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Metformin Arrests Growth and Induces Apoptosis of Neuroblastoma Cells

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Metformin Arrests Growth and Induces Apoptosis of Neuroblastoma Cells

by

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Submitted in Partial Fulfillment of the Requirements

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DEDICATION

I dedicate my thesis with special thanks to The Higher Committee for Education Development in Iraq (HCED) who has encouraged me and supported me financially throughout my career.

I dedicate this work to my professors at Tikrit University College of Medicine in Iraq who worked hard helping me succeed in my career.

I dedicate this work to the best people I have ever in my life, my parents, Tawfeeq Al-Sammarraie and Faten Shihab, for their endless love and encouragements throughout my life pushing me always toward success .

I dedicate my thesis with great love to my wonderful husband, Mohammed Mahmood, who was patient, supportive, and helpful throughout my mission, thanks for encouraging me and for having me in your life. Besides, I dedicate this work to my lovely precious kids Abdulrahman, Dina, and Rahaf who fill my life with joy and happiness and motivate me every day to work hard and succeed.

I dedicate this work to my beloved brothers and their families with great thanks for their support and encouragement.

Finally, I dedicate this thesis to the honor of all neuroblastoma patients hoping my work will help in finding safe cure for this aggressive disease in the near future.

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ABSTRACT

Neuroblastoma is one of the most common solid neoplasms in children, which affects sympathetic neurons with a wide range of genetic abnormalities, especially MYCN amplification, a molecular marker of poor prognosis. Despite advance therapy, still the mortality rate for advance disease is high with frequent relapse and resistance to cytotoxic therapy with evidence of p53 mutation. Metformin, an oral hypoglycemic medication, is a relatively safe and cost effective drug with wide clinical applications, mainly for treatment of Type 2 diabetes. Recent studies have showed promising anticancer effect of metformin on various cancer types, especially on p53 mutant cells. In our research, we performed in vitro study testing the effect of metformin on neuroblastoma cells, as little is known about its effect on this disease. Our results demonstrate that metformin at 10 and 20 mM has significant growth and survival inhibitory effects on both N-myc amplified and N-myc non-amplified and p53 mutant cells. Metformin induces apoptosis in both cell types revealed by morphological changes and detection of cleaved caspase3 by western blot analysis. Metformin's anticancer effect may be independent on AMPK and AKT pathways as our initial results show no marked change relative to control. Metformin decreases total RhoA protein expression in both cell lines and total Rac1 protein expression in N-myc amplified cells. Our results suggest that metformin can be a good and safe candidate for treatment of neuroblastoma.

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LIST OF ABBREVIATIONS

4E-BP1	eukaryotic initiation factor 4E-binding protein-1
ACC	acetyl-CoA carboxylase enzyme
ADP	Adenosine diphosphate
ALK	Anaplastic lymphoma receptor tyrosine kinase
ALL	Acute lymphocytic leukemia
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BAX	BCL2-associated X protein
BCA	Bicinchoninic acid protein
bHLHZ	Basic-helix-loop-helix-zipper
BSA	Bovine serum albumin
CD	Cluster of differentiation
CNS	Central nervous system
COG	Children's Oncology Group
CT	Computed tomography
DAPI	4',6-diamidino-2-phenylindole
DI	DNA Index
DMEM	Dulbecco's modified Eagle medium
DMs	Double-minute chromatin bodies
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
EFS	Event free survival
FBS	Fetal bovine serum

FITC..... Fluorescein isothiocyanate
 GD2..... Disialoganglioside
 GDP..... Guanosine-5'-diphosphate
 GTP..... Guanosine-5'-triphosphate
 HSRs Homogeneously staining regions
 INRGSS International Neuroblastoma risk group staging system
 INSS International Neuroblastoma Staging System
 LKB1..... Liver Kinase 1
 MAPK..... Mitogen-Activated Protein Kinase
 MATE Multidrug and toxin extrusion transporter
 MAX..... MYC associated factor X
 MDM2..... Mouse double minute 2 homolog
 MIBG Metaiodobenzylguanidine
 MKI Mitosis and karyorrhexis index
 MRI..... Magnetic resonance imaging
 mTORc1..... Mammalian target of rapamycin complex1
 MTT Methylthiazol tetrazolium
 MW Molecular Weight
 MYCN.... v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
 NAFLD Non- alcoholic fatty liver diseases
 OCT3..... Organic Cation Transporters
 P Chromosome short arm
 p14^{ARF} Alternate reading frame (ARF) product of the CDKN2A locus
 p21..... Cyclin-dependent kinase inhibitor 1A
 p53..... Tumor protein p53
 PBS Phosphate buffered saline
 PCOS..... Polycystic ovarian diseases
 PHOX2B..... Paired-like homeobox2b
 PI3 Phosphatidylinositol-3-kinase
 pAkt..... phosphorylated v-akt murine thymoma viral oncogene homolog 1

PMAT plasma membrane monoamine transporter
PVDF polyvinyl difluoride
q..... Chromosome long arm
Rac1 Ras-related C3 botulinum toxin substrate 1
RAP1 RAS-related protein 1
REED1 Regulated development and DNA damage response
Rheb Ras homologue enriched in brain
RhoA ras homolog family member A
ROCK Rho-associated protein kinase
S6K ribosomal protein S6 kinase
SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIRT1 NAD⁺-dependent deacetylase sirtuin1
SREBP-1 sterol regulatory element-binding protein-1
TBST Tris-Buffered Saline and Tween 20
TEMED..... Tetramethylethylenediamine
TSC2 tuberous sclerosis complex2

CHAPTER 1

INTRODUCTION

1.1 Neuroblastoma:

Neuroblastoma is the third most prevalent childhood malignancy, after acute lymphocytic leukemia (ALL) and CNS and brain tumors, and it is the commonest neoplasm encountered in children below one year of age (American Cancer Society, 2014; Chakrabarti, Abou-Antoun, Vukmanovic, & Sandler, 2012). Neuroblastoma represents 7% of all childhood malignancy and up to 15% of cancer related mortality (American Cancer Society, 2014; Davidoff, 2012). In the United States, around 700 new cases of neuroblastoma are diagnosed each year with approximate incidence of 10.2 cases per 1,000,000 children below 15 years old (Davidoff, 2012; Cheung & Dyer, 2013).

Neuroblastoma is a heterogeneous embryonic tumor affecting postganglionic sympathetic neurons with wide variety of clinical presentations and genetic aberrations (Normand, Michon, Janoueix-Lerosey, Delattre, & Schleiermacher, 2011). It generally tends to affect males slightly more than females and white children more than other races (American Cancer Society, 2014). Neuroblastoma disease could in some cases regress spontaneously or it can undergo differentiation into more benign form ganglioneuromas (Weinstein, Katzenstein, & Cohn, 2003). However, up to half of cases are associated with metastatic presentations at time of diagnosis with overall survival rate around 40%

(Matthay, Weiss, et al., 2012). Most cases of neuroblastoma are sporadic with various genetic abnormalities, and no definitive environmental factors have been found predisposing to its development (Weinstein et al., 2003). On the other hand, only 1- 2% of neuroblastoma are familial cases with early onset of the disease and with multiple primary tumor foci detected at time of diagnosis (Maris et al., 2002).

Despite the increase in the survival rates of neuroblastoma in general (around 79% survival rate during 2003-2009 compared to the 54% in the previous years), the survival rate of high risk neuroblastoma patients remains low, with increased morbidity due to complications of intensified chemotherapeutic treatment (American Cancer Society, 2014).

1.1.1 Pathogenesis and genetic abnormalities:

Neuroblastoma is a malignant tumor involving sympathetic neurons, mainly adrenal medulla and paraspinal sympathetic nerves (Normand et al., 2011). Neuroblastoma originates from sympathoadrenal precursor cells which are derived from neural crest cells (Cheung & Dyer, 2013). Neural crest cells are group of embryonic cells which are found in neural plate border region of developing embryo (Dupin & Sommer, 2012). These neural crest cells can give rise to different cell types, such as: melanocytes of epidermis, smooth muscle cells of vessels, craniofacial bone, glia and Schwann cells, enteric and peripheral neurons, olfactory cells, adipocytes, meninges and adrenal medulla (Cheung & Dyer, 2013; Dupin & Sommer, 2012). During embryonic development, some of neural crest cells migrate from their original site to the dorsal aorta where they become sympathoadrenal lineage cells (Cheung & Dyer, 2013). In turn, sympathoadrenal

precursor cells further differentiate, in response to many signals, into paraspinal sympathetic ganglia or chromaffin cells comprising adrenal medulla(Cheung & Dyer, 2013).

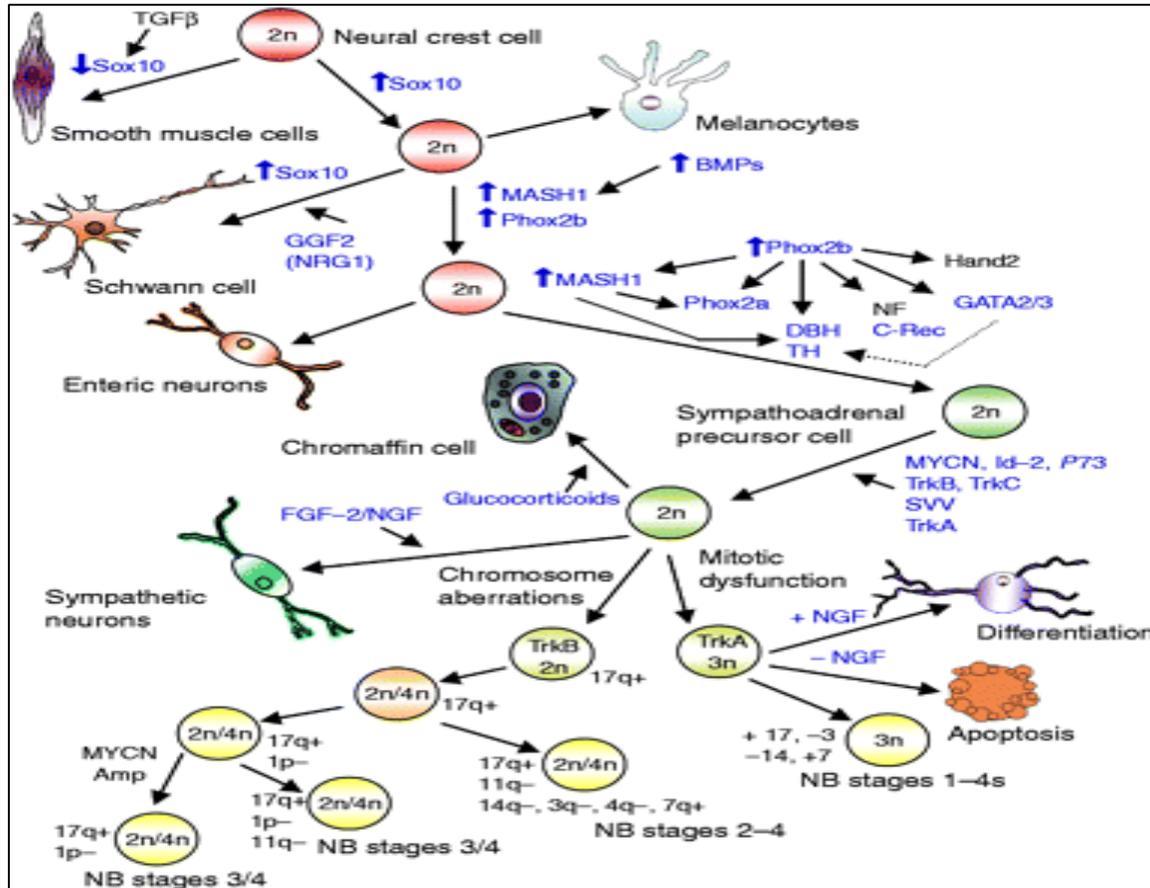


Figure 1.1 “Development of the lineages derived from neural crest cells and the origin of the different stages of neuroblastoma (NB)”. Reused from “Neuroblastoma therapy: what is in the pipeline?” by Verissimo, C. S., Molenaar, J. J., Fitzsimons, C. P., & Vreugdenhil, E. (2011). *Endocr Relat Cancer*, 18(6), R213-231, figure 1. doi: 10.1530/ERC-11-0251 Copyright © [2011] [BioScientifica Ltd]. Reused with permission.

Several signaling pathways and genes are well regulated during embryogenesis and contribute to accurate differentiation of these precursor cells to become specialized neuronal cells, such as: paired-like homeobox2b (PHOX2B), which is important for neuronal cells differentiation, and anaplastic lymphoma receptor tyrosine kinase (ALK)

gene, which control MAPK and RAS-related protein 1 (RAP1) signaling pathways in order to regulate neuronal cell proliferation and differentiation (Cheung & Dyer, 2013; Verissimo, Molenaar, Fitzsimons, & Vreugdenhil, 2011). Similarly, MYCN proto-oncogene, located on chromosome 2p24, is found to be involved in neuronal precursor cells proliferation and inhibition of their differentiation during embryogenesis (Davidoff, 2012; Verissimo et al., 2011).

Although the exact genetic causes of neuroblastoma are not well understood, disruption or overexpression of these signaling factors are linked to the development of neuroblastoma, besides other genetic aberrations (Santo et al., 2013). In familial neuroblastoma, two common mutations can be found: mutation in paired-like homeobox2b (PHOX2B) gene, or more commonly ALK gene mutation in germ line which is also found as a somatic cell mutation or amplification in approximately 8% of sporadic neuroblastoma cases (Cheung & Dyer, 2013; Van Roy et al., 2009). Besides, several genetic abnormalities are encountered in most neuroblastoma cases, especially in advance stages, including: DNA ploidy, chromosomal loss, mutations of specific genes, and most importantly MYCN amplification (Davidoff, 2012). N-myc proto-oncogene, a transcription factor belongs to the basic-helix-loop-helix-zipper (bHLHZ) class, is amplified in about 22% of neuroblastoma cases, predominately in metastatic disease, and associated with poor prognosis (Knoepfler, Cheng, & Eisenman, 2002; Cheung & Dyer, 2013). MYCN amplification in neuroblastoma can be found in two different forms: extra-chromosomal fragments as double-minute chromatin bodies (DMs), or homogeneously staining regions (HSRs) within the chromosome (Davidoff, 2012). N-myc, which is commonly amplified in advance neuroblastoma, can heterodimerize with MAX protein in

DNA specific sequences and promote transcription (Knoepfler et al., 2002). However, the exact contribution of MYCN amplification to the development of aggressive neuroblastoma, which is associated with poor outcome, remains unknown (Van Roy et al., 2009). Other genetic abnormalities commonly detected in neuroblastoma include: change in the number of chromosomes or individual chromosomal abnormalities, which associate with various outcomes (Normand et al., 2011). DNA ploidy generally refers to the DNA content within the cell (2 copies of each 23 chromosomes are normally found in diploid cells) (Davidoff, 2012). In primary neuroblastoma disease, up to 55% of cases contain near triploid or triploid cells, while the rest 45% cases harbor near-diploid or near-tetraploid cells (Davidoff, 2012). Similarly, chromosomal abnormalities frequently encountered in neuroblastoma are: gain of chromosome 17q and loss of chromosomal regions 1p, 11q, and 3p (Normand et al., 2011). Chromosome 17q gain are found in up to 80% of cases due to unequal translocation with other chromosomes (chromosome 1p or chromosome 11q) in tumor cells (Van Roy et al., 2009). Another common chromosomal abnormality is loss of chromosome 1p in up to 25-35% of cases, which may contain cancer suppressor genes (Van Roy et al., 2009). Deletion of chromosome 1p is frequently detected in neuroblastoma cases, particularly in advanced disease when it commonly correlate with MYCN amplification (Davidoff, 2012; Van Roy et al., 2009). Moreover, several other chromosomal loss, which might contain cancer suppressor genes, are frequently found in neuroblastoma tumor without association with MYCN amplification, these include: chromosome 11q loss commonly found in high stage neuroblastoma, and chromosome 3p loss in association with loss of chromosome 11q commonly found in older patients (Van Roy et al., 2009).

Genetic mutations in neuroblastoma is not only confined to the primary tumor but also can be found after cytotoxic therapy in relapsed neuroblastoma (Carr-Wilkinson et al., 2010; Tweddle, Malcolm, Bown, Pearson, & Lunec, 2001). Mutations in p53, a tumor suppressor gene located on short arm of chromosome 17, are rare in primary neuroblastoma at time of diagnosis (Tweddle et al., 2001). However, several studies have shown that p53 mutations could be found in relapsed neuroblastoma after chemotherapy (Tweddle et al., 2001; Carr-Wilkinson et al., 2010). P53 is a transcription factor that binds specific DNA sequence and promotes transcription of p21, MDM2 and BAX genes in response to DNA damage, which in turn involve in regulation of cell cycle and apoptosis (Tweddle et al., 2001; Carr-Wilkinson et al., 2010). In neuroblastoma cells from relapsed cases, it was found that p53 is frequently inactivated by genetic mutation or the p53/MDM2/p14^{ARF} pathway that control p53 in the cell is mutated. (Tweddle et al., 2001; Carr-Wilkinson et al., 2010). These genetic abnormalities are used clinically as prognostic markers, especially MYCN amplification, in diagnosis, staging, and prediction of prognostic outcome of the patient (Lonergan, Schwab, Suarez, & Carlson, 2002). In addition they are used to determine the appropriate strategy for treatment, which range from observation and surgery alone for low risk patients to the use of more intensified cytotoxic therapy for high risk disease (Davidoff, 2012).

1.1.2 Clinical presentations of neuroblastoma:

Neuroblastoma is the most frequently encountered extracranial solid malignancy in children, and it is mainly diagnosed during infancy and in children below 5 years (around 90%) (Van Roy et al., 2009; Verissimo et al., 2011). The median age of diagnosis is around 18 months for sporadic cases, while in familial neuroblastoma it is

around 9 months with frequent presentation of bilateral or multifocal malignancy (Brodeur, 2003). Neuroblastoma can develop in different places along the sympathetic neurons but the majority arises in the lumbar sympathetic neurons and adrenal medulla (around 65%), whereas the rest cases can occur anywhere through the sympathetic neurons in chest, neck, or pelvic regions in a ratio of 4/1/1, respectively (Cheung & Dyer, 2013).

The clinical presentations of neuroblastoma vary depending on the effect of primary tumor and distant metastasis (Maris, 2010; Papaioannou & McHugh, 2005; Papaioannou & McHugh, 2005). The primary tumor usually presents as palpable mass in the abdomen, especially in case of adrenal involvement, or mass in the neck or chest due to involvement of corresponding sympathetic neurons (Maris, 2010). In addition, neuroblastoma growth may involve vital structures or press the surrounding tissues, blood vessels, and nerves, especially in high stage disease, which can lead to various signs and symptoms, including: blood vessels involvement such as renal arteries, Horner's syndrome due to involvement of cervical neurons, and pressure effect of growing tumor on spinal cord which leads to various neurological defects (Papaioannou & McHugh, 2005).

In neuroblastoma cases with metastasis, infiltration of surrounding tissues can lead to various presentations, such as: liver enlargement especially in stage 4S disease, blindness due to involvement of optic nerve, lymph node enlargement, and infiltration of bone marrow with fever, malaise, anemia and bone pain. (Papaioannou & McHugh, 2005).

1.1.3 Diagnosis, Staging and risk group classification:

Diagnosis of neuroblastoma is based mainly on histopathological examination, bone marrow aspiration, laboratory tests and radiological investigations which also help in disease staging and predicting patient outcome (Weinstein et al., 2003). Ultrasonography is the initial technique commonly used to detect abdominal neuroblastoma mass and determine its location, similarly, plain radiograph is helpful in the detection of primary tumor and metastasis (Papaioannou & McHugh, 2005). However, CT scan and MRI are the main techniques used for definitive diagnosis and staging of the disease, beside, Scintigraphy ,using ^{99m}Tc -MDP and ^{123}I -MIBG, is commonly used for diagnosis of primary tumor and searching for metastatic lesions (Papaioannou & McHugh, 2005; Weinstein et al., 2003). Laboratory tests frequently used in diagnosis of neuroblastoma include: histochemical testing of bone marrow sample and urinary testing of catecholamine level(Papaioannou & McHugh, 2005). Histological examination of neuroblastoma generally reveals presence of undifferentiated small round neuronal cells (Neuroblastoma cells), and in some cases tumor cells exhibits mild degree of histological differentiation (ganglioneuroblastomas) (Brodeur, 2003). On the other hand, in the more mature form of the disease the histological examination shows mature neuronal cells within Schwann rich stroma (Brodeur, 2003).

Generally, neuroblastoma is classified into favorable and unfavorable histopathological prognostic groups based on Shimada histopathological classification in 1984(Weinstein et al., 2003). This classification takes into account age of the patient at time of diagnosis (<1.5 years,1.5–5 years, or >5 years) and histologic criteria including: presence of schwannian stroma whether rich (mature) or poor (immature), degree of

cellular differentiation (undifferentiated, differentiated or mixed), and the mitosis and karyorrhexis index (MKI) (low, intermediate and high) (Loneragan et al., 2002; Weinstein et al., 2003). MKI represents the summation of tumor cells with mitosis and karyorrhexis per 5,000 cells sample (Loneragan et al., 2002). According to this classification, patients with favorable histopathology are those who are < 1.5 years old with at least partially differentiated tumor and low or intermediate MKI or those who are 1.5–5 years old with differentiated tumor and a low MKI, however, the remaining groupings are classified as unfavorable histopathology (Loneragan et al., 2002).

Furthermore, neuroblastoma is commonly classified into different stages based on clinical, surgical and radiologic criteria of the International Neuroblastoma Staging System (INSS), established in 1988 and modified in 1993 (Loneragan et al., 2002; Weinstein et al., 2003). In this staging system, the classification of neuroblastoma generally ranges from stage 1 disease, in which tumor is localized and carry good prognosis, to stage 4 disease, in which tumor is metastasized and carry poor prognosis. (Loneragan et al., 2002; Weinstein et al., 2003). In addition, there is another stage called stage 4S (stage 4 “special”) represents 7-10% of neuroblastoma cases and includes the following criteria: presence of small primary tumor, age <12 months, and evidence of distance metastasis confined to the liver, skin and <10% of bone marrow, which generally carries good prognosis with disease free survival rate up to 57–87% (Schleiermacher et al., 2003).

In 2009, International Neuroblastoma risk group staging system (INRGSS) classifies neuroblastoma into 4 stages (L1, L2, M, and MS) using radiographic investigation, and this classification is based mainly on extent, stage, and risk factors of

the tumor (Cohn et al., 2009; Van Roy et al., 2009). L1 and L2 denote local disease, M for metastatic tumor, and MS for tumor encountered in children below 18 months with similar stage 4S metastatic criteria (Cohn et al., 2009; Van Roy et al., 2009). Based on this staging system and molecular prognostic factors, neuroblastoma is further classified according to International Neuroblastoma Risk Group (INRG) Pretreatment Classification into 4 main categories (very low, low, Intermediate, and high risk) with 16 pretreatment groups (A-R), to determine overall prognosis and suitable treatment for patients (Cohn et al., 2009). According to this classification, the overall 5 years event free survival (EFS) for patients with very low risk group (A, B, C) about 85%, low risk group (D, E, F) 75%-85%, intermediate risk group (G, H, I, J) 50% -75%, and high risk group (K, N, O, P, Q, R) below 50% (Cohn et al., 2009).

1.1.4 Treatment of neuroblastoma:

Current treatment of neuroblastoma is based on children's oncology group (COG) neuroblastoma risk classification, in which patients are categorized into 3 major groups: low risk group, intermediate risk group, and high risk group (Davidoff, 2012). This risk group classification is based on various prognostic criteria, including: stage of the tumor, age of the patient at time of diagnosis, presence of MYCN amplification, DNA ploidy status, histological features (favorable or unfavorable histology), and others including the percent of tumor which is resected (Henderson et al., 2011). Patients with low risk neuroblastoma are those with stage 1, stage 2A/2B with > 50% of tumor resected, or stage 4S diseases with favorable histology and DNA Index (DI) >1, and all these stages without evidence of MYCN-amplification (Davidoff, 2012; Henderson et al., 2011). Intermediate risk group includes patients up to one year old with stage 2A/2B disease and the tumor

undergo biopsy only or <50% resected, patients up to 1.5 years with stage 3 tumor, those between 1.5-12 years old with stage 3 disease and favorable histology, patients 1-1.5 years with stage 4 disease and favorable histology with DI >1, or symptomatic patients with stage 4S and unfavorable histology or DI equal to 1, and all these stages without MYCN amplification (Davidoff, 2012; Henderson et al., 2011). High risk group are those with stage 4 disease and > 1.5 years old, patients with stage (2A/2B,3,4, or 4S) with evidence of MYCN amplification, patient with stage 3 with unfavorable histology and >1.5 years old, or patients between 1-1.5 years old with advanced stage 4 disease that has either unfavorable histology or DI=1 (Davidoff, 2012; Henderson et al., 2011). Accordingly, low risk group patients are generally treated with surgery alone, or sometimes observation and biopsy, especially for infant with favorable stage 4S disease (Davidoff, 2012). Intermediate-risk groups are treated with chemotherapy and surgical removal of resectable tumor, while those with high-risk illness generally require intensive chemotherapy (myeloablative therapy), stem cells transplantation, radiotherapy and reducing tumor mass surgically (Davidoff, 2012).

However, more than 50% of high risk cases relapse and develop resistance to chemotherapy after initial response (Carr-Wilkinson et al., 2010). In recent years, more efforts have been made to develop targeted therapy in order to improve the treatment of neuroblastoma, which ultimately may lead to increase overall survival of the patients (Matthay, George, & Yu, 2012). One of these approaches depends on using small molecules to inhibit ALK, a tyrosine kinase commonly mutated in familial neuroblastoma and in up to 6-10% of sporadic cases (Cheung & Dyer, 2013; Matthay, George, et al., 2012). The second approach is by immune destruction of cancer cells using antibodies

specific for GD2 a (Disialoganglioside), a specific cell surface antigen presenting in around 98% of neuroblastoma cases(Davidoff, 2012);(Cheung & Dyer, 2013; Matthay, George, et al., 2012). Another recent approach is to use ¹³¹I-metaiodobenzylguanidine (MIBG) (norepinephrine analogue) commonly used for staging of neuroblastoma disease (Matthay, Weiss, et al., 2012). This therapy targets norepinephrine transporter expressed in around 90% of neuroblastoma cancer cells, then these cancer cells are destroyed using radiotherapy(Matthay, George, et al., 2012; Matthay, Weiss, et al., 2012). These targeting therapies for the treatment of neuroblastoma disease are being used in early clinical trials with promising results, however, each one of these therapy may cause considerable side effects, such as the risk of radiation accompany MIBG therapy and cell toxicity accompany anti GD2 immunotherapy, which require further evaluation of these new approaches to minimize their adverse effects (Matthay, George, et al., 2012).

1.2 Metformin:

Metformin (*N,N'-dimethylbiguanide*) is the most commonly used oral hypoglycemic medication in the treatment of non-insulin dependent (type2) diabetes mellitus worldwide (Ben Sahra, Le Marchand-Brustel, Tanti, & Bost, 2010; Gong, Goswami, Giacomini, Altman, & Klein, 2012). Metformin belongs to biguanide family, which also includes two other compounds phenformin and buformin (Dowling, Goodwin, & Stambolic, 2011). Metformin is originally derived from the French Lilac herb (*Galega officinalis*) and was used as a herbal tea during the medieval Europe and ancient Egypt to treat symptoms similar to type 2 diabetes, including polyuria and halitosis (Dowling et al., 2011). Biguanide contains guanidine active component that was first discovered in 1900s to have antidiabetic proprieties, which eventually lead to the synthesis of

metformin and phenformin to be used clinically as antidiabetic drugs(Quinn, Kitagawa, Memmott, Gills, & Dennis, 2013). Phenformin was used in the USA from 1957-1970s when it was discontinued due to its side effect of sever lactic acidosis (Quinn et al., 2013). On the other hand, metformin has been used to treat diabetic patients in Britain since 1958 and in Canada since 1972, while it was first approved in the united states in 1995, due to its effectiveness and safety with the fact that the risk of lactic acidosis associated with metformin treatment is very low (less than 1 per 10,000), mainly in patients with impaired renal function (Ben Sahra et al., 2010; Dowling et al., 2011). Besides, more than 120 million prescriptions per year of metformin are used worldwide, and up to 40 million prescriptions of metformin were recorded in 2008 in the United States alone, due to its important role in treating diabetes and improving the survival of diabetic patients (Dowling et al., 2011; Kourelis & Siegel, 2012).

1.2.1 Pharmacological proprieties of metformin:

Metformin is given usually as an oral tablet,(Metformin HCL with MW 165.63),in a dose ranging between 1500-2250 mg/day for adult, with an average plasma half-life about 5 hours after administration (Graham et al., 2011; Dowling et al., 2011; Gong et al., 2012). Metformin is positively charged molecule with hydrophilic properties, so it requires transporters to enter cell membrane (Graham et al., 2011). After administration, it is absorbed by gut plasma membrane and this may be through plasma membrane monoamine transporters(PMAT), which are found mainly on luminal side of enterocytes, or may be through OCT1 or OCT3 Organic Cation Transporters (Gong et al., 2012). Peak plasma level of metformin in diabetic patients after one dose is about 0.5-2 μ g/ml or around 4-15 μ M, and then metformin is distributed and accumulated in different parts of

body tissues, including: kidneys, salivary gland, duodenum, stomach, and liver (Quinn et al., 2013). Metformin is distributed to the liver mainly through OCT1 transporters which are found on the basolateral side of plasma membranes of hepatocytes, and may be through OCT3 transporters which are also localized on basolateral side of these cells (Gong et al., 2012). Metformin is distributed to other body tissues including heart, muscle, and adrenal glands which express OCT1 and/or OCT3 transporters (Graham et al., 2011). In addition, metformin uptake by the kidney is mainly through OCT2 transporters presented on basolateral side of plasma membrane of renal tubular cells, and is excreted from the kidney via MATE1 and MATE2-K located on the apical membrane of the renal proximal tubules, when metformin is then eliminated in the urine as unchanged drug (Graham et al., 2011; Gong et al., 2012).

1.2.2 Metformin's role in diabetic and non-diabetic insulin resistance diseases:

Metformin is commonly used to treat type2 diabetes, which is characterized by insulin resistance in peripheral tissue and associated hyperglycemia, without causing considerable hypoglycemia or weight gain compared to sulfonylurea (Graham et al., 2011; Pollak, 2012). Metformin exerts its antidiabetic action through reduction in hepatic gluconeogenesis, increasing in peripheral tissue sensitivity to insulin, and increasing in tissues use of glucose, particularly in muscle and adipose tissues (Gong et al., 2012; Ben Sahra et al., 2010).

In the liver, metformin reduces gluconeogenesis by targeting mitochondrial oxidative phosphorylation mainly through affecting respiratory chain complex 1, or possibly by other indirect mechanisms (Dowling et al., 2011; Pollak, 2012). Ultimately

metformin treatment leads to decrease in ATP production and increase in AMP and ADP level (Quinn et al., 2013). The elevated AMP/ATP or ADP/ATP ratio leads to activation of AMP-activated protein kinase AMPK, which can sense low energy condition (Quinn et al., 2013). AMPK is a heterotrimeric serine/threonine protein kinase that has three distinct catalytic and regulatory subunits including: α -subunit (with 2 isoforms), β subunits (with 2 isoforms) and γ subunit (with 3 isoforms) (Quinn et al., 2013; Towler & Hardie, 2007). AMP nucleotides activate AMPK by both allosteric binding to the enzyme and by making it a good substrate to be phosphorylated by Liver Kinase 1 (LKB1), which is the AMPK upstream kinase (Towler & Hardie, 2007). The activated (phosphorylated) AMPK in turns affects different downstream targets to reduce mainly gluconeogenesis and fatty acid and protein synthesis (Dowling et al., 2011). Activated AMPK leads to inhibition of major gluconeogenesis genes and glucose production, inhibition SREBP-1 gene expression (lipogenic transcription factor) and decrease lipid synthesis, and inhibition ACC (acetyl-CoA carboxylase enzyme) and fatty acid synthesis (Quinn et al., 2013). However, recent study suggests that metformin can directly inhibit glucagon/PKA signaling pathway that leads to inhibition of gluconeogenesis independent on AMPK activation (Quinn et al., 2013).

In addition to its role in diabetes, metformin in many studies help in reducing overall hyperinsulinemia and insulin resistance commonly found in patients with non-diabetics insulin resistance diseases, including : the use of metformin in the management of polycystic ovarian diseases (PCOS), which is characterized by anovulation, excess androgen and polycystic ovaries, and in the clinical trial for non- alcoholic fatty liver diseases (NAFLD), which is characterized by fatty liver changes accompanied metabolic

syndrome in absence of high alcohol consumption (Lashen, 2010; Mazza et al., 2012). Moreover, recent clinical trial regarding the use of metformin in obese children with insulin resistance demonstrated that metformin treatment associated with moderate effect on body weight and composition in these children (Yanovski et al., 2011).

1.2.3 Metformin's role in cancer:

In recent years, several epidemiological studies have linked metformin treatment to lower incidences of cancer and cancer related mortality among diabetic patients (Ben Sahra et al., 2010). Besides, these studies compare incidence of cancer risk among those who are on metformin treatment with those who are on other antidiabetic medication (Ben Sahra et al., 2010). In 2005, Evan et al published a pilot case-control study regarding metformin treatment and cancer risk in patients with type 2 diabetes who resided in Scotland, Tayside during 1993-2001 years, and the results suggested that metformin treatment was correlated with low cancer risk among those patients (Viollet et al., 2012; Evans, Donnelly, Emslie-Smith, Alessi, & Morris, 2005). These results are followed by several epidemiological studies to test the role of metformin as anticancer medication, for instance, in 2006, a published population-based cohort study found that patients with type2 diabetes on sulfonylurea or insulin therapy have higher risk of cancer death rate compared to those using metformin (Bowker, Majumdar, Veugelers, & Johnson, 2006). Moreover, In 2012, Noto et al published a systemic review and meta-analysis of several published articles regarding metformin and cancer risk, and they found that metformin was markedly associated with reduced incidence of cancer among diabetic patients (Noto, Goto, Tsujimoto, & Noda, 2012). Similarly, In 2013, another detailed systemic review was published including wide range of studies, conducted

during 1966-2012, regarding the use of metformin and other hypoglycemic agents among diabetic patients and their correlation with cancer risk (Franciosi et al., 2013). This review showed that metformin might have a correlation with lower incidence of different cancer types and associated cancer death (Franciosi et al., 2013).

In addition, several *in vitro*, *in vivo*, and clinical studies have been conducted in recent years, exploring the role of metformin as anti-cancer agent for different cancer types, and investigating the underlying molecular mechanisms (Pollak, 2012). *In vitro* studies using metformin, with dose typically ranging between 5-30 mM, demonstrated that metformin was able to inhibit cell growth in different cancer cell types such as lung, colon, and pancreatic cancers (Ben Sahra et al., 2010). Besides, some *in vitro* studies showed that metformin can induce apoptosis in different cell lines including triple negative breast cancer and pancreatic cancer (B. Liu et al., 2009; Kourelis & Siegel, 2012; Ben Sahra et al., 2010).

Likewise, *in vivo* results using different cancer animal models demonstrated that metformin treatment was able to reduce tumor growth significantly in wide range of cancers, for instance, lung, colon, prostate, and triple negative breast cancer (Ben Sahra et al., 2010; Kourelis & Siegel, 2012). Moreover, metformin at very low concentrations specifically reduced growth of CD133+ pancreatic cancer stem cells (Gou et al., 2013). In addition, metformin treatment was selectively able to impair cell growth in p53 mutant colonic cancer (colon cancer) established in xenograft mice than p53 non mutant. (Buzzai et al., 2007).

The overall *in vitro* and *in vivo* studies suggest that metformin inhibits cell growth and/or induces apoptosis through two possible ways: indirect (insulin dependent) and direct (insulin independent) mechanisms (Dowling et al., 2011). Indirect mechanism depends on the systemic effect of metformin in lowering insulin level through reduction of hepatic glucose output and increasing tissue sensitivity to insulin (Dowling et al., 2011; Kourelis & Siegel, 2012).

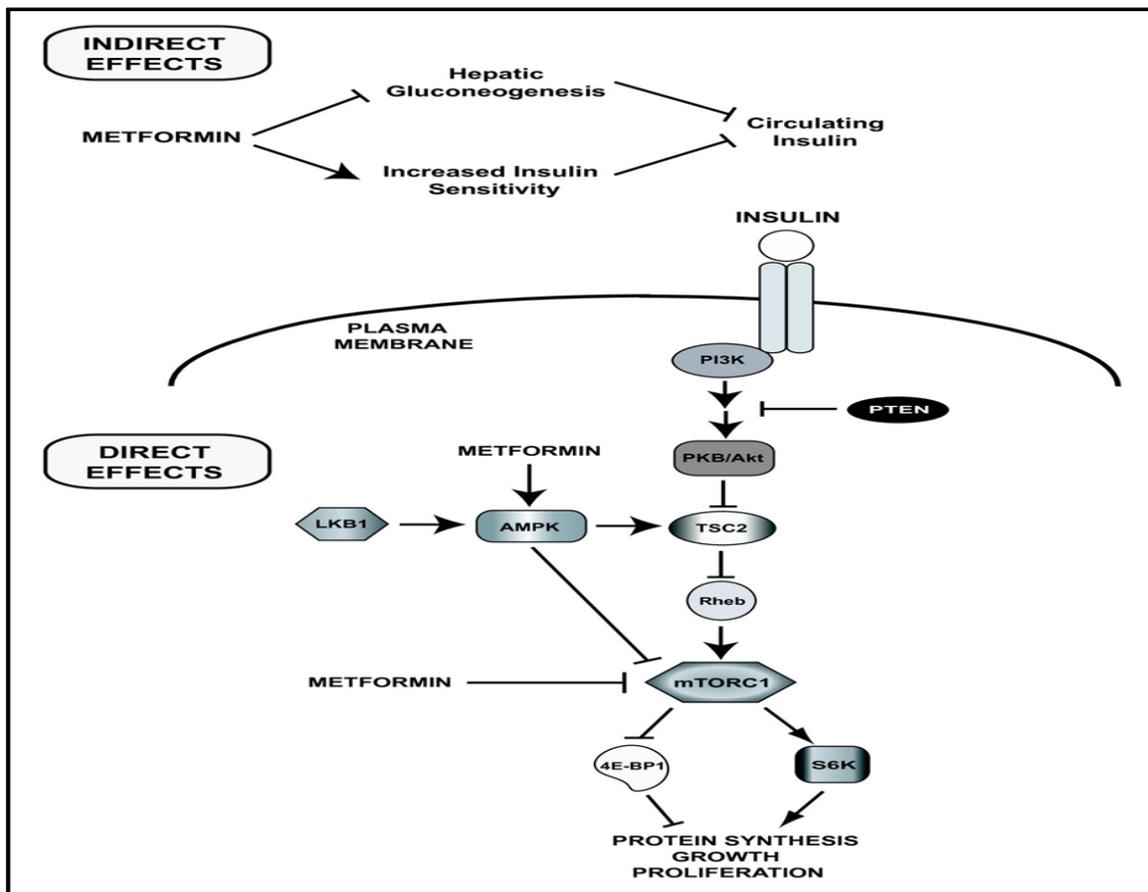


Figure 1.2 “Direct and indirect effects of metformin on cancer” reused from “Understanding the benefit of metformin use in cancer treatment” by Dowling, R. J., Goodwin, P. J., & Stambolic, V. (2011). *BMC Med*, 9:33, figure 1. doi: 10.1186/1741-7015-9-33. © 2011 Dowling et al; licensee BioMed Central Ltd.

In many cancer types, Insulin level is found to be elevated and the related receptor tyrosine kinase signaling pathway is upregulated (Quinn et al., 2013). Insulin causes activation of these receptors which send growth and survival signals to the cells through PI3/AKT pathway (Dowling et al., 2011). So, metformin may reduce tumor growth indirectly by reducing insulin dependent growth promoting signals to the cell (Dowling et al., 2011). On the other hand, the direct mechanism is independent on insulin and metformin may target different cancer cell pathways, including: AMPK-dependent pathway and AMPK-independent pathway (Dowling et al., 2011; Quinn et al., 2013). In AMPK dependent mechanism, metformin can work through affecting AMPK/mTORc1 pathway to inhibit protein synthesis and arrest cell growth (Quinn et al., 2013). In this mechanism, metformin activates AMPK which in turn inhibits mTOR (the mammalian target of rapamycin), a major regulator of cell growth and metabolism (Dowling et al., 2011; Quinn et al., 2013). Activation of AMPK can inhibit mTOR by two main mechanisms: it can cause phosphorylation of tuberous sclerosis complex2 (TSC2) which leads to buildup of Rheb-GDP (inactive form) and ultimately mTOR inhibition, or it can phosphorylate raptor (a regulatory molecule bound to mTOR and a substrate for AMPK) which subsequently lead to inhibition of mTORc1 (Quinn et al., 2013). mTORc1 controls several downstream targets to regulate protein synthesis, transcription process, and autophagy in the cell (Duran & Hall, 2012). mTORc1 activation leads to activation of S6K (ribosomal protein S6 kinase) and inhibition of 4E-BP1 (eukaryotic initiation factor 4E-binding protein-1), the two main downstream targets of mTORc1 (Duran & Hall, 2012). S6k is important for protein synthesis, and 4E-BP1 is particularly important for controlling cell metabolism and proliferation (Duran & Hall, 2012). So, inhibition of

mTOR pathway through AMPK activation can lead to inhibition of cell proliferation and protein synthesis (Dowling et al., 2011). On the other hand, metformin can inhibit mTOR independent on AMPK activation by affecting REDD1 expression (regulated development and DNA damage response) and Rag GTPase proteins (Quinn et al., 2013). Besides, it was shown that metformin treatment can be more toxic to p53 mutant cancer cells, in which metformin-induced AMPK activation can lead to autophagy or apoptosis depends on presence or absence of functioning p53. (Buzzai et al., 2007; Quinn et al., 2013). Thus p53 mutant cancer cells may be more sensitive to energetic stress exerted by metformin. (Quinn et al., 2013).

Furthermore, a recent clinical trial using metformin therapy in women with breast cancer, metformin demonstrated inhibitory effect on cancer cell growth evident by biological markers (Hadad et al., 2011). Likewise, nowadays several preclinical and clinical trials are being conducted to test the role of metformin, as a promising anticancer medication, on different cancer types (Ben Sahra et al., 2010; Quinn et al., 2013).

1.3 Published Data:

Despite the great efforts which have been conducted in last years to elucidate the effect of metformin on various cancer types, not much is known about its effect on neuroblastoma growth and metastasis. According to previous research published in 2006, AMPK was activated in SH-SY5Y cell line in response to metformin treatment (Garcia-Gil et al., 2006). Besides, these cells demonstrated reduction in survival and early morphological changes suggestive of apoptosis, with significant effect was found 72 hours post metformin treatment (Garcia-Gil et al., 2006). Besides, a published data in

2013 testing the effect of metformin and other mitochondrial inhibitors (MIBG and phenformin) on different neuroblastoma cell lines, metformin was able to destabilize MYC/MYCN, enhance histone H3 acetylation in neuroblastoma cancer cell lines, and promotes expression of several genes encoded for biomarkers associated with favorable prognosis (Wang et al., 2014).

1.4 Hypothesis and specific aims:

In our research we propose that metformin, a relatively safe, effective and inexpensive medication, could be a good candidate for the treatment of pediatric neuroblastoma. In the current research, we focus mainly on studying the possible *in vitro* anticancer effects of metformin on two different human neuroblastoma cell lines, SH-SY5Y and the chemo-resistant SKNBE(2). Our research is conducted under following specific aims:

Specific Aim 1: to test the effect of metformin on neuroblastoma cells growth and survival.

Specific Aim 2: to determine the effect of metformin treatment on the tumorigenicity of neuroblastoma cells.

Specific Aim 3: to further investigate possible mechanisms underlie the antineoplastic effect of metformin on neuroblastoma cells including: AMPK, Akt, and Rho GTPases signaling pathways.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines and culture conditions:

Human neuroblastoma cell lines SH-SY5Y and SKNBE (2) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM ; HyClone Laboratories, South Logan, Utah) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, GA) and 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Antibiotic-Antimycotic; Atlanta Biologicals, GA), and allowed to grow at 37°C in humidified incubator with 5% CO₂. Similar preparation for complete culture medium was used to grow neuroblastoma cells for all experiments in this research.

2.2 Metformin and Antibodies:

Metformin Hydrochloride (MP Biomedicals; Solon, OH) was dissolved in a sterile triple distilled water to make 1 Molar stock solution, which is further diluted in culture media to obtain the desired concentrations (1,10, 20 mM), respectively, for each experiment. Monoclonal primary antibodies and the compatible secondary antibodies for western blot analysis and Immunofluorescence assay were obtained from the following sources: Cleaved Caspase-3 Rabbit Ab, Beta- Actin Rabbit Ab, p-AMPK alpha Rabbit

Ab, Total AMPK alpha Rabbit Ab, p-Akt Rabbit Ab, and total Akt Mouse Ab (Cell Signaling Technology, Danvers, MA) Beta- Actin Mouse Ab, goat anti-rabbit IgG-HRP Ab, goat anti-mouse IgG-HRP Ab, Secondary antibodies conjugated with fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Dallas, TX)

2.3 Cell proliferation quantification using Trypan blue dye:

Each cell line was cultured in 60 millimeter plates with density of 5×10^5 cells/plate supplemented with complete culture medium. After 24 hours, the media were removed, and cells were treated with metformin 1, 10, 20 mM diluted in medium, while the control received complete medium without metformin. Cells were allowed to grow under humidified condition for 2, 4, 6 days for SH-SY5Y cells and 2 and 4 days for SK-N-BE (2), respectively. At indicated time points, cells were harvested by trypsinization and centrifuged at 1200 rpm for 5 minutes to get a cell pellet. Cell pellets were suspended in 1xPBS and live cells (unstained cells) were counted under light microscope using hemocytometer and trypan blue dye (Sigma-Aldrich).

2.4 MTT colorimetric cell viability assay:

According to manufacture protocol (Roche diagnostics corporation; Indianapolis, IN), 5×10^3 cells/well of each cell line were seeded in 96 wells culture plate in triplicate with complete culture medium. After 24 hours, the medium was removed and cells were treated with metformin (1, 10, 20 mM), respectively, diluted in 100 μ l of complete culture medium, while the control received medium without metformin. Cells were treated for 2, 4, 6 days for SH-SY5Y cells and 2 and 4 days for SK-N-BE (2), and at time of analysis 10 μ l/well of MTT (3-4,5-Dimethylthiazol-2-Yl-2,5-Diphenyltetrazolium

Bromide) labeling reagent was added and incubated at 37 °C for 4 hours, followed by overnight incubation with 100 µl/well of solubilization solution. Cell viability was measured based on the ability of active viable cells to yield formazan product (purple color) as a result of cleavage of the yellow Methylthiazol tetrazolium (MTT) salt (Roche diagnostics corporation). The resulting colored solution was read by spectrophotometer at 550 nm (the absorbance of formazan product which is directly related to the density of live cells), while the background reference absorbance was read at 690 nm.

2.5 Soft agar colony formation assay:

Approximately 5×10^4 cells/plate of each cell line were mixed with 0.35% agarose gel (Amersham) in DMEM medium supplemented with 10% FBS. The suspension (cells and 0.35% agarose gel) was laid on the top of a solidified bottom layer of 0.8% agarose gel supplemented with the same culture medium and plated in 35 millimeter cell culture plates. After the top layer was solidified, the two-layer agarose gel plates (containing cells) were kept at 37°C in a humidified incubator. After 24 hours, cells were treated with or without metformin (1, 10, 20 mM) diluted in 500µl /plate complete DMEM medium. Cells were fed each 4-5 days with DMEM medium containing 10% FBS with or without (for control) the indicated concentrations of metformin. After 45 days, colonies were stained with 0.1% crystal violet dye and the number of colonies was counted under the light microscope.

2.6 Immunofluorescence analysis:

Each cells line was grown in Lab-tek II chamber slides (Thermo scientific) in complete culture medium with 10% FBS. After cells were reached 20-30% confluent,

they were treated with or without 20mM metformin diluted in medium and kept at 37C in a humidified incubator. At indicated time points, the growth medium was removed and cells were washed with 1x PBS and fixed with 4% paraformaldehyde in PBS for approximately 20 minutes. Cells then were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and washed again with 1x PBS. Cells were incubated with blocking reagent of 10% immunoglobulin free-bovine serum albumin (Jackson Immune Research Laboratories, PA) in 1 x PBS for overnight at 4°C. Cells were incubated for overnight with cleaved caspase-3 primary antibody in 2.5% IgG-free BSA/PBS at 4°C. FITC (Santa Cruz Biotechnology, TX) secondary antibody in 2.5% IgG-free BSA/PBS was used to detect cleaved caspase-3 after 2 hours incubation at room temperature. Slides then washed with 1 x PBS followed by adding of antifade Vectashield mounting media (Vector Laboratories, CA), and the nucleus was counterstained with DAPI (4',6-diamidino-2-phenylindole;Sigma). Signals for cleaved caspase3 and nucleus were visualized under fluorescence microscope (Nikon-E600, Tokyo, Japan).

2.7 Western blots analysis:

Cells were plated in 100 millimeter plates with DMEM culture medium supplemented with 10% FBS. After they reached 20-30% confluent, cells were treated with metformin (1, 10, 20 mM) diluted in 10 ml complete culture medium for 4 and 6 days for SK-N-BE (2) and SH-SY5Y cells, respectively, while the control received culture medium without metformin. At indicated time points, cells were harvested and lysed in RIPA lysis buffer and protein concentration was estimated using bicinchoninic acid protein assay (BCA assay; Thermo scientific, Rockford, IL). Equal amount of protein samples were denatured by heating with Laemmli loading dye containing 2-Mercaptoethanol(Bio-Rad

laboratories; Hercules, CA). Protein samples were resolved by electrophoresis using 10-14% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis or SDS-PAGE (30% acrylamide/Bis solution, 1.5M Tris-HCl buffer pH 8.8(Bio-RAD laboratories), 10% sodium dodecyl sulfate SDS (Biomedicals Inc), 10% Ammonium per Sulfate, Tetramethylethylenediamine or TEMED (Fisher BioReagents)). Proteins were transferred to polyvinyl difluoride (PVDF; Amersham, Buckinghamshire, NA) membrane and then blocked with 5% non-fat dry milk for 2 hours at room temperature or 10% Bovine serum Albumin or BSA for overnight at 4°C. Western blots membranes were incubated with either monoclonal primary antibodies diluted in 2.5% nonfat dry milk/TBST (1:1000 dilution) or 5% BSA/TBST (1:1000 dilution) for overnight at 4 °C.

After washing with 1xTBST, a compatible secondary antibody was added (1:2000-4000 dilution in milk or BSA/TBST) and incubated for 1-2 hours at room temperature followed by wash with 1xTBST. ECL substrate (Enhanced Chemiluminescence; Bio-Rad laboratories) were used to reveal protein of interest and the signal was detected using radiographic film (Thermo Scientific).

2.8 Statistical analysis:

Statistical analysis was performed using GraphPad InStat software (GraphPad Software, Inc. La Jolla, CA). For data from 3 experiments, the average results were calculated and the error bar represents the standard deviation of the mean. One-way ANOVA was performed followed by Tukey-Kramer test to compare significance among treated group relative to control (p-value < 0.05 was considered significant).

CHAPTER 3

RESULTS

3.1 Metformin exerts growth and survival inhibitory effects on neuroblastoma cell lines:

To test the growth inhibitory effect of metformin on SH-SY5Y cell line and the chemo-resistant SK-N-BE2 cell line, cells were grown in complete culture media supplemented with 10% FBS in presence or absence of metformin (1,10,20 mM) for 6 and 4 days, respectively. The results of viable cells count using trypan blue dye demonstrate that metformin significantly inhibit cell proliferation at concentrations of (10 and 20 mM) in time and dose dependent pattern relative to control, in both cell lines, while no significant effect were found at the lower concentration (1 mM) (Figure 3.1 A&B). Besides, the morphological changes of treated cells were examined daily under light microscope and compared to the control. We noticed morphological changes suggestive of apoptosis at (10 mM and 20mM) metformin treated groups, which started at day 2 in SK-N-BE2 cells and at day 4 in SH-SY5Y cells, while more drastic changes were noticed at day 4 for SK-N-BE2 and day 6 for SH-SY5Y cells, when marked number of cells became rounded and started lifting, (Figure 3.1 C), as reported in our previous work (Kumar, Al-Sammarraie, DiPette, & Singh, 2014). To further confirm our results, MTT colorimetric assay was performed to check the enzymatic activity of both cell lines, which directly correlated with cell viability (Roche diagnostics corporation; Indianapolis,

IN). Our results show that metformin was significantly able to inhibit cells viability at (10 and 20 mM) in time and dose dependent manner for both N-MYC amplified and non N-MYC amplified cells. However, no significant effect on cell viability was noticed at (1 mM) treated group compared to the control (Figure 3.2 A&B).

3.2 Metformin inhibits the anchorage-independent colony formation of both neuroblastoma cell lines:

In order to test the ability of metformin to inhibit anchorage-independent malignant growth of neuroblastoma cells *in vitro*, we monitored colony formation of SH-SY5Y and SK-N-BE2 cells seeded on a two-layered agarose gel and treated with metformin at different concentrations (1, 10, 20 mM) ,versus the untreated control group, over 45 days. Cells were fed every 4-5 days with fresh complete medium containing the indicated concentrations of metformin, while control group received culture media without metformin. At indicated time point, the number of established colonies was counted under the light microscope, and the number of colonies in treated groups was compared relative to the control. Our preliminary data suggest that metformin was able to inhibit colony formation of neuroblastoma cells at (10 and 20 mM) respectively in both cell lines (figure 3.3A&B). However, no significant effect on colony formation was found at the lower concentration (1mM) treated group.

3.3 Metformin induces apoptosis in neuroblastoma cell lines:

In order to check the anti-survival effect of metformin on neuroblastoma cell lines, SK-N-BE (2) and SH-SY5Y cells were grown in presence or absence of 20 mM metformin for 4 and 6 days, respectively. At the indicated time points, immunofluorescent staining was performed using anti-cleaved caspase3 antibody, which

detect the active form of caspase3. Our results show that metformin was able to induce apoptosis through activation of caspase 3 in both cell lines reflected by the green cytoplasmic signal of the cleaved active form, especially when counter staining of the nucleus with DAPI was performed (Figure 3.4 A). Besides, western blot analysis was performed to detect cleaved caspase 3 band using whole cell lysates of SK-N-BE(2) and SH-SY5Y cells treated with 1,5,10, or 20 mM metformin, for 4 and 6 days respectively, compared to untreated control. Our results demonstrate that metformin induced marked activation of caspase 3 in both cell lines at (10 and 20 mM) revealed by western blot bands, while no difference was found at 1mM relative to control, (Figure 3.4.B), as indicated in our previous work (Kumar et al., 2014).

3.4 Metformin's anti-proliferative and anti-survival effects may be independent on AMPK and AKT pathways:

To further check the possible pathways by which metformin could exert its antineoplastic effects, SK-N-BE (2) and SH-SY5Y were treated with(1, 10, 20 mM) metformin for 4 and 6 days, respectively, and the level AMPK phosphorylation was measured using western blot analysis of pAMPK (phosphorylated AMPK). We observed slight increase in the level of pAMPK in the treated group of both cell lines relative to control. We further measured the total AMPK to get the ratio of pAMPK/total AMPK. Our results show that the level of phosphorylation of AMPK in both cell lines was statistically insignificant relative to the control, (Figure 3.5 A&B), as reported in our previous work (Kumar et al., 2014). Similarly, we checked the effect of metformin on Akt survival pathway, in which Akt activation is frequently associated with high stage neuroblastoma (Santo et al., 2013). Our preliminary data suggest that metformin treatment has no marked effects on pAkt or total Akt proteins expression, demonstrated by western blot

analyses of whole cell lysates of treated cells relative to control, (Figure 3.6), similar to previous work (Kumar et al., 2014).

3.5 Metformin effect on Rho GTPases protein expression:

Rho GTPases are small G-proteins belong to Ras superfamily and play vital role in cell growth and proliferation (Shang et al., 2012). Metformin anticancer effect noticed in many cancer types works mainly through inhibition of mTOR/S6k pathway, and S6k is regulated by Rho GTPases (Chou & Blenis, 1996; Dowling et al., 2011). In this study, we performed western blot analysis using whole cell lysates of SK-N-BE (2) and SH-SY5Y cells to detect a change in total RhoA and Rac1 small GTPases proteins expression post metformin (1,10, 20 mM) treatment, for 4 and 6 days, respectively. Our initial data suggest that metformin markedly reduced total RhoA expression in both cell lines at 10 and 20mM concentrations relative to control (Figure3.7A). Similarly, total Rac1 expression was reduced in response to metformin (10 mM and 20 mM) treatment in SK-N-BE(2), while no marked change was detected in SH-SY5Y total Rac1 protein expression compared to control (Figure3.7B).

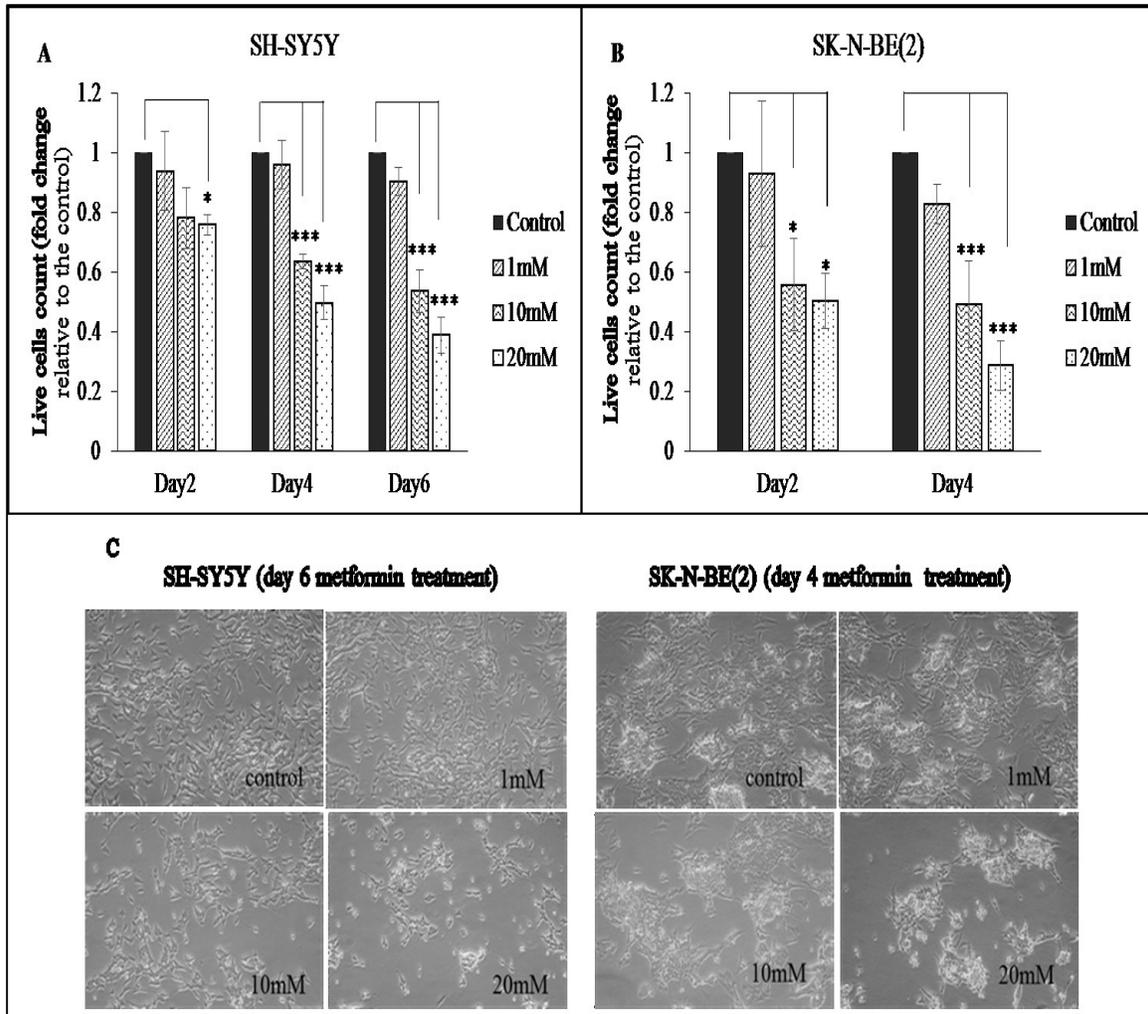


Figure 3.1 The effect of metformin on neuroblastoma cells proliferation (A) Live cells count of SH-SY5Y cells treated with metformin (1, 10, 20 mM) for 6 days duration, N=3, error bars represent \pm SD of the mean. (B) Live cells count of SK-N-BE2 cells treated with metformin (1, 10, 20 mM) for 4 days duration, N=3, error bars represent \pm SD of the mean. (C) Representative images of morphological changes accompanied metformin treatment on both cell lines.

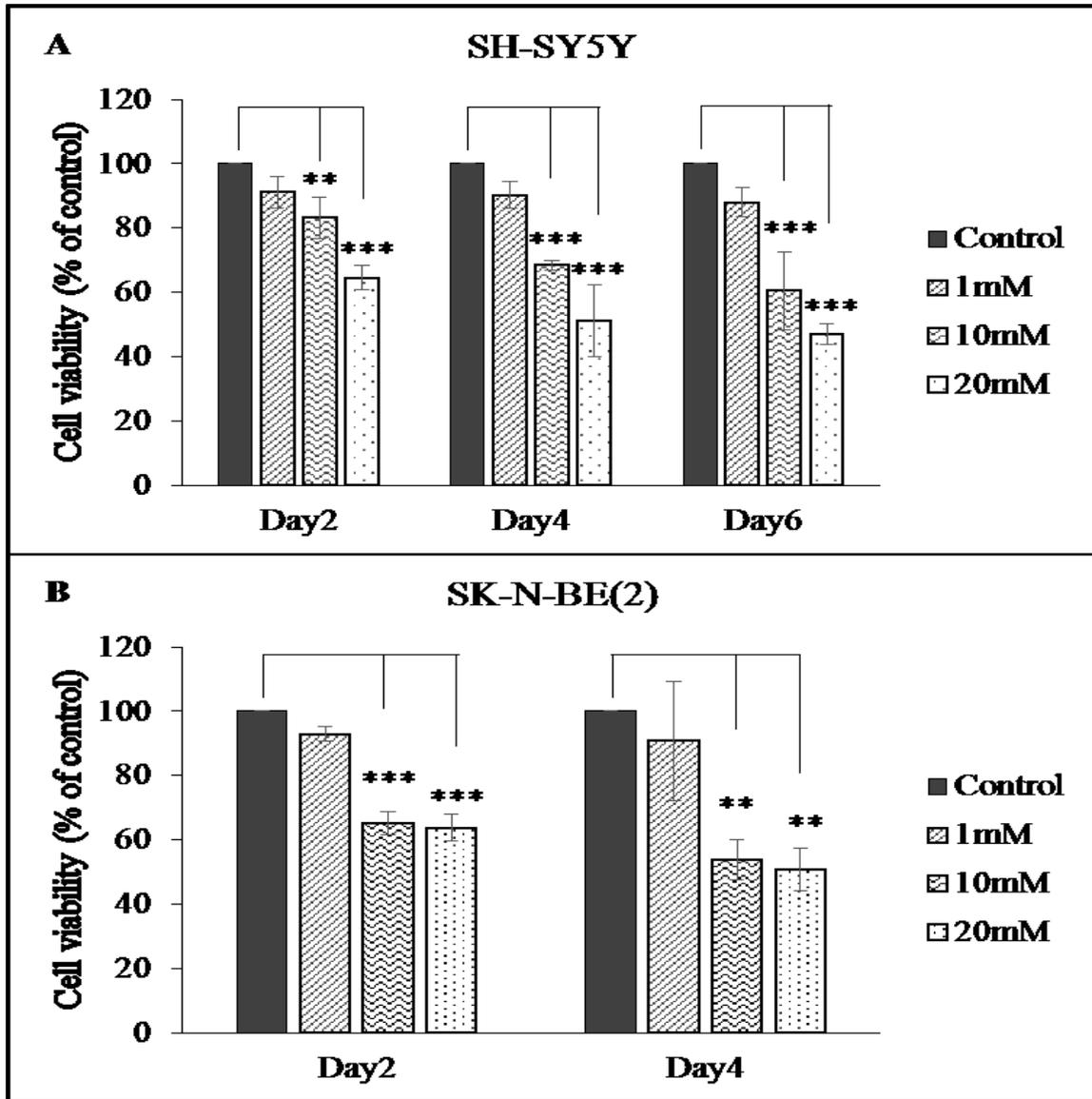


Figure 3.2 The effects of metformin on neuroblastoma cell viability (A) Cell viability estimated by MTT assay of SH-SY5Y cells treated with metformin (1, 10, 20 mM) for 6 days duration, N=3, and error bars represent the \pm SD of the mean. (B) Cell viability estimated by MTT assay of SK-N-BE (2) cells treated with metformin (1, 10, 20 mM) for 4 days duration, N=3, and error bars represent the \pm SD of the mean.

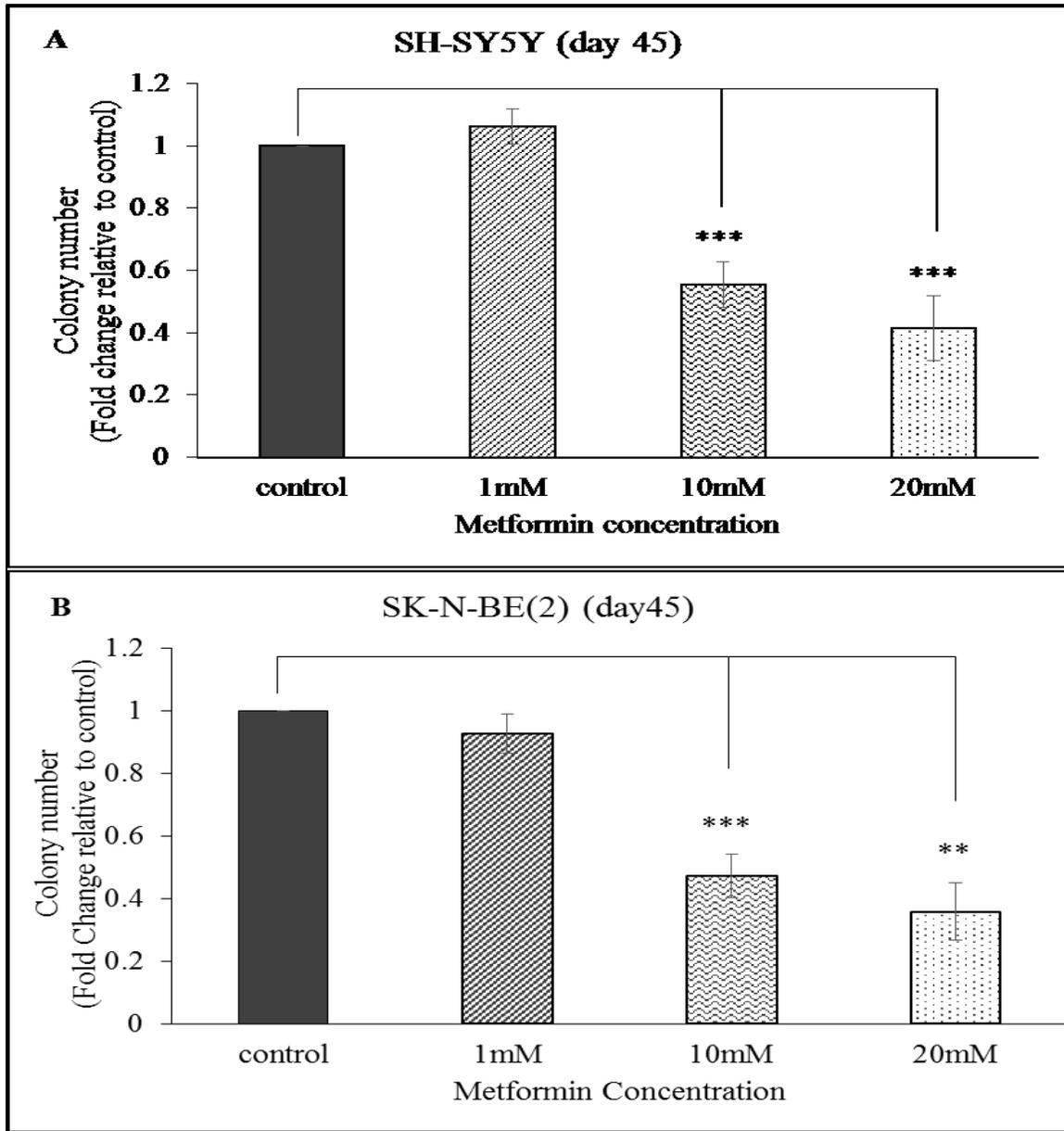


Figure 3.3 The effects of metformin on neuroblastoma colony formation. (A) Number of colonies of SH-SY5Y cells treated with metformin for 45 days and expressed as fold change relative to control, N=triplicate, and error bar represent \pm SD of the mean. (B) Number of colonies of SK-N-BE(2) cells treated with metformin for 45 days and expressed as fold change relative to control, N=triplicate, and error bar represent \pm SD of the mean.

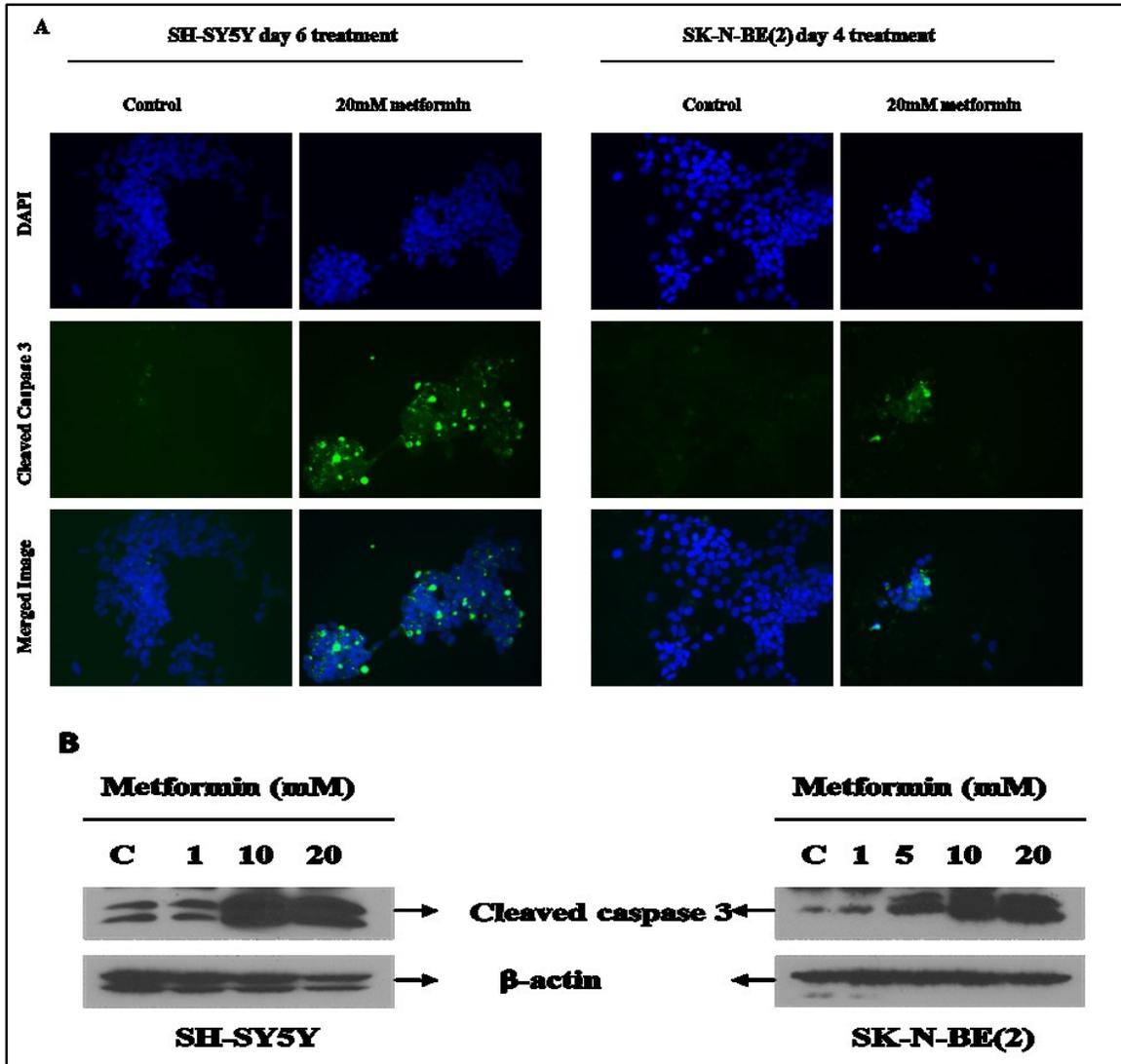


Figure 3.4 Metformin induces apoptosis of neuroblastoma cell lines (A) Immunofluorescence staining for cleaved caspase 3 detection in SH-SY5Y and SK-N-BE(2) treated with 20mM metformin for 6 and 4 days respectively. (B) Western blot analysis for cleaved caspase 3 in SH-SY5Y and SK-N-BE(2) cells treated with 1,5,10, or 20 mM metformin for 6 and 4 days respectively, relative to control N=3.

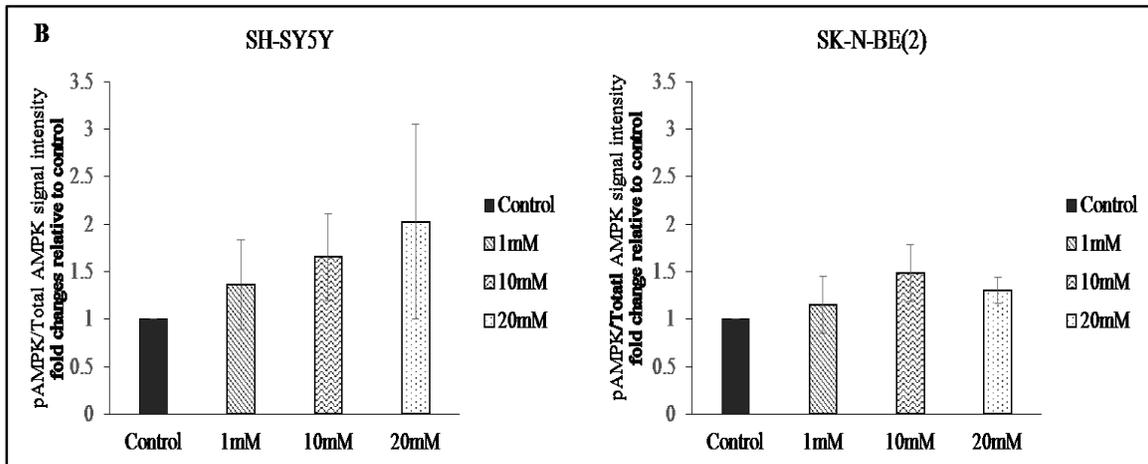
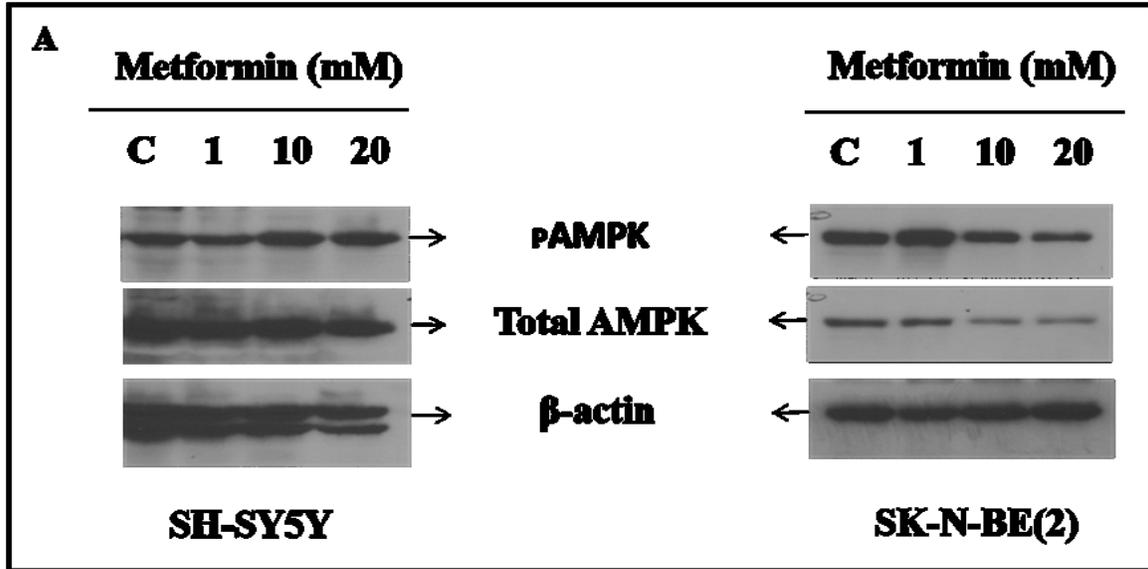


Figure 3.5 The effect of metformin on AMPK activation in neuroblastoma cells: (A) Western blot analysis of AMPK (phosphorylated and total protein) in SH-SY5Y and SK-N-BE (2) treated with 1, 10, or 20mM metformin for 6 and 4 days, respectively, relative to control (B) Corresponding signal density of the ratio of pAMPK /Total AMPK measured by Image J software, N=3, and error bars represent \pm SD of the mean.

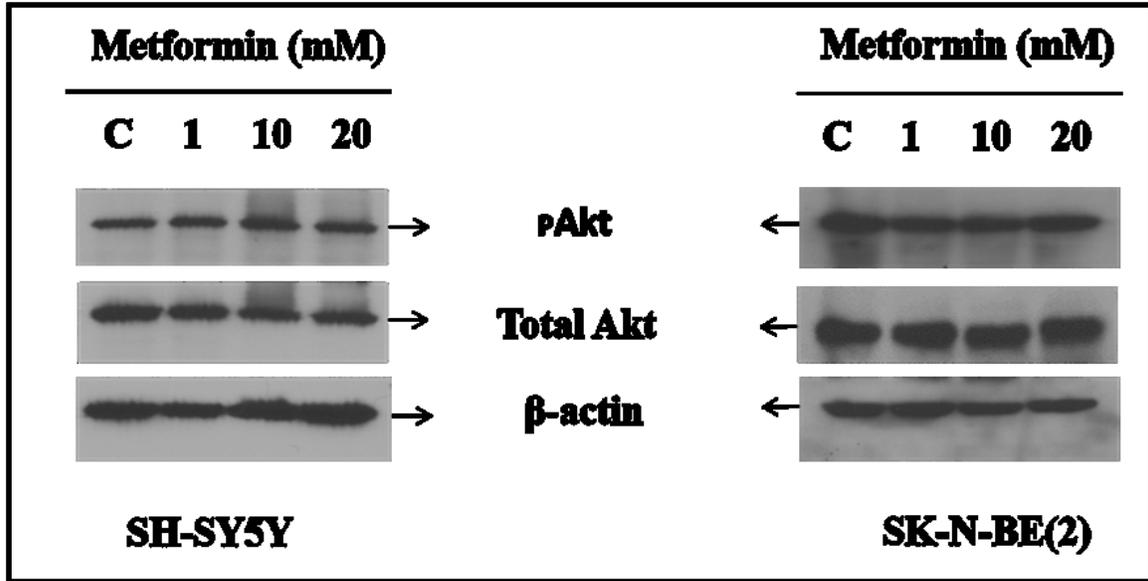


Figure 3.6 The effect of metformin on AKT activation in neuroblastoma cells. Western blot analysis of AKT (phosphorylated and total protein) in SH-SY5Y and SK-N-BE (2) treated with 1, 10, or 20mM metformin for 6 and 4 days, respectively, relative to control, N=1-2.

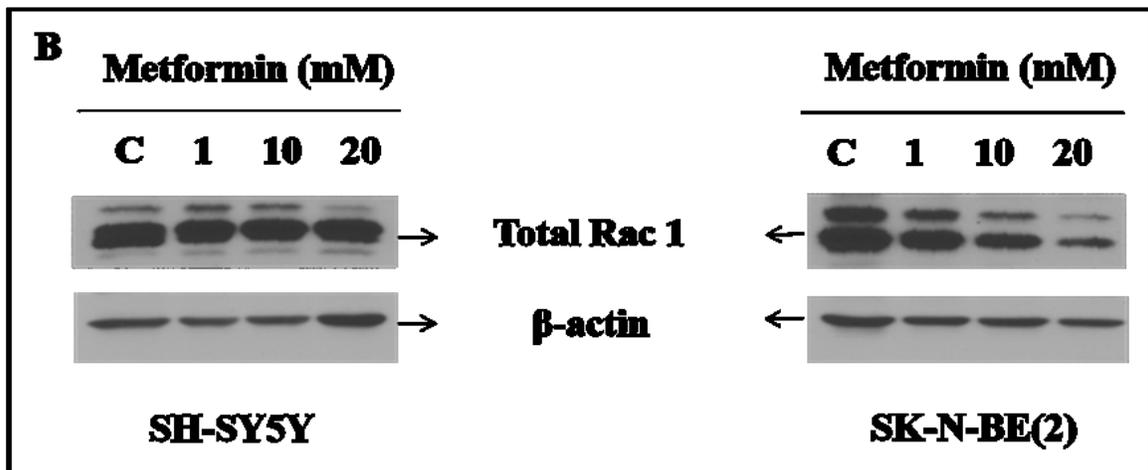
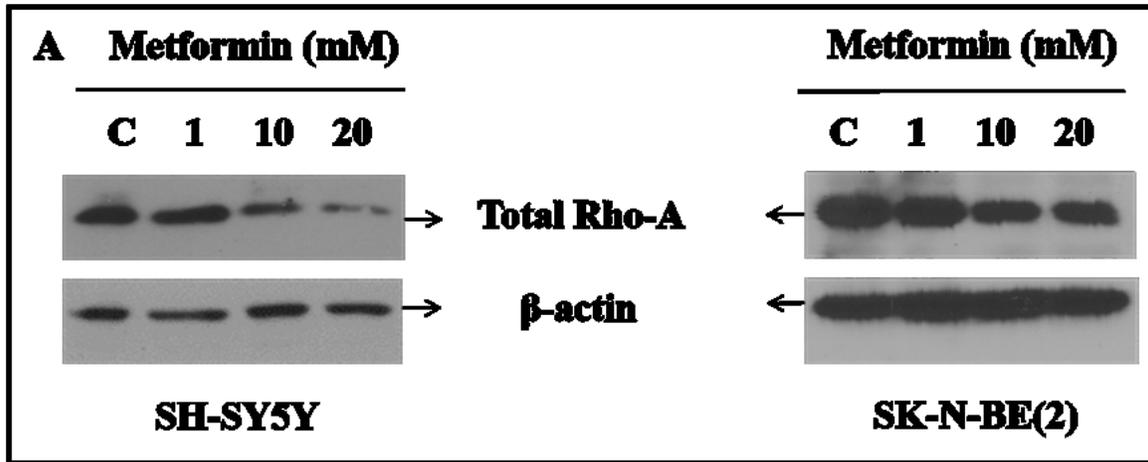


Figure 3.7 The effect of metformin on Rho GTPases protein expression in neuroblastoma cells: (A) Western blot analysis of RhoA total protein expression in SH-SY5Y and SK-N-BE (2) treated with (1, 10, or 20mM) metformin for 6 and 4 days, respectively, relative to control, N=1-2. (B) Western blot analysis of Rac1 total protein expression in SH-SY5Y and SK-N-BE (2) treated with (1, 10, 20mM) metformin for 6 and 4 days, respectively, relative to control, N=1-2.

CHAPTER 4

DISCUSSION

Neuroblastoma is a heterogeneous tumor with combination of several molecular and genetic abnormalities (Davidoff, 2012). These genetic aberrations can develop both in primary tumor and after chemotherapy which make the tumor difficult and sometime unresponsive to the treatment (Tweddle et al., 2001; Van Roy et al., 2009). Despite the combination of different modalities for treatment of neuroblastoma, still the survival rate low for high-risk group (American Cancer Society, 2014). Besides, the current treatment for high risk neuroblastoma may depend on intensified chemotherapy, which itself can carry serious complications for patients (American Cancer Society, 2014). In recent years, several laboratory studies have been performed testing the effect of metformin on different cancer types, which demonstrate marked anti-cancer effect of metformin on different cancers both *in vitro* and *in vivo* (Quinn et al., 2013). Moreover, metformin is worldwide approved first line medication for treatment of type 2 diabetes, due to the fact that it is relatively safe with mild side effects and rare complication of lactic acidosis (Ben Sahra et al., 2010; Rojas & Gomes, 2013). Besides, in recent years, clinical trials using metformin treatment in children and adolescence with type 2 diabetes have been conducted and showed that metformin treatment reduce blood glucose level (fasting) and body weight, without evidence of severe side effects, such as hypoglycemia or lactic acidosis (Rojas & Gomes, 2013). So, recently great work have been performed

studying the effect of metformin therapy on various cancer types, however, there is not much known about the effect of metformin on neuroblastoma.

In this study, *in vitro* experiments were performed to test the effect of metformin on neuroblastoma cell lines, as metformin relatively safe drug with remarkable *in vivo* and *in vitro* anticancer effects and, in some cancer types, works more effectively on p53 deficient cancer cells (Ben Sahra et al., 2010; Quinn et al., 2013). In this research, metformin anticancer effect on neuroblastoma cells was conducted using two different cell lines to mimic the heterogenic nature of neuroblastoma : SH-SY5Y, a subline of SK-N-SH cells derived from bone marrow aspirate of neuroblastoma lesions, which is N-myc non-amplified (Xie, Hu, & Li, 2010; Wang et al., 2014), and SK-N-BE(2), a cell line derived from bone marrow biopsy of relapsed neuroblastoma lesions, which is N-myc amplified and p53 mutant (loss of function mutation) (Tweddle et al., 2001; Wang et al., 2014). We first tested the effect of metformin on cell proliferation and survival using low and high concentrations of metformin (1-20 mM), which is similar to *in vitro* concentrations used in other studies (Ben Sahra et al., 2010). Our results suggest that metformin at 10 and 20 mM was markedly able to inhibit growth and survival of both neuroblastoma cell lines, similar to our previous work (Kumar et al., 2014) and to many *in vitro* studies testing the effect of metformin on different cancer types, such as breast cancer and pancreatic carcinoma (Ben Sahra et al., 2010).

In addition, we observed morphological changes in cells being smaller and rounded which were more marked at day 4 and 6 for SK-N-BE (2) and SH-Y5Y, respectively, as reported previously (Kumar et al., 2014). Our finding is similar to previous study when metformin was able induce morphological changes suggestive of

apoptosis in SH-SY5Y cells with more significant effect noticed after 72 hours from treatment (Garcia-Gil et al., 2006). Besides, in some *in vitro* studies, metformin was able to induce apoptosis such as in triple negative breast cancer with caspases activation (B. Liu et al., 2009). So, we performed immunofluorescence staining to check the expression of cleaved caspase-3 protein, an apoptotic marker indicative of the active effector caspase-3 (Fan & Bergmann, 2010), in 20 mM metformin treated SK-N-BE (2) and SH-SY5Y cells for 4 and 6 days, respectively, versus the control. Our results suggest that caspase-3 was activated in treated group which revealed by presence of green immunofluorescence cytoplasmic signals. We further confirmed our results by detecting cleaved caspase-3 band, using western blot analysis, and it was found to be markedly activated in 10 and 20 mM treated cells at same time points, as indicated in our previous work (Kumar et al., 2014).

The next part of our study focused on understanding the molecular pathways by which metformin may work as anticancer medication. We used western blot analysis of whole cell lysates of SK-N-BE (2) and SH-SY5Y treated with metformin for 4 and 6 days respectively, to check different possible pathways by which metformin could inhibit growth and/or survival of neuroblastoma cells, including: AMPK activation, AKT inhibition, and change in Rho GTPases protein expressions. These preliminary data could be helpful as a start point for our ongoing and future work exploring the anticancer effect of metformin on neuroblastoma.

In many studies, metformin has been found to work through AMPK dependent pathway to inhibit cell growth of different cancer cell types (Dowling et al., 2011; Viollet et al., 2012). In previous study using SH-SY5Y neuroblastoma cell line, AMPK was

activated in these cells 3 hours post metformin treatment (Garcia-Gil et al., 2006). Our results demonstrate that AMPK phosphorylation in metformin treated cell lines for longer time periods was statistically insignificant compare to control (Kumar et al., 2014). However, further study is needed to test whether metformin inhibit AMPK acutely at early time points that might lead to inhibition of cell growth and survival.

On the other hand, several studies have shown that metformin may work through targeting AKT pathway, via AMPK dependent or independent mechanism. Xiaona Liu et al reported that metformin inhibits cell growth of Glioblastoma cells by promoting PRAS40 (AKT substrate) association with RAPTOR, which ultimately inhibited mTOR independent on AMPK activation (X. Liu et al., 2014). Besides, *in vivo* study testing the effect of metformin on prevention of tobacco induced lung cancer; metformin was able to inhibit AKT activation and other pathways in lung tissue independent on AMPK, leading to attenuation of lung tumorigenesis. (Memmott et al., 2010). However, Zakikhani et al show that metformin was able to induce apoptosis in breast cancer cells due to the effect of metformin on inhibition of AKT pathway, beside mTOR inhibition, through AMPK dependent mechanism (Zakikhani, Blouin, Piura, & Pollak, 2010). AKT is a serine/threonine kinase which plays important role in regulation of cell growth and survival (Megison, Gillory, & Beierle, 2013). Akt activation has been found to be associated with high stage neuroblastoma, and found to be important for MYCN post-translation stabilization; hence, it is important for cancer cell growth and proliferation (Santo et al., 2013). So, to test if metformin can inhibit AKT pathway in neuroblastoma cells, we checked pAkt expression using western blot analysis to see whether metformin treatment can inhibit this survival pathway to arrest growth and induce apoptosis. Our

preliminary data suggest that metformin works in an Akt-independent mechanism, as no marked change was noticed on pAKT protein expression in treated groups of both cell lines compared to control, similar to previous work (Kumar et al., 2014).

Similarly, we tested the effect of metformin on Rho GTPases proteins expression, as they are pivotal signaling molecules involved in cell growth, cell cycle progress and metastasis. (Shang et al., 2012). Metformin is known to affect S6 kinase pathway, and this pathway is regulated by Rho GTPases (Chou & Blenis, 1996; Dowling et al., 2011). So, we tested whether metformin could be targeting Rho GTPases signaling to inhibit growth and survival of neuroblastoma cells. RhoA and Rac1 are small GTPases proteins belong to Ras superfamily and they are well regulated in the cell in response to different cell conditions (Shang et al., 2012). Upon activation, with aid of Rho guanine nucleotide exchange factors (GEFs), they switch from GDP-bound inactive state to GTP-bound active state (Shang et al., 2012). Rho GTPases activities, especially RhoA, have been found to be upregulated in various cancer types and associated with cancer metastasis and growth; hence, targeting this vital pathway that control cell growth has been conducted in recent research (Shang et al., 2012). Rac1 is a small GTPase protein which controls S6kinase activity that plays role in cell passage from G1 to S phase through cell cycle (Chou & Blenis, 1996). It is also important for regulation of cell migration and cytoskeleton organization(Dirat et al, 2014). In recent study testing the effect of metformin on metastasis of prostatic cancer cells in nude mice, metformin was able to inhibit Rac1 GTPases activity by affecting several upstream signaling pathways, which in turn reduced cancer cells migration and metastasis (Dirat et al, 2014). In our studies we checked whether metformin affect expression of Rac1 using western blot analysis. Our

preliminary data suggest that metformin was able to inhibit total Rac1 protein expression in SK-N-BE (2) cells, while no marked difference is found on Rac1 protein expression in SH-SY5Y cells compared to control. Likewise, we checked the effect of metformin on Rho/ROCK pathway, which is important for cell proliferation, migration, differentiation, malignant transformation and apoptosis (Ohta et al., 2012). In a published data regarding human ovarian cancer cells, inhibition of Rho/ROCK pathway, using fasudil inhibitor, promoted the anticancer effect of cisplatin in these cell lines (Ohta et al., 2012). Similarly, we tested the effect of metformin on this vital pathway using western blot analysis to check the total RhoA protein expression in treated cells. Our results demonstrated that metformin reduces the total RhoA protein expression in both cell lines, which may play a role in its anticancer effects. However, further *in vitro* studies are important to test the effect of metformin on Rho GTPases activity by measuring the relative ratio of active protein form to the total expression. In conclusion, our results imply that metformin, a relatively safe drug with wide clinical applications, could be a promising anticancer candidate for treatment of neuroblastoma in the future.

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APPENDIX A: PERMISSION LETTER

11/4/2014

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