Chapter 2:
in vivo Study
2. Chapter II: *in vivo* study

2.1 Introduction:

Glioblastoma Multiforme (GBM) is an aggressive form of Astrocytoma prevalent in adults. Glioblastoma accounts for 82% of cases of malignant glioma (Dolecek, Propp et al. 2012) and is characterized histologically by considerable cellularity and mitotic activity, vascular proliferation, and necrosis. Because cells in these tumors vary in size and shape, i.e., they are pleomorphic, glioblastomas were called *glioblastoma multiforme*. Glioblastoma and other malignant gliomas are highly invasive, infiltrating surrounding brain parenchyma, yet they are typically confined to the central nervous system (CNS) and do not metastasize. Malignant gliomas arise in a multistep process involving sequential and cumulative genetic alterations resulting from intrinsic and environmental factors. Gliomas are more common in men than women and in white rather than black populations. A number of rare hereditary syndromes are associated with an increased risk of glioma, including Cowden, Turcot, Li-Fraumeni, neurofibromatosis type 1 and type 2, tuberous sclerosis, and familial schwannomatosis (Hottinger and Khakoo 2007, Alcantara Llaguno, Chen et al. 2009). A family history of glioma is rarely observed but, when present, is associated with a 2-fold increase in the risk of developing glioma. Genome-wide association studies have identified a few susceptibility variants such as 20q13.33 (*RTEL*), 5p15.33 (*TERT*), 9p21.3 (*CDKN2BAS*), 7p11.2 (*EGFR*), 8q24.21 (*CCDC26*), and 11q23.3 (*PHLDB1*), but these genes are only weakly associated with glioma, possibly reflecting multiple molecular subsets (Shete, Hosking et al. 2009, Rajaraman, Melin et al. 2012, Omuro and DeAngelis 2013). Gliomas are inversely associated with the presence of atopic diseases such as asthma, eczema, and hay fever (Frojdo, Cozzone et al. 2007). Preventive measures, such as lifestyle changes, are ineffective in averting gliomas. Early diagnosis and
treatment unfortunately do not improve outcomes, precluding the utility of screening for this disease. Headaches are relatively frequent, present in about 50% of patients at diagnosis, but usually with a nonspecific pain pattern (Forsyth and Posner 1993); progressive severity, unilateral localization, and new-onset headache in a patient older than 50 years are some of the features that may distinguish a tumor-associated headache from a benign headache. Papilledema is associated with significantly intracranial pressure and is now rarely seen because imaging is usually obtained at earlier disease stages. Cognitive difficulties and personality changes may develop and are often mistaken for psychiatric disorders or dementia, particularly in elderly individuals. Gait imbalance and incontinence may be present, usually in larger tumors with significant mass effect. Focal signs such as hemiparesis, sensory loss, or visual field disturbances are common and reflect tumor location. Occasionally, the development of symptoms is rapid, mimicking a stroke. Language difficulties may be mistaken for confusion or delirium. Seizures are the presenting manifestation in about 20% to 40% of patients, and usually a focal onset is reported (Glantz, Cole et al. 2000). Brain magnetic resonance imaging (MRI) with and without contrast is the diagnostic modality of choice when a brain tumor is suspected (class I, level B) (Bradley, Waluch et al. 1984); CT scan is reserved for patients unable to undergo MRI (eg, those with pacemakers). Malignant gliomas typically enhance with gadolinium and may have central areas of necrosis; they are characteristically surrounded by white matter edema. Tumors are often unifocal but can be multifocal. Findings on MRI can be indistinguishable from brain metastases. GBM has very few chemotherapeutic options (Carmustine Wafers and temozolomide), largely involving surgical removal. Symptomatic relief ultimately relies on the efficacy of specific antitumor therapies, but corticosteroids may temporarily alleviate neurologic symptoms caused by peritumoral edema. Dexamethasone is often used because of its low
mineralocorticoid activity. Initial doses are typically 12 to 16 mg/d in divided doses; given the high bioavailability, oral use is comparable with intravenous. Unfortunately, corticosteroid adverse effects can be substantial, and early tapering is indicated whenever possible. The presence of primary CNS lymphoma (PCNSL) should be considered before initiating corticosteroids because these agents are lympholytic and may obscure identification of lymphoma cells on histologic examination. On MRI, PCNSL usually displays a more uniform pattern of contrast enhancement, often described as “cotton” or “snowball,” which tends to disappear rapidly with corticosteroids (Buhring, Herrlinger et al. 2001). In patients who present with seizures, initiation of antiepileptics is required, but there is no evidence to support prophylactic use of antiepileptics in patients without seizures (class III, level B). (Buhring, Herrlinger et al. 2001). Levetiracetam is often preferred because it has a favorable toxicity profile, both oral and intravenous formulations are available, and it has no drug-to-drug interaction with most chemotherapeutic agents (class I, level B) (Maschio, Dinapoli et al. 2011). Other nonenzyme liver inducers may be used, such as topiramate, lamotrigine, valproic acid, and lacosamide. If possible, potent liver enzyme inducers such as phenytoin, carbamazepine, and phenobarbital should be avoided because they may decrease the effectiveness of some chemotherapeutic agents (Perucca 2006) and preclude participation in most clinical trials. After neuroimaging, patients with suspected malignant glioma should be considered for surgical resection, aiming at relieving mass effect, achieving cytoreduction, and providing adequate tissue for histologic and molecular tumor characterization. In suspected low-grade gliomas, early surgical resection may also be indicated, providing more reliable tumor grading (Jakola, Myrmel et al. 2012). In inoperable tumors, stereotactic biopsy may be performed for histologic diagnosis, but the limited amount of tissue acquired may preclude full molecular
characterization. Whenever possible, patients should be referred for surgery in tertiary care facilities, which provide optimized surgical tools (advanced intraoperative monitoring, awake mapping, and functional and intraoperative MRI) (Sherman, Hoes et al. 2011) and allow for adequate handling, processing, and storage of the tissue, including comprehensive molecular characterization and tissue profiling that may guide subsequent treatments. Even post-surgical removal, fatality is certain in most of the patients, because of high degree of invasiveness exhibited by GBMs (Preusser, de Ribaupierre et al. 2011).

Though efficacy of a drug is proven in vitro, proving the same in an animal model (in vivo) is very important to carry forward the therapy to clinical trials. The rat has been one of the most widely used experimental animals, and rat brain tumor models have been used extensively since the mid-1970s (Barth and Kaur 2009). Until recently, murine models (Fomchenko and Holland 2006) were used less frequently than rat models, but the ability to produce genetically engineered cell lines (Lampson 2001) has increased the use of murine models over the past five years. The advantages of rat models over mice models are summarized below:

Table 2.1
Advantages and disadvantages of rat brain tumor models compared to mouse models

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<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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<td>1. Larger size of the rat brain (compared to the mouse brain ~1200 mg vs ~400 mg) permits more precise stereotactic implantation than in mice, a longer interval of time until death and a thicker skull essentially eliminates osseous invasion and s.c. growth.</td>
<td>Rat brain tumor models cannot be as easily genetically engineered and manipulated as mouse models in order to elucidate the importance of genetic factors, signaling pathways, cell types and stroma in tumor growth and invasion.</td>
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<td>2. Larger tumor size prior to death permits better in vivo localization and imaging by a variety of diagnostic modalities in the rat.</td>
<td>The potential to produce genetically engineered tumor cell lines is less in the rat than in the mouse.</td>
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<td>3. Larger tumor size prior to death permits the administration of larger amounts of various therapeutic agents, especially if administered i.c. by CED and more critical evaluation of their effectiveness.</td>
<td>There are a smaller number of mAbs directed against rat surface antigens and chemokines compared to the mouse.</td>
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<td>4. More extensive literature on in vitro and in vivo studies of rat brain tumors compared to mouse tumors.</td>
<td>Rats are more expensive to purchase and maintain than mice.</td>
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Feline and canine models have been used less frequently (Krushelnycky, Farr-Jones et al. 1991, Kimmelman and Nalbantoglu 2007), but nevertheless, still provide an intermediate between rodent models and humans. It was first reported in the early 1970s that central nervous system (CNS) tumors could be induced reproducibly and selectively in adult rats that had been given repeated, weekly intravenous injections of \( N \)-methylnitrosourea (MNU) or a single dose of \( N \)-ethyl-\( N \)-nitrosourea (ENU). These studies led to the development of a number of rat brain tumor models that were highly reproducible and did not require the topical application of a chemical carcinogen to the brain, but nevertheless, still provide an intermediate between rodent models and humans. It was first reported in the early 1970s that central nervous system (CNS) tumors...
could be induced reproducibly and selectively in adult rats that had been given repeated, weekly intravenous injections of N-methylnitrosourea (MNU) or a single dose of N-ethyl-N-nitrosourea (ENU). These studies led to the development of a number of rat brain tumor models that were highly reproducible and did not require the topical application of a chemical carcinogen to the brain (Barth 1998). The utility of these models notwithstanding, it is important to recognize that no currently available animal tumor model exactly simulates human high grade brain tumors such as glioblastoma multiforme (GBM) or anaplastic astrocytomas. The cellular signaling pathways important for the genesis of brain tumor are multiple, with feedback mechanisms that can dramatically affect the efficacy of molecularly targeted therapeutic strategies. The heterogeneous composition of human high grade gliomas, which consists of tumor stem cells and differentiated tumor cells with varying characteristics, further complicates their susceptibility to treatment. Brain tumors also can evolve within their microenvironment, adapting to changes that produce epigenetic effects thereby altering their biology, but concomitantly providing additional targets for therapeutic intervention. Finally, genetic variations between individuals can dictate how tumors initiate, progress, and respond to treatment. Rat brain tumor models have provided a wealth of information on the in vitro and in vivo responses to various therapeutic modalities. The larger rat brain (~1200 mg) compared to that of the mouse (~400 mg) allows for more precise tumor cell implantation, and relatively larger volumes (~20 μl) that can be injected versus mice. Mouse models, on the other hand, have allowed researchers to rigorously test hypotheses developed from examining human tumors by genetically manipulating them and controlling specific variables such as environmental influences, in order to better understand the roles of different pathways, cell types, stromal factors and genetic variation (Reilly, Rubin et al. 2008). Mouse tumor models also have allowed researchers to test hypotheses derived from examining
human tumors, in a controlled environment with specific genetic alterations and controlled environmental influences. There is a general consensus that valid brain tumor models should fulfill the following criteria: (1) they should be derived from glial cells; (2) it should be possible to grow and clone them in vitro as continuous cell lines and propagate them in vivo by serial transplantation; (3) tumor growth rates should be predictable and reproducible; (4) the tumors should have glioma-like growth characteristics within the brain including neovascularization, alteration of the blood-brain barrier (BBB), an invasive pattern of growth, and lack of encapsulation; (5) host survival time following i.c. tumor implantation should be of sufficient duration to permit therapy and determination of efficacy; (6) for therapy studies, the tumors should be either non or weakly immunogenic in syngeneic hosts; 7) they should not grow into the epidural space or extend beyond the brain and finally (8) their response or lack thereof to conventional treatment should be predictive of the response in human brain tumors. In studies carried out prior to the 1970s, either cells or tumor fragments were injected i.c. using a free hand approach, which generally lacked reproducibility and precision. A stereotactic implantation procedure using suspensions of tissue-culture-derived brain tumor cells was more successful (Barker, Hoshino et al. 1973). This procedure was further improved by the use of concentrated cell suspensions in small volumes, improved injection needles, better stereotactic localization to structures deeper in the white matter such as the caudate nucleus, the use of slower injection rates, 0.5–1.0% low gelling agarose to prevent backflow of tumor cells through the injection track (Kobayashi, Allen et al. 1980)and cleansing of the operative field with a solution of Betadine. Finally, rinsing the surface of the brain with sterile water destroys extravasated tumor cells by osmosis prior to closure of the skull with bone wax has also been recommended (Landen, Hau et al. 2004). This implantation procedure resulted in high success
rates of i.c. tumor growth with the elimination of spinal and extracranial dissemination. The C6 glioma was produced by Benda et al. (Benda, Lightbody et al. 1968) and Schmidek et al. (Schmidek, Nielsen et al. 1971), in Sweet’s laboratory at the Massachusetts General Hospital (MGH) by repetitively administering MNU to outbred Wistar rats over a period of approximately 8 months. When animals developed neurological signs, they were euthanized, and the tumors were excised and explanted into tissue culture. Among these was a tumor designated as “#6”, which was subsequently cloned by Benda et al. and was shown to produce S-100 protein. Following cloning, it was re-designated “C6” (Pfeiffer, Herschman et al. 1970). The C6 glioma is composed of a pleomorphic population of cells with variably shaped nuclei. There is focal invasion into contiguous normal brain. Initially, the tumor was histopathologically classified as an astrocytoma, and eventually it was designated as a glial tumor following accession by the American Type Culture Collection, Rockville, MD (ATCC# CCL-107). The cells have been reported to have a mutant p16/Cdkn2a/Ink4a locus (Schlegel, Piontek et al. 1999) with no expression of p16 and p19ARF mRNAs, and a wildtype p53 (Asai, Miyagi et al. 1994). More recent molecular characterization, which compared changes in gene expression between the C6 glioma and rat stem cell-derived astrocytes, revealed that the changes in gene expression observed in the C6 cell line were the most similar to those reported in human brain tumors (Sibenaller, Etame et al. 2005). Compared to astrocytes, they also had increased expression of the PDGFβ, IGF-1, EGFR and Erb3/Her3 genes, which are frequently overexpressed in human gliomas (Morford, Boghaert et al. 1997, Guo, Hu et al. 2003). Similar to the increased activity of the Ras pathway observed in human gliomas (Nakada, Niska et al. 2005), C6 cells also had an increase in both Ras expression and Rasguanine triphosphate activator protein. However, contrary to what has been reported for human GBM, there was an increase in
expression of Rb in these cells. A subclone of C6 cells, stably expressing β-galactosidase, subsequently was described (Lampson, Lampson et al. 1993) and this has permitted in vivo immunohistochemical analysis of these tumors in the brain. This clone is available through the ATCC (# CRL-2303). However, it must be noted that the β-galactosidase marker protein itself can serve as a tumor antigen, and immunization of rats against the reporter gene protected the animals against tumor growth. The C6 rat glioma model has been widely used in experimental neuro-oncology to evaluate the therapeutic efficacy of a variety of modalities, including chemotherapy (Doblas, Saunders et al. 2008), anti-angiogenic therapy (Solly, Fish et al. 2008), proteosome inhibitors (Ahmed, Jacob et al. 2008), treatment with toxins (Zhao, Zhang et al. 2008), radiation therapy (Sheehan, Ionescu et al. 2008), photodynamic therapy (Mannino, Molinari et al. 2008), oncolytic viral therapy (Beer, Zetterberg et al. 2004), and gene therapy (Tanriover, Ulu et al. 2008). Since this tumor arose in an outbred Wistar rat, however, there is no syngeneic host in which it can be propagated. This is a very serious limitation that diminishes its usefulness for survival studies since the tumor is immunogenic, even in Wistar rats. The C6 glioma has been demonstrated to be immunogenic in Wistar and BDX rats (Parsa, Chakrabarti et al. 2000), and it therefore is not useful for evaluating the efficacy of immunotherapy. This problem is exemplified by prior studies in which C6 glioma cells were transfected with an antisense cDNA expression vector that downregulated the constitutive production of IGF-1 (Trojan, Johnson et al. 1993). Not recognizing that the tumor was of Wistar origin, the authors unfortunately used BD IX rats, which they thought was the strain of origin, due to some ambiguity in the literature. Subsequently, it was reported that BD IX rats, which had been immunized with the C6 anti-sense IGF-1 transfected cells, were resistant to both s.c. and i.c. challenge of the C6 glioma. Similarly, Wistar rats, bearing C6 gliomas (s.c. or i.c.),
developed potent humoral and cellular immune responses to the tumor, and rats challenged simultaneously with s.c. and i.c. tumors, had a survival rate of 100%. Since C6 glioma cells are allogeneic in all inbred strains, this should provide a strong cautionary note for studies employing this tumor model and they should not be used for immunotherapy studies. Despite this limitation, the C6 glioma model continues to be used for a variety of studies related to brain tumor biology (Karmakar, Olive et al. 2007). These have included studies on tumor growth, invasion, migration, BBB disruption, neovascularization, growth factor regulation and production, and biochemical studies (Assadian, Aliaga et al. 2008, Valable, Barbier et al. 2008). Finally, single-cell clonal analysis has revealed that C6 cells also have cancer stem cell-like characteristics, including self-renewal, the potential for multi-lineage differentiation in vitro and tumor formation in vivo (Shen, Shen et al. 2008). Therefore, we explored the Wistar Rat model in this study for developing Glioblastoma. The originator cells for GBM have mostly remained a mystery with neural progenitor cells doubted as tumor pioneers (Alcantara Llaguno, Chen et al. 2009). Recent studies have brought a family of cells called Brain Tumor Stem Cells (BTSC) to light as one of the tumor causing candidates but lack of proper molecular markers has made it difficult to design a strategy to neutralize BTSC (Singh, Hawkins et al. 2004, Murat, Migliavacca et al. 2008). Efforts to look into molecular etiology has thrown up candidates like EGFR/RAS/NF1/PTEN/PI3K Pathway, TP53/MDM2/MDM4/p14ARF Pathway and p16INK4a/CDK4/RB1 Pathway, and chromosomal aberrations like Loss of Heterozygosity (LOH) at 10p, 10q, 13q, and 22q as well as deletion of 1p and 19q arms in homo sapiens. Among these the deregulation in EGFR/RAS/NF1/PTEN/PI3K pathway contributes to malignancy in most cases (Ohgaki and Kleihues 2009). Studies also indicate involvement of metabolic machinery changes along with protein deregulation and chromosomal aberrations especially hypoxia aiding enzyme
suppression like IDH1 (Ohgaki and Kleihues 2009, Zhao, Lin et al. 2009, Venneti and Thompson 2013). Nitric oxide synthase (iNOS) has also been implicated lately in BTSC tumorigenesis downstream of EGFR/STAT3 signaling (Jahani-Asl and Bonni 2013). All the above proteins have a problem while targeting, that they belong to only one or couple of the total number of signaling pathway spectrum. In EGFR driven Glioblastoma, Pyruvate kinase M2 (PKM2) though, targets a central factor - c-Myc gene, by translocating itself to the nucleus and in turn phosphorylates histone 3, thereby attracting transcription factors to c-Myc promoters.

The blood–brain barrier (BBB) prevents entry into the brain of most drugs from the blood. The presence of the BBB makes difficult the development of new treatments of brain diseases, or new radiopharmaceuticals for neuroimaging of brain. All of the products of biotechnology are large molecule drugs that do not cross the BBB. While it is assumed that small molecules are freely transported across the BBB, ~98% of all small molecules are not transported across the BBB (Pardridge 2005). The BBB is the principal interface between blood and the ISF that bathes synaptic connections within the parenchyma of the brain. A separate barrier system in the brain is localized to the epithelial cells of the choroid plexus, which form the blood–cerebrospinal fluid (CSF) barrier (BCSFB). The choroid plexus is the principal interface between the blood and CSF that bathes the surface of the brain. The BBB is the fundamental problem blocking progress in the development of new therapeutics for brain disorders, or the development of new radiopharmaceuticals for imaging brain. This review will discuss both the principals of BBB drug transport, and strategies for the reengineering of drugs to enable BBB transport. The classical approaches to drug delivery to the brain, transcranial drug delivery or small molecules, are discussed, and the limitations of these strategies are reviewed. The endogenous carrier-mediated transport (CMT) and RMT systems within the BBB are
discussed, and strategies are reviewed for the reengineering of pharmaceuticals that penetrate the brain from blood via access of the CMT and RMT systems within the BBB. Drug distribution into CSF is frequently used as an indicator of BBB transport. This is a misconception that stems from the lumping of the BBB and BCSFB systems as a single brain barrier. In fact, the BCSFB is leaky compared with the BBB, and all molecules in blood enter the CSF at a rate inversely related to molecular weight (MW) (Reiber and Felgenhauer 1987). The finding of drug penetration into CSF is expected for any molecule, and does not provide information on rates of drug penetration across the BBB at the brain capillary endothelium. Thus, drug distribution in CSF is not an index of BBB transport, but rather is simply a measure of transport across the choroid plexus at the BCSFB. The idea that CSF composition reflects BBB transport is 100 years old, and finds its origins in Goldman’s vital dye experiments published in 1913 (1913). Before 1913, it was known that certain acidic vital dyes, such as trypan blue, are excluded from entry into the brain following peripheral administration, and this observation was explained, not within the context of a barrier between blood and brain, but rather because the brain lacked the ‘receptors’ that bind the dye and sequester the molecule within the tissue. Goldman injected the trypan blue into the lumbar sac, or subdural space, of rabbits and observed vital dye staining of spinal cord and brain tissue, respectively. The idea that nutrient flux from blood to brain occurred across the capillaries within the parenchyma of brain was not accepted in 1913. By the 1930s, studies with brain-penetrating basic vital dyes showed that the dye entered the brain from blood across the walls of capillaries perfusing brain parenchyma. Broman(Broman 1941) and Friedemann (Pardridge 2012) clearly observed that the BBB was localized to the capillary wall in brain, and that drug entry into the CSF across the choroid plexus was an entirely separate problem from drug transport across the BBB. These ideas form the basis for bypassing the BBB.
with intrathecal drug delivery to the brain. In 2013, or 100 years after the publication of Goldman’s experiment defining the BBB, there are multiple ongoing clinical trials of intrathecal delivery to the human brain of drugs that do not cross the BBB. The brain has no lymphatic system. The CSF of the brain is produced at the choroid plexi of the ventricles, moves over the surface of the brain, and is absorbed into the general circulation across the arachnoid villi into the superior sagittal sinus of the venous bloodstream (Oldendorf 1972). In the human brain, there is about 100 to 140 mL of CSF, and this entire volume is turned over completely every 4 to 5 hours, or 4 to 5 times per day (CUTLER, PAGE et al. 1968). In the mouse brain, there is about 40 μL of CSF, and the entire volume is turned over every 2 hours, or about 12 times per day (Rudick, Zirretta et al. 1982). Cerebrospinal fluid is a fluid compartment in rapid equilibrium with the blood, which is due to the rapid rate of bulk flow (convection) of CSF from brain to blood. Conversely, drug movement into brain tissue from the CSF flow tracts occurs via diffusion. The differential rates of convection and diffusion create the paradox that drug injected into the CSF distributes easily to blood and poorly to brain beyond the ependymal surface. The poor distribution of drug into brain parenchyma is shown by the autoradiogram of rat brain prepared 20 hours after a single injection of [125I]-BDNF (brain-derived neurotrophic factor) into a lateral ventricle (Yan, Matheson et al. 1994). In contrast to the relative rapid rate of bulk flow of CSF out of brain, the transport of drug from the CSF to brain tissue is slow, and limited by diffusion. Diffusion decreases with the square of the distance. The limited distribution into brain from the CSF is also observed for small molecules. Small molecule drug concentration in the brain decreased logarithmically with each mm of distance from the CSF surface. There is a ~10-fold decrease in drug concentration of small molecule with each mm of distance removed from the brain surface. In the case of a lipid soluble small molecule, which can diffuse into brain cells,
there is a 10-fold decrease in drug concentration with each 500 $\mu$m of distance into the brain (Fung, Shin et al. 1996). Fishman and Christy (Fishman and Christy 1965) observed nearly 50 years ago that an intrathecal injection of drug ‘is, in effect, equivalent to no more than a prolonged IV injection.’ The rapid movement of CSF to blood means that drug injected into the CSF compartment will also rapidly move to the general bloodstream. This creates the paradox that drugs injected into the CSF may have a pharmacological effect within brain parenchyma, but only after transport of the drug from CSF to blood and then to brain across the BBB. Drug delivery to the brain via the CSF is limited by the slow diffusion of drug from the ependymal surface of the brain. So as to replace diffusion with bulk flow, drug delivery to the brain has been attempted with CED (convection-enhanced diffusion). In this approach, drug is placed in a peripheral reservoir connected to a pump, which is in line with a transcranial catheter. In theory, drug will penetrate the brain tissue by bulk flow, which should produce high drug levels in the brain at sites remote from the intracerebral catheter. In practice, drug concentrations in the brain decrease logarithmically with each mm of distance removed from the tip of the CED catheter in the brain. A logarithmic decrease in the brain concentration of GDNF (glial-derived neurotrophic factor) is observed following the CED infusion of the neurotrophin in the monkey brain (Salvatore, Ai et al. 2006). Almost all drugs for the brain presently in clinical practice are lipid soluble small molecules with an MW $<$400 Da. These drugs fit the dual criteria for lipid-mediated free diffusion across the BBB, which are (1) MW $<$400 Da threshold and (2) high lipid solubility, which is equivalent to low hydrogen bonding. In practice, very few small molecule drug candidates for the brain fit these dual criteria, and do not cross the BBB. Of $>$6,000 drugs in the Comprehensive Medicinal Chemistry database, only 6% are active in the brain, and these drugs treat only a select group of brain diseases, comprised of affective disorders and insomnia.
Blood–brain barrier permeation decreases 100-fold when the size of the drug is increased from an MW of 300 Da, which corresponds to a surface area of 50 square angstroms, to an MW of 450 Da, which corresponds to a surface area of 100 square angstroms (Barth 1998). The molecular mechanism of the MW threshold phenomenon is consistent with the observation that transient water pores form in biological membranes, which are caused by the transient fluctuations in the conformation of the fatty acyl side chains of membrane lipids (Marrink, Jähnig et al., Träuble 1971). The first evidence of saturable or Carrier Mediated Transport of a solute across the BBB was demonstrated by Crone (Crone 1965) for D-glucose using the indicator dilution technique, which is a venous sampling-carotid artery injection method. Terasaki and colleagues (Ohtsuki, Uchida et al. 2011) determined the mass of transporters in isolated brain capillaries using the methods of LC–MS (liquid chromatography mass spectrometry). Capillaries were isolated from brain followed by digestion with trypsin. The tryptic peptides were separated and identified by LC–MS. Transporter-specific peptides were identified and quantitated by solid-phase synthesis of synthetic peptide standards. These peptide standards, which enable the quantification of the transporter expression, could be synthesized based on the known amino-acid sequences of the cloned transporters, as well as standard programs for predicting the sequence of tryptic peptides based on known amino-acid sequence of the target protein. The transporter concentrations in isolated human brain capillaries, Ccap, are GLUT1, MCT1, LAT1, and CAT1. The drug JQ1 (MW = 457), dealt in the present study is a lipophilic agent and we currently examine the permeability across the BBB. The recently reported BET domain inhibitor, JQ1 (tert-butyl 2-((6S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetate) (Filippakopoulos, Qi et al. 2010, Delmore, Issa et al. 2011) from the stables of James Bradner Lab, Dana Farber Cancer Institute,
Boston, USA, is an effective \( c\text{-Myc} \)-epigenetic silencer. JQ1 acts by displacing BET bromodomains from chromatin by competitive inhibition of the acetyl-lysine pocket (Bandopadhayay, Bergthold et al. 2013, Belkina, Blanton et al. 2013, Horn, Ziepert et al. 2013).

\( c\text{-Myc} \) is a very important onco–protein, as it acts as a master switch, from being an upregulator of a myriad of growth promoting genes (Dang 1999) to causing manipulation in cellular biochemistry (Zeller, Zhao et al. 2006) to suit the tumor micro-environment. Deregulation of \( c\text{-Myc} \) is one of the best strategies, a tumor cell can remain malignant, because of the array of genes it regulates that promote cell cycle. How does a transformed cell ‘know’ that it needs to keep dividing, dodging all the negative elements thrown at it for cell cycle down-regulation by the cellular machinery itself or therapeutically? Like bookmarks in a novel, navigating a reader to recognize the page to be read, certain ‘bookmarks’ on the genome of the tumor cell, anchor transcription factors to specific genes, to be transcribed at a very high rate. One such bookmark is the chromatin binding protein BRD4, belonging to bromodomain and extraterminal (BET) subfamily. BRD4 helps anchor \( c\text{-Myc} \) promoter and subsequently signals \( c\text{-Myc} \) transcription. As there are few efficient direct inhibitors of \( c\text{-Myc} \) because of an absence of clear ligand binding site on the protein, an approach to transcriptionally inactivate \( c\text{-Myc} \) seems to be a fair strategy (Darnell 2002).

Hence, this study aims to target GBMs with JQ1, in an effort to silence \( c\text{-Myc} \) expression, initiated by the EGFR/PKM2 cascade and examine molecular/biochemical changes as well as morphological changes to cellular ultra-structure and also to analyze the lasting effects, post cessation of JQ1 treatment, especially to assess how the cell cycle machinery reacts to an initial suppression followed by discontinuation of the suppressor.

2.2 Materials and Methods:
2.2.1. Animal Cell Culture

Reagents

7. DMEM medium: 10 g of DMEM was dissolved in 800 ml of sterilized double distilled water. To this solution, 20 ml of (1.5 g/L) sodium bicarbonate was added. This was followed by the addition of 10 ml of sodium pyruvate and 10 ml of penicillin-streptomycin cocktail (100 units/ml penicillin and 100 μg/ml streptomycin) and mixed thoroughly. The pH was adjusted to 7.4 using 1 N HCl and 1 N NaOH. It was then made up to 1000 ml with sterilized double distilled water. Then the medium was sterile filtered using (0.22 μ) membrane filter. The medium was then dispensed into sterilized containers and stored at 4°C.

8. HAM’s F12 medium: 10 g of HAM’s F12 was dissolved in 800 ml of sterilized double distilled water. To this solution, 20 ml of (1.5 g/L) sodium bicarbonate was added. This was followed by the addition of 10 ml of sodium pyruvate and 10 ml of penicillin-streptomycin cocktail (100 units/ml penicillin and 100 μg/ml streptomycin) and mixed thoroughly. The pH was adjusted to 7.4 using 1 N HCl and 1 N NaOH. It was then made up to 1000 ml with sterilized double distilled water. Then the medium was sterile filtered using (0.22 μ) membrane filter. The medium was then dispensed into sterilized containers and stored at 4°C.

9. Growth medium (DMEM:HAM’s F12 (1:1) with 10% FBS): 100 ml of growth medium was prepared by adding 10 ml FBS in 90 ml DMEM:HAM’s F12 (45ml of DMEM + 45ml of HAM’s F12) medium and stored in a sterile container.

10. Phosphate Buffered Saline (PBS; pH 7.4): 0.63 g of sodium dihydrogen phosphate(NaH₂PO₄), 0.17 g of disodium phosphate dibasic (Na₂HPO₄) and 4.5 g of
sodium chloride (NaCl) were dissolved in 500 ml of sterile double distilled water. pH was adjusted to 7.4 with 0.1 N NaOH, sterile filtered (0.22 μ) and stored in refrigerator.

11. Trypsin, PBS, Versene, Glucose solution (TPVG): 84 ml of PBS, 5 ml of 2% Trypsin, 10 ml of 0.2% Ethylene Diamine Tetra Acetic acid (EDTA), 0.5 ml of 10% Glucose and 0.5 ml of penicillin and streptomycin were dissolved in 100 ml of sterile double distilled water. pH was adjusted to 7.4 with 0.1 N NaOH and stored in -20°C.

12. Cell line: N2A cell line was procured from the National Centre for Cell Science (NCCS), Pune. The cells were grown in T-75 culture flasks containing MEM

Procedure
The medium from the culture flask was aspirated. The flask was rinsed with 2.0 ml of PBS and aspirated again quickly. 5.0 ml of TPVG solution was added and incubated at 37°C for about 3-5 min until cells started lifting. As soon as the cells were loose, using a transfer pipette the trypsinized medium containing cells were transfer to the tube and centrifuged at 1000 rpm for 5 min. The medium was carefully aspirated off. Care was taken not to put the pipette tip in the bottom of the tube, where the cells were pelleted. The cells were gently resuspended in fresh DMEM: HAM’s F12 medium with 10% FBS by pipetting up and down 5-8 times. From the cell suspension, a drop was placed to the edge of the cover slip of Neubauer haemocytometer. The drop was left to run under the cover slip by capillary action. Care was taken not to "force" the liquid and the entry of air bubble was avoided. The cells were then gently re-suspended in fresh growth medium and transferred to sterile T-75 flasks and the volume of medium was made up to 10 ml with growth medium/flask. Cells were grown on 25mm² flasks at 37°C under humidified 5%CO₂ - 95% air mixture.
2.2.2.Animals

Male Wistar Rats, weighing between 80 - 100 g, 4 to 6 weeks older were purchased from Kings Institute, Guindy, Chennai, India and maintained under controlled environmental conditions. The animals were provided with Gold Mohor rat feed, M/s. Hindustan Lever Ltd., Mumbai and water ad libitum. This study was conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and by Animal Ethics Committee Guidelines of our Institution (Devan and Janardhanam 2011). Animals were housed in groups of 5 under standard conditions at a temperature of 24 ± 1°C and a 12-h light/dark cycle.

2.2.3.Tumor Xenograft Development

Materials

- Ketamine/xylazine cocktail (see recipe)
- Hair shaver or depilatory cream (optional)
- Ophthalmic ointment or mineral oil
- Betadine
- Bone wax
- Mouse stereotaxic frame (Cartesian Research) equipped with:
  - Sighting scope
  - Drill with #74 bit
  - Monojector with 24-G Hamilton syringe (model #8800 for injections of up to 5 µl)
- Heating pad
- Forceps
- Scalpel
- Surgical scissors
- Agricola-style retractors (Fine Science Tools)
- Cotton swabs
- Sutures

Procedure
Rats weighing 150-200 g aged 10 - 12 weeks were selected for the experiment. Before implantation, 85 to 95% confluent C6 cells were trypsinized, rinsed with DMEM + 10% fetal bovine serum, and centrifuged at 1000 rpm for 4 min. The cell pellet was resuspended in DMEM and placed on ice. Concentration of viable cells was adjusted to $2 \times 10^4$ cells/1 µl of DMEM. Each rat was anesthetized with Kitamine:Xylazene (80-120 mg/kg:10-16 mg/kg IP) and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA) a hole was drilled at anteroposterior 0.0, right -3.0 relative to bregma according to the stereotaxic atlas of König and Klippel (1963). Tumor cells were injected at a rate of 1.0 µl/s, using a 10 µl syringe (Hamilton Co., Reno, NV) with a 26s-gauge needle mounted on a stereotactic holder at a depth of 5 mm (Grommes, Landreth et al. 2006). Thereafter, the skull was cleaned, and the incision was sutured. The animals were then segregated into 4 groups – Group 1: Control (injected with 10µl of DMEM alone), Group 2: Tumor Control (injected with 10ul of DMEM containing C6 Glioma Cells), Group 3: Treated (C6 Glioma Cells + 7mg/kg bw JQ1) for 60 days, Group 4: Treated (C6 Glioma Cells + 7mg/kg bw JQ1) for 15 days and observed from there on, Group 5: Drug Control (7mg/kg bw JQ1 alone; utilised only for survival rate experiments). Treatment was oral and drug administered using a cannula fixed to a 2ml syringe, once a day, starting from day 1 after the stereotaxic surgery.

2.2.4. High Pressure Liquid Chromatography (HPLC)

JASCO HPLC system consisting of LC-20AD pump, JASCO 2010 Plus Multiwavelength Detector (PDA), SIL-20A auto-sampler, CTO-20A column oven and DGU-20A3 solvent degasser fitted with Fortis C18 column (250x4.6mm) coupled with a guard cartridge was selected for qualitative analysis of the given sample. The mobile phase consisted of Acetonitrile (HPLC Grade) (eluent A) and Water (HPLC Grade) (eluent B), which were applied in the
gradient elution as follows: linear gradient from A–B (90:10, v/v). Prior to each run, the column was equilibrated to the starting conditions for 15 min. The chromatogram was monitored at 254 nm, the column temperature set at 30°C, the flow rate at 1.0 ml/min, and the sample injection is performed using a Hamilton Syringe, consisting of 10μl volume in each run. The data were collected and analyzed with Shimadzu LC solution software.

2.2.5. RT (Reverse Transcriptase) – PCR

2 groups of GBM induced animals were treated with 7mg/kg bodyweight of JQ1 (Group 3 and Group 4), keeping one group as control (Group 1) and one group as tumor control (untreated) (Group 2).

Isolation of RNA

Total cellular RNA extraction from C6 Glioma cell was performed using TRIZOL reagent (Life technologies cat no.15596026), which is based on the acidic phenol chloroform method. About 1X10^5 C6 Glioma cell was homogenized in 1.0 ml of ice-cold TRI reagent. The homogenate was cleared off by centrifugation at 12,000 rpm for 5 min. To the supernatant 0.2 ml of ice cold chloroform was added and shaken vigorously for 2x10 Sec. The contents were centrifuged at 12,000 rpm for 15 min, and decant the supernatant carefully into a fresh tube. To this, two volumes of ice cold isopropanol was added and mixed gently by repeated inversion of the tubes. RNA precipitation was enhanced by storing at -20°C for ~3 hour. After incubation, RNA was pelleted out by centrifugation at 12,000 rpm. The RNA pellet was washed once with 70% ice cold ethanol and allowed to air dry to remove ethanol. Resuspend the pellet in 50 μl of RNase free-H2O. Store at -80°C. All centrifugation steps were carried out at 4°C unless otherwise stated.
Determination of RNA Purity and Concentration

The final preparation of total RNA is essentially free of DNA and proteins and has a 260/280 ratio 1.6 – 1.8.

RNA concentration (μg/ml) = \( \text{OD}_{260} \times 40 \times \text{dilution factor} / \text{OD}_{260} \)

Where, 40 = standard quantity of RNA/1 (OD260)

Dilution factor = 100 (10μl made up to 1.0 ml)

Amplification of gene by RT-PCR

Polymerase chain reaction (PCR) analysis was performed in order to detect, \( c\text{-Myc} \), \( Bcl-2 \), \( Akt \), \( Cyclin D1 \) and \( Caspase 3 \) gene expressions using a thermal cycler with \( \beta\text{-Actin} \) as an internal control. Primers were obtained from Eurofins and used for amplification.

<table>
<thead>
<tr>
<th>PRIMERS FOR PCR:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NAME</td>
<td>SENSE PRIMER</td>
<td>ANTI SENSE PRIMER</td>
<td>PRODUCT SIZE</td>
</tr>
<tr>
<td>BRD 4</td>
<td>GATGGCGTGAAACTGAACCT</td>
<td>CTTTCGCTGCACAATCATA</td>
<td>271</td>
</tr>
<tr>
<td>c-MYC</td>
<td>TCAAGAGGGCAACACACAAC</td>
<td>GGCCCTTTTCATGGTTTTCCA</td>
<td>268</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>CGACTTTGCAAGATGTCCA</td>
<td>ATGCCGATTCAAGTACTCAG</td>
<td>186</td>
</tr>
<tr>
<td>Akt</td>
<td>AAGAAGCTTGGCTGCATAAA</td>
<td>GTCCATTCTTCCGCTCTTC</td>
<td>277</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>GACCTGGATGCTGGAAGTGT</td>
<td>TTGTTCAACCAGCAGCAGTTC</td>
<td>258</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>GCATGGATAGCGGCTATTAT</td>
<td>TGCGCATTTCATATTTTACG</td>
<td>183</td>
</tr>
</tbody>
</table>

RT-PCR for mRNA expression was done according to the manufacturer’s guidelines (GeNetBio (R-4000) (Daejeon, South Korea) one step RT-PCR mix). Briefly the reaction mixture contained 10 μl of 5x GeNetBio one step RT-PCR buffer containing final concentrations of 2.5 mM MgCl\(_2\), 2 μl of dNTPase mix (0.4 mM of each dNTP as final concentration), 5 μl of each sense and antisense primer of \( c\text{-Myc} \), \( Bcl-2 \), \( Akt \), \( Cyclin D1 \) and \( Caspase 3 \), respectively for each reaction, 0.75 μg of template RNA, 2 μl of GeNetBio one step RT-PCR enzyme mix and made up to 50 μl with...
RNAse free water. Thermal cycling conditions were started for reverse transcription reaction for 1 h at 50°C followed by initial activation of Taq DNA polymerase for 15 min at 94°C. Then cDNA amplification started with 3 step cycling with denaturation at 94°C for 30 sec, extension at 60°C for 45 sec and extension for 15 sec at 72°C. A total of 40 cycles was performed. Final extension was carried out at 72°C for 5 min. Then the reaction was terminated by holding the temperature at 4°C. Preliminary experiments were conducted with each gene to ensure that the number of cycles represented a linear portion for the PCR OD curve for the C6 cell samples. After amplification, the RT-PCR products were electrophoresed on 2% agarose gels, and stained with Ethidium bromide. Band intensity was measured using Gel Doc EZ System, BioRad.

2.2.6. Western Blot Analysis

Sample Preparation for SDS-PAGE

C6 Glioma cell was harvested and then homogenized using ice-cold lysis buffer (1% NP40, 50mM Tris pH 7.4, 150mM NaCl), then centrifuged at 14,000g for 10 min at 4°C the supernatant fluid is the total cell lysate. For SDS-PAGE all the extracts of individual groups were pooled together and using the Lowry’s method with BSA as standard quantified protein content.

Reagents

1. lysis buffer (1% NP40, 50mM Tris pH 7.4, 150mM NaCl),
2. 2x sample buffer with reducing agent (Sample solubilising buffer, SSB) [125mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.004% bromophenol blue]
3. 1x Tris buffered saline (TBS, pH 7.4: 0.2M Tris in 0.89% sodium chloride)
4. Wash buffer (TBST): 0.1% Tween 20 in 1xTBS
5. Blocking buffer: 5% non fat dry milk powder and 0.1% Tween 20 in 1xTBS

Thesis: Vishal Rajagopalan
6. Antibody dilution buffer: 5% BSA and 0.1% Tween 20 in 1xTBS
7. PVDF membrane.
8. ECL kit (Millipore)

Procedure
The C6 Glioma cells were washed and homogenates with lysis buffer (1% NP40, 50mM Tris pH 7.4, 150mM NaCl) extracts were then centrifuged for 5 min at 14,000 r.p.m. at 4°C. The samples were boiled for 5 min before loading onto gels and the equal amount of protein (60μg) was loaded and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membranes were then blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 for 1 hr at room temperature, and probed with the following primary antibodies: c-Myc [mouse monoclonal antibody at a dilution of 1:500]; Bcl-2 [rabbit polyclonal antibodies at a dilution of 1:1000] and Akt [rabbit polyclonal at a dilution of 1:1000]; β-actin [mouse monoclonal antibody at a dilution of 1:2000] overnight at 4°C. The blots were then extensively washed with Tris-buffered saline with 0.1% Tween 20 and then incubated with respective [anti rabbit and anti mouse] HRP labeled secondary antibody (Genei, Bangalore, India) at dilution of 1:1000 for 1 hr at room temperature. After extensive washes in TBS-T, The bound antibodies were visualized using an enhanced chemiluminescence detection kit (Millipore, USA) in Chemi Doc image scanner from alphadigdoc gel documentation system and the band intensity was quantified.

2.2.7. Immunohistochemistry
Reagents

3. Sodium Citrate buffer
4. Ice cold methanol
5. 0.1% Triton X-100 in PBS

Procedure

Sections of the brain tissue were deparaffinised and hydrated, post which they were subjected to antigen retrieval by incubating in Sodium Citrate buffer for 20 mins at 98°C (Zirath, Frenzel et al. 2013). Then, the slides were washed in PBS, fixed for 2 min in cold methanol and washed twice in PBS. Slides were washed in PBS for three changes at RT for 5 min each. Tissue sections were blocked for 1 hr in 10% normal goat serum (Invitrogen). Monoclonal antibodies (from Santa Cruz, Catalog No.sc-40, sc-492, sc-101629 for c-Myc, Bcl-2, Akt Ser-473 respectively) were diluted in PBS/1.5% normal goat serum and incubated overnight at 4°C. Slides were washed in PBS for three changes at RT for 5 min each. Secondary antibody (Anti-mouse FITC and anti-Rabbit HRP conjugate) was applied and incubated at RT for 1 hr. Nuclei were visualized with Haematoxylin. Slides were washed well in PBS and mounted with Gold Mounting Media for FITC labelled slides and DPX for HRP labelled slides.

2.2.8. Transmission Electron Microscopy

TEM analysis was performed by the method of Chappel and Zaban (Chappel and Zaban 2002):

Reagents

3. Karnovsky’s fixative: 4:1 of Para formaldehyde and glutaraldehyde
4. Embedding medium: the following ingredients such as Araldide CY212 (10 ml), DDSA (Dodecenyl Succinic Anhydride) (10 ml), Tri-dimethyl aminomethyl phenol (DMP) (0.4 ml) and Plasticizer (dibutyl phthalate) (1.0 ml) were mixed and stirred vigorously. The excised tissue samples were first fixed with a mixture of 4:1 4% formaldehyde and 1% glutaraldehyde in 0.1 M Phosphate Buffer, overnight at 4°C. Then, the tissue is washed with 0.1M Phosphate Buffer. Post fixation the tissue was treated with 1% osmium tetroxide in 0.1 M PB 1-2 hours at room temperature. Tissue was then subjected to dehydration and embedded in beam capsules. The tissue was subsequently sectioned and stained using Uranyl acetate and lead citrate. The stained sections were viewed for cellular ultra-structure changes using Morgagni 268D (Fei Electron optics) Transmission Electron Microscope at the Sophisticated Analytical Instrument Facility (SAIF), Department of Anatomy, All India Institute of Medical Sciences (AIIMS), New Delhi, India.

2.2.9. Weight loss

Body weight loss is a common factor in cancer patients. Glioblastoma experimental animals were monitored for their body weight changes post treatment of JQ1 and notable changes were found constantly during the study period.

2.2.10. Survival Rate

The Wistar Rats were divided into four groups (as in the material & methods section) of six animals each and examined for survival rate. The survival rate was calculated as follows.
MST (Mean Survival Time) = (Day of first death + day of last death)/2

ILS (Increase in Life Span) (%) = [(Mean survival time of treated group/ mean survival time of control group 1)] x100

2.3 Results:

The Molecule passes the BBB

The molecule JQ1 passes the Blood Brain Barrier easily was confirmed by the HPLC results (Fig 2.1).

Down regulation of c-Myc and associated genes

The group 2 animals when compared to group 3 animals show marked decrease in expression of c-Myc, both transcriptional and translational as evidenced through Semi-quantitative PCR studies (Fig 2.2), immunohistochemical studies (Fig 2.3) and Western Blot studies (Fig 2.4). The group 4 animals exhibited very high expression of c-Myc. The other genes that were examined were Bcl-2 and ser-473-Akt. Although there is no established direct relationship between c-Myc and Bcl-2, there are reports of synergic activity in tumors (Fanidi, Harrington et al. 1992, Kihara-Negishi, Yamada et al. 1998, Bandopadhayay, Bergthold et al. 2013). And Bcl-2 being an oncogenic protein made it a suitable candidate to be examined. ser-473-Akt was chosen as it is known to be involved in cell proliferation and also c-Myc’s regulation. The group 3 animals showed a marked decrease in the expression of ser-473-Akt but not so prominent decrease with respect to Bcl-2 as it has many upstream regulators. The group 4 animals exhibited sharp increase in Bcl-2 and Akt expression similar to that of group 2 tumor control.
Accumulation of Glycogen and Lipid and External appearance among groups

Studies (Pescador, Villar et al. 2010, Favaro, Bensaad et al. 2012) indicate that tumor cells accumulate glycogen as conditions gradually turn to hypoxia. Transmission Electron Microscopy (TEM) shows sharp glycogen accumulation in both group 3 (Fig 2.5) and group 4 animal tissue samples. May be absence of glycogen in group 2 tissue samples is because of cytoplasm being eclipsed by the large malignant nucleus. The treated groups both Group 3 and Group 4 exhibit lipid accumulation along with glycogen. This is because of c-Myc inhibition as demonstrated by studies (Zirath, Frenzel et al. 2013); but at the pathological level the cellular ultra-structure is visibly destroyed in group 4 animals as compared to group 3 animals. This is more eminent when analysing the external pathology (Fig 2.6) as group 4 animals show severe nasal and eye bleeding. Group 2 tissues exhibit a large anaplastic nucleus, almost the size of the cell itself, but compared to group 4 animals, external appearance of group 2 animals seems healthier.

Bodyweight and Survival Rate

A 3% gain in body weight of Glioblastoma induced wistar rats treated with JQ1 (Group 3) was found in this study as compared to Tumor Control (Group 2) which exhibited a 8% loss in weight, further investigating revealed a whopping 21% weight loss in semi-treated JQ1 group (Group 4), while Control Group (Group 1) and Drug Control (Group 5) exhibited a 14% weight gain over a 30 day period (Table 2.2). A significantly increased body weight gain and a reduced tumor size was observed in JQ1 treated rats (Group 3) as compared to tumor control animals (Group 2) and semi-treated animals (Group 4) were found.

Table 2.2
Repeated experiments proved tumor mass size in untreated group (Group 2) to be approx 3mm at day 14 and fatality at around 45 days after implantation. Therefore, all animals were sacrificed by day 90 and the survival rates calculated. The group treated by JQ1 for a period of 60 days (group 3) showed an increase in survival rate of 30% approx. The group that received only 15 days of JQ1 (group 4) treatment developed serious pathology by day 25 and expired by day 29-30 displaying a total survival rate of 32%, a decrease of 20% when compared with the untreated group (Group 2) (Fig 2.7).

Statistical Analysis

All data obtained were analyzed by Students-t-test using MS-Excel, represented as mean ± SD for six animals in each group. The results were computed statistically (SPSS/10 Software Package; SPSS Inc., Chicago, IL, USA) using one-way ANOVA. Post-hoc testing was performed for inter comparisons using the LSD. In all tests, the level of statistical significance was set at $p<0.05$. 

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Weight (gms)</td>
<td>200</td>
<td>210</td>
<td>196</td>
<td>203</td>
<td>199</td>
</tr>
<tr>
<td>Final Weight (gms)</td>
<td>228</td>
<td>185</td>
<td>202</td>
<td>160</td>
<td>230</td>
</tr>
<tr>
<td>Percentage Gain/Loss (%)</td>
<td>+14</td>
<td>-8</td>
<td>+3</td>
<td>-21</td>
<td>+14</td>
</tr>
</tbody>
</table>
Fig 2.1 The HPLC analysis shows the presence of JQ1 in the Rat CSF (B), administered at 7mg/kg bodyweight for 7 days as against control CSF (A).
Fig 2.2: Reverse Transcriptase-PCR (RT-PCR) results of **a**. c-Myc **b**. Bcl – 2 and **c**. Akt respectively. The table illustrates significant variation in expression of group 2 and 4 when compared to Group 1. The almost negligible or almost nil variation of expression between Group 1 and Group 3 exhibits the JQ1’s tumor suppressive effect almost uniformly on all the pro-oncogenic candidates.
c-Myc Silencing in Glioma cells and Glioblastoma induced Rats

**c-MYC**

Group 1

Group 2

Group 3

Group 4

**Bcl-2**

Group 1

Group 2

Group 3

Group 4
Fig 2.3: Immunohistochemical image of FITC tagged anti-c-Myc secondary antibody. Group 2 and Group 4 tissue samples show very high expression while Group 3 exhibits reduced expression (Black arrows indicate c-Myc expressing cells in the tissue). Immunohistochemical image of Horse Raddish Peroxidase tagged anti-Bcl-2 and Akt Ser-473 secondary antibody. Group 2 and Group 4 tissue samples show very high expression while Group 3 exhibits reduced expression (White arrows indicate Bcl-2 expressing cells).
**Fig 2.4:** Western Blot analysis agreed with the results of Immunohistochemistry

<table>
<thead>
<tr>
<th>In vivo</th>
<th>c-Myc</th>
<th>Bcl-2</th>
<th>p-Akt</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1</td>
<td>0.05139±0.007</td>
<td>0.01153±0.357</td>
<td>0.07±0.123</td>
</tr>
<tr>
<td>GROUP 2</td>
<td>0.38876±0.456</td>
<td>0.5305±0.468</td>
<td>0.34403±0.135</td>
</tr>
<tr>
<td>GROUP 3</td>
<td>0.08365±0.876</td>
<td>0.1581±0.791</td>
<td>0.11975±1.001</td>
</tr>
<tr>
<td>GROUP 4</td>
<td>0.47621±0.984</td>
<td>0.61606±0.098</td>
<td>0.46622±0.246</td>
</tr>
</tbody>
</table>
Fig 2.5: TEM image of Group 1 tissue sample showing normal intact nucleus, organelles and cellular ultra-structure. TEM image of Group 2 tissue sample showing enlarge anaplastic nucleus. Most of the organelles are being degraded due to autophagy and glycogen accumulation (white arrows) witnessed because of Warburg Effect. TEM image of Group 3 tissue sample showing shrinking nucleus compared to Group 2 accompanied with lipid droplets (L) and glycogen (white arrows). Large vacuoles adjacent to the nucleus are also observed (V). Cellular ultra-structure especially the cell membrane seems dissolved. TEM image of Group 4 tissue sample showing shrinking nucleus compared to Group 2 accompanied with lipid droplets (L) and glycogen (white arrows). The lipid droplets are unusually large compared to Group 3 tissue samples and are in greater number. The cellular ultra-structure seems more degraded compared to Group 3 and lipid droplets have invaded extracellular spaces.
c-Myc Silencing in Glioma cells and Glioblastoma induced Rats

GROUP 1

GROUP 2

ajagopalan
Fig 2.6: The external appearances of Group 1, Group 2, Group 3 and Group 4 animals. As can be observed, the external appearances of Group 1 and Group 3 animals appear normal, whereas the external appearance of Group 4 animals seem very pathological because of extensive nasal and ocular bleeding. They also seem very weak. Group 2 animals displayed external weakness but exhibited no bleeding.
**Fig 2.7:** Graphical Illustration of survival rates of all the groups (Group 1: Control (injected with 10µl of DMEM alone), Group 2: Tumor Control (injected with 10ul of DMEM containing C6 Glioma Cells), Group 3: Treated (C6 Glioma Cells + 7mg/kg bw JQ1) for 60 days, Group 4: Treated (C6 Glioma Cells + 7mg/kg bw JQ1) for 15 days and observed from there on, Group 5: Drug Control (7mg/kg bw JQ1 alone; utilised only for survival rate experiments). Compared to the Tumor Control (Group 2) the JQ1 treated group (Group 3) shows an increase of 30% in lifespan. In contrast Group 4 shows a drop of 20% lifespan compared to Group 2. Group1 and Group 5 (Drug Control) are very similar proving JQ1 doesn’t have any effect on lifespan. Also shown is the tumor size comparison of group 2 and Group 4 after 15 days.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 vs Group 2</td>
<td>***P &lt; 0.001</td>
</tr>
<tr>
<td>Group 1 vs Group 3</td>
<td>***P &lt; 0.001</td>
</tr>
<tr>
<td>Group 1 vs Group 4</td>
<td>***P &lt; 0.001</td>
</tr>
<tr>
<td>Group 1 vs Group 5</td>
<td>P &gt; 0.05 (Not Significant)</td>
</tr>
</tbody>
</table>

*** Significant at P < 0.001
2.4 DISCUSSION:

c-Myc’s role in metabolism and role of Bcl-2 and Akt in tumor prognosis

c-Myc appears to promote alterations in metabolism, especially by supporting mitochondrial biogenesis (Kim, Lee et al. 2008, Fan, Dickman et al. 2010, Morrish, Noonan et al. 2010, Graves, Wang et al. 2012, Zirath, Frenzel et al. 2013) and thereby initiate acetyl CoA production for lipid biosynthesis through induction of oxidative glycolysis (Morrish, Isern et al. 2009, Morrish, Noonan et al. 2010). But observation suggests degeneration of cellular organelles in both group 3 and group 4 tissue samples as examined under TEM. TEM examination also shows very high degree of lipid accumulation, especially in Group 4 tissue samples and to a lesser degree in Group 3 animals. This would mean that the c-Myc initially induces large scale production of lipids and it does by acting on lipogenic ACL, ACC and FASN and by increasing glutamine transporters and glutaminase 2 expressions respectively (Zhang and Du 2012). But subsequent degeneration of mitochondria as observed may result in these lipid particles accumulating in the cell. But the large aggregation of glycogen contradicts lipid metabolism as glycogen is produced in response to hypoxia and lipid through oxidative glycolysis. This co-existence of lipid and glycogen particles can be explained as a result of initial import of glycogen to satisfy the Warburg Effect and later degeneration of mitochondria through autophagy. Glucose being a preferred moiety for metabolism gets generated from different quarters, starting with translocation of glycogen from liver and subsequent utilization by glycolysis. This burning of glucose is up by about 150 – 200 times in tumor cells to compensate for the high demand of ATP necessary for continuous generation of cells. As the utilization of ATP increases, it leads to increase in AMP to ATP ratio which in turn is sensed by AMPK (AMP activated Protein Kinase) (Dang 2012). AMPK in turn inhibits malanoyl CoA ceasing lipid synthesis and triggers
Autophagy by activating ULK-1 (serine/threonine protein kinase) to recycle cellular resources for further energy production (Rabinowitz and White 2010, Singh and Cuervo 2011). But higher c-Myc expression means promotion of mitochondrial regeneration leading to oxidative glycolysis leading to lipogenesis. The stability of c-Myc is further enhanced by the expression of ser-473-Akt through GSK – 3 down regulation (Sears, Nuckolls et al. 2000). Therefore higher the expression of ser-473-Akt, higher is the Myc accumulation/stability leading to higher rate of lipid metabolism. But this to and fro play of hypoxia and oxidative glycolysis may lead to accumulation of glycogen and fat droplets as observed in the TEM images. The resulting accumulation and metabolic imbalance may lead to the breaking down of cellular ultra-structure.

With respect to fat accumulation, fats may be largely imported by the growing tumor cells as a form of fuel and not generated de novo, through Myc induction. This arises from the in vitro TEM data, where TEM images of in vivo samples treated with JQ1 show large number of lipid droplets as compared to in vitro TEM images. Therefore, Myc may be involved in initiating β-oxidation by promoting mitochondrial genesis and also regulate lipid import from tissues such as adipose to satiate growing energy needs during malignancy. May be a co-culture of tumor cells with adipose cells could prove this phenomenon. The observed lipid droplets in vitro could be a fruitless attempt at anabolism by the fast dividing tumor cells to yield lipid through lipogenesis.

Another study indicates higher Akt expression rescuing c-Myc from JQ1 induced suppression in Glioblastoma (Cheng, Gong et al. 2013), whereas this study indicates the converse, where c-Myc down regulation effects Akt down regulation. Akt also induces Myc activated apoptosis up on stimulation by antitumor drug Temozolomide (De Salvo, Maresca et al. 2011). All these studies indicate a strong interplay between Akt and c-Myc genes, both in promotion of proliferation and cell death. With respect to higher expressions of Bcl-2, there are
two schools of thought; one which says Bcl-2 as an anti-apoptotic agent, which in synchrony with other pro-oncogenic proteins like c-Myc advances the tumor through promotion of survival in spite of organelle autophagy but the other thought is c-Myc is able to override the effort of Bcl-2 to inhibit cell cycle entry (Evan and Littlewood 1998, Cory, Vaux et al. 1999, Cory, Huang et al. 2003).

All together the findings of this study give a map of protein – protein communication involved in metabolic alterations during oncogenesis in Glioblastoma Multiforme, especially re-emphasizing c-Myc’s ability to control metabolism, in turn regulating cell survival and proliferation. It also demonstrates Bcl-2’s co-regulation with that of c-Myc, though the action may not necessarily be agonistic. And although the net expression of all the three candidates’ c-Myc, Bcl-2 or ser-473-Akt is higher in tumor tissue (Group 2) than the transiently suppressed tissue (Group 4), the tumor aggressiveness is striking in case of Group 4 animals, when examining the pathology, both in the external appearance (Fig 4) of the animals and tumor size – approx 3mm (group 2) & approx 5mm (group 4) (Fig 5). But the extent of ultra-structure degeneration in Group 4 and to an extent in group 3 proves BET (BRD 4) domain targeting of c-Myc, as an effective therapy, given, the therapy is long enough for tumor to completely degenerate or it would back fire aggravating the tumor as in Group 4.

Concluding, the study suggests BRD4 being a epigenetic target, it is not only responsible for c-Myc expression alone, but depending on the tissue type it is also responsible for expression of genes like α-Smooth Muscle Actin, Collagen 1A, PAI 1, IL-6, etc (Bandopadhayay, Bergthold et al. 2013), which are responsible for de novo functioning of normal tissues. Therefore, care has to be taken to study long term effects of JQ1 treatment in clinical trials with respect to tissue specific effects, optimal drug targeting, clearance rate, Pharmacokinetics and drug metabolism.
Route of administration would remain oral for JQ1, when combined with a carrier such as Hydroxypropyl-β-Cyclodextrin.

**Fig 2.8** Summary of Myc functions apparent from silencing by JQ1. Myc seems to promote lipid metabolism through induction of FASN, ACC, ACL and Glutamine transporter. Myc also promotes mitochondrial regeneration in turn turning the autophagy wheel for cell survival. It also seems to enhance lipid import from neighbouring tissues into tumor tissue. Myc also seems to affect both Akt and Bcl-2 expression in a positive way as it’s down-regulation seems to cause Akt and Bcl-2 down-regulation. By inducing Akt, Myc is able to regulate its own half-life. Ultimately, Myc, Bcl-2 and Akt seem to working together towards cell proliferation in a tumor micro-environment and simultaneously work towards down-regulating apoptosis.