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Casein Kinase 2: A Novel Player in Glioblastoma Therapy and Cancer Stem Cells

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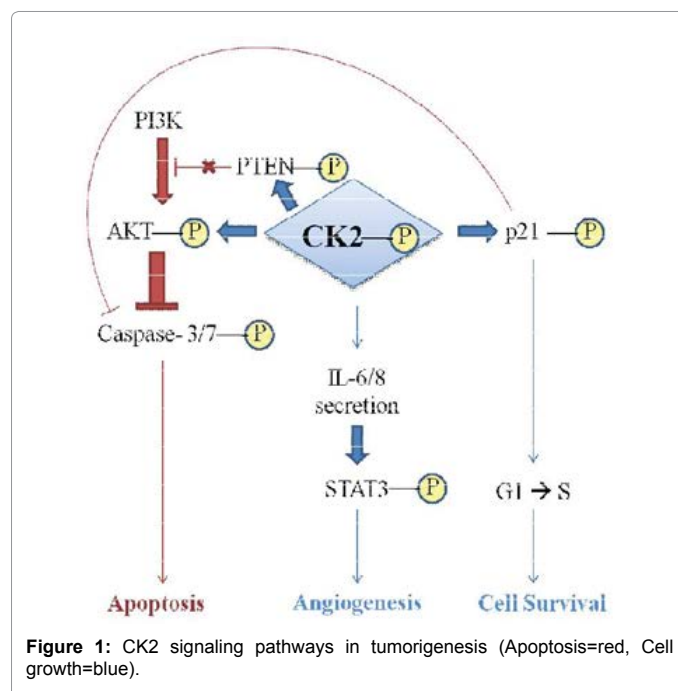*Department of Neurosurgery, Stanford University Medical Center, Stanford, CA 94305, USA*

Abstract

Casein kinase 2 (CK2) is an oncogenic protein kinase which contributes to tumor development, proliferation, and suppression of apoptosis in multiple cancer types. The mechanism by which CK2 expression and activity leads to tumorigenesis in glioblastoma (GBM), a stage IV primary brain tumor, is being studied. Recent studies demonstrate that CK2 plays an important role in GBM formation and growth through the inhibition of tumor suppressors and activation of oncogenes. In addition, intriguing new reports indicate that CK2 may regulate GBM formation in a novel manner; CK2 may play a critical role in cancer stem cell (CSC) maintenance. Since glial CSCs have the ability to self-renew and initiate tumor growth, new treatments which target these CSCs are needed to treat this fatal disease. Inhibition of CK2 is potentially a novel method to inhibit GBM growth and reoccurrence by targeting the glial CSCs. A new, orally available, selective CK2 inhibitor, CX-4945 has had promising results when tested in cancer cell lines, *in vivo* xenograft models, and human clinical trials. The development of CK2 targeted inhibitors, starting with CX-4945, may lead to a new class of more effective cancer therapies.

Introduction

Glioblastoma (GBM) is the most common and fatal primary brain tumor in humans. Since no cure currently exists, and the median overall survival of afflicted patients remains between 14 to 15 months, the need for new and better therapies is urgent [1]. GBM is most common in the sixth and seventh decade of life, and more commonly occurs in men than women. All patients with GBM universally experience disease recurrence [2]. Currently, the standard treatment approaches for patients with GBM include safe optimal surgical resection, followed by radiotherapy, and chemotherapy [3]. GBMs, however, are notoriously known to resist conventional tumor therapies [4].

Recent evidence points to casein kinase 2 (CK2) as a promising therapeutic target for GBM. CK2 is a messenger-independent serine/threonine oncogenic protein kinase composed of two catalytic alpha subunits (CK2α and CK2α') and two regulatory beta subunits (CK2 β) [5]. Over 400 substrates have been identified for CK2 indicating that this kinase can regulate a multitude of cellular pathways including: proliferation, survival, apoptosis, tRNA and rRNA synthesis and cellular transformation [6]. CK2 is known to phosphorylate tumor suppressors such as AKT, PTEN, and p53 causing inhibition of apoptosis in cancer cells [5]. On the other hand, p21, STAT3 (indirectly), NF-kB/p65, and c-Myc represent known oncogenes that CK2 activates leading to cell survival and proliferation in cancers (Figure 1) [5]. Apart from its key involvement in regulating normal cell growth and development, enhanced CK2 activity was observed in a variety of cancers, including breast, prostate, lung, leukemia, and brain [7,8]. Numerous reports have recently demonstrated that the subunit, CK2α, plays an important role in GBM tumorigenesis. CK2α (CSNK2a1) had frequent gene dosage gains in more than fifty percent of the tissue in human GBM cases that correlated with increased mRNA and protein levels [9,10]. Many experiments have also confirmed that down-regulation of CK2α by siRNA induced cell death in GBM cell lines [11]. Recently a phospho-proteomic study indicated that a variant of the epidermal growth factor receptor (EGFRvIII) may regulate the activity of CK2α in GBM through the ERK1/2 pathway, however, the downstream mechanism by which CK2α increases susceptibility to GBMs is still being uncovered.

Figure 1: CK2 signaling pathways in tumorigenesis (Apoptosis=red, Cell growth=blue).

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Recently a new concept has evolved suggesting that CK2 could induce tumorigenesis in a novel method. Numerous reports indicate that CK2 may be involved in the growth and maintenance of cancer stem cells (CSC). CSCs are a subpopulation of tumor cells that possess stem-like characteristics such as self-renewal and the ability to differentiate into different cell types found within the heterogeneous tumors. There are a number of different pathways that were found to be involved in maintaining glial CSC phenotypes [12] (Figure 2). Our review will discuss what is known about the CK2-CSC connection and focus on three major CSC pathways, Gli1, Notch, and Wnt/β-catenin. In addition, a great deal of research has focused on identifying inhibitors to CSCs with the hope of preventing tumor reoccurrence. If CK2 does play an essential role in GBM CSC maintenance, then a CK2 inhibitor could be an integral therapeutic for GBM. Consequently, we will also discuss a potent and selective CK2 inhibitor, CX-4945, that is currently being evaluated and tested in preclinical non-GBM and GBM studies and non-GBM clinical trials.
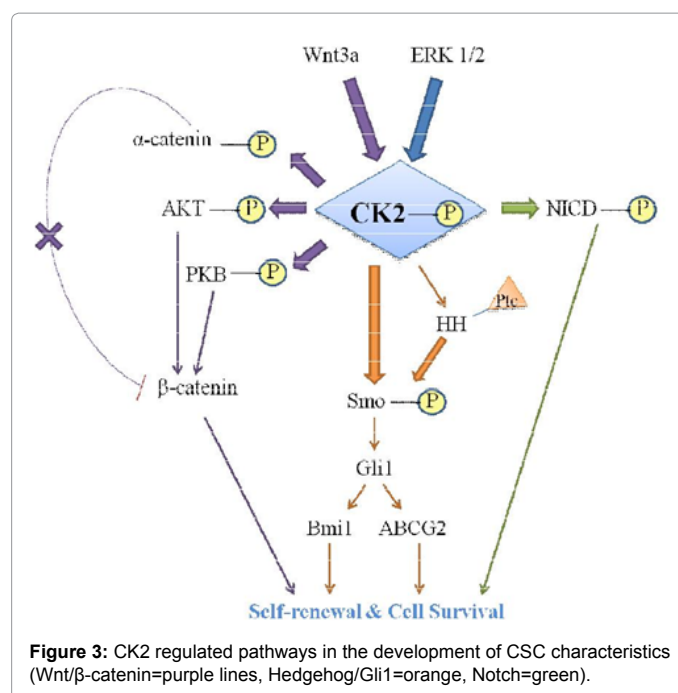
CK2 and Cancer Stem Cells

Recent evidence suggests that CK2 is involved in maintaining a subpopulation of cells in the tumor that are responsible for initiating and maintaining tumor growth. This subpopulation, known as CSCs is capable of self-renewal, asymmetric division and multi-lineage differentiation [13,14]. The "cancer stem cell hypothesis" poses that the CSCs are responsible initiating the tumor and for the heterogeneous population of cells found in the tumor [15,16]. Consequently it is necessary (and sufficient) to eradicate CSCs for therapeutic efficacy in multiple forms of cancer [15,17].

CSCs were first identified in hematopoietic malignancies [18,19], multiple myelomas [20], colorectal, prostate and hepatocellular carcinomas [21-24]. CSCs in GBM have been widely studied, yet studies are still being conducted to further elucidate the pathways in glial stem cells. Recent reports suggest that CK2 could play a vital role in GBM CSC growth and maintenance, but currently more studies are needed. CK2 was found to regulate the expression and/or activity of important stem cell factors and markers in a variety of different types of cancers. We have highlighted three major pathways that CK2 was found to regulate that are well known players in GBM CSCs: Hedgehog/Gli, Notch, and β-catenin (Figure 3).

Hedgehog/gli1 pathway

The hedgehog (HH) signaling pathway plays important roles in mammalian development and in stem cell maintenance during

Figure 2: Pathways in glial cancer stem cell maintenance.

Figure 3: CK2 regulated pathways in the development of CSC characteristics (Wnt/β-catenin=purple lines, Hedgehog/Gli1=orange, Notch=green).

embryonic development [25]. The HH pathway is initiated at the cell surface by the HH ligand binding to its receptor Patched (Ptc), resulting in de-repression of the G-protein-coupled receptor, Smoothened (Smo) [26]. Ultimately, Smo activates the Gli family of transcription factors and target genes [27,28]. Hyperactivation of this pathway or activation of Gli1, by either mutation or deregulation, has recently been recognized to cause tumorigenesis in a wide variety of tissues. For instance, enhanced Gli1 activity was discovered in non-small cell lung cancer (NSCLC), leukemia, lung, gastrointestinal, lung, ovarian, breast and prostate cancers [27] and GLI1 is amplified in human glioma in basal cell carcinoma [29,30]. Recently HH/Gli signaling was found to be involved in maintaining GBM CSCs. For example, human gliomas including GBM displayed enhanced Gli1 signaling and HH-Gli1 signaling regulates self-renewal, sustained growth and survival of GBM CSCs [31]. In addition Gli1 was shown to regulate key factors in maintaining GBM CSCs including: Bmi1, a transcriptional repressor of polycomb group of transcription factors and known regulator of the self-renewal of glioma stem cells [32-34] and ATP-binding cassette transporter member 2 of G family protein (ABCG2) [35,36].

Two recent investigations have demonstrated that CK2 can regulate the HH/Gli1 pathway. Jia et al. [37] initially showed that in Drosophila CK2 can positively regulate HH/Gli signaling through the phosphorylation and activation of Smo. In addition, inhibition of CK2α expression decreased HH target gene expression and resulted in the loss-of-Hh wing phenotype consistent with mutation of Smo [37]. Zhang et al. expanded on Jia et al's discovery and determined that CK2 can regulate the stem-like side population in human lung cancer cells [38]. Zhang et al. discovered that inhibition of CK2α using small molecule inhibitors or siRNAs reduced expression of Gli1 mRNA and protein, and also decreased Gli1 transcriptional activity [38]. In addition, decreasing CK2α expression altered ABCG2 expression which consistently led to a reduction in the cancer stem cell-like side population in the lung cancer cells.
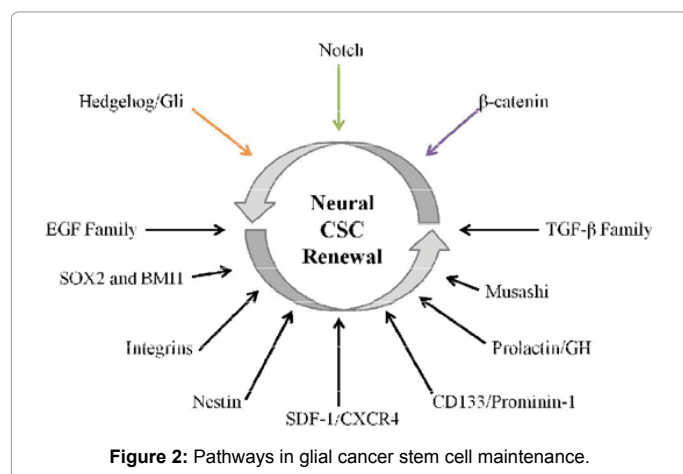
Notch pathway

Notch signaling has a critical role in regulating cell-to-cell communication during embryogenesis, cellular proliferation, differentiation, and apoptosis [39]. Notch signaling is also critical for normal hematopoiesis, breast development, colorectal epithelial maturation, immune regulation, and neural stem cell survival [40]. Mammalian-membrane-bound Notch ligands consist of two structurally distinct families, delta-like ligands (DLLs) and jagged ligands, which interact with the Notch family receptors. Once ligand–receptor binding occurs, the Notch receptor undergoes a conformational leading to its cleavage and release of the active Notch intracellular domain (NICD) into the cytoplasm. NICD undergoes nuclear translocation and binding to the transcription initiation complex and core binding factor-1 (CBF-1), thus modulating Notch-specific gene expression.

The oncogenic role of Notch is highlighted by the presence of activating mutation and amplification in the Notch pathway in variety of human CSCs including GBM. Over-expression of a constitutively active Notch1 protected GBM stem cells from radiation and inhibition of Notch1 impaired xenograft tumor formation [41]. Previous reports demonstrate that Notch activation in neural stem cells leads to increased self-renewal and survival [42-45], while inhibition of Notch pathway by γ-secretase inhibitor treatment attenuated proliferation and self-renewal of GBM stem cells and induced neuronal and astrocytic differentiation [46].

CK2 has been demonstrated to be a regulator of the Notch pathway through the phosphorylation of NICD. Using mapping and mutational studies, Ranganathan et al. identified multiple CK2 phosphorylation sites, located in the ankyrin domain of Notch [47]. Phosphorylation of both sites resulted in decreased binding of NICD to DNA and consequently lower transcriptional activity. Subsequent studies have confirmed that CK2 regulates Notch1 signaling; however, in lung cancer cells CK2 was found to be a positive regulator of Notch1. Zhang et al. found that Notch1 protein levels were reduced after CK2α expression was silenced in lung cancer cells [38]. In addition, inhibition of CK2 using a small molecule inhibitor decreased Notch1 transcriptional activity and reduced the stem-cell like CD44+/CD24- cell population.

Wnt/Beta-catenin

The Wnt/β-catenin pathway regulates stem cell pluripotency and cell fate decisions during development. The Wnt family of proteins serves as ligands for the Frizzled (Fz) and low-density-lipoprotein-related protein (LRP) 5/6 transmembrane receptors [48]. Wnt binding to the Fz receptors initiates three distinct signaling cascades, the most well known being the canonical pathway. In the absence of Wnt, the destruction complex (comprising of GSK3β, adenomatosis polyposis coli (APC), Axin) phosphorylates β-catenin targeting it for proteasomal degradation. Upon Wnt binding to Fz and LRP5/6, the scaffolding protein Dishevelled (Dvl) becomes phosphorylated by GSK3b and Casein-Kinase Iγ. Consequently, the destruction complex components are recruited to the receptor complex, leading to β-catenin stabilization. Stabilized β-catenin translocates to the nucleus, where it binds to lymphoid enhancer factor-1 (Lef-1)/T-cell factor (Tcf) transcription factors and regulates expression of Wnt target genes. Aberrant Wnt signaling has been reported in gliomas [28,49,50], and was shown to have a negative correlation with patient prognosis [51,52]. Together these results show the importance of Wnt/β-catenin in GBM tumorigenesis. The canonical Wnt signaling also was found to have a key role in the regulation of tissue self-renewal [53] and has been well studied in GBM CSCs. β-catenin was demonstrated to regulate numerous genes associated with GBM CSC including OCT4 and NANOG and enhanced β-catenin activity lead to increased CD133+ GBM cells [54].

Numerous reports have demonstrated that CK2 plays an important role in regulating the expression and transcriptional activity of β-catenin. *In vitro* mapping studies identified the Thr393 site in the central armadillo repeat domain of β-catenin as a CK2 phosphorylation site [55]. Mutation of Thr393 site reduced β-catenin transcriptional activity and induced proteasomal-dependent degradation. Additionally studies suggest that CK2 can regulate β-catenin activity indirectly as well. *In vitro* studies show that CK2 can phosphorylate AKT/PKB, a known activator of β-catenin [56,57]. When CK2α is over-expressed, AKT/PKB becomes hyperactivated leading to an increase in β-catenin subcellular localization and transcriptional activity, while also enhancing cell resistance to apoptosis. CK2 was also found to prevent the inhibitory effects of α-catenin on β-catenin by phosphorylating α-catenin at Ser641 [58]. These studies suggest that CK2 may positively regulate β-catenin activity in a direct or indirect manner. Interestingly, multiple regulators of CK2 were found to dictate CK2-dependent β-catenin activity. In the more canonical Wnt/β-catenin pathway, Wnt3a was found to activate CK2 kinase activity through Dvl thereby increasing β-catenin transcriptional activity [59]. However, the mitogen activated protein kinase (MAPK) ERK1/2 was also shown to regulate β-catenin activity by phosphorylation of CK2α [59].

CK2 Inhibitors

The intricate, yet still uncharted role that CK2 plays through these pathways in maintenance of CSCs in a wide array of human cancers amplifies the need for an effective and selective CK2 inhibitor in order to decrease tumor proliferation and differentiation. One class of inhibitors, ATP-competitive CK2 inhibitors, includes derivatives of TBB (4,5,6,7-tetrabromobenzimidazole) and IQA (indoloquinazoline-based compounds), both of which are being studied [60]. Researchers, however, are trying to identify non-competitive CK2 inhibitors that may prove to be more selective; examples include inhibitors that disrupt CK2α and CK2β subunit interactions (Pc peptide or W16) [61], specifically target the CK2β subunits (P1 Peptide) [62], allosterically inhibit CK2 (polyoxometalates, or POMs) [63], or target the substrates of CK2 (P15 peptide) [64] (Figure 4). Within the first class of inhibitors, attention is now being focused on CX-4945, an ATP-binding site CK2 inhibitor [65].

Among the various available CK2 inhibitors, CX-4945 has proven to be the most selective inhibitor available. CX-4945 is a "first-in-class," CK2α small molecule ATP-binding site inhibitor, also known as 5-(3-chlorophenylamino) benzo[c][2,6] naphtylridine-8-carboxylic acid (Figure 5) [66]. According to a recent study, ATP-site directed inhibitors of CK2 fall into the following five chemical categories: (i) Flavonoids; (ii) Derivatives of hydroxyantraquinones/xantenones; (iii) Derivatives of

Figure 4: Small molecule inhibitors to inhibit the activity of the CK2 tetramer.
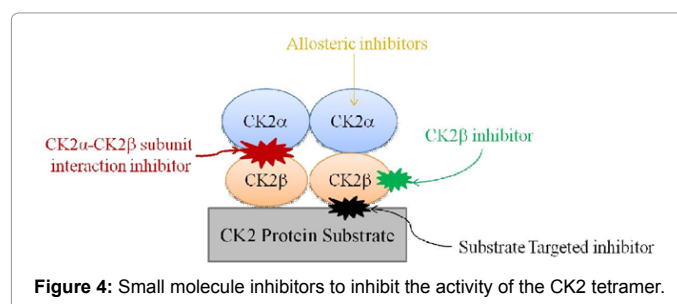
hydroxycoumarines; (iv) Derivatives of tetrabromotriazole/imidazole; (v) Derivatives of indoloquinazolines [67]. Compared to the two most prominent CK2 inhibitors being researched, TBB and IQA, CX-4945 is the only one with high bioavailability [68].

Most of the recent *in vitro*, *in vivo* and clinical research on CX-4945 inhibition of CK2 has been conducted in non GBM cancer cells. Following these studies, there has only been one pre-clinical study in which CX-4945 has been evaluated specifically in GBM. We have highlighted the major experiments that have been conducted in pre-clinical *in vitro* cancer cell lines and *in vivo* mice and human xenograft tumor models, and in human clinical trials that have been conducted most recently (Figure 6) [5,7,66,69,70].

Pre-clinical studies: *in vitro* and *in vivo*

Recent *in vitro* studies in a broad spectrum of human cancer cell lines demonstrated significant inhibition of survival and angiogenesis in the cells when treated with CX-4945. CX-4945 functioned by suppressing the PI3K/Akt signaling in breast, human umbilical vein endothelial cells (HUVEC), and CLL cancer cells [10,69,71] and by inactivating PTEN in chronic lymphocytic leukemia (CLL) [70]. In an *in vivo* study involving pancreatic cancer and breast cancer-inoculated xenograft models, administration of CX-4945 exerted partial or complete anti-tumor efficacy [66,72,73]. Following these studies, similar experiments were done in GBM cell lines and GBM orthotopic models using primary human GBM xenografts in mice.

In human immortalized GBM cell lines, treatment with CX-4945 decreased cell adhesion and migration, and caused a retracted and rounded phenotype in affected cells [10]. Moreover, these reports showed that CX-4945 played a strong regulatory role through

Figure 5: CX-4945 competes with ATP for binding in the ATP-binding pocket of CK2α. (blue=CK2α ATP-binding pocket).

Figure 6: CX-4945 treatment and results (research in GBM and non-GBM=blue, research only in non-GBM=orange).

activation of several inhibitors of cell cycle progression and initiators of apoptosis. CX-4945 also dephosphorylated inhibitor proteins p21 and p27 and reinitiated apoptotic activity in cells by activating caspase 3/7 [66]. Among the pre-clinical *in vivo* studies, researchers found that when GBM was injected into the flank in a mice xenograft model, the treatment significantly inhibited tumor growth. In the same study, intracranial xenograft models in mice revealed even more significant results with regard to CX-4945 inhibition of CK2 in GBM. Following tumor implantation in mice, CX-4945 treatment increased median survival time from 38 days (95% confidence interval: 35.6-40.4) to 59 days (95% confidence interval: 50.2-67.8). Along with this, CX-4945 treatment was shown to significantly deactivate the STAT-3, NF-κB p65, and AKT pathways, all of which are known to cause GBM growth [10].

The findings from these pre-clinical studies show that specific targeting of CK2 using CX-4945 could be the most strategic method of CK2 inhibition due to the effect on multiple downstream pathways [10]. These studies confirm the efficacy of CX-4945 and that it is well tolerated in several primary human xenograft models. Treatment with CX-4945 did not affect body weight, white and/or red blood cell counts, or the level of hemoglobin in these cases. Furthermore, the results of several pharmacokinetics studies show that the profile of CX-4945 boasts acceptable features such as long half-life and high oral bioavailability. The CK2 inhibitor also exhibited non-mutagenicity, nongenotoxicity and non-cardiac toxicity in these pre-clinical studies providing evidence for future success in clinical trials [66,71,74].

CX-4945 clinical trials

Cylene Pharmaceuticals has developed an orally available CX-4945 treatment with high potency (Ki=0.38 nM) and is currently planning phase II clinical trials, after two conclusive phase I trials were completed (http://clinicaltrials.gov, NCT00891280). The two phase I trials were conducted in a total of 44 patients with advanced solid tumors in successive escalating dose cohorts. Tumor types evaluated in this study included prostate, lung, breast, thyroid, ovarian pancreatic, colorectal, and eight others. Oral administration of CX-4945 followed two different dosing schedules (2 or 4 times daily) for the first 3 consecutive weeks of the 4 week cycle. Patient safety and pharmacokinetic/pharmacodynamic analyses were evaluated regularly throughout the study.

Inhibition of CK2 was evaluated based on specific biomarker measurements for phospho-proteins in peripheral blood mononuclear cells. The downstream inhibition of Akt and p21 pathways, along with reduction in circulating tumor cells (CTCs) and interleukin 6 and 8 (IL-6/8) (angiogenesis promoters) levels in peripheral blood were evaluated to assess the impact of CX-4945. CTCs are cells that have been shed from a primary tumor and subsequently begin circulating in the bloodstream. The reduction in CTCs in this study gives evidence to the role CX-4945 may play in inhibiting the metastatic potential of cancer cells [75].

Therapy continued until the patient showed signs of intolerance to CX-4945, or evidence of disease progression. Though generally well-tolerated, two cases of diarrhea and one case of hypokalemia provided the dose limiting toxicities (DLTs). These toxicities were reversible with drug discontinuation, anti-diarrheal use, and potassium supplementation. Twenty percent of the treated patients showed signs of stable disease for at least 16 weeks, with the most durable stabilization in patients with the highest percentage decreases in IL-6 and IL-8 levels. Beyond the 16 week marker, nine patients demonstrated disease stabilization. Following these phase I trials, Cylene Pharmaceuticals is planning rational drug combination phase II trials in multiple cancers.
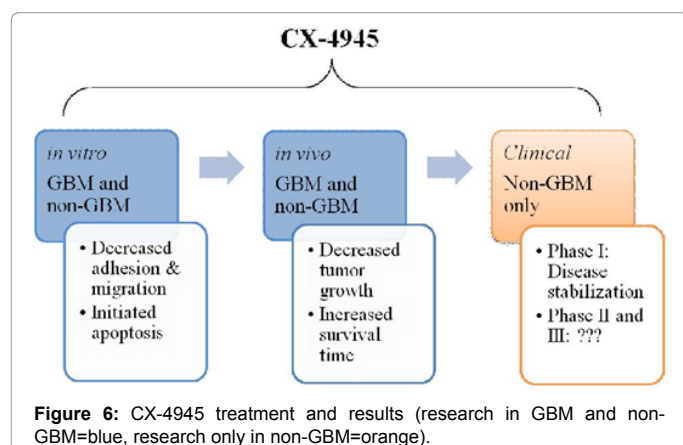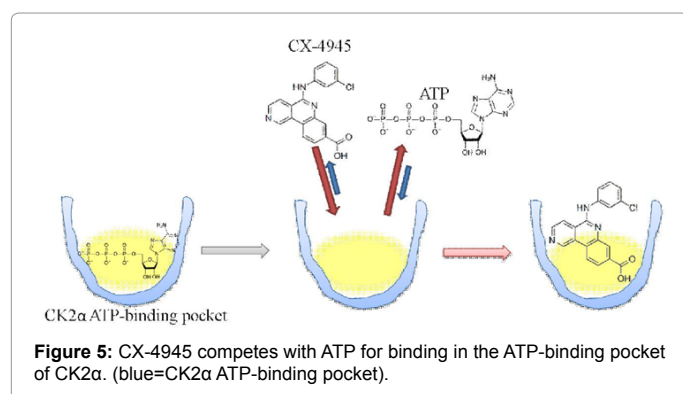
Conclusion

Cancer stem cells in GBM are both chemoresistant and radioresistant, leading to unhindered tumor progression and reoccurrence even with the most aggressive GBM treatments. GBM is highly aggressive and is diffusely infiltrating, meaning that the tumors invade into normal brain tissues, preventing successful surgical resection [76]. Many researchers are developing new methods of targeting CSCs to treat GBM; whether it is a new chemotherapeutic agent specific to CSCs, radiosensitizers to enhance traditional radiotherapy, or an agent that promotes CSCs to differentiate into normal cells [77]. One promising avenue, however, is the ongoing research targeting specific signal pathways known to regulate glioma CSC growth. This review has discussed one such pathway. CK2 is a serine/threonine protein kinase that has been shown to be critical in the formation and maintenance of many cancer types. *In vitro* and *in vivo* experiments have demonstrated an important role for CK2 in gliomagenesis. Our manuscript has reviewed the role of CK2 in the maintenance of the highly differentiable and self-renewable cancer stem cells. We have highlighted three CSC maintenance pathways that CK2 is known to regulate: Hedgehog/Gli, Notch, and β-catenin. While there are therapeutics that singularly target each of these pathways, the novelty of targeting CK2 is that all three pathways will be affected, and the therapeutic effect may be multiplicative. Further research studying the role CK2 plays in GBM specific CSCs is ongoing.

CX-4945 is the first CK2 inhibitor to reach clinical stage testing for the treatment of multiple types of cancer. CX-4945 represents a new class of highly selective ATP-binding site competitive CK2 inhibitors. In a recent study, two analogues of CX-4945 (CX-5011 and CX-5279) demonstrated even greater specificity in inhibiting CK2 [78]. In this review we have discussed the preclinical and clinical testing of CX-4945 for GBM and non GBM cancers. We look forward to the results from the phase II studies using this novel therapeutic.

Acknowledgements

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