General Introduction and Objective
A glioma is a type of tumor that starts in the brain or spine. It is called a glioma because it arises from glial cells. The most common site of gliomas is the brain. Gliomas make up ~30% of all brain and central nervous system tumors and 80% of all malignant brain tumors.

Gliomas are classified by cell type, by grade, and by location. Gliomas are named according to the specific type of cell they share histological features with, but not necessarily originate from. The main types of gliomas are:

- Ependymomas—ependymal cells.
- Astrocytomas—astrocytes (glioblastoma multiforme is the most common astrocytoma).
- Oligodendrogliomas—oligodendrocytes.
- Brainstem glioma — develop in the brain stem
- Optic nerve glioma — develop in or around the optic nerve
- Mixed gliomas, such as oligoastrocytomas, contain cells from different types of glia.

Malignant brain tumors are among the most feared types of cancer, not only for their poor prognosis, but also because of the direct repercussions on quality of life and cognitive function. Prevalence studies estimate that 138,054 patients had a diagnosis of a primary malignant brain tumor in the United States in 2010 (Porter, McCarthy et al. 2010). In contrast, most of the epidemiological data in India is based on population-based cancer registries, which represent only a small proportion of the population. The situation is even more difficult for the less common CNS tumors. Epidemiological information about these tumors in India, therefore, has mainly been based on reports from individual centers.

A prospective study from the Tata Memorial Hospital provided a one-year demographic data and relevant tumor-related information on all 656 patients registered in the Neuro-oncology
Clinic (Jalali and Datta 2008). Astrocytomatas were the most common primary tumors. Gliomas constituted 38.7% (254 cases) of CNS tumors, with high-grade gliomas comprising 151 (59.5%) and low-grade gliomas 79 (33.1%) cases. Pilocytic astrocytoma was seen commonly in children. Among the 19 oligodendrogliomas, 12 cases were grade-II and seven were anaplastic. Most of these tumors were seen in middle-aged males. Ependymomas presented either with posterior fossa mass or as a spinal tumor. Most of the cases were below 18 years of age (43.5%). All spinal ependymomas were grade-II, whereas, eight intracranial ependymomas were of anaplastic histology. Eighteen patients presented with clinical and radiological signs of brain stem glioma. To be specific, the median age of presentation of pilocytic, fibrillary and, anaplastic astrocytomas, and glioblastomas were 16, 35, 36, and 50 years as compared to the corresponding values of 23, 33, 49, 62 years recorded in developed countries (Wrensch, Rice et al. 2006, Munshi and Jalali 2009).

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, ie, they are pleomorphic, glioblastomas were called glioblastoma multiforme, a term no longer in use. 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, Gu, Liu 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).

Ionizing radiation is an established environmental risk factor for glioma development. This association was demonstrated in studies of children receiving cranial irradiation for cancer therapy and Tinea capitis and in individuals exposed to atomic bombs and nuclear weapons testing. Most studies of radiation used in diagnostic procedures found no increased risk of glioma (Bondy, Scheurer et al. 2008). Glioma risk is not increased from exposure to cell phones and other types of electromagnetic fields, head injury, foods containing N-nitroso compounds, aspartame, occupational risk factors, pesticides, or season of birth. Cell phone risks have captured the public’s attention, but associations between cell phone usage and glioma are not consistent (Hardell, Carlberg et al., Corle, Makale et al. 2012). Biological effects of radiofrequency on the brain include ipsilateral increases in cerebral blood flow (Huber, Treyer et al. 2005) and glucose metabolism (Volkow, Tomasi et al. 2011) during cell phone exposure, but most case-control studies have failed to demonstrate a relationship with the development of brain tumors.

Although numerous genetic alterations have been described in GBM (von Deimling, Louis et al. 1995, Watanabe, Tachibana et al. 1996), such markers have proven to be of marginal utility in predicting outcome or guiding decisions regarding disease management. In general, the molecular characterization of GBM should provide a better understanding of the genomic landscape of GBMs and more efficacious means for rapid, high-throughput analyses.
of tumor cells and tissues. Despite common clinical presentations and histology, it has been clearly demonstrated that GBM is a highly anaplastic and morphologically highly heterogeneous tumor. The presence of microvascular proliferation and/or necrosis is an essential criterion for the diagnosis of GBM (Steck, Lin et al. 1999). The diagnosis of GBM has been based on a complete clinicopathological assessment and this has been an extremely valuable approach. The pathognomonic features that characterize GBM at the tissue level are the presence of areas of necrosis with surrounding pseudopalisades and microvascular hyperplasia, which are believed to be instrumental to its accelerated growth (Brat and Van Meir 2004).

With our current understanding of the expression of specific molecular biomarkers, the use of methylguanyl methyltransferase (MGMT) promoter methylation status for routine diagnostic or therapeutic purposes is considered to be a promising molecular factor that is predictive of the response of GBM patients to treatment (Shah, Lin et al. 2011). It is associated with prolonged progression-free and longer overall survival in patients with GBM who receive alkylating chemotherapy with carmustine, lomustine or temozolomide (TMZ). The MGMT gene is located on chromosome 10q26 and encodes a DNA-repair protein that removes alkyl groups from the O6 position of guanine, a significant site of DNA alkylation (Hegi, Diserens et al. 2005). For chemotherapy in patients with GBM, as a standard alkylating agent, TMZ-induced injury is repaired by the DNA repair enzymes, including MGMT, which is a unique DNA repair enzyme in the context of alkylating chemotherapy that removes the DNA methylation that is produced by TMZ. It is believed that alkylating agents cause cell death by forming cross-links between adjacent strands of DNA, owing to alkylation at the position of O6-guanine in DNA. Epigenetic silencing of this gene by promoter methylation is associated with loss of its expression and diminished DNA-repair activity. It becomes permanently inactivated and depleted in the process. Hypermethylation
of the MGMT promoter decreases the expression of the protein and, as a result, DNA damage from alkylating agents is not repaired, leading to tumor cell death. The epigenetic silencing of the MGMT gene through promoter methylation is correlated with a median survival in patients who receive radiotherapy (RT) with TMZ for the treatment of GBM. It is now commonly recognized that silencing of the MGMT gene promoter by methylation is associated with improved GBM response to combination treatment with radiation and TMZ (Palanichamy, Erkkinen et al. 2006). The current standard of care to improve the survival of patients who have GBM begins with surgical removal as the initial treatment of choice, followed by radiation and then chemotherapy. Besides establishing a definitive histopathological diagnosis, gross or near-total resection, if feasible, may lead to rapid improvement of clinical symptoms and a reduction of steroid doses or dehydrating agents. In turn, this will dictate subsequent therapy options and significantly improve survival (Lacroix, Abi-Said et al. 2001). Following surgery, a combination of RT and TMZ followed by adjuvant therapy continues to be the most effective therapy available for patients with GBM. Mineo et al showed that 80% of patients received RT prior to chemotherapy. The median survival rate was longer when RT and chemotherapy were combined versus chemotherapy alone (16 months versus 11 months) (Mineo, Bordron et al. 2007). The clinical literature confirms that GBM is a highly vascularized tumor that relies on angiogenesis, the formation of new blood vessels. The vascular structure of GBM is disorganized, tortuous and functionally abnormal, which leads to hypoxia, acidosis, increased interstitial pressure, blood brain barrier disruption and cerebral edema or tissue necrosis (Chi, Sorensen et al. 2009). Bevacizumab (BV), a recombinant humanized IgG1 monoclonal antibody (MAb) that targets VEGF (a key regulator of tumor angiogenesis) is the first antiangiogenic therapy approved for use in cancer, and received FDA approved for the treatment of rGBM following primary therapy in 2009. BV produces response rates of approximately 20 to 40% in GBM and
increases 6-month progression-free survival (PFS6) to approximately 30 to 50% (Wen 2010), which is superior to the 21% PFS6 rate reported for TMZ (Yung, Albright et al. 2000). These reports demonstrated that BV therapy leads to rapid reductions in peritumoral edema, often permitting a decrease in dose or even cessation of corticosteroid use. The MAb study proved that multivalent proteins are engineered to have high selectivity and affinity to antigenic epitopes, and are capable of functioning on the eliminable side of blood vessels without a need to traverse the blood-brain barrier. This may be effective in the treatment of brain tumors (Sathornsumetee and Rich 2008).

The latest in the line of molecules targeting tumors is from the Triazolothienodiazepine compounds family. It is 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) from the stables of James Bradner Lab, Dana Farber Cancer Institute, Boston, USA. The Triazolothienodiazepine compounds have been used as agonists of Platelet Activating Factor (Walser, Flynn et al. 1991), for prophylaxis or suppression of transplant rejection (Adachi 2011), against myocardial infarction, stroke and Alzheimer’s (Kempen 2013) and lately against tumors (Miyoshi 2013). JQ1 acts by displacing BET bromodomains from chromatin by competitive inhibition of the acetyl-lysine pocket (Bandopadhyay, Bergthold et al. 2013, Belkina, Blanton et al. 2013, Horn, Ziepert et al. 2013).

![Structure of JQ1](image)
Superimposition of the mouse BRD4(1)-H3K14ac peptide complex with the human BRD4(1)-(1)-JQ1 complex structure. The hydrogen bond formed to the conserved asparagine (N140) in the peptide complex is shown as yellow dots. (Above) The acetyl-lysine binding pocket of BRD4(1) is shown as a semi-transparent surface with contact residues labelled and depicted in stick representation. Carbon atoms in (1)-JQ1 are coloured yellow to distinguish them from protein residues. Distinguishing surface residues are shown in red; the family conserved asparagine is shown in blue (below). Courtesy: (Filippakopoulos, Qi et al. 2010)
Myc being the primary target of this study has to be justified as a tumor target. MYC belongs to a family that includes MYCL (L-Myc) and MYCN (N-Myc) (Brodeur, Seeger et al. 1984, Nau, Brooks et al. 1985, Maris 2010). Whereas the role of L-Myc is less well understood, N-Myc expression is tissue restricted, and N-Myc can substitute for c-Myc in murine development. The protooncogene MYC lies at the crossroads of many growth-promoting signal transduction pathways and is an immediate early response gene downstream of many ligand-membrane receptor complexes (Kelly, Cochran et al., Armelin, Armelin et al. 1984). The road to MYC's discovery was paved by early studies of fulminant chicken tumors caused by oncogenic retroviruses, leading to the identification of the v-myc oncogene that causes myelocytomatosis (leukemia and sarcoma) (Duesberg and Vogt 1979, Hu, Lai et al. 1979, Sheiness and Bishop 1979). The v-myc oncogene was co-opted from the host cellular genome containing the protooncogenic version or c-myc (Vennstrom, Sheiness et al. 1982). MYC is frequently translocated in multiple myeloma (Shou, Martelli et al. 2000) and is one of the most highly amplified oncogenes among many different human cancers (Beroukhim, Mermel et al. 2010). MYC is downstream of deregulated Notch signaling pathways found in T cell leukemia (Palomero, Lim et al. 2006). Hence, alterations of MYC are commonly found on the path to cancer.

Early in vitro studies of MYC revealed its potential to transform normal embryonic fibroblasts in cooperation with other oncogenes (Land, Parada et al. 1983). These studies set the stage for transgenic mouse studies that provided the evidence that deregulated expression of MYC is sufficient to drive tumorigenesis in a number of transgenic mouse tissues (Chesi, Robbiani et al., Adams, Harris et al. 1985). Retroviral insertional mutagenesis further
identified c-Myc as a major murine oncogene (Akagi, Suzuki et al. 2004). *MYC* is documented to play a role in tumor initiation; however, whether it also participates in tumor maintenance had previously been unclear. Knockdown of *MYC* in established cancer cell lines in vitro appears to uniformly reduce cell proliferation and in some instances induce apoptosis (Cappellen, Schlange et al. 2007, Wang, Mannava et al. 2007). Myc dimerizes with Max to bind DNA and mediates many of its functions (Blackwood and Eisenman 1991, Amati, Dalton et al. 1992, Grinberg, Hu et al. 2004). Myc biology is further complicated by the finding that a cytoplasmic cleavage product of Myc (Myc-nick), which lacks the nuclear localization signal and DNA-binding domain, can promote alpha-tubulin acetylation by recruiting GCN5 and promote cell differentiation in a nontranscriptional manner (Conacci-Sorrell, Ngouenet et al. 2010). Myc also appears to recruit DNA replication licensing factors to catalyze DNA replication, although whether its transcriptional function at replication origins is part-and-parcel of its DNA replication activity is not yet clear (Dominguez-Sola, Ying et al. 2007). Lastly, Myc appears to function even in the absence of functional Max protein as documented in PC12 cells (Hopewell and Ziff 1995). Moreover the sheer array of genes involved under transcription by Myc makes it an inviting tumor target (Dang 1999).
Myc also seems to play a huge role in regulating cellular metabolism so as to regulate tumor micro-environment. The growth of a normal mammalian cell depends on growth factor signaling and nutrient availability (Cantor and Sabatini 2012, Dang 2012). In the case of yeast, the availability of nutrients is sufficient to trigger a transcriptional program of growth without the need for growth factors. There are, however, no clear yeast orthologs for MYC:MAX. In mammalian cells, it is envisioned that growth factor receptor signaling, in the presence of adequate nutrients, would induce cascades of gene expression responses that should result in (1) import of key nutrients, (2) generation of ATP and NTPs, (3) generation of key building blocks for the synthesis of macromolecules, (4) stimulation of protein synthesis and the synthesis of macromolecules, (5) biogenesis of key cellular organelles, (6) orchestration of the cell-cycle machinery and stimulation of cell growth and division, and (7) regulation of cell- type-specific differentiation from the stem cell compartments. With this

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Regulation</th>
<th>Pathway</th>
<th>Functional relevance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21&lt;sup&gt;MM&lt;/sup&gt;</td>
<td>Down</td>
<td>DNA-damage response, APC pathway</td>
<td>Checkpoint failure, cell differentiation</td>
<td>21,57,91</td>
</tr>
<tr>
<td>p15&lt;sup&gt;MM&lt;/sup&gt;</td>
<td>Down</td>
<td>TGFβ pathway</td>
<td>Resistance to TGFβ-mediated proliferation arrest</td>
<td>55,56</td>
</tr>
<tr>
<td>ODC, ROL, HMG1/4, PMTA</td>
<td>Up</td>
<td>Transformation of rat fibroblasts</td>
<td>Anchorage-independent growth</td>
<td>149-152</td>
</tr>
<tr>
<td>HDAC2</td>
<td>Up</td>
<td>APC pathway</td>
<td>Suppression of differentiation</td>
<td>153</td>
</tr>
<tr>
<td>CCND1, CCND2, CDK4</td>
<td>Up</td>
<td>Growth-factor response, proliferation of cerebellar neuronal precursors, skin carcinogenesis</td>
<td>G1 progression in response to mitogenic signals</td>
<td>93,99,154,155</td>
</tr>
<tr>
<td>E2F2</td>
<td>Up</td>
<td>Growth-factor-dependent proliferation</td>
<td>Required for Myc-induced proliferation</td>
<td>156</td>
</tr>
<tr>
<td>IGF2, H-ferritin</td>
<td>Up, down</td>
<td>Iron metabolism</td>
<td>Required for Myc-induced proliferation and transformation</td>
<td>4</td>
</tr>
<tr>
<td>LDHA</td>
<td>Up</td>
<td>Glycolysis</td>
<td>Required for Myc-induced transformation</td>
<td>101</td>
</tr>
<tr>
<td>N-cadherin, integrins</td>
<td>Down</td>
<td>Adhesion of stem cells to their niche in skin and bone marrow</td>
<td>Exit from the stem-cell compartment</td>
<td>20</td>
</tr>
<tr>
<td>SHMT</td>
<td>Up</td>
<td>C1 metabolism</td>
<td>Myc-induced proliferation and growth</td>
<td>105</td>
</tr>
</tbody>
</table>

Selected downstream targets of c-Myc (Adhikary and Eilers 2005)
picture in mind, MYC is surmised to affect different genes quantitatively in a fashion that allows for the stoichiometric production of key components of the growing cell, supported by an adequate supply of energy and building blocks. Metabolism is up-regulated as cell growth increases and is therefore not just along for the ride. As the cell is induced to grow, glycolysis and glutaminolysis are induced through increased expression of glucose and amino acid transporters (Vander Heiden 2011). Glucose is imported through transporters into the cell and is subsequently phosphorylated by hexokinase to form glucose-6-phosphate, which is subsequently split into 3-carbon molecules, releasing ATP and culminating in the production of pyruvate. The glycolytic intermediate 3-phosphoglycerate is a substrate for the synthesis of lipids, as well as a precursor of serine and glycine (Chaneton, Hillmann et al. 2012). High metabolic flux through glycolysis hence provides growing cells with building blocks for macromolecular synthesis from glucose as well as ATP. Glucose could also be shunted into the pentose phosphate pathway for the production of NADPH and ribose for reductive biosynthetic reactions and nucleotide synthesis, respectively (Cantor and Sabatini 2012). The final product of glycolysis, pyruvate, could be further oxidized in the mitochondrion through its conversion to acetyl-CoA, which combines with oxaloacetate to produce citrate, for subsequent degradation in the tricarboxylic acid (TCA) cycle. Citrate can also be exported to the cytosol and converted to acetyl-CoA by acetyl-CoA citrate lyase (ACLY) for fatty acid synthesis. In growing cells, a significant amount of pyruvate is converted to lactate, a phenomenon known as aerobic glycolysis or the Warburg effect (Zaidi, Swinnen et al. 2012). The shunting of pyruvate to lactate, catalyzed by lactate dehydrogenase (LDH), is necessary for the rapid regeneration of NAD$^+$ from NADH. This NAD$^+$ could be reused by glyceraldehyde 3-phosphate dehydrogenase (GADPH), one of the intermediate steps of the glycolytic pathway, to maintain glycolytic flux (Le, Cooper et al. 2010). Glutamine is imported into growing cells through the activation of transporters such as ASCT2.
(DeBerardinis and Cheng 2009). Once in the cytosol, glutamine can be imported into the mitochondrion, converted to glutamate by glutaminase, and subsequently metabolized to α-ketoglutarate by either glutamate dehydrogenase or glutamate-oxaloacetate transaminase. α-Ketoglutarate is further oxidized in the TCA cycle to generate ATP or provide the carbon skeleton for the production of aspartate, which is a key substrate for nucleotide biosynthesis. Glutamine in the cytosol could also be converted to glutamate by cytosolic glutaminase or exported through the antiporter SLC7A5, which extrudes glutamine in exchange for import of branched-chain amino acids such as leucine. In contrast to the false belief that metabolism is along for the ride as cells grow and divide, the finding that MYC directly regulates genes involved in glucose metabolism as well as those in ribosome biogenesis suggest that increased production of metabolic enzymes as a cell grows is far from being a passive phenomenon. Rather, enhanced metabolic capacity of a growing cell is essential for biomass accumulation and high-fidelity DNA replication. This enhanced capacity requires rewiring of the metabolic capacity of a resting cell into that of a growing and proliferating cell.

The normal hepatocyte, for example, can undergo gluconeogenesis as part of its normal role to store glucose when nutrient is ample. However, the proliferating hepatocellular carcinoma cell tends to drive glucose down the glycolytic pathway so that glucose could be used to generate building blocks and ATP for the growing cell. In this regard, MYC stimulates virtually all genes involved in glycolysis relative to its induction of genes involved in gluconeogenesis, which is diminished in liver cancer cells (Osthus, Shim et al. 2000). For many glycolytic genes, MYC binds phylogenetically conserved MYC consensus E boxes (CACGTG) to activate these genes (Kim, Zeller et al. 2004).
In addition to the ability of MYC to induce glycolytic genes, MYC has also been implicated in promoting RNA splicing for the expression of pyruvate kinase M2 (PKM2), which has been associated with proliferating cells, versus the alternative form, PKM1 (Beroukhim, Mermel et al. 2010). MYC also stimulates genes involved in glutamine metabolism at the transcriptional and posttranscriptional levels (Wise, DeBerardinis et al. 2008). Intriguingly, MYC induction of glutamine metabolism appears to be important for cell survival under glucose or oxygen-deprived conditions (Le, Lane et al.). The ability of MYC to induce glycolysis and glutaminolysis supports the cell’s need for ATP and building blocks. Both glucose and glutamine are required for nucleotide biosynthesis. MYC has been shown to activate many, but not all, genes involved in nucleotide metabolism (Liu, Li et al. 2008,
Glucose and glutamine are also essential for fatty acid synthesis. Citrate generated from glucose and/or glutamine through the TCA cycle is exported into the cytosol and converted by ACLY to acetyl-CoA. Although ACLY is not highly induced by MYC, ACACA (acetyl-CoA carboxylase; ACACA converts acetyl-CoA to malonyl-CoA), FASN (fatty acid synthetase; FASN adds two carbons from acetyl-CoA to the growing fatty acid chain to form longer chains), and SCD (stearoyl-CoA desaturase; SCD introduces a double bond into longer chain fatty acids such as palmitate to form the unsaturated fatty acid oleate) are highly responsive to MYC as documented in the MYC target gene database and more recent studies (Lovén, Orlando et al., Zeller, Jegga et al. 2003). Functionally, MYC induces fatty acid synthesis partially through the use of glucose carbons. MYC was shown to induce mitochondrial biogenesis apparently through the direct activation of many genes involved in mitochondrial biogenesis, specifically PGC-1β (Kim, Lee et al. 2008, Morrish and Hockenbery 2014). Induction of MYC expression is accompanied by increased mitochondrial mass, which is diminished by siRNA-mediated knockdown of PGC-1β expression. Overexpression of MYC also increases the expression of its target C1QBP or p32, which is involved in mitochondrial protein synthesis (Fogal, Richardson et al. 2010). Therefore, Myc seems a good target to inhibit tumor growth. The associated targets that can be analysed are PCNA, Bcl-2 and Akt.

**PCNA** is an evolutionarily well conserved protein found in all eukaryotic species from yeast to humans, as well as in archaea. PCNA functions are associated not only with DNA replication, but also with other vital cellular processes such as chromatin remodelling, DNA repair, sister-chromatid cohesion and cell-cycle control. PCNA was originally described as an antigen for autoimmune disease in systemic lupus erythematosus patients, detected only in the proliferating-cell populations (Miyachi, Fritzler et al. 1978). PCNA plays
a role in replication of damaged DNA. The current model suggests that, at the stalled replication forks, PCNA becomes mono-ubiquitylated in a Rad6A (or Rad6B)–Rad18 dependent manner on an evolutionarily conserved position: a lysine residue at position 164. This is a signal for recruitment of a special polymerase, which is able to continue DNA replication even on a damaged template. Humans, together with other eukaryotes, have several such polymerases commonly referred as TLS polymerases (Loeb and Monnat 2008). TLS is one of the two main pathways of DNA-damage avoidance during replication. The role of PCNA in replication seems to be important in many aspects; however, that is not the only function of the protein. PCNA is an indispensable part of several repair pathways such as MMR (mismatch repair), NER (nucleotide excision repair) and BER (base excision repair) (Stoimenov and Helleday 2009). More enigmatic is the role of PCNA in chromatin assembly and maintenance. The organization of genetic material in eukaryotes is a real barrier for many aspects of DNA metabolism, which imposes dynamic changes in the chromatin structure, especially while the cell is dividing. During DNA replication, chromatin is completely disrupted in front of the replication fork, but is quickly restored when the fork passes. At present, it is not clear how big a region around the fork is remodelled; however, it is known that at least the nucleosomal organization is restored once the replication machinery leaves behind a couple of hundred nucleotides (Lucchini, Wellinger et al. 2001). This suggests a very tight regulation between chromatin remodelling and DNA replication. Indeed, among the many chromatin-remodelling factors, several are believed to function around the forks. One of them is the histone chaperone complex known as CAF1 (chromatin assembly factor 1). Interestingly enough, a protein of the complex (CAF1 subunit A) possesses a PIP-box and is found to interact with the front side of PCNA. There have been studies exploring that interaction and also suggesting a mechanism for coupling of chromatin remodelling with DNA replication. The working model is based on the observation that PCNA might function
as a double trimer in vivo (Naryzhny, Zhao et al. 2005, Naryzhny, DeSouza et al. 2006). There are several other PCNA-interacting proteins, whose function is dedicated to chromatin remodelling and maintenance, although the role of the corresponding interactions has not been clearly defined. Successful replication of the genome is one of the important steps required in cell division, while the next step is a segregation of the genetic material into progeny cells. A special structure is involved in keeping the homologous chromosomes together, termed the sister-chromatid cohesion complex (or simply cohesin). Cohesin is already established in S-phase of the cell cycle, coupling the DNA replication to sister-chromatid cohesion. An essential protein for cohesin assembly in yeast is yEco1 [in humans, the family contains ESCO (establishment of cohesion) 1 and 2] and that protein was shown to bind PCNA via a defined PIP-box (Moldovan, Pfander et al.). Cell-cycle regulators such as CDK–cyclins as well as the inhibitors of the cell cycle such as p21 and p27 are crucial components of the checkpoint system, a barrier very often overcome in the progression of cancer. It seems that extensive cross-talk, mediated by PCNA, exists between cell-cycle regulation, DNA replication and repair.

Alterations in Bcl-2 family proteins expression and function contribute to the pathogenesis and progression of human cancers, thus providing targets for drug discovery that are currently being explored in human clinical trials. Abundant examples exist where the regulation of genes encoding either antiapoptotic or proapoptotic Bcl-2-family proteins is altered in cancers. In fact, the founding member of the gene family, BCL-2, was discovered because of its involvement in t(14;18) chromosomal translocations observed in non-Hodgkin's lymphomas (Tsujimoto, Cossman et al. 1985). Defects in the expression of proapoptotic members of the BCL-2 family also occur in cancer, resulting in loss of the tumor suppressor function of these killer genes. The best documented is BAX, where homozygous
deletions or inactivating mutations have been identified, particularly in cancers that arise with microsatellite instability because of defective DNA mismatch repair (Rampino, Yamamoto et al. 1997). Overexpression of the Bcl-2 and related antiapoptotic proteins has been demonstrated to inhibit cell death induced by many stimuli, including growth factor deprivation, hypoxia and oxidative stress. However, it is the ability of antiapoptotic Bcl-2-family proteins to suppress cell death induced by cytotoxic anticancer drugs that makes these proteins particularly interesting as potential targets for cancer drug discovery. Regardless of the primary mode of action, whether single or double-strand DNA breaks, whether microtubule depolymerization or aggregation, whether nuclear hormone receptor activation (glucocorticoid receptor) or inhibition (estrogen and androgen receptors), essentially all traditional anticancer drugs appear to depend in large measure on Bcl-2/Bax-dependent mechanisms for killing cancer cells (Reed 2008). Thus, Bcl-2 operates at a distal point in a conserved cell death pathway utilized by most anticancer drugs, constituting a form of intrinsic chemoresistance, distinct from the previously identified mechanisms involving drug efflux, drug metabolism, drug inactivation and related mechanisms. The central pathway involved in daily (‘normal’) programmed cell death in most tissues involves mitochondria, energy-producing organelles that play critical roles in both cell life and death (Green and Kroemer 2004). Several Bcl-2-family proteins, both antiapoptotic and proapoptotic, have C-terminal transmembrane domains that insert in the outer membrane of mitochondria. While much research has focused on homo- and heterodimerization among Bcl-2-family proteins, many if not most of these proteins have other interaction partners that regulate their activity and that link them to a wide variety of cellular pathways, giving the impression that Bcl-2-family proteins operate as critical nodes in complex networks to integrate information and make ultimate life/death decisions.
The AKT/PKB (protein kinase B) kinases, which include AKT1, AKT2, and AKT3, are key intermediates of signaling pathways that regulate cellular processes controlling cell size/growth, proliferation, survival, glucose metabolism, genome stability, and neo-vascularization. A large body of literature has documented frequent hyperactivation of AKT kinases in a wide assortment of human solid tumors and hematological malignancies (Bellacosa, Testa et al. 1991, Bellacosa, Kumar et al. 2005). Moreover, genetically modified mice have been used as in vivo models to demonstrate that aberrant AKT signaling can contribute to malignancy, either alone or in cooperation with other genetic alterations (Luo, Manning et al.). Since the AKT signaling cascade is frequently deregulated in many types of cancer and, in some malignancies, has implications with regard to tumor aggressiveness (Mitsiades, Mitsiades et al. 2004), there is potential utility in molecularly targeting components of the AKT pathway for cancer therapy and, possibly, cancer prevention. AKT is now known to be a central node in a signaling pathway consisting of many components that have been implicated in tumorigenesis, including upstream phosphatidylinositol 3-kinase (PI3K), PTEN (Phosphatase and Tensin homologue deleted on chromosome Ten), NF1 and LKB1, and downstream tuberous sclerosis complex 2 (TSC2), Forkhead Box Class O (FOXO) and eukaryotic initiation factor 4E (eIF4E). Several of these proteins (AKT, eIF4E, and both the p110α catalytic and p85α regulatory subunits of PI3K) can behave as oncoproteins when activated or overexpressed, while others (PTEN, FOXO, LKB1, TSC2/TSC1, NF1, and VHL) are tumor suppressors. Somatic genetic and/or epigenetic changes involving genes encoding these AKT pathway components have been reported in various sporadic cancers. Moreover, germline mutations in PTEN, LKB1, TSC2/TSC1, NF1, and VHL are linked with five different dominantly inherited cancer syndromes characterized by numerous scattered hamartomas, which are benign tumors with normal differentiation but disrupted architecture, and predisposition to certain
malignancies (Boudeau, Sapkota et al. 2003, Eng 2003, Kwiatkowski 2003). Each of these tumor suppressors is a negative regulator of the AKT-mTOR pathway, which, when deregulated, results in altered translation of cancer-related mRNAs that regulate cellular processes such as cell cycle progression, autocrine growth stimulation, cell survival, invasion, and communication with the extracellular environment (Mamane, Petroulakis et al. 2004).

It has been known that Bcl-2 and Akt express in a pattern associated with Myc.

**Objectives:**

- To assess if JQ1 application in C6 Glioma cell lines affects proliferation using MTT Assay
- To authenticate that JQ1’s target is c-Myc using RT (Reverse Transcriptase) - PCR
- To examine if other associated oncogenes PCNA, Bcl-2 and Akt are affected by JQ1’s suppression of c-Myc using RT-PCR
- To cross relate gene expression with protein expression for Myc, PCNA, Bcl-2 and Akt using Immunocytochemistry (Fluorescent Microscopy) and Western Blotting
- To evaluate the changes in cell cycle post JQ1 treatment using FACS (Fluorescent Activated Cell Sorting)
- To examine the external pathology of tumor and treated (JQ1) cells using Scanning Electron Microscopy (SEM)
- To visualize the intracellular changes in the C6 cells, both pre-treatment and post-treatment by JQ1 using Transmission Electron Microscopy (TEM)
- Comet Assay was performed to examine apoptosis
➢ After the *in vitro* studies prove that JQ1 is a good anti-proliferative candidate, test if JQ1 can cross the Blood-Brain Barrier (BBB) by examining CSF and brain tissue extract using HPLC.

➢ To create a Glioma Rat model using cranial implantation of C6 Glioma Cells

➢ Orally administer the drug for 30 days

➢ To evaluate if the *in vivo* gene expression patterns of *Myc*, *PCNA*, *Bcl-2* and *Akt* are in agreement with *in vitro* results using RT-PCR

➢ To correlate the *in vivo* gene expression with protein expression using Immunohistochemistry and Western Blot

➢ Evaluate intracellular changes for pre and post-JQ1 treatment using Transmission Electron Microscopy (TEM)

➢ To assess tumor size and weight loss between Control, JQ1 Treated and Tumor Control Groups

➢ To evaluate lifespan between the control, tumor control and JQ1 Treated groups

➢ To evaluate the biochemical changes in CSF and Serum for both pre and post-JQ1 treated groups for the following parameters:

a. Glucose

b. Lactate

c. Total Lipid

d. Total Protein
➢ To evaluate the biochemical parameters for the Control, Tumor and JQ1 treated brain tissues, namely the following:

   a. Glucose
   b. Total Protein
   c. Urea
   d. Uric acid
   e. Cholesterol
   f. Free Fatty Acid
   g. Phospholipid
   h. Triglycerides
   i. Creatinine Kinase
   j. Lactate Dehydrogenase
   k. Glucose – 6 – Phosphate Dehydrogenase
   l. 5’ – Nucleotidase

➢ To evaluate the antioxidant parameters for the Control, Tumor and JQ1 treated brain tissues, namely the following:

   a. Superoxide Dismutase (SOD)
   b. Catalase (CAT)
c. Glutathione – S- Transferase (GST)

d. Glutathione Peroxidase (GPX)

➢ To evaluate the brain tissue morphology using H&E staining for Control, Tumor Control and JQ1 Treated Groups

➢ To evaluate acute and chronic liver toxicity for JQ1 using H&E Staining

It is anticipated that the study may yield an alternative drug regime for the neglected but fast growing tumor like glioma which has very few therapeutic options.

**SCHEME:**

- To assess anticancer property using MTT Assay

- Assess gene expression and examine cellular morphology and intracellular pathophysiology

- Assess gene expression and examine cellular morphology and intracellular pathophysiology
  - Examine CSF, Serum and Tissue Biochemistry and tissue morphology

- Suggest both upside and the downside of the molecule of JQ1 and advise accordingly for clinical application
The image on the left illustrates the pathway that triggers Myc induction. A signal initiated by the GPCR is routed through RAS-MEK-ERK pathway to p-TEFb. P-TEFb in turn triggers BRD4 to transcribe Myc. Upon translation Myc induces Cell Growth, Cell division and ‘differentiation (except in tumor)’. Myc also takes control of cellular metabolism and biochemistry by effecting mitochondrial regeneration during autophagy and mitochondrial regulation.

The image on the right illustrates the predicted effects of Myc suppression due to BRD4 inhibition by JQ1, the molecule under the scanner in this study. C-Myc suppression may lead to Cell growth and Cell division arrest, imbalance in tumor autophagy due to repression of mitochondrial regeneration, which may also lead to metabolic imbalance and subsequent induction of cell death. Although there is not enough evidence of direct interaction between Bcl-2, Akt or PCNA, the possible crosstalk will be