Fall 12-12-2022

Bis-Indolyl Compounds and the Induction of Apoptosis in T98G Glioblastoma Multiforme Cells

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Bis-Indolyl Methane (BIM) compounds and the Induction of Apoptosis in T98G Glioblastoma Multiforme Cells

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Submitted in partial fulfillment of the requirements for the
degree of Master of Science in Biology from the
Department of Biological Sciences of Seton Hall University
December 2022

Seton Hall University
Department of Biological Science

College of Arts and Sciences
Department of Biological Studies
APPROVAL FOR SUCCESSFUL DEFENSE

Margot Chloe Brown has successfully defended and made the required modifications to the text of the Master's thesis for the M.S. during this Fall Semester, 2022

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Acknowledgements

First, I would like to thank my loved ones for providing me with unwavering support as I have moved through my educational career. To my dad, hero, and best friend. The unwavering support and encouragement you have provided me with throughout this journey is truly something I'll never forget. You have always set the example for what it means to be an incredibly strong, yet kindhearted person. To my mom, thank you for always being my sounding board, and for teaching me how to navigate through life. And to my sister Cedra, you have always been somebody that I have greatly admired. You are the embodiment of what it means to work hard to achieve your dreams. I love you all more than words can describe.

I would also like to thank my mentors Dr. Suzanne Gantar and Dr. Daniel B. Nichols. Thank you both for your guidance throughout this process. The knowledge that you have passed down to me is invaluable, and I am incredibly grateful that I was able to work with you.

I would like to thank my committee members Dr. Angela Klaus and Dr. Constantine Bitsaktsis. I greatly appreciate you taking the time to help me complete my studies here at Seton Hall University.

Table of Contents:

List of Figures:

Abstract:

1,1-bis(3'idolyl)-1(aryl)methane compounds (BIM compounds) have been shown to have anti-cancer properties in colon cancer, bladder cancer, and leukemia cells. The purpose of this work was to determine if BIM compounds could be an effective treatment of glioblastoma multiforme. Sulforhodamine B (SRB) assays showed that 20μM of the BIM compounds could inhibit cellular proliferation of the T98G glioblastoma multiforme cell line over 72 hours. Then immunoblotting was used to analyze the molecular pathway induced by BIM compounds. An increase in the expression of both BAX and cleaved caspase 3 suggest BIM compounds activate programmed cell death, or apoptosis in glioblastoma cells. In conducting this work, I am hoping to contribute to the creation of a new treatment for glioblastoma multiforme that will minimize the negative side effects of traditional cancer treatments.

List of Key Terms

1. Analysis of Variance (Anova)
2. BAX (Bcl-2 associated protein)
3. Cleaved Caspase 3 (active caspase protein)
4. Glioblastoma Multiforme
5. Isocitrate Dehydrogenase 1 (IDH1)
6. Peroxisome Proliferation Receptor Gamma (PPARγ)
7. T98G (glioblastoma multiforme cell line)

Introduction:

Glioblastoma multiforme is a brain tumor categorized as a stage IV astrocytoma. Glioblastoma multiforme differs from astrocytoma in that it is made up of more than one type of glial cell, primarily astrocytes and oligodendrocytes, whereas astrocytoma solely consists of cancerous astrocytes (National Cancer Institute). With a recurrence rate of 90% in those who seek treatment, as well as a five-year survival rate of 6.8% (National Brain Tumor Society), glioblastoma multiforme, is the deadliest cancer that can develop in adults in the United States. Additionally, individuals over the age of 65 who are diagnosed with glioblastoma multiforme have a survival rate of only 2% (Wirsching et. al 2016), indicating that age is a predominant risk factor in the poor prognosis of glioblastoma multiforme (Wirsching et. al 2016).

Glioblastoma multiforme not only have a poor prognosis but are also associated with a plethora of symptoms including edema, epilepsy, memory loss, loss of motor function, and generalized destruction of brain tissue (Wirsching et. al 2016). Methods of obtaining a sample of the tumor for extensive analysis such as performing stereotactic biopsy or microsurgical resection of the tumor is needed to diagnose glioblastoma (Wirsching et. al 2016).

Glioblastoma multiforme can be divided into two subtypes consisting of primary or secondary glioblastoma multiforme tumors. Primary, or *de novo* glioblastoma tumors are growths which originate in the brain. Secondary glioblastoma multiforme tumors are typically deposited in the brain when malignant and metastatic cells from a tumor originating somewhere else in the body breaks off and enter the individual's nervous system. Additionally, secondary glioblastoma multiforme is a more common diagnosis among adolescence as it is developed from a cancer independent of the nervous system, known as astrocytoma. Isocitrate dehydrogenase 1 (IDH1) genetic mutations are commonly associated with a secondary

glioblastoma multiforme diagnosis (Ohgaki 2013). IDH1 is typically found in the cytoplasm of somatic cells and is primarily responsible for fat metabolism and the breakdown of molecules which could be harmful to the cell (National Cancer Institute). Loss of function mutations in the IDH1 gene may lead to abnormal cell development which may cause some cells to become cancerous (National Cancer Institute).

Current treatment of glioblastoma multiforme consists of radiotherapy as well as surgical resection. Both radiotherapy and surgical resection present a variety of negative side effects which can be detrimental towards the physical wellbeing of the individual seeking treatment. Regardless of treatment, glioblastoma multiforme has an extremely poor prognosis (Taylor et. al 2019).

1,1-bis(3'idolyl)-1(aryl)methane compounds, or "BIM" compounds have anti-cancerous properties (Shorey et. al, 2012) in a variety of cell lines such as colon cancer, bladder cancer, and acute myelogenous leukemia (Contractor, 2005; Kassouf, 2006: Chintharlapalli, 2005). BIM compounds are also used for their anti-fungal and anti-inflammatory properties, as well as their ability to contribute to cardiovascular health by way of inhibiting the enzyme cyclooxygenase which causes edema (Kaishap, 2013).

The peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear hormone receptor which may play a role in cancer suppression (Vandoros et. al 2005). Activation of PPARγ have been proven to moderate lipid and glucose metabolism, cell growth, immune function, cell differentiation, and adipogenesis (Vandoros et. Al, 2005). PPARγ activation has also been shown to induce intrinsic apoptotic pathways by facilitating the synthesis of pro-apoptotic proteins such as cleaved caspase 3 (Garcia-Bates et. al 2008). PPARγ is targeted in this research due to its high level of expression in various cancer types including multiple myeloma as well as primary

metastatic breast cancer (Wang, 2013; Garcia-Bates, 2008). BIM compounds and their derivatives have been proven to be PPARγ receptor agonists (Chintharlapalli, 2005; Kassouf, 2006: Contractor, 2005).

Side effects of existing cancer treatments are detrimental to the health of the individual. The purpose of the present study is to investigate the BIM compounds as a potential new treatment for glioblastoma multiforme. New treatments are needed to improve patient survival rates and reduce the negative side effects of current cancer treatments.

Methods:

Cell Culture

T98G glioblastoma multiforme cell line (ATCC, Manassas, VA) was maintained using Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich,St. Louis, MO). The final DMEM product used by researchers contained 10% Fetal Bovine Serum, 1% non-essential amino acids, 1% Penicillin Streptomycin, and 1% L-glutamine (Gibco, Gaithersburg, MD). Incubation of T98G cells took place at 37^{o}C and 5% CO_2.

BIM Compounds

BIM compounds were produced by the Badillo lab at Seton Hall University using a minor photoacid catalysis reaction (Salem, et. al). BIM compounds were received from the Badillo lab in a solid form and were consequently dissolved in DMSO to 20mM concentration before being used in this research.

Sulforhodamine (SRB) Assay

Cell proliferation was measured using a procedure previously established by Esteban A Orellana and Andrea L Kasinkski (Orellana and Kasinski, 2016). Cells were plated in 96-well

plates in quintuplicates with a density of 3,500 cell per well. Following a 3-hour incubation period, the media was aspirated out of each of the wells and 100 µL of the desired BIM compound was placed in each well at various concentrations, ranging from 5 µM to 50 µM. BIM compounds were dissolved in DMSO and stored at 4^{o}C. Plates were left to incubate at 37^{o}C for 72 hours. Following the 72-hour incubation period, the BIM solution was aspirated from each of the wells, and cells were washed with 1X Phosphate Buffer Saline (PBS). 20% trichloroacetic acid (TCA) was used to then fix the cells to the plate (Sigma-Aldrich). Following cell fixation, plates were rinsed with tap water and cells were stained using a 1% acetic acid and Sulforhodamine B v/w solution (Sigma-Aldrich). Plates were dried and cells were resuspended in 1mM Tris. Imaging the absorbance of the plates was conducted at a 505nm wavelength using a Varioskan™ LUX multimode microplate reader which was obtained from Sigma-Aldrich.

Treating Cells for Immunoblotting

Cells were plated in T75 flasks or 6 well plates and allowed to reach 40% confluency. Treatments were diluted in DMEM. Control groups consisted of DMSO (0.025%), rosiglitazone (20µM), and cycloheximide/TNF-alpha (10ng/mL: 1µL/mL) (Sigma-Aldrich,St. Louis, MO). Treatments were allowed to run for 6.5 hours. Media was then aspirated, and cells were washed with PBS. The washing solution was aspirated from the cells, at which point cells were trypsinized and their pellets were collected.

Immunoblotting

A RIPA buffer solution containing a protease inhibitor (Pierce, Rockford, IL) was used to lyse cells. Concentration of protein in the lysate was established by BCA assay (Pierce, Rockford, IL). 30 µg of protein was separated using 10% SDS page running buffer, then transferred to a nitrocellulose membrane. Transfer was facilitated using Trans-Blot Turbo

Transfer System™ (Philadelphia, PA). 5% milk in TBS-T was used to block the membrane at room temperature for 1 hour on a rotator.

For a manual transfer, the gel membrane was removed from plastic encasement and submerged in a transfer buffer for ten minutes. Transfer buffer was made using tris base (Sigma-Aldrich,St. Louis, MO), glycine (Sigma-Aldrich,St. Louis, MO) and methanol. Two pieces of filter paper and two square sponges were also submerged in the same transfer buffer for ten minutes. PVDF transfer membrane was allowed to sit in 100% methanol for approximately two minutes. The materials were removed from the transfer buffer. A gel holder cassette was used to hold the materials together during transfer. First, a square sponge was placed on the gel holder cassette, followed by a piece of filter paper, then the gel membrane. The PVDF membrane was then placed on top of the gel membrane, followed by a second piece of filter paper and a second square sponge. The gel cassette was then placed in an electrode assembly in a buffer tank with one ice pack. The tank was filled to the top with transfer buffer, and transfer was allowed to take place for 1hour at 90mV.

Following transfer, a 5% milk in TBS-T was used to block the membrane at room temperature for 1 hour on a rotator. The block was aspirated from the membrane and the membrane was then incubated overnight with a primary antibody at 4^{o}C on a rotator. BAX (Cell Signaling Technology, Danver, MA), cleaved caspase 3 (Cell Signaling Technology, Danver, MA), and β-actin antibodies (Cell Signaling and Santa Cruz Biotechnology, Dallas, TX) were used at a 1:1000 dilution. Both anti-mouse and anti-rabbit horseradish peroxidase-linked antibodies were used as secondary antibodies at a 1:1000 dilution (Cell Signaling Technology) when probing for BAX, while the anti-rabbit horseradish peroxidase-linked secondary antibody was used at a 1:10,000 dilution when probing for cleaved caspase 3. The appropriate secondary

antibody was placed on the membrane, and the membrane was incubated overnight at 4ºC on a rotator. SuperSignal West Femto substrate was used in detection. Images of the western blots were taken on a FluorChem™ E system (ProteinSimple, Santa Clara, CA).

Quantification of Western Blot

The NCBI ImageJ software was used in quantification of the western blot. The lanes in which BAX and cleaved caspase 3 bands appeared in western blot imaging were selected and consequently differentiated. Using the ImageJ software, the lanes with the corresponding graphs were plotted, producing graphs. The graph appeared as a curve and the surface area of the curve corresponded to the amount of protein in the band, which was then given a percentage value depending on the total BAX or cleaved caspase 3 expression appeared in the given lane. Band quantification was then repeated for β-actin expression in each lane. The data was then normalized, and the ratio of BAX or cleaved caspase 3 to β-actin for each band was established.

Statistical Analysis

GraphPad Prism software (La Jolla, CA) was also used to calculate EC_{50} values from the SRB absorbance data that was previously collected. Microsoft excel (Redmond, WA) was used to conduct one-way anova testing following western blot quantification. P-values less than 0.05 were considered statistically significant.

Results:

BIM compounds inhibit T98G cell proliferation

Previous research has shown that BIM compounds inhibit cellular proliferation in cancers such as bladder cancer (ZMS1-109 and ZMS1-120) and leukemia (ZMS1-146) (Kassouf et. al; Contractor et. al) **(Figure 1).** The effect of BIM compounds on the cellular proliferation of

glioblastoma multiforme cells has not previously been measured. The adherent cell line T98G derived from a 61-year-old white male was obtained from the ATCC. Cell density of T98G cells was measured 72 hours after BIM treatment using a Sulforhodamine B (SRB) assay. As seen in **Figure 2**, cell proliferation was significantly inhibited by all three BIM compounds at 20μM concentration compared to DMSO control.

Absorbance was compared to the log concentration of BIM compounds to calculate EC_{50} values. EC_{50} values correspond to the concentration of the given BIM compound in which half-maximal effect was measured. Compounds ZM-109, ZM-120 and ZM-146 had EC50 values of 16.72 μM, 13.34 μM, and 11.07 μM respectively (**Figure 3, Table 1**).

BIM compound 146 inhibits T98G proliferation through activation of apoptosis

BIM compounds induce apoptosis the bladder cancer cell lines KU7 and 253J-BV through activation of PPARγ (Kassouf et. al). T98G cells have been shown to express PPARγ (LIU, et. al), which was confirmed here (**Figure 4**).

Next, expression of BAX was measured in the T98G cell line. Cells were treated cells with 5uM of ZMS1-146 for 6.5 hours. To ensure that DMSO did not have a cytotoxic effect on cells, T98G cells were treated with DMSO alone. Rosiglitazone was used as a PPARγ agonist in this research.

***All molecular structures in this paper were generated by the author of this paper and all subsequent images were generated by the author of this paper.**

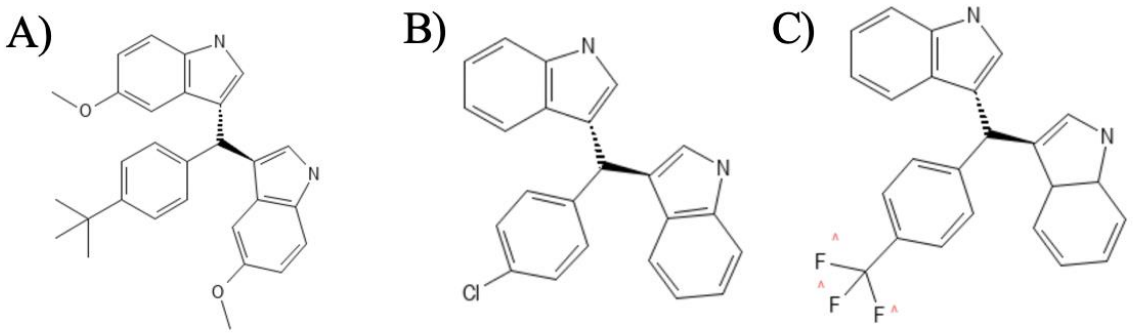


Figure 1. Molecular structure of 1,1-bis(3'idolyl)-1(aryl)methane compound ZMS1-146 **(A)**, ZMS1-120 **(B)**, AND ZMS1-109 **(C)**

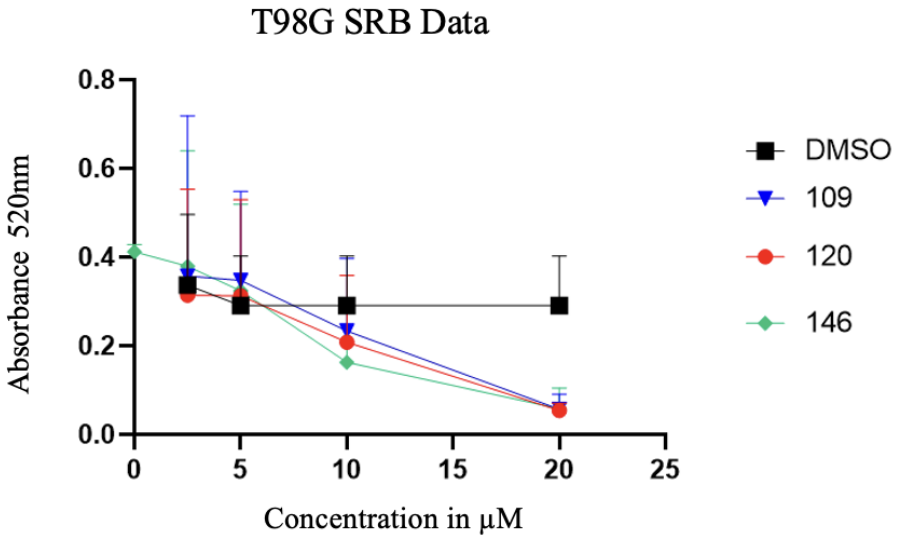
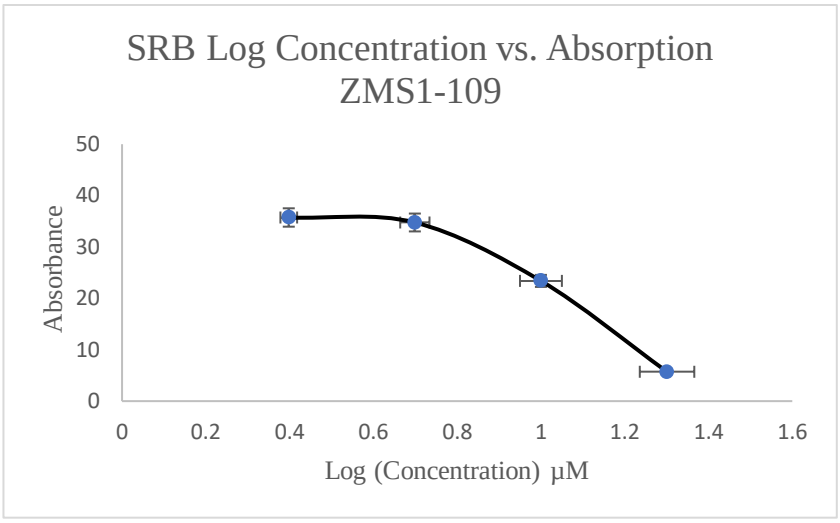


Figure 2. The concentration of BIM compound used to treat T98G cells compared to its absorbance under 520nm after 72 hours of treatment.

A)



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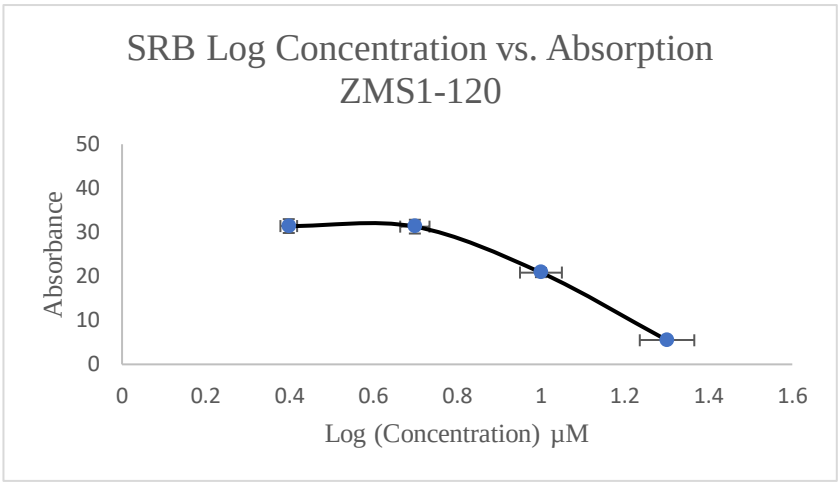

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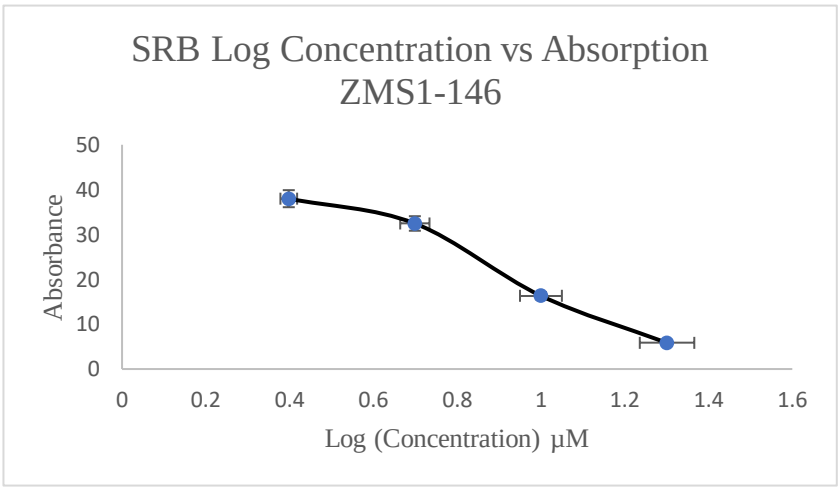

Figure 3. SRB Log Concentration vs absorption for BIM compounds **A)** ZMS1-109 **B)** ZMS1-120 **C)** ZMS1-146

BIM Compound	EC_{50} Value
ZMS1-109	16.72μM
ZMS1-120	13.34μM
ZMS1-146	11.07μM

Table 1. EC_{50} values for the BIM compounds.

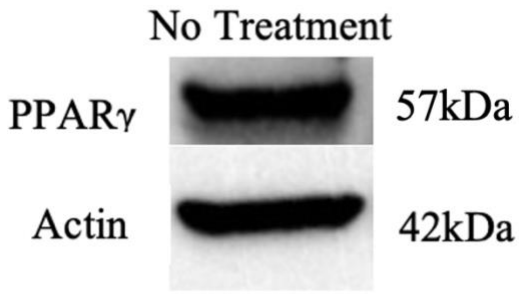

Figure 4. Western blot analysis of PPARγ in T98G cells. This figure was generated by Gantar Lab undergraduate researcher Matthew Sunda.

As seen in **Figure 5**, BAX was more highly expressed in cells treated with the BIM treatment compared to the control groups. A one-way ANOVA test was used to calculate a p-value for this experiment. The p-value was determined to be 0.0012.

Expression of cleaved caspase 3 was measured in the T98G cell line. Cells were treated cells with 5μM of ZMS1-146 for 6.5 hours. As seen in **Figure 6**, cleaved caspase 3 was more highly expressed in our BIM treatment group than it was in the groups treated with cycloheximide/TNF-alpha and 0.025% DMSO. A one-way ANOVA test was used to calculate a p-value for this experiment. The p-value was determined to be 0.0359.

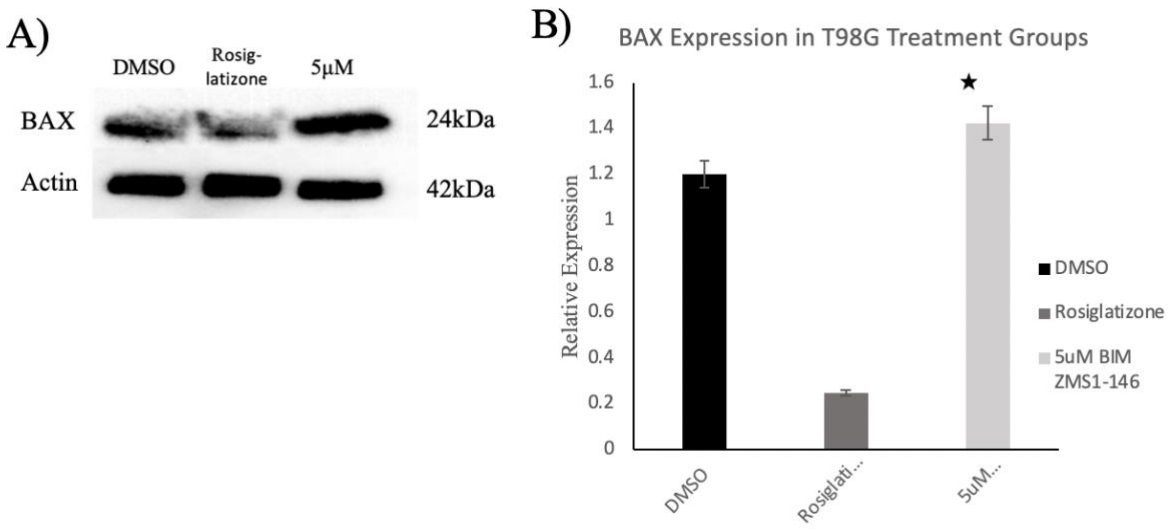

Figure 5. Western blot analysis of BAX in T98G cells **A)** BAX was more highly expressed in T98G cells that were treated with 5 μM ZMS1-146 compared to DMSO and Rosiglitazone. **B)** Quantification and consequent statistical analysis of the bands produced by western blot proved that the expression of BAX in the experimental group is higher than that of the control groups to an extent of being statistically significant. A one-way ANOVA test produced a p-value of 0.0012.

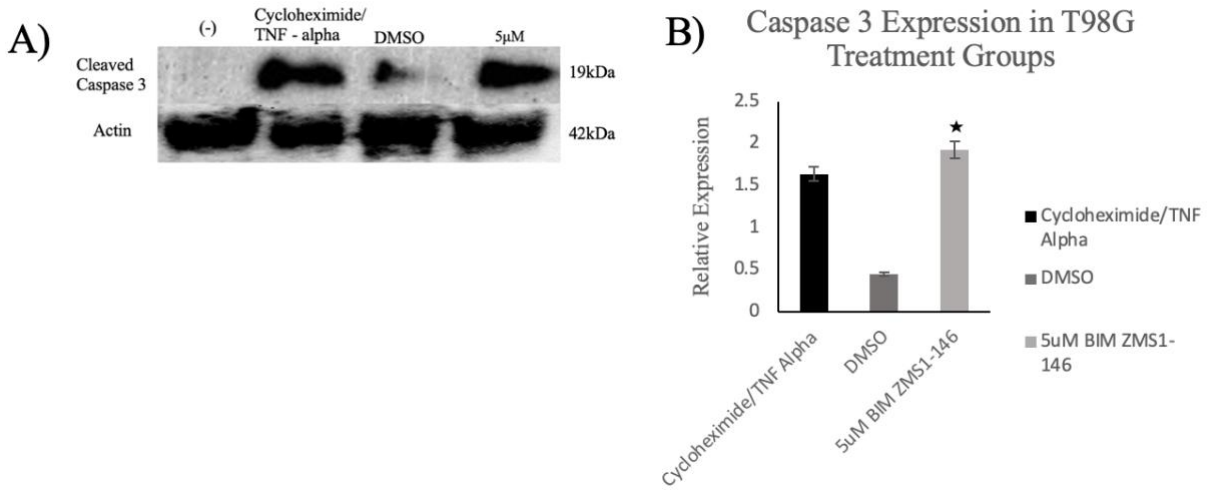

Figure 6. Western blot analysis of cleaved caspase 3 expression in T98G cells **A)** Cycloheximide/TNF-alpha treated cells acted as the positive control. Cleaved caspase 3 was more highly expressed in T98G cells that were treated with ZMS1-146. **B)** Quantification and consequent statistical analysis of the bands produced by western blot proved that the expression of cleaved caspase 3 in the experimental group is higher than that of the control groups to an extent of being statistically significant. A one-way ANOVA test produced a p-value of 0.0359.

Discussion and Conclusions:

This research sought to determine if BIM compounds could be used as a therapeutic against glioblastoma multiforme. All three BIM compounds tested inhibited proliferation of T98G glioblastoma multiforme cells. Compound 146 was then further examined to determine how BIMs inhibit proliferation. BAX and cleaved caspase 3 protein levels increase after 6.5 hours of treatment with 5μM BIM suggesting activation of apoptosis. The BIM compound may be signaling through the PPARγ which is present in the cells.

The EC_{50} values calculated for the three compounds that were focused on in this experiment ranged from 11.07μM to 16.72μM. A low EC_{50} value indicates that a smaller amount of drug is needed to inhibit cellular proliferation, while a high EC_{50} value indicates that the drug is less potent. In a study conducted by A. Saveanu et. al, researchers determined the effects of BIM compounds binding to different receptors in human pituitary somatotroph adenoma cells. The purpose of their research was to determine if BIM-23A387 was capable of suppressing growth hormone production in human pituitary somatotroph adenoma cells. Researchers tested the effects of the BIM compound of interest on receptors sst_1-sst_5. EC_{50} values were produced ranging from 0.1M (sst_1), to 1000M (Saveanu et. al). Generally, the concentration of the BIM compound used in this research to produce a half maximal response in the cancer of interest far exceeded that which was observed in the present study. The larger EC_{50} values obtained by Saveanu et. al may be a result of their research utilizing *in vivo* studies. Should the present study be continued in the future and moved from *in vitro* to *in vivo* testing, the EC_{50} values may be larger.

In research conducted by Lyndsey E. Shorey et. al, scientists investigated the effects of 3,3-diindolylmethane (DIM) compounds on acute lymphoblastic leukemia (Shorey et. al).

Researchers conducted an *in vitro* study using cell lines CEM and SUP-TI (Shorey et. al). In conducting this study, researchers hypothesized that a decrease in cellular proliferation would be observed upon exposing acute lymphoblastic leukemia cells to the DIM compounds of interest. Moreover, researchers observed that the expression of cell division protein kinases such as CDK4 and CDK6 significantly decreased following exposure to DIM compounds (Shorey et. al). CDK4 and CDK6 expression was observed via western blotting. In addition to measuring the expression of cyclin dependent protein kinases, Shorey et. al established that the cells were undergoing apoptosis following DIM exposure using ViaCount Assay + (Shorey et. al). The ViaCount Assay + was also used by researchers to examine cellular proliferation following exposure to the DIM compounds of interest. EC_{50} values were then calculated for the experiment. The EC_{50} for cell line CEM was determined to be 15µM, while the EC_{50} value for cell line SUP-TI was 13µM (Shorey et. al). The data pertaining to cellular proliferation and EC_{50} values collected in this research is similar to that which was obtained in the present study.

Expression of BAX and cleaved caspase 3 in T98G cells treated with the BIM compound of interest suggest that an intrinsic apoptotic pathway was activated in the cancer cells. Intrinsic apoptotic pathways refer to a stress response within the cell that causes apoptosis, while an extrinsic apoptotic pathway refers to the extracellular environment dictating that the cell is no longer able to live (Thermo-Fischer Scientific). Expression of BAX in T98G cells treated with ZMS1-146 was significantly greater than the expression of BAX in the control groups. Expression of BAX in cells treated with rosiglitazone was lower than expected. Rosiglitazone is known to be a PPARγ agonist (Cuzzocrea et. al), however in this experiment rosiglitazone treatment produced less BAX expression compared to the negative control DMSO. Similar results were obtained when observing cleaved caspase 3 expression in T98G cells. Again,

DMSO was tested on the T98G cells to ensure that it did not have a cytotoxic effect on the cancer cell line. Cycloheximide/TNF-alpha was used as the positive control group because these compounds are known to induce apoptosis (Rath and Aggarwal; Dai et. al). Expression of cleaved caspase 3 in BIM treated T98G cells was significantly greater than that of either one of the control groups, suggesting that an intrinsic apoptotic pathway was activated in the T98G cells upon exposure to ZMS1-146.

Previous research, as well as preliminary docking studies have suggested that BIM compounds bind to the PPARγ receptor, consequently inducing apoptosis in the cell. Future studies could be done to determine if BIM compounds are binding to the PPARγ receptor to induce apoptosis. T98G cells could be manipulated to over-express the PPARγ receptor. Researchers could then observe whether the BIM compounds are more effective in inducing apoptosis in cells with the over-expression. Moreover, the PPARγ could be entirely deleted from the T98G cell line, and researchers could test whether BIM compounds are still capable of inducing apoptosis in the cell line.

Throughout this research, BIM compounds have been shown to increase BAX and cleaved caspase 3 expression in T98G glioblastoma multiforme cells. The increased expression of these pro-apoptotic proteins was observed via western blotting. Moreover, BIM compounds decrease cellular proliferation of T98G glioblastoma multiforme cells, which was measured using SRB assay.

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