Tetra-o-methyl nordihydroguaiaretic acid and Temozolomide work synergistically on Glioblastoma Multiforme tumors

By Nusaiba Baker

A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science

Baltimore, MD
April 2014
Abstract

Glioblastoma multiforme (GBM) is a grade IV astrocytoma and one of the most malignant brain tumors. GBM responds poorly to the current treatment methods, temozolomide (TMZ) and radiation. Tetra-O-methyl-nordihydroguaiaretic acid (M4N), a global transcription repressor of genes, is potent when used on GBM cell lines. Here, we investigate the effects of M4N in combination with TMZ on GBM cell lines. Cell proliferation measurements and drug combination analyses using different schedules of administration were performed on GBM cell lines. We found that M4N worked synergistically with TMZ to decrease tumor cell proliferation. M4N also induced apoptotic cell death, and our results suggest future clinical applications of M4N in combination with TMZ for GBM treatment.

Thesis readers and advisors: Dr. Ru Chih Huang, Dr. Robert Horner
# Table of Contents

Abstract ..................................................................................................................... ii

Introduction ............................................................................................................... 1

Materials and Methods .......................................................................................... 7

Reagents .................................................................................................................. 7

Results ..................................................................................................................... 10

Discussion .............................................................................................................. 20

Acknowledgements ............................................................................................... 23

References .............................................................................................................. 24

Curriculum Vitae ................................................................................................. 28
Introduction

Tetra-O-methyl norhydroguaiaretic acid (M4N) is a semi-synthetic derivative of nordihydroguaiaretic acid (NDGA), isolated from the creosote bush Larrea tridentate, found in the deserts of Mexico and south-western United States. NDGA derivatives have been shown to inhibit the production of human immunodeficiency virus (Gnabre, 1995), Herpes simplex virus (Chen, 1998), and human papillomavirus (Craigo, 2000) by interfering with the activity of the Sp1 transcription factor. (Grossman, 2012) M4N is hypothesized to induce reversible G2 cell-cycle arrest in mammalian cells without cytotoxicity and exhibit anti-tumor activities in vivo. (Heller, 2001) It is also known to be selectively tumoricidal in animal cancer models. M4N has been shown to induce growth arrest in tumor cells and when injected intratumorally into tumors, exhibits tumoricidal activity. (Park, 2005)

M4N has been reported to arrest the proliferation of several tumor cell lines in culture as well as in a C3 cell-induced mouse tumor model system. (Huang, 2006) Similarly, M4N was shown to inhibit growth of both murine and human melanomas and human colon cancer in vivo. (Lambert, 2001) Due to its bioavailability, M4N may be administered intratumorally, systemically, or as an oral chemotherapeutic. M4N may thus be a viable treatment option for more aggressive tumors.
Glioblastoma multiforme (GBM) is the most common primary tumor of the central nervous system (Adamson, 2009) in adults and a devastating disease, with life expectancy following diagnosis being a year or less. In the United States alone, there are approximately 10,000 new cases of GBM per year. (Wen, 2008) GBM is classified as a grade IV astrocytoma (Turnher, 2007) and is characterized by particularly low prognosis, tumor recurrence, and resistance to therapy (Walker, 1978). Recent successes have led to advances in treatment of select patients with GBM that have resulted in life expectancies exceeding 21 months. (Hegi, 2005) The current methods of treatment for GBM include a combination of surgery, radiation, and chemotherapy using Temozolomide (TMZ), which have increased the median survival from 12.1 months using radiotherapy alone to 14.6 months. (Stupp, 2005) Given the increased life expectancy, studies have begun to focus on combinatorial treatments as the ideal method for treating GBM. Optimizing currently available treatment options and investigating other regimens is necessary to further improve treatment for GBM.

TMZ, a DNA-alkylating agent, is an orally administered drug developed in the 1980s, and is widely used in the treatment of high-grade gliomas. Treatment with this drug reveals good bioavailability with oral administration, good tissue penetration, and minimal side effects. (Leschenko, 2013) TMZ has been used in brain tumor therapeutics, including gliomas, astrocytomas, cutaneous T-cell lymphoma, and acute myeloid lymphoma. Although progress has been made in treatment of these high-grade glioma with surgery and radiation, tumor recurrence and resistance to therapy
continues to occur. Developing novel treatment methods with improved efficacy and lower variability across patients is critical to increasing the patient survival.

Recent studies have shown that combining TMZ with other agents has suggested that in order to enhance efficacy, it is crucial to apply drug combinatorial therapies in treatment of such an aggressive tumor. (Yang, 2014) This way, synergistic effects between the drugs will cause enhanced results and work more efficiently than single drugs alone. In addition, the intention to explore molecular differences across multiple GBM-derived cell lines and understand the differences by use of transcriptional biomarkers will create better knowledge of the disease mechanisms. New therapeutic strategies will provide insight into new experimental methods to initiate tumor regression and prevent relapse after treatment.

Recently, it has been shown that M4N and TMZ may exhibit combinatorial effects by reducing GBM tumor cell viability in vitro. (Castro-Gamero, 2013) We were interested in identifying whether the two drugs work together to produce synergistic effects. We investigated the variable effects of the combinatorial treatment consisting of M4N and TMZ on different cell lines. Drug-drug interactions were quantified for the combination of M4N and TMZ in U87MG and LN-229 cell lines. Using this pair of drugs, strong interactions between the drug combination and each cell line were defined.
Materials and Methods

Cell culture

The human glioblastoma cell lines LN229 were obtained from American Type Culture Collection, USA. The cells were maintained in Dulbecco’s minimal essential medium (DMEM) (Gibco, USA), supplemented with penicillin (100 units/mL) and 10% head-inactivated fetal bovine serum (FBS) at 37°C under 5% CO2 in a humidified chamber.

The human glioblastoma cell lines U87MG were maintained in DMEM supplemented with 10% Febtal Bovine Serum and Penicillin (50 U/ml) at 5% CO2 in a humidified incubator at 37°C.

Cell Viability Assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT (Santa Cruz Biotechnology), assay of attached cells. LN-229 and U87MG cells were seeded at 1x10^4 cells/ml in a 24 well microplate. After attachment, cells were treated with serial doses of M4N alone, TMZ alone, or both for 24 hours. MTT assay was performed at the end of the treatment period according to the manufacturer’s protocol. The resulting product was measured using multiplate reader at 450 nm. Cell viability was calculated relative to untreated cells.
**Combination drug analysis**

CalcuSyn software was used to quantify the interaction between simultaneous treatment between M4N and TMZ on LN-229 and U87 cells. Treatments of 24 hours were used for simultaneous exposure of the two drugs. Combination indexes (CIs) were generated and analyzed for each cell line. This data was analyzed for synergistic (CI<1), additive (CI=1), or antagonistic effects (CI<1) using the Chou and Talalay equation. (Chou TC, Talalay P; 1984) The IC$_{50}$ value, at which the drug concentration inhibits cell proliferation by 50%, was calculated using CalcuSyn software. This allowed for analysis of the interaction between M4N and TMZ.

**Reagents**

Tetra-O-methyl nordihydroguaiaretic acid (M4N) was obtained from Dr. Ru Chih Huang and dissolved in dimethylsulfoxide (DMSO) at 50 mM. Temozolomide (TMZ) was also obtained from Dr. Ru Chih Huang and was also diluted in DMSO at a stock concentration of 200 mM.

**Western Blotting**

LN-229 and U87 cells were cultured onto 100 mm plates and cultured for 24 hours. After this time, M4N and TMZ were added alone or in combination to LN-229 and U87 cells. Two plates did not receive treatment by either M4N or TMZ and were used as controls. Once confluency was reached, the cells were washed
with PBS and harvested with 10 mmol/L EDTA. The washed cells were pelleted and resuspended in RIPA buffer containing a protease inhibitor cocktail. The lysate was cleared by centrifugation and protein concentrations were determined using a BSA standard curve. The absorbances of each protein were read at 595 nm. 20 micrograms of protein were separated on a 10% SDS-PAGE gel. This was electroblotted to an enhanced chemiluminescence nitrocellulose membrane using a semidry electroblot apparatus. Primary rabbit polyclonal antibodies against Myc (Cell Signaling Technology) and primary mouse polyclonal antibody against actin (Cell Signaling Biotechnology) were used at a final concentration of 0.2 ug/ml. Secondary antibodies were anti-rabbit or anti-mouse immunoglobulin G conjugated to horseradish peroxidase. The chemiluminescence filters were placed against X-ray film for detection of protein bands.
Results

M4N inhibits MYC expression in prostate cancer, but not in GBM

To investigate the response of molecules involved in cell proliferation to M4N treatment, we focused on c-Myc signaling. A prostate cancer cell line, LNCaP, and the glioblastoma multiforme cell line, LN-229, were treated with various concentrations of M4N after 24 and 72 hours. (Figures 1 and 2). Our results show decreasing levels of Myc expression with increasing levels of M4N in the prostate cancer cell line after 24 and 72 hours. Myc levels in LN-229, however, remain unchanged in response to treatment. This would suggest that M4N does not affect the same pathway or upstream effectors of Myc in the prostate cancer cell line and the glioblastoma cell line.
Figure 1. LNCap, prostate cancer cell line, and LN-229, glioblastoma cell line treated with 0, 30, 60 uM M4N after 24 hours

<table>
<thead>
<tr>
<th>LnCaP</th>
<th>0 uM</th>
<th>30 uM</th>
<th>60 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN229</td>
<td>0 uM</td>
<td>30 uM</td>
<td>60 uM</td>
</tr>
</tbody>
</table>

Figure 2. LNCap, prostate cancer cell line and LN-229, Glioblastoma cell line, treated with 0, 30, 60 uM M4N after 72 hours

<table>
<thead>
<tr>
<th>LnCaP</th>
<th>0 uM</th>
<th>30 uM</th>
<th>60 uM</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN229</td>
<td>0 uM</td>
<td>30 uM</td>
<td>60 uM</td>
</tr>
</tbody>
</table>
M4N inhibited GBM cell line proliferation

The GBM cell lines U87 and LN-229 were treated with M4N, TMZ, and the combined treatment of M4N with TMZ at increasing concentrations. After 24 hours, the cells were tested for cell viability (Figure 3,4). Inhibition of cell proliferation was observed in a dose- and time-dependent manner in both U87 and LN-229 cell lines (Fig 5). Our results demonstrate that at higher concentrations, M4N and TMZ resulted in more tumor cell death than M4N or TMZ alone. Interestingly, at lower drug concentrations, there appeared to be a spike in cell growth. This may be attributed to slow action of the two drugs or a prolonged internal molecular response to treatment.
Table 1. Cell Viability after treatment with M4N, TMZ, and M4N + TMZ in LN-229 cells

<table>
<thead>
<tr>
<th>[Drug] (uM)</th>
<th>Relative cell viability after M4N treatment</th>
<th>Relative cell viability after TMZ treatment</th>
<th>Relative cell viability after M4N + TMZ treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>1.25</td>
<td>0.994</td>
<td>0.962</td>
<td>1.097</td>
</tr>
<tr>
<td>2.5</td>
<td>0.953</td>
<td>0.942</td>
<td>1.200</td>
</tr>
<tr>
<td>5</td>
<td>0.870</td>
<td>0.995</td>
<td>1.161</td>
</tr>
<tr>
<td>10</td>
<td>0.931</td>
<td>1.154</td>
<td>1.083</td>
</tr>
<tr>
<td>20</td>
<td>0.876</td>
<td>1.038</td>
<td>0.719</td>
</tr>
<tr>
<td>40</td>
<td>0.875</td>
<td>1.026</td>
<td>0.560</td>
</tr>
<tr>
<td>80</td>
<td>0.854</td>
<td>0.914</td>
<td>0.547</td>
</tr>
</tbody>
</table>

LN-229 cells treated with M4N Only, TMZ Only, or M4N and TMZ at various concentrations. Cell viability is measured relative to viable cells in control sample treated with no drug.
Figure 3. Cell viability assay of LN-229 Human glioblastoma cells

Cell viability assay of LN-229 cells treated with M4N Only, TMZ Only, or M4N and TMZ at various concentrations
Table 2. Cell Viability after treatment with M4N, TMZ, and M4N + TMZ in U87 cells

<table>
<thead>
<tr>
<th>[Drug] (µM)</th>
<th>M4N</th>
<th>TMZ</th>
<th>M4N + TMZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>1.25</td>
<td>0.896</td>
<td>1.026</td>
<td>0.942</td>
</tr>
<tr>
<td>2.5</td>
<td>0.878</td>
<td>0.971</td>
<td>0.901</td>
</tr>
<tr>
<td>5</td>
<td>1.235</td>
<td>1.016</td>
<td>1.346</td>
</tr>
<tr>
<td>10</td>
<td>1.081</td>
<td>1.111</td>
<td>1.095</td>
</tr>
<tr>
<td>20</td>
<td>0.726</td>
<td>1.058</td>
<td>0.683</td>
</tr>
<tr>
<td>40</td>
<td>0.709</td>
<td>0.930</td>
<td>0.566</td>
</tr>
<tr>
<td>80</td>
<td>0.756</td>
<td>0.965</td>
<td>0.534</td>
</tr>
</tbody>
</table>

U87MG cells treated with M4N Only, TMZ Only, or M4N and TMZ at various drug concentrations. Cell viability is measured relative to viable cells in control sample treated with no drug.
Figure 4. Cell viability assay of U87MG Human glioblastoma cells

Cell viability assay of U87MG cells treated with M4N Only, TMZ Only, or M4N and TMZ at various concentrations
Figure 5. Dose-effect curves for M4N, TMZ and their combinations in LN-229 and U87 cells.

A. M4N alone, TMZ alone, and M4N and TMZ (M + T) in LN-229 cells

B. M4N alone, TMZ alone, and M + T in U87 cells

The x-axis represents the drug dosage in umol/L and the y-axis represents Fa, fraction of cells affected (growth inhibition).
**M4N and TMZ work synergistically in GBM cells**

Simultaneous exposure to both M4N and TMZ for 24 hours showed synergism between the two drugs in both U87 and LN-229 cell lines (Figures 3 and 4). These data suggest that M4N and TMZ combined caused a synergistic effect in GBM cells. These results were confirmed by CompuSyn analysis. (Table 3)

The median effect analysis of Chou and Talalay (Chou and Talalay, 1984) was also used to calculate the CI for each drug combination. In the U87 cell line, the M4N and TMZ combination was strongly synergistic across the entire range of doses (CI < 1). The LN-229 cell line was also very synergistic (CI < 1), except for the ED95 value (CI > 1).

The DRI determines the fold dose-reduction allowed for each drug in synergistic combinations. This allows for dose-reduction results that cause reduced toxicity while maintaining the ideal efficacy. (Chang, 2006) The DRI exhibited a dose reduction in the LN-229 cells for M4N and TMZ treatment and exhibited a large dose-reduction for M4N and TMZ in U87 cells as a result of their synergism. The DRI indicated that the concentration of M4N necessary to inhibit the growth of LN-229 cells by 75% (ED$_{75}$) could be decreased by 6.11-fold and that at the ED$_{50}$, it could be reduced by 15.44-fold. Similarly, the ED$_{75}$ of TMZ in U87 cells could be decreased by 5.21 x 10$^7$-fold.
Table 3. Dose-effect relationships of M4N, TMZ, and M4N in combination to TMZ in human glioblastoma cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Drugs</th>
<th>CI(^1) (combination index)</th>
<th>DRI(^2) (fold dose reduction) value at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ED50</td>
<td>ED75</td>
</tr>
<tr>
<td>LN-229</td>
<td>M4N</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TMZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M4N + TMZ</td>
<td>0.12918</td>
<td>0.32658</td>
</tr>
<tr>
<td>U87</td>
<td>M4N</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TMZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M4N + TMZ</td>
<td>0.04579</td>
<td>0.13047</td>
</tr>
</tbody>
</table>

\(^1\) CI is the combination index (CI<1 indicating synergism, CI=1 indicates additive effect, and CI>1 indicates antagonism)

\(^2\) DRI dose reduction index (shows the fold dose reduction by the doses needed to achieve a specific degree of inhibition when using the drug as a single agent and in combination.)
Discussion

Glioblastoma multiforme (GBM) is an aggressive brain tumor with patient life expectancy after diagnosing being around 12-14 months. Treatment options are limited for GBM and the development of new therapies is crucial to enhancing the life span of patients. (Khasraw, 2010) In this study, we report the effects of M4N, a transcriptional inhibitor of Sp1-dependent genes, on GBM in combination with temozolomide, the current treatment for GBM.

We analyzed the effects of M4N on two different cell lines, the human prostate cancer cell line, LnCaP, and the human glioblastoma cell line, LN-229. Myc is an important factor in cell proliferation that enables G₁/S cell cycle progression. (Steiner, 1995) Thus, we investigated the levels of Myc in both LN-229 and LnCaP after 24 hours of treatment with M4N. Interestingly, Myc protein expression levels in LN-229 remained the same with increasing concentrations of M4N. Myc expression levels in LnCaP were found to decrease within 24 hours of M4N treatment. This data may be explained by differing internal responses to the drug, or a more prolonged effect in LN-229 cell lines.

In order to study the efficacy of M4N on the aggressive GBM cell lines, we investigated the role of two drugs, M4N and TMZ, on GBM. We performed cell viability assays using the LN-229 and U87MG human glioblastoma cell lines. We
observed that, while M4N and TMZ alone had tumoricidal effects, the simultaneous combination of M4N and TMZ exhibited significant synergistic effects, resulting in greater cell death in both LN-229 and U87 samples with the concomitant treatment. This was confirmed using CompuSyn analysis, where we found the CI (combination index) values of the U87 cell lines all below 1 (CI<1) using median-effect analysis. The combination results obtained allowed for the demonstration that M4N has potent chemo-sensitizing effects when used in combination with TMZ.

Interestingly, the combination of M4N and TMZ significantly increased the sensitivity of both GBM cell lines used compared to drug treatment alone and significantly increased inhibition of cell proliferation. This data confirms that M4N and TMZ work synergistically on the LN-229 and U87 GBM cell lines in vitro.

In conclusion, this study showed that M4N and TMZ work synergistically to induce growth arrest and apoptosis of GBM cells. The drug combination analysis supported the potential of M4N as a chemosensitizing agent in GBM. This suggests that future understanding of the M4N mechanisms of action may be of clinical use in GBM therapy. Thus, we propose that M4N may be a promising tool for GBM therapy and for enhancing the efficacy of conventional treatment modalities for GBM.
GBM has proven difficult to treat using conventional methods. It is of utmost necessity to understand the reasons behind tumor resistance to therapy and recurrence. Our study is crucial in identifying a novel potential concomitant treatment for this aggressive tumor. Recently, studies have shown that methylation of the O6-methylguanine-DNA methyltransferase (MGMT) gene is associated with good response to chemoradiation therapy. (Lalezari, 2013) We are interested in furthering our studies by conducting methylation-specific PCR in order to understand methylation levels of MGMT activity and how they contribute to resistance to M4N and TMZ treatment. Ultimately, identifying novel treatments and developing biomarkers will be paradigm-shifting in treating GBM.
Acknowledgements

I would like to extend my thanks and gratitude to my advisor and PI, Dr. Ru Chih Huang, for her incredible guidance and for providing me the lab space that made this thesis possible.

In addition, thank you to the other members of the Huang lab, who taught me so much and helped me whenever I needed it: Dr. David Mold, Dr. Ibrahim Abd-Elazem, Dr. Jong-Ho Chun, Tiffany, and Christine.

Lastly, I thank my family and friends for being so compassionate and understanding throughout this year.
References


5. Adamson C, Kanu OO, Mehta AI, Di C, Lin N, Mattox AK, Bigner DD (2009) Glioblastoma multiforme: a review of where we have been and where we are going. Expert Opin Investig Drugs 2009;19:1061-1083


**Curriculum Vitae**

Nusaiba Baker grew up in Fresno, California and attended Bullard high school. After graduating in three years, she attended Johns Hopkins University in Baltimore, MD, where she majored in Neuroscience and Molecular and Cellular Biology. She is currently in the BS/MS program in the Biology department at Johns Hopkins University and will likely be attending the Emory MD/PhD program next year in Atlanta, Georgia.