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Cancer is a major health concern for the world, with children's cancers being particularly devastating. Children's brain cancers are treatable, but those treatments often leave behind developmental impairments and so it is vital to seek out novel cancer treatment options. Examining old drugs for cancer treatment saves considerable cost and time in drug development, and so it is an important option to explore. The focus of this practicum was learning *in vitro* methods of evaluating potential anti-cancer drugs, using cell viability, Western Blotting, and apoptosis analyses. These methods are useful for quickly measuring changes in cancer cell death, exploring reasons for those changes via protein analysis, and can lead to further investigations. This thesis discusses current problems with medulloblastoma treatment and investigates the potential use of metformin in medulloblastoma treatment. The results of this practicum conclude that metformin is likely to be an inhibitor of medulloblastoma viability and should be further investigated.

IN VITRO METHODS FOR EVALUATING POTENTIAL ANTI-CANCER DRUGS

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CHAPTER I

MEDULLOBLASTOMA, CANCER THEAPIES, AND METFORMIN

INTRODUCTION

The drug discovery process is long and expensive. It requires multiple stages of early screening using *in vitro* models, which can lead to *in vivo* studies, that may eventually be approved for clinical testing, however, the process is prone to failure. An alternative strategy for this process is to repurpose existing drugs. By using already existing drugs, it is possible to expedite the process, as much of the pharmacokinetic information and safety profiles of the drug will already be known, and time and financial investment may be reduced for any necessary changes to the drug. One such drug that is being investigated for repurposing is the anti-diabetes drug metformin.

In vitro studies use toxicity screenings, high throughput screening, bioavailability studies, and other assessments to characterize drugs of interest. These methods of study often serve as one of the first steps in the drug discovery process, being used to develop a basic profile of how the drug(s) of interest will interact with various kinds of cells. (Barile, Dierickx et al. 1994, Chidambaram, Kandasamy et al. 2011, Katt, Placone et al. 2016, Ediriweera, Tennekoon et al. 2019). For this internship practicum, it was decided to investigate the potential use of the anti-diabetic drug metformin as an anti-cancer drug, using *in vitro* cell cultures of medulloblastoma as the model.

Medulloblastoma is a type of embryonal cell brain cancer that forms at the base of the brain at the cerebellum. It uses the cerebrospinal fluid of the brain to migrate through the spine and brain, and it impedes muscle coordination, movement, and balance, and has many detrimental symptoms and will eventually lead to death if untreated. Medulloblastoma is one of the most common forms

of brain cancer in children, representing nearly 10% of childhood brain tumors (Staff) (Brun, Markant et al. 2015, Millard and De Braganca 2016). Treatment typically consists of radiation and adjuvant chemotherapies, followed by surgery to remove the remaining tumor cells. Even with current treatments the morbidity rates for patients remain as high as 1 in 3 on average, with some specific subtypes of medulloblastoma having much worse survival rates (Millard and De Braganca 2016). Those patients that do survive treatment, however, are often left with life-long, debilitating neurological side effects. There is a strong interest in finding additional novel cancer treatments, and to this end there is interest in investigating existing drugs with the goal of repurposing them as anti-cancer therapies.

Metformin is a well characterized and widely available drug that has been used to treat type 2 diabetes for decades; it is well tolerated and safe within the human body and its pharmacokinetics are well known (Shaw, Lamia et al. 2005, Podhorecka, Ibanez et al. 2017). Given the availability of both the drug and information about it, as well as a growing body of evidence exists supporting the notion that metformin has anti-cancer properties, there is interest in studying metformin's effectiveness as a potential cancer treatment (Podhorecka, Ibanez et al. 2017, Chen, Li et al. 2020).

A potential avenue of cancer therapies for reducing the severity of their side effects is to find treatments that can sensitize cancer cells to radiation and chemotherapy treatments, which could reduce the necessary dosages of those treatments. A promising target in achieving this goal is survivin. Survivin is an Inhibitor of Apoptosis Protein (IAP) that has strong associations with cancer cell survival and resistance to anti-cancer therapies. Survivin is present in most cells at negligible levels, as it is a small component of the cell cycle, but it is overexpressed in cancer cells compared to non-cancer cells. Based on this association between cancer cell survival and survivin expression, it is possible that pharmacological inhibition of its expression would inhibit the

viability of cancer cells as well as increase their susceptibility to radiation and chemotherapies (Ambrosini, Adida et al. 1997, Chakravarti, Zhai et al. 2004, Brun, Markant et al. 2015, Wheatley and Altieri 2019).

BACKGROUND AND LITERATURE

Medulloblastoma

Treatment of medulloblastoma typically consists of surgery, followed by radiation and adjuvant chemotherapy using drugs such as vincristine. Current practices for treatment consist of surgery to remove as much of the tumor as safely possible, followed by radiation and adjuvant chemotherapy to destroy any remaining cells. Treatment of patients has a 70% chance of success, but the long-term neurological effects of treatment are often detrimental (Leary and Olson 2012, Millard and De Braganca 2016).

The side effects of medulloblastoma treatments and surgeries are well documented, though not always well understood. Posterior fossa syndrome (PFS), also known as cerebellar mutism syndrome, is present in as many as 1 in 4 recovering patients and is a result of surgical intervention; it is characterized by diminished speech, ataxia, emotional lability, and axial hypotonia. Radiation and chemotherapies tend to cause neuronal and endocrinal disorders in the short and long term. Patients who have undergone craniospinal radiation therapy have increased risk of stroke and other neurovascular diseases later in life, while chemical treatments like vincristine and cisplatin have neurotoxic side effects- like distal paresthesia, loss of deep tendon reflexes, and other autonomic neuropathies- that patients will gradually recover from (Millard and De Braganca 2016). Given that quality of life is an important consideration for patients after they have survived, it is important

that we find ways of reducing the severity of the side effects of our treatments, especially for young children.

Part of the treatment of medulloblastoma cells is the categorization of the severity of the tumors within the patient, including by risk stratification and by identification of molecular transcription pathways. Risk stratification is largely categorized based on the age of the patient, metastatic status, and the volume of postoperative tumor in the patient. The characterization of medulloblastoma based on their transcription pathways falls into four general categories: Wingless (wnt), sonic hedgehog (SHH), Group 3/C, and Group 4/D (Packer, Cogen et al. 1999, Leary and Olson 2012, Taylor, Northcott et al. 2012, Millard and De Braganca 2016, Institute 2020).

The risk stratifications that patients are grouped into are "standard-risk patients," "high-risk patients," and "patients under the age of 3", with standard risk consisting of patients over the age of 3, with localized tumors and <1.5 cm^2 of tumor present after surgical resection and high-risk patients having >1.5 cm^2 of tumors after resection and/or disease dissemination. The third group of patients, those under the age of 3, are separated out because they cannot safely receive upfront radiation treatment, due to the risk of neurological impairment. This means that generally, infant patients will rely on high-intensity chemotherapy treatments with the goal of avoiding radiation therapy as much as possible (Packer, Cogen et al. 1999, Leary and Olson 2012, Millard and De Braganca 2016). Studies found that in standard risk patients, it is often possible to administer relatively lower doses of radiation therapy combined with adjuvant chemotherapy, while still maintaining an average 70% five-year survival rate in patients (Packer, Cogen et al. 1999, Packer, Goldwein et al. 1999, Gajjar, Chintagumpala et al. 2006, Packer, Gajjar et al. 2006). This is potentially promising, as reducing the dosages of radiation and chemotherapy should reduce the overall toxicity of medulloblastoma treatments to the patients.

High-risk patients generally have a worse prognosis and lower survival rate when compared to standard risk patients, and so many different treatment options have been explored for improving patient outcome in survival rates and/or reduced neurological damage. A general trend in current treatments for the high-risk category is to increase the intensity of treatments to increase the effectiveness. Studies have shown that using high intensity chemotherapy over a shorter time or using multiple regiments of high intensity treatments can achieve similar rates of survival in high-risk patients to standard risk treatments. Conversely, attempting to lower the dosage for these patients may result in a significant decrease in survival rate, as much as from 70% to 40% (Gajjar, Chintagumpala et al. 2006, Packer, Gajjar et al. 2006, Jakacki, Burger et al. 2012, Leary and Olson 2012). While increased dosage treatments can improve the survival rates of high-risk patients, they can also increase the detrimental effects of those treatments (Sung, Lim et al. 2013).

The molecular subtyping of medulloblastoma has four major categories: wingless (WNT/beta-catenin) sonic hedgehog (SHH), group 3, and group 4, each of which have certain biological markers in their gene expression (Packer, Cogen et al. 1999, Leary and Olson 2012, Taylor, Northcott et al. 2012, Millard and De Braganca 2016, Institute 2020). The Wnt/beta-catenin pathway is composed of secreted glycoproteins that control aspects of embryonic development through signal transduction normally, but unregulated activation of the pathway leads to an accumulation of beta-catenin, which leads to upregulation of the *CTNNB1* gene and oncogenesis (Leary and Olson 2012, Millard and De Braganca 2016). Wnt pathway medulloblastoma are the least common, being approximately 10% of the disease, and are characterized by monosomy 6, *CTNNB1* mutations, and are positive for nuclear beta-catenin (Leary and Olson 2012, Millard and De Braganca 2016). Wnt pathway medulloblastoma occurs

primarily in patients over 3 years old, and patients tend to have high survival rates, exceeding 90%. Given the relative stability and high survival rate of this subtype, it is favored for investigating reduced radiation therapy treatments to reduce the side effects to patients, but it is still important to find ways of improving patients 5-year survival rates (Cho, Tsherniak et al. 2011, Leary and Olson 2012, Millard and De Braganca 2016).

The SHH subtype of medulloblastoma represents approximately 30% of spontaneous medulloblastoma, while having a bimodal representation, appearing within patients younger than 3 years and older than 16 more than in any group between them (Kool, Koster et al. 2008, Cho, Tsherniak et al. 2011, Northcott, Korshunov et al. 2011, Millard and De Braganca 2016). It is associated with a genetic predisposition syndrome that results in germline mutations in the patched-1 gene (PTCH1) or the suppressor of fused gene (SUFU), which causes a loss of tumor suppressor effects, but it is also characterized by other SHH pathway mutations such as PTCH2, SMO, GL11, and GL12 mutations (Millard and De Braganca 2016). They have lower survival rates than Wnt subtypes, with a higher propensity for metastasizing, though infants with desmoplastic/nodular histology tend to have the highest ten-year survival rate at around 77%, while children and adults have ten-year survival rates as low as 51% and 34% respectively for the same histology (Kool, Korshunov et al. 2012, Millard and De Braganca 2016).

Groups 3 and 4 are the most serious forms of medulloblastoma, with the lowest survival rates and are the least understood forms. They are characterized firstly by a lack of Wnt or SHH mutations driving their development, with the underlying genetic drivers often not having been determined yet (Kool, Koster et al. 2008, Cho, Tsherniak et al. 2011, Northcott, Korshunov et al. 2011, Kool, Korshunov et al. 2012). Group 3 represents approximately 30% of medulloblastoma tumors, while Group 4 represent 35% of medulloblastoma tumors and is the largest subgroup.

Group 3 typically has amplified expression of *MYC* and MYC protein, with high rates of dissemination- metastasis being detected in approximately 30% of patients at time of diagnosis. They are most prominent in infants and children and have the lowest survival outcomes being between 20-50% within these groups (Cho, Tsherniak et al. 2011, Northcott, Korshunov et al. 2011, Kool, Korshunov et al. 2012, Leary and Olson 2012, Millard and De Braganca 2016). Group 4 tumors have a prognosis that is between Wnt and Group 3 subgroup tumors and is considered similar to SHH subgroup, occurring most commonly in late childhood and early adolescents. They are otherwise the least well known or understood group and require further research to identify their genetic markers (Cho, Tsherniak et al. 2011, Northcott, Korshunov et al. 2011, Kool, Korshunov et al. 2012, Leary and Olson 2012, Taylor, Northcott et al. 2012, Millard and De Braganca 2016).

Metformin

Metformin has a well characterized pathway of action in treating diabetes; it reduces the amount of blood glucose while decreasing fasting plasma insulin levels, thereby decreasing blood glucose levels without causing hypoglycemia (Morales and Morris 2015). It does this by inhibiting the respiratory chain complex 1 in mitochondria, decreasing the production of ATP in hepatocytes, which leads to an increase in the intracellular adenosine monophosphate (AMP) to adenosine triphosphate (ATP) ratio (Morales and Morris 2015, Podhorecka, Ibanez et al. 2017). The increase in the ratio of AMP to ATP causes the AMP-activated protein kinase (AMPK) to activate, causing the cells to shift to a catabolic state which affects the cancer cells' gluconeogenesis, lipogenesis, and proteins synthesis. AMPK also serves as metabolic tumor suppressor, helping to activate tumor suppressor genes, such as p53, serine-threonine liver-based kinase 1(LKB1), and tuberculosis

sclerosis complex 2 (TSC2) as part of its direct inhibition pathway. TSC2 forms a complex with TSC1 that inhibits the mammalian target of rapamycin (mTor) complex 1, which regulates protein translation important for cell growth (El-Mir, Nogueira et al. 2000, Vander Heiden, Cantley et al. 2009, Viollet, Guigas et al. 2009, Faubert, Boily et al. 2013, Morales and Morris 2015, Podhorecka, Ibanez et al. 2017).

While they are not as well understood and characterized as the diabetes pathways, there are several potential anti-cancer mechanisms that metformin is thought to have, often called direct and indirect based on their pathway, that primarily focus on the inhibition of growth stimuli and metabolic processes (Morales and Morris 2015, Podhorecka, Ibanez et al. 2017). One of metformin's indirect pathways of inhibiting cancer growth is through the reduction of available blood glucose, insulin, and insulin-like growth factor 1 (IGF 1), which can reduce the availability of growth signals available for certain cancer cells and therefore will inhibit their ability to proliferate (Morales and Morris 2015, Podhorecka, Ibanez et al. 2017).

Metformin's direct pathways concern its AMPK-dependent and -independent mechanisms and are typically concerned with affecting transcription (Morales and Morris 2015, Podhorecka, Ibanez et al. 2017). AMPK is known to inhibit the activation of the mTOR signaling pathway in cells, which is associated with integrating growth factors and signals, as well as inhibiting cell autophagy. It is possible that by suppressing the activation of mTOR, this may allow autophagy mechanisms to become active in cancerous cells, and it may also inhibit the ability of cancer cells to transcribe genes associated with glycolysis (Kim, Kundu et al. 2011, Faubert, Boily et al. 2013, Morales and Morris 2015, Podhorecka, Ibanez et al. 2017).

Several studies have already searched for a relationship between metformin usage and a decrease in cancer growth, proliferation, and/or viability, as well as increased sensitivity to

chemotherapy and radiation therapy in multiple types of cancer cells (Morales and Morris 2015, Podhorecka, Ibanez et al. 2017). Based on this evidence of metformin as a cancer inhibitor, there is also interest in evaluating its effectiveness on medulloblastoma specifically, and so this internship had the goal of exploring metformin's effects on cancer cell viability and the expression of certain cancer-associated proteins, such as survivin and Sp1 (Ashinuma, Takiguchi et al. 2012, Rizos and Elisaf 2013, Morales and Morris 2015, Podhorecka, Ibanez et al. 2017, Chen, Li et al. 2020).

Survivin

Survivin is an inhibitor of apoptosis protein (IAP) that is associated with mitosis and resistance to apoptosis within cancer cells. Member proteins of the IAP family are typically thought to inhibit the activity of caspase enzymes, which are responsible for enacting apoptosis. Survivin has been identified as a member of the IAP family, has been associated with increased cancer cell survivability when exposed to chemo- and radiation therapy treatments, and is known to be highly expressed in many types of cancer cells, including lung, colon, pancreas, prostate, and breast cancers (Ambrosini, Adida et al. 1997, Chakravarti, Zhai et al. 2004). Under normal circumstances, survivin is associated with the Chromosomal Passenger Complex (CPC) during mitosis but is otherwise largely unexpressed, whereas it is highly abundant in cancer cells (Velculescu, Madden et al. 1999) . Given the evidence that survivin plays an important role in cancer cell survival, it has become a priority target for anti-cancer research. A major goal of this internship is to investigate if metformin inhibits the expression of survivin, and to for me to learn methods of investigating any relationship between metformin dosage and survivin expression in

medulloblastoma cells (Chakravarti, Zhai et al. 2004, Brun, Markant et al. 2015, Wheatley and Altieri 2019).

The regulatory mechanisms of survivin expression are generally not very well known, but studies have indicated that regulation predominantly occurs at the transcription level, with Sp1 binding sites having been identified in the survivin promoter (Chen, Wang et al. 2011). Sp1 has been found to have strong associations with survivin expression and evidence also suggests that inhibiting the expression of Sp1 protein will lead to the inhibition of the expression of survivin; therefore, it stands to reason that Sp1 may be a target for inhibiting survivin in medulloblastoma cells. Additionally, studies have shown that metformin may be useful as an inhibitor of Sp1 transcription factors (Xu, Zhang et al. 2007, Chen, Wang et al. 2011, Nair, Pathi et al. 2013).

CHAPTER II

EFFECTS OF ANTI-CANCER DRUGS ON CELL VIABILITY

INTRODUCTION

The primary purpose of this internship was to learn and develop ways of applying i*n vitro* techniques for studying potential anti-cancer therapies. *In vitro* experiments with cancer cells can be done in models of varying complexity, depending on the goals of the experiments, and they can allow for a great degree of control over the variables being measured. The complexity of the model can range from relatively simple tumor-derived cell lines to 3D models of tumor microenvironments. Cells with well understood profiles and characteristics can be obtained from sources such as the American Type Culture Collection (ATCC) (Katt, Placone et al. 2016). In this case, two medulloblastoma cell lines, DAOY and D283, were grown and studied over the course of the internship.

In vitro models of studying cells have distinct advantages and disadvantages that make them suitable for different tasks, especially when compared with *in vivo* models. The physiochemical environmental conditions (e.g., pH, temperature, O_2 to CO_2 levels) and the physiological conditions of the cells (e.g., growth factors, hormones, nutrient concentrations) are easier to control (Freshney, Capes-Davis et al. 2016). *In vitro* models are also often more economic than *in vivo* modeling, requiring less use of reagents as they can be directly applied to the cell cultures, and reducing the amount of animal experiments required in order to establish properties of the drugs (Chidambaram, Kandasamy et al. 2011, Freshney, Capes-Davis et al. 2016). The basis of *in-vitro* anticancer studies is the concept of "basal" cytotoxicity of the compounds or drugs of interest, which will affect the functionality of cells and leads to cellular damage (Chidambaram,

Kandasamy et al. 2011). Cell viability is a measure of the proportion of healthy, living cells within a population and is an important metric for determining the cytotoxicity of a drug. It can be measured based on the amount of cell damage within a population or by measuring the activity of the living cells of a population, either of which can be measured using cell viability assays.

SPECIFIC AIMS

The aims of this section were to perform experiments to investigate the effects of the anti-diabetic drug metformin on medulloblastoma cells, and to learn different methods and techniques that could be employed to this end. For this purpose, extensive cell culturing was performed in the lab, and multiple viability dose response curves were studied to determine the IC_{50} value for metformin. The end goal of this line of investigation is to investigate the potential use of metformin in combination with existing cancer therapies

MATERIALS AND METHODS

Materials

MB cell lines DAOY and D283 were procured from American Type Culture Collection (ATCC, Manassas, VA). DAOY cells are adherent with polygonal morphology, while D283 are semi-adherent, having adherent and suspension cells. Both cell lines were grown in Eagle's Minimum Essential Media (EMEM) from Corning (Corning, NY), supplemented with fetal bovine serum (FBS). Metformin and DMSO was obtained from Sigma-Aldrich (St. Louis, MO). Trypsin, PBS, and FBS were purchased from HyClone (Logan, UT). The viability assays were performed using white walled 96-well plates from Greiner Bio (Monroe, NC) and CellTiter-Glo Luminescent Cell

Viability Assay from Promega (Madison, WI). Luminescence intensity was measured using a SynergyHT Microplate reader from BioTek (Winooski, VT).

Background and Methods

2.1 Cell Culturing

Cell culturing is the process of growing cells in an artificial and controlled environment, typically with the use of growth media. By maintaining certain environmental conditions and providing the necessary nutrients, isolated cells can be maintained even separated from their original source. Once a culture has been established, it is possible to maintain a sub-culture of that particular line of cells, making it possible to perform multiple experiments with the same cell culture over a long period of time. It is important to distinguish between cancer and non-cancer cell lines being used for culturing, as cancer cells have a near indefinite number of replications, whereas healthy cells have a far more limited number of replications before they will die by a process known as senescence (Freshney, Capes-Davis et al. 2016). Freshney et al. differentiate between primary culturing and subculturing, as primary culturing is the process by which a cell line is established from a tissue source while subculturing is the process of continuing an established primary culture (Freshney, Capes-Davis et al. 2016). For the purposes of this internship, only subculturing was performed.

2.2 Thawing frozen cells (DAOY and D283)

1. Remove cryovial containing frozen DAOY or D283 cells from liquid nitrogen storage and immediately place it into 37°C water bath.

2. Quickly thaw the DAOY or D283 cells (< 1 minute) by gently swirling the vial in the 37°C water bath until there is a small amount of ice left in the vial
3. Transfer the vial into a biosafety cabinet. Wipe the outside of the vial with 70% ethanol before opening.
4. Transfer the thawed cells dropwise to a centrifuge tube containing the desired amount of pre-warmed growth media appropriate for the cell line.
5. Centrifuge the cells at 300 g for 5 minutes.
6. After centrifugation, decant the supernatant without disturbing the cell pellet.
7. Gently resuspend the cell pellet in complete growth media, so that the total volume is 10 mL, then transfer the cells into appropriate T-25 flask or 100 mm plate and place them into incubator. Incubate at 37°C and 5% CO_2.

Cultured cells may or may not adhere to the container they are grown in, thus there are differences in how adherent and non-adherent cells are subcultured.

2.3 Subculturing of DAOY cells (adherent cells)

1. Remove and discard old culture media from the 100 mm plate.
2. Wash cells with PBS. Place pipette tip against the side of the plate and slowly add approximately 4 mL of PBS, then rock the plate back and forth several times.
3. Remove and discard the PBS.
4. Add 1 mL of warmed trypsin to the plate and gently rock it for complete coverage.
5. Incubate at 37°C until > 90% of cells have detached from the plate surface. Tap the plate to expedite the process of detachment, if necessary.

6. Add 5 mL of pre-warmed media to the plate to neutralize the trypsin, then mix the media and cells several times, including pipetting media onto the plate to dislodge still attached cells. The purpose of this step is to further detach remaining cells and separate them into single cells.
7. Transfer 0.5 mL of cells to a new plate with 10 mL of growth media added and incubate at 37°C and 5% CO_2. The volume of cells added to each plate can be changed based on the cell count.

2.4 Subculturing of D283 cell (semi-adherent)

1. Use cell scraper to detach adhering cells from the surface of the culture plate. Collect cell suspension into 15 ml conical tube. Make sure to remove at least 90% of surface cells.
2. Centrifuge cells at 300 g for 5 minutes, then decant supernatant under biosafety cabinet without disturbing the cell pellet.
3. Resuspend cell pellet in 5 mL fresh EMEM media.
4. Perform a cell count after resuspension.
5. Transfer appropriate volume of cell suspension (about 10^6 cells) based on calculations to a 100 mm plates that have corresponding volumes of growth media (total 10 mL). Gently shake plates to ensure proper distribution of cells. Incubate at 37°C and 5% CO_2.

2.5 Cryopreservation of DAOY or D283 cells

Cryopreservation is a process by which subcultured cells of a particular cell line can be preserved for later use for a variety of reasons and is an essential process for *in vitro* studying of cells. According to Freshney et al., there are several reasons for cryopreservation including; 1) freezing

to prevent genotypic drift from genetic instability, 2) preventing the extinction of a primary cell line from senescence, 3) minimize changes in cell phenotype from dedifferentiation, 4) avoid cross contamination by other cell lines, 5) to limit the investment of resources used for growing cells that are not being experimented on, and 6) to allow cells from a cell line to be distributed for other uses (Freshney, Capes-Davis et al. 2016).

The optimal method of freezing cells needs to reduce intracellular ice crystal formation and cryogenic damage from high concentration of solutes formed during the freezing process. To achieve this, it is necessary to (1) freeze slowly, allowing water to leave the cell but not slowly enough for ice to form, (2) use a hydrophilic cryoprotectant to sequester water (glycerol or DMSO), (3) store the cells at the lowest possible temperature, minimizing the effects of high salt concentrations on protein denaturation, and (4) thawing rapidly to minimize ice crystals and generation of solute gradients formed as residual intracellular ice melts (Freshney, Capes-Davis et al. 2016).

Protocol for cryopreservation of cells

1. Perform appropriate protocol to harvest the cells, as in methods section 2.3 or 2.4.
2. Collect cells into a centrifuge tube and centrifuge at 300 g for 5 minutes. Decant under sterile biosafety cabinet.
3. Resuspend cell pellet in complete media containing 5% DMSO. Resuspend pellet in enough volume to get at least 1 million cells/mL of suspension.
4. Transfer 1 mL of suspension to cryotubes, transferring all of cell suspension.
5. Place cryotubes into isopropanol freezer storage containers, and store at -80°C.
6. Move to liquid nitrogen for long term storage

2.6 Preparation of metformin stock solution and dilutions for treatment

1. Equilibrate the metformin and EMEM media to room temperature
2. Dissolve 500 mg of metformin in 3 mL. Add 2.9 mL of media to the contents of the bottle of metformin (500 mg), and ensure they are dissolved by shaking the bottle up and down until all visible metformin is dissolved. This results in a 1000 mM stock solution.
3. Dispense metformin stock into microcentrifuge tubes and store at -20°C.
4. For viability assays, metformin concentrations used were 1, 5, 10, 20, 40, 80 mM, and diluted as shown below.

2.7 Preparing 96-well plates for viability assay

1. Harvest cells using the appropriate method of detaching them (see the related sub-culturing protocol) and collect them in centrifuge tubes. Perform a cell count using either a hemocytometer or an automated cell counter.
2. Dilute cells in of media to make the appropriate concentrations of cells per mL.
    a. For DAOY: 300,000 cells/mL (3,000 cells per well in 50 µL).
    b. For D283: 1,000,000 cells/mL (5,000 cells per well in 50 µL)
3. Add 50 µL of cell suspension to each well of a white walled 96-well plate (or 25 µL if using half-area plates). Incubate cells at 37°C and 5% CO_2 for 24 hours.
4. After incubation period, begin treatment of cells.
5. Calculate and prepare the appropriate volumes and concentrations of drugs for experiments.

6. Add 50 µL (or 25 µL for half area plate) of treatment per well. Total volume should be 100µL or 50µL depending on plate size. The addition of drug treatments in equal volume to the cells in each well serves as the final dilution for the drug treatments, so be sure to account for this.
7. Incubate plates at 37°C and 5% CO_2.

2.8 Viability Assays

Viability assays are methods of measuring the proportion of living, healthy cells within a population, and there are multiple ways of measuring it. Commonly used factors for viability assays include changes in mitochondrial activity and metabolic products like ATP, and indicators of cellular damage such as lactate dehydrogenase enzyme concentrations and uptake of fluorescent dyes after DNA fragmentation. The principles behind each method differ, but they generally use a substrate or dye that will react with intracellular enzymes, ATP, DNA, or cellular membrane components that can then be observed and recorded (Barile, Dierickx et al. 1994, Nagata 2000, Chidambaram, Kandasamy et al. 2011, Ediriweera, Tennekoon et al. 2019).

2.8a Tetrazolium Salts

Tetrazolium salts are a type of reagent commonly used to measure cell viability, particularly the activity of the mitochondria of cells. There are several different types of tetrazolium salts, including: INT, XTT, MTS, WST, and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), with the MTT assay being the most commonly used variant, wherein yellow, water soluble MTT is converted into purple water insoluble formazan. The principle of Tetrazolium salts is that anticancer drugs and/or cytotoxic compounds damage cancer

cells and change their mitochondrial activity; tetrazolium salts will be taken into the mitochondria due to their net positive charge and the mitochondria's membrane potential. In the mitochondria, the tetrazolium salts are reduced to formazan dye by the NADH+ dependent reaction catalyzed by the mitochondrial succinate dehydrogenase enzymes (MSDE). The amount of formazan produced is proportional to the number of living cells. MTT assays are useful in that they can be measured directly from the plate and are rapid and simple to use, however they also have some drawbacks. The assay salts require a solvent, such as DMSO, to be used, which can potentially interact with the drugs being tested, and the assay results can be influenced by concentrations of glucose within the media at the time of measurement and the length of exposure to MTT. The kinetics of MTT formazan formation also vary by cell line, requiring additional standardization and optimization of lab practices per cell line. (Mosmann 1983, Fanning, Biddle et al. 1990, Husøy, Syversen et al. 1993, Harbell, Koontz et al. 1997, Young, Phungtamdet et al. 2005, Chidambaram, Kandasamy et al. 2011, Sachin Kumar 2016, Ediriweera, Tennekoon et al. 2019).

2.8b Lactate Dehydrogenase Assay

Cell death by apoptosis causes the release of several cytoplasmic enzymes, such as lactate dehydrogenase (LDH). LDH is an enzyme that is present in all tissues, as it is part of the anaerobic metabolic pathway, and it remains stable following a loss of membrane integrity, which allows it to be used as a measure of cytotoxicity within the microenvironment of cells. An anti-cancer drug damages cancerous cells, causing them to release cytoplasmic LDH, which are extracted by Triton X-100. The freed LDH is treated with substrates containing lactate, NAD+, diaphorase and a dye; the LDH catalyzes lactate into pyruvate while also producing NADH from reduced NAD+. Diaphorase transfers H/H+ from NADH/H+ to the dye, which reduces to a fluorescent product,

measurable at 490-520 nm, and with the amount of color being proportional to the lysed cells (Legrand, Bour et al. 1992, Bonfoco, Krainc et al. 1995, 2005, Chidambaram, Kandasamy et al. 2011).

2.8c DNA Fragmentation Assay

DNA fragmentation is one of the later steps in the process of apoptosis, caused by the activation of calcium- and magnesium-dependent nucleases which degrade DNA endonucleases; the DNA Fragments range from ~300kb to 50bp. The assay is based on Terminal Deoxynucleotidyl Transferase (TdT)-mediated dTUP nick-end labelling. The breaking of the DNA exposes a large number of 3'-hydroxyl ends, to which 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdU), is attached by TdT. After its incorporation into the DNA, BrdU can be detected by an antibody using immunohistochemical techniques, fluorescence microscopy, or flow cytometry (Fehsel, Kolb-Bachofen et al. 1991, Kraupp, Ruttkay-Nedecky et al. 1995, Negoescu, Lorimier et al. 1996, Negoescu, Guillermet et al. 1998, Chidambaram, Kandasamy et al. 2011).

2.9 ATP assays and CellTiter-Glo assay

ATP is an important chemical energy reservoir that is only produced in living cells and is rapidly decreased in cytoplasmic concentrations by cell injuries and oxygen depletion. ATP is therefore a key indicator of cellular activity and is used to measure cell viability and cytotoxicity (Maehara, Anai et al. 1987, Cree and Andreotti 1997, Chidambaram, Kandasamy et al. 2011).

The CellTiter-Glo assay is a chemiluminescence based assay that detects cellular ATP as a means of measuring cellular activity and viability and is combined with 96-well plates to allow high throughput screening (HTS). The CellTiter-Glo® reagent causes cell lysis and uses a form of

luciferase to cause reactions with ATP that generate a "glow-type" luminescent signal, allowing the number of cells present in a well to be quantified, as the amount of ATP is directly proportional to the number of cells. The reagent uses the reaction of beetle luciferin and ATP reacting with luciferase and Mg^{2+} to produce oxyluciferin, AMP, and light. Compared to standard ATP assays, which make use of luciferase purified from the firefly species *Photinus pyralis* (LucPpy), the CellTiter-Glo assay uses a more stable form of luciferase based on the gene from the firefly species *Photuris pennsylvanica* (LucPpe2) that allows improved performance in ATP assays. The reagent is robust, sensitive and stable, and therefore is easy to use while still being effective (Promega 2015). Using this assay, we can determine the correlation between decreased cell viability and increased dosages of metformin treatment.

2.9a Promega CellTiter-Glo Reagent Preparation

1. Thaw CellTiter-Glo Buffer and Substrate (lyophilized enzyme/substrate) to room temperature.
2. Transfer appropriate volume of Buffer into amber bottle containing the Substrate. This forms the CellTiter-Glo Reagent.
3. Gently mix the CellTiter-Glo Reagent until it is homogenous. Store reagent at -20 °C.

2.9b Promega CellTiter-Glo Viability Assay

1. Prepare white walled 96-well plate with mammalian cells (DAOY and D283) in culture medium, as described in the preparing 96-well plates protocol (Section 2.7).
2. Prepare control wells containing medium without cells to obtain a value for background luminescence.

3. Add test compound (increasing concentrations of metformin) to experimental wells and incubate at 37°C and 5% CO_2.
4. Add a volume of CellTiter-Glo Reagent equal to the volume of cell culture media present in each well (100µL reagent for 96-well plates or 50µL for half-area plates).
5. Allow plate to incubate at room temperature for 10 to 20 minutes to stabilize luminescence signal.
6. Record luminescence using a luminescence plate reader (BioTek, Synergy HT)

(Promega 2015)

2.10 IC_{50} Values

An IC_{50} value is the "inhibitory concentration" of a drug at which 50% inhibition of a biological process is achieved. For the purposes of this internship and report, it is a measure of the concentration of metformin that will result in a 50% inhibition of cell viability of DAOY or D283 medulloblastoma cell lines. The IC_{50} can be determined by using a non-linear regression analysis of the data, plotted as percentage activity versus Log (metformin concentration). This analysis can be performed using GraphPad Prism graphing software. The equation used by the GraphPad Prism for log(inhibitor) vs. normalized response with variable slope is Y=100/(1+10^((LogIC50-X)*Hill Slope))).

2.11 Statistical Analysis

The data was analyzed using a one-way analysis of variance (ANOVA) test, which is used to determine if there are statistically significant differences between the means of three or more independent groups. The Dunnett's test was applied as a post-hoc test on the one-way ANOVA

results, The Dunnett's test measures experimental groups against a control group to determine which pairs of means from an ANOVA are statistically significant. GraphPad Prism was used to process the data and perform the statistical tests

Difficulties and Complications

During the course of the internship, there were difficulties and failures with some of the cells cultured. At various points, plates of cells became contaminated while being subcultured, necessitating their removal and replacement, a process that sometimes took weeks to fix because of how long it takes to subculture new plates to sufficient cell numbers. Unexpected results were observed during the course of the DAOY viability assays- several assays showed a sharp drop in cell viability to near zero levels, typically at 5-10 mM, with a sudden spike at 20 mM. This problem was frequent enough to cause disruptions in viability testing but lacked enough consistency for proper diagnosis.

The D283 cells thawed after returning from the COVID-19 lockdown displayed a tendency to develop sudden fungal contamination between one to two weeks after thawing and beginning new subcultures. It is difficult to say the exact cause of the contamination in these cases but given that the exact same contamination would appear in each plate further subcultured, it is possible that the originally frozen cells were contaminated at their time of freezing. This seems a likely explanation, as the problem was eliminated after thawing a different batch of D283 cells.

RESULTS AND DISCUSSION

The effect of metformin on medulloblastoma cell growth was assessed using Luminescent CellTiter-Glo Cell Viability Assay. DAOY and D283 cells were plated in 96-well plates at a 3000

and 5000 cells/well in 50 µl media respectively, as mentioned above. After 24h incubation the cells were treated with increasing doses of metformin (0,1, 5, 10, 20, 40, 80 mM) in triplicate. Cell viability was assessed at 24- and 48-hours post-treatment using CellTiter-Glo Luminescent Viability Assay. The data was recorded as relative light units (RLUs) and was normalized against the no metformin control, to obtain percentage viability. The triplicate values were represented as mean+/- SD. The percentage viability was then plotted against the metformin dose, for both DAOY and D283 cells. The experiments were repeated at least 3 times. To obtain the IC_{50} values, the metformin concentrations were transformed into log values and plotted against the percentage viability; the data was then processed using a non-linear regression model with a logarithmic transformation, as described in section 2.10.

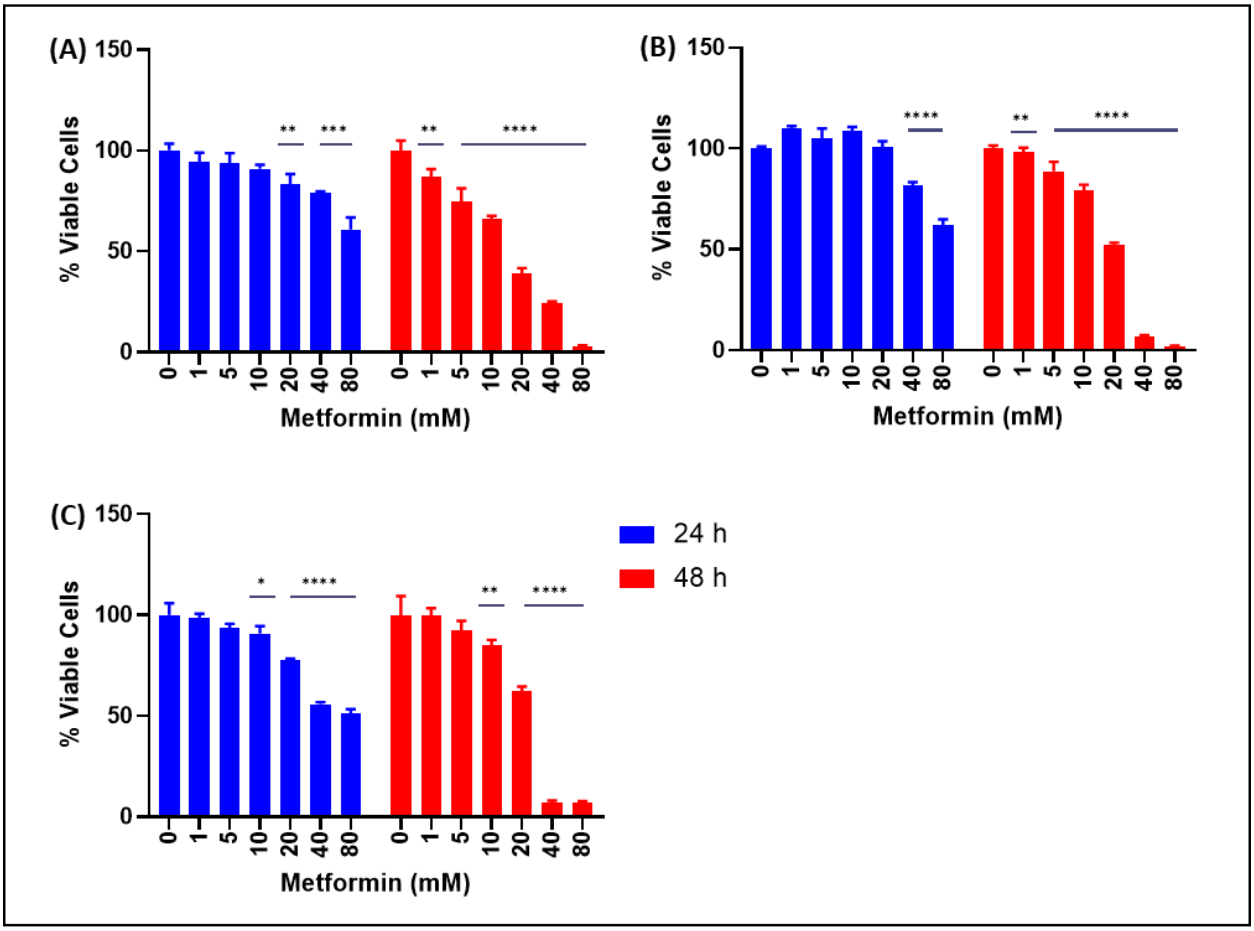
As seen in Figure 2.1 and 2.2, a dose and time dependent decrease in cell viability was observed with metformin treatment for both medulloblastoma cell lines. At 24-hour time period, there was little change in viability except at high dosages, a trait shared across both DAOY and D283 cells. The viability for DAOY at 24 hours at 40 mM metformin was between 80-55%, typically not reaching 50% inhibition. At 48-hour, however, DAOY showed a significant decrease in cell viability, ranging from 62-39% viability with 20 mM metformin. Higher doses displayed significantly lower cell viability, with an average of 13% viability at 40 mM, and almost complete loss of viability at 80 mM. Results for the D283 cells were similar to that for DAOY cells. The D283 cells displayed a consistent decrease in cell viability, correlating with an increase in metformin dose, and time, although the decrease in viability was less than in DAOY cells for the same doses. This decrease was slight enough that at 24-hours, the viability at the highest dose of metformin 80 mM was 52- 39% of control untreated cells. After 48-hours, the D283 cells showed

significant decreases in viability, with the averages ranging from 46 % to 22% cell viability at 40 mM and 80 mM metformin dose respectively.

The dose response curves of the DAOY and D283 cell lines indicate that there is a correlation between an increase in doses of metformin and a decrease in cell viability. To quantitate the dose response of metformin on DAOY and D283 cells we calculated the IC_{50} values. The values were calculated using non-linear regression analysis using GraphPad Prism software, as shown in Figures 2.3 and 2.4. For DAOY cells the average IC_{50} at 48h was 17.47mM± 2.78mM based off of six independent dose response curves as shown in Figure 2.3. The average IC_{50} at 48h for D283 was 38.62mM± 4.22mM, based on four dose response curves as shown in Figure 2.4. The IC_{50} values of metformin for D283 were higher than for DAOY, indicating that the D283 cell line is more resistant compared to DAOY. The r-squared values for both the 24- and 48-hour data were all calculated to be over 0.95, indicating a very high goodness of fit for the graphs.

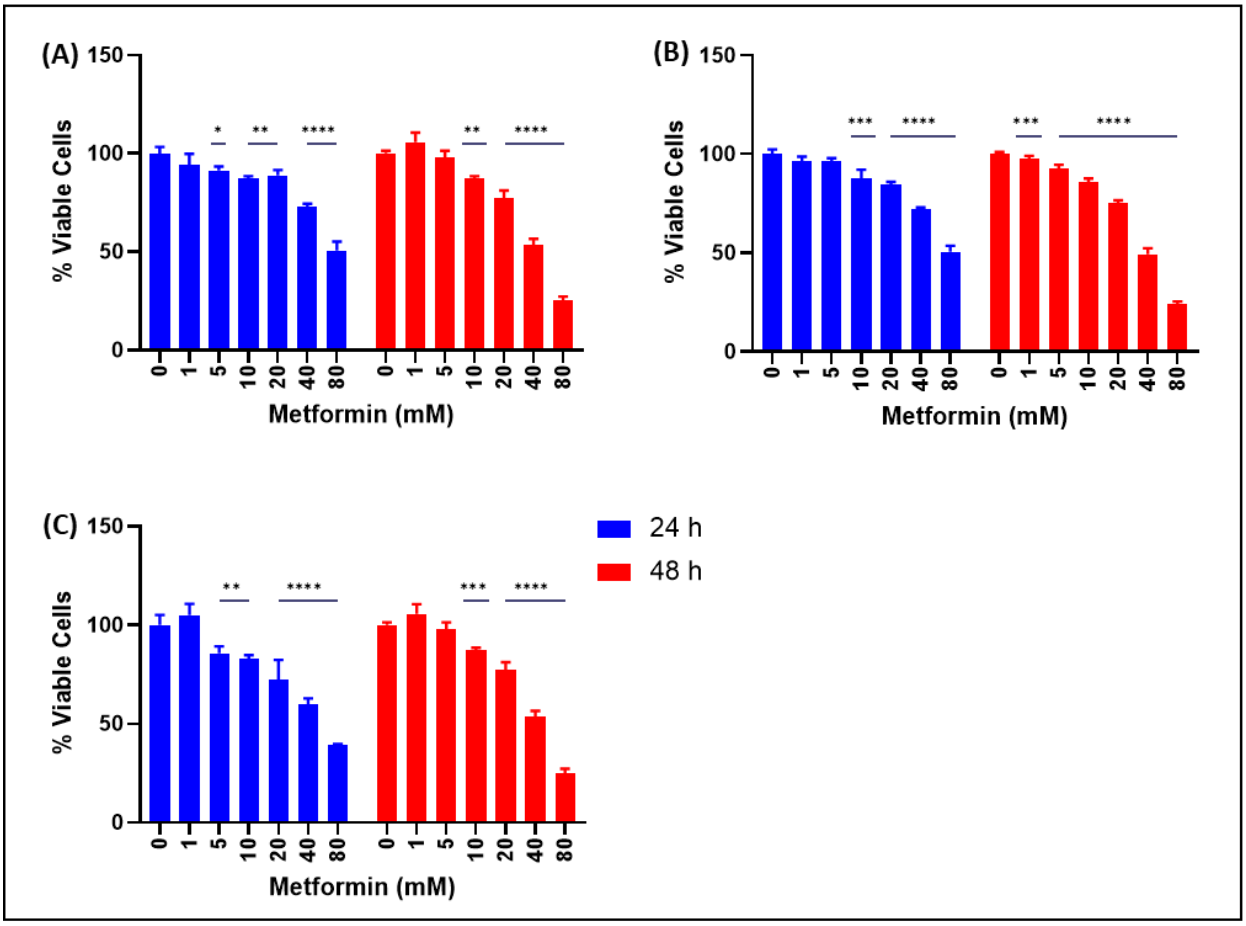
Data from other studies suggests that several different types of cancer are susceptible to inhibition by metformin treatments, such as Cantrell et al. showing ICC-1 and Ishikawa endometrial cancer cells having an IC_{50} of 1 mM over a 72-h period and in breast cancer cells (Cantrell, Zhou et al. 2010, Deng, Wang et al. 2012, Esparza-López, Alvarado-Muñoz et al. 2019).

Figure 2.1 DAOY Viability assays



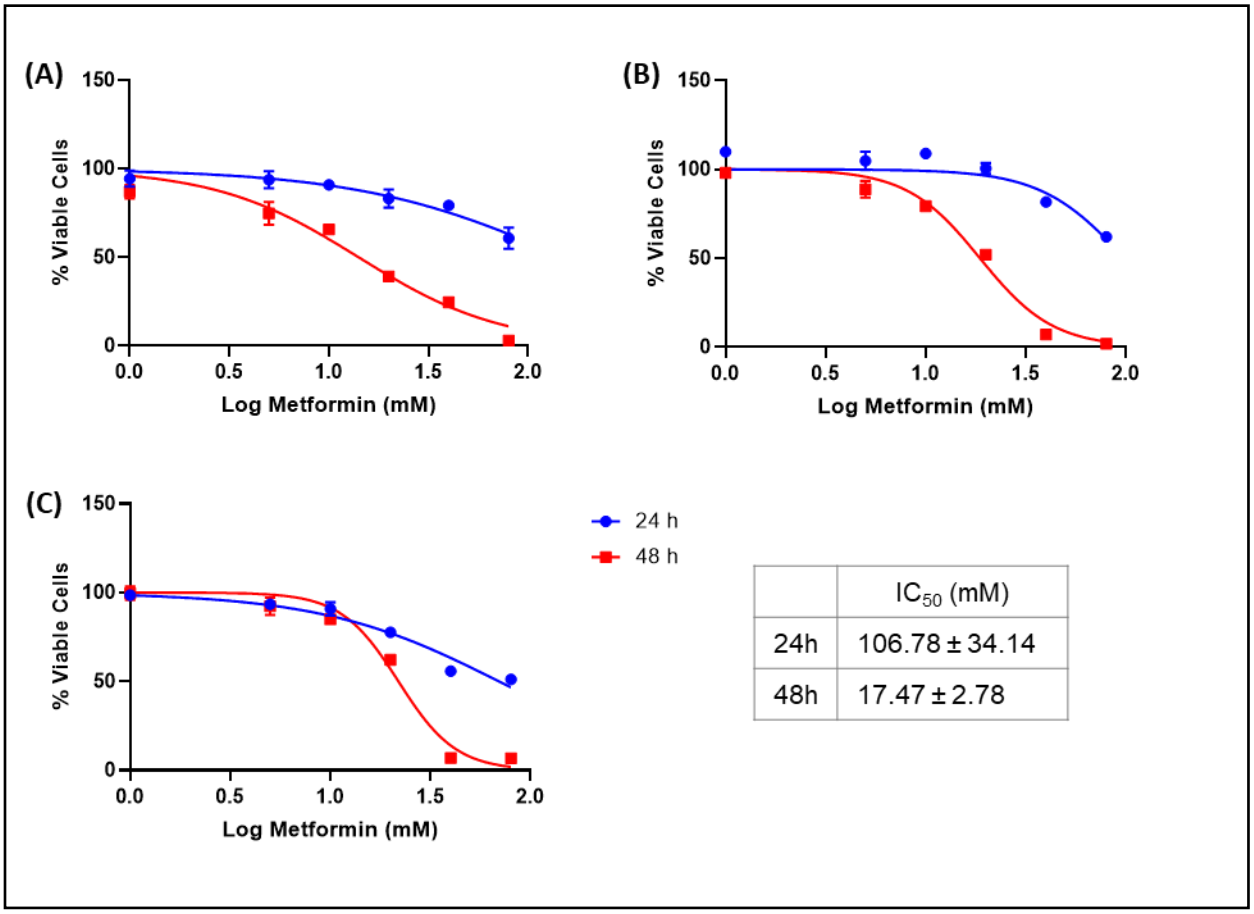
DAOY cells were treated with increasing dose of metformin (0-80 mM). Cell viability was measured using CellTiter-Glo kit at 24 and 48h post treatment. Percent viable cells compared to control untreated cells were calculated and plotted against metformin dose. Treatments were done in triplicate and data shown is the mean ±SD. A, B, and C represent three independent experiments. Statistical analysis was done using a one-way ANOVA test with Dunnett's multiple comparison test. The * indicates p values and the degree of statistical significance. * p≤.05, ** p≤.01, *** p≤.001, **** p≤.0001.

Figure 2.2 D283 Viability assays.



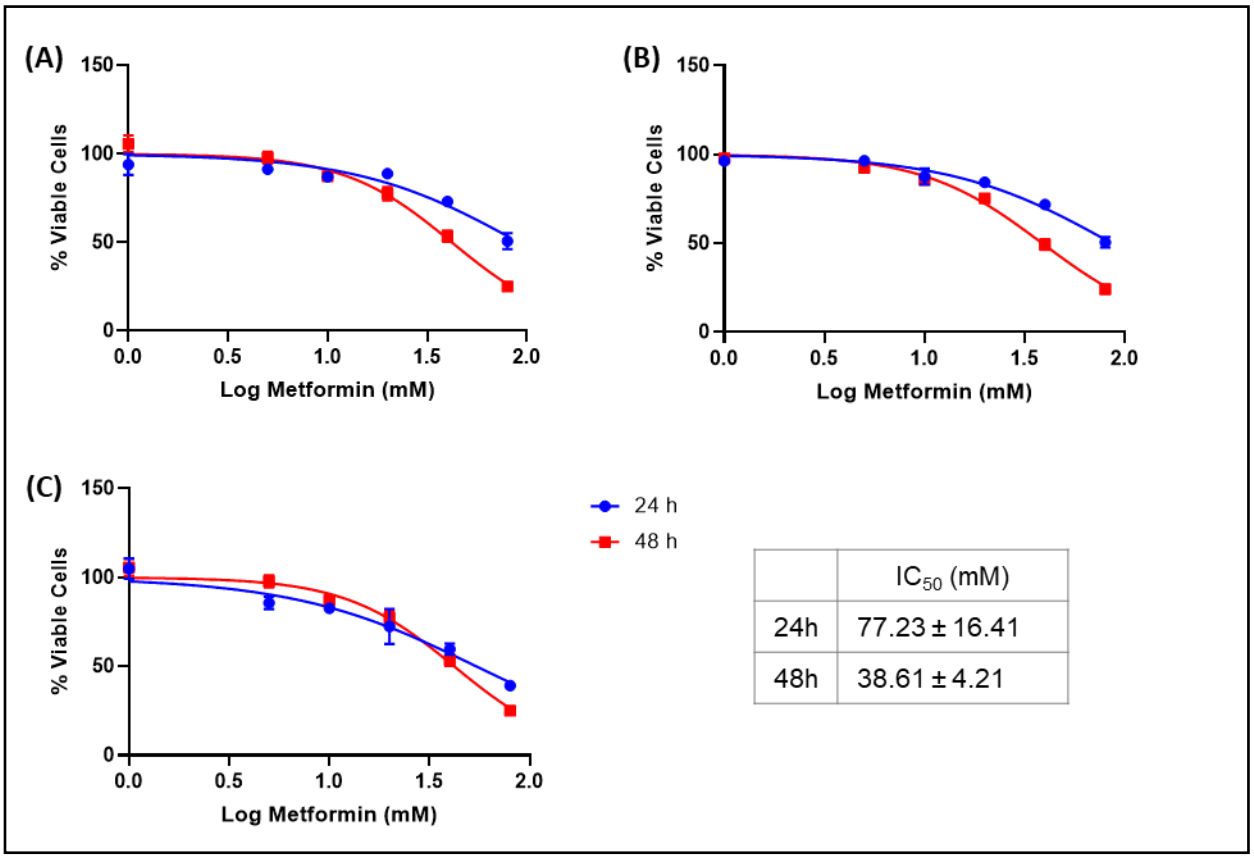
D283 cells were treated with increasing dose of metformin (0-80 mM). Cell viability was measured using CellTiter-Glo kit at 24 and 48h post treatment. Percent viable cells compared to control untreated cells were calculated and plotted against metformin dose. Treatments were done in triplicate and data shown is the mean ±SD. A, B, and C represent three independent experiments. Statistical analysis was done using a one-way ANOVA test with Dunnett's multiple comparison test. The * indicates p values and the degree of statistical significance. * p≤.05, ** p≤.01, *** p≤.001, **** p≤.0001.

Figure 2.3 DAOY dose response curves and IC_{50} values.



DAOY cells were treated with increasing dose of metformin (0-80 mM). Cell viability was measured using CellTiter-Glo kit at 48h post treatment. Percent viable cells, compared to control untreated cells, were calculated and plotted against Log of metformin dose. Treatments were done in triplicate and data shown is the mean ±SD. IC_{50} values were calculated using GraphPad Prism software and the non-linear regression curve fitting model. A, B, and C represent three independent experiments.

Figure 2.4 D283 dose response curves and IC_{50} values.



D283 cells were treated with increasing dose of metformin (0-80 mM). Cell viability was measured using CellTiter-Glo kit at 48h post treatment. Percent viable cells compared to control untreated cells were calculated and plotted against Log of metformin dose. Treatments were done in triplicate and data shown is the mean ±SD. IC_{50} values were calculated using GraphPad Prism software and the non-linear regression curve fitting model. A, B, and C represent three independent experiments.

CHAPTER III

WESTERN BLOTTING AND PROTEIN ANALYSIS

INTRODUCTION

Western Blotting is a method of protein analysis that allows for the detection of specific proteins within a cell sample and is useful for examining the effects of drug treatments. Western Blotting is a multi-stage method of analysis that begins with extraction of protein followed by three key elements: (1) the separation of proteins based on size by electrophoresis, (2) the transfer of proteins to a durable porous membrane, (3) the detection of target proteins by primary and secondary antibodies (Mahmood and Yang 2012). By using antibodies, it is possible to take advantage of their antigen specificity to make sure that only the proteins of interest are detected, allowing for high accuracy and specificity.

SPECIFIC AIMS

The aims of this section were to analyze the effects of metformin on survivin and Sp1 protein expression in medulloblastoma cells, as well as other potential proteins of interest, such as pAkt, and Akt. By measuring the changes in the expression of these proteins, it may be possible to quantify cancer cell viability inhibition as it relates to the reduced expression of cancer associated proteins. The We hypothesize that metformin treatments will correlate with a decrease in the expression of Sp1 and/or survivin, which will correlate with a decrease in cell viability.

METHODS AND MATERIALS

Materials

Antibodies used in this study were purchased from Cell Signaling Technology (Danvers, MA), Santa Cruz Biotechnology (Dallas, TX), or R&D Systems (Minneapolis, MN). All primary antibodies were used at a dilution of 1:1000 unless otherwise noted. Secondary anti-rabbit and anti-mouse antibody was used at a dilution of 1:3000.

Background and Methods

3.1 Protein extraction

1. Use a scraper to gently remove any adhering cells from the plate without damaging them. Premature damage to the cells may cause loss of protein and may affect results.
2. Collect cells and media into a centrifuge tube; keep harvested cells on ice for the duration of the extraction process.
3. Centrifuge the collected cells for 5 minutes at 300 g. A visible cell pellet should form during this step.
4. Decant the supernatant and resuspend the cell pellet in 1 mL PBS. Transfer cell suspension to microcentrifuge tube. Do not leave any cells behind.
5. Centrifuge cells at 2000 rpm for 5 minutes.
6. Remove all PBS from tube without disturbing the cell pellet (cell pellets can be stored at -20 °C for later use).
7. Prepare cell lysis buffer according to manufacturer's instructions (Invitrogen) by adding appropriate amount of protease inhibitor.

8. Resuspend harvested cell pellets in 100 µL of cell lysis buffer and incubate on ice for 45 minutes (volume of buffer should be adjusted for number of cells). During incubation period mix the pellet with micropipette to break up the cell pellet, then vortex every 7-9 minutes. This ensures the pellet is fully lysed.
9. Centrifuge for 15 minutes at 12,000 rpm, at 4°C.
10. Transfer the clear supernatant (protein extract) into a fresh tube on ice, store at - -80°C.
11. Perform protein estimation.

3.2 BCA Protein Estimation

An essential step in performing Western Blotting is to quantify the amount of protein extracted and used for electrophoresis. The Thermo Scientific Pierce BCA Protein Assay Kit was used for protein estimation. The kit uses bicinchonic acid (BCA) for the colorimetric detection and quantification of total protein. This method uses the reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline medium, with the highly sensitive and selective detection of the cuprous (Cu^{+1}) ion using a reagent that contains BCA. The reaction of the reagent with cuprous causes a purple colored reaction that has strong absorbance at 562 nm that is nearly linear with increasing protein concentrations; thus, it is possible to estimate the amount of protein based on the strength of the signal from the reaction (ThermoFisherScientific 2020).

3.2a Protein estimation assay

1. Dilute extracted protein; 1:10 dilution for DAOY cells (6 µL of extracted protein into 54 µL of H_2O).

2. Add 25 µL of diluted protein, BSA standards and water (blank) in duplicate into a 96-well plate.
3. Add 200 µL of BCA reagent to each well and incubate at 37°C for 30 minutes.
4. Measure absorbance in microplate reader using BCA protocol.
5. Generate a standard curve using the measured absorbance and known BSA standard. Extrapolate concentration of unknown proteins using linear regressing analysis.

3.3 SDS-PAGE

Proteins naturally have differing electrical charges, 3-dimensional structures, and molecular weights due to their varying molecular compositions; as such, it is possible to sort them based on different factors and in different ways. SDS-PAGE is a method of separating proteins based solely on their molecular weight by denaturing the proteins and overriding the natural charges of the proteins with a constant one. SDS is a detergent that denatures proteins and binds to their backbone at a constant molar ratio; if combined with a reducing agent that cleaves disulfide bonds, such as mercaptoethanol, the denatured proteins will unfold into linear chains with an electrical charge proportional to their length. Polyacrylamide gel forms a mesh-like structure, where the size of the holes can be adjusted by changing the percentage of polyacrylamide in the gel, and allows the proteins to be separated based on their length (MBLInternationalCorporation).

3.3a Casting the resolving gel (10%)

1. Assemble the gel casting apparatus and prepare the gel casting plates. Make sure that the seal between them is watertight. This can be checked by pouring water between them and observing for leakage.

2. In a centrifuge tube, mix together 4.9 mL H_2O, 2.5 mL 40% acrylamide, 2.5 mL resolving buffer, and 0.1 mL 10% SDS for a total volume of 10 mL. This recipe creates one10% resolving gel.
3. Add APS and TEMED. The gel will begin to polymerize after both of these have been combined, so it is important to proceed quickly.
4. Pipette the combined solution into the gel casting mold. Add a small layer of water on top of the gel to help it set faster and more evenly, then remove the water once the gel has set (time may vary but is typically 15-30 minutes).

3.3b Casting the stacking gel (4%)

1. In a centrifuge tube, add 3.2 mL H_2O, 0.5 mL 40% acrylamide, 1.25 mL stacking buffer, and 0.05 mL 10% SDS for a total volume of 5 mL, doubling all volumes if making two gels at once.
2. Add APS and TEMED, then immediately pipette the stacking gel on top of the resolving gel, filling to the top of the mold, insert comb, and allow to set.

After both parts of the gel have been cast, either refrigerate at 4°C if storing, or proceed with Western Blotting.

3.3b Sample Preparation

1. Prepare protein dilutions in microcentrifuge tubes, tubes, consisting of protein, 2X loading buffer, and water. The total loaded volume will vary based on the concentration of the protein extract. Typically, 25-30 µg of protein was used in our experiments.

2. Heat the samples at 100°C for 5 minutes in a dry plate to denature the proteins, keeping the microcentrifuge tubes covered so the lids do not pop open.
3. Briefly microcentrifuge samples at high speed to ensure their contents settle.

3.3c Electrophoresis

1. After the resolving and stacking gels have polymerized, place them into the electrophoresis assembly clamp, and place the clamp into an electrophoresis chamber. Two gels can be run simultaneously, use a placeholder plate in the opposite side of the clamp if running only one gel.
2. Fill the clamp with enough 1X running buffer to fill to the top of the plates, then fill the electrophoresis chamber at least half-way with 1X running buffer.
3. Carefully remove the combs from the gels. Using a micropipette, load the prepared protein samples into the wells.
4. Connect the apparatus to its power supply. Run the gel at 80 V until the proteins enter the resolving gel, then increase the voltage to 100 V. Run the gel until the dye reaches the green line at the bottom of the gel.

3.4 Western Blotting

Western Blotting is a protein identification technique that is useful for identifying the presence or absence of certain known proteins within cells, using antibody-associated markers. After using SDS-PAGE to separate the proteins, they are transferred to a membrane, nitrocellulose or another material, through a process of electro transfer. After the proteins have been transferred, the membrane is blocked with non-specific proteins from a source such as non-fat dry milk; the

blocking process prevents antibodies from binding non-specifically to the membrane. After blocking the membrane, it is treated with antibodies that are specific to the proteins of interest, referred to as primary antibodies, and incubated overnight. After the primary antibody incubation phase is done, a secondary antibody specific to the species of the primary antibody is added. The secondary antibody is conjugated to horseradish peroxidase (HRP) or a fluorescent small molecule for detection.

3.4a ThermoFisher iBlot Dry Blotting System

1. Open the lid of the iBlot Gel Transfer Device. Ensure the blotting surface is clean.
2. Remove the iBlot Anode Stack, Bottom from the package. Remove the laminated sealing of the iBlot Anode Stack, Bottom and keep the stack in the plastic tray.
3. Open the cassette and immerse the pre-run gel briefly in deionized water to facilitate easy positioning of the gel on top of the transfer membrane.
4. Place the pre-run gel on the transfer membrane of the anode stack. Make sure there are no bubbles trapped between the gel and the membrane.
5. In a clean container, soak one iBlot Filter Paper in deionized water and place on the pre-run gel. Use the Blotting Roller to remove any air bubbles between the membrane and gel.
6. Remove the iBlot Cathode Stack, Top from the package and place on top of the presoaked filter paper with the copper electrode side facing up (and agarose side facing down) and aligned to the right of the bottom stack. Remove any air-bubbles using the Blotting Roller. Place the iBlot Disposable Sponge on the inner side of the lid. Close the iBlot lid and secure the latch. Ensure that the correct program is selected and start transfer

1. After the transfer, carefully remove and discard the gel and filter paper by peeling them off of the membrane. Remove the transfer membrane from the stack and proceed with the blocking procedure. (ThermoFisherScientific 2012)

3.5 Primary and Secondary Antibodies and Detection

1. Prepare 5% milk from instant non-fat dry milk and 1X TBST buffer and block the membrane for at least 1 hour.
2. Discard the milk and wash the membrane once with 1X TBST buffer.
3. Dilute antibodies with 1X TBST.
4. Add primary antibody to membrane and incubate overnight at 4°C.
5. Remove the primary antibody.
6. Wash the membrane with 1X TBST buffer at least 3-4 times with 5-10-minute interval between wash. Discard wash buffer, making sure there is none in the container before adding secondary antibody. (All washing steps should be done at room temperature on an orbital shaker to ensure even agitation.) (Mahmood and Yang 2012)
7. Add the HRP-linked secondary antibody to the membrane, enough to cover it within the container, and allow it to incubate for 1 hour at room temperature.
8. Remove the secondary antibody.
9. Wash the membrane with 1X TBST wash buffer 3-4 times with 5-10-minute intervals between wash. Discard the buffer, making sure there is none left on the membranes.
10. Prepare the SuperSignal West Dura Extended Duration Substrate by mixing equal parts the Luminol/Enhancer Solution and the Stable Peroxide Buffer.

11. Add 1 mL of SuperSignal West Dura Extended Duration Substrate to the membrane and incubate for 5 minutes.
12. Analyze the membrane using an Imaging System.

RESULTS AND DISCUSSION

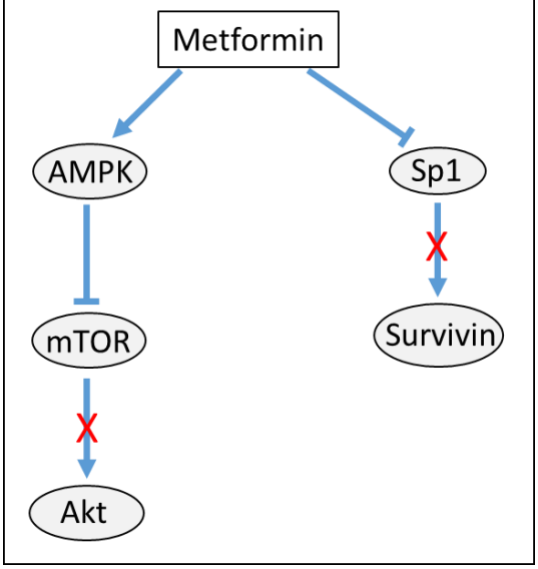
DAOY cells were used for Western Blotting. Cells were grown in 100 mm plates and treated with increasing doses of metformin (0, 5, 10 and 15 mM). The cells were harvested at 24h and 48h post-treatment. Proteins were extracted using Cell Lysis Buffer (Invitrogen), and quantitated using BCA protein assay as described in methods section 3.1. Western blot analysis was carried out using these extracts to determine expression of proteins of interest such as Sp1, survivin, actin, apoptotic proteins and cell signaling proteins.

The protein extracts (25 µg) were separated a on 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane using iBlot transfer system. Membranes were first blocked with blocking buffer (5% Milk in TBST) and then probed with specific primary antibodies overnight at 4 °C. Membranes were then washed and incubated at room temperature with secondary antibody for 1 hour (HRP conjugated anti-rabbit or anti-mouse antibody). The blots were washed before being developed using SuperSignal West Dura Extended Duration Substrate. The images were acquired using BioSpectrum Imaging System (UVP, Upland, CA).

The Western Blotting was carried out to determine the effect of metformin on the expression of with Sp1 and Survivin proteins. Expression of β-actin was used as a loading control. The amount of the protein expressed is proportional to the intensity of the band on the nitrocellulose membrane.

Survivin is an important cancer associated protein that plays a role in both apoptosis and cell cycle. Its overexpression in cancer cells is associated with poor prognosis and resistance to chemotherapy and radiation. Sp1 is a transcription factor that has been demonstrated to regulate the expression of survivin (Xu, Zhang et al. 2007, Chen, Wang et al. 2011). Previous studies have shown that metformin has the ability to downregulate Sp1 (Nair, Pathi et al. 2013). Here, we evaluated the effect of metformin on the expression of Sp1 and survivin in medulloblastoma cells. As seen in Figure 3.2, there was a decrease in both Sp1 and survivin proteins upon metformin treatment. At 24h post-treatment there is no change in the expression of either of the two proteins regardless of the metformin dose. However, at 48h post-treatment, there was noticeable decrease in expression of Sp1 and survivin. Akt is a protein whose expression is affected by metformin as part of its anti-cancer activity. Akt signaling pathway is associated with cell proliferation and is upregulated in cancer cells (Yung, Chan et al. 2013, Becher, Millard et al. 2017, Yang, Shi et al. 2020). Akt is shown to be phosphorylated by the mTOR complex (Yung, Chan et al. 2013). The phosphorylated state of Akt (pAkt) is its active form, which can be detected using Western Blotting.

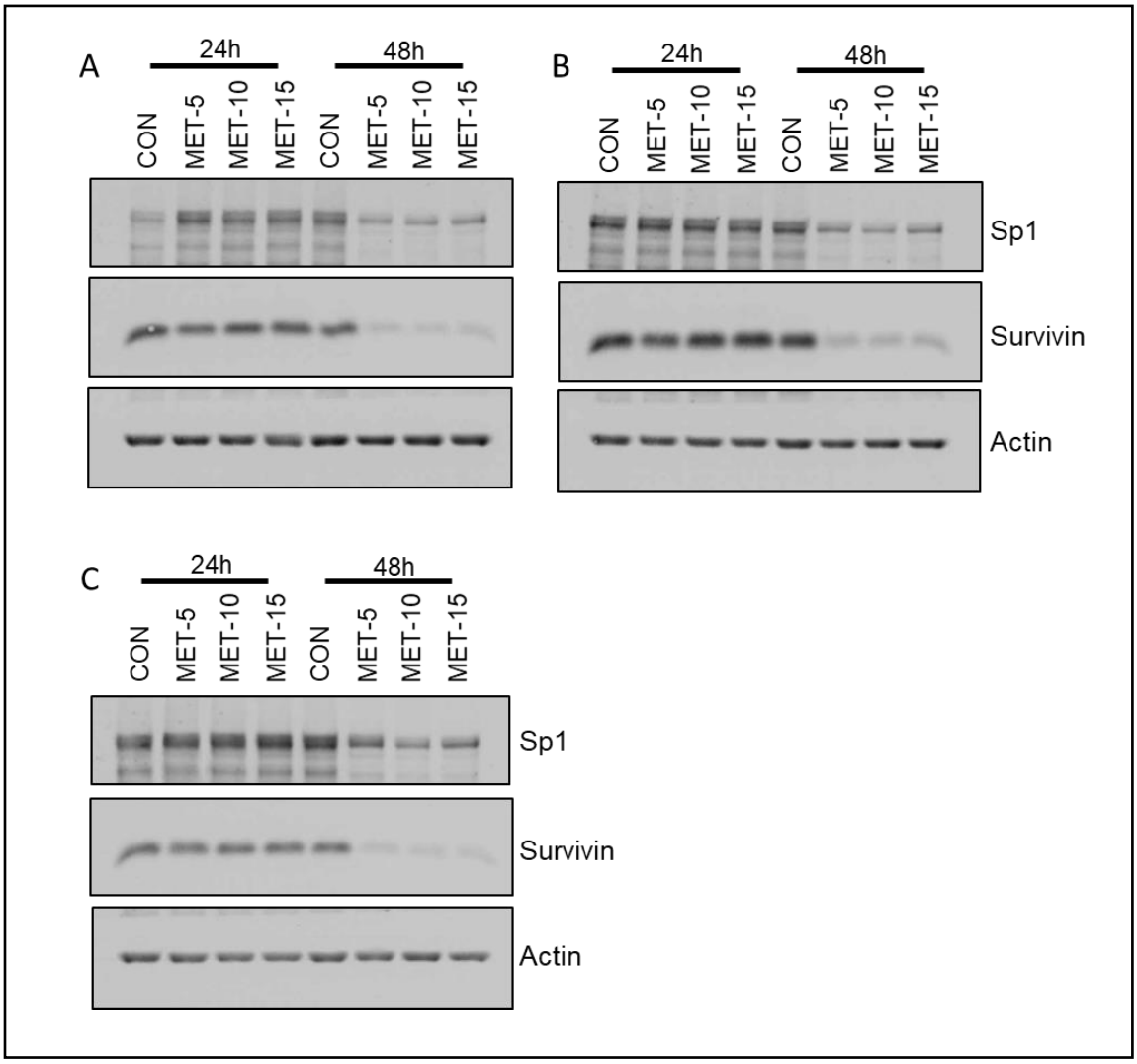
Figure 3.1 Possible mechanism of survivin and pAkt regulation by metformin



Based on information from literature that Sp1 can regulate survivin expression, it is possible the down regulation of Sp1 by metformin leads to a decrease in the expression of survivin in medulloblastoma cells (Xu, Zhang et al. 2007, Chen, Wang et al. 2011, Nair, Pathi et al. 2013). It is also possible that metformin could be acting independent of Sp1 pathway. It is documented in

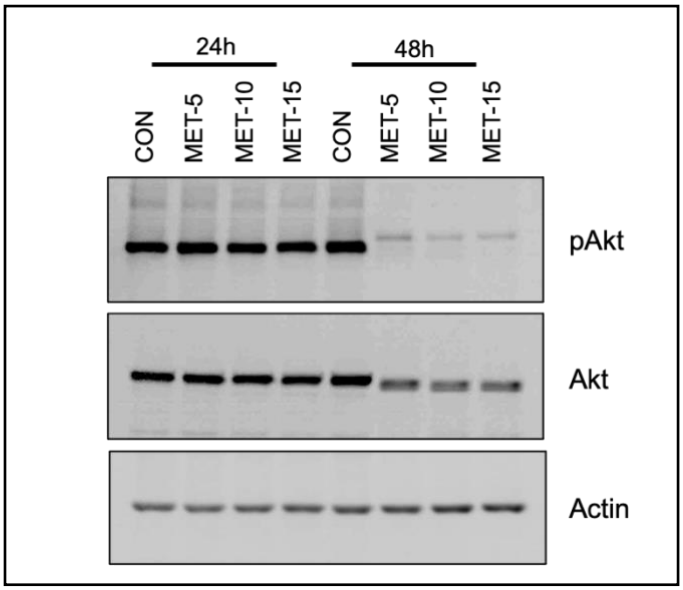
literature that Akt is phosphorylated by the mTOR Complex 2. It has also been demonstrated that metformin activates the AMPK, which in turn results in the inhibition of mTOR complex 2 (Cantrell, Zhou et al. 2010, Yang, Shi et al. 2020). The reduced expression of pAkt seen in DAOY cells treated with metformin (Figure 3.3) can be attributed to metformin inhibiting the activity of the mTOR pathways (Faubert, Boily et al. 2013, Morales and Morris 2015). Figure 3.1 illustrates the possible mechanism of metformin inhibiting Sp1while activating AMPK as a means of inhibiting medulloblastoma viability. Further experiments using promoter assays will need to be done to demonstrate the regulation of survivin by Sp1 in DAOY cells.

Figure 3.2 Effect of metformin on survivin, Sp1, and actin



DAOY cells were treated with increasing dose of metformin (0, 5, 10, 15 mM). Cells were harvested at 24- and 48-hours post-treatment and their proteins extracted. 25 µg of total protein was separated on SDS-PAGE, transferred to a nitrocellulose membrane and probed with antibodies for Sp1, survivin, and β-actin, which was used as a loading control. Blots were then probed with HRP-conjugated secondary antibody and detected using Supersignal Chemiluminescent Substrate. A. B. and C. represent three independent experiments.

Figure 3.3 Effect of metformin on pAkt and Akt



DAOY cells were treated with increasing dose of metformin (0, 5, 10, 15 mM). Cells were harvested at 24- and 48-hours post-treatment and their proteins extracted. 25 µg of total protein was separated on SDS-PAGE, transferred to a nitrocellulose membrane and probed with antibodies for pAkt, Akt, and β-actin, which was used as a loading control. Blots were then probed with HRP-conjugated secondary antibody and detected using Supersignal Chemiluminescent Substrate.

CHAPTER IV

APOPTOSIS ASSAYS AND CELL CYCLE

INTRODUCTION

Apoptosis is the process by which cells undergo programmed cell death, a necessary part of the cell cycle and cell health and is the safer method of cell death within the body. Apoptosis can be caused by intrinsic or extrinsic pathways, but both pathways activate the effector caspases 3 and 7, which cause cell death by cleaving macromolecules downstream. Given that these caspases are activated through either pathway, it is possible to measure apoptotic activity within cell cultures by measuring the activity of caspases 3 and 7 and determine if a particular drug treatment has had an effect on the rate of cell death (Wheatley and Altieri 2019).

SPECIFIC AIMS

The aims of this section of the internship were to study the effects of metformin on cell apoptosis, and apoptosis-related gene expression, and to correlate this to cell viability and survivin expression. The second part of the aim was to investigate the effects of metformin on the cell cycle using flow cytometry analysis, however, this aim was unable to be met due to circumstances and external factors. It was hypothesized that treating medulloblastoma cells with increasing doses of metformin would result in increased cell apoptosis, as well as increased expression of apoptosis related proteins and enzymes, such as cPARP and caspases 3 and 7.

METHODS AND MATERIALS

Materials

Caspase 3/7-Glo assay was purchased from Promega (Madison, WI), cPARP antibody was purchased from Cell Signaling (Danvers, MA).

Background and methods

4.1 Apoptosis

As mentioned above, apoptosis can be caused by intrinsic or extrinsic pathways. The intrinsic pathway is usually activated by internal stress factors or irradiation, leading to a loss of mitochondrial membrane potential and release of cytochrome *c*; this activates caspase 9, which is the initiator caspase. The extrinsic pathway is activated by ligand binding to the "death" receptors of the cell such as the TNF receptor, which activates the caspase 8 pathway. Both pathways activate the effector caspases 3 and 7, which cause cell death by cleaving macromolecules (Wheatley and Altieri 2019). While there are assays to study each pathway specifically, this internship focused on studying changes in caspase 3/7 activity.

There are also certain proteins associated with the apoptosis pathways that will be expressed in higher amounts in certain forms, such as cleaved poly (ADP-ribose) polymerase (cPAPR). PARP is a polymerase associated with DNA repair from environmental factors, and serves as a target of several caspases, such as caspase 3. Because of this, it is possible to use cPARP as an indicator of apoptotic activity using Western Blotting (Satoh and Lindahl 1992, Lazebnik, Kaufmann et al. 1994, Cohen 1997). Based on this information, increased levels of apoptosis should result in a measurable increase in cPARP levels, as measured by Western Blotting.

4.2 Caspase-Glo 3/7 assay

The caspase family of enzymes are essential components of the intrinsic and extrinsic apoptosis pathways, with caspases 3 and 7 being activated by both pathways. As a result, we can use an assay designed to detect the presence and quantity of caspases 3 and 7 to measure increases in apoptosis compared to a base line expression. For the purposes of this internship, Promega Caspase-Glo 3/7 was used. The assay contains a tetrapeptide sequence DEVD in a reagent that measures caspase and luciferase activity, as well as causing cell lysis. The process can be done in a simple "add-mix-measure" format that results in cell lysis, then caspase cleavage of the substrate and the generation of a "glow-type" luminescent signal from the luciferase. The luminescence of the cells, which is proportional to the amount of caspase activity present, is measured by a luminometer after an hour-long incubation period, which allows the enzymes to reach peak activity and then remain at a steady state for several hours (Promega 2020).

4.2a Preparation of Caspase-Glo 3/7 Reagent

1. Equilibrate the Caspase-Glo 3/7 Buffer and lyophilized Substrate to room temperature.
2. Transfer the contents of the Buffer bottle into the amber bottle containing the substrate. Mix by swirling or inverting the contents until the substrate is thoroughly dissolved to form the Caspase-Glo 3/7 Reagent.
3. If necessary, freeze unused reagent and store at -20° until use; reagent that has been frozen for 1 week will have approximately 75% signal strength compared to fresh reagent

(Promega 2020)

4.2b Detection of Caspase-3 and -7 Activities in Cell-Based Assays

1. Before starting, allow the Caspase-Glo 3/7 reagent to equilibrate to room temperature
2. Remove 96-well plates containing treated cells from incubator and allow to equilibrate to room temperature. See section 2.7 for details on preparing 96-well plates.
3. Add 100 µL of caspase3/7 reagent to the wells of interest (treated wells, blanks, and controls) and allow to incubate at 37°C for 1 hour.
4. After incubation, measure luminescence in microplate reader. Record and analyze results. (Promega 2020)

4.3 Statistical Analysis

The data was analyzed using a one-way analysis of variance (ANOVA) test, which is used to determine if there are statistically significant differences between the means of three of more independent groups.

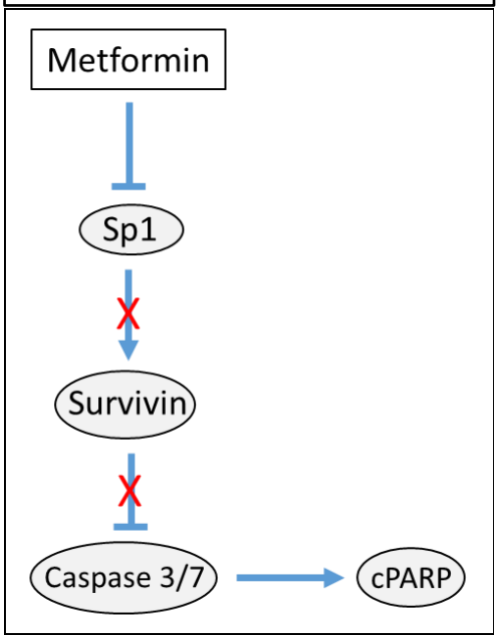
The Dunnett's test was applied as a post-hoc test on the one-way ANOVA results. The Dunnett's test measures experimental groups against a control group to determine which pairs of means from an ANOVA are statistically significant. GraphPad Prism was used to process the data and perform the statistical analysis tests.

RESULTS AND DISCUSSION

Metformin has the ability to decrease viability and down regulate Sp1 and survivin protein levels in medulloblastoma cells (DAOY) To better understand the mechanistic details of metformin's action, its effect on apoptosis was studied. Activity of effector caspases 3 and 7 was studied using

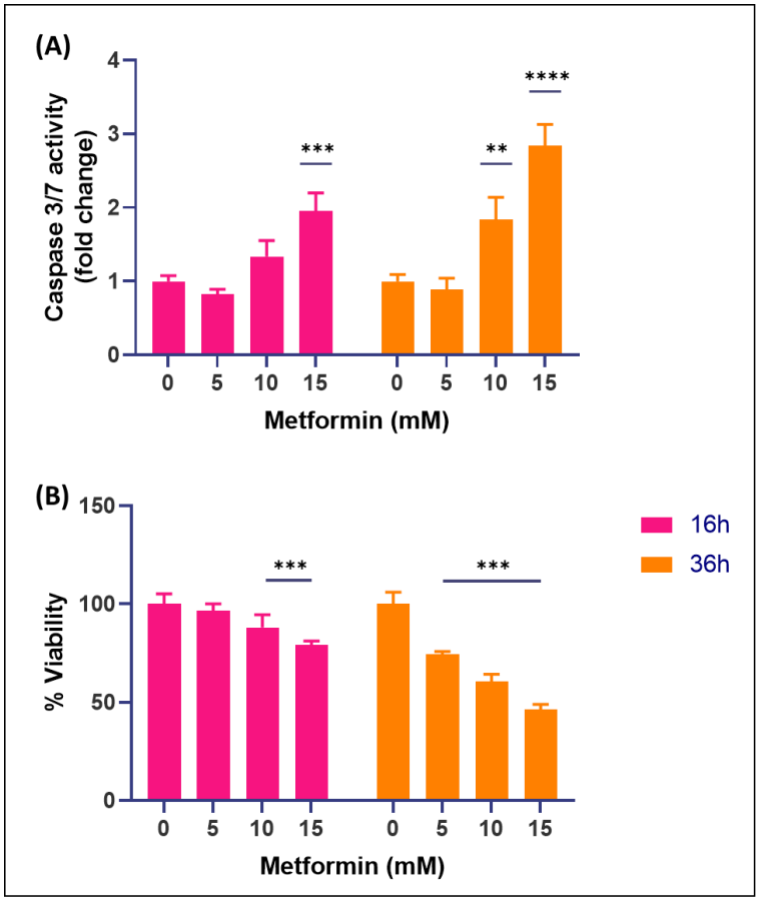
the Caspase-Glo 3/7 assay (Promega). DAOY and D283 cells in 96-well plates were treated with increasing doses of metformin (0, 5, 10, and 15 mM). Activity of caspase 3/7 was measured at 16h and 36h post-treatment using Caspase-Glo 3/7 assay. The data obtained as relative light units (RLU) was normalized to untreated control and to the corresponding viability data. As seen in Figure 4.1, there is a dose and time dependent increase in caspase 3/7 activity, with a corresponding decrease in viability. At 16h, up to 2-fold increase in activity was observed, at the highest dose of metformin. At 36h, the caspase activity increased to 3-fold with 15 mM metformin. Apoptotic activity was also determined by the cleavage of PARP (cPARP, poly-ADP ribose polymerase) protein which is a downstream target of the activated caspases. Western blot analysis was used to determine the expression of cPARP. As seen in Figure 4.3, significant cPARP was detected at 48h post-treatment with metformin which correlated with increased caspase activity. These two results taken together suggest that metformin has the ability to induce apoptosis in medulloblastoma cells, as illustrated in figure 4.1. Our results are similar to what has been observed in literature with other cancer types, such as colon cancer, breast cancer, bile duct cancer, and ovarian cancer (Yasmeen, Beauchamp et al. 2011, Gao, Liu et al. 2016, Sena, Mancini et al. 2018, Lee, Hong et al. 2019). Apoptosis is routinely analyzed by flow cytometry using the Annexin V assay, which detects the membrane phosphatidylserine that translocates from the cytosolic side to the outside. This section of experiments was left largely unfinished, compared to the original aims, due to time constraints and unexpected difficulties during my internship practicum. As a result,

Figure 4.1 Mechanism of induction of apoptosis by metformin



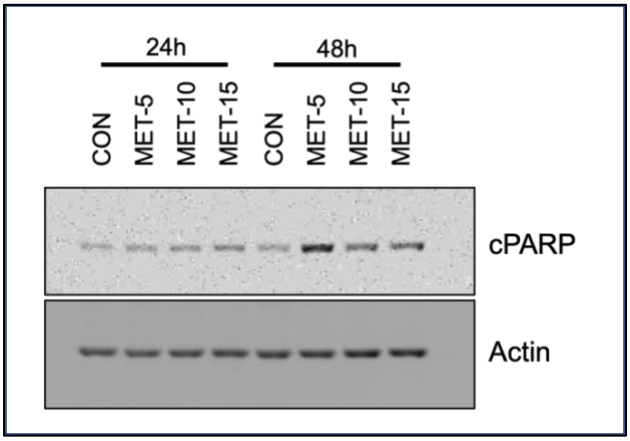
only one major caspase experiment was performed on the DAOY and D283 cell lines, and the flow cytometry analysis and experiments were unable to be performed.

Figure 4.2 Caspase-Glo 3/7 assay and viability analysis



DAOY cells were treated with increasing doses of metformin (0, 5, 10, 15 mM). Cell viability was measured using CellTiter-Glo and Caspase 3/7 kits at 16 and 36 h post treatment. Percent viable cells and percent caspase 3/7 activity compared to control were calculated and plotted against metformin dose. Treatments were done in triplicate and data shown is the mean+/-SD. Statistical analysis was done using a one-way ANOVA test with Dunnett's multiple comparison test. The * indicates p values and the degree of statistical significance. * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$, **** $p \leq .0001$.

Figure 4.3 cPARP Western Blot analysis



DAOY cells were treated with increasing dose of metformin (0, 5, 10, 15 mM). Cells were harvested and proteins extracted. 25 µg of protein were used for Western Blot analysis. The blot was probed with anti-cPARP antibody, Beta-actin was used as loading control.

CHAPTER V

SUMMARY AND CONCLUSIONS

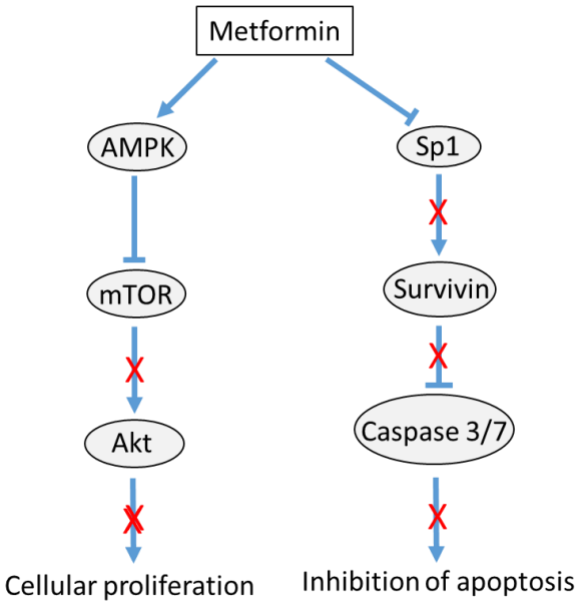
The experiments performed during this internship practicum examined the properties of metformin as a potential anti-cancer drug by studying its effects on cancer cell viability, cancer associated protein expression, and apoptosis levels. The results obtained suggest that metformin works as an inhibitor of medulloblastoma viability, with treated cells displaying a time- and dose-dependent response curve. Metformin also shows evidence of being an inhibitor of Sp1, survivin, and pAkt expression in medulloblastoma cells, and increasing caspase activity in a dose and time-dependent response curve.

The conclusion reached during this internship practicum project is that metformin decreases viability and increases apoptotic activity in medulloblastoma cells through a combination of factors, particularly through the activation of the AMPK pathway's inhibitory mechanisms and through the inhibition of survivin expression by the downregulation of Sp1 transcription factor. pAkt is associated with cell proliferation and is phosphorylated by mTOR, which is inhibited by the AMPK pathway. Metformin activates AMPK, which inhibits mTOR from phosphorylating Akt into its active state, which is also seen in our experimental results; this indicates that metformin may enhance AMPK's role as a metabolic tumor suppressor (Shaw, Lamia et al. 2005, Cantrell, Zhou et al. 2010, Kim, Kundu et al. 2011, Faubert, Boily et al. 2013, Yung, Chan et al. 2013, Lee, Hong et al. 2019).

The decreased levels of Sp1 and survivin indicate that metformin inhibits Sp1 expression, which can also be tied to the decrease in survivin as Sp1 is known to regulate survivin's expression

in cancer (Xu, Zhang et al. 2007, Chen, Wang et al. 2011, Nair, Pathi et al. 2013). The decreased survivin levels also correlated with an increase in apoptotic activity, as measured in the experiments. As survivin is an inhibitor of apoptosis protein (IAP) that inhibits the activity of caspase enzymes, it is, therefore, likely that the increase in apoptotic activity and cPARP protein observed in metformin treated cells is a result of the reduced survivin levels, which the experimental data supports. The proposed mechanism is illustrated in Figure 5.1

Figure 5.1 Possible mechanism of action of metformin in medulloblastoma.



Combined with the evidence from studies that support these findings, the overall conclusion reached during this internship is thus: metformin shows potential as an anti-cancer therapy option. This means that metformin could potentially be used to reduce the severity of the side effects of therapy regiments used to treat medulloblastoma by reducing the doses needed to achieve results in patients. It could also potentially improve the outcome for patients suffering from medulloblastoma subtypes with poor prognoses, such as groups 3 and 4.

As a follow up to the experiments performed in this internship, the next steps would be to perform further testing and profiling on more cell lines as well as performing combination studies to examine how metformin interacts with current therapies. Further *in vitro* testing of metformin should be performed in additional cell lines of medulloblastoma to verify if it remains effective, especially in cell lines that represent the molecular subtypes. Additionally, it is important to

evaluate metformin as part of a combination therapy option for current medulloblastoma treatments, as increasing their effectiveness should allow less harsh treatment, which could reduce the detrimental side effects suffered by medulloblastoma patients.

INTERNSHIP EXPERIENCE

My internship was undertaken at the University of North Texas Health Science Center, with Dr. Umesh Sankpal, Ph.D. as my major professor.

Work during the internship was heavily disrupted in March 2020, due to the outbreak of COVID-19 and the subsequent lockdowns imposed state-wide. As a result, there was a long stretch between March and June during which no lab work could be conducted. By late June, work in the lab resumed, with limited hours and accessibility, though the several months long interruption meant that any previously in-progress experiments had to be restarted.

I am very grateful for the opportunities I was given for this internship, as I have gained valuable laboratory experience. The techniques I have learned to use for cell culturing and viability assays will be useful to me in the future, as I can now reliably grow human cell cultures and freeze new stocks for later use. Additionally, I am now familiar with the principles of using viability assays to assess cytotoxicity and anti-cancer activity, should I need to apply them in the future.

APPENDIX A

Weekly Journal

Week 1: 2/3/2020 - 2/6/2020

Began internship. Received DAOY medulloblastoma cells from Dr. Sankpal. Subcultured and harvested DAOY cells, prepared 96-well plates (for viability assay) and 10 cm plates (for protein extraction). Cells were treated with 0, 1, 5, 10, 20, 40, and 80 mM metformin for 96-well plates and 0, 5, 10, 15 mM for 10 cm plates, over 24 and 48h. Prepared 6-well colony formation assay. DAOY cells (1000, 500 cells) were treated with metformin and plated in 6-well plates in triplicates. Colonies formed after 2-weeks will be stained and counted. Contamination in some of the stock plates 10 cm plates.

Week 2: 2/10/2020 – 2/14/2020

DAOY cells were plated in 96-well plates for viability assay. Cells were treated with metformin doses (0-80 mM) for 24 and 48 h and viability determined using assay. Proteins were extracted from metformin treated cells from week 1. Thawed D283 medulloblastoma cells, these cells are semi-adherent unlike DAOY which are adherent. Performed protein estimations for Western Blotting. Viability assay results indicate that there is an early dip and sudden drop off at 40 mM metformin dose.

Week 3: 2/17/2020-2/21/2020

DAOY and D283 cells were plated for viability assays in 96-well plates, and for Western blotting in 10 cm dish. Cell were treated over 24 and 48h with metformin (0-80 mM). for viability and 0,

5, 10, 15 mM metformin for Western blotting. Extracted proteins from week 2 DAOY cells. Performed first Western Blots using treated cells from 2/4/2020. Viability assay results have steady decline, then drop off at 40 mM

Week 4: 2/24/2020-2/28/2020

DAOY and D283 subcultured in 10 cm plates, treated with metformin (0-15 mM), and harvested after 24 and 48 h. D283 96-well plate viability assays performed (0-80 mM). Ran Western Blots from remaining previous protein extracts (2/12, 18, and 21) and analyzed. Stained colony assay plates with crystal violet-methanol/PBS. Viability assay results show early increase followed by sudden drop, not great.

Week 5: 3/3/2020-3/6/2020

Performed D283 viability assays (0-80 mM metformin) and extracted proteins from treated cell pellets (from week 4). Ran 2 sets of Western Blots for treated cells (weeks 2 and 3). Analyzed IC_{50} values of DAOY and D283 viability assays. Viability assay results are odd- an early dip followed by steady decrease. Western Blots showed no signs of Sp1, possible issues with membranes (transfer) or with antibody.

Week 6: 3/10/2020-3/13/2020

Plated DAOY and D283 cells for metformin treatments (0-15 mM) (Western Blotting) and harvested after 24 and 48 h. Performed DAOY and D283 96-well plate viability assays over 24 and 48 h (0-80 mM). Developed Western Blot membranes for week 5 protein extracts. IC_{50} data

analysis. Lost a plate of D283 cells. Viability assay results for DAOY show steady decline in viability. D283 shows early sharp drop, then gradual decline.

Week 7: 3/16/2020-3/19/2020

DAOY and D283 viability assays performed over 24 and 48 h (0-80 mM metformin). Subcultured DAOY and D283 to prepare them for treatment and protein extraction. Lockdowns have begun to affect my work. Viability assay results for DAOY have steady decline followed by sharp drop at highest doses. D283 shows steady decline at all points

3/23/2020-6/4/2020: Lockdown period

Work interrupted due to lockdown. Worked from home on whatever I could, mostly doing research and data analysis. Unable to conduct experiments. Prepared research proposal for committee

6/4/2020

Presented research proposal to committee members.

6/5/2020-6/17/2020: Lockdown continues

Continued working from home.

Week 8: 6/18/2020 and 6/23/2020-6/25/2020

Returned to lab with limited operability. Previous cell subcultures had to be discarded. Thawed new DAOY and D283 cells on 18th. Starting from 23rd, subcultured DAOY cells and D283 cells.

Week 9: 6/29/2020-7/2/2020

Subcultured and froze DAOY cells, D283 cells showed signs of fungal contamination and had to be discarded and replaced. Thawed new D283 cells.

Week 10: 7/6/2020-7/10/2020

Subcultured DAOY and D283 cells, treated with metformin for Western Blotting (0, 5, 10, 15), and harvested after 24 and 48 h. DAOY viability assays performed over 24 and 48 h (0-80 mM metformin). D283 cells were lost to bacterial contamination, had to thaw new D283 cells. DAOY viability results are showing steady decrease over 24 hours, but total loss of viability followed by spike at 20 mM after 48 hours. Need more data.

Week 11: 7/13/2020-7/16/2020

Subcultured DAOY and D283 cells and performed DAOY viability assays over 24 and 48 h (0-80 mM metformin). DAOY viability results show more stable loss of viability across all points at both times. D283

Week 12: 7/20/2020-7/24/2020

Subcultured DAOY and D283 cells and froze D283 cells. Performed 96-well viability assays for DAOY and D283 (0-80 mM metformin) over 24 and 48 h. Harvested DAOY and D283 cells treated with metformin (0, 5, 10, 15.mM) after 24 and 48 h. Unusual DAOY viability assay data this week. DAOY viability results show early increase then very slight decrease at 24 hours, with early dip and late total loss after 48 hours. D283 shows decline across all doses and both time points. Personal error is suspected as cause of 24-h DAOY viability results.

Week 13: 7/27/2020-7/31/2020

Subcultured DAOY and D283 cells and froze D283 cells. Performed DAOY and D283 viability assays (0-80 mM metformin) over 24 and 48 h (48-hour assays performed over the weekend by Dr. Sankpal). Some DAOY and D283 culture plates show contamination, probably from poor handling and technique, but did not lose entire stock. Subcultured non-contaminated plates while keeping an eye out for contamination. DAOY viability results show return of the total loss then spike at 20 mM over 48 hours seen in week 10. D283 shows similar results to week 12.

Week 14: 8/3/2020-8/7/2020

Subcultured DAOY and D283 cells. DAOY and D283 viability assays (0-80 mM metformin) performed and analyzed over 24 and 48 h. Made new metformin for treatments and compared its results against old stock; DAOY viability results are almost exactly the same for both metformin treatments, but they show similar loss of viability with spike at 20 mM seen in weeks 10 and 13. D283 is continuing to show results of steady decline with dose and time response.

Week 15: 8/10/2020-8/13/2020

Subcultured DAOY and D283 cells. Performed 96-well viability assays for DAOY and D283 (0-80 mM metformin) over 24 and 48 h. Subcultured cells were behaving strangely, going to thaw new cells to investigate/compare against next week. Lost at least one 10 cm plate of D283 to contamination. DAOY viability results show the total loss then spike at 20 mM. D283 showed some slight fluctuations over 24 hours, but a general decrease with time and dose.

Week 16: 8/17/2020-8/20/2020

Performed 96-well viability assays for DAOY and D283 (0-80 mM metformin) over 24 and 48 h at the beginning of week. Prepared more Reagent for experiments. New DAOY and D283 cells were thawed and subcultured; previous DAOY cells have strange viability response, going to compare against new batch of DAOY. Performed 96-well viability assays for new DAOY and D283 cells, same metformin treatments, 48-hours only at the end of the week. DAOY viability results show the total loss then spike at 20 mM, again. D283 is back to a stable decrease with time and dose.

Week 17: 8/24/2020-8/28/2020

D283 plates had fungal contamination, thawed new D283. Subcultured DAOY and new D283 cells. Prepared 96-well viability assay for DAOY (0-80 mM metformin) for Sunday, 8/30/2020 48 h viability assay, handled by Dr. Sankpal. DAOY viability results show a dose and time response, with near total loss at the highest doses.

Week 18: 8/31/2020-9/4/2020

Analyzed 48-hour DAOY viability assay from Sunday. Froze D283 cells. Subcultured DAOY and D283 cells and treated them with metformin (0, 5, 10, 15 mM) for Western Blotting. Performed 96-well viability assays for DAOY and D283 (0-80 mM metformin) over 24h and 48h and harvested 24- and 48-hour cell cultures. 48 h viability assays were performed on Saturday, 9/5/2020 by Dr. Sankpal. D283 results continue to show dose and time response. DAOY results once again show the total loss followed by a spike at 20 mM pattern.

Week 19: 9/8/2020-9/11/2020

Subcultured DAOY and D283 cells and treated them with metformin for Western Blotting, (0, 5, 10, 15 mM). Performed 96-well viability assays for DAOY and D283 (0-80 mM metformin) over 24 and 48 h. Performed protein extractions on harvested cell pellets from week 18. Froze D283 cells. Will be moving to a new lab soon. DAOY viability results show dose and time response. D283 is similar to before with slight increase at low doses over 24 hours.

Week 20: 9/14/2020-9/18/2020

Subcultured D283 and DAOY cells and treated them with metformin for Western Blotting, (0, 5, 10, 15 mM). Harvested treated cell cultures over 24 and 48 h. Performed 96-well viability assays for DAOY and D283 (0-80 mM metformin) over 24 and 48 h. Performed protein extractions. Autoclaved paper towels for usage in new lab. Ran out of metformin, so prepared new stock. DAOY viability results show loss of viability followed by spike at 20 mM. D283 shows dose and time response.

Week 21: 9/21/2020-9/25/2020

Subcultured DAOY cells in 10 cm plates. D283 fungal contamination appeared again, had to thaw new D283 cells. Moved to new lab on Friday, 9/25/2020, and subcultured DAOY and D283 cells after transporting them on ice to new biosafety cabinet.

Week 22: 9/28/2020-10/2/2020

D283 cells from old lab showed contamination and mass death. DAOY cells were growing unusually slowly, then showed contamination. Thawed new DAOY and D283 cells and worked on internship practicum report

Adjusting to new lab equipment and requirements. The new microscope is more difficult to use without a low magnification lens and with an uneven observation stand.

Week 23: 10/5/2020-10/9/2020

Subcultured DAOY and D283 cells in 10 cm plates. Performed protein estimation on previously collected protein extracts and estimated proteins with BCA protein estimation assay. No signs of contamination in new cells.

Week 24: 10/12/2020-10/16/2020

Subcultured DAOY and D283 cells and treated them with metformin (0, 5, 10, 15 mM) for Western Blotting. Harvested cells after 24 and 48 h. Performed protein extractions on previously harvested cell pellets. Performed DAOY and D283 96-well Caspase 3/7 assays (treated with 0, 5, 10, and 15 mM) and viability assays over 16, 24, and 36 h (0-80 mM metformin). Ran and analyzed Western Blots for survivin and Sp1 protein extracts. Froze D283 to help replenish stocks. There were problems with the APS for the SDS-PAGE gels, had to replace it with new APS. Prepared new stock of metformin. Both caspase assay results show a dose and time dependent increase in caspase activity. The viability assays of DAOY and D283 both show a decrease in viability that is dose and time dependent.

Week 25: 10/19/2020-10/23/2020

Stripped Western Blot membranes and tried to analyze apoptosis proteins- didn't work. Subcultured DAOY and D283 cells, treating them with metformin (0, 5, 10, 15 mM) for 24 and 48 h, and froze more D283 cells. Performed DAOY and D283 viability assays and DAOY caspase 3/7 assay over 24, 40, and 48 h. Ran remaining sets of Western Blots for Sp1 and survivin, and new Blots for cCasp3, pAkt, and CyclinD3. Analyzed IC_{50} values for viability assays. Worked on practicum report. DAOY viability assay results show the 48-hour loss of viability followed by the spike at 20 mM, while the D283 results show the steady dose and time dependent decrease. The DAOY caspase assay is strange, showing an increase and spike in caspase activity, followed by a decline (probably due to a lack of living cells).

Week 26:10/26/2020-10/30/2020

Worked on internship practicum report. Viability assays for DAOY and D283. The DAOY viability results show a stable dose and time response, with typical loss of viability at high doses. The D283 results show a stable dose and time response loss of viability.

11/2/2020-11/6/2020

Continued to work on internship practicum report and submitted it for review.

11/9/2020-11/13/2020

Worked on defense presentation and defended internship practicum.

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