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Metformin inhibits 2D and 3D motility in Astrocytoma Cancer Cells

By

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DEDICATION

To my Beloved Parents

My whole life

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Marwa F. Al Hassan

ABSTRACT

Metformin is an oral anti-hyperglycemic agent in the biguanide class which lowers blood glucose concentrations and has been used to treat type 2 diabetes mellitus. Metformin is highly used in polycystic ovary syndrome (PCOS), premature puberty, and non-alcoholic fatty liver disease (NAFLD). A brain tumor is an intracranial solid neoplasm which is a tumor within the brain or the central spinal canal. According to the World Health Organization (WHO), primary brain tumors are classified according to their histological characteristics and cellular origin. In addition, gliomas, which belong to the neuroepithelial brain tumors, include oligodendroglioma, astrocytoma, ependymoma, mixed glioma, and glioblastoma. Previous studies showed a cytotoxic effect of metformin on ovarian cells. This prompted us, in our current study, to investigate the effect of metformin on the proliferation and metastasis astrocytoma. When this drug is applied, it led to a relative decrease in the proliferation of astrocytoma cancer cells. Metformin treatment also resulted in a drastic inhibition of 2D cell motility and an increase in cellular adhesion. Hence, our study shows that Metformin could be used as an anti-metastatic drug in brain tumor.

Keywords: Metformin, Cell Proliferation, Cell Adhesion, Cell Motility,
Astrocytoma.

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LIST OF ABBREVIATIONS

WHO: World Health Organization

GBM: Glioblastoma multiforme

M: Mitosis phase

G1: Gap phase 1

S: Synthesis phase

G2: Gap phase 2

DNA: Deoxyribonucleic acid

CDK: Cyclin-dependent kinase

PI3K: Phosphatidylinositol 3-kinase

Akt: Acutely transforming retrovirus

RTK: Receptor tyrosine kinase

GPCR: G-protein-coupled receptor

PI(4,5)P₂: Phosphatidylinositol (4,5)-diphosphate

PI(3,4,5)P₃: Phosphatidylinositol (3,4,5)-triphosphate

PH: Pleckstrin homology

mTOR: Mammalian target of rapamycin

PDK1: Phosphoinositide-dependent kinase-1

TORC2: transducer of regulated CREB activity 2

ECM: Extracellular matrix

FAK: Focal Adhesion Kinase

NAFLD: Non-alcoholic fatty liver disease

PCOS: Polycystic ovary syndrome

GLUT4: Glucose transporter type 4

AMPK: Adenosine monophosphate-activated protein kinase

LKB1: Liver kinase B1

S6K1: S6 kinase 1

IGF-1: Insulin-like growth factor 1

IRS-1: Insulin receptor substrate 1

IGF1R: insulin-like growth factor 1 receptor

ERK1/2: Extracellular signal-regulated protein kinases 1 and 2

mTORC1: mammalian target of rapamycin complex 1

MO25: Mouse protein-25

STRAD: STe20-Related Adapters

Raf: Rapidly Accelerated Fibrosarcoma

MEK: MAPK/ERK kinase

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal Bovine Serum

PBS: Phosphate Buffered Saline

WST-1: Water-soluble tetrazolium salt

ELISA: Enzyme-linked immunosorbent assay

SEM: Standard error of the mean

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

PVDF: Polyvinylidene fluoride

ECL: Enhanced chemiluminescence

Chapter I

LITERATURE REVIEW

1.1. Brain tumors

1.1.1. Introduction

A brain tumor is an intracranial solid neoplasm which is a tumor within the brain or the central spinal canal. Brain tumors are created by an abnormal and uncontrolled cell division. These tumors can be either malignant which means cancerous or benign (non-cancerous). In fact, the combination of different factors like the type of tumor, its size, its state of development, and its location can determine the tumor threat level (Barrow Neurological Institute, 2013).

The incidence rate in the United States for primary brain and nervous system tumors in 20 years old adults or older is estimated to be 27.4 per 100,000 persons (Ostrom, Gittleman, & Farah, et al., 2013). Approximately one-third of tumors in these different cases are malignant and the remainder is benign or even borderline malignant (Kohler, Ward, & McCarthy, et al., 2011; Ostrom, Gittleman, & Farah, et al., 2013).

1.1.2. Classification

According to the World Health Organization (WHO), primary brain tumors are classified according to their histological characteristics and cellular origin. Thus, WHO classifies primary brain tumors into five different classes which include, lymphomas and hematopoietic neoplasms, tumors of the cranial and paraspinal nerves, neuroepithelial

tumors, tumors of the meninges, and an important fifth class that includes germ cell tumors and tumors of the sellar region (Louis, Ohgaki, Wiestler, & Cavenee, 2007). Gliomas, which belong to the neuroepithelial brain tumors, include oligodendroglioma, astrocytoma, ependymoma, mixed glioma, and glioblastoma (CBTRUS, 2011). In addition, gliomas are categorized based on their grade, so they can be either low-grade gliomas or high-grade gliomas. Low grade gliomas are well-differentiated, benign, and carry better prognosis, whereas high-grade gliomas are malignant and carry worse prognosis. Approximately, gliomas make about 30% of brain and central nervous system tumors and about 80% of all malignant brain tumors (Goodenberger, & Jenkins, 2012).

1.2. Astrocytoma

Astrocytoma develops from glial cells called astrocytes. They may occur in different parts of the brain and most commonly in the cerebrum (Ohgaki, & Kleihues, 2009). The World Health Organization (WHO) classified astrocytoma according to a grading scheme which is based on the presence of specific characteristics such as mitosis, necrosis, atypia, and endothelial proliferation. The four grades include: grade I which is Pilocytic astrocytoma, grade II (low-grade diffuse astrocytoma), grade III (Anaplastic astrocytoma), and grade IV (Glioblastoma multiforme, GBM) (Ohgaki, & Kleihues, 2009).

In addition, grade II astrocytoma are well differentiated tumors that seldom grow but show an intrinsic susceptibility for diffuse infiltration of the different neighboring brain structures. Thus, Grade III and IV astrocytoma are considered malignant accounting for the majority of the patients' mortality (Watanabe, et al., 2003).

1.3. Cell cycle is controlled by PI3Kinase/Akt pathway

A pro-survival signaling pathway in a cell includes the “Phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway”. Cellular stimuli and toxic insults activate this signaling pathway. In fact, PI3K/Akt signaling pathway regulates different cellular functions, which include proliferation, apoptosis, translation, growth, and cell cycle progression (Maddika, et al., 2007). This family of lipid kinases catalyzes the phosphorylation of phosphoinositides (Dbouk & Backer, 2010).

PI3Ks are classified into three different classes depending on their sequence homology and the specificity of their substrates. Thus, RTKs or Receptor Tyrosine Kinases and the GPCRs or G-protein coupled receptors are upstream of Class I PI3Ks which phosphorylate PI(4,5)P₂ to produce PI(3,4,5)P₃ that recruits proteins which includes Pleckstrin homology (PH) domain. In fact, the two main classes of PI3Ks are Class IA and Class IB. The first class includes heterodimer PI3Ks of P110 α catalytic subunit and with regulatory subunits including P85, P50 and P55. However, Class IB PI3Ks include dimers with a P110 catalytic subunit and regulatory subunits that are P101 and P87. This classification allows Class IA of PI3Ks to be downstream of receptor tyrosine kinases (RTKs) and Class IB of PI3Ks to be downstream of G-proteins coupled receptors (GPCRs) (Dbouk & Backer, 2010). Therefore, this will allow the activation of different intracellular proteins included in many signaling pathways to regulate cell proliferation and motility (Vivanco & Sawyers, 2002).

Akt is one of the main downstream targets of Phosphatidylinositol 3-kinase. When the PH domain of Akt binds to the PI3K phospholipid products, Akt will be translocated into the plasma membrane. Hence, at this stage, Akt is activated by the phosphorylation of upstream kinases mTOR and PDK-1. Thus, upon its activation, Akt activates many proteins that are included in different cellular responses such as cell survival (Dugourd et. al, 2003).

Figure 1: Akt promotes cell survival and proliferation. Downstream of PI3K, the activated Akt phosphorylates and activates cytosolic different effectors which lead to cell growth and proliferation. **Source: (Echeverria & Sellers, 2008).**

1.4. Glioma Invasion

Glioma invasion into parts of the brain is a multistep process which consists of a number of steps that include integrated extracellular and intracellular interactions (Nakada, et al., 2007). First of all, glioma cells should detach from the original site which is the emerging tumor mass. Thus, the subsequent step is the binding of those cancer cells to the ECM, extracellular matrix. Then, degradation of the ECM is triggered by the secretion of matrix proteinases or activators of matrix proteinases from the glioma cells. These glioma cells migrate through creating a space which is formed by the degradation of the ECM to finalize the invasion process (Giese, et al., 2003).

1.4.1. Factors included in glioma invasion

In order for the glioma cell to detach, downregulation of some adhesion molecules involving E-cadherin and NCAM, neural cell adhesion molecules is required (Teodorczyk & Martin- Villalba, 2010). In fact, homophilic binding is mediated by NCAM which corresponds to the superfamily of glycoprotein immunoglobulin receptors. Also, as the astrocytic tumors progresses, the NCAM expression decreases. Another necessity for the detachment requires the cadherin switch proposed to occur during the conversion from a benign tumor to a malignant invasive tumor. This shift includes E-cadherin downregulation and N-cadherin upregulation (Teodorczyk & Martin- Villalba, 2010). The metastatic and invasive properties of cells are directly related to their adhesive state. This mechanism is reflected by the increase of cells' adhesion to mesenchymal cells and a decrease of adhesion to their neighboring epithelial cells (Hulit, et al., 2007). In fact, CD44, an adhesion molecule that is a main part of the initiation of invasion is upregulated in human gliomas (Ranuncolo, et al., 2002). This adhesion molecule is a transmembrane glycoprotein and also a member of the immunoglobulin receptor superfamily.

The next step involved in invasion is the attachment to the ECM through transmembrane proteins called integrins which form focal adhesions. These focal adhesions are known to be mature focal complexes which represent integrin receptor aggregation on the cell surface (D'Abaco & Kaye, 2007). Furthermore, many classes of proteases such as matrix metalloproteinases (MMPs) specifically MMP-2, MMP-9, and membrane-type MMP (MT-MMP) and ADAMs (a disintegrin and metalloproteinase)

are responsible for the ECM remodeling. Plasmin, which is included as a leading factor in this multistep process, is needed to activate MMP protoenzymes. Moreover, the generation of plasmin from plasminogen is directly related to urokinase (uPA) which is activated by attaching to its receptor uPAR (Ranuncolo, et al., 2002).

Finally, the effective migration of glioma cells through the ECM finalizes the invasion process.

Figure 2: Process of glioma invasion. Glioma invasion involves the detachment from the original site, attachment to ECM, degradation of ECM, and cell migration. (Source: Nakada, et al., 2007)

1.5. Cell Motility

Various biological processes such as inflammation, cancer cell invasion and metastasis, tissue regeneration involve cell migration (Leonithas et. al, 2014). Cell motility usually occurs in response to chemoattractants or different growth factors present in the ECM. This action is called chemotaxis (Lauffenburger & Horwitz, 1996). Cells move in a polarized manner, and undergo actin polymerization which allows it to

extend an important actin- rich protrusion, most likely as lamellipodia or filopodia, in the direction of the chemoattractant (Bailly, Condeelis, & Segall, 1998). There are steps that follow in order to achieve the motility cycle that involve formation of the adhesion structures which stabilize the protrusion, progression of a contractile force that transfers the cell body to the forward direction, liberation of the adhesion structures at the cell end and retraction of the cell to the direction of the movement (Condeelis, et al., 2001). Thus, the Rho family of GTPases plays an important role in regulating the cell motility cycle by reorganizing the actin cytoskeleton (Condeelis, et al., 2001).

Figure 3: The cell motility cycle. For cell motility to be achieved, a cell must protrude, form focal adhesions in order to stabilize the protrusion, disassemble the old adhesion in the tail, and contract so the cell body can move forward. **Source: (Alexander & Chernoff, 2003).**

1.6. Metformin

1.6.1. Medical Uses

Metformin, known as (N, N-dimethylbiguanide) is an oral anti-hyperglycemic agent in the biguanide class which lowers blood glucose concentrations and has been used to treat type 2 diabetes mellitus (Miyoshi, et al., 2013; Saito, et al., 2013). Metformin was well-known as a glucose-lowering drug and came to be available for human intake in UK (Witters, 2001). Metformin became of international interest; in 1995, it was FDA approved in the US (Kourelis & Siegel, 2011). Metformin is highly used in polycystic ovary syndrome (PCOS), premature puberty, and non-alcoholic fatty liver disease (NAFLD) (Lord, Flight, & Norman, 2003; Marchesini, et al., 2001; Ibáñez, et al., 2006).

1.6.2. Mechanism of action

Metformin primarily decreases hyperglycemia by suppressing glucose production by the liver (Kirpichnikov, McFarlane, & Sowers, 2002). In addition, metformin increases insulin sensitivity, decreases the absorption of glucose from the gastrointestinal tract, and enhances the peripheral glucose uptake by inducing the phosphorylation of GLUT4 enhancer factor (Collier, et al., 2006). Thus, metformin, which is an insulin-sensitizing drug, is used to manage type2 diabetes and to improve glycemic control in insulin resistant patients. Inhibiting the secretion of hepatic glucose which decreases hyperinsulinemia is the important metabolic action of metformin. In fact, this mechanism is conveyed by the activation of AMPK, an energy sensing AMP-

activated protein kinase in hepatocytes via the LKB1, the liver kinase B1 signaling pathway (Karnevi, Said, Andersson & Rosendahl, 2013).

Moreover, metformin shows an important antiproliferative action on many cancer cells, including pancreatic, colon, lung, breast, ovarian and prostate cancer cell lines. Furthermore, in vitro research proposed that metformin prevents the growth of cancer cells by activating AMPK which is adenosine monophosphate protein kinase, rendering mammalian target of rapamycin (mTOR) inactive, and diminishing the activity of S6K1 which is an mTOR effector (Sarfstein et. al, 2013). Therefore, metformin induces the AMPK- dependent inhibition of the IGF-1/insulin pathway. This inhibition is done through the phosphorylation of the IRS-1 at Ser794 residue, which, in contrast with the IGF1R/R- mediated phosphorylation, promotes the decrease in IRS-1, AKT, and mTOR1 activation (Figure 4). It's valuable to highlight that metformin disrupts the important cross-talk between the GPCR and IGF-1/insulin pathways and blocks ERK1/2 through the LKB1-mediated suppression of mTOR1 (Pierotti et. al, 2013).

Figure 4: Action of Metformin on the IRS-1 and G protein-coupled receptor (GPCR) pathways. Explained in the above section. 1.7.2. Source: (Pierotti et. al, 2013).

1.7. Purpose of the study

In the current study, we aim to investigate the role of metformin on the motility and the proliferation of brain cancer cell lines. As a first step, we studied metformin's effect on the cellular proliferation by treating the cells with the drug for 24 hours and performing different assays. Then, we studied the effect of metformin on cellular motility. Finally, we examined the possible effect of metformin on the PI3K activity through its effect on the activity of the anti-apoptotic protein AKT.

Chapter II

MATERIALS & METHODS

2.1. Cell Culture

Human astrocytoma cell line SF268 was cultured in DMEM medium supplemented with 10% FBS and 100U penicillin/streptomycin at 37°C and 5% CO₂ in a humidified chamber.

2.2. Antibodies and reagents

The following primary antibodies were used in the study: rabbit monoclonal antibody against pan-Akt (Abcam Inc, Cambridge, UK) and rabbit monoclonal antibody against Akt1 phosphorylated at S473 (Abcam Inc, Cambridge, UK) purchased from Abcam.

Furthermore, anti-rabbit HRP- conjugated secondary antibody was obtained from Promega (Promega, CO., Wisconsin).

Collagen was purchased from Invitrogen. Metformin (1, 1-dimethylbiguanide hydrochloride) was purchased from Sigma-Aldrich. 500mg pill was dissolved in 1L PBS.

2.3. Cell proliferation reagent (WST-1)

Cells were seeded in 96-well plates (growth area: 0.6cm²) at a concentration of 1x10⁵cells/ml. Depending on the experiment, cells were treated with metformin (2.5mM) with a control (only media). Following treatment period, 10µl of Cell

Proliferation Reagent (WST-1; Roche, Germany) was added to each well. The plates were incubated in a humidified incubator (37°C) in 95% air and 5% CO₂ for 2 hours. WST-1 is a tetrazolium salt that on contact with metabolically active cells is cleaved to produce formazan dye by mitochondrial dehydrogenases. Quantitation of formazan is done colorimetrically at 450nm. The absorbance of the each blank well was subtracted from the corresponding sample well. The results were normalized to the corresponding controls, and the percent of cell proliferation was reported.

2.4. Wound healing assay

Cells were grown to confluence on culture plates and a wound was made in the monolayer with a sterile pipette tip. After wounding, the cells were washed to remove debris and new medium was added. Phase-contrast images of the wounded area were taken at 0 and 24 hours after wounding. Wound widths were measured at 12 different points for each wound, and the average rate of wound closure was calculated in $\mu\text{m/hr}$.

2.5. Western blotting

Cell lysates were prepared by scraping the cells in a sample buffer consisted of 4% SDS, 10% β -mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris-HCl at a pH of 6.8. The resulting lysates were boiled for 5 minutes. Protein samples were separated by SDS-PAGE on 8% or 15% gels and transferred to PVDF membranes overnight at 30V. The membranes were then blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 for 1 hour at room temperature and incubated with primary antibody at a concentration of 1:500 for 2 hours at room temperature. After

the incubation with the primary antibody, the membranes were washed and incubated with secondary antibody at a concentration of 1:1000 for 2 hour at room temperature. The membranes were then washed, and the bands visualized by treating the membranes with western blotting chemiluminescent reagent ECL (GE Healthcare). The results were obtained on an X-ray film (Agfa Healthcare). The levels of protein expression were compared by densitometry using the ImageJ software.

2.6. Invasion Assay

Cells were treated with metformin and invasion assay was performed after 48hrs treatment using the collagen-based invasion assay (Millipore) according to manufacturer's instructions. In short, 24hrs before doing the invasion assay, cells were starved with serum-free medium. Then, cells were harvested, centrifuged and resuspended in quenching medium (media without serum). Then, cells were brought to a concentration of 0.6×10^6 cells/mL. By this time, inserts were prewarmed with 300 μ L of serum free medium for 30min at room temperature. After rehydration, 250 μ L of media was removed from inserts and 250 μ L of cell suspension was added. Then, inserts were placed in a 24-well plate, and 500 μ L of complete media (with 10% serum) was added to the lower wells. Plates were incubated for 24hrs at 37°C in a CO₂ incubator. Following incubation period, inserts were stained for 20min at room temperature with 400 μ L of cell stain provided with the kit. Stain was then extracted with extraction buffer (also provided). 100 μ L of extracted stain was then transferred to a 96-well plate suitable for colorimetric measurement using a plate reader. Optical Density was then measured at 560 nm.

2.7. Adhesion Assay

96-well plates were covered with collagen using Collagen Solution, Type I from rat tail (Sigma) overnight at 37°C and then washed with washing buffer (0.1% BSA in DMEM). The plates were then blocked with 0.5% BSA in DMEM at 37°C in a CO2 incubator for 1 hour. Then, Plates were washed and put on ice. At the same time, cells were trypsinized and counted to 4x10⁵cell/mL. 50µL of the cells were then added in each well and incubated at 37°C in a 5% CO2 incubator for 30 minutes. Plates were then shaken and washed for 3 times. Fixation of the cells took place with 4% paraformaldehyde at room temperature for 10 minutes. Cells were washed and stained with crystal violet (5mg/mL in 2% ethanol) for 10 minutes. After staining, plates were washed very well with water, and left aside to dry completely. Crystal violet was solubilized by incubating the cells with 2% SDS for 30 minutes. The absorption of the plates was read at 550nm using ELISA.

2.8. Statistical Analysis

All the results reported represent average values from three independent experiments. All error estimates are given as $\pm$ SEM. All the results showed statistical significance with a p-value $\leq$ 0.05.

Chapter III

RESULTS

3.1. Metformin treatment decreases astrocytoma cells viability

We wanted to study the effect of metformin on astrocytoma cancer cell viability. First, we treated the cells with metformin with different concentrations and then proliferation assay was performed using the cell proliferation reagent (WST-1). Thus, WST-1 reagent showed around 50% decrease in cellular proliferation of cells treated with metformin at 2.5mM concentration (Figure 5).

Figure 5: Treatment with metformin decreases cell viability. Cells were treated with metformin over a range of different concentrations. The duration of the treatment was 24hrs. Using WST-1 reagent, 50% decrease in the cellular proliferation was observed at 2.5mM concentration. Data are the mean $\pm$ SEM from 3 experiments.

3.2. Metformin decreases cellular motility

After we proved that metformin has a main role in the proliferation of cancer cells, we wanted to further investigate the effect of this same drug in 2D motility. Hence, we treated the cells with metformin and studied its effect using wound healing assay. Thus, results showed a decrease in motility of around four and a half folds in terms of wound closure in the cells with metformin treatment at 2.5mM ($0.22\mu\text{m/hr}$) with respect to the control cells ($1\mu\text{m/hr}$) (Figure 6 A&B).

Figure 6: Metformin inhibits cellular motility. Cells (SF 268) were treated with metformin. A) Cells from the control and treatment were grown in plates and formed monolayer then wounded and pictured directly (upper micrographs). After 24 hours, pictures were imaged at the same frame (Lower micrographs). B) Quantitation for A. Width of single wound was measured at 12 distinct points, and average rate of wound closure was calculated in $\mu\text{m/h}$. Data are the mean $\pm$ SEM from 3 different wound healing experiments. The results were significant with $p=0.02$.

3.3. Metformin treatment decreases cellular invasion

After we proved that metformin treatment has a role in astrocytoma cancer cell proliferation, we next wanted to examine the effect of metformin treatment on cellular invasion. We used an in vitro collagen-based invasion assay through which FBS was used as a chemoattractant. The results showed around 30% decrease in cell invasion in the cells treated with metformin at 2.5mM compared to the control cells (Figures 7A-B).

Figure 7: Metformin treatment decreases cellular invasion. **A.** Representative micrographs that show invaded cells on the bottom side of the membrane which is stained with the cell stain crystal violet according to the assay instructions. **B.** Extraction of cell stain was done and colorimetric measurements using ELISA were taken at 560nm. Data is measured in arbitrary units and normalized to the control. Data are the mean $\pm$ SEM from 3 experiments.

3.4. Metformin treatment increases cellular adhesion to collagen

Since metformin treatment showed a decrease in the cellular motility, we wanted to further investigate the effect of this treatment on the adhesion of these astrocytoma cancer cells to collagen, which is an important component of the ECM. The results showed around a 35 percent increase in the adhesion of these cells with treatment as compared to the control cells (Figures 8 A-B).

Figure 8: Metformin treatment increases cellular adhesion to collagen. **A.** Representative micrographs of cells that were fixed and stained with crystal violet to detect adhesion. **B.** Quantitation of the micrographs. Crystal violet was solubilized. Absorption was taken at 550nm using ELISA. Data is measured in arbitrary units and normalized to the control. Data are the mean $\pm$ SEM from 3 experiments. The results were significant with $p=0.02$.

3.5. Metformin treatment inhibits the activity of anti-apoptotic protein Akt

Another important aspect was to study the effect of Metformin treatment on cellular proliferation through its effect on the activity of the anti-apoptotic protein AKT. Cells were treated with metformin for 24 hours. Then, cells were lysed and the total proteins were extracted. Thus, immunoblotting was done using a phosphospecific antibody which is anti-AKT antibody. Results showed that the phosphorylation of the AKT protein is partially inhibited (31%) upon Metformin treatment at 2.5mM (Figure 9 A&B).

Figure 9: Metformin treatment inhibits the activity of anti-apoptotic protein Akt. Cells were plated and treated with metformin for 24 hours. **A.** Cells were lysed and then immunoblotted by western blotting analysis for P-Akt (Lower gel) and for Akt (Upper gel). As seen in the lower gel, metformin led to the inhibition of phosphorylation of Akt. **B.** The bands in the above gels were quantified using the ImageJ software, and

then were normalized to the control. Data are the mean $\pm$ SEM from 3 different experiments. The results were significant with $p=0.03$.

CHAPTER IV

DISCUSSION

Metformin, 1, 1 dimethylbiguanide hydrochloride, is a universally used anti-diabetic agent which has been proved to perform an anti-cancerous activity in many tumor models (Levesley, et. al, 2013). In our study, an inclusive characterization of metformin in astrocytoma cancer cells is provided, regarding its effect on the viability and proliferation of cells, motility and invasion, as well as its role on the expression of the anti-apoptotic protein Akt.

We examined an in vitro model of astrocytoma cancer cell line i.e. SF-268, to initially look at the effect of metformin on cellular proliferation and viability. In fact, using the WST-1 proliferation assay, results showed that the treatment of metformin in this cell line led to a decrease in cellular viability. These results were consistent with a recent study which showed that metformin diminishes cell viability and triggered apoptotic morphological alterations in the T98G glioblastoma multiforme cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Ucbek et.al, 2014). In addition, another study showed that 2.5 mM of metformin, which is the same concentration that we used, suppresses 46% U87MG proliferation, and inhibits 92% T98G and 99% U251 proliferation respectively (Kennedy, et.al, 2013). Furthermore, another study showed that metformin resulted in a strong inhibition of the cellular proliferation in all gastric cancer cell lines (Kato, et.al, 2012).

After proving that metformin has an important role in decreasing the cellular proliferation and viability of astrocytoma cancer cells, we then needed to study the effect of metformin on the 2D cell motility. Indeed, the treatment of metformin in the SF-268 cell line inhibited cell motility by 80% at 2.5mM concentration using wound healing assay. This was consistent with previous studies in which treatment with metformin repressed the growth and migration of many cancer cell lines including pancreatic, breast, renal cell, colon, lung, ovarian, glioma, and prostate (Isakovic, et.al, 2012; Zakikhani, et.al, 2006; Rattan, et.al, 2011) .

To further examine the suppression of the 2D cell motility caused by metformin treatment, an adhesion assay was performed, which as expected indicated a foremost increase in the steadiness and adhesion of the cells to collagen upon metformin treatment. This means metformin is keeping RhoA active, preventing the dissolution of focal adhesions required for cells to move. Also, this increase in adhesion could have prohibited the retraction of the tail, which we greatly suspect is the major incident that inhibited cell motility. This increase in adhesion was consistent with previous work in our lab on astrocytoma and breast cancer cell lines through STARD13, a Rho GAP, through which the leading active RhoA and StarD13 knockdown led to the formation of greater focal adhesions (Khalil et. al, 2011; Hanna et. al, 2012). Hence, this hardens our results with metformin treatment, where we normally have an increase in the activity of RhoA, and thus stimulating cellular adhesion.

After showing the mechanism through which metformin might influence the random 2D cellular motility, it was exciting to us to investigate its effect on the 3D motility, which means cellular invasion. We treated the cells with metformin and examined a collagen-based trans-well invasion assay. Knowing that metformin clearly

inhibited the cellular motility in 2D, it was expected that it would also suppress cellular invasion. Thus, our expectation was in its place, by which metformin treatment decreased cellular invasion, regardless of the fact that metformin increases cellular adhesion. Moreover, previous study supported our results by showing a decrease in cellular invasion in many cell lines. Thus, in vitro approaches have shown that metformin inhibits the cellular invasion in melanoma (Cerezo, et.al, 2013). Another study has shown that Metformin strongly blocked U251 brain cancer cell invasion (Gao, et.al, 2013). Furthermore, another study has shown that metformin decreases the cellular invasion and migration of ovarian cancer cells (Wu, et.al, 2012).

After proving that metformin has an important and direct effect on the proliferation and motility of astrocytoma cells, we wanted to further investigate the possible effect of metformin on the activity of the anti-apoptotic protein AKT. Our results showed that metformin inhibits the phosphorylation of Akt, as by western blot analysis, which means it's possibly inhibiting the PI3K activity. These results were consistent with previous studies which showed that the effects of Metformin in glioblastoma cells were correlated with a potent inhibition of Akt-dependent cell survival pathway (Würth, et.al, 2013). So, our data showed that metformin inhibits PI3K, which means Rac is being inhibited. This is consistent with previous work which showed that Rho and Rac works in an antagonistic manner (Shoval & Kalcheim, 2012).

CONCLUSION

In our present study, we have designated the role of metformin in astrocytoma cancer cell proliferation and motility. Our results revealed that metformin deleteriously affects cellular viability and proliferation. Metformin has different roles in the 2D and 3D motility. Metformin treatment inhibited the 2D cell migration. However, this effect of metformin has increased cellular adhesion to collagen but decreased cellular invasion. Last but not least, metformin treatment inhibited the phosphorylation of Akt, which possibly means blocked the PI3K activity.

Auxiliary experiments are still to be performed to further investigate the role of Metformin on the viability and proliferation by flow cytometry. Other supplementary studies include finding the possible interaction of metformin with Rac1, Rho, and CDC42, and their influence on migration and invasion of brain cancer cells by performing pull down assay.

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