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INHIBITION OF MIR26A MICRORNA RESTORES SENSITIVITY TO TEMOZOLOMIDE

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by

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Inhibition of MiR26a MicroRNA Restores Sensitivity to Temozolomide

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ABSTRACT

Inhibition of miR26a microRNA restores sensitivity to temozolomide

by Angela Grace Stewart

MicroRNAs have been associated with tumorgenesis of Glioblastoma Multiforme (GBM). A microRNA (miRNA) is a small RNA (22-25 nucleotides) that can prevent the translation of messenger RNA (mRNA) into protein by binding the mRNA (Huse et al. 2008, Zamore and Haley 2005). The miRNA/mRNA complex is then degraded. PTEN is a tumor suppressor protein which regulates the AKT/PIP3 pathway, which is active in many cellular regulatory pathways. The microRNA miR-26a is known to bind PTEN mRNA. The loss of PTEN promotes ideal conditions for the unregulated growth of tumor cells and Huse et al. (2008) demonstrated that miR-26a is frequently amplified in human glioma. Jiang and colleagues (2007) reported that the lack of PTEN influences the cells ability to resist chemotherapy treatment, specifically temozolomide (TMZ). TMZ is the current chemotherapy standard of care for GBM patients. This study investigated a possible link between miR-26a inhibition and TMZ sensitivity. LN229 glioma cell line (wild-type PTEN and miR-26a positive) was treated with a miR26a oligo inhibitor, and subsequent TMZ treatment. Cellular proliferation was assayed. We demonstrated that treatment of LN229 glioma cells with the combination of microRNA inhibitor and TMZ resulted in a marked decrease in cell proliferation and viability.

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ABBREVIATIONS

> ……..……Greater than

ΔRn ……..……. Change in fluorescence signal

µg …….Microgram

µL ……Microliter

AKT……..Serine/threonine-specific protein kinase

Anti_has_miR-26a-1 ……...MiR26a inhibitor

ATP…….Adenosine-5'-triphosphate

B7-H1 …….Programmed cell death 1 ligand 1 (B7 homolog)

C ……..Celsius

CD28 ……Cluster of Differentiation 28 (Co-stimulatory T cell signal)

cDNA …… Copied DNA

de novo …….From a new

DGCR8/Pasha……... Protein localized to nucleus, component of RISC

Dicer ……..Endoribonuclease, cleaves pre-microRNA

DMEM …….Dulbecco's Modified Eagles Medium

DNA ……..…… Deoxyribonucleic acid

Drosha …… Class 2 RNase III enzyme

dsRNA ……..Double stranded RNA

EC_{50}.....Effective concentration that kills 50% of experimental cells

FBS ……..Fetal Bovine Serum

g ……..The force of gravity

GDP ……..Guanosine diphosphate

GBM ……..……..Glioblastoma multiforme

GTP ……..Guanosine-5'-triphosphate

HEPES……..4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HS_miR-26-a-1_1 ……..….Primer for miR26a

In vivo …… In living organism conditions

LN229 …….Glioma cell line

LOH ……..……Loss of Heterozygosity/Haploinsufficient

miRISC ……MiRNA-induced silencing complex

miRNA ……MicroRNA

miRNP …….. MicroRNA-containing ribonucleoprotein complexes

mL ……Milliliter

mM…….MilliMolar

MMR……. Mis-Match Repair

mRNA …….Messenger RNA

ms …….Milliseconds

nmol…….Nano mole

Oligonucleotide ……Short nucleic acid polymer

OncomiR …… MicroRNA associated with cancer

PCR ……Polymerase chain reaction

pg ……Pico gram

PI3K ……Phosphatidylinositol 3-kinases

PIP2……. Phosphatidylinositol (4, 5)-bisphosphate

PIP3……..….....Phosphatidylinositol (3, 4, 5)-triphosphate

Pre-miRNA ……Precursor miRNA

pri-miRNA ……Primary miRNA

PTEN……Phosphatase and Tensin Homolog

RISC…… RNA-induced silencing complex

RNA ……...Ribonucleic acid

RNAse…… Ribonuclease, catalyzes degradation of RNA

RT ……..Reverse transcription

S …….Seconds

SDS-Page…….. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS……... Sodium dodecyl sulfate

SYBR Green ……..Asymmetrical cyanine dye

T cells ……. T lymphocytes (white blood cells, originating in the Thymus)

TMZ …….Temozolomide

UTR ……Untranslated Region of DNA

v …… Voltage

WHO……..…..……World Health Organization

INTRODUCTION

Glioblastoma multiforme (GBM) is one of the most common and aggressive types of brain tumors (Huse et al. 2008). The World Health Organization (WHO) classifies GBM as a grade IV tumor due to its poor prognosis and median survival of 14 months after diagnosis. GBMs are heterogeneous; a single tumor may have a variety of cell types with distinct alterations to specific signal transduction pathways. GBM requires aggressive treatment including surgery, chemotherapy and radiation, however there has been little advancement in GBM treatments since the introduction of temozolomide as a treatment option.

Inactivation of PTEN has been shown to be involved in the formation of heritable and sporadic cancers (Planchon 2007). It has been demonstrated that the AKT signaling pathway can be continuously activated in GBMs, and in some cases this activation is due, in part, to the loss of Phosphatase and Tensin Homolog (PTEN) expression. PTEN contains both a protein phosphatase and a tensin-like region involved in cell adhesion and is a negative regulator of the AKT pathway. AKT is a serine/threonine protein kinase with several important roles in multiple cellular processes including; cell proliferation, cell survival, transcription and cell migration (Wang 2012). Phosphatidylinositol 3, 4, 5 tri-phosphate (PIP3), is a signaling molecule that activates AKT, setting in motion a cascade of signaling pathways. PTEN negatively regulates the AKT signaling

pathway by dephosphorylating PIP3 to phosphatidylinositol 4, 5 bi-phosphate (PIP2) and PIP2 cannot activate AKT thus preventing unregulated cell growth. With the loss of PTEN, PIP3 can accumulate within the cell creating a consistent stream of activation for AKT.

Additionally a recent review paper by Parsa et al. (2007) showed a connection between the loss of PTEN and an increase in B7-H1 cell surface receptors on GBM cells. B7 is traditionally a receptor on antigen presenting cells, that when paired with the CD28 receptor of T cells causes a stimulatory signal to illicit an immune response. B7-H1 (B7 homolog) which is up regulated with the loss of PTEN assists the tumor cells in evading the immune system. The signal produced when T cells interact with B7-H1 is inhibitory and prevents a response. PTEN deficient cells were thus rendered less susceptible to cytotoxic T lymphocyte-mediated targeting (Waziri et al. 2010). Tumor specific T cells were found to lyse human glioma targets expressing wild-type PTEN more effectively than those expressing mutant PTEN; this data identifies an unrecognized mechanism linking loss of the tumor suppressor PTEN with immunoresistance (Parsa et al. 2007). Treatment of patients with drugs that selectively target cancer cells with loss of PTEN could improve response to T cell mediated immunotherapy by decreasing local immunoresistance (Parsa et al. 2007). The results of Parsa et al. (2007) could impact immunotherapy trials by defining the loss of PTEN function as important molecular screening criteria.

Recent studies have shown that PTEN can be epigenetically silenced. Epigenetics is a way of altering gene expression that does *not* involve changes in the nucleotide sequence of DNA or RNA. Epigenetic regulation is manifested as methyl groups added to DNA/RNA following replication or miRNA binding to mRNA preventing translation. Another epigenetic regulator of PTEN is miR-26a microRNA.

MicroRNAs (miRNAs) are small (~22 nucleotides), post-transcriptional regulators that bind to complementary sequences in the three prime untranslated regions (3' UTR) of target messenger RNA transcripts (mRNAs), usually resulting in gene silencing (Huse et al. 2008). MiRNAs are small noncoding RNA molecules that regulate protein expression by targeting the mRNA of protein coding genes for either cleavage or repression of translation (Chan et al. 2005). The miRNA works to sequester the mRNA by binding to it and preventing translation. Most miRNA genes are found in intergenic regions (between gene clusters) making it an endogenous molecule. The miRNA genes contain their own promoter and regulatory units. MiR-26a in particular acts on the mRNA of PTEN, acting as a miRNA associated with cancer or oncomiR that negatively regulates PTEN protein. Huse et al. (2008) demonstrated that miR-26a is frequently amplified in human glioma, and miR-26a-mediated PTEN repression in a murine glioma model enhances *de novo* tumor formation. The biogenesis of miRNA requires several posttranscriptional steps to yield the functional mature

miRNA (Esquela-Kerscher and Slack 2006). MiRNAs are created by the microprocessor complex of Drosha and DGCR8 (Esquela-Kerscher and Slack 2006). Drosha is in the RNAse-III family and is activated when bound to the nuclear protein DGCR8 (which is also known as pasha).This complex cleaves the correct 70 nucleotides from the miRNA gene creating the primary miRNA (pri-miRNA). The pri-miRNA is then cleaved into a double stranded stem and loop conformation with a 2 nucleotide overhang, resulting in what is known as the preliminary miRNA (pre-miRNA). The pre-miRNA is exported out of the nucleus by exportin-5, which recognizes the 2 nucleotide overhang, and Ran-GTP. Ran-GTP or Ras associated nuclear protein transports molecules in/out of the nucleus. This action of transport is dependent on the hydrolysis of guanine triphosphate (GTP) to guanine diphosphate (GDP). Once in the cytoplasm the pre-miRNA complex binds to Dicer. Dicer is an endoribonuclease that cleaves the pre-miRNA duplex. The now single strands of miRNA are considered mature. When the mature miRNA is combined with the RNA-induced silencing complex (RISC) the miRISC is formed (Esquela-Kerscher 2006). The miRISC is the machinery that binds to mRNA transcripts allowing the miRNA to interact with its target mRNA. Degradation of the target mRNA only occurs if there is perfect complementary binding. Some miRNA-mRNA binding complexes are not perfectly complementary; the result is steric hindrance of the translational machinery preventing mRNA translation into a functional protein (Figure 1).

PTEN is often referred to as a haploinsufficient gene (Salmena et al. 2008). Following the loss of one allele the remaining allele is not sufficient to produce a functional protein. Epigenetic alterations such as miR-26a binding to the mRNA of PTEN, prevents the production of a functional protein without loss of the PTEN gene. It has also been demonstrated in a murine model that over expression of the miRNA can functionally substitute for the loss of heterozygosity at the PTEN locus, leading to the haploinsufficient state of the gene (Huse et al. 2008). Even with an intact PTEN locus, the over expression of miR-26a creates the same haploinsufficient state as if the locus were deleted or mutated. The roles of miRNAs in lineage determination and proliferation as well as the location of several miRNA genes at sites of translocation breakpoints or deletions has led to the speculation that miRNAs could be important factors in the development or maintenance of the neoplastic state (Chan et al. 2005). As of yet, no microRNA has been definitively shown to either enhance or suppress the process of glioma formation *in vivo*. However, it has been demonstrated that miR-26a reduces PTEN levels by directly targeting the PTEN mRNA transcript, by binding to one of three known binding sites (Huse et al. 2008). With the loss of a tumor suppressor gene, such as PTEN, the cell might be more likely to undergo transformation. Additionally if PTEN has a role in TMZ functionality its loss would increase chemotherapy resistance.

Jiang and colleagues (2007) reported that decreased PTEN expression plays a role in the cell's ability to resist chemotherapy treatment, specifically temozolomide. They reported that inducing PTEN expression down regulated AKT activity and enhanced cell sensitivity to TMZ. The exact mechanism for TMZ resistance due to PTEN loss is unknown at this time however it is speculated that the level of phosphorylated AKT might play a role. Temozolomide is the current standard of care for GBM chemotherapy and acts as an alkylating agent that adds a methyl group to the O6 or N7 position on guanine residues in the cell's DNA. This alteration to the DNA activates the cells mis-match repair system (MMR) (Chamberlain and Mrugala 2008). Continued mis-match by the cell's MMR mechanism creates accumulations of double stranded breaks. Ultimately cell proliferation is halted by either apoptosis or termination of cell division, due to the accumulation of DNA damage. An intact mis-match repair mechanism is required for TMZ induced cytotoxicity (Chamberlain and Mrugala 2008).

PROJECT OBJECTIVES

The GBM cell line LN229 (ATCC#CRL-2611) is reported to contain the wild-type PTEN gene, but also expresses the miRNA miR-26a (Huse et al. 2008, Furnari et al. 1997). The LN229 cell line was established from a female glioblastoma patient in 1979 (ATCC). The presence of miR-26a has an effect on PTEN's tumor suppressor ability by preventing translation of the genetic information into a protein (Huse et al. 2008). If the presence of PTEN in the cell is thought to increase the potency of its tumor suppression ability, the knockdown of miR-26a should increase the amount of PTEN protein and increase the LN229 cells sensitivity to chemotherapy agents such as TMZ. This research examined the hypothesis that silencing of miR-26a will allow PTEN to be translated into protein increasing the cells sensitivity to TMZ, resulting in a decrease in the cells viability. The following steps were used to support this hypothesis: LN229 glioma cells were confirmed to contain miR-26a using RT-PCR. The miRNA was silenced with the transfection of an anti-miR-26a oligonucleotide producing a decrease in cell viability. Relative levels of PTEN protein were examined using a western blot, before and after transfection with anti-miR26a. Temozolomide was then combined with the anti-miR-26a treatment to verify further decrease in cell viability.

Chapter 1

PTEN: a tumor suppressor

Structure and Function of PTEN

Phosphatase and Tensin homolog (PTEN) is a lipid phosphatase tumor suppressor protein. A ubiquitous inhibitor of the AKT pathway by dephosphorylation of PIP3, PTEN assists in regulation of the cell cycle to prevent unregulated growth (Leslie and Downes 2004). A member of the protein tyrosine phosphatase family, PTEN removes phosphates from phosphorylated tyrosine residues on proteins. Removal of the phosphate in the 3 position on the inositol ring of tyrosine prevents activation of the AKT pathway by termination of PIP3 signaling (Wang et al. 2012). The AKT pathway is responsible for cellular survival mechanisms: glucose metabolism, cellular proliferation, prevention of apoptosis, and cell migration (Wang et al. 2012). PIP3 binds AKT activating the protein; once activated AKT moves to the cytoplasm and nucleus allowing for stimulation of several downstream targets involved in cellular functions (Wang et al. 2012). The level of PIP3 is very low in resting cells that contain wild type PTEN. Cells that lack PTEN have elevated levels of PIP3, even in the resting state. (Sulis and Parsons 2003). Functional PTEN maintains the cell cycle within normal operating parameters; however a loss of functional PTEN can allow unregulated cellular growth with the potential to become cancerous.

Mechanisms for loss of PTEN in Glioblastoma

The PTEN gene, which encodes for the PTEN protein, is located on chromosome 10 and in some glioblastoma cases the locus for the PTEN gene is mutated or deleted. Even partial loss of the PTEN gene prevents protein production due to a haploinsufficient state (Leslie and Downes 2004 Salmena et al. 2008). Haploinsufficiency is when one locus is not suitable to produce adequate amounts of functional protein. The loss of functional protein can result from mechanisms other than gene deletion. Leslie and Downes (2004) noted that 54% of glioblastomas have a loss of expression of PTEN, meaning there could be an intrinsic mechanism preventing protein production. Epigenetic alteration of the PTEN mRNA transcript would alter the amount of protein expressed in the cell. Epigenetics manifest as methyl groups added to DNA/RNA or miRNA binding post-transcriptionally. MicroRNAs are post-transcriptional regulators that prevent translation of mRNAs and ultimately protein production. PTEN has two well-known microRNAs found in GBMs that bind and prevent its protein production; miR-21 and miR26a (Leslie and Foti 2011). The latter miRNA is known to have 3 binding sites on the PTEN mRNA transcript, making it a more attractive target for therapy (Huse et al. 2008).

Materials and Methods

Western Blot

LN229 cells were plated at a density of $5x10^4$ cells/well in a 24-well plate. The cells were then harvested 24 hours later to create a whole cell lysate. Sample lysis buffer was placed in boiling water to warm. The sample lysis buffer was composed of 20 mM HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*), 1% SDS with a pH of 7.3. One hundred to 150 microliters of sample lysis buffer was administered to each of the 24 wells to lyse the cells. The lysate was pipetted to mix, collected and pooled, resulting in 4 mL total volume. The LN229 lysate was determined to contain 1.1 μg/μL of protein using a BCA protein assay (Bio-Rad). The lysate sample was combined with Laemmli sample buffer and pipetted into a Bio-Rad SDS-Page pre cast gel in 15 μL and 50 μL volumes. The gel was run in 1x tris/Glycine/SDS running buffer at a constant voltage (39 v) for 55 minutes. The gel was then placed on a nitrocellulose sheet in a Bio-Rad transfer cassette. The transfer cassette was placed in western transfer solution (Tris 48 mM, Glycine 39 mM, SDS 0.04%, 200 mL methanol and 800 mL dH20) in the BIO-RAD Power Pack machine at constant amperage 0.10 A, 39 v and 4 w for 10 hours. After the transfer, the nitrocellulose paper was placed in a 3% nonfat milk blocking buffer for 30 min. The primary antibody, mouse monoclonal anti-PTEN (Calbiochem), was applied in a 1:200 dilution and incubated for 11 hours.

The secondary antibody, goat anti-mouse labeled with horseradish peroxidase (Thermo scientific), was applied in a 1:1000 dilution, for 1 hour. Between the primary and secondary antibody applications the membrane was washed 3 times for 15 min each in TBST (Table 1). Following final antibody incubation the membrane was washed 3 more times in TBST prior to visualization.

Visualization

Protein was visualized using SuperSignal ELISA Pico Chemiluminescent Substrate (Bio-Rad). A 1:1 ratio (undiluted) of the substrate was applied to the nitrocellulose paper. The proteins were imaged immediately using Kodak 1D image system (Figure 2).

Results/Discussion

PTEN was confirmed to be present within the LN229 cell line as indicated by well-defined bands in the western blot (Figure 2). This result was expected as the LN229 cell line was previously reported to be wild-type for the PTEN protein (Furnari et al. 1997). PTEN expression was expected due to the intact wild-type gene. However if miR-26a is present the amount of protein should be decreased but not necessarily completely eliminated. A decrease in PTEN protein may be sufficient to alter the tumor suppressor role of PTEN and to alter the cells response to the chemotherapy agent TMZ.

Chapter 2

Temozolomide: Current Standard of Care

Action of Temozolomide

Temozolomide (chemical structure: 3, 4-dihydro-3-methyl-4-oxoimidaso [5, 1-d]-1, 2, 3, 5-tetrazine-8-caboxamide) is the current standard of care for chemotherapy treatment of GBM. The drug has been used since August of 1999 in the United States. TMZ is efficiently absorbed after oral administration, and readily crosses the blood-brain barrier (Chamberlain and Mrugala 2008). The therapeutic benefit of TMZ depends on its ability to alkylate/methylate DNA. TMZ adds a methyl group to either the O6 or N7 position on guanine residues. The presence of the methyl group on the O6 position of the guanine residues causes mis-matching during DNA replication, the guanine is matched with a thymidine as opposed to a cytosine (Chamberlain and Mrugala 2008). The mis-match triggers activation of the cell's mis-match repair system (MMR). The MMR system removes the incorrect base pair match and the replication is resumed. However, the same mis-match occurs again, due to the ongoing presence of the methyl group. This mis-match and the futile repair keep occurring until numerous double-stranded DNA breaks accumulate in the cell. It is the accumulation of these double-stranded DNA breaks that leads the cell to apoptosis.

Loss of PTEN and chemotherapy resistance

In breast cancer, reduction of PTEN by antisense oligonucleotides conferred trastuzumab resistance *in vitro* and *in vivo*. Patients with PTEN-deficient breast cancers had significantly poorer responses to trastuzumab-based therapy than those with normal PTEN (Nagata et al. 2004). It has been demonstrated that loss of PTEN decreases cells sensitivity to chemotherapy treatments in several types of cancer and chemotherapies. In GBM decreased PTEN levels reduced cell sensitivity to TMZ and induction of PTEN expression was found to enhance cell sensitivity to TMZ (Jiang et al. 2007). PTEN can sensitize glioma cells to chemotherapy and also induce apoptosis (Wang et al. 2002). When PTEN is present it can sensitize cells to TMZ, allowing TMZ to have a greater affect in decreasing cell viability compared to cells where PTEN is not present. Jiang et al. (2007) also found that by inducing PTEN, in PTEN-deficient cells, TMZ sensitivity was restored through a decrease in AKT pathway activity. The induction of wild-type PTEN in U251-wtPTEN cells led to a statistically significant increase in sensitivity to a range of temozolomide doses from 10 to 50 μmol/L (Jiang et al. 2007). The exact mechanism for the increase of sensitivity to TMZ when functional PTEN is present has yet to be elucidated. It has been suggested that a deficiency in MMR coupled with the haploinsufficiency of

PTEN can account for resistance to TMZ and tumor progression. It has been shown that MMR deficiency can accelerate tumorgenesis in PTEN +/- mice (Wang et al. 2002). Another cellular mechanism that confers resistance to TMZ is the presence of O6-methylguanine-DNA methyltransferase (MGMT). As stated, TMZ is an alkylating agent that acts on the O6 or N7 positions on guanine residues in DNA. The DNA changes created by TMZ are a major carcinogenic lesion. The cell has mechanisms to repair such lesions; one mechanism is the enzyme MGMT. MGMT is a suicide repair protein that removes the cytotoxic O6 guanine produced by the alkylator-based chemotherapy, thereby inducing resistance (Chamberlain and Mrugala 2008). The repair protein is not regenerated once the alkyl group is removed. It has been reported that LN229 cells do not express MGMT (Davis 2008). The lack of MGMT expression in LN229 cells rules out MGMT as a mechanism for resistance.

Chapter 3

MicroRNA: Small RNAs with big impact

Biogenesis, Structure and Function

MicroRNAs are endogenous small RNAs that bind the 3' UTR of target mRNA transcripts. The miRNA genes possess all the necessary machinery to produce a functional mature miRNA. The biogenesis of miRNA begins with the RNAse-III enzyme Drosha and its cofactor DGCR8 (Esquela-Kerscher and Slack 2006). DGCR8 is also known as pasha; a protein localized to the nucleus. Pasha binds to Drosha forming the microprocessor complex. Pasha is thought to stabilize the pri-miRNA for binding by Drosha (Esquela-Kerscher and Slack 2006). The Drosha and DGCR8 complex recognizes the pri-miRNA sequence and cleave the strand into a 70 nucleotide dsRNA with a 2 nucleotide overhang (Zamore and Haley 2005). Once cleaved the pri-miRNA has a stem-loop conformation with an overhang, this is now known as the pre-miRNA (Turner et al. 2010). The pre-miRNA overhang is recognized by exportin-5 and Ran-GTPase which exports the pre-miRNA, from the nucleus. Exportin-5 mediates transport of cargo from the nucleus into the cytoplasm by interacting with Ran. Ran-GTPase transports molecules in/out of the nucleus during interphase of mitosis. This action of transport is GTP dependent, meaning the energy from the hydrolysis of GTP to GDP is necessary. Once in the cytoplasm the pre-miRNA is further

digested by Dicer (Turner et al. 2010). Dicer is an endoribonuclease in the RNase III family that cleaves the pre-miRNA into short double-stranded RNA fragments. Cleavage by Dicer creates a mature miRNA (Turner et al. 2010). Dicer then catalyzes the first step in the miRNA mediated interference of protein expression. Formation of the RNA-induced silencing complex (RISC) allows the mature miRNA to bind the target mRNA. RISC is also known as a microRNA ribonucleoprotein complex (miRNP) and RISC with incorporated miRNA is sometimes referred to as "miRISC" (Zamore and Haley 2005). The miRNP RISC can either perfectly match the target mRNA transcript resulting in degradation of the whole complex or bind and prevent translation due to steric hindrance if not completely complementary (Esquela-Kerscher and Slack 2006; Figure 1).

Materials and Methods

Cell Culture

The glioblastoma cell line LN229 (ATCC#CRL-2611) was used. LN229s were cultured in Dulbecco's modified Eagle's medium (DMEM), 5% FBS. The cell line was maintained without antibiotics, at 37°C in a humidified atmosphere containing 5% CO_2.

Determination of miR-26a presence in LN229 cell line was accomplished by SYBR Green miScript Reverse Transcription PCR.

Reverse Transcription

RNA Isolation/Purification

RNeasy Mini Kit (Qiagen) was used to isolate and purify total RNA, including miRNA from the LN229 glioma cells following the manufacturer's protocol. Briefly, cells were grown in a culture flask as a monolayer until 90% confluent. The cells were treated with trypsin-EDTA and pelleted prior to lysis. Cells were disrupted by adding 600 μL of Buffer RLT to the pellet and then vortexed. The lysate was pipetted directly into a QIAshredder spin column placed in a 2 mL collection tube, centrifuged for 2 minutes at >8000 x g. One volume (600 μL) of 70% ethanol was added to the homogenized lysate, and mixed by pipetting. Up to 700 μL of the sample was transferred to an RNeasy spin column in a 2 mL collection tube. The lid was closed securely and centrifuged for 15 seconds (s) at >8000 x g. The flow through was discarded. Buffer RW1 (700 μL) was added to the RNeasy spin column. The sample was centrifuged again for 15 s at >8000 x g. Buffer RPE (500 μL) was added to the RNeasy spin column and centrifuged for 15 s at > 8000 x g, the flow through was again discarded. An additional 2 minute centrifugation was performed with RPE buffer (500 μL) to wash the spin column

membrane (> 8000 x g). The RNeasy spin column was placed in a new 1.5 mL collection tube and centrifuged for 1 minute at > 8,000 x g to elute miRNA.

Reverse transcription (RT)

A master mix was created using the guidelines provided in the miScript PCR System Handbook (Qiagen) (Table 2). The master mix was placed on ice until the RNA reaction was prepared. The RNA template was prepared as follows: For each 20 µL RT reaction, master mix was combined with total RNA in the ratio of 1 µL master mix: up to 1 µg total RNA (10pg to 1µg per reaction). MiScript RT buffer (4 µL) was combined with a pre-designed PTEN primer from Qiagen (UUCAAGUAAUCCAGGAUAGGCU) and RT master mix; incubated for 60 minutes at 37^0C then incubated for 5 minutes at 95^0C to inactivate miScript Reverse Transcriptase mix. Tubes were stored at -20^0C if not proceeding directly to PCR amplification of cDNA.

Real Time-PCR Amplification

A reaction volume of 20 µL was pipetted into a 48-well plate using the following: Volume/reaction: 10 µL QuantiTect SYBR Green PCR Master Mix (Qiagen), 1 µL Product from RT reaction (cDNA), 2µL 10x miScript Universal Primer(Qiagen), 2 µL 10x HS_miR-26a-1_1 miScript Primer Assay(Qiagen), 5 µL of Nuclease-free water (Table 3). Twenty microliters of each sample was placed

into the wells of a 48-well plate. The plate was centrifuged briefly to mix the reagents thoroughly. The tubes were held at 95°C for 15 minutes, and then the following thermal cycling conditions used were: 15 seconds at 94°C to denature, 30 seconds at 55°C to anneal 30 seconds at 70°C for extension (Figure 3). There were 40 cycles run.

Results

The results for the amplification of miR-26a are shown in Figure 4. Each sample well tested showed positive amplification for miR-26a compared to the negative control. This data confirms the presence of the target miRNA within the LN229 glioma cell line.

Discussion

The LN229 cell line was found to contain the miRNA in question, miR-26a. This result was expected as the LN229 glioma cell line had previously been reported to contain the microRNA miR26a (Huse et al. 2008). Given these results it could be predicted that knockdown of miR26a should have an effect on the cellular level of PTEN protein and the viability of the LN229 glioma cell line.

Chapter 4

MicroRNA Inhibition

Background

Studies using LN229 GBM cell lines indicate PTEN is "silenced" in a post-translational mechanism by miR-26a miRNA (Nicoloso and Calin 2008). Silencing of miR-26a was accomplished via transfection of a miScript miRNA inhibitor, Anti-has-miR-26a-1 oligonucleotide (Qiagen). Anti-has-miR-26a-1 is a modified antisense single–stranded oligonucleotide (Qiagen). It is designed to be complementary to the miR-26a miRNA and specifically inhibits its expression by binding to miR-26a. Binding of Anti-has-miR-26a-1 oligonucleotide to miR-26a prevents the miRNA from binding to the PTEN transcript. Transfection was completed using the Neon transfection system (Invitrogen) using electroporation. Electroporation is a method used to increase the electrical conductivity and permeability of the cell plasma membrane, by an externally applied electrical field. This method is used to introduce new or foreign substances into a cell, such as anti-miR26a oligonucleotide. The viability of the LN229 cells was determined by quantitation of the amount of ATP. The luminescence signal generated correlates with the amount of ATP present, which is directly proportional to the number of live cells in culture. The smaller the luminescent signal the fewer the number of live cells present. Western blot was employed to detect increased presence of

PTEN protein. Knocking down/silencing miR-26a should allow PTEN mRNA to be translated and potentially increase protein expression.

Materials and Methods

Transfection

Twenty wells of a 96-well plate were used to create and incubate the transfected cell suspensions (Table 4). Four different miR26a inhibitor concentrations were used to treat each cell suspension. Untreated cells were electroporated with buffer only. Inhibitor treated cells were electroporated with the following concentrations of inhibitor: 50 μM (1.5 μL distilled water, 0.50 μL anti-has-miR-26a-1); 75 μM (1.25 μL distilled water, 0.75 μL anti-has-miR-26a-1); 100 μM (1.0 μL distilled water, 1.0 μL anti-has-miR-26a-1); and 200 μM (2.0 μL anti-has-miR-26a-1). The original stock concentration of the anti-has-miR-26a-1 was 100 nmol. LN229 cells were diluted to $5x10^3$ cells per well in 56 μL of resuspension buffer R (Invitrogen). Anti-has-miR-26a-1 miScript miRNA inhibitor (Qiagen) treatment was accomplished by electroporation using the Neon Transfection system (Invitrogen). Ten microliters of cell suspension including anti-has-miR26a-1-miScript miRNA was placed in the Neon pipette station with 3 mL of electrolytic buffer E (Invitrogen). The LN229 cells were treated with the following

parameters; Pulse voltage 1050 v, and 40 ms width. This should provide a transfection efficiency of 73% and a cell viability of 80% as previously determined by Invitrogen (Table 5). The cell suspension post-pulse was placed into complete media and incubated at 37°C for a 24 hour period.

CellTiter-Glo Luminescent Assay

After 24 hours of incubation the plate was equilibrated to room temperature for 30 minutes. CellTiter-Glo reagents were added to each well in an amount equal to the cell culture media (100 μL was used). Contents were mixed for 2 minutes, the plate was then incubated at room temperature for 10 minutes to stabilize luminescent signal (Figure 5). Luminescence was recorded at an integration time of 1 second per well on the Turner Biosystems Modulus microplate luminometer. Luminescence was measured in relative light units, which correlate with the number of active cells within the sample.

Statistics

Statistical analysis of data was performed using IBM SPSS Statistics 19 and Microsoft Excel. A one way ANOVA with Tukey post hoc test was performed on the luminescence data to determine if there was a significant difference between the luminescence amounts in the five treatment groups: untreated, 50 μM, 75 μM, 100 μM and 200 μM.

Western Blot

LN229 cells were plated at a density of 5 x 10^4 cells/well in a 24-well plate. Ten microliters of LN229 cells were transfected with anti-has-miR26a-1 at a concentration of 100 μM with resuspension buffer R (Invitrogen) using the Neon Transfection system (Invitrogen) as previously described. The cells were then harvested 24 hours later by direct application of sample lysis buffer to each well. The lysed cells were used to create a whole cell lysate. Lysis buffer contained 20 mM HEPES and 1% SDS at a pH of 7.3. Sample lysis buffer was placed in boiling water to warm. One hundred to 150 μL of sample lysis buffer was administered to each of the 24 wells to lyse the cells. The lysate was pipetted to mix, collected and pooled in a 15 mL tube, resulting in 4mL total volume. The LN229 lysate was determined to contain 0.457 ng/μL of protein (Figure 6). The lysate sample was combined with Laemmli sample buffer and pipetted into a Bio-Rad SDS-Page pre cast gel in 10 μL and 50 μL amounts. The gel was run in 1x tris/Glycine/SDS running buffer at constant voltage (39 v) for 55 minutes. The gel was then placed on a nitrocellulose sheet in a transfer cassette (BIO-RAD). The transfer cassette was placed in western transfer solution (Tris 48 mM, Glycine 39 mM, SDS 0.04%, 200 mL methanol and 800 mL dH20) at constant amperage 0.10 A, 39 v and 4 w for 10 hours. After the transfer the nitrocellulose paper was placed in a 3% nonfat milk blocking buffer for 30 min. The primary antibody, mouse monoclonal anti-PTEN was applied in a 1:200 dilution and

incubated for 11 hours. The secondary antibody, goat anti-mouse labeled with horseradish peroxidase (Thermo scientific), was applied in a 1:1000 dilution for 1 hour. Between the primary and secondary antibody applications the membrane was washed 3 times for 15 min each in TBST (Table 1). Following final antibody incubation the membrane was washed 3 more time in TBST prior to visualization.

Visualization

Protein was visualized using SuperSignal ELISA Pico Chemiluminescent Substrate. A 1:1 ratio (undiluted) of the substrate was applied to the nitrocellulose paper. The proteins were imaged using Kodak 1D (Figure 7).

Results

A range of anti-miR26a concentrations were tested; 50, 75, 100 and 200 μM to determine which concentration had the greatest effect. Each treatment amount of anti-miR26a had a significant effect (P=0.0004) compared to untreated cells (Figure 8). The anti-miR26a treatment was consistent in decreasing the LN229 cell line viability through all concentrations, over a 24 hour period. The electroporation alone did not have a significant effect on cell viability; all untreated cells were electroporated in subsequent experiments. The western blot

showed a greater amount of PTEN protein in the anti-miR26a treated LN229 cells, determined by visual inspection (Figure 7).

Discussion

It is clear the inhibition of miR-26a reduced the LN229 cell viability and PTEN protein levels. Tumor suppressor genes are subject to numerous regulatory mechanisms including epigenetic effects and posttranslational modifications that ultimately govern protein levels, activity, binding partners and function (Salmena et al. 2008). It was demonstrated in studying three human glioblastoma samples that miR-26a directly targets PTEN and enhances the AKT pathway; transfecting miR-26a into a murine model showed that over expression enhances gliomagenesis (Turner et al. 2010). In this study it was demonstrated that inhibition of miR26a in a cell line that is expressing it, was successful in decreasing the cells viability. The decrease in cell viability could be due to an increased amount of PTEN protein. The western blot showed an increase in the amount of PTEN protein within the LN229 cells treated with anti-miR26a (Figure 7). The quantification of the increase in PTEN protein amount was based upon visual inspection. It can be inferred that the increase of PTEN protein resulted in the decrease in cell viability, possibly due to a reduction in active AKT, but further research would need to be completed to confirm this.

Chapter 5

Combination Temozolomide and miR-26a inhibitor Treatment

Background

If the demonstrated inhibition of miR-26a was successful in restoring functional PTEN protein it is likely to make the LN229 cells more sensitive to chemotherapy treatment with TMZ. A combination treatment with the anti-miR-26a inhibitor and 400 uM (the EC_{50} dose of TMZ) (Moroz et al. 2011) was tested.

Materials and Methods

Transfection

Thirty wells of a 96 well plate were used to incubate anti-has-miR-26a-1 and TMZ treated LN229 cells at a density of $5x10^3$ cells/well in 56 μL of resuspension buffer R (Invitrogen). Cell suspension (100 μL per well) was placed in a 96 well plate as described in Table 6. Anti-has-miR-26a-1 concentrations used were as follows: 75 μM (1.25 μL distilled water, 0.75 μL anti-has-miR-26a-1); 100 μM (1.0 μL distilled water, 1.0 μL anti-has-miR26a-1); and 200 μM (2.0 μL anti-has-miR26a-1. The 50 μM treatment was not used in combination treatment due to inconsistent results. Transfection of anti-has-miR26a was accomplished by electroporation using the Neon Transfection System (Invitrogen). Ten microliters

of cell suspension (cells and resuspension buffer R), including anti-has-miR26a-1, was placed in the Neon pipette station in 3 mL of electrolytic buffer E (Invitrogen). The following treatment parameters were applied; Pulse voltage 1050 v, and 40 ms width. This should provide a transfection efficiency of 73% and a cell viability of 80% as previously determined by Invitrogen (Table 5). The cell suspension post-pulse was placed into complete media containing EC_{50} dosage of TMZ (400uM) (Moroz et al. 2011). The cells were incubated at 37°C for a 24 hour period.

CellTiter-Glo Luminescent Assay

After 24 hours of incubation the plate was equilibrated to room temperature for 30 minutes. CellTiter-Glo reagents were added to each well in an amount equal to the cell culture media of each well (100 μL was used). Contents were mixed for 2 minutes, the plate was then incubated at room temperature for 10 minutes to stabilize luminescent signal. Luminescence was recorded at an integration time of 1 second per well on the Turner Biosystems Modulus microplate luminometer. This test should show if indeed the presence of functional PTEN within the cell is beneficial for increasing a chemotherapy agent's effect and decreasing the LN229 cells viability. Three different trials were completed; each was conducted as a separate experiment to ensure results were consistent.

Statistics

Statistical analysis of data was performed using IBM SPSS Statistics 19 and Microsoft Excel. A one way ANOVA with Tukey post hoc test was performed on the luminescence data to determine if there was a significant difference between the luminescence amounts in the three treatment groups: untreated, inhibitor only treated, TMZ only and combination (TMZ and inhibitor) treatment.

Results

Compared to anti-has-miR26a-1 treatment only the combination treatment of anti-has-miR26a-1 and TMZ significantly decreased cell viability over a 24 hour period in trial 1 (Figure 9). Trial 2 (Figure 10) shows the combination of Anti-has-miR26a miRNA and TMZ treatment had a greater effect on decreasing cell viability than the inhibitor only.

Discussion

Statistical analysis of data was performed to determine significant between miR26a inhibitor treated LN229 cells and untreated LN229 cells. A one-way ANOVA with Tukey post-hoc test confirmed a significant difference ($P=0.0001$) between the treatment groups (inhibitor, TMZ, and combination). The decrease in cell viability is thought to be due to an increase in functional PTEN protein,

this was supported by the western blot results (Figure 7). The combination of TMZ and miR-26a inhibitor had a greater effect on LN229 cell viability then either inhibitor or TMZ only in trial 3 (Figure 11). The inhibitor only treatment reduced cell viability and appeared to cause an increase in PTEN protein. Even with the PTEN protein increase the inhibitor only treatment was not as effective at reducing cell viability as the TMZ only or combination treatments in trials 2 and 3. The inhibitor only might have been less effective because of residual activated AKT present in the cell. The results of the combination treatment showed further reduction in cell viability which appeared to be greater than either the anti-miR26a or TMZ treatments alone. These results indicate that miR-26a is a viable target to increase the efficacy of TMZ treatment. The electroporation did not have a significant effect on the cell growth.

SYNOPSIS AND CONCLUSIONS

This study showed the inhibition of miR-26a when combined with TMZ treatment results in an increased TMZ response and a decrease in cell viability. This suggests that the presence of the anti-miR26a oligo binds miR-26a and inhibits its ability to decrease the translation of PTEN mRNA into protein. Down regulation of miR-26a could be confirmed by real time PCR, but was not in this study. The up regulation of PTEN protein was visually confirmed by the western blot of the anti-has-miR-26a miRNA treated cells. The LN229 cells were successfully transfected using the Neon transfection system (Invitrogen) through electroporation. The electroporation did not have a significant effect on the LN229 cells viability. Other means of PTEN detection could be used to verify up regulation. PCR for the mRNA PTEN transcript could be used to compare pre and post transfection levels. Immunocytochemistry could be used to stain the cells directly for PTEN protein presence. MiR-26a has a complex role in cancer. It has been demonstrated in hepatocellular carcinoma miR26a has reduced expression compared to the high levels of normal expression in diverse tissue (Kota et al. 2009). A balance is needed for miRNAs to help regulate normal cellular proliferation. The literature has support for both sides of the tumor suppressor/oncogenic argument, indicating the need for a clearer picture of miRNAs involvement in tumorgenesis. Clearly inhibition of miR26a has an effect

on the viability of LN229 cells. This is a beneficial finding because inhibition of miR-26a allows the current standard of GBM care, TMZ, to work more effectively. All results indicate miR-26a miRNA is a plausible target in GBM therapy.

FUTURE DIRECTIONS

Other studies that could be performed to further this research would be to compare these results with similar tests performed on PTEN mutated cells, such as the U87 GBM cell line. Comparing wild-type PTEN cells with mutated PTEN cells would give a greater indication that miR26a is in fact inhibiting PTEN protein production. Additionally looking at downstream effects of anti-miR26a application would be helpful in clarify the miRNAs function. Targets such at AKT activity after treatment with anti-miR26a might help create a clearer picture of the effect miR26a has on the cell cycle. Exiqon has been developing a protocol to sample amounts of miRNA within serum samples from patients. This protocol has the potential to be used as a prognostic factor for the severity of one's tumor; currently the protocol only involves colo-rectal cancer. This is a potential stepping stone for a non-invasive test to determine a patient's tumor severity, possible even how a tumor would react to chemotherapy treatment with TMZ. For example if a patient had a blood sample taken and miR26a was found in a substantial amount it could be inferred that the tumor might be less sensitive to TMZ than if there was no miR26a present in the sample. The fact that miRNA overexpression in cancer has a pathogenic effect provides the rationale for using miRNAs as potential therapeutic targets in cancer. A robust increase in miR-21 expression was found in six commonly used model cell lines derived from human

glioblastoma, this represents only initial studies that should be followed by large trials in patients with brain cancers to support the biomarker roles for miRNAs (Nicoloso & Calin 2008).

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APPENDIX A

Figures and Tables

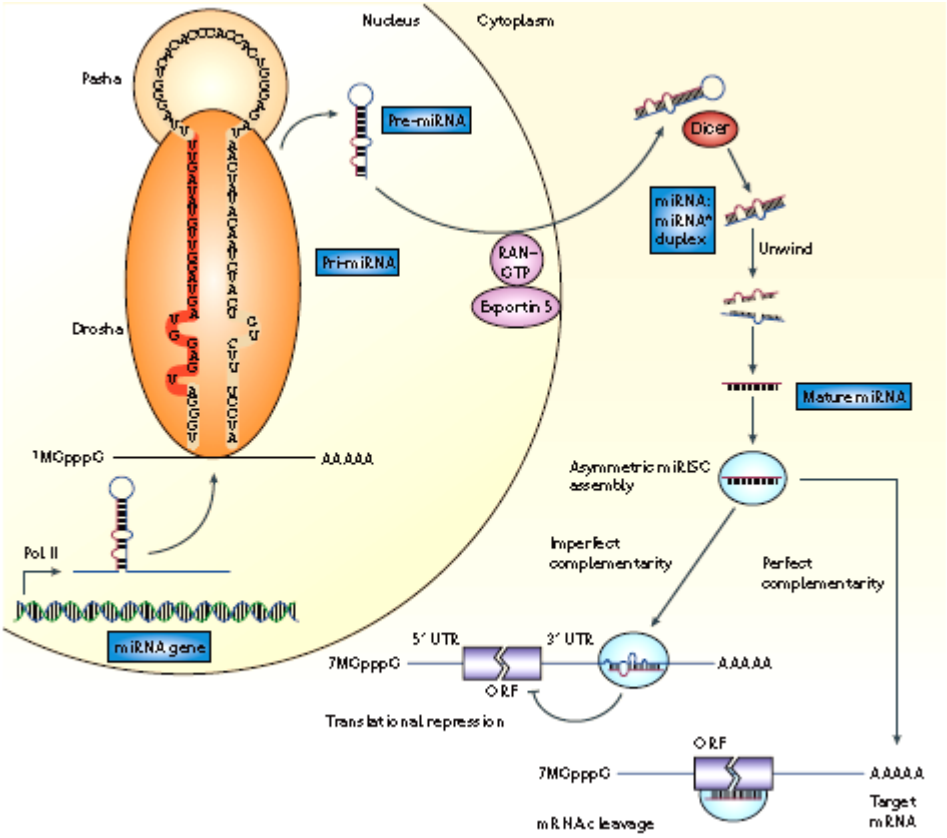


Figure 1.Biogenesis of microRNA. Begins in the nucleus with Drosha and Pasha. Once in the cytoplasm Dicer cleaves the double stranded miRNA complex to create a mature miRNA. Mature miRNA can then assemble miRISC. If imperfect complementarity translation is repressed, perfect complementarity is cleaved and degraded (Esquela-Kerscher and Slack 2006).

Figure 2. Western Blot for PTEN. The clear bands imaged confirm PTEN protein within LN229 lysate. The lysate amount from left to right is 15 μL, 50 μL, 15 μL and 50 μL.

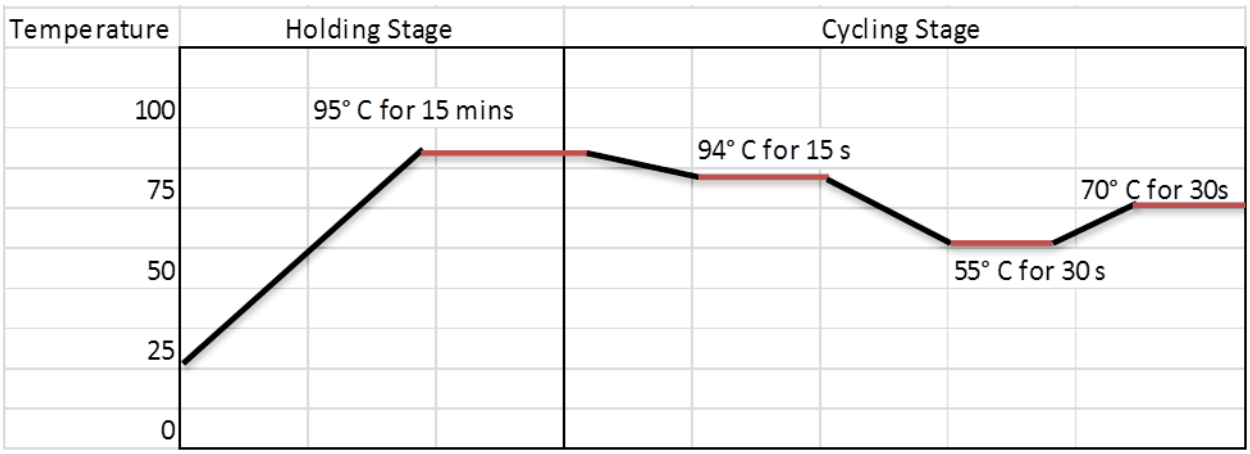


Figure 3. **RT-PCR** Cycling conditions for RT- PCR using StepOne Real-Time PCR System, 40 cycles were completed.

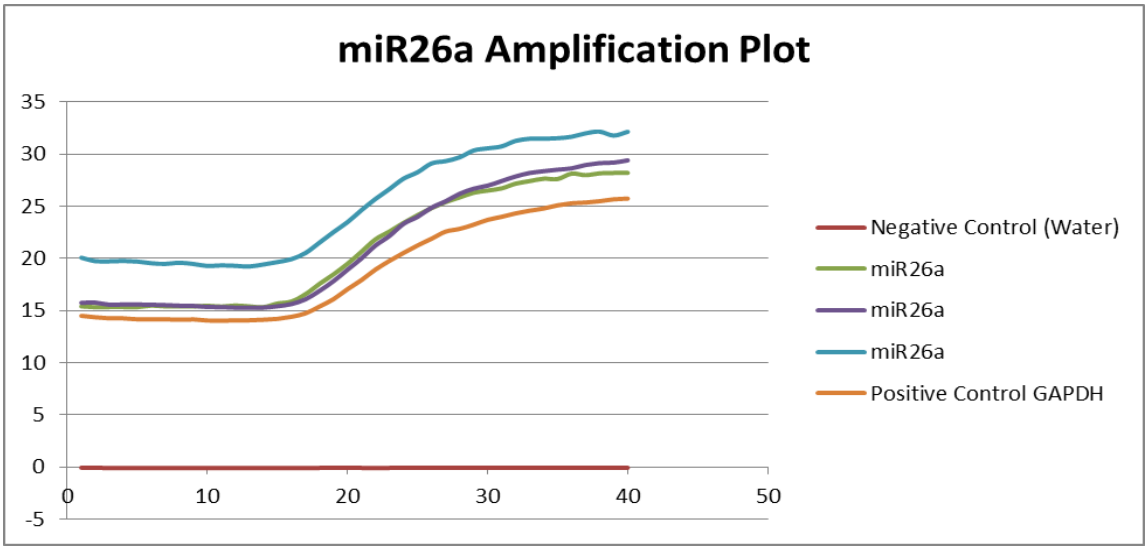


Figure 4. Amplification of miR-26a using RT-PCR. Primer RNA for miR-26a (UUCAAGUAAUCCAAGGAUAGGCU) was combined with RT buffer and RT master mix to create cDNA for miR-26a. The cDNA was combined with SYBR Green PCR master mix and HS_miR-26a1_1 primer to produce the above amplification plot. The change in Rn shows an increase in miR-26a as cycles continue; this confirms the presence of miR-26a in the LN229 cells.

Figure 5. Cell-Titer Glo Reaction. Cell-Titer Glo measures cell viability by presence of ATP, and is directly proportional to the amount of active cells present in the suspension. The reaction shows how luminescence is created to provide quantitation of ATP present.

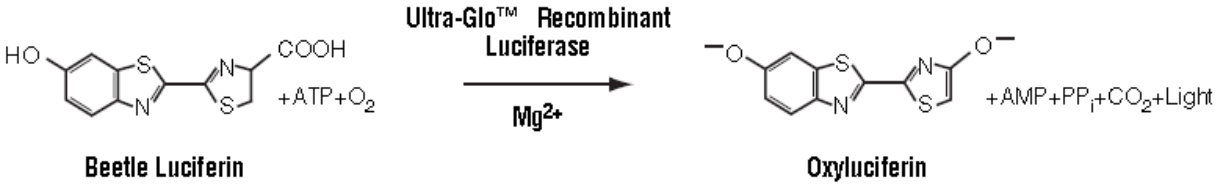
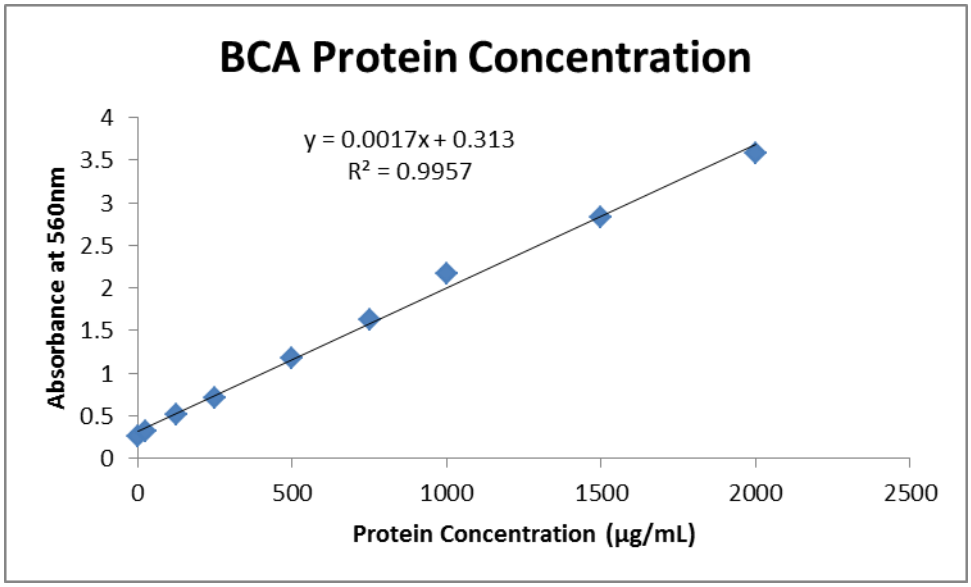


Figure 6. BCA Protein Concentration. Standard graph of protein concentrations used to determine amount of protein in LN 229 lysate sample (0.457 µg/ µL)



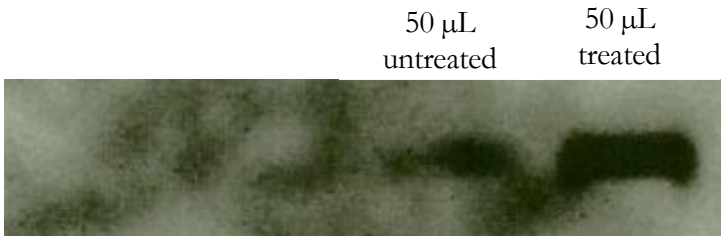


Figure 7. Western Blot of PTEN: post transfection. Visually there was an increase in the amount of PTEN in the treated cells compared to the untreated cells.

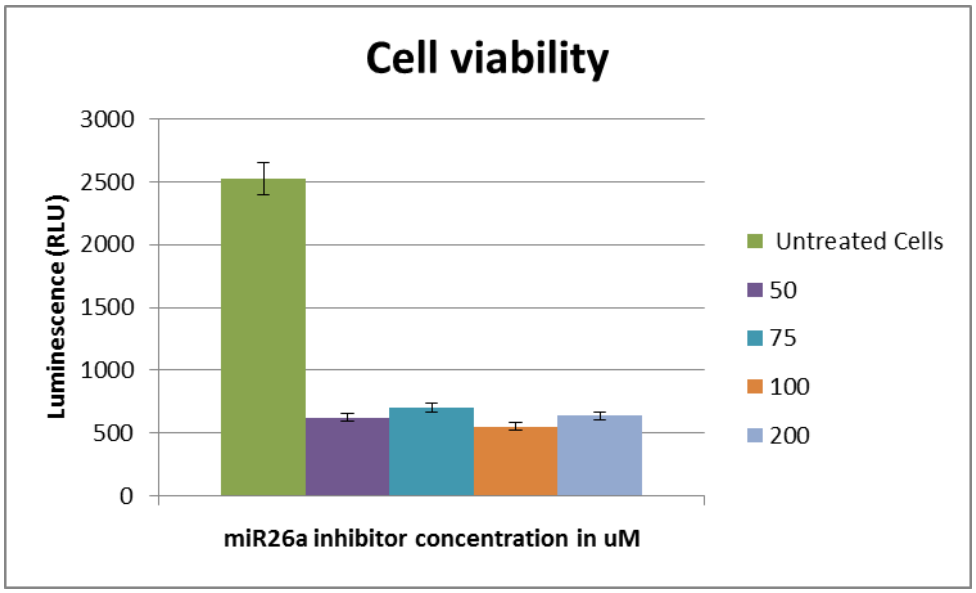


Figure 8. Cell viability after treatment with anti-miR-26a oligo. There is a significant decrease in cell viability in the LN229 cells treated with all anti-miR-26a oligo concentrations compared to the untreated LN229 cells at all inhibitor concentrations (P=0.0004). There was no significant difference between the concentration amounts.

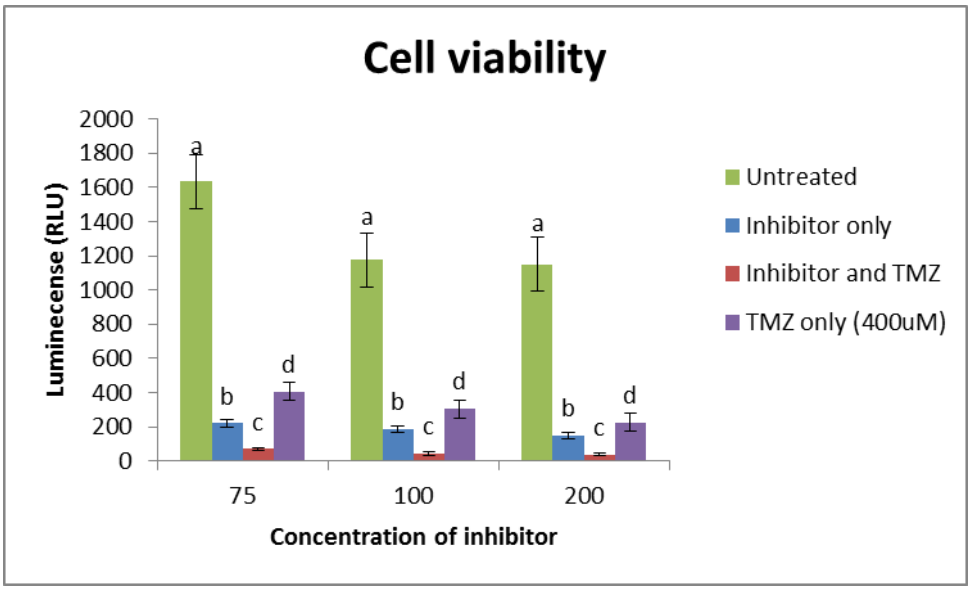


Figure 9. Silencing of miR-26a 24 hours post transfection trial 1. Untreated cells had the greatest luminecense indicating more viabile cells were present in that treatment. Inhibitor only had less luminecense then TMZ only. The combination treatment had the least amount of luminecense indicating the least amount of active cells. The bars with the same letters do not differ, the bars with differing letters are significantly different (P=0.00001).

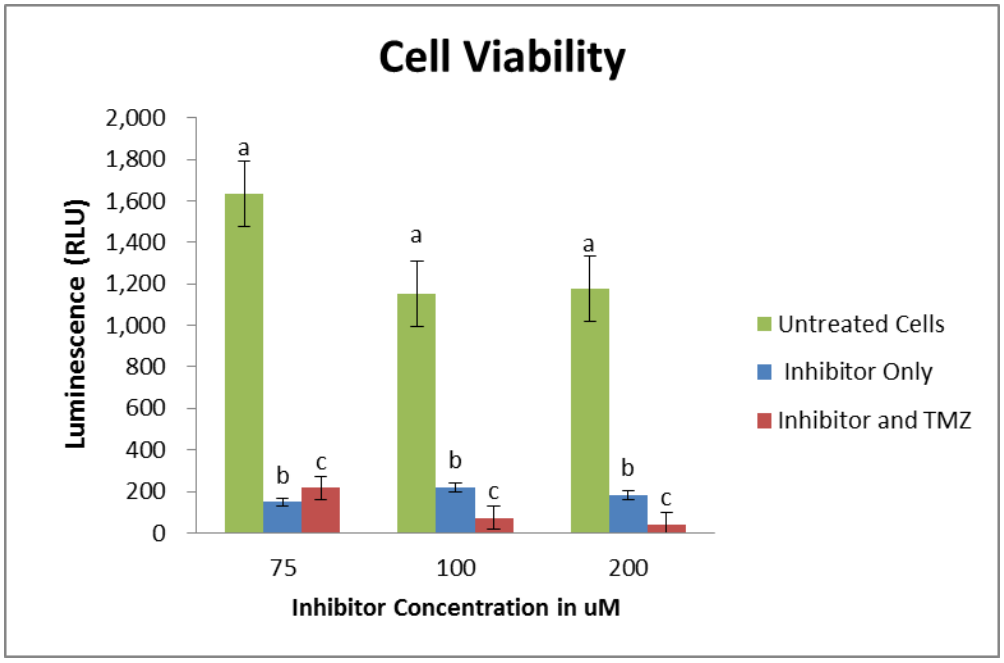


Figure 10. LN229 inhibition of miR-26a combined with TMZ trial 2. It is clear the combination of miR26a inhibitor with TMZ had a greater effect on LN229 cell viability compared to inhibitor alone, the different letters indicate significant difference between treatments (P=0.0001). A one way ANOVA was performed with Tukey post-hoc (P=0.0001), indicating there is a significant difference between the treatment groups (untreated, inhibitor only, and combination. The bars with the same letters do not differ.

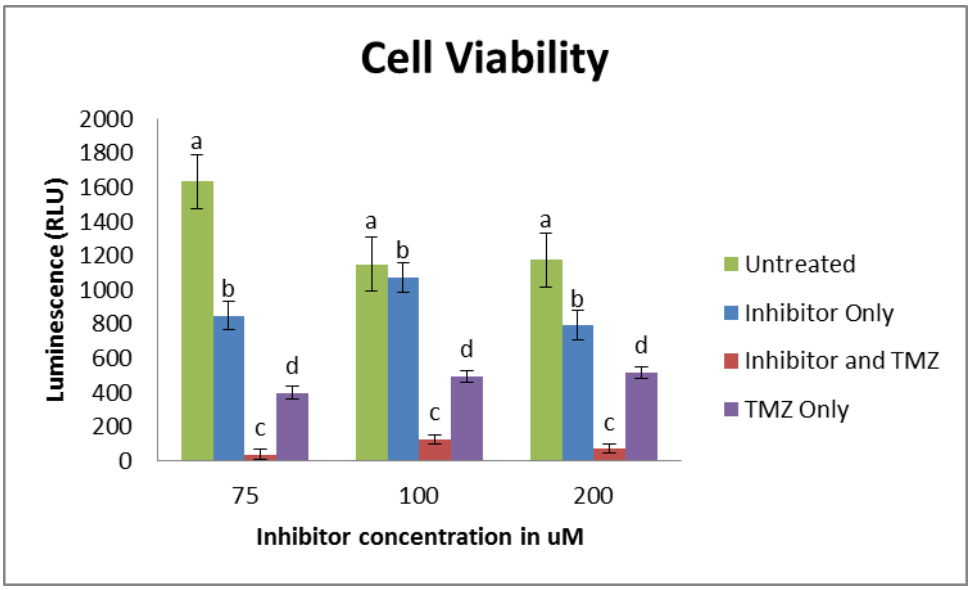


Figure 11. Cell viability of LN229 cell line after miR-26a inhibition plus TMZ trial 3. Once again the combination treatment (miR26a inhibitor and TMZ) had a significant effect on LN229 cell viability after 24 hours of application compared to the TMZ and anti-miR26a only treatments ($P<0.001$). Differing letters indicate the significant difference between treatments (untreated, inhibitor only, TMZ only, and inhibitor and TMZ).

Table 1. TBST Solution. Western transfer solution components.

Component	Concentration / Amount
Tris	48 mM (5.8g)
Glycine	39 mM (2.9g)
SDS	0.04% (0.37g)
Methanol	200 mL
Distilled H_2O	800 mL

Table 2. Reaction components for reverse transcription. Reverse transcription was performed on total extracted RNA from LN229 cells. The resulting cDNA was used in PCR for amplification of miR-26a, confirming presence in the cell line.

Component	Volume 1 reaction (µ L)
miScript RT Buffer, 5x	4
RNase-free Water	14
miScript Reverse Transcriptase mix	1
Template RNA	1 µg
TOTAL	20

Table 3. Reaction components for PCR Assay. PCR was performed in the StepOne Real-Time PCR cycler. Amplification of miR26a confirmed its presence in the LN229 cell line.

Component	Volume 1 reaction (µL)
2x QuantiTect SYBR Green PCR Master Mix	10
10x miScript Universal Primer	2
10x miScript Hs_miR-26a-1_1 Primer Assay	2
RNase-free Water	5
Template cDNA	1
TOTAL	20

Table 4. Well Plate Template. Schmatic of 96-well plate set up for cell incubation after Neon Transfection of miR-26a inhibitor. MiR-26a inhibitor wells contained 10 uL of cell suspension and 90 uL of complete media. Untreated cells were electroporated and placed into complete media; they contained no inhibitor.

	Anti-miR-26a	**Anti-miR-26a**	**LN229 Cells**	**LN229 Cells**
A	0 µ M	0 um	Untreated	Untreated
B	50 µ M	50 µ M	Untreated	Untreated
C	75 µ M	75 µ M	Untreated	Untreated
D	100 µ M	100 µ M	Untreated	Untreated
E	200 µ M	200 µ M	Untreated	Untreated

Electroporation parameters

Pulse voltage (v)	Pulse width (ms)	Pulse number	Cell density (cells/ml)	Transfection efficiency	Viability	Tip type
1,050	40	1	5×10^6	73%	80%	10 µl

Table 5. Electroporation parameters. Parameters for electroporation of glioma cell lines as previously determined by Invitrogen, for successful transfection**.**

	miR26a Inhibitor	miR26a Inhibitor			miR26a Inhibitor &TMZ	miR26a Inhibitor &TMZ	
A	0 µ M	0 um	Untreated	Untreated	0 µ M	0 µ M	TMZ only
B	75 µ M	75 µ M	Untreated	Untreated	75 µ M	75 µ M	TMZ only
C	100 µ M	100 µ M	Untreated	Untreated	100 µ M	100 µ M	TMZ only
D	200 µ M	200 µ M	Untreated	Untreated		200 µ M	TMZ only

Table 6. Combination treatment plate setup. The following 96 well plate schematic illustrates the setup for transfection with miR-26a inhibitor plus TMZ treatment. The plate was read using the Cell-Titer Glo Cell Viability assay 24 hours after treatment.