JOVANNI AHMAD
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EDUCATION

MS University of Maryland, Baltimore, CMBS May 2021
Resident GPA: 3.83

Relevant Coursework: Mechanisms in Biomedical Sciences, Current Topics in Vascular and Stem Cell Biology, Fundamentals of Biostatistics, Cell and Systems Physiology, Master's Thesis Research, Cancer Biology: From Basic Research to the Clinic, Muscle Cell Biology and Development, Master's Thesis Research

BS Centre College, Biochemistry and Molecular Biology May 2019
Minor in Spanish
Studied Abroad August 2017-December 2017
Resident GPA: 3.03

Relevant Coursework: Introduction to Genetics, Introduction to Biochemistry and Cell Biology, Organic Chemistry I, Organic Chemistry II, Macromolecules, Biochemistry Lab Techniques, Cellular Metabolism, Molecular Genetics, Cell Biology, Senior Seminar, Lab in Molecular Genetics, Histology, Introduction to Physics, Introduction to Physics for the Life Sciences

HONORS AND AWARDS

2020 MS Scholar Award, University of Maryland, Baltimore October 2020
Greek Leader of the Year, Centre College Greek Life Office April 2018

LABORATORY AND TEACHING EXPERIENCE

Research Assistant, Dr. Charles Hong Laboratory June 2019 to May 2021
Department of Cardiovascular Research**,** University of Maryland, Baltimore, Baltimore, MD

Identified potential mechanisms for the sensitization of temozolomide in glioblastoma multiforme using a novel GPR68 inhibitor via various genetic and protein techniques. Conducted data analysis that determined differences in morphology and contractility of iPSC-derived cardiomyocytes using different protein concentrations in basement membrane mediums. Contributed figures, statistics, and methods to upcoming publication. Independently developed and optimized protein purification protocols for producing in-house iPSC growth medium. This medium will save significant resources for the lab if successful.

Laboratory Teaching Assistant, Dr. Stephanie Dew Jan. 2017-May 2017 & Jan. 2019-May 2019
Centre College, Danville, KY

Answered questions and guided students through lab protocols in Introduction to Biochemistry and Cell Biology Lab. Administered lab assessments to students. Cleaned and prepared benches with necessary reagents for next lab section.

PUBLICATIONS AND POSTERS

Ahmad, J. D., Keyser, B. D., Hong, C. C., & Williams, C. H. Inhibition of GPR68 Sensitizes GBM to Temozolomide Treatment via the NFκ-B Pathway. University of Maryland, Baltimore 43rd Graduate Research Conference; 26 Mar 2021; Baltimore, MD

WORK EXPERIENCE

Senior Certified Pharmacy Technician August 2013 to Present
Walgreens Boots Alliance, Baltimore, MD
(Certified November 2015; received senior status August 2017)
Previous position: Customer Service Associate (August 2013-March 2014).

Input patient information and prescription data into IC+ operating system. Assist pharmacists with compounding and dispensing medications. Train and mentor incoming pharmacy technicians and pharmacy interns. Manage inventory of non-controlled and controlled substances. Consult insurance companies for rejections, certificates of medical necessity, and prior authorizations. Contact patients regarding MTM of their maintenance medications. Maintain Yuyama Automatic Vial Filling Machine. Register patients into EHR and administer the COVID-19 vaccine.

Clinic Intern, Orthopedics and Oncology August 2017 to December 2017
Onkort Centro Médico y Cirugía Avanzada, Merida, YUC, MX

Observed Dr. Herrera and Dr. Reyes perform various orthopedic and oncological procedures such as knee replacements and tumor removals. Studied procedures and medical terminology in English and Spanish. Participated in mild orthopedic procedure involving removal of contaminated intramedullary rod.

PrEP Organizing Intern, Public Policy May 2018 to Aug 2018
Planned Parenthood of Indiana and Northern Kentucky, Louisville, KY

Connected and coordinated with volunteers and supporters during PPINK related events. Researched HIV/AIDS statistics and demographics in the state of Kentucky with focus on the Louisville Metro Commonwealth and Lexington. Worked closely with staff members and other interns to staff outreach events. Promoted advocacy campaigns to patients at health center. Collaborated with the Kentucky Department of Health in identifying different health fair events across the state.

TECHNICAL SKILLS

GraphPad Prism 8, ImageJ, Microsoft Office Software, Adobe Photoshop

LICENSES AND CERTIFICATIONS

Certified Pharmacy Technician Certificate, Pharmacy Technician Certification Board
License Number: 10099159, Exp: 12/31/21

Pharmacy Technician License, Maryland Board of Pharmacy
License Number: T22648, Exp: 08/31/21

Pharmacy Technician License, Kentucky Board of Pharmacy
License Number: PT00077774, Exp: 03/31/22

Basic Life Support, American Red Cross
Certificate ID: 00HNAU2, Exp: 02/12/23

Certified Immunizing Pharmacy Technician, American Pharmacists Association
Exp: 02/09/2024

LEADERSHIP POSITIONS

Student Representative August 2020-Present
University of Maryland, Baltimore Student Advisory Committee, Baltimore, MD

Participate in monthly student advisory committee meetings. Advise GPILS leadership on all matters relating to graduate student enrichment on campus. Plan events for graduate students such as community outreach and volunteer work.

President April 2018-April 2019
Centre College Interfraternity Council, Danville, KY

Oversaw operations of the Interfraternity Council. Coordinated with Greek Life Office and chapter presidents regarding Greek Life related affairs. Served on social event approval committee with other members of IFC and the Panhellenic Council. Facilitated conversations and changes regarding social policy amendments to ensure that IFC and its respective chapters were in compliance with college and national guidelines.

Program Director January 2018-January 2019
Centre College After School Program, Danville, KY

Previously served as Tutor (January 2016-May 2017), Marketing Coordinator (September 2016-December 2016), School Coordinator (January 2017-May 2017). Oversaw operations of Centre College After School Program. Managed 96 volunteers and 76 students who participated in the program. Maintained budgeting and finances across general account and scholarship fund. Coordinated with Centre College and other 3rd party affiliates regarding funding and program development.

Philanthropy and Service Chairman January 2018-January 2019
Phi Kappa Tau - Delta Chapter, Danville, KY

Created and executed philanthropy events in various capacities with other Greek and non-Greek organizations on campus. Coordinated with community partners in the Danville/Boyle Community to develop community service events and promote service efforts in the Greek Life community. Total money raised for charities and organizations of 01/04/2019: $5327

Summer Intern May 2017-August 2017
Centre College Bonner Scholars Program, Danville, KY

Organized and facilitated annual fall retreat that focused on immigration. Prepared and facilitated New Bonner Orientation. Conducted community partner surveying to expand community outreach efforts. Organized a breakfast networking and appreciation event for our local community partners.

Vice President of Risk Management April 2016-April 2017
Phi Kappa Tau - Delta Chapter, Danville, KY

Worked with college administration of Centre College to promote safety during social events hosted by the fraternity on and off campus. Networked with Centre College and the Danville/Boyle County community to develop programs regarding topics such as sexual abuse, fire prevention and safety, drug and alcohol abuse, etc. with qualified presenters (Fire Marshall, Department of Public Safety, and Danville Police Department).

COMMUNITY SERVICE

Bonner Scholar August 2015-May 2019
Centre College Bonner Scholar Program, Danville, KY

Completed 10 hours of community service per academic week. Traveled to different colleges and universities to meet with other students and discuss topics such as poverty and improving education through active community service and civic engagement. Total accumulated service hours as of 05/19/2019: 1502 hours.

Canine-Human Acclimation August 2015-May 2016
Danville-Boyle County Humane Society, Danville, KY

Worked with rescued canines in human interaction to prepare them for adoption.

Activities Facilitator August 2014-May 2015
Kentucky Science Center, Louisville, KY

Prepared and facilitated group activities for kids during school field trips

Garden Maintenance August 2013-May 2014
Kentucky Americana Center, Louisville, KY

Removed weeds and other invasive species in the local garden

Warehouse Organizer August 2012-May 2013
Kentucky Refugee Ministries, Louisville, KY

Sorted through donated items and prepared move-in kits for refugee families

LANGUAGES

English: Native Language
Spanish: Advanced Reading, Writing and Listener, Intermediate Speaker

Abstract

Glioblastoma Multiforme (GBM) remains as one of the most aggressive and lethal cancer types, often resulting in poor prognosis. Currently, Temozolomide (TMZ) is the standard chemotherapy for combating GBM. However, GBM's upregulation of O-6-Methylguanine-DNA Methyltransferase (MGMT) mitigates the alkylating effects of TMZ treatment, generating a dire new need for novel or adjuvant therapy. U138MG is a TMZ-resistant GBM cell line used for the development of new chemotherapy. Here, we investigated whether inhibition of proton sensing GPR68 would decrease MGMT expression and sensitize U138MG cells to TMZ treatment. Using various genetic, protein, and cell-based assays we determined that inhibition of GPR68 may be decreasing MGMT protein expression and sensitizing U138MG to TMZ via the Gq/NF-kB pathway. Furthermore, we identified that co-administration of TMZ and OGM resulted in a synergistic decrease in cell growth compared to OGM treatment alone.

Inhibition of GPR68 Sensitizes GBM to Temozolomide Treatment via the NF-kB Pathway

by
Jovanni D. Ahmad

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Master of Science
2021

Acknowledgements

I would like to thank Dr. Charles Hong and Dr. Charles Williams for their guidance and support throughout this project. I entered their lab with a very entry-level skill set and this project has been my first independent research project. Their knowledge and patience have allowed me to expand my understanding of research in basic science and I look forward to continuing research throughout my medical career. I would also like to thank the other members of my lab. Urmila, Brittany, Ajoke, Rebeca, and Leif, have unconditionally supported me throughout this project and answered all my questions with hesitation. Completion of this project wouldn't have been possible without their support and friendship.

I would also like to thank my core course friends, Dr. Ivy Dick, and Elice Garcia-Baca. Despite losing a year of memories due to the COVID-19 pandemic, their support was invaluable throughout my master's degree and I look forward to staying in contact with them even after leaving UMB. While there was no laboratory collaboration with these individuals, their emotional support and friendship had a huge impact in completing this project.

Lasty, I would like to thank Trong Phung who has constantly encouraged me to give my 100% effort in any project or challenge that I face. Despite my stubbornness, she continues to be my biggest supporter and always helps me see the light at the end of the tunnel no matter how long that tunnel may seem.

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List of Abbreviations

EMSA	Electrophoretic Mobility Shift Assay
GBM	Glioblastoma Multiforme
IR	Ionizing Radiation
MGMT	O-6-methylguanine-DNA methyltransferase
OGM	Ogremorphin
TMZ	Temozolomide
TPM	Transcripts per Million

Chapter I: What is Glioblastoma Multiforme?

In combating all forms of cancer, chemotherapy has been one of the primary means of treatment. Different classes of drugs have been developed to either promote apoptosis or inhibit cell proliferation in cancerous tissues while attempting to minimize damage to healthy cells. While these drugs have been effective in tackling various types of cancers, some cancer types have developed drug-resistant mechanisms through a series of pro-oncogenic mutations. These drug-resistant mechanisms make some cancer types such as glioblastoma multiforme (GBM) nearly impossible to treat (Holland, 2000; Hanif *et al*., 2017; Bahadur *et al.,* 2019)

GBM is a form of glioma that presents as an accumulation of tumors that arises from precursor cells in the central nervous system and is the most aggressive form of glioma to treat. Gliomas account for 80% of all malignant primary tumors originating from the brain and is the most commonly occurring tumor type of the CNS (Hanif *et al.*, 2017). Most patients who develop GBM often left with 14 to 15 months of life expectancy (Holland, 2000; Ohka *et al.* 2012). Although GBM is a relatively rare cancer with a global incidence of approximately 10 per 100,000 people, it accounts for 50% of gliomas across all patients with most incidents occurring between 55 to 60 years of age. Malignant tumors account for 2.5% of deaths due to cancers and is the most lethal cancer in individuals between 15 and 34 years of age (Hanif *et al.,* 2017). Unfortunately, little is known about the carcinogenic causes of gliomas. The only confirmed cause is high doses of ionizing radiation (IR) with approximately 100 cases of GBM occurring due to IR since 1960 (Salvati *et al.*, 2003).

GBM exhibits both genetic and physical characteristics that make it incredibly difficult to treat and remove. It's integration throughout the brain makes surgical resection and removal a very intricate task. Due to its highly proliferative and aggressive nature, it's nearly impossible to

completely remove the glioma. GBM is also difficult to treat through chemotherapy treatments due to the high genetic variation throughout the multiforme. Various point mutations and indels lead to activation of signal transduction pathways that promote cell proliferation and loss of function in tumor suppressor genes, notably p53 (Holland, 2000). This multiforme characteristic results in the need to constantly reassess chemotherapeutic treatments and look for novel therapies.

Recently, G-protein-coupled receptors (GPCRs) have become a new drug target to consider when treating various cancer types. One GPCR, GPR68 (OGR1), has been shown to be upregulated in various cancer types, including GBM (Hertzig *et al.*, 2019; Wiley *et al.* 2019; Zhang *et al.,* 2020). GPR68 is a proton sensing GPCR that stimulates the Gq/11 pathway and promotes cell survival. Wiley *et al.* (2019) measured GPR68 expression across numerous different cancer types via RNA-seq. Measured in transcripts per million (TPM), GPR68 expression in glioblastomas had a TPM value of 7+/-2 compared to other common cancers such as lung adenocarcinoma which only had a TPM value of 3+/-1 for GPR68 expression, making GPR68 a good candidate for drug targeting.

Chapter II: FDA-Approved Temozolomide and GBM Resistance

Currently, Temozolomide (4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo [4.3.0] nona-2,7,9-triene-9-carboxamide) (TMZ) is the only FDA-approved drug for treating GBM. TMZ is a prodrug that becomes active at physiological pH. Once active, TMZ generates a methyl group and acts as an alkylating agent to DNA bases, most notably the methylation of guanine at the O^6 position to produce the O^6-methylguanine. These cells then undergo apoptosis as the O^6-methylguanine preserves double-strand breaks during DNA mismatch repair (Zhang *et al.*, 2012). Unfortunately, due to the high genetic variation of GBM, drug resistance to TMZ occurs is incredibly common. These forms of GBM over express an enzyme known as O-6-methylguanine-DNA methyltransferase (MGMT) that counteracts the alkylating effects of TMZ. MGMT is a small enzyme that removes the methyl group from the O^6-methylguanine and is then irreversibly inactivated and proteolytic degraded. Currently, this is the most widely accepted mechanism for TMZ resistance in GBM, making it a popular drug target to combat this lethal cancer type (Zhang *et al.*, 2012; Lee *et al.*, 2017; Jiaper *et al.*, 2018).

Since the acquisition of TMZ-resistance of GBM is well known, researchers have actively looked for novel treatments and adjuvant therapies to combat temozolomide resistance. Groups such as Yuan et al. (2012), have studied resveratrol as an adjuvant therapy with GBM to study the ROS dependent mTOR signaling pathways. Their results demonstrated an increase in cell cycle arrest in the G2/M phase from 36.67% using TMZ alone to 61.62% when combining 10 μM resveratrol with TMZ in SHG44 cell line. Other groups such as Scicchitano et al. (2018) have investigated combining already FDA-approved drugs such as levetiracetam, a common antiepileptic drug, with TMZ for adjuvant therapy. These researchers demonstrated that not only did the TMZ + levetiracetam treatment decrease cell proliferation, introduction of high doses of

levetiracetam alleviated the need to have high doses of TMZ and was even more effective when lower doses of TMZ were used, mitigating the negative side effects associated with this chemotherapy.

There have been no studies regarding whether GPR68 affects MGMT expression in GBM cell lines; however, given that GBMs acidify their microenvironment to promote cell survival and proliferation, increased GPR68 activity may be a root cause of increased MGMT expression (Hielmeland *et al.,* 2011; Sanderlin *et al.,* 2015; Wiley *et al.,* 2019, Zhang *et al.,* 2020). Therefore, introducing a selective inhibitor could decrease tumor growth and survival. In a study by Williams *et al.* (2016), an unbiased screen of roughly 30,000 small molecules was conducted to assess phenotypic changed in morphology in zebrafish. 5-ethyl-5'-naphthalen-1-ylspiro[1H-indole-3,2'-3H-1,3,4-thiadiazole]-2-one, now referred to as Ogremorphin (OGM), is a small-molecule compound that induced aberrant pigmentation, ventral curvature, craniofacial defects, among other changes. Changes in the activities of 158 GPCRs were assessed in the presence of OGM8345, an analog of OGM. OGM8345 showed to be a highly specific reversible inhibitor of GPR68 and could be used to study the effects of MGMT expression in TMZ-resistant GBM cell lines (Williams *et al.*, 2019).

Chapter III: Potential Mechanism of MGMT Expression via GPR68

As previously mentioned, acidification of GBM's microenvironment promotes cell survival and proliferation through stimulation of GPR68. Given this information, we can employ our novel GPR68 inhibitor on glioma cells and conduct cell viability assays as well as spheroid size assays to gain a better physiological understanding of how OGM may be affecting these glioma cells. GPR68 is known to act through the Gq/11 pathway, therefore, it is important to determine if there is a link between the Gq/11 pathway and MGMT expression (Chandra *et al.*, 2016). One potential lead shown in figure 1 is the phosphorylation of Akt through the Gq pathway that allows NF-κB to translocate into the nucleus and upregulate its downstream targets (Caporali *et al.*, 2012; Yu *et al.*, 2019). Verified by EMSA, Lavon *et al.* (2007) demonstrated that NF-κB had two specific binding sites on the promoter region of MGMT further strengthening our hypothesis that our novel GPR68 inhibitor could decrease MGMT expression in temozolomide resistant glioma cells and decrease cell survival and proliferation. IL-8 (CXCL8), a gene known to be highly expressed in GBM, is also heavily modulated by NF-κB, therefore we can also measure its gene expression to assess the effects of OGM treatment (Guo, *et al.*, 2017; Hasan *et al.*, 2019). Here, we can utilize various Gq inhibitors as well as NF-κB inhibitors to compare decreases in MGMT expression with our novel GPR68 inhibitor.

Additionally, we can generate gene expression profiles using qRT-PCR arrays specific to cancer stem cell related genes. Given the robust number of downstream targets of the Gq/11 pathway, it is possible that there may be other genes that are dysregulated by OGM that would inhibit cell survival and proliferation. Availability of these gene expression profiles can open avenues for further investigation of GPR68's role in cancer proliferation and survival.

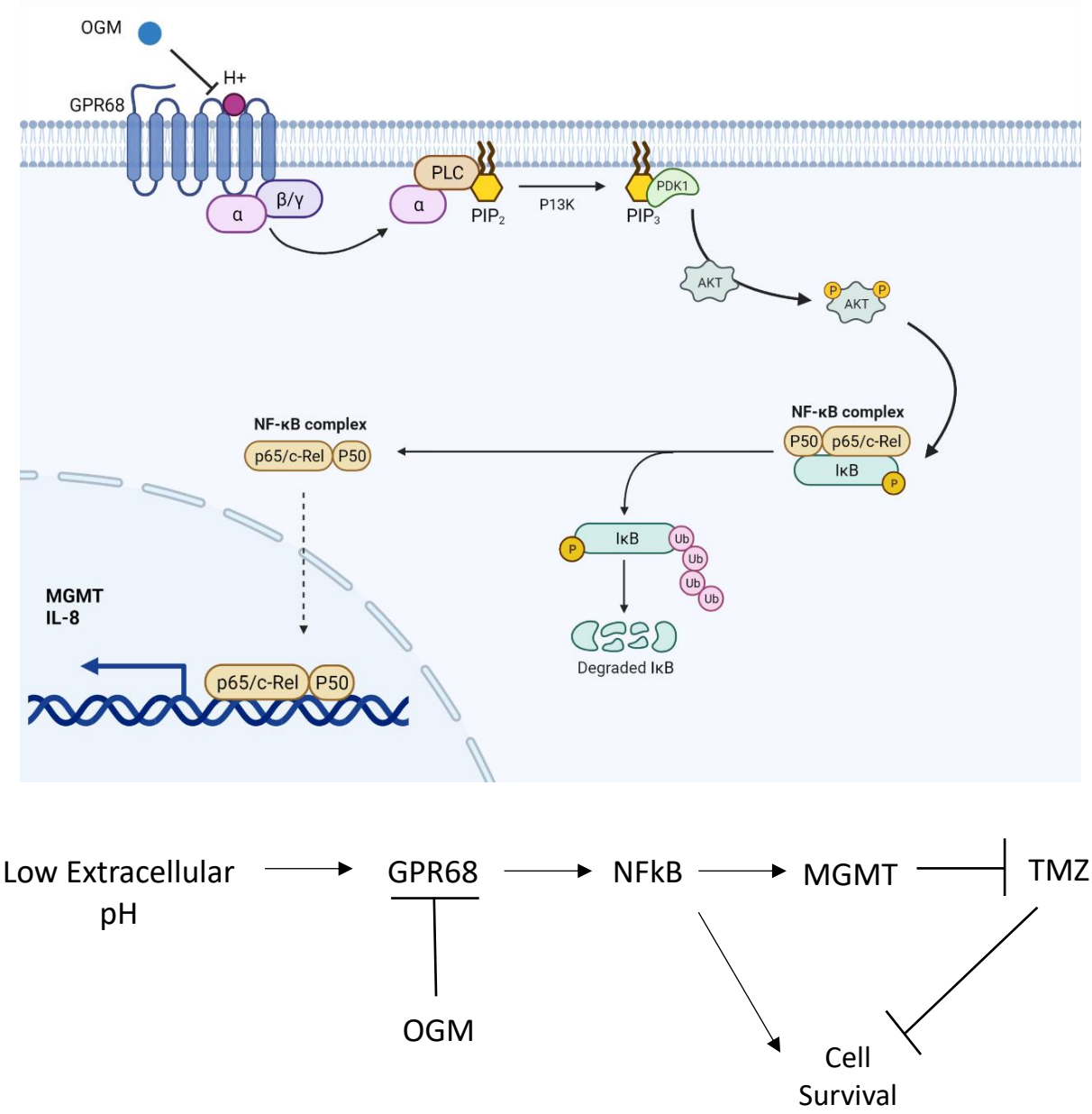


Figure 1: Proposed Mechanism for the Inhibition of MGMT Expression via NF-KB. We hypothesize that inhibition of proton-sensing GPR68 will prevent the phosphorylation of Akt and retain NF-KB in the cytosol. Retention of NF-KB in the cytosol should decrease MGMT and IL-8 expression which can be measured via qRT-PCR. Created with biorender.com

Chapter IV: Materials and Methods

Drugs and chemicals

OGM8345 was synthesized as previously described (Williams et al., 2019). Temozolomide was purchased from TOCRIS bioscience (Cat No. 2706). ReadyProbes™ Cell Viability Imaging Kit, Blue/Green was bought from ThermoFisher Scientific (Cat No. R37609). All the cell culture media and reagents were obtained from Gibco Company.

Cell line and culture conditions

Human temozolomide sensitive glioblastoma cell line (U87) was a kind gift from Rebecca Ihrie (Vanderbilt University, Nashville, TN). Human temozolomide insensitive glioblastoma cell line (U138MG) was a purchased from ATCC (HTB-16). Cell culture for both were maintained in the DMEM (Dulbecco's minimal essential medium) supplemented with 10% of the fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator containing 5% CO2.

Cell viability assay

1,000 U138MG cells were plated per well in a standard 96 well plate and allowed to attach for 24 hours prior to exposure to concentrations of vehicle, OGM8345, or Temozolomide. Cells were treated for 72 hours and then stained with DAPI. Wells were imaged with 10x magnification lens on LionheartFX (Biotek) and stitched together with Gen5 software (Biotek). Automated nuclei counting was done within Gen5 software.

Cell spheroid assay

U138MG cells were plated at 1000 cells per well in an ultra-low attachment round bottom 96 well plate and spheroids were allowed to form for 3 days. Wells were then exposed to concentrations of vehicle, OGM8345, or Temozolomide or a combination for 3 days. Spheroids were imaged in brightfield at 10x using z-stacks that were collapsed into z-projections in the Gen5 software using the LionheartFx (Biotek). Automated measurement of the area of the spheroid was measured within Gen5 software.

Western Blotting

U138MG cells were grown to 80-90% confluency and treated either with 10 μM vehicle, 10 μM OGM8345, 2 μM BAY 11-7082 (selleckchem, Cat No. S2913), or 10 μM U73122 (R&D Systems, Cat No. 1268) for 24 hours in the previously described medium at pH 6.4. Cells were lysed in ice-cold RIPA buffer (Thermofisher, Cat No. 89900) containing protease inhibitor for 30 minutes. Supernatant was isolated following centrifugation at 14000x g at 4 ˚C for 15 minutes. Protein concentrations were measured using a BCA assay (Thermofisher, Cat No. 23225). 20 micrograms were resuspended in SDS sample buffer and then denatured at 85 ˚C for 5 minutes. Samples were fractionated by electrophoresis on a 4-12% Bis-Tris precast polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane using a semi-dry transfer system. The membrane was incubated with primary antibodies for α-tubulin (1:1000, Thermofisher, Cat No. A11126) and MGMT (1:1000, Thermofisher, Cat No. MA5-13506) overnight at 4 ˚C. The membrane was then incubated with DyLight 550 and DyLight 650 (abcam) secondary antibodies for 2 hours at RT. Protein bands were visualized with a ChemiDoc MP Imaging System (BioRad). Densitometry analysis of gels was performed using ImageJ from the NIH.

qRT-PCR of MGMT, NFKB1, NFKB2, and IL-8 (CXCL8)

U138MG cells were grown to 80-90% confluency and treated either with 10 μM vehicle or 10 μM OGM8345 for either 24 hours at pH 6.4. RNA was isolated using RNeasy Mini Kit (Qiagen, Cat No. 74104) per manufacturer protocol. cDNA was generated using the isolated RNA and the High-Capacity cDNA Reverse Transcription Kit (Thermofisher, Cat No. 4368814) per manufacturer protocol. 100 ng of cDNA were loaded onto a 384-well qPCR plate along with 9 uL TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems, Cat. No 4440040) and 1 uL of the following Taqman gene expression primer: 18S (Thermofisher, Cat. No Hs03003631_g1), MGMT (Thermofisher, Cat. No Hs01037698_m1), NFKB1 (Thermofisher, Cat. No Hs00765730_m1), NFKB2 (Thermofisher, Cat. No Hs01028901_g1), and IL-8 (CXCL8) (Thermofisher, Cat. No Hs00174103_m1). qRT-PCR was conducted on a QuantStudio 5 system (Thermofisher) per TaqMan™ Gene Expression Profile manufacturer protocol. Results were assessed via the QuantStudio 5 software (Thermofisher). Fold change values were calculated using the log2 method and normalized to 18S.

qRT-PCR of RT^2 Profiler Cancer Stem Cell Assay

U138MG cells were grown to 80-90% confluency and treated either with 10 μM vehicle, 10 μM OGM8345, 2 μM BAY 11-7082 or 10 μM U73122 for either 24 or 48 hours at pH 7.8 or 6.4. RNA was isolated using RNeasy Mini Kit (Qiagen, Cat No. 74104) per manufacturer protocol. cDNA was generated using the isolated RNA and the High-Capacity cDNA Reverse Transcription Kit (Thermofisher, Cat No. 4368814) per manufacturer protocol. 100 ng of cDNA and PowerUp™ SYBR™ Green Master Mix (Thermofisher, Cat. No A25741) was loaded per well onto the RT^2

Profiler™ PCR Array Human Cancer Stem Cells (Qiagen, GeneGlobe ID: PAHS-176Z) per manufacturer protocol. qRT-PCR was conducted on a QuantStudio 5 system (Thermofisher) per RT^2 Profiler™ PCR Array manufacturer protocol. Results were assessed via the GeneGlobe RT^2 Profiler PCR Data Analysis Program (Qiagen). Fold change values were calculated using the log2 method and normalized to the geometric means of RPLP0, B2M, HPRT1, GAPDH, and ACTB.

Chapter V: Results

2D Cell Viability Assay of U138MG Cells Treated with OGM8345 or TMZ

In order to determine whether our novel compound did inhibit cell survival and proliferation in the temozolomide-resistant glioblastoma cell line, we conducted a cell viability assay at varying concentrations of OGM8345 and TMZ (Figure 2). To no surprise, there was no decrease in relative cell number at most concentrations of TMZ treatment until dose saturation at over 100 μM. However, under OGM treatment, we observed a dose-dependent decrease in relative cell number with an EC50 of roughly ~1 μM and an 80% decrease in relative cell number at 100 μM.

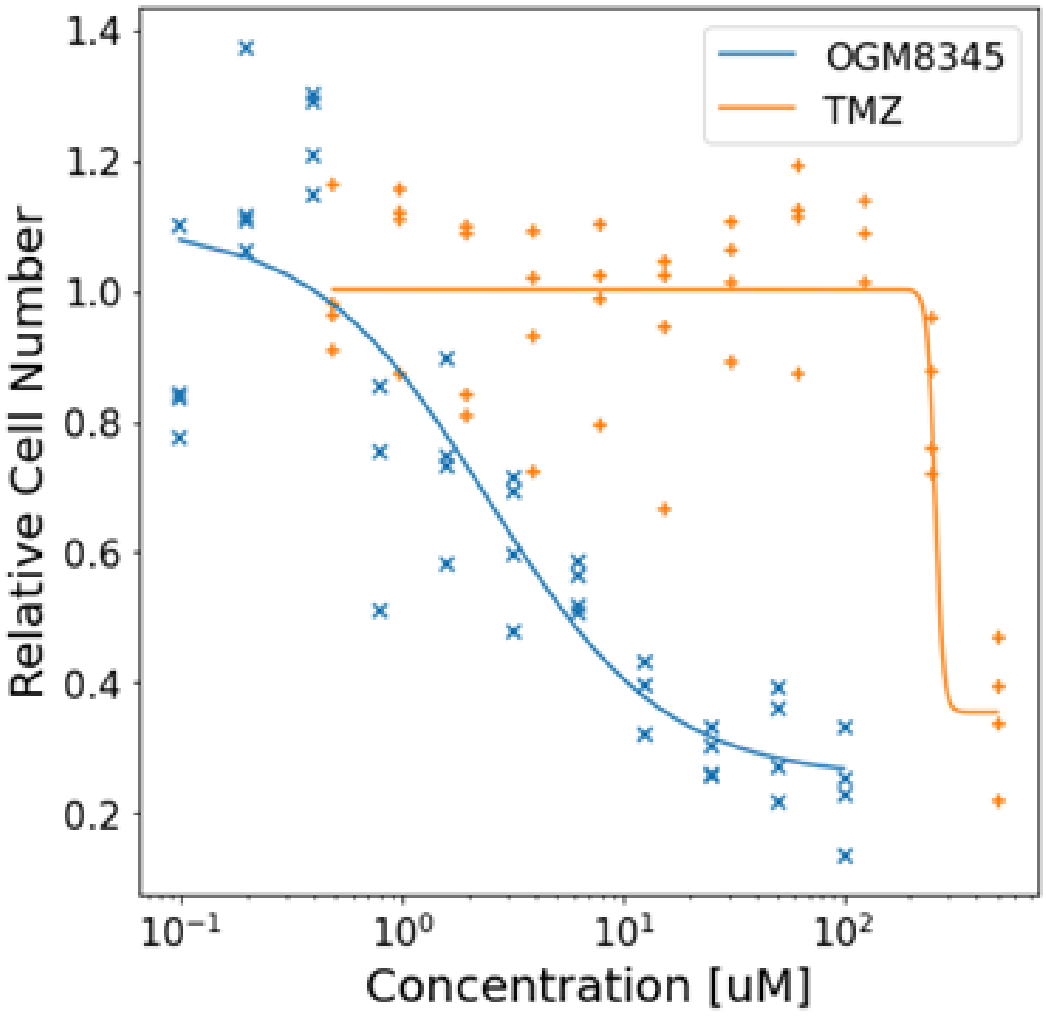


Figure 2: Cell Viability Assay of U138MG Cells Following TMZ or OGM8345 Treatment. Relative cell number of TMZ-resistant U138MG cells remained constant for TMZ concentrations of 0.5 µM until 100 µM at dose saturation. U138MG cells that underwent OGM8345 treatment experienced a dose-dependent decrease in relative cell number.

3D Cell Viability Assay of U138MG Cells Treated with OGM8345 or TMZ

While the 2D cell viability assay demonstrated that OGM was decreasing relative cell number in a dose dependent manner, it does not adequately reflect how the cells would grow or respond to treatment in a physiological manner. Given extravasation of this cancer type throughout the brain, it is incredibly important to know whether our compound could restrict tumor growth. Following varying concentration of TMZ treatment, there were no changes in relative spheroid size further validating that this cell line did not respond to TMZ treatment as shown in Figure 3. Similar to the 2D cell viability assay, the relative size of these spheroids did decrease in a dose-dependent manner of OG8345 treatment. Combined with the previous data, we were confident that GPR68 activity did indeed promote cell survival and proliferation and inhibiting its activity halted tumor growth.

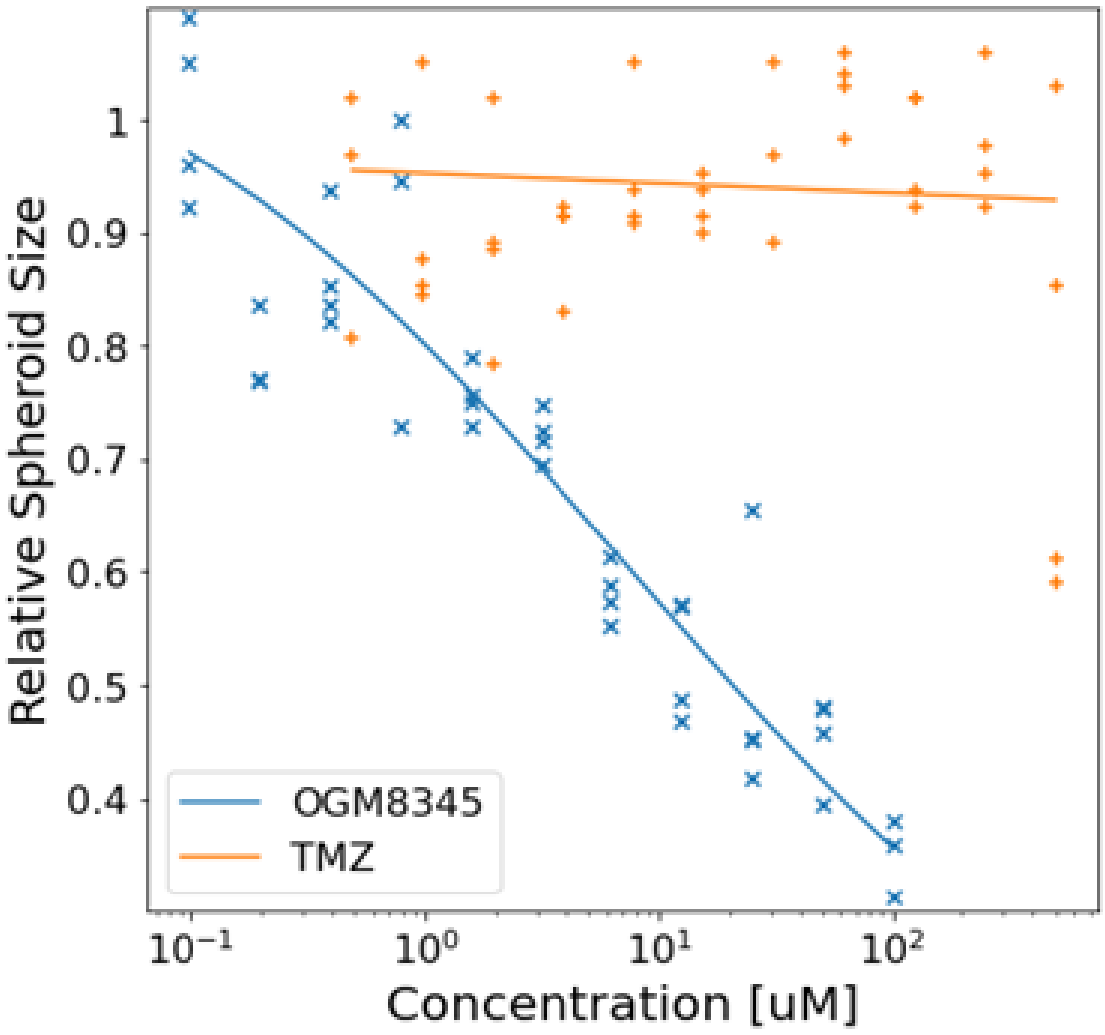


Figure 3: 3D Cell Spheroid Assay of U138MG Cells Treated with TMZ or OGM. U138MG spheroids were formed to better mimic the physiological characteristics of this cancer type. Spheroids treated with TMZ maintained spheroid size well past 100 µM while U138MG spheroids treated with OGM experienced a decrease in size in a dose-dependent manner.

OGM8345 Treatment Sensitizes U138MG Cells to TMZ Treatment

Currently, TMZ must be administered frequently and at high doses in order to effectively treat this cancer type. Given TMZ's unselective nature, patients undergoing TMZ treatment often experience a significant decrease in quality of life with most not surviving past 15 months, generating a dire need for either novel or adjuvant therapy (Holland, 2000; Hanif *et al.*, 2017). To assess whether OGM8345 affected the response of U138MG cells to TMZ treatment, we treated U138MG cells with either DMSO, TMZ, a suboptimal concentration of OGM, or a combination of OGM and TMZ for three days. We performed a cell viability assay and normalized the relative cell numbers to our cells treated with the DMSO negative control shown in Figure 4. The relative cell number of U138MG cells treated with TMZ exhibited no change compared to the DMSO control. The U138MG cells treated with a sub-optimal concentration of OGM experienced a non-significant decrease in relative cell number as expected. However, when the sub-optimal dose of OGM and TMZ were co-administered, there was a significant decrease (p-value = <0.01) in relative cell number compared to the DMSO control. This data suggests that not only does OGM treatment decrease tumor growth in U138MG cells, but it also sensitizes these cells to TMZ treatment opening opportunities for it to be used as an adjuvant therapy with TMZ.

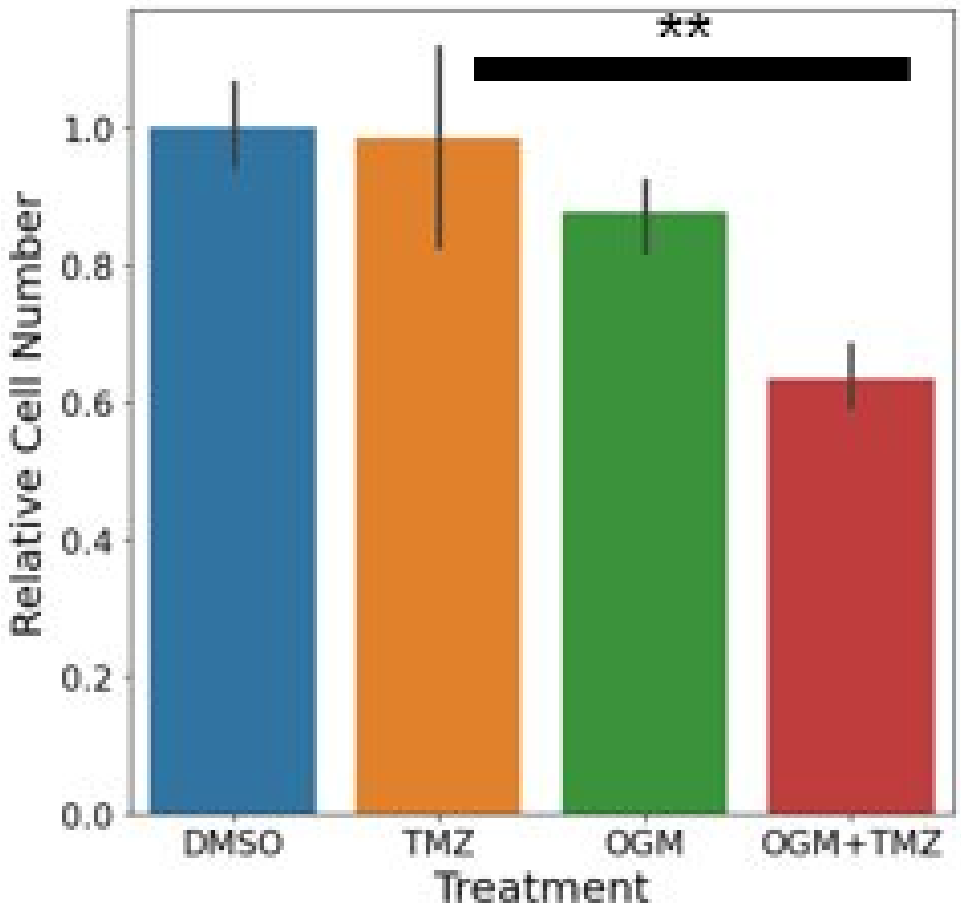


Figure 4: Quantified Cell Viability Assay of U138MG. U138MG cells treated with either TMZ or a suboptimal concentration of OGM resulted in an insignificant decrease in relative cell number. Cells treated with OGM+TMZ exhibited a significant decrease in relative cell number (p-value = <0.01).

MGMT Expression Decreases Following OGM Treatment

As previously mentioned, upregulation of MGMT in gliomas is the primary accepted mechanism for TMZ resistance. To determine whether MGMT expression may be partially modulated by MGMT expression, we treated U138MG cells with either DMSO, OGM8345, BAY 11-7082 and U73122 for 24 hours at pH 6.4 to stimulate GPR68 activity. BAY 11-7082 prevents the phosphorylation of IKBa and inhibits ubiquitin-specific proteases (Lee *et al.*, 2012). U73122 is a well-accepted PLC inhibitor used to modulate changes of downstream targets of the Gq/11 pathway. After 24 hours, the cells were lysed, and protein was collected to measure MGMT protein expression following the respective treatments. MGMT expression was normalized to the DMSO negative control treatment (Figure 5). All three treatments exhibited significant decreases in MGMT expression compared to DMSO with the BAY 11-7082 treatment having the most significant decrease (p-value = <0.001). OGM8345 and U73122 elicited similar decreases in MGMT expression (p-value = <0.01). This data supports our proposed mechanism in Figure 1 while putting significant emphasis on NF-κB's role in MGMT expression.

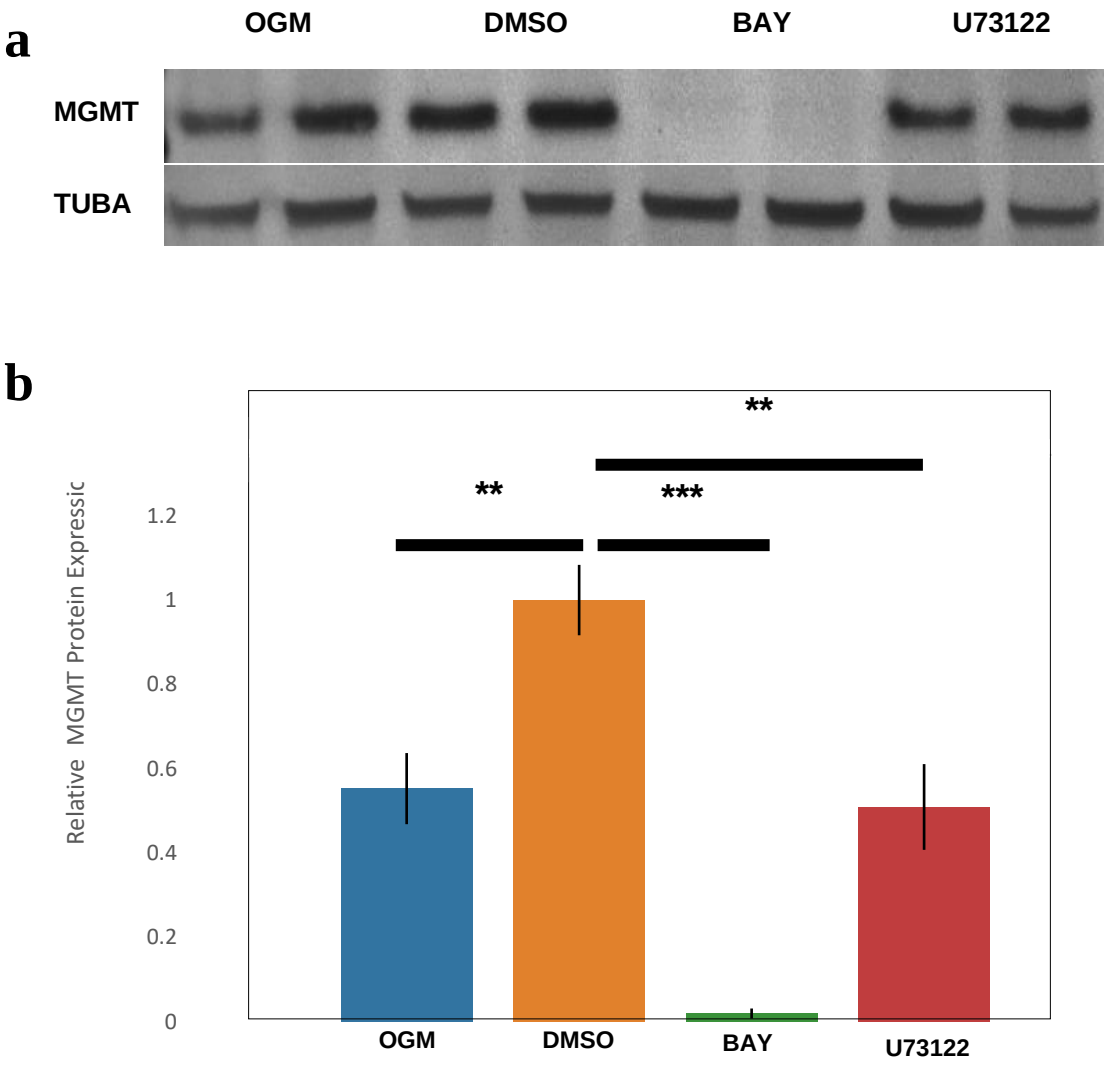


Figure 5: Relative MGMT Protein Expression in U138MG Cells Following Vehicle, DMSO, BAY 11-7082, or U73122 Treatment. **(A)** Western blot of MGMT and tubulin expression following 24 hour treatment. **(B)** Quantified MGMT Expression in U138MG Cells Following 24 hour treatment. MGMT Expression was normalized to vehicle. There was a significant decrease in MGMT expression following all treatments compared to vehicle treatment: OGM (p-value = <0.01), BAY 11-7082 (p-value = <0.001), U73122 (p-value = <0.01) (n = 3 per treatment).

OGM Treatment Downregulates MGMT, NF-κB-genes, and IL-8 in U138MG Cells

To ensure that the decrease in MGMT protein expression was caused by downregulating the MGMT gene, we conducted a qRT-PCR using Taqman Gene Expression Primers for 18S, MGMT, NFKB1, NFKB2, and IL-8. Cells were treated with either DMSO or OGM in pH 7.8 or 6.4 for 24 hours. Fold change values were normalized to the 18S CT value of DMSO treated cells at pH 7.8. Stimulation of GPR68 via media acidification resulted in a significant increase in MGMT, NFKB1, NFKB2, and IL-8 gene expression. Cells incubated in pH 7.8 medium and treated with OGM exhibited no significant change in gene expression compared to DMSO treated cells at pH 7.8. However, acidified cells treated with OGM had significant decreases in all tested genes to either undetectable or near-baseline levels (Figure 6).

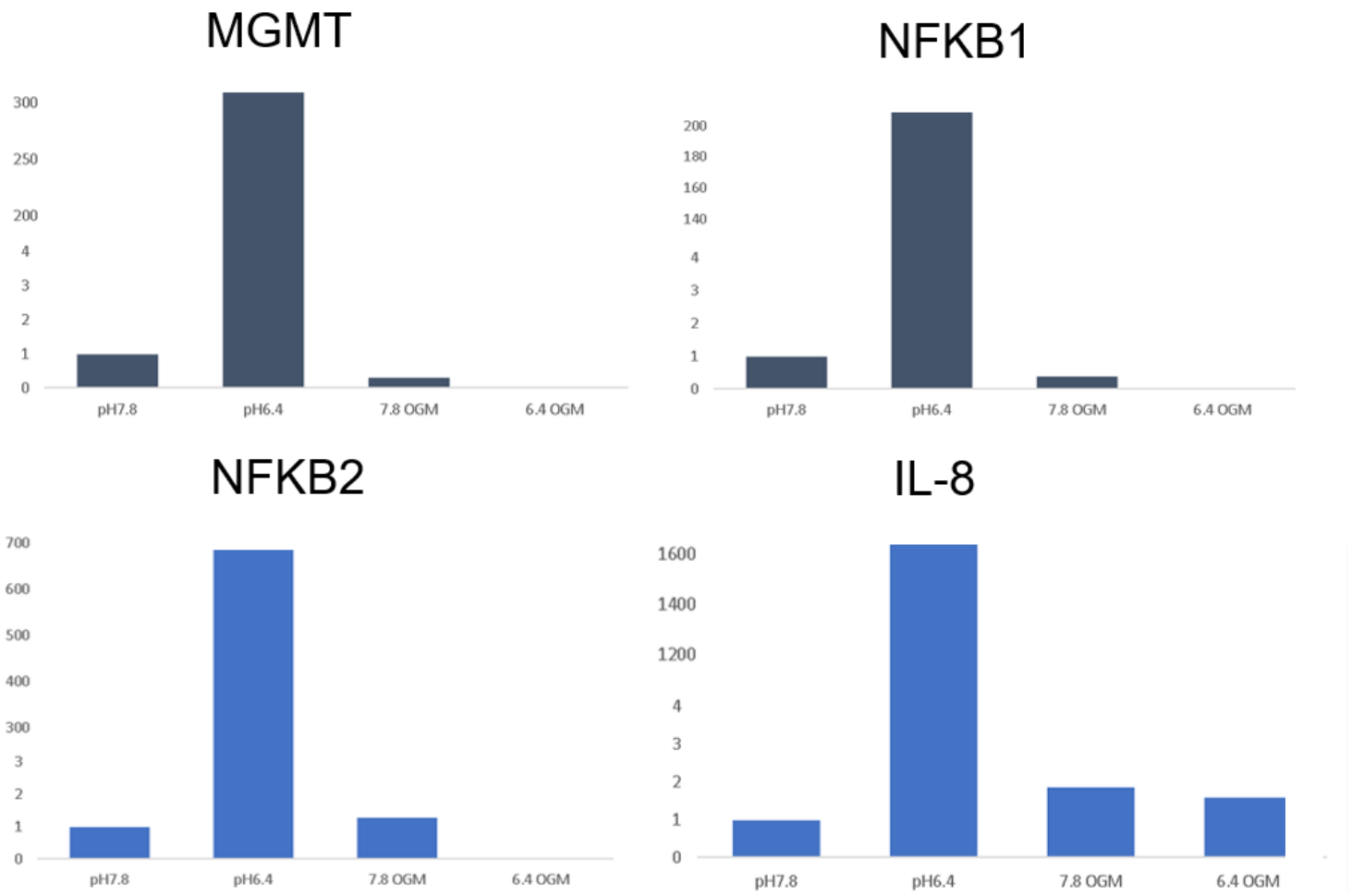


Figure 6: qRT-PCR of MGMT, NFKB1, NFKB2, and IL-8 Gene Expression in U138MG Cells. U138MG Cells were treated with either DMSO or OGM for 24 hours in pH 7.8 or 6.4. Gene expression of all genes were normalized to the gene expression profile of DMSO treated cells at pH 7.8. MGMT, NFKB1, NFKB2, and IL-8 gene expression significantly increased when cells were acidified in the 6.4 pH medium. Cells in pH 7.8 medium and treated with OGM had insignificant changes in respective gene expression. In cells treated with OGM in pH 6.4 medium, gene expression of the respective genes was either undetectable or returned to near baseline expression.

OGM Treatment Dysregulates Other Genes in U138MG Cells

Activation of the Gq/11 pathway is known to up- and downregulate several downstream targets related to cell survival and proliferation. To assess potential changes in gene expression, we generated gene expression profiles using a Qiagen RT2 Profiler^{TM} PCR Array that measures cancer stem cell-related genes. Samples were treated with either DMSO or OGM and qRT-PCRs were run in triplicate. Shown in Figure 7 is the Clustergram of genes dysregulated by OGM treatment in two biological replicates compared to a DMSO-treated sample and normalized to RPLP0, B2M, HPRT1, GAPDH, and ACTB. Of the 84 genes tested, 8 genes were upregulated, while 11 genes were downregulated when compared to DMSO treated cells.

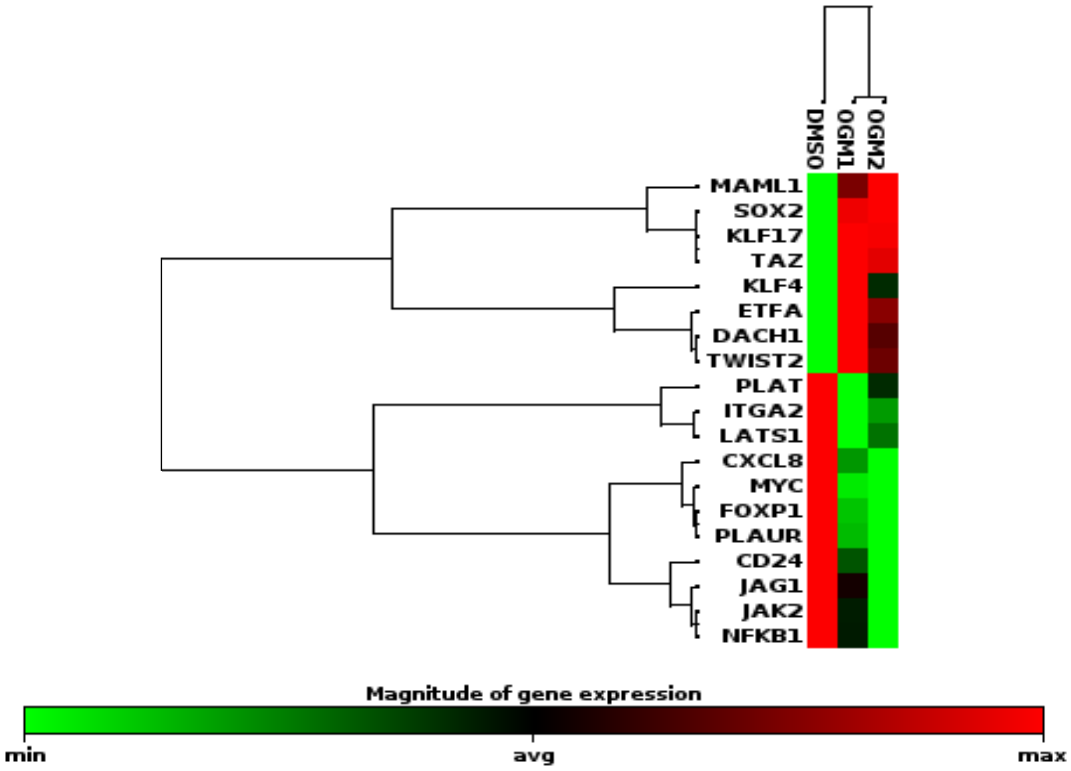


Figure 7: Clustergram of genes dysregulated by OGM Treatment in U138MG Cells. 8 genes were upregulated by OGM treatment when compared to cells treated with DMSO (n = 3). 13 genes were downregulated by OGM treatment when compared to DMSO treated cells (n = 3).

Chapter VI: Discussion

OGM as an Adjuvant Therapy for TMZ-Treatment in TMZ-Resistant Glioma Cells

Glioblastoma Multiforme remains as one of the most aggressive and lethal cancer types. Currently, temozolomide is the only FDA-approved drug treatment for GBM; however, upregulation of MGMT in GBM has resulted in TMZ resistance. There have been attempts at generating novel or adjuvant therapy to tackle this TMZ resistance, but previous inhibitors have either been ineffective or highly unselective, resulting in excessive damage to healthy cells. Here, we employ a GPR68-selective competitive inhibitor, OGM, that not only decreases MGMT expression through the Gq/11-NF-κB pathway. Additionally, suboptimal concentrations of OGM sensitize U138MG cells to TMZ in a synergistic manner (Figure 3). This may be due to the mechanism of MGMT itself. When MGMT removes a methyl group from alkylated DNA, it is subsequently ubiquitinated and proteolytically degraded thereby further decreasing cumulative MGMT in the cancer cell (Fan *et al.,* 2013). Given this information, introduction of OGM as an adjuvant therapy with TMZ may eliminate or hinder the need for high doses of TMZ during chemotherapy.

OGM Downregulates NF-κB-regulated genes in Acidified Cells

It is well understood that MGMT expression is heavily modulated by NF-κB activation making NF-κB inhibitors a strong potential therapy to combat TMZ-resistant glioblastoma cells (Avci *et al.,* 2020). However, NF-κB modulates the expression of a large array of genes that are essential for regulating the immune system (Hayden *et al.,* 2006). Despite the significant decrease in MGMT expression in U138MG cells treated with BAY 11-7082, complete inhibition of NF-κB in all cells could result in increased tumor growth and metastasis, causing more problems that are

being remedied (Perkins & Gilmore, 2006). Use of PLC inhibitors to combat TMZ-resistant GBM cells would also cause significant dysregulation to healthy cells, especially those that are heavily modulated by the Gq/11 pathway (Mizuno & Itoh, 2009). Here we show that OGM treatment only downregulated MGMT, NFKB1, NFKB2, and IL-8 in acidified U138MG cells while insignificantly dysregulating respective gene expression in U138MG cells treated at physiological pH (Figure 6). This makes OGM a stronger potential candidate for adjuvant therapy in TMZ-resistant GBMs as it appears to be specific to acidified cells, a hallmark characteristic of glioma cells.

OGM Treatment May Have Other Chemotherapeutic Targets

Using qRT-PCR, we generated gene expression profiles of 84 genes associated with cancer stem cells in DMSO and OGM treated U138MG cells. We found 19 genes to be dysregulated following OGM treatment shown in Figure 7. These genes are involved in the following cellular properties or mechanisms: stem cell markers, pluripotency, asymmetric division, cell migration and metastasis, loss of stemness, Hippo signaling, Notch signaling, and STAT/NF-κB signaling. Although our focus in this study surrounded MGMT expression through Gq/11 pathway, we suspect that GPR68 stimulation may be involved in other pathways related to tumor growth and survival. Of note is the downregulation of IL-8 (CXCL8) in OGM treated cells in acidified pH. It has been reported that TMZ treatment increased IL-8 mRNA levels in various cell lines, including gliomas, which significantly increased self-renewing capacity in GBM (Hasan *et al.*, 2019; Kadowaki *et al.,* 2019). IL-8 is also heavily modulated by NF-κB binding in cancer cells, therefore selective inhibition of GPR68 via OGM treatment may also be employed to mitigate IL-8 upregulation following TMZ treatment (Guo, *et al.* 2017). Although the qRT-PCR may have to be

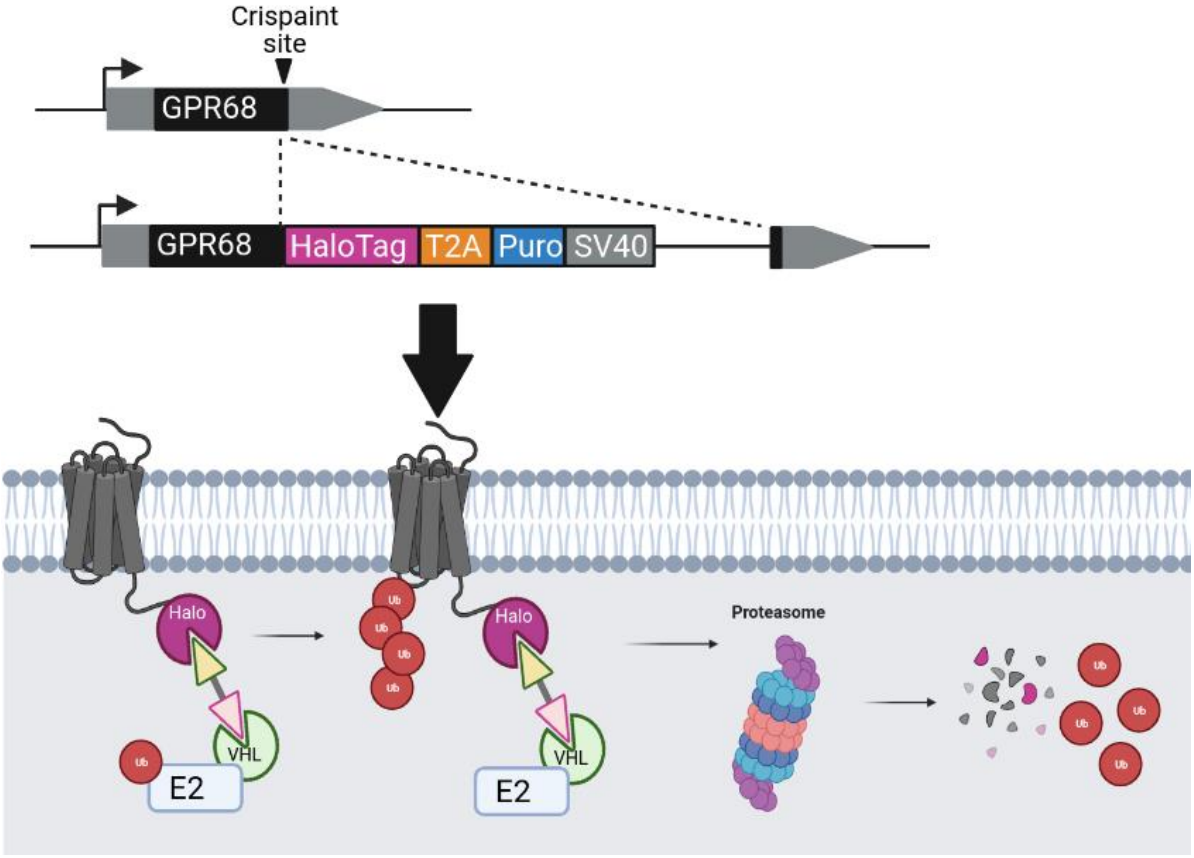
repeated to ensure accuracy, JAK2 was also seen to be downregulated following OGM treatment. JAK2 codes for Janus Kinase 2 which is responsible for the phosphorylation and activation of signal transducer and activator of transcription 3 (STAT3). STAT3 is a key regulator in the production of blood cells from bone marrow and angiogenesis. When dysregulated, STAT3 also plays a role in gliomagenesis (Kim *et al.*, 2013). Given the initial results, inhibition of GPR68 with our novel selective inhibitor may also be downregulating JAK2 gene expression to decrease cell growth and proliferation.

Chapter VII: Conclusion

In this study, we demonstrated that GPR68 inhibition using OGM resulted in the downregulation of MGMT, the primary mechanism for TMZ resistance in glioblastoma multiforme. Using western blot analysis and qRT-PCR, we demonstrated that this downregulation was being modulated by inhibiting NF-κB activity through the Gq/11 pathway. Additionally, we show that using a suboptimal concentration of OGM sensitized TMZ-resistant glioma cells and may be caused by downregulating either MGMT or IL-8 gene expression among other genes that were dysregulated by OGM treatment. To our knowledge, this is the first study using a novel GPR68 competitive inhibitor to decrease tumor growth and survival.

GBM among other cancer types over express GPR68 to promote tumor growth and survival. In this study we used a selective competitive inhibitor to downregulate GPR68 activity. An alternative method would be to selectively degrade GPR68 itself. Currently, we are utilizing CRISPAINT to endogenously tag GPR68 with a HALO protein tag. He we can employ a HALO specific PROTAC to selectively target and degrade GPR68 (Schmid-Burgk *et al.*, 2016) (Supplemental Figure 1). At this time, we do not know if we can specifically target GPR68 in cancer cells; however, we have demonstrated that HALO PROTAC does decrease tumor growth in another glioma cell line, U87MG, after endogenously tagging GPR68 with HALO. If successful in U138MG cells, we can then look to determine other tumor growth and survival mechanisms that are being promoted by GPR68 activity.

Supplemental Figures:



Supplemental Figure 1: Schematic of HALO-PROTAC Mechanism. Using CRISPaint, we can endogenously tag GPR68 with a Halo Tag allowing us to use a HALO-PROTAC protocol to proteolytically degrade GPR68.

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