Targeted Treatment on PTEN Mutated GBM Cell Lines Using Metformin and Chlorpromazine

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Abstract

Glioblastoma multiforme (GBM) is among the most aggressive and lethal of all human cancers. Characteristically, GBM is genetically heterogenous with different tumors portions exhibiting vastly different genetic profiles. Due to this, GBM patients are plagued with poor prognosis and high recurrence rates. Within the last decade there has been an increase in knowledge of the molecular finger print of GBM but improvement in patient outcome has been slow as personalized treatment regimens have not been linked to significant improvement. However, there is hope with feasible multiagent personalized regimens as well as expanding the amount of treatment options with repurposed agents and immunologic modulators improving patient outcomes. One hypothesized gene of interest in tumor development and progression is PTEN. In this study we investigate two repurpose agents Metformin and Chlorpromazine which are thought to depress downstream oncogenic proteins specific to the PTEN pathway using a cell culture model. For this study four Xenograft cell lines with differing PTEN status were treated with titrated concentrations of Metformin and Chlorpromazine. After treatment, results were quantified by SiRNA function using cell-titer glo assay a marker for cell viability. Results showed no difference cell susceptibility in regards to PTEN status when treated with chlorpromazine. Metformin appear to spare PTEN wild type cell lines while inconsistently targeting PTEN mutated cell lines. We hope that our data will add to the growing knowledge of Molecular understanding the molecular mechanism PTEN targeted therapy with metformin within GBM patients.

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Introduction

The importance and challenges in treating GBM

Glioblastoma multiforme (GBM) is among the most aggressive and lethal of all human cancers. Despite great interest, the root pathophysiology of glioblastoma remain largely unknown. The cell types that are thought to spawn these highly destructive and aggressively invading tumors are believed to originate from glial, astrocytes, oligodendrocyte, progenitor cells or neural stem cells. Out of approximately 70,000 primary CNS tumors that are diagnosed annually in the US, GBM is the most frequent. This high-grade glioma has an incidence of 3–4/100,000 (1). It is also one of the most difficult cancers to treat with little effective current options and essentially all tumors progress or reoccur. The lack of treatment efficacy is thought to arise from several reasons one being genetic heterogeneity has cells and tumors exhibit an array of mutations. For instance, tumor genomics vary widely in different areas of the tumor as different parts are classified separately such as the “core” or the “edge”. This allows for multiple avenues for cancer development, progression and resistance to treatment. In quantifying the array of mutations, it is thought that approximately 35 non synonymous mutations exist within an average GBM tumor (2). These characteristics are thought to play a role in plaguing patients with high reoccurrence rates, inconsistent responses to treatment, and poor prognoses. Currently, the median survival rate for new onset GMB patients is 14.6 months under the current gold standard of care which includes surgical resection, radiation, and concurrent low dose temozolomide (TMZ) (3). The median survival for patients with recurrent GBM retreated with gold standard anti-angiogenic agent bevacizumab is between eight to nine months (1). Approximately 10% of patients go on past 5-year survival after diagnosis (4). Although the long-term survival for GBM patients as been incrementally improving over the last 12 years patient outcomes is still currently dismal. This may be due to current standards of treatment being effective on certain cell types, but the diversity of GBM tumors may allow other cell types to repopulate the tumor and allow for a reoccurrence of disease. Given the heterogeneity of the genetic presentation of GBM and the expanding knowledge of the use of target therapy, the formulation of personalized treatment regimens

that are more effective than current treatment protocols are being developed. To allow for progress in treatment of GBM an increase an understanding of the molecular pathways and investigating novel therapeutic approaches is of great importance.

Current and future treatment modalities

Within the last decade there has been a significant increase in the understanding of genetic and molecular fingerprints of GBM tumor development and resistance to therapy. Using microarray and genetic panels, GBM tumors can be classified into different subgroups with varying prognostic properties. Genetic profiling separate tumors based on which spawn from low-grade gliomas vs primary GBM tumors (5). Analyzing the genetic variants in GBM tumors has outlined the diverse array of specific genetic and epigenetic changes expressed in GBM tumors. However reoccurring commonality in the deregulation of PTEN, PI3K, TP53, RB, IDH1, ATRX, CDKN2A, NF1 pathways is seen throughout almost all GBM tumor types (3). Developing a more robust understanding of these pathways will hopefully help deicer disease etiology and allude to better treatment. As discussed early genomic profiling has shown to be a prognostic indictor with hopes of this knowledge progressing to better treatment options. For instance, Isocitrate dehydrogenase, IDH, is present in approx. 80% of secondary GBM tumors but only 5% of primary tumors. Although it is thought to be a pivotal in gene in early tumor formation it is seen has a better prognostic indicator (5). The understanding of the interplay between these genetic pathways and how to halt the progression of cancer has been difficult but has shown promise. For instance, 40-50% of GBM tumors exhibit EGFR gene amplification. However, trials with the treatment of single agent Tyrosine kinase inhibitor which target this pathway uniformly disappointing, Current trials looking at a subgroup of EGFR mutations with wild type PTEN do respond to targeting the EGFR pathway highland of prescreening for which patients well respond and which will not (6).

However, progress in the treatment of GBM has been largely stagnant with the role of molecular profiling being questioned. Retrospective studies suggest that most primary glioblastoma tumors possess potentially actionable genomic altercations. (7) (8). However, despite tumor profiling of potential treatment targets, clinical trials have not shown encouraging patient responses to tumor specific single-agent genomics-based treatment. In fact currently 30% of patient with GBM receive targeted treatment based on profiling with essentially no survival benefit (9). Tumor and cellular heterogeneity along with limited brain penetration of potential therapies are likely the culprit for the limit response rates.

However, clinical trials involving multiple agents paired with molecular profiling have become feasible with improvement in scientific knowledge and technological improvement (3). The ability to complete tumor genome-wide exome and RNA sequencing analysis and come up treatment recommendations that contain multiagent treatment protocols has shown to be feasible for GBM patients. For instance, a recent prospective trail 13/14 patient received treatment recommendations within a 35 calendar day window and where treated with three-four agents on average. Of the fourteen patient who received treatment recommendations seven pursued tailored treatment. Of these seven, two remained free of disease progression for more than 365 days (3). This new process has several advantages including allowing patients to be treated with targeted cancer therapies, chemotherapies, immunotherapies and repurposed agents. Treatment with multiple agents allows for blockade of multiple pathways which may be more effective at combating cellular and tumor heterogeneity. With personalized multiple agent treatment protocols continue to be developed the role of novel therapeutic agents and how they affect certain cellular pathways is of great importance.

Cell culture studies and repurpose drugs

In order improve the efficacy of these multiagent trails, expanding on the knowledge of molecular pathways and novel treatment agents is necessary. One method of better understanding these agents and what molecular pathways they effect is through xenograft and cell culture models with pharmaceutical response rates between different cell lines. This method is relatively low cost and allows controlled treatment conditions to discern the roles of different variables. In addition, the use of already approved FDA pharmaceuticals, offers many advantages. The regulations involving the approval of novel therapeutics is strenuous and a slow process that is very expensive and hard for patient to access. The method of repurposing already FDA approved drugs with established safe record may provide an innovative solution to this process and this method is commonly used in the field of neuro-oncology. These is because agents can be selected based on tolerance, blood brain barrier penetration, and pre-clinical suggestions of potential activity in cancer (10)

Research Scope

PTEN is a tumor suppressor protein that is present in approximately 20% of GBM tumors. It is thought to regulate the PI3/AKT pathway, which is involved in both preventing apoptosis via caspase 9 and initiation of translation of protein production needed for cell growth via activation of MTOR and s6 proteins (11). The way in which PTEN is thought to regulate this pathway is through dephosphorylation of PIP3 to PIP2 rendering this pathway inactive. Thus PTEN is thought to increase apoptosis, and halt the translation of a subset of genes involved in cell growth and replication. The Role of PTEN the development of multiple cancer types including glioblastoma is well studied (11).

The Role of Chlorpromazine and metformin in the treatment of glioblastoma has been long since postulated. Chlorpromazine which is classified as a first-generation antipsychotic has been found to be experimentally effective on multiple cancer types including Gilomas (11). For instance, a laboratory cell culture investigation found that Chlorpromazine triggered increased autophagy in mutated PTEN GBM cell line U-87 through inhibition of the 3-phosphoinosite-

dependent protein kinase (11). However, this study did not compare treatment to PTEN wild type cell lines and given that Chlopromazine is hypothesized to be cytotoxic through multiple avenues. including the inhibition of mitotic kinesin and inhibiting Cytochrome C oxidase within mitochondria, additional insight into the mechanism of action of cell death initiation by chlorpromazine is warranted to help uncover the mechanism.

Metformin which is classified as a biguanide and is first line diabetic medication, has been shown to exhibit anti-tumoral effects. This has been postulated to occur through the inhibition of mitochondrial electron transport chain complex I (ETCI) and Liver Kinase B1 (LKB1)-dependent activation of AMPK, a regulator cell metabolism that is upstream from in modulating MTOR through potentially independent or dependent pathway with respect to PI3/AKT (12). A Cell culture study using cell lines with different PTEN status including U87 and SF767 looking at the effects of metformin was performed. Their findings included evidence that metformin has potential to augment the cytotoxic effects of TMZ and radiotherapy. Additionally, Sesen and colleagues work also suggested that metformin was found to decreases mitochondrial oxygen consumption and increases lactate and glycolytic ATP production in GBM cell lines (11). This was though to occur by decreased proliferation, cell cycle arrest, autophagy, and apoptotic cell death in vitro in part via AMPK Inhibition and decreasing mTOR activation that was dependent on PTEN status (9). Thus, replicating these results on additional cell lines without the use of other adjuvant therapy is warranted to investigate the potential therapeutic benefits of Metformin.

This investigation into PTEN mutated GBM cells response to metformin and Chlorpromazine may offer additional information about the plausibility of using a targeted treatment approach with these agents. Chlorpromazine and metformin were chosen as potential pharmacologic agents based on strict criteria including blood brain barrier penetration and as well as a demonstrative efficacy on targeting the specific genetic alteration expressed by the respective patient tumor xenograft within the PTEN pathway (13) (14). Thus we hypothesize that PTEN mutated cells lines will be sensitive to these agents when compared to PTEN wild type cell lines. Through a cell culture model, we hope to quantify pharmacological response

in cell types with different PTEN status including null, wild type, and other mutated PTEN cell types. Thus, the data collected and analyzed with regards to cell susceptibility may lead to a better understanding of PTEN's role in certain tumor types and potentially allow for further personalized treatment that is catered to molecular classification of a patient tumors. Ultimately improving treatment outcomes in GBM patients with mutated PTEN status.

This experiment will consist of treatment of differing PTEN status cell types at incremental concentration of Metformin, Chlorpromazine and LY29004. Metformin and Chlorpromazine will be compared to the control agent Ly29004 which is a known PI3 inhibitor within the PTEN/Akt pathway (15). Results will be compared by quantification of SiRNA function using cell-titer glo assay representing cell viability. Additionally, Metabolic production of P-S6 which is a downstream product of PTEN/IP3 pathway will be detected in treated cells using Western-Blot Immuno-assay for agents. Thus we will investigate mechanisms behind the cytotoxic effects of Chlorpromazine and Metformin to delineate the molecular mechanisms involved in these agents suspected cytotoxic effects.

Methodological approach

Cell types involved

Xenograft cell lines that express unique genetic alteration that is exhibited in four actual patient tumors have been obtained from Mayo Clinic and have been genetically verified by the Translational Genomics Research Institute. These cell types include A172, with wild type PTEN status and three mutated cell lines SF767, U87, and T98G. Cell lines used is this study are immortalized cell types that have established protocol within cell culture models.

Plating cell lines and treatment

To increase statistical significance, two trails of plating and testing of cell viability were performed and averages of results were in then graphed. The cell lines were plated in Phenol red-free complete DMEM:F12 media in 96 white bottom well plates at a concentration of 5,000 cell/well in 80uL. Pharmaceutical dilutions were prepared from 49.7mg Metformin and resuspended in 3mL of PBS. (100mM), 100mM Chlorpromazine and 10mM LY29004 drugs as stock solutions. Diluted drugs were then placed in 12-well reservoir and added 20uL/well on each plate. Tables below outline the tested concentrations at each dosages. These concentrations were chosen based on pharmacological based dosages corresponding to estimate *en vivo* dosages. (see tables 1-3).

Quantitation of viable cells using Cell titer Glo reagent

To establish ID50's of the of the agents used or to assess toxicity of siRNA delivery protocols, a microplate assay will be used to count viable cells. Five to ten thousand cells will be seeded on a 96-well plate then Cell Titer Glo reagent was added to each plate and incubated for 20 min (5 min of a slow shaker prior to obtaining results.

Chlorpromazine

DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only

Table 1.1 Dosages for chlorpromazine

Metformin

DMSO	DMSO	20mM	10mM	2mM	1mM	0.2mM	0.1mM	20uM	10uM	20uM MG132	Media Only
DMSO	DMSO	20mM	10mM	2mM	1mM	0.2mM	0.1mM	20uM	10uM	20uM MG132	Media Only
DMSO	DMSO	20mM	10mM	2mM	1mM	0.2mM	0.1mM	20uM	10uM	20uM MG132	Media Only
DMSO	DMSO	20mM	10mM	2mM	1mM	0.2mM	0.1mM	20uM	10uM	20uM MG132	Media Only
DMSO	DMSO	20mM	10mM	2mM	1mM	0.2mM	0.1mM	20uM	10uM	20uM MG132	Media Only
DMSO	DMSO	20mM	10mM	2mM	1mM	0.2mM	0.1mM	20uM	10uM	20uM MG132	Media Only
DMSO	DMSO	20mM	10mM	2mM	1mM	0.2mM	0.1mM	20uM	10uM	20uM MG132	Media Only
DMSO	DMSO	20mM	10mM	2mM	1mM	0.2mM	0.1mM	20uM	10uM	20uM MG132	Media Only

Table 1.2 Dosages metformin

Ly29004

DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only
DMSO	DMSO	50uM	25uM	5uM	2.5uM	0.5uM	0.25uM	50nM	25nM	20uM MG132	Media Only

Table 1.3 Dosages for control agent Ly29004

Results

Our results show inconsistencies in cell susceptibility to agents of chlorpromazine and metformin in PTEN mutated cell lines when compared to PTEN wild type cell lines. As seen in Figure 1.1, Chlorpromazine showed no difference in Cell susceptibility to PTEN wild type (A172) compared to mutated cell lines. However, Metformin did appear to spare PTEN wild type cell lines and appeared to reduce cell susceptibility in PTEN mutated SF767 and U87 cell lines while sparing PTEN mutated T98G cell lines (see figure 1.2). Cell response to control agent Ly29004, a known PI3 inhibitor shows susceptibility to of all PTEN mutant cell types and sparing A172 wild type cell line.

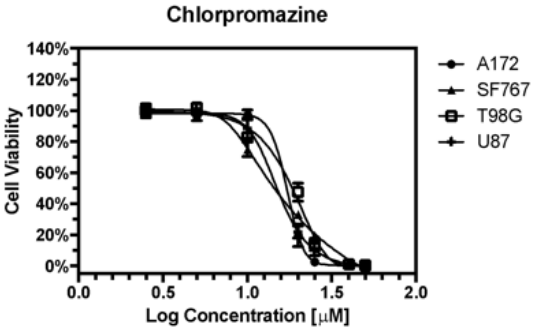

Figure 1.1 Quantitative analysis of cell viability using cell titer-glo assay for chlorpormazine

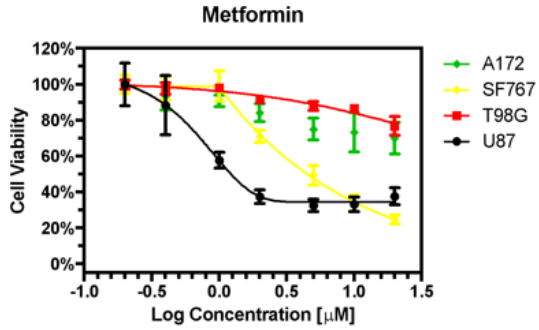

Figure 1.2 Quantitative analysis of cell viability using cell titer-glo assay for control metformin

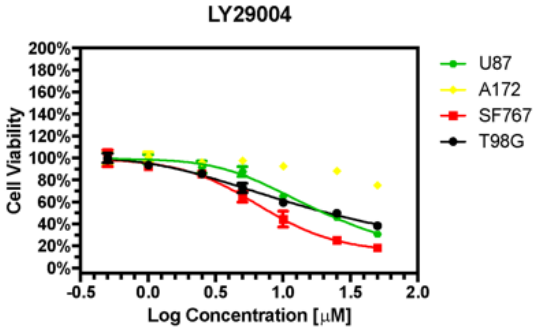

Figure 1.3 Quantitative analysis of cell viability using cell titer-glo assay for control agent Ly29004

Western blot analysis for varying concentrations of chlorpromazine shows decreased S6 protein production with the concentration of other upstream cell signaling proteins such as AKT and MTOR remaining constant in wild type PTEN status cell lines, see figure 2.1. In comparison to the westernblot of metformin, which shows not only decreased s6 protein but also akt appears to be mildly decreased. See figure 2.1.

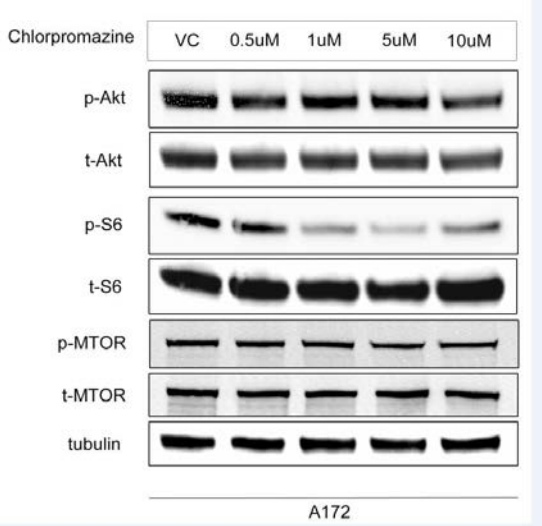

Figure 2.1 western blot for chlorpromazine

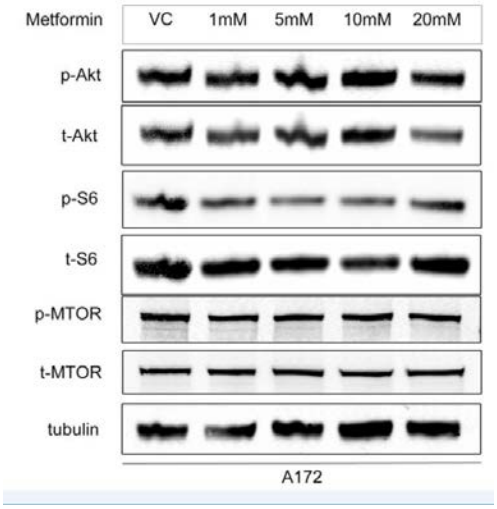

Figure 2.2 Western blot for metformin

Discussion

This is an exciting time in neuro-oncology. Discoveries elucidating the molecular mechanisms of oncogenesis and the molecular subtypes of glioblastoma multiforme (GBM) have led to new diagnostic and classification schemes with more prognostic power than histology alone. Molecularly profiling is commonly done. Despite the frequent use of tumor genetic classifications and early attempts at personalized treatment regimens, benefits to actual patient has been slow. However, there is much development in the understanding of distinct tumor genomic and feasible protocols into multi drug treatment regimens which are currently in their infancy. During this experiment we hoped to be investigated the potential actionable targets in PTEN mutated cell with repurpose drugs Metformin and Chlorpromazine for the potential use in selected patient populations. Our results show a no significant different in response rate of different cell types when treated with Chlorpromazine and only partial differences to Metformin in mutated PTEN status cell lines. Specifically, with Metformin certain cell types such as T98G did not appear to be effect while PTEN mutated cell line such U87appeared to have a dose specific response. Thus there is limited evidence to suggest that targeted blockade is independent to PTEN status with the Chlorpromazine. Likely the toxicity with Chlorpromazine occurs for reasons that have already been postulated such as mitochondrial Cytochrome C oxidase.

When analyzing the experimental results of metformin, we visualized some targeted response to PTEN mutated vs PTEN wild type cell lines suggesting a specific response to PTEN status as noted above. The potential incomplete response may be secondary to heterogenous nature of mutations within the pathways regulated by tumor suppressor protein PTEN possibly specific to IP3/AKT- MTOR pathway in the different cell lines. However, our data is comparable with another cell culture study showing that different PTEN status Glioblastoma cell lines treat withmetformin exhibiting a dosage response with augmented treat with the TMZ and radiotherapy in vitro (11). There is however the possibility that metformin cellular toxicity effects is independent of the PTEN pathway.

Limitations of this study include the treatment model being limited to only cell culture investigations. Thus, the response to tumor heterogeneity was not assessed. Also, although PTEN status was known of each cell line, there was limited control over other cellular mutations within tested cell lines thus the presence of other significant mutations involved with the PTEN pathway cannot be excluded. Additionally, measured controls for other cellular toxicity involving other mechanisms including cytochrome C toxicity was not controlled for. This means that the net effect of metformin and chlorpromazine could have been a response of other involvement outside of the PTEN pathway. Given these limitations additional studies into the response and mechanism of Chlorpromazine and Metformin are warranted.

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