral

regulators of these pathways or signal transducers involved in a multitude of cellular processes.

2. Materials and Methods

2.1 Cell Lines used

Human GBM stem cell lines 6.22 and 11.1 were previously established according to (Sherry, Reeves, Wu, & Cochran, 2009) in the laboratory from patient tumor samples from the Tufts Medical Center (Goel, 2016). MGG8 cells were previously acquired from the laboratory of Dr. Hiroaki Wakimoto (Wakimoto et al., 2012).

2.2 Cell Culture

GBM stem cells were cultured in 100mmX20mm Falcon tissue culture dishes (ThermoFisher, Waltham, MA) in Gibco DMEM/F-12 + GlutaMAX media (ThermoFisher) supplemented with Gibco Pen Strep antibiotic (ThermoFisher), Gem21 NeuroPlex (Gemini Bio-Products, West Sacramento, CA), Animal-Free Recombinant Human FGF-basic and Animal-Free Recombinant Human EGF (PeproTech, Rocky Hill, NJ) and referred to as Stem Cell Media (SCM). The SCM mixture was then filtered through Millipore Stericup 0.22µm pore-size filtration unit (Merck, Kenilworth, NJ). Weekly, cells were transferred into 15mL Falcon conical tubes, spun down, supernatant aspirated, and resuspended in 1mL Accutase cell dissociation reagent before incubating in a 37°C water bath for 5-10 minutes. The tubes were then spun down again, the Accutase aspirated, and the pellet resuspended in 1mL pre-warmed SCM. A portion of this single-cell suspension was then plated in 7-10mL fresh SCM in a new culture dish, before being returned to the incubator.

2.3 Cell viability Assay

2,500 to 5,000 cells per well were plated as single a cell suspension in Corning Costar tissue-culture treated 96-well clear-bottomed plates (MilliporeSigma, Burlington, MA). DMSO or Inhibitors at 1µM or 5µM were added to each sample well and incubated for 6 days in normoxia or 8 days in hypoxia (≤1% O_2). At the end of this incubation period, 40x Trypsin was used to separate the neurospheres into a single cell suspension by adding 5µL per well, mixing by pipetting up and down 5 times, and incubating at 37°C for a period of 10 minutes. The plates were then removed from the incubator and 20µL of Invitrogen PrestoBlue Cell Viability Reagent (ThermoFisher) was added to each well before mixing 5 times using a ViaFlo 300µL Multichannel electronic pipette (INTEGRA Biosciences Corp., Hudson, NH). The plates were then incubated at 37°C for 30 minutes. Fluorescence was then read from plate bottom using a Tecan SPECTRAFluor Plus with the excitation filter wavelength set to 544nm and the emission filter wavelength set to 590nm.

2.4 Kinase Inhibitor Library

The Kinase Inhibitor library was obtained from Cayman Chemical (Ann Arbor, MI) and it was made up of 152 specific and non-specific kinase inhibitors covering a variety of targets and pathways. The inhibitors are listed with their item numbers and targets in Tables 2.1 and 2.2.

Well #	Inhibitor Name	Cayman Item #	Target
A2	TG003	10398	Clk family kinases
A3	pkc412	10459	PKC, Syk, FLK1, Akt, PKA, c-Kit, C-Fgr, c-Src, FLT3, PDGFRβ, VEGFR1, VEGFR2
A4	Doramapimod	10460	p38 MAPK
A5	Paclitaxel	10461	disrupts multipolar spindle formation

A6	Erlotinib	10483	EGFR
A7	NVP-BEZ235	10565	PI3K, mTOR
A8	Phthalazinone pyrazole	10735	Aurora A kinase
A9	AG-879	10793	HER-2, TrKA, STAT3, ETK, FLK1
A10	1-NA-PP1	10954	v-Src-as1, c-Fyn-as1, c-Abl-as2, cdk2-as1
A11	Torin 1	10997	mTORC1, mTORC2
B2	BIBF 1120	11022	VEGFR, PDGFR, FGFR
B3	SMI-4a	11029	Pim Kinases
B4	CAY10657	11140	IKK2
B5	AS-703026	11226	MEK1/2
B6	Chelerythrine(cholride)	11314	protein kinase C, Bcl-xL
B7	Tunicamycin	11445	N-linked glycosylation
B8	AZD 7762	11491	Chk 1, Chk2
B9	GSK1059615	11569	PI3Kα
B10	Ruxolitinib	11609	JAK1 and JAK2
B11	Necrostatin-1	11658	RIP1 Kinase
C2	SB-505142	11793	ALK5, ALK4
C3	INK128	11811	TORC1/TORC2
C4	Canertinib (hydrochloride)	12076	EGFR, HER2, HER4
C5	SB-431542	13031	ALK5, ALK4
C6	PD 173074	13032	FGFR1 autophosphorylation
C7	Valproic Acid (sodium salt)	13033	Class I HDACs
C8	PD 0325901	13034	MEK
C9	SB 203580	13067	p38MAPK
C10	VX-702	13108	p38MAPK
C11	2-Hydroxyestradiol	13019	angiogenesis via the HIF-1A pathway
D2	CHIR99021	13122	GSK3α, GSK3β
D3	BIO	13123	GSK3α, GSK3β
D4	Imatinib (mesylate)	13139	c-Abl, PDGFR, KIT, BCR-ABL
D5	Sunitinib (malate)	13159	FLK1, PDGFRβ, FLT3
D6	Gefitinib	13166	EGFR
D7	PP2	13198	p56lck, p59fynT, Hck, Src
D8	3-Methyladenine	13242	PI3K
D9	Bisindolylmaleimide I	13298	PKC, GSK3, 5-HTR
D10	Bisindolylmaleimide IV	13299	PKC, PKA
D11	Bisindolylmaleimide V	13300	p70s6k/p85s6k (S6K)
E2	NSC 663284	13303	Cdc25A, Cdc25B2, Cdc25C
E3	D 4476	13305	CK1
E4	NU 7026	13308	DNA-PK
E5	Gö 6983	13311	PKC
E6	H-9 (hydrochloride)	13312	PKG , PKA
E7	Indirubin-3'-monoxime	13314	GSK3β, Cdk1/cyclin B, Cdk2/cyclin A, Cdk2/cyclin E, Cdk4/cyclin D1, Cdk5/p35
E8	NU 6102	13317	Cdk1, Cdk2
E9	KN-62	13318	CaMKII
E10	KN-93	13319	CaMKII
E11	CGP 57380	13322	MNK1
F2	Iso-Olomoucine	13325	inactive control
F3	(S)-Glycyl-H-1152 (hydrochloride)	13332	ROCKII
F4	Bisindolylmaleimide VIII	13333	PKC
F5	Bisindolylmaleimide IX (mesylate)	13334	PKC
F6	ST638	13337	tyrosine kinases
F7	SU6656	13338	Src, Yes, Lyn, Fyn
F8	LY364947	13341	TGF-β RI, TGF-β RII
F9	SB 203580	13344	p38 MAPK
F10	CAY10621	13371	SPHK 1
F11	YM-201636	13576	PIKfyve
G2	ZM 447439	13601	Aurora Kinases
G3	AS-041164	13622	PI3K
G4	NVP-AEW541 (hydrochloride)	13641	IGF-1R

G5	PP242	13643	mTORC1, mTORC2, PKC, PI3K, JAK2
G6	ABT-869	13653	VEGFR, PDGFR
G7	CAY10622	13687	ROCK-I, ROCK-II
G8	17β-hydroxy Wortmannin	13812	PI3K
G9	CAY10626	13838	PI3K, mTOR
G10	SU 6668	13873	PDGFRβ, VEGFR2, FGFR1
G11	PHA-767491	18218	Cdc7 Kinase
H2	N,N-Dimethylsphingosine	62575	SPHK
H3	LY294002	70920	PI3K
H4	U-0126	70970	MEK1, MEK2
H5	Staurosporine	81590	PKC
H6	KN-92 (hydrochloride)	9000890	inactive control
H7	AS-605240 (potassium salt)	9000980	PI3K
H8	PD 166326	9000988	c-Abl, BCR-ABL
H9	O-1918	10004914	GPR18, GPR55
H10	Y-27632 (hydrochloride)	10005583	p160ROCK, ROCK-II, PRK2
H11	Leelamine	10006148	PDK

Table 2.1: Plate 1 of the Cayman Chemical Kinase Inhibitor library.

Well #	Inhibitor Name	Cayman Item #	Target
A2	PD 98059	10006726	RAF, MEK
A3	PD 169316	10006727	p38 MAPK
A4	TGX-221	10007349	PI3K
A5	(S)-H-1152 (hydrochloride)	10007653	ROCK
A6	AS-605240	10007707	PI3K
A7	Sphingosine	10007907	PKC
A8	JNJ-10198409	10008131	PDGFR
A9	Leelamine (hydrochloride)	10008614	PDK
A10	Arachidonic Acid Leelamide	10008617	PDK
A11	Lauric Acid Leelamide	10008618	PDK
B2	AS-252424	10009052	PI3K
B3	CAY10505	10009078	PI3Kγ
B4	PI-103	10009209	PI3K, mTORC1, mTORC2
B5	PIK-75 (hydrochloride)	10009210	PI3K
B6	Sphingosine Kinase Inhibitor 2	10009222	SPHK
B7	Piceatannol	10009366	IκBα kinase
B8	SC-1	10009557	RasGAP, ERK1
B9	(R)-Roscovitine	10009569	Cdk2/cyclin E
B10	BAY 43-9006	10009644	Raf-1, B-Raf
B11	CAY10561	10010043	ERK2
C2	AS-604850	10010175	PI3K
C3	PI3-Kinase α Inhibitor 2	10010177	PI3K
C4	CAY10567	10010233	Akt1
C5	ML-9	10010236	Akt
C6	Triciribine	10010237	Akt
C7	Erbstatin analog	10010238	EGFR
C8	Kenpaullone	10010239	GSK3β, Cdk1/cyclin B, Cdk2/cyclin A, Cdk5/p25, Lck
C9	Olomoucine	10010240	CDC2/cyclin B, Cdk2/cyclin A, Cdk2/cyclin E, Cdk5/p35 kinase, ERK1/p44 MAPK
C10	AG-494	10010242	EGFR
C11	AG-825	10010243	Her2/Neu, EGFR, PDGFR
D2	AG-1478	10010244	EGFR
D3	SB-216763	10010246	GSK3α
D4	SB-415286	10010247	GSK3α
D5	AG-17	10010248	EGFR
D6	H-8 (hydrochloride)	10010249	cyclin C/Cdk8, cyclin H/Cdk7/p36 CTD kinase
D7	LFM-A13	10010265	BTK, Plk
D8	SC-514	10010267	IKK2

D9	Apigenin	10010275	CK2
D10	AG-18	10010300	EGFR
D11	DRB	10010302	CK2, Cdk7, Cdk8, Cdk9
E2	RG-13022	10010309	EGFR
E3	RG-14620	10010310	EGFR
E4	AG-490	10010311	JAK2
E5	AG-82	10010312	EGFR
E6	AG-99	10010313	EGFR
E7	AG-213	10010314	EGFR
E8	AG-183	10010315	EGFR
E9	Lavendustin C	10010329	EGFR
E10	ZM 336372	10010367	Raf-1
E11	5-Iodotubercidin	10010375	PKA, phosphorylase kinase, CK1, CK2, PKC
F2	SB 202190	10010399	P38 MAPK
F3	CAY10571	10010400	CSAID-binding protein
F4	Nilotinib	10010422	BCR-ABL
F5	SP 600125	10010466	JNK1, JNK2, JNK3
F6	L-threo-Sphingosine	10010541	PKC
F7	H-89 (hydrochloride)	10010556	PKA
F8	HA-1077 (hydrochloride)	10010559	ROK-II, PRK2, MSK1
F9	AG-370	10010568	PDGFR
F10	Wortmannin	10010591	PI3K
F11	AG-1296	10010592	PDGFR
G2	WHI-P131	10011246	JAK3
G3	CAY10574	10011247	Cdk9
G4	CAY10575	10011248	IKK-ε
G5	CAY10576	10011249	IKK-ε
G6	NH125	10011250	eEF-2K
G7	TWS119	10011251	GSK3β
G8	NSC 210902	10011255	CK2
G9	CAY10577	10011256	CK2
G10	CAY10578	10011264	CK2
G11	PD 184161	10012431	MEK1, MEK2
H2	CCT018159	10012591	HSP90
H3	Myricetin	10012600	TBAR formation

Table 2.2: Plate 2 of the Cayman Chemical Kinase Inhibitor library.

2.5 Cell Lysate Preparation

Cultured cells were dissociated by incubation in 1mL of Accutase at 37°C for 10min. They were then counted using a Scepter 2.0 handheld automated cell counter (MilliporeSigma) with 60µm tips attached. Cells were then plated at a density of 400,000 cells per 2mL of SCM in 6-well plates and incubated for 90-120min with inhibitors selected from the Cayman Chemical KI library. After incubating, cells were transferred with media to sterile 2mL centrifuge tubes and spun down for 5min at 4°C in an Eppendorf 5418 microcentrifuge (Hamburg, Germany). The supernatant media was then

aspirated, and the cells were resuspended in 40µL of Pierce RIPA Lysis buffer (Thermofisher) supplemented with protease and phosphatase inhibitors. After incubating for 15min on ice, the samples were briefly vortexed and spun down at 14,000rpm for 10min at 4°C. The supernatant was then transferred to new 1.5mL microcentrifuge tubes and the BCA assay was used to determine the protein concentration of the samples. 6X loading dye was then added to each sample before boiling for 5min and storing in the -20°C freezer.

2.6 Western Blotting

Cell lysates were loaded onto a 12-well SurePAGE gel (Genscript, Piscataway, NJ) with Precision Plus Protein Standards (Bio-Rad, Hercules, CA) and run in MOPS buffer at 120-150V for 1hr. The gel cassette was then opened, the gel sandwiched between blotting paper and a nitrocellulose membrane, air bubbles were removed by rolling out the gel sandwich with a Pasteur pipette, and the blot was transferred in 1X transfer buffer with 20% methanol at 100V with ice and stirring for 1-1.5hr. The blot was then treated with Ponceau stain (Sigma-Aldrich, St. Louis, MO) to confirm clean transfer before rinsing with DI H_2O and blocking with 5% nonfat Milk in TBST for 45min at room temperature with shaking. The blot was then rinsed three times with TBST for 5min with shaking and then incubated in primary antibody solution overnight at 4°C on a tilting shaker. The following day, the blots were removed from the antibody solution, rinsed three times with TBST for 5min with shaking and then incubated in secondary antibody conjugated with HRP (1:1000 or 1:3000 in 5% milk in TBST) for 45min covered at RT with

shaking. The blots were then rinsed three times in TBST for 5min with shaking and treated with a 1:1 mixture of Pierce ECL Western Blotting substrate reagents A and B (ThermoFisher Scientific) and placed in autoradiography cassettes. These were then brought to a dark room and exposed to Hyblot CL autoradiography film (Denville Scientific, Holliston, MA) for varying amounts of time. The film was then developed using a Mini-Medical 90 film processor (AFP Manufacturing, Peachtree City, GA).

2.7 Antibodies

Antibodies targeting phospho-p65 (#3033), p65 (#6956), p70S6K (#2708), phospho-S6 (#4858), phospho-ERK (#9106), and total ERK (#4695) were purchased from Cell Signaling Technology (Danvers, MA).

3. Results

3.1 Reproducibility of the Kinase Inhibitor Screen

In order to verify the validity of the data generated by the KI screen, an R-squared value was calculated for each set of duplicate plates by linear regression of paired values. The average of all values is 0.7967 and they range from 0.6397 to 0.9373. Additionally, the average of all slopes was 1.0561, with values ranging from 0.9037 to 1.245. A full statistical break down of the R-squared analysis can be seen in Table 3.1.

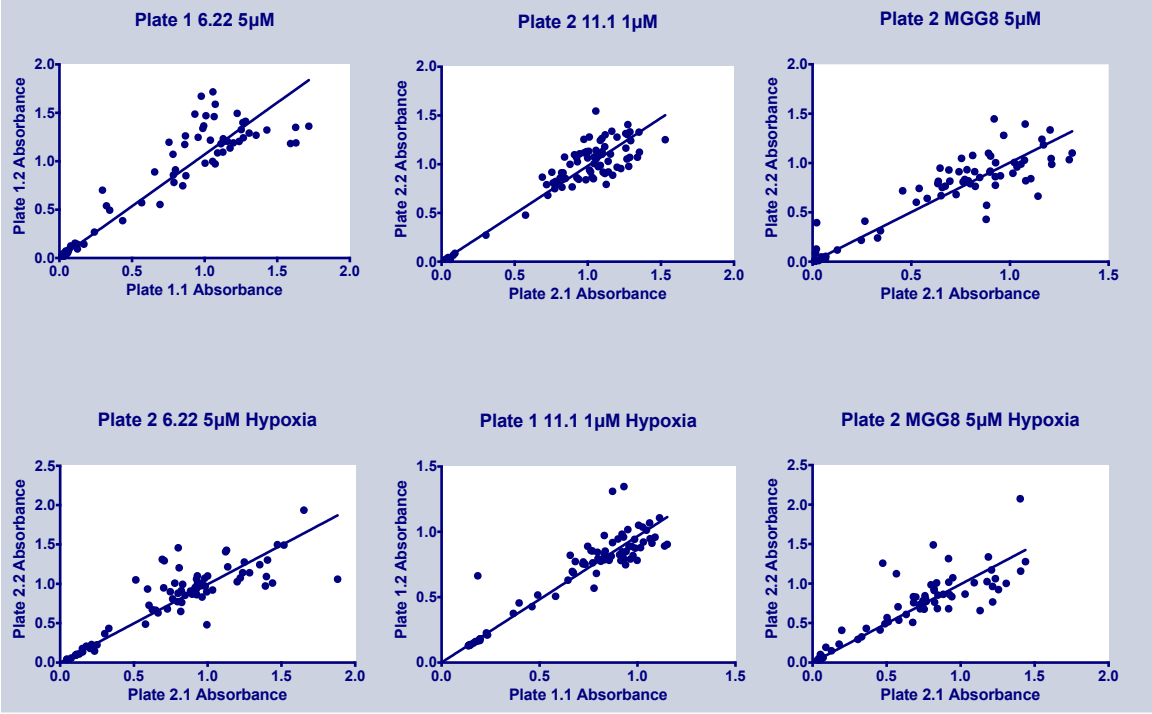


Figure 3.1: Representative R-squared regressions for each cell line in both normoxia and hypoxia.

	R-squared	Slope
Average	0.797	1.056
Average Absolute Deviation	0.068	0.065
Median	0.801	1.043
Median Absolute Deviation	0.063	0.054
Variance	0.007	0.007
Standard Deviation	0.081	0.081
Minimum	0.640	0.904
Maximum	0.937	1.245

Table 3.1: Statistics for R-squared analysis of duplicate plates.

3.2 KI Inhibitor Screen Results

The KI screen was conducted by plating cells with either DMSO or an inhibitor from the Cayman Chemical KI library. A diagram of this outline can be seen in figure 3.2. Each plate was performed in duplicate and incubated at 37°C in normoxia (pO_{2atm}=20%) for 6 days or in hypoxia ($pO_2 \leq 1\%$) for 8 days. At the end of this incubation, a cell viability assay was performed to determine the extent of the effect of each KI. Resulting values were standardized by subtracting an average background value from the media only wells; then the values of the KI treated samples were normalized relative to the average of each plate's DMSO control values. Paired duplicate values were then averaged. See the appendix for how to access the primary data.

Plate 1

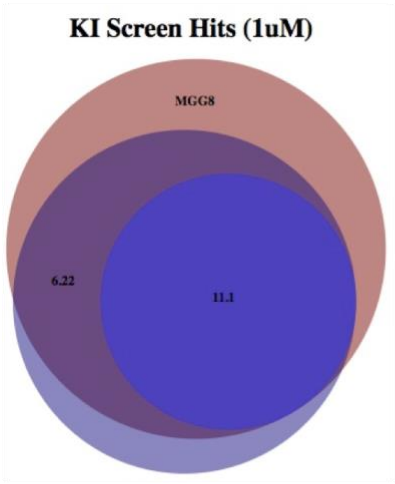
	A	B	C	D	E	F	G	H	I	J	K	L
1	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
2	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
3	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
4	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
5	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
6	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
7	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
8	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO

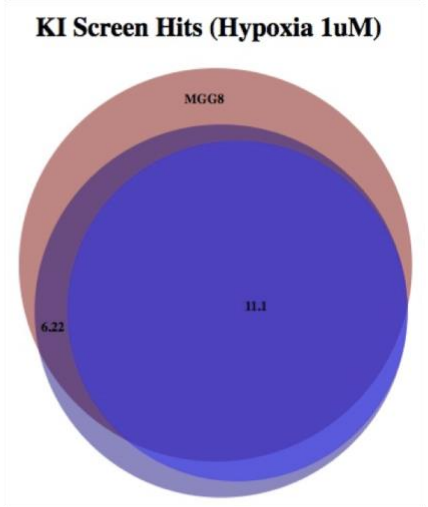
Plate 2

	A	B	C	D	E	F	G	H	I	J	K	L
1	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
2	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
3	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
4	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
5	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
6	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
7	DMSO	KI	KI	KI	KI	KI	KI	KI	KI	KI	KI	DMSO
8	DMSO	KI	KI	Media	Media	Media	DMSO	DMSO	DMSO	DMSO	DMSO	DMSO

Figure 3.2: Diagram of 96-well plates used in Kinase Inhibitor Screen.

For the purposes of this screen, we considered hits to be any compound with a growth inhibition of 80% or more (20% or less growth relative to DMSO controls) at either 1µM or 5µM. As can be seen in figures 3.3 and 3.4, the majority of hits were in common between the three GSC lines. These findings are in contrast to results in previous screens by our group, using shRNA and siRNA to target approximately 10,000, where the majority of hits were unique to each cell line and only select genes were identified as common hits (Kulkarni, 2016).





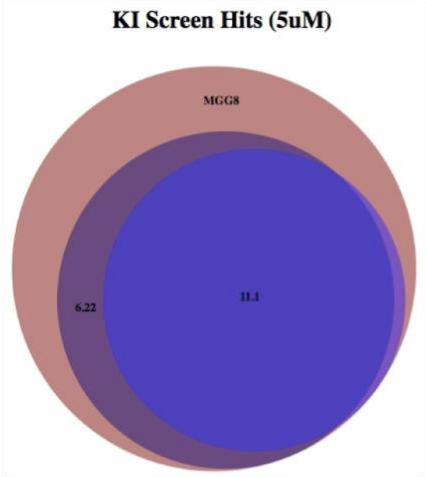
1µM Hits	MGG8	6	11
Total Hits	23	21	17
Unique Hits	5	1	0

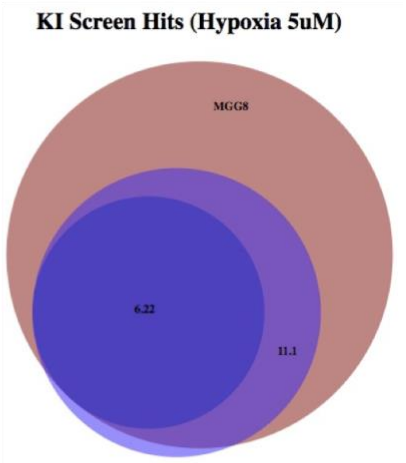
MGG8/6.22	3	
MGG8/11.1	0	
6.22/11.1	2	
All 3 Lines	15	57.7%

1µM Hypoxia Hits	MGG8	6	11
Total Hits	20	18	15
Unique Hits	4	1	0

MGG8/6.22	2	
MGG8/11.1	0	
6.22/11.1	1	
All 3 Lines	14	63.6%

Figure 3.3: Distribution of KI screen hits at 1µM inhibitor concentration. Venn Diagrams were generated with the BioVenn web application (Hulsen, de Vlieg, & Alkema, 2008).





5µM Hits	MGG8	6	11
Total Hits	59	41	33
Unique Hits	17	0	0

MGG8/6.22	9	
MGG8/11.1	1	
6.22/11.1	0	
All 3 Lines	32	54.2%

5µM Hypoxia Hits	MGG8	6	11
Total Hits	66	34	46
Unique Hits	21	0	1

MGG8/6.22	0	
MGG8/11.1	11	
6.22/11.1	0	
All 3 Lines	34	50.7%

Figure 3.4: Distribution of KI screen hits at 5µM inhibitor concentration. Venn Diagrams were generated with the BioVenn web application (Hulsen et al., 2008).

When comparing the hits from the cells incubated in normoxic versus hypoxic conditions, there was relatively little difference. Moreover, the compounds that were unique hits for one condition or the other targeted kinases that were also targeted by compounds that were found to be effective in both conditions. In 6.22 cells, there is seemingly the most variation in hits between conditions. Upon closer analysis, there are only a couple targets that are only affected under one condition. Glycogen synthase kinase 3 beta (GSK3β) was a hit in 6.22 cells under hypoxic conditions (80.8% growth inhibition) but not with normal oxygen (45.7% growth inhibition). A couple of mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitors were more inhibitory under normoxia than hypoxia (more than 80% in normoxia vs. 67.5% and 63.4% in hypoxia). The rest of the hits not shared by both compounds were hits in normoxia but missed the hit qualification mark by less than 10 percentage points, such as tunicamycin, an inhibitor of N-linked glycosylation (76.6% growth inhibition under hypoxia).

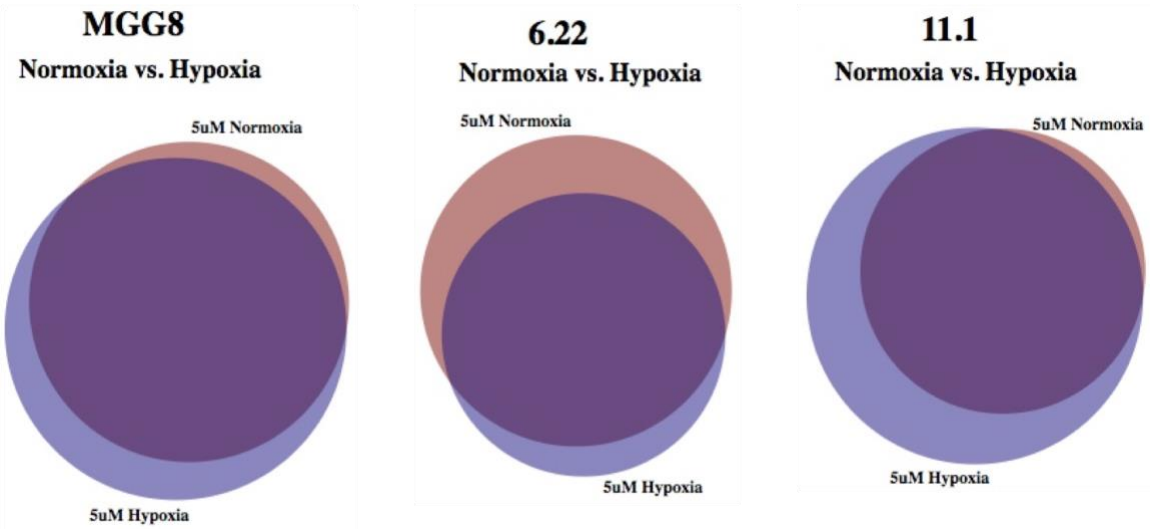


Figure 3.5: Comparison of hits from normoxia and hypoxia incubations.

Among the most effective compounds across all cell lines (figure 3.6), there are unsurprisingly a number of RTK/Ras/PI3K pathway inhibitors. Among the other compounds that make up the top of this list, there are a couple targets that are more surprising. Eukaryotic Elongation Factor 2 Kinase (eEF-2K) is a protein kinase that negatively regulates eEF2 and has been found to contribute to cancer by limiting translational elongation, protecting cells in nutrient depleted environments (Wang, Xie, & Proud, 2017). AZD 7762 is a compound that was discontinued by AstraZeneca due to issues with cardiotoxicity, however there are a number of other Checkpoint Kinase 1 and 2 (CHK1, CHK2) still being pursued by other firms (Weng et al., 2015). It was somewhat surprising to find Protein Kinase C (PKC) so highly ranked among the various targets of this KI library but the compounds targeting the various isoforms were many of the most effective inhibitors tested. There has not been much research into the role of PKC in GBM. However, based on our results, it is clear that PKC does play a significant role. And while Staurosporine is known to be a less than specific inhibitor of PKC (Karaman et al., 2008), the Bisindolylmaleimide (BIM) compounds are much more specific.

Inhibitor Name	Target	Average	Average Deviation	Median	Median Deviation	Variance	Standard Deviation
PIK-75 (hydrochloride)	PI3K	0.035	0.017	0.030	0.011	0.001	0.025
NH125	eEF-2K	0.036	0.018	0.031	0.013	0.001	0.024
Staurosporine	PKC	0.047	0.034	0.027	0.015	0.002	0.045
Bisindolylmaleimide IX (mesylate)	PKC	0.052	0.035	0.034	0.014	0.002	0.042
SC-1	RasGAP, ERK1	0.055	0.030	0.061	0.026	0.001	0.033
CAY10626	PI3K, mTOR	0.059	0.041	0.042	0.020	0.003	0.051
JNJ-10198409	PDGFR	0.060	0.030	0.051	0.021	0.002	0.040
Tunicamycin	N-linked glycosylation	0.069	0.053	0.042	0.032	0.004	0.066
Paclitaxel	disrupts multipolar spindle formation	0.071	0.048	0.053	0.035	0.003	0.058
AZD 7762	Chk 1, Chk2	0.072	0.046	0.058	0.034	0.003	0.054

Table 3.2 : Most effective KIs ranked by average growth relative to DMSO control.

We sorted the results by target in order to determine an average inhibition value for each targeted kinase across all conditions. The levels of inhibition for the most commonly targeted kinases (more than one inhibitor) are summarized in figure 3.7. Among these are many members and downstream targets of the RTK/Ras/PI3K pathway. Importantly, when the values from each individual cell line are compared, there are some significant differences to make note of. In addition to being generally more sensitive to the various inhibitors, MGG8 cells were significantly more sensitive to PDGFR inhibitors than both other cell lines (80.2% median inhibition compared to 5.4% in 6.22 and 22.5% in 11.1). This is consistent with the amplification of PDGFRA in this cell line (Suvà et al., 2014; Wakimoto et al., 2012).

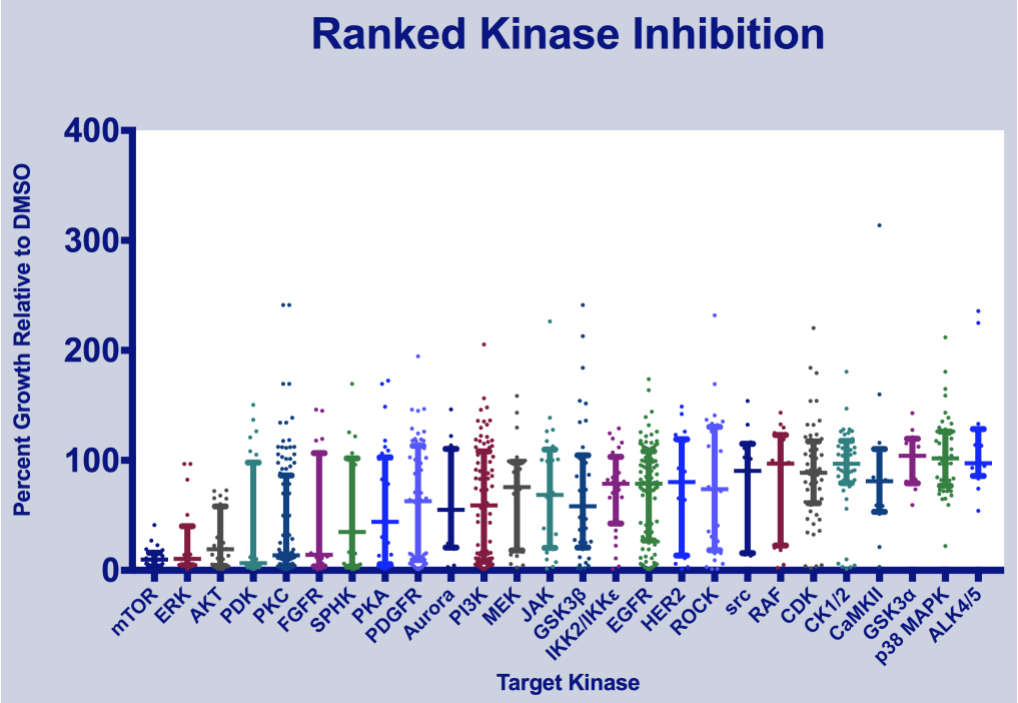


Figure 3.6: Inhibition of frequently targeted kinases, ranked by average inhibition value across all cell lines and oxygen conditions at 5µM. Bars represent median value and interquartile range. Points represent individual data from one cell line with one inhibitor in either normoxia or hypoxia.

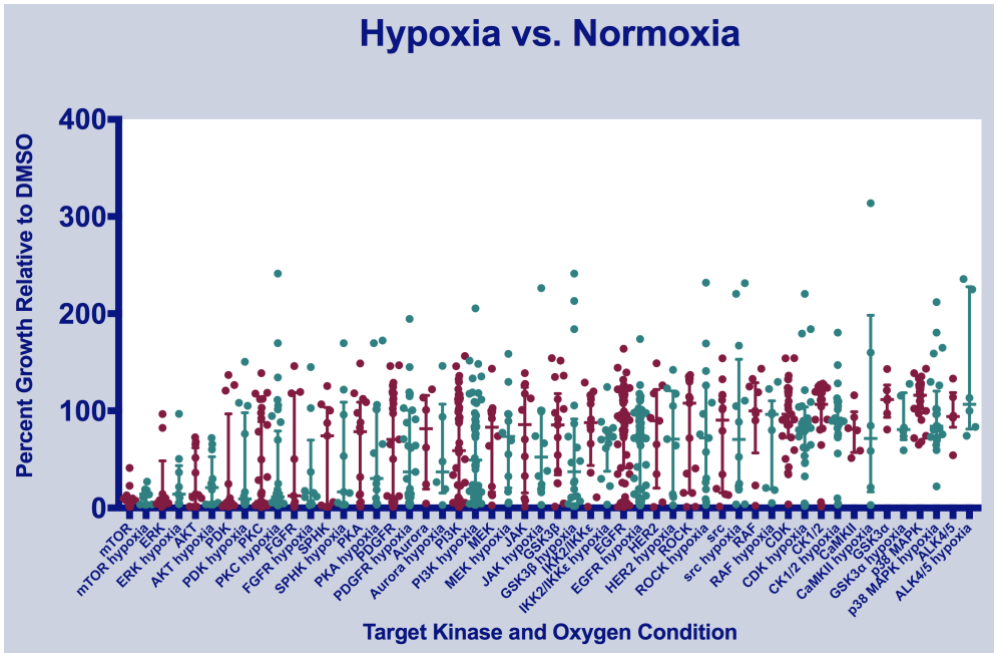


Figure 3.7: Differential inhibition of GSCs under normoxia or hypoxia. Bars represent median value and interquartile range. Red bars and points represent normoxia at 5µM whereas green bars represent hypoxia at 5µM.

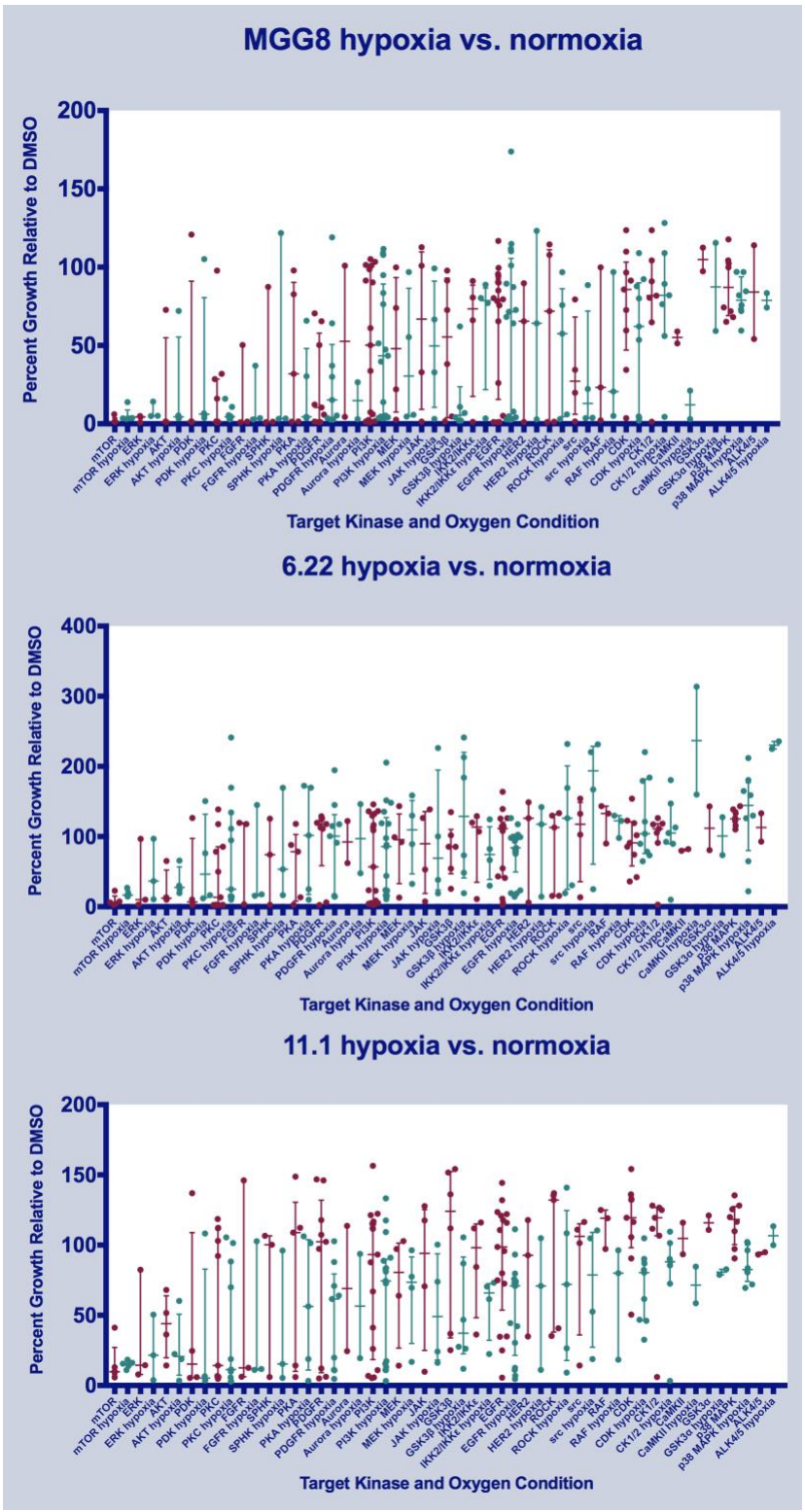


Figure 3.8: Differential inhibition under normoxia and hypoxia for each cell line. Bars represent median value and interquartile range. Red bars and points represent normoxia at 5µM whereas green bars and points represent hypoxia at 5µM.

Inhibitors of the RTK/Ras/PI3K signaling pathway members were high among the most effective inhibitors from this library, consistent with the importance of this pathway with regard to the survival of GBM. Our data shows that the MGG8 cells were more sensitive to inhibition by PDGFR inhibitors as well as the Raf/MEK/extracellular-signal related kinase (ERK) inhibitors. It is important to note that all three FGFR inhibitors tested in this library also targeted either vascular-endothelial growth factor receptor (VEGFR) or VEGFR and PDGFR. What appears to be weak growth under FGFR inhibition is likely actually the result of PDGFR or VEGFR inhibition.

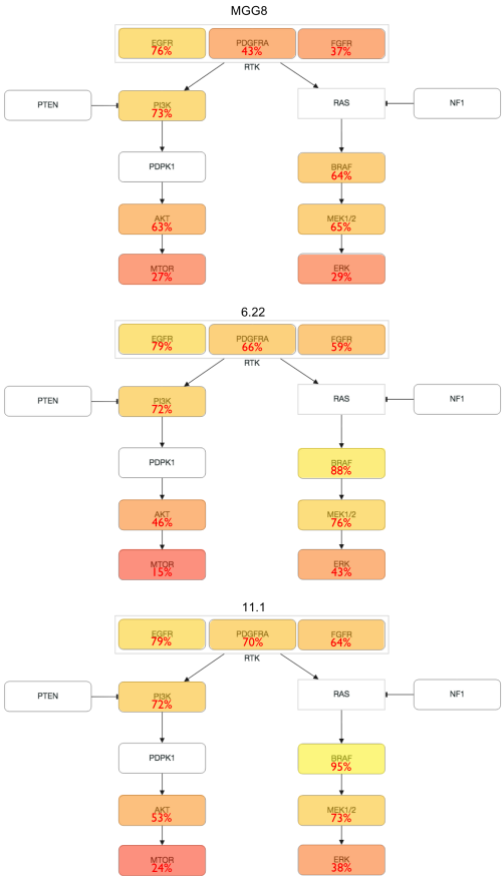


Figure 3.9: The RTK/Ras/PI3K signaling pathway in our GSC cells. Values are average percent growth relative to DMSO control when subjected to inhibitors of the highlighted kinase.

Interestingly, EGFR inhibitors were not particularly effective at inhibiting growth in GSCs. It is well documented that EGFR amplification and mutation are some of the most important genetic alterations that contribute to the etiology of GBM. Across 17 different inhibitors, there was an average inhibition of 21.5% and the few inhibitors that did show significant inhibition all targeted at least one other RTK.

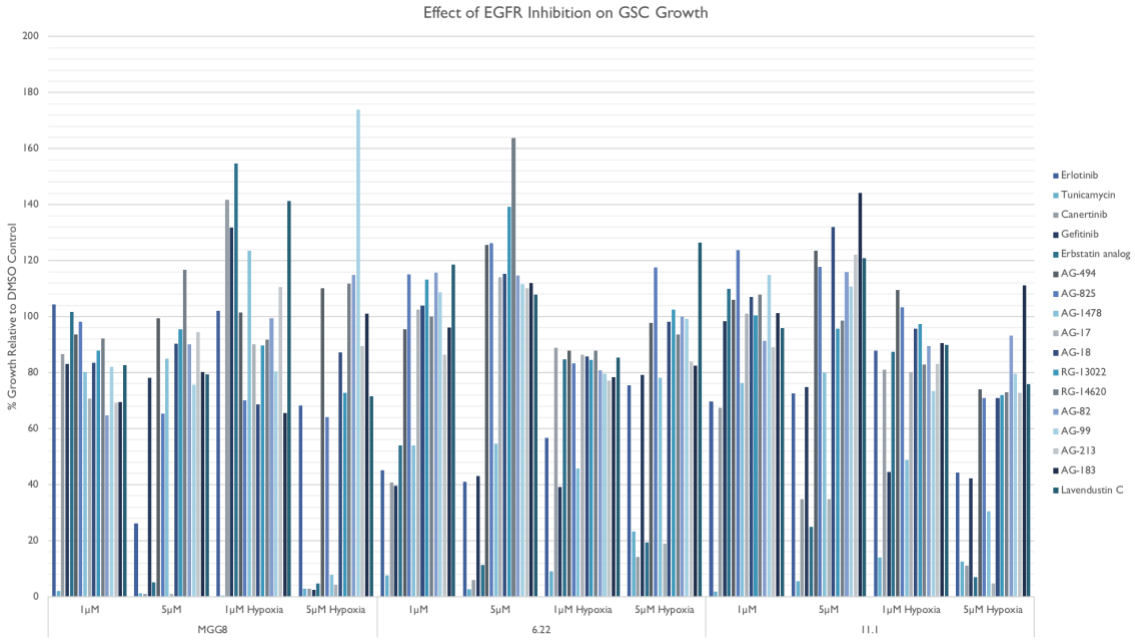


Figure 3.10: Effect on GSC growth of the inhibition of EGFR.

3.3 PKC Western Blotting

Among the most interesting results from the KI library screen of our GSC lines is the strong effect PKC inhibitors have on the growth of our GSC cells. The research into the role of PKC in GBM is fairly limited. A PubMed search including the terms “PKC” and “GBM” yields fewer than 50 results. There are, however, several thousand articles discussing the role of PKC in many types of cancer, including many articles suggesting it

as a target for the treatment of cancers such as that of the lung and pancreas (Storz, 2015; Zhang et al., 2015).

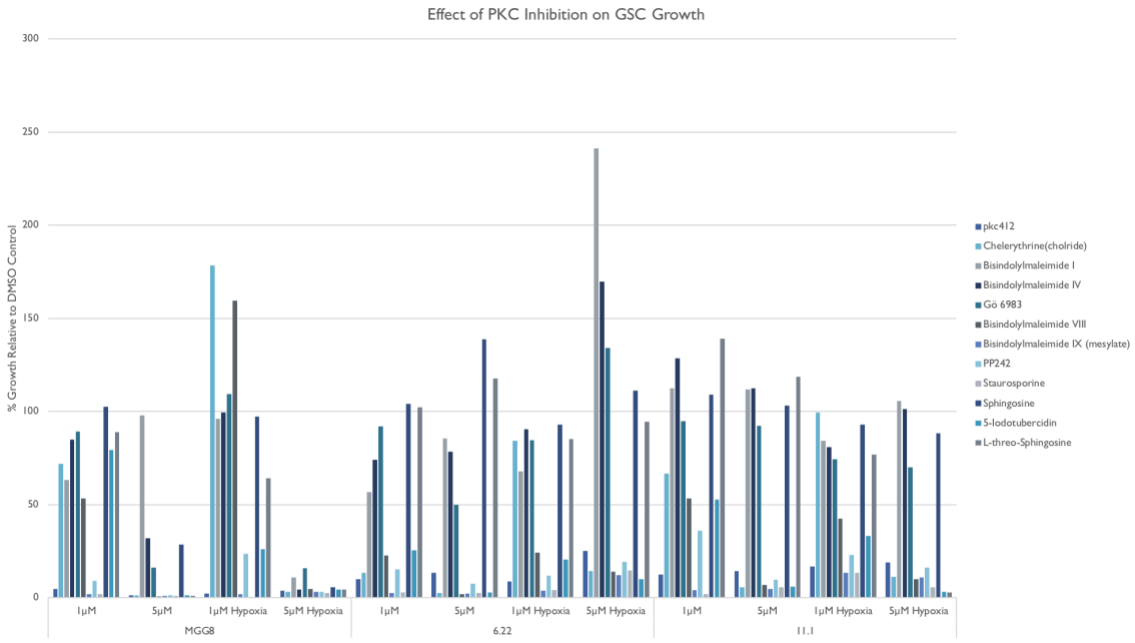


Figure 3.11: Effect of PKC inhibition on GSC growth.

The PKC family of proteins is made up of ten isozymes sorted into three classes based on activation constraints. “Conventional” PKCs require diacylglycerol (DAG), Calcium (Ca^{2+}), and negatively charged phospholipids for activation (Martiny-Baron & Fabbro, 2007). “Novel” isoforms need only DAG and “atypical” PKCs require neither DAG nor Ca^{2+} for activation. Each of these isozymes contributes to cellular function in a multitude of ways, frequently dependent on the cell type. In order to figure out exactly why our GSC lines are so susceptible to PKC inhibition, we decided to evaluate the various downstream targets to determine which one or ones are being activated by PKC to induce cellular growth.

One downstream target of PKC is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) subunit p65. When ubiquitylated at Lys321, PKC-ε can phosphorylate and activate inhibitor of NF-κB kinase (IKK2), an activator of p65 (Yang et al., 2012). According to the findings of Yang et al., this activation leads to the transcription and upregulation of pyruvate kinase M2 (PKM2) contributing to tumorigenesis in GBM. When PKC was inhibited, there was no effect on the phosphorylation of p65 in 6.22 cells as can be seen figure 3.12. We were not able to detect this phosphorylation in MGG8 cells, even when pretreating a positive control group with tumor necrosis factor-α (TNF-α) (not shown).

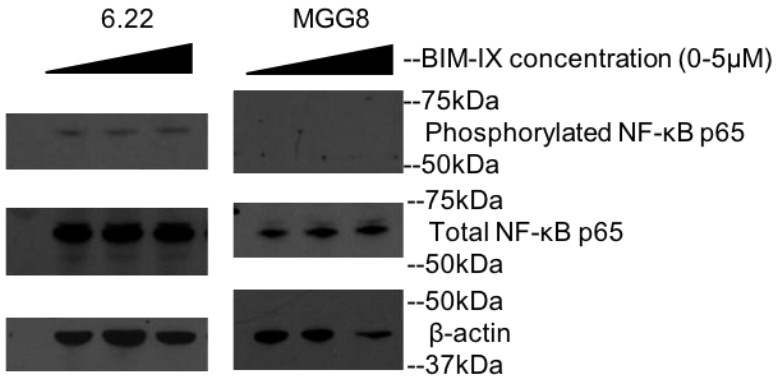


Figure 3.12: Western Blot of phosphorylated and total NF-κB p65 in MGG8 and 6.22 cells after treatment with 0, 2.5, or 5μM BIM-IX.

ERK1 and ERK2 are members of the MAPK family of enzymes and are associated with cell proliferation, differentiation, and many other cellular processes in a significant way (Sun et al., 2015). PKC can act as part of a feed-forward loop for RTK/Ras signaling by activating Ras and RAF1 upstream of ERK. However, there are more than one regulator of this pathway. As can be seen in figure 3.13, inhibiting PKC is not sufficient to decrease ERK phosphorylation and therefore activation in our cell lines.

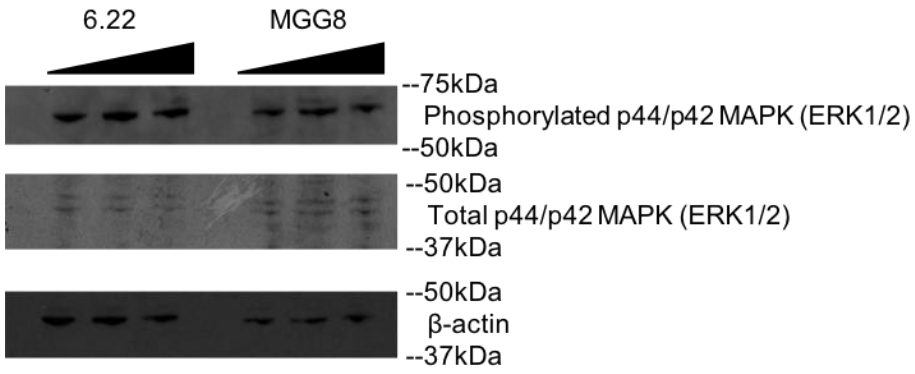


Figure 3.13: Western Blot of phosphorylated and total ERK in MGG8 and 6.22 cells after inhibition of PKC by treatment with 0, 2.5 or 5μM BIM-IX.

Ribosomal protein S6 (S6) is an essential component of the 40S ribosomal subunit and it has been implicated in tumorigenesis in Pancreatic Ductal Adenocarcinoma (Alliouachene et al., 2008). S6 is activated by phosphorylation from p70 S6 kinase and its nuclear counterpart p85 S6 kinase, among others as a result from signaling pathways including the RTK/Ras/PI3K pathway (Meyuhas, 2015). It has been demonstrated that the phosphorylation of p70 S6K by PKC inhibits its translocalization to the nucleus so that it can then go on to activate S6 in the cytoplasm (Valovka et al., 2003). Upon inhibition of PKC, we found that phosphorylation of S6 is also prevented, suggesting this activation is required for cellular growth in GSCs.

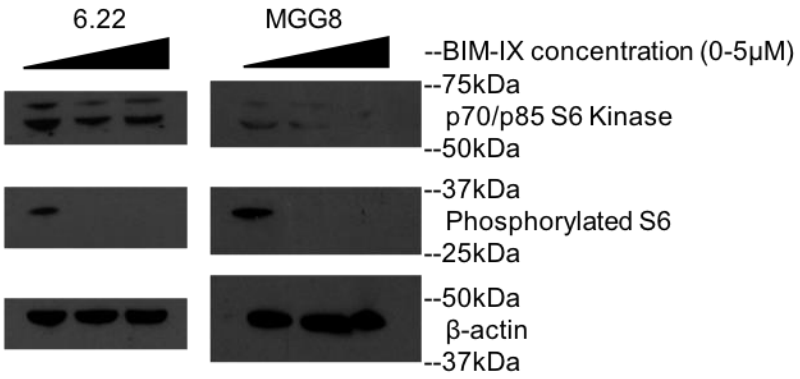


Figure 3.14: Western Blot of p70 S6 Kinase and phosphorylated Ribosomal protein S6 in MGG8 and 6.22 cells after treatment with 0, 2.5, and 5μM BIM-IX.

4. Discussion

4.1 KI Library Screen of GSCs

GSCs are at the core of not only the etiology of GBM, but also the key to being able to effectively treat the disease. By virtue of their resistance to chemo- and radiotherapy, these cells survive the treatments conventionally utilized, allowing them to generate new tumors time after time. Understanding the mechanisms that cause these cells to arise in the first place and that they use to survive is essential to any hope of being able to successfully treat this disease.

There are a number of limitations to our screen worth discussing. For one, most of the KIs included in our library and that are used in research in a variety of fields are not unispecific. Kinases are key regulators of cell signaling and for this reason, many have been duplicated over the course of our evolution. They are highly conserved but the genes that give rise to them are different enough that they can be uniquely targeted by shRNAs or CRISPR gene editing. For instance, the existence of 10 PKCs means that if only one isoform is silenced genetically, some of the others may compensate for the loss of signaling. On the other hand, KIs are rarely able to target only one isoform, possibly revealing the functions of families of kinases that are not revealed by shRNA or CRISPR targeting of individual family members. Many of these KIs are also flawed by virtue of their mechanism of action. KIs typically act by targeting an ATP-binding pocket or some other structural feature of a kinase. These structures are at the root of kinase

functionality and therefore it is difficult to design KIs that are target specific (Klaeger et al., 2017).

Another limitation to this study is the make-up of our KI library. The vast majority of the inhibitors target members of the RTK/Ras/PI3K signaling pathway. And while this is an important pathway to study our cell lines, this bias makes it difficult to conclude much from the results of the few inhibitors of other pathways. For instance, NH125 was the second most potent inhibitor out of all 152 tested. It was also the only KI targeting eEF-2K which makes it impossible to compare with kinases being targeted by up to 17 different inhibitors, as in the case of both PI3K and EGFR. The amount of time it would have taken to generate a more balanced KI library was prohibitive to this study but in a future KI screen, it would be better to take longer and explore the inhibition of a larger variety of kinases and signaling pathways.

A limitation of the design of this screen is the duplication and not triplication of the samples. Unfortunately, the generation of triplicate data came with logistical constraints that were prohibitive to achieving the same level of data production in a timely manner. Nevertheless, the R2 scores of our duplicates indicated a reasonable degree of reproducibility of our assay which was further reinforced by consistent results from multiple inhibitors of the same kinases and by consistency between the normoxia and hypoxia results.

There were some interesting results that were seemingly at odds with our findings from previous shRNA screens. In past shRNA screens, there has been little

overlap of the hits of the different cell lines. In the screen of about 10,000 genes performed by Dr. Shreya Kulkarni, of the hits required for the survival and proliferation of GSCs in either hypoxia or normoxia, only approximately 25% were required in both conditions (Kulkarni, 2016). In the results from our screen, more than 66% percent of compounds were considered hits in both conditions for any given cell line. In the RNAi screen of 501 kinases by Dr. Surbhi Goel, only 12 kinases or about 17% of hits were common to all three GSC lines (Goel, 2016). In each set of conditions in this study, over 50% of compounds qualified as hits were shared by all three cell lines. Despite the higher variety of the genes targeted in the shRNA screens, neither of them detected PKC as a target. Our observation of growth inhibition by PKC inhibitors is likely the result of small molecule KI inhibitors acting on multiple family members at once.

Our screen was consistent with much of what is known about the genetics of signaling in GBM. Keeping the bias of our KI library in mind, the growth inhibition that was achieved with inhibitors of the RTK/PI3K/Akt signaling pathway was significant in all of our cell lines whereas the RAF/MEK/ERK pathway inhibitors were far more effective in MGG8 than either 6.22 or 11.1 cells. And while EGFR inhibition was surprisingly ineffectual, this result served to highlight the robustness of this pathway. In GBM, Ras and PI3K signaling is amplified not only by the deletion or mutation of their negative regulators but also by the abundance of upstream RTKs. Besides EGFR, GBM cells experience increased signaling from amplification or alteration to PDGFR, MET, and

VEGFR, likely ensuring continued signaling through this pathway despite inhibition of one of them.

Within the RTK/Ras/PI3K signaling pathway a pattern was evident across all three cell lines. Whatever the inhibition level of a given kinase of this pathway, the inhibition of the next downstream member (i.e. mTOR relative to Akt) had a stronger effect of GSC growth. An explanation of this pattern might be the specialization of the downstream members. Upstream targets like the RTKs are numerous and all serve to activate the same pathway or pathways. The role of the kinases in the next step of the pathway is signal transduction, a cascade through which an extracellular signal is carried through the cell to its eventual target or targets. The kinases in this part of the pathway, such as Akt, almost always have multiple downstream targets and share those targets with other kinases. This allows for compensation to occur as the downstream targets continue to be activated by other kinases. Inhibition at either of these first levels has a smaller impact on GSC growth because the numerous kinases that are involved in these first two steps compensate for any loss of signaling. The next level down concerns kinases such as mTOR which are downstream of and integrate signaling from multiple different signaling pathways (Magnuson, Ekim, & Fingar, 2012). Therefore, by inhibiting these key kinases, we are affecting many signaling pathways at once.

4.2 Protein Kinase C in GBM

Results outside of the RTK pathway were less predictable from the genetics. PKC inhibitors were among the most effective compounds in the entire screen. While some

of these inhibitors had other target kinases, half of them were specific for the PKC family of enzymes and just as effective. How PKC regulates GSC growth and survival remains unclear. In order to better understand the ways that PKCs contribute to GSC survival, we investigated the likely downstream targets. Our results show that inhibition of PKC had no effect on ERK or p65 signaling. On the other hand, PKC signaling was essential for phosphorylation of Ribosomal Protein S6.

S6 is a ribosomal protein that makes up part of the 40S small ribosomal subunit and has been the focus of much research. S6 was first described nearly 50 years ago as the first known protein in ribosomes to undergo activation by phosphorylation (Kabat, 1970). It is activated by dedicated kinases S6K1 (p70/p85) and S6K2 in addition to being phosphorylated by Protein Kinase A and Casein Kinase 1 (Meyuhas, 2015). S6 has been demonstrated as having a role in global protein synthesis and cell size regulation and is important for regulating cell proliferation. Mouse embryo fibroblasts (MEFs) with substituted Serine residues were smaller in size and had much shorter G1 phases than wild type MEFs indicative of deficient growth and the importance of phosphorylation to the role of S6 (Ruvinsky et al., 2005).

The PI3K/Akt pathway is another important activator of S6K. In the late 90s, it was demonstrated that phosphorylation by mTOR was an essential step in the activation of S6K (Burnett, Barrow, Cohen, Snyder, & Sabatini, 1998).

4.3 Future Directions

The human kinome is made up of more than 600 kinases. Our screen targeted only 96 of these and only a third were targeted by more than one compound. Kinase inhibitors are commercially available for a wide range of cellular targets and are useful tools for investigating cellular signaling mechanisms. The screen performed in this study should be expanded to cover many more targets and the statistical power should be increased in order to obtain a more complete understanding of cellular signaling in GSCs.

The role of PKC in GSCs needs to be further elucidated. There is some research into the role of various isozymes but very little to suggest how truly important this protein family is in this disease. We have shown that the phosphorylation of S6 is controlled by PKC activity and integral to cellular proliferation. The mechanism through which this occurs is still unknown. Of the ten PKC isoforms, there may be as few as one or as many as all of them involved in regulating this process. Our results seem to indicate that the classical PKC isoforms (α, βI/II, and γ) are contributing to this result as they have been implicated in EGFR signaling through mTOR (Fan et al., 2009) but this should be investigated further.

5. Appendix

Primary data from this study can be accessed at https://drive.google.com/file/d/1rbTg7NwHNtFT9KnlRJmJEE0l4KfVHLkT/view?usp=sharing or with a Tufts login at the following URL: https://mynotebook.labarchives.com/share/Cochran%2520-%2520Playground/MTY2LjR8MjgzMzczLzEyOC9UcmVlTm9kZS8yNjI3NzEyOTkzfDQyMi40

Data separated by inhibitor target can be accessed at https://drive.google.com/file/d/1mbGLUpiKzA6lkGHKZvILREGHhSIx_MEB/view?usp=sharing

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