Exploration of Synergistic Eg5 Inhibitors for Glioblastoma Treatment

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Abstract

This study aims to investigate the potential of Eg5, a kinesin-5 motor protein, as a target in glioblastoma (GBM) therapy, building on preliminary research on its yeast homolog, *CIN8*. Previous work established the functional and biochemical properties of *CIN8* in yeast and bacteria, focusing on its ATPase activity and motor function, which are pivotal in mitotic spindle dynamics and cell division. A homology between *CIN8* and the human Eg5, particularly in their role in mitosis, led to the literature-backed hypothesis that Eg5 inhibition could be a viable strategy against GBM. The overexpression of Eg5 in GBM cells and its essential role in mitotic spindle formation make it a promising target for cancer therapeutics. The future direction of this research will delve into the synergistic effects of Eg5 inhibitors combined with other therapeutic agents, as such combinations could enhance therapeutic efficacy and target not only GBM cell proliferation but also migration and invasion. Molecular analyses will be conducted to identify changes in gene expression and protein levels, an approach informed by the insights from *CIN8* studies, which offer a foundational understanding of kinesin-5 proteins.

Background and Significance

Eukaryotic chromosome segregation relies on the mitotic spindle, a microtubule-based structure whose movements are dictated by microtubule-based motor proteins. Two such highly conserved proteins, expressed by the yeast *Saccharomyces cerevisiae*, are Cin8p and Kip1p. Though they serve overlapping functions, both are essential in spindle assembly and provide the force for spindle elongation during anaphase, the third, shortest stage of mitosis (1).

CIN8 and *KIP1* belong to kinesin-5, a family of molecular motors that use ATP hydrolysis to move along microtubules towards specific ends of a dividing cell (2). They operate as homotetramers that cross-link and slide antiparallel spindle microtubules, facilitating cell division. Despite being functionally analogous, deletion of *CIN8* has been experimentally shown to cause more severe phenotypes than *KIP1*, indicating its priority in successful spindle function. As a result of this importance, a characterization of *CIN8* and its homologs was deemed to be highly relevant in understanding cell division mechanisms, and their potential inhibition to combat proliferative diseases such as cancer.

In the preliminary phase of investigation, Cin8p's *in-vivo* and *in-vitro* activity was analyzed in both yeast (S. *cerevisiae*) and bacteria (C. *thermophilum*) (3). *In-vitro* studies in bacteria provided a controlled environment to study the biochemistry and structure of Cin8, providing a closer look into its motor activity and microtubule interactions. Comparatively, examining *CIN8* in yeast offered insights into its function within a cellular context, including its involvement in spindle formation and localization with other eukaryotic components, while its *in-vivo* functionality could be further explored via phenotypic analysis. Furthermore, truncated protein sequences of both ScCIN8 and CtCIN8 were used to isolate the functionality of the protein's motor domain and study its mechanism more directly.

In light of the fundamental roles of Cin8p and Kip1p in cell division, the exploration of their human homologs presents a significant opportunity for understanding and potentially manipulating cell division in human cells. According to the Saccharomyces Genome Database, *CIN8*'s human homolog is kif11, a gene encoding for Eg5, a motor protein essential for mitotic

spindle formation and function (4). Like Cin8p, Eg5 is a homotetramer that cross-links and slides microtubules, playing a crucial role during mitosis. Its overexpression or dysfunction is often linked to cancer progression, making it a target for cancer therapeutics (5).

Glioblastoma (GBM) is the most common malignant brain tumor, characterized by rapid proliferation and invasion into brain tissue. There remains no prognosis, and previous efforts have failed for a variety of specific reasons, including the presence of the blood brain barrier and effects on the central nervous system (CNS) (6). Exploring Eg5 inhibition in GBM therapy raises a pivotal question: Can targeting Eg5, particularly alongside other treatments, effectively combat GBM's aggressive characteristics (7)? An important benefit to this approach is that kinesins, unlike microtubules themselves, do not display neurotoxic effects (8). Our preliminary work, which involved studying the kinesin-5 protein *CIN8* in yeast and bacteria, has laid the foundation for this inquiry. Leveraging insights from *CIN8* studies, I will focus on Eg5 in GBM, specifically investigating the effects of its inhibition combined with other therapeutic agents. This research will lead to a relatively unexplored area of combatting GBM.

Preliminary Results

The initial step of the experiment involved a genomic analysis of *CIN8* in *S. cerevisiae* (subsequently referred to as ScCIN8), specifically related to the conservation of sequences between kinesins, in order to select the appropriate genes and proteins to carry out characterization. Using SGD, the closest homolog to *CIN8* in *S. cerevisiae* was KIP1, corroborated by their overlapping functionalities described in literature. An alignment was also executed with the *CIN8* homolog from the bacteria *Chaetonium thermophilum* (subsequently referred to at CtCIN8), yielding a 34% similarity in protein sequence. The gene sequences of CtCIN8 and ScCIN8, and their truncated sequences of only their motor domains, were isolated using BLAST in order to identify locations of forward and reverse primers to be used for PCR. Lastly, using SGD data, *CIN8* and *KIP1* expression patterns showed peaks during the S and G2 phases of the cell cycle, confirming activation during the anaphase.

After relevant genes and proteins were identified, these sequences needed to be cloned, purified, and transformed into a host organism for further experiments. The vectors being cloned into were pET24b, GFP-C-FUS, and pΔGFP. The former is a bacterial vector with a C-terminal Histidine tag to facilitate protein purification via nickel-affinity chromatography and contains a kanamycin resistance marker (?) for selection. GFP-C-FUS is a yeast vector with a GFP tag (for protein visualization via fluorescence microscopy), CIN8 promoter, Ampicillin resistance marker (for selection), and URA-3 gene (selection after transformation). The latter is analogous to GFP-C-FUS without the GFP tag. These vectors, along with the inserts (CtCIN8, ScCIN8, and their truncated sequences), were cleaved with restriction enzymes HindIII and BamHI, before being treated with phosphatase to prevent self-ligation of the vector. The sequences were then purified using a commercial column purification kit, ligated, and transformed into *E. coli* DH5α, a prolific DNA transformation recipient, via heat shock. Plasmid DNA was then recovered, purified with a miniprep kit, and characterized by restriction digestion analysis on agarose gel, after which the constructs were transformed via electroporation into E. coli BLR(DE3)pGroRIL(2) (insert + pET24b), *S. cerevisiae* DDY904, and *S. cerevisiae* Y119.

The driving force in kinesin movement along microtubules is the hydrolysis of ATP to ADP, so a Malachite green assay (also referred to as an ATPase assay) was developed to measure the activity of Cin8p through measuring the free phosphate released from ATP by Cin8p. First, an extinction coefficient was experimentally derived through a standard curve of absorbances

(Figure 1) measured at 650 nm based on varying free phosphate concentrations in a solution of malachite green and sodium citrate. The group calculated an epsilon value of 95.7 μg/mL, though the class average was 93.9 μg/mL. Next, protein activity of human heavy chain kinesin-1 was measured, once again through various absorbances at 650 nm, but with differing reaction components—namely, the inclusion and exclusion of ATP, MT, and Kinesin-1—in a 1 mL solution containing buffer, malachite, and sodium citrate. The calculated specific activity of Cin8p in the presence of microtubules, 1.35 $\mu molmin^{-1}mg^{-1}$, represented an 18.5 extent of simulation of ATPase activity compared to the activity without microtubules, indicating their role in inducing a conformational shift in kinesins upon binding to increase their affinity for ATP. This observation was reflected across all groups in the class, depicted by Figure 2. Furthermore, an NADH-coupled ATPase assay was performed to reinforce the exploration of *CIN8* ATPase activity in a continuous manner to account for kinetic considerations. Various concentrations of ATP were prepared in solutions with kinesin, microtubules, and buffer, gathering plate readings at 340 nm every 20 seconds for 30 minutes.

After successful cloning of *CIN8* into pET24b, purification by affinity chromatography (leveraging the histidine tags) was done to study the biochemical properties of Cin8p *in-vitro*. SDS-Page and Western Blotting were used to separate and specifically identify the presence of Cin8p. During purification, Bradford assay was performed to determine Cin8p concentration of various elutions from the lysate reaction column. 800 μL of Bradford Reagent was combined with varying amounts of ddH_2O and BSA (1 mg/ml) and transferred to a cuvette to create a standard curve (Figure 3) based on absorbances measured at 595 nm. Based on the curve, it was determined that the pool—the most concentrated samples of Cin8p during elution—had a concentration of .367 μg/mL, implying 9.8 μL of solution necessary for 2.6e-11 moles of CtCIN8 construct for future experiments.

Future Directions

The preliminary phase of investigation provided the groundwork for experiments into Cin8's biochemistry and *in vitro* and *in vivo* functionality—the next phase aims to delve deeper not only *CIN8* localization and phenotypic analysis, but also into the conservation and divergence of kinesin functions, especially in the context of neuronal growth and potential applications in glioblastoma treatment.

To further understand Cin8p's mechanism of action, a knowledge of its subcellular localization is necessary. Fluorescence microscopy is one method that will be tested, utilizing the GFP tag on the yeast construct to visualize proteins relative to dividing cells. Another fluorescent tag that will be used is on the *S. Cerevisiae* DDY904 strain, where a TUB1-mCherry gene (red color) resides in addition to wild type TUB1 gene (an alpha-tubulin gene), allowing visualization of the location and appearance of the mitotic spindle. This will also allow for a localization comparison between CtCIN8 and ScCIN8, providing clues to the movement of Eg5 in humans.

Furthermore, a phenotypic analysis of the Cin8p constructs will be performed to test their *in-vivo* functionality. Endogenous *CIN8* has an AID tag, which, in the presence of auxin, signals for the degradation of CIN8. Therefore, *CIN*8 expression should be drastically reduced. In contrast, GFP-tagged cin8 constructs do not have the AID tag, so auxin should not affect transcription. This is important to the Y119 yeast strain, which has a non-functional KIP gene—it must have the *CIN8* construct without the AID tag so it has a protein that facilitates cell division, otherwise it would no longer grow. Cincreasin, a drug that acts as a spindle checkpoint inhibitor, will also be used as a control to monitor *CIN8* function, while the potential effects of

the GFP tag on the *CIN8* construct will also be measured. These experiments will provide phenotypic responses that should roughly correspond to those exhibited by Eg5, especially during its inhibition. Further ATPase activity, in addition to those already explored, will also be measured via a linearity assay to test for optimized biochemical conditions for activity.

Building upon current understanding of the Cin8p motor activity and its cellular functions, the next phase of research extends to its human homolog, Eg5, specifically focusing on glioblastoma models. Given the overexpression of Eg5 in GBM and its pivotal role in mitotic spindle formation, I hypothesize that targeting Eg5, especially in combination with other therapeutic agents, could significantly impede GBM progression. This hypothesis will be tested by correlating data from *CIN8* studies with the functionality of Eg5 in GBM, enhancing our understanding of kinesin motor proteins in cancer biology.

In this experiment, I plan to use fluorescence microscopy to compare the subcellular localization of GFP-tagged Cin8p in yeast cells with that of Eg5 in GBM cells. This comparison will provide insights into the similarities and differences in the localization patterns of these kinesins, potentially revealing new targets for therapy. Additionally, a phenotypic analysis that includes monitoring the effects of spindle checkpoint inhibitors on *CIN8* constructs will offer a parallel to Eg5 inhibition in GBM cells. The aim is to observe if the *CIN8* constructs can mimic the phenotypic responses expected from Eg5 inhibition in GBM, particularly in terms of cell division and spindle dynamics.

More ambitiously, the synergistic effects of combining Eg5 inhibitors with other therapeutic agents in GBM treatment could be explored—GBM cell lines will be cultured and treated with Eg5 inhibitors, both alone and in combination with BRD4 inhibitors and P-glycoprotein inhibitors (9). Treatment effects on cell proliferation, migration, and invasion could be assessed using a Transwell assay, and Western blotting will be conducted to identify changes in gene expression and protein levels induced by these treatments.

This research and proposed experiments will serve to shine new light on GBM treatments, with the potential to contribute to the development of more effective, targeted therapies to improve patients' quality of life.

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