Metabolic Targeting of Glioblastoma Cells

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

In the 1920s, Otto Warburg and his colleagues showed that normal liver tissue exhibited the "Pasteur effect," which is inhibition of fermentation in the presence of oxygen, whereas tumour tissue continued to produce lactate even in the presence of oxygen (aerobic glycolysis). Warburg showed that tumour tissue consumed ten-fold more glucose than accounted for by respiration and twice as much lactate as produced by normal tissue. This phenomenon was described as the "Warburg effect". Upregulated glycolysis has not only been shown to provide intermediates for biosynthesis, but studies have also demonstrated that it plays a vital role in transformation, cell cycle progression, proliferation, resistance to cell death, metastasis, angiogenesis and maintenance of stemness; the hallmarks of cancer.

The study aimed to target metabolic dependence of cancer cells on glucose. We have shown that glucose deprivation led to significant cell death in glioma cell lines whereas it did not affect untransformed human fibroblasts. We have also demonstrated that glucose deprivation led to an atypical form of cell death in glioma cells which was not mediated by apoptosis, autophagy or necrosis. We have also shown that free radical scavenger N-acetylcysteine was able to suppress glucose withdrawal-induced cell death. Our data indicate that glucose deprivation led to energetic and oxidative stress. We have also shown that metformin potentiates glucose withdrawal-induced cell death in glioma cells which was again not mediated by apoptosis, autophagy or necrosis. We have demonstrated that metformin potentiates glucose deprivation-induced energetic stress whereas it suppresses glucose deprivation-induced unfolded protein response leading to significant cell death. Finally, we have shown that metformin and 2-deoxyglucose combination treatment led to significant cell death in glioma cells.

As both metformin and 2-deoxyglucose are in human use, our study may pave the way for future preclinical work to see if combination treatment can suppress glioma cell growth *in vivo.*

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Abbreviations

ACC - acetyl CoA carboxylase

ACL - ATP citrate lyase

ADP - adenosine diphosphate

AICAR- 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside

AIF - apoptosis inducing factor

ALL - acute lymphoblastic leukaemia

AML - acute myeloid leukaemia

AMP - adenosine monophosphate

AMPK - 5' Adenosine monophosphate-activated protein kinase

AOA - amino-oxyacetic acid

APS - ammonium persulphate

APAF1 - apoptotic protease activating factor 1

ATF - activating transcription factor

Atg - autophagy related genes

ATP - adenosine triphosphate

BAX - Bcl-2 associated X protein

BH - Bcl-2 homology

BIK - Bcl-2 interacting killer

BPTES - bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide

BSO - buthionine sulfoximine

CAD- caspase-activated DNase

CAMKK2 - calcium/calmodulin-dependent protein kinase kinase 2

CDK - cyclin dependent kinase

CHC - alpha-cyano-4-hydroxycinnamate

CHOP - CCAAT/enhancer-binding protein homologous protein

CLL - chronic lymphocytic leukaemia

CNS - central nervous system

CO_2 - carbon dioxide

CPT1 - carnitine palmitoyl transferase 1

CREB - cAMP response element-binding protein

CSC - cancer stem cells

DCA - dichloroacetic acid

DISC - death inducing signalling complex

DMSO - dimethyl sulphoxide

DNA - deoxy ribonucleic acid

EGCG - epigallocatechin gallate

EGF - epidermal growth factor

EGFR - epidermal growth factor receptor

eIF4E - eukaryotic translation initiation factor 4E

EMT - epithelial mesenchymal transition

ER - endoplasmic reticulum

Erk 1/2 - extracellular regulated kinase 1/2

ETC - electron transport chain

F1,6-BP - fructose 1, 6-bisphosphate

F2,6-BP - fructose 2, 6-bisphosphate

FAO - fatty acid oxidation

FAS - fatty acid synthase

FDA - Food and Drug Administration

FDG - 2-deoxy-2-(^{18}F)fluoro-D-glucose

FH - fumarate hydratase

FoxO3 - forkhead box O3

G6PD - glucose 6-phosphate dehydrogenase

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GAP - GTPase activating protein

GBM - glioblastoma multiforme

GDP - guanosine diphosphate

GLS - glutaminase

GLUT - glucose transporters

GPI - glucose 6-phosphate isomerase

GPNA - gamma-l-glutamyl-p-nitroanilide

GRP78 - glucose regulatory protein 78

GSH - glutathione

GSK3β - glycogen synthase kinase 3β

GSSG - glutathione disulfide

GTP - guanosine-5'-triphosphate

H_2O_2 - hydrogen peroxide

HCC - hepatocellular carcinoma

HIF-1 – hypoxia-inducible factor-1

HK - hexokinase

HNSCC - head and neck squamous cell carcinoma

HRE - hypoxia response elements

Hsp - heat shock protein

IDH - isocitrate dehydrogenase

IGF-1 - insulin-like growth factor 1

IL-1 - interleukin-1

IMM - inner mitochondrial membrane

IRE1 - inositol-requiring enzyme 1

JNK - c-Jun N-terminal kinase

KEAP - kelch-like ECH -associated protein

LDH - lactate dehydrogenase

LDL - low density lipoproteins

LKB1 - liver kinase B1

LOX - lysyl oxidase

MAPK - mitogen-activated protein kinase

MCT - monocarboxylate transporter

MDM2 - murine double minute 2

MDR - multi-drug resistance

MMP- matrix metalloproteinase

MOMP - mitochondrial outer membrane permeabilization

MPTP - mitochondrial permeability transition pore

MRI - magnetic resonance imaging

mRNA - messenger RNA

MtDNA - mitochondrial DNA

mTOR - mammalian target of rapamycin

NAC – N-acetylcysteine

NEAA - non-essential amino acids

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NMR - Nuclear magnetic resonance

Nrf2 - nuclear factor (erythroid-derived 2)-like 2

NSCLC - non-small-cell lung cancer

OAA - oxaloacetic acid

OMM - outer mitochondrial membrane

OS - overall survival

PCR - polymerase chain reaction

PDGF - platelet derived growth factor

PDH - pyruvate dehydrogenase

PDK1 - pyruvate dehydrogenase kinase 1

PEP - phosphoenolpyruvate

PERK - protein kinase R (PKR)-like endoplasmic reticulum kinase

PET - positron emission tomography

PFK - phosphofructokinase

PFS - progression free survival

PGK - phosphoglycerate kinase

PGM - phosphoglycerate mutase

PI3K - phosphoinositide 3-kinase

PK - pyruvate kinase

PKM2 - pyruvate kinase M2

PPAR-γ - peroxisome proliferation activated receptor gamma

PPP - pentose phosphate pathway

PTEN - phosphatase and tensin homolog

RCC - renal cell carcinoma

REDD1 - regulated in development and DNA damage responses 1

Rheb - ras homolog enriched in brain

ROS - reactive oxygen species

RPTK - receptor protein tyrosine kinase

S6K1 - S6 kinase 1

SDH - succinate dehydrogenase

SEGA - subependymal giant cell astrocytoma

SOD - superoxide dismutase

SREBP1 - sterol regulatory element-binding protein 1

TCA - tricarboxylic acid

TET - ten-eleven translocation

TGF - transforming growth factor

TIGAR - TP53-inducible glycolysis and apoptosis regulator

TKTL - transketolase like enzyme

TMZ - temozolomide

TNF - tumour necrosis factor

TPI - triosephosphate isomerase

TPZ - tirapazamine

TRAIL - TNF-related apoptosis-inducing ligand

TRAP1 - tumour necrosis factor receptor-associated protein 1

TSC - tuberous sclerosis complex

TUNEL - terminal dUTP Nick End -Labelling

TXNIP - thioredoxin interacting protein

UCP - uncoupling protein

ULK1 - UNC-51 like kinase 1

UPR - unfolded protein response

VDAC - voltage dependent anion channels

VEGF - vascular endothelial growth factor

WHO - world health organization

VHL - von Hippel-Lindau

α-KG - α-ketoglutarate

2DG - 2-Deoxyglucose

2HG - 2-hydroxyglutarate

2-ME - 2-methoxyestradiol

3-BP - 3-Bromopyruvate

4E-BP1 - eukaryotic translation initiation factor 4E binding protein 1

5-FU - 5-Fluorouracil

6-AN - 6-Aminonicotinamide

17-AAG - 17-N-allylamino-17-demethoxygeldanamycin

Chapter 1

1.1 Glioblastoma

1.1.1 Introduction

Brain tumours account for less than 2% of all primary cancers; however, 1860 new cases of malignant gliomas are diagnosed each year in England and Wales (NICE guidelines 2007). As per World Health Organization (WHO) 2007 classification system, astrocytomas are classified into low grade (grade I and II) and high-grade malignant gliomas (Grade III and IV) (Louis 2007). Malignant gliomas are the most common primary malignant tumour in the brain and are usually seen in the 6^{th} to 8^{th} decade of life (Dolecek 2012). Grade III malignant gliomas include anaplastic astrocytoma, anaplastic oligodendroglioma and anaplastic oligoastrocytoma whereas grade IV malignant glioma is also called glioblastoma. As per 2016 WHO classification, gliomas are further subdivided on the basis of their cytogenetic properties (Louis 2016).

The term Glioblastoma was coined in 1926 by Percival Bailey and Harvey Cushing. Multiforme was later added to denote pleomorphic appearances of the cell in the tumour though it is no longer used and grade IV gliomas are simply termed glioblastomas (Omuro 2013). Glioblastoma arises from the supporting glial cells of the central nervous system (CNS).

Glioblastoma can be further sub-divided into primary and secondary forms. Primary glioblastoma arises *de novo*, is usually seen in older patients and may be associated with epidermal growth factor receptor (EGFR) amplification or mutation, phosphatase and tensin homolog (PTEN) loss of function and loss of heterozygosity on chromosome 10. Secondary glioblastoma arises as a result of the transformation of grade II or grade III glioma, is usually seen in younger patients, is associated with isocitrate dehydrogenase (IDH) mutation and may also be associated with TP53 mutation (Kleihues 2002).

1.1.2 Clinical presentation

Patients may present with a headache, nausea, vomiting or drowsiness as a result of raised intracranial pressure. Depending upon the location of a tumour, patients may also present with hemiparesis, visual or speech disturbance, seizures, personality change or gait disturbance.

1.1.3 Diagnosis

Magnetic resonance imaging (MRI) with and without gadolinium contrast is the imaging modality of choice. Glioblastoma appears as heterogeneously enhancing lesion with surrounding vasogenic oedema. However, diagnosis can only be confirmed from histopathology.

1.1.4 Treatment

Standard care of treatment for patients with glioblastoma is surgery followed by adjuvant radiotherapy and chemotherapy. Given the infiltrative nature of the tumour, surgery is not curative, and the goal of the operation is cytoreduction while preserving function. Post-surgery, typical radiotherapy dose is 60 Gy divided in 30 fractions (Omuro 2013). Deoxy ribonucleic acid (DNA) alkylating agent temozolomide (TMZ) is also administered along with radiotherapy. Glioblastoma is a highly aggressive and infiltrating tumour, and with advances in radiotherapy, chemotherapy and surgical technique, there has been some improvement in patient survival, but a cure is still not possible (Lamborn 2004). Patients with glioblastoma may initially respond to treatment, but the tumour invariably recurs after a variable duration of time. Cause of death in glioblastoma patients is usually multifactorial and includes cerebral herniation as a result of the mass effect, systemic illness and surgical complications (Silbergeld 1991).

As a cure for glioblastoma multiforme (GBM) remains elusive, it is essential to identify new treatment modalities as well as modify existing therapies to possibly change malignant gliomas from a deadly disease into a chronic one (Pyrko 2007).

1.2 Warburg effect and cancer

1.2.1 Introduction

Metabolism is defined as the sum total of all biochemical reactions taking place inside a cell or an organism which either produce or consume energy (Ferreira 2010, Deberardinis 2012). Normal mammalian cells enter the cell cycle and proliferate only when they are stimulated by growth factor-mediated signalling pathways. In the absence of growth factor signalling, mammalian cells fail to take up nutrients and turn to autophagy to supply energy for survival (Lum 2007). On the other hand,

rapidly proliferating cells take up nutrients in excess of their bioenergetic needs and shunt metabolites into biosynthetic pathways for growth, proliferation and survival (Bauer 2005). This shows that in such cells signalling pathways which coordinate growth, proliferation and survival are wired to metabolic pathways (Deberardinis[a] 2008).

1.2.2 Warburg effect

In the 1920s, Otto Warburg and his colleagues showed that normal liver tissue exhibited the "Pasteur effect," which is inhibition of fermentation in the presence of oxygen, whereas tumour tissue continued to produce lactate even in the presence of oxygen (aerobic glycolysis). Warburg showed that tumour tissue consumed ten-fold more glucose than accounted for by respiration and twice as much lactate as produced by normal tissue. This phenomenon was described as the "Warburg effect". According to Warburg, this occurred as a result of impaired or damaged mitochondrial respiration in the tumour cells (Warburg1927, Warburg 1956).

The period from the 1920s to the 1960s has been described as the golden age of biochemistry as there was rapid development in the field of study of metabolism (Deberardinis 2012). Then in the 1980s, there was a rapid explosion in the field of genetics, and the focus shifted to genetic alterations seen in cancer cells. It was thought that activation of oncogenes or loss of tumour suppressor genes modulated signal transduction pathways during carcinogenesis affected growth, proliferation, survival, evasion of cell death as well as metabolic alterations seen in cancer cells (Shaw 2006). However, in the last few years, there has been renewed interest in tumour metabolism, and manipulation of altered tumour metabolism to target cancer cells as a result of growing realization that signalling pathways in the cell and metabolism are interconnected (Bayley 2012, Deberardinis 2012).

Epidemiological studies have also shown a relationship between altered metabolism and cancer. Diabetes is associated with an increased risk of bladder, breast, colorectal, endometrial, liver, pancreatic and renal cancer (Rizos 2013, Morales 2015). Studies have also shown that diabetes is associated with increased mortality in patients with breast and colon cancer as compared to non-diabetic patients with these cancers (Rizos 2013). Insulin resistance and hyperinsulinemia have been proposed as one of the causes for an increased incidence of cancer in people with diabetes (Rizos 2013). Insulin has also been shown to decrease the level of insulin-like growth factor 1 (IGF-1) binding protein in the plasma, which leads to increased free IGF-1 in the blood which exhibits anti-apoptotic and mitogenic properties (Samani 2007, Vander Heiden 2011, Morales 2015). Diabetes is also

characterised by increased oxidative stress which can lead to DNA damage, low-grade inflammation and accumulation of advanced glycation products, all of which can promote carcinogenesis (Abe 2008, Morales 2015). Fatty tissues produce adipokines which cause insulin insensitivity which in turn leads to increased blood glucose levels, which further stimulates the production of insulin and IGF-1 from the pancreas (Khandekar 2011). On the other hand, calorie restriction can lead to decreased IGF-1 levels, enhanced mitochondrial efficacy as a result of clearing cells of defective mitochondria leading to reduced oxidative stress and reduced mutagenesis (Youle 2011, Dang 2012).

Initially, the six recognised hallmarks of cancer were unlimited replicative potential, growth factor independence, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis (Hanahan 2000). However, since then, altered metabolism has also been recognised as a hallmark of cancer, contributing to initiation, growth and maintenance of tumours (Hanahan 2011). The Warburg effect has been identified as a move by the cancer cells to promote growth, proliferation and survival in a microenvironment where there may not be sufficient availability of nutrients and oxygen (Qian 2014) (Figure 1.1).

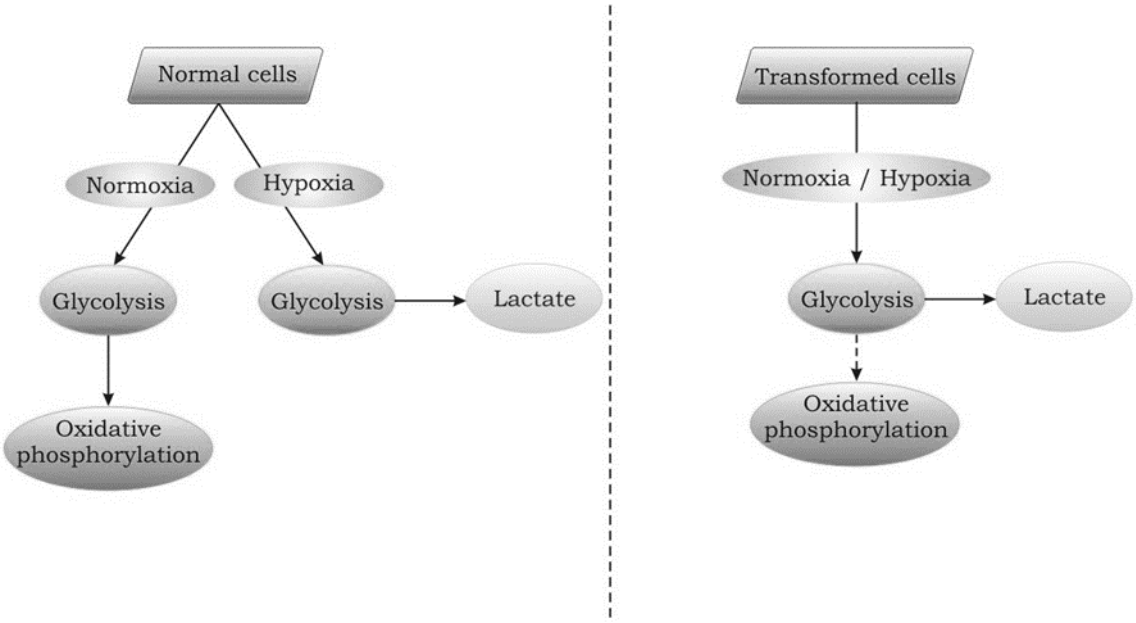


Figure 1.1 The Warburg effect: In non-transformed cells, in the presence of oxygen, glucose is converted into pyruvate via glycolysis. Pyruvate is then transported into the mitochondria, where it undergoes oxidative phosphorylation. Oxygen acts as the final electron acceptor. When non-transformed cells are exposed to hypoxia, glucose is converted into pyruvate via glycolysis which is then converted into lactate. Oxidative phosphorylation is downregulated as it requires oxygen. Otto Warburg and his colleagues showed tumour tissue-maintained lactate production even in the presence of oxygen. They showed that tumour tissue consumed tenfold more glucose than accounted for by respiration and twice as much lactate as produced by normal tissue. This phenomenon was described as the "Warburg effect". Figure adapted from the article by Vander Heiden et al.

"Understanding the Warburg effect: the metabolic requirements of cell proliferation." Science. 2009 May 22;324(5930):1029-33. doi: 10.1126/science.1160809.

Upregulated glycolysis can provide metabolic intermediates which can be used in protein, lipid and nucleotide biosynthesis. Whereas if glucose was catabolised entirely via oxidative phosphorylation for energy production, then the biosynthetic needs of the proliferating cells could not be met. Increased pyruvate produced as a result of upregulated aerobic glycolysis can also serve multiple purposes. Besides being metabolised via the tricarboxylic acid (TCA) cycle to generate energy, pyruvate has been shown to scavenge reactive oxygen species (ROS). Pyruvate can also be converted into non-essential amino acids (NEAA) and lactate (Porporato 2011). Upregulated glycolysis is also an essential source of NADPH which plays a vital role in biosynthetic reactions as well as anti-oxidant defence. ^{13}C Nuclear magnetic resonance (NMR) spectroscopy study has shown that glioblastoma cells in culture convert 90% of glucose and 60% of glutamine to lactate and alanine, with NADPH being a significant by-product in these reactions (Deberardinis 2007). Data from other studies suggest that though glycolysis only produces two adenosine triphosphate (ATP) molecules, the rapid flux of glucose through glycolysis can compensate for this. Hence in non-glucose limiting conditions, ATP generation through glycolysis is not an issue (Cairns 2011). Studies have also shown that the percentage of cellular ATP produced from glycolysis can exceed that produced from oxidative phosphorylation (Guppy 1993). Scholnick *et al*. showed that glycolysis in proliferating cells is limited by the rate of ATP consumption rather than ATP production (1973). Upregulated glycolysis can also liberate cancer cells from dependence on oxygen for ATP generation which may be relevant in the context of tumour hypoxia (Kim 2006).

Mitochondria of cancer cells may have defects in mitochondrial DNA (mtDNA), mutations in enzymes of TCA cycle or downregulation of enzymes of oxidative phosphorylation and in such cases, upregulated glycolysis can play an essential role in meeting the energetic demands of the cancer cells (Herling 2011).

Some authors consider enhanced glycolysis as an adaptation to tumour hypoxia (Cairns 2011). However, others argue that switching to aerobic glycolysis is an early event in tumorigenesis rather than being an adaptation to tumour hypoxia. Leukemic cells in the blood, as well as lung tumours arising in the airways, have access to oxygen, but they still exhibit the Warburg effect (Vander Heiden 2009, Tennant 2010). The Pasteur effect is a shift to glycolysis in the absence of oxygen. However, if the Warburg effect was merely a form of the Pasteur effect, then it cannot explain the fact that leukaemia and lung cancer cells bathed in oxygen or during *in vitro* experiments, where cells are cultured in the presence of 20% oxygen, tumour cells continue to utilise aerobic glycolysis (Fyles 1988).

Hence tumour hypoxia, though an important factor in tumour biology, is a late event and may not be a significant player in the switch to aerobic glycolysis (Vander Heiden 2009).

Non-glycolytic functions of glycolytic enzymes such as the anti-apoptotic effect of hexokinase (HK) II, cell cycle-dependent transcription regulation by lactate dehydrogenase (LDH) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enhanced cell motility by glucose 6-phosphate isomerase (GPI) can also contribute to tumorigenesis (Kim 2006). Studies have also shown that aerobic glycolysis can also play a role in cellular immortalisation. GPI and phosphoglycerate mutase (PGM) have been shown to enhance glycolytic flux, decrease oxidative damage and extend the lifespan of cells whereas their silencing by small interfering ribonucleic acid (siRNA) induces premature senescence (Kondoh 2005, Kondoh 2007). Other data suggest that glycolytic intermediates can also be involved in regulating the expression of genes involved in metabolism. Pyruvate has been shown to influence histone acetylase and histone deacetylase leading to increased expression of glucose transporters (GLUTs) and enzymes of glycolysis (Buchakjian 2010).

Recently, Samudio *et al.* have proposed that it is mitochondrial uncoupling rather than its impairment which is responsible for increased glycolysis seen in cancer cells. They have shown that mitochondrial uncoupling protein (UCP) 2 is elevated in many cancer cell lines (2009). Uncoupling results in increased reliance of cells on glycolysis, decreased ATP production via oxidative phosphorylation which further reinforces glycolysis, decreased ROS generation and increased resistance to cell death (Derdak 2008, Wang 2010).

Altered metabolism in cancer cells may also help them to evade cell death. The p53 target TP53-inducible glycolysis and apoptosis regulator (TIGAR) has been shown to lower fructose 2,6-bisphosphate (F2,6-BP) levels in cells which results in inhibition of glycolysis and diversion of intermediates towards the pentose phosphate pathway (PPP) to generate NADPH and an overall decrease in intracellular ROS levels (Bensaad 2006). Similarly, GAPDH has been shown to prevent caspase-independent cell death which may be due to the fact that GAPDH upregulates glycolysis and promotes autophagy (Colell 2007). Most somatic cells have limited replicative potential as a result of telomere shortening, and they finally undergo permanent cell cycle arrest which is called replicative senescence (Hayflick 1961). Increased ROS accumulation is seen during replicative senescence. Hence enhanced glycolysis via NADPH may protect cells from oxidative damage, prevent senescence and promote immortalisation (Lee 1999, Chen 2001, Kondoh 2005) (Figure 1.2).

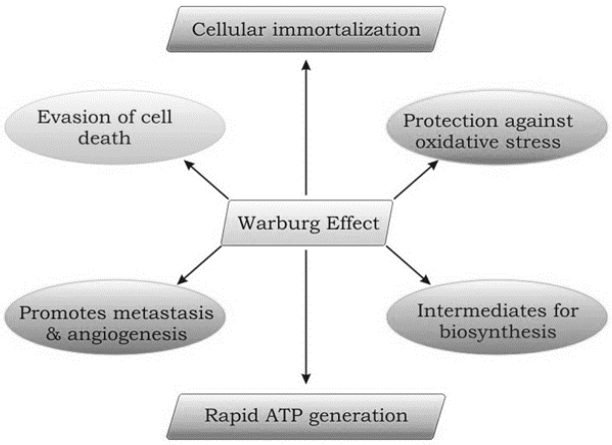


Figure 1.2 Biological effects of upregulated glycolysis: The Warburg effect has been recognised as a move by the cancer cells to promote growth, proliferation and survival in a microenvironment with constraints of nutrition and oxygen. Upregulated glycolysis can provide with intermediates which can be used for biosynthesis. Glycolysis, as compared to oxidative phosphorylation, is a faster process and hence the Warburg effect may be a mean by cancer cells for rapid ATP generation. Increased lactate produced as a result of upregulated glycolysis creates an acidic microenvironment which promotes proliferation, migration, invasion of cancer cells and help cells to evade apoptosis. Upregulated glycolysis via TIGAR, GAPDH and association of HK II to voltage-dependent anion channels (VDAC) may also help cancer cells to evade cell death. Upregulated aerobic glycolysis can also play a role in cellular immortalisation. Enhanced glycolysis via NADPH may also protect cells from oxidative damage, prevent senescence and promote immortalisation.

However, other authors argue that there is conflicting evidence that the Warburg effect offers resistance against cell death as TMZ resistant glioma cells have been shown to have decreased glucose consumption and lactate production, which are the hallmarks of the Warburg effect. Oliva *et al.* showed that increased efficiency of mitochondrial coupling resulted in reduced ROS generation and oxidative stress as a result of TMZ treatment (2011). Similarly, cancer stem cells (CSC) have been shown to exhibit increased reliance on oxidative phosphorylation as compared to aerobic glycolysis to meet their energetic needs. These CSC exhibits reduced ROS generation and increased resistance to radiotherapy and chemotherapy (Vlashi 2011).

1.2.3 Reverse Warburg effect

Studies have shown that a tumour may contain areas which are well oxygenated whereas some other regions may be poorly oxygenated. Poorly oxygenated cells can generate lactate via glycolysis and release it in the extracellular space via monocarboxylate transporter4 (MCT4) which can then be taken up by other cells in oxygenated areas of a tumour via MCT1. These cells can then convert lactate into

pyruvate and use it for oxidative phosphorylation (Sonveaux 2008). Pavlides *et al*. proposed that epithelial cancer cells induce the Warburg effect in neighbouring stromal fibroblast cells which in turn secrete lactate and pyruvate in the tumour microenvironment. Epithelial cancer cells can take up these energy-rich metabolites and use them in the TCA cycle of the mitochondria for oxidative phosphorylation; so, called reverse Warburg effect (2009) (Figure 1.3).

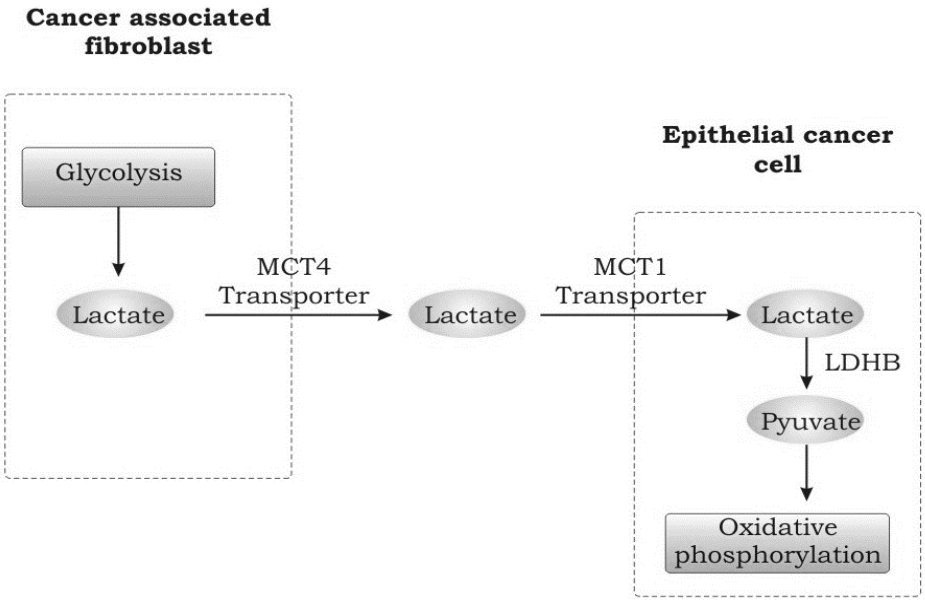


Figure 1.3 Reverse Warburg effect: Epithelial cancer cells induce the Warburg effect in neighbouring stromal fibroblast cells which in turn secrete lactate in the tumour microenvironment. Epithelial cancer cells can then take up lactate and use it in the TCA cycle of the mitochondria for oxidative phosphorylation. Figure from the description of Pavlides et al. in the article "The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma." Cell Cycle. 2009 Dec;8(23):3984-4001. Epub 2009 Dec 5.

1.2.4 Metabolism in non-transformed cells

Glucose metabolism can be divided into glycolysis, TCA cycle and oxidative phosphorylation. Glycolysis occurs in the cytosol of the cell during which glucose is converted into pyruvate. During this process, two molecules of ATP are generated per glucose molecule. Pyruvate then enters mitochondria where it is converted into acetyl-CoA which then proceeds through TCA cycle and oxidative phosphorylation to generate 36 molecules of ATP in total.

During the process of glycolysis, glucose is taken up by the cell with the help of GLUT. In the cytosol of the cell, glucose is phosphorylated to glucose 6-phosphate by HK which is then converted into fructose 6-phosphate by GPI. Phosphofructokinase 1 (PFK1) then phosphorylates fructose 6-phosphate to

fructose 1, 6-biphosphate (F1,6-BP). F1,6-BP is then converted into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by aldolase. Glyceraldehyde 3-phosphate is then converted into 1,3-bisphosphoglycerate by GAPDH, and phosphoglycerate kinase (PGK) converts 1,3-bisphosphoglycerate to 3-phosphoglycerate. In the presence of PGM , 3-phosphoglycerate is converted into 2-phosphoglycerate and enolase converts 2-phosphoglycerate into phosphoenolpyruvate (PEP). PEP is then converted into pyruvate by pyruvate kinase (PK). In normoxia, pyruvate proceeds to TCA cycle for further metabolism. However, in hypoxia, which may be seen in muscles during exercise, pyruvate is converted into lactate by LDHA.

1.2.5 Metabolism in transformed cells

Studies have shown that in cancer cells, as compared to normal cells there is an altered expression of enzymes of glucose metabolism which may favour a switch to aerobic glycolysis (Figure 1.4).

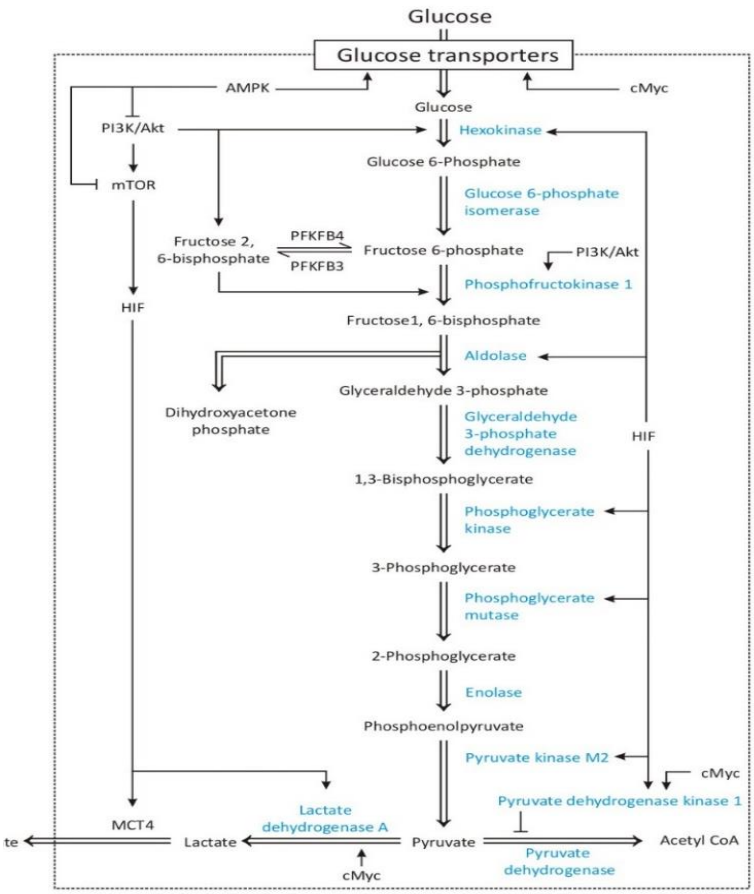


Figure 1.4 Regulation of glycolysis in transformed cells: During the process of glycolysis, glucose is taken up by the cell with the help of GLUTs. Various regulatory molecules such as hypoxia-inducible factor (HIF), *myc* and 5' adenosine monophosphate-activated protein kinase (AMPK) fine-tune the glycolytic pathway. In the cytosol of the cell, glucose is phosphorylated to glucose 6-phosphate by HK which is then converted into fructose 6-phosphate by GPI. PFK1 then phosphorylates fructose 6-phosphate to F1,6-BP. F1,6-BP is then converted into glyceraldehyde 3-phosphate and

dihydroxyacetone phosphate by aldolase. Glyceraldehyde 3-phosphate is then converted into 1,3-bisphosphoglycerate by GAPDH, and PGK converts 1,3-bisphosphoglycerate to 3-phosphoglycerate. In the presence of PGM , 3-phosphoglycerate is converted into 2-phosphoglycerate and enolase transforms 2-phosphoglycerate into PEP. PEP is then converted into pyruvate by PK. In normoxia, pyruvate proceeds to TCA cycle for further metabolism. However, in hypoxia, which may be seen in muscle during exercise, pyruvate is converted into lactate by LDH A. Lactate can be transported out of the cell by MCT4. Pyruvate generated during normoxia is carried inside the mitochondria when it is converted into acetyl CoA by the action of enzyme pyruvate dehydrogenase (PDH).

1.2.5.1 GLUTs

GLUTs are plasma membrane-associated transporters which are essential for the transport of glucose across the plasma membrane of the cells, along with its concentration gradient. Cancer cells may show upregulation of GLUT1 and downregulation of insulin-sensitive GLUT4 which can make these cells insensitive to growth signals and allow them to uptake nutrients more than what is needed to meet their bioenergetics needs (Herling 2011, Zheng 2012).

1.2.5.2 HK

HK is a key glycolytic enzyme which catalyses the conversion of glucose to glucose 6-phosphate. Phosphorylation converts non-ionic glucose into anionic glucose 6-phosphate which is trapped inside the cell (Pelicano 2006). There are four different isoforms of HK in humans. Untransformed cells express HK I whereas tumour cells preferentially express HK II (Herling 2011, Bayley 2012). HK II is also bound to mitochondria where it interacts with voltage dependent anion channels (VDAC) and inhibits binding of pro-apoptotic Bcl-2 associated X protein (BAX) to VDAC leading to suppression of apoptosis (Herling 2011). Depletion of HK II in glioblastoma cells has been shown to inhibit aerobic glycolysis, restore oxidative phosphorylation with the resultant increase in oxygen consumption along with increased sensitivity to radiation and TMZ (Wolf 2011). HK II is induced by hypoxia (Porporato 2011).

1.2.5.3 GPI

GPI catalyses the conversion of glucose 6-phosphate to fructose 6-phosphate. GPI can also function as an autocrine motility factor, and it is secreted by tumour cells to promote motility, invasion and metastasis (Funasaka 2005, Pelicano 2006, Herling 2011). GPI is overexpressed in many cancers and is also upregulated by hypoxia (Funasaka 2005, Herling 2011).

1.2.5.4 PFK1

PFK1 converts fructose 6-phosphate to F1,6-BP and is a rate-limiting enzyme of glycolysis. ATP is a potent inhibitor of PFK1 whereas F2,6-BP is a potent activator. F2,6-BP can override inhibition of PFK1 by ATP allowing for glycolysis to proceed even in the presence of adequate ATP (Marie 2011). F2,6-BP is regulated by bidirectional 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1-4 (PFKFB1-4) which can result in the formation of F2,6-BP from fructose 6-phosphate or breakdown of F2,6-BP into fructose 6-phosphate. PFKFB3 which is commonly upregulated in tumours has a prominent kinase activity resulting in increased F2,6-BP (Herling 2011). Upregulation of F2,6-BP leads to enhanced glycolysis through activation of PFK1 whereas the diminished activity of F2,6-BP, suppresses glycolysis and leads to increase shunting of glucose 6-phosphate into the PPP (Hers 1982, Yalcin 2009).

1.2.5.5 Aldolase

Aldolase catalyses the conversion of F1,6-BP to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Aldolase has been shown to be upregulated in cancers such as pancreatic ductal carcinoma whereas it may be downregulated in some other tumours such as hepatocellular carcinoma (HCC) and glioblastoma (Herling 2011, Zheng 2012).

1.2.5.6 GAPDH

GAPDH catalyses the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. During this reaction, NAD^+ is reduced to NADH. GAPDH can also bind to DNA and ribonucleic acid (RNA) and play a role in regulating a number of cellular processes such as nuclear tRNA transport, endocytosis and DNA repair and replication (Pelicano 2006). GAPDH is regulated by hypoxia and p53 (Herling 2011).

1.2.5.7 PGK

PGK catalyses the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate. During this process, a molecule of ATP is generated. PGK1 has been shown to be upregulated in patients with pancreatic ductal carcinomas (Herling 2011, Zheng 2012).

1.2.5.8 PGM

PGM catalyses the conversion of 3-phosphoglycerate to 2-phosphoglycerate. PGM is overexpressed in cancers such as lung, liver, colon and breast and negatively regulated by p53 (Herling 2011, Zheng 2012).

1.2.5.9 Enolase

Enolase catalyses the conversion of 2-phosphoglycerate to PEP. Enolase is upregulated in a number of cancers such as brain, liver, lung, breast, cervix prostate, pancreas, kidney and gastric. It has been shown to be upregulated by *myc* (Herling 2011, Zheng 2012).

1.2.5.10 PK

PK catalyses the conversion of PEP to pyruvate. During this process, adenosine diphosphate (ADP) is converted into ATP. Four isoforms of PK have been described in humans. PKM1 is found in non-transformed cells whereas cancer cells express a tumour specific pyruvate kinase M2 (PKM2). PKR is found in erythrocytes, and PKL is found in liver and kidney. PKM2 can be present in tetrameric form with high affinity for PEP or a dimeric form with a low affinity for PEP. Hence depending upon the needs of cancer cells, PKM2 can drive the glycolysis forward to generate ATP or slow it down leading to shunting of glycolytic substrates into PPP (Dombrauckas 2005, Herling 2011).

1.2.5.11 LDH

LDHA catalyses the conversion of pyruvate to lactate and in the process, generates NAD^+ from NADH which is essential to maintain the glycolytic flux. Many tumours preferentially overexpress LDHA which is a target of HIF and *myc* (Herling 2011, Zheng 2012). Inhibition of LDHA has been shown to force cells to revert to oxidative phosphorylation leading to impaired cellular proliferation (Fantin 2006).

1.2.5.12 Pyruvate dehydrogenase

Pyruvate dehydrogenase (PDH) catalyses the conversion of pyruvate into acetyl-CoA which then undergoes oxidative phosphorylation.

1.2.5.13 Pyruvate dehydrogenase kinase 1

Pyruvate dehydrogenase kinase 1 (PDK1) inhibits PDH thereby allowing pyruvate to be converted into lactate and NAD^+, which allows glycolysis to continue. Inhibition of PDH by PDK1 also prevents oxidative stress as a result of ROS generation in the respiratory chain of the mitochondria (Porporato 2011). PDK1 is upregulated by HIF (Porporato 2011), and its increased expression correlates with poorer outcome in head and neck cancer (Wigfield 2008).

1.2.5.14 MCT transporter

MCT4 which transports lactate out of the cell and MCT1 which is involved in lactate uptake by the cells is upregulated in certain cancers (Herling 2011). MCT4 is a target of hypoxia inducible factor-1 (HIF-1).

1.2.6 Lactate

Pyruvate, the end product of glycolysis is converted into lactate by LDHA. During this process, NADH is converted into NAD^+ which is essential for glycolysis to continue. Cancer cells transport lactate and H^+ to the extracellular space via MCT and Na^+/H exchangers leading to acidic microenvironment and slightly alkaline cytosol. Cancer cells have been shown to upregulate MCT (Gatenby 2004) because if lactate was overproduced as compared to its secretion, then low intracellular pH would inhibit PFK1 and glycolysis (Erecińska 1995).

Lactate has also been shown to stabilise HIF-1 in glioma cell lines in normoxia and reinforce the Warburg effect (Lu 2002). Low pH can also induce changes in the chromosomes by inhibiting DNA repair mechanisms, thereby promoting transformation (Morita 1992, Gatenby 2004). The acidic tumour microenvironment also promotes proliferation, migration, invasion of cancer cells and help cells to evade apoptosis. Lactate has been shown to play an essential role in the immune escape of tumour cells (Gottfried 2006, Hirschhaeuser 2011). Lactate has also been shown to stimulate vascular endothelial growth factor (VEGF) production by endothelial cells leading to endothelial cell migration and angiogenesis (Beckert 2006). Data from studies also suggest that lactate promote expression of matrix metalloproteinase-2 (MMP-2), hyaluronidase and lysosomal proteases which in-turn encourage migration (Stern 2002, Baumann 2009).

Export of lactate outside the cell results in slightly alkaline intracellular pH, which has been shown to promote cell proliferation and inhibit caspases which require an acidic pH for activation (Webb 2011, Zheng 2012). A number of drugs such as doxorubicin and vincristine are weak bases; thus, by maintaining an intracellular alkaline pH, drug uptake is also impaired (Webb 2011, Zheng 2012).

Lactate is also known to scavenge free radicals and hence may play a role in resistance to radiotherapy and some chemotherapy drugs which act by generating ROS (Groussard 1985, Hirschhaeuser 2011).

1.2.7 Lipid metabolism

Fatty acids play an essential role in cell growth, proliferation, survival and signalling as they can be used for energy production and membrane biosynthesis as well as to modify membrane-bound proteins. Untransformed cells take up low density lipoproteins (LDL), and free fatty acid from the bloodstream and *de novo* synthesis of fatty acid and cholesterols is limited to only a few tissues such as liver, adipose tissue and lactating breast. In contrast, majority of fatty acid in cancer cells is derived from *de novo* fatty acid synthesis (Menendez 2007), and most of the carbon for fatty acid synthesis is derived from glucose (Ookhtens 1984, Deberardinis[b] 2008). Glucose is converted into pyruvate which is then acted upon by PDH in the mitochondrial matrix to form acetyl CoA. Acetyl CoA combines with oxaloacetic acid (OAA) to form citrate. Under conditions of high ATP/ADP and NADH/NAD^+ ratio which is found in most proliferating cells, citrate is transported out of the cell and acted upon by ATP citrate lyase (ACL) to form acetyl CoA and oxaloacetate. Acetyl CoA is converted into malonyl CoA by acetyl CoA carboxylase (ACC) which is then acted upon by fatty acid synthase (FAS) to form fatty acids (Vander Heiden 2009). OAA is used for the synthesis of NEAA (Marie 2011). ACL reinforces the Warburg effect as it breaks down citrate in the cytoplasm which is an allosteric inhibitor of glycolysis (Deberardinis[b] 2008).

Ongoing fatty acid synthesis generates malonyl CoA which inhibits carnitine palmitoyl transferase (CPT) 1 which is involved in fatty acid oxidation (FAO). FAO leads to ROS generation, and it may be that cancer cells show low FAO to evade ROS mediated cell death (Ferreira 2010).

Increased lipogenesis is seen in a number of cancers (Herling 2011, Ru 2013). Tumour cells show increased expression of enzymes involved in fatty acid synthesis such as ACL, ACC and FAS, all of which have been targeted for cancer therapy (Kuhajda 1994, Brusselmans 2005, Hatzivassiliou 2005, Ru 2013). FAS is overexpressed in gliomas, and its expression correlates with histological grade (Grube 2014).

1.2.8 PPP

At times, depending upon the need of the cell and redox status, glucose 6-phosphate may be diverted into the PPP instead of proceeding through glycolysis. During the oxidative phase of PPP, glucose 6-

phosphate is sequentially converted into ribulose 5-phosphate and two molecules of NADPH. The first step of this reaction is catalysed by glucose 6-phosphate dehydrogenase (G6PD). During the non-oxidative phase of PPP, three molecules of ribulose 5-phosphate is converted into two molecules of fructose 6-phosphate and one molecule of glyceraldehyde 3-phosphate. Besides xylulose 5-phosphate can also be converted into glyceraldehyde -3-phosphate by transketolase like enzyme (TKTL)1 which can then undergo further glycolysis (Wamelink 2008). PPP generates NADPH which plays an essential role in defence against oxidative stress (Ahmad 2005, Deberardinis 2008, Marie 2011).

Increased flux through the PPP, overexpression of G6PD and TKTL1 is seen in many tumours. G6PD has been shown to play a critical role in survival, proliferation, and metastasis of some cancer cells (Zhang 2014, Herling 2011) and G6PD or TKTL1 known-down has been shown to inhibit tumour growth (Herling 2011).

1.2.9 Positron emission tomography imaging

2-Deoxy-2-(^{18}F) fluoro-D-glucose positron emission tomography (FDG-PET) imaging is based on the fact that cancer cells show increased glucose uptake as compared to normal cells. FDG is taken up by the cells via GLUT1 and is phosphorylated by HK to FDG 6-phosphate. While glucose 6-phosphate can either be acted upon by enzymes to form fructose 6-phosphate via glycolytic cycle or to 6-phosphogluconolactone via PPP, FDG 6-phosphate cannot be further metabolized due to lack of an oxygen atom at the C2 position. FDG 6-phosphate is hence trapped inside the cell and rate of accumulation depends upon the activity of GLUTs and HK which provides a mean to assess glucose uptake by the cell (Gambhir 2002, Kelloff 2005). Cancer cells which consume glucose at a faster rate as determined by PET have been shown to have a poorer prognosis as compared to cancer which takes up less glucose (Kunkel 2003, López-Ríos 2007).

1.2.10 Conclusion

Gradually a molecular basis of the Warburg effect is emerging (Shaw 2006). Knowledge about cellular signalling pathways and genetics gathered over last few decades is being integrated with both existing as well as new understanding about cell metabolism to create a complete picture (Bayley 2012).

However, one has to recognise that the Warburg effect is not a feature of all cancer cells and even in glycolytic cancer cells, oxidative phosphorylation is not entirely redundant (Moreno-Sánchez 2007, Cairns 2011). Studies have shown that rather than being impaired, the mitochondrial function may be

reprogrammed to meet the biosynthetic needs of rapidly proliferating cells (Weinhouse 1976, Fantin 2006, Moreno-Sánchez 2007, Ward 2012) and such cells can utilise fatty acids, lactate and amino acids as fuel for their energetic needs (Cairns 2011). Besides, rapidly proliferating normal cells such as lymphocytes also use aerobic glycolysis to meet their energetic and biosynthetic needs (Fox 2005, Deberadinis[a] 2008). One also has to remember that not all the tumours show a specific metabolic pattern and there may be several different metabolic profiles which vary from tumour to tumour, which can make targeting of tumour metabolism difficult.

Hence one should regard the Warburg effect as a positive modifier of cancer. Though it does not lead to tumorigenesis, it facilitates tumour progression (Kim 2006) and further studies into the complex relationship between oncogenic signalling pathways and altered tumour metabolism is needed.

1.3 Targeting tumour metabolism

1.3.1 Why target deranged glucose metabolism of tumour cells

Metabolism has already been successfully targeted by 5-fluorouracil (5-FU), methotrexate, gemcitabine and fludarabine which inhibit nucleotide biosynthesis (Vander Heiden 2011). Even though cancer cells and rapidly proliferating normal cells share the same metabolic profile, an essential difference between them is the fact that normal proliferating cells, unlike tumour cells do not exhibit activation of oncogene or loss of tumour suppressor genes (Dang 2011). Normal proliferating cells also possess cell cycle checkpoints which may be a reason that such cells are resistant to antimetabolites (Vander Heiden 2011). This points to the fact that metabolic differences must exist between the cancer cells and normal proliferating cells (Vander Heiden 2011). Antibiotics in routine use have also been shown to target the metabolism of micro-organisms without affecting normal cells, which provides further proof that deranged metabolism can be selectively targeted (Vander Heiden 2011).

Efforts have been made to target genetic alterations seen in cancer cells. However, mutations affecting cancer can be very heterogeneous. Different mutations are seen in different cancers, and even the same cancer in two different organisms can have different mutations. Cells within a cancer can also exhibit different mutations. As a result of this, targeting tumour specific mutation poses a significant challenge (Pelicano 2006). As discussed earlier, upregulated aerobic glycolysis in cancer cells not only plays a vital role in meeting the bioenergetics and biosynthetic needs of the cell, it also helps in survival. Hence, in theory, targeting glycolysis will abolish the survival advantages conferred to cancer cells and lead to cell death. However numerous studies have shown that though inhibition

of glycolysis is well tolerated by normal tissues, it has limited efficacy as monotherapy in cancer cells (Tennant 2010). This may be due to the fact that altered metabolism seen in cancer cells can be very heterogeneous and hence poses a challenge in targeting. Poor efficacy of glycolysis inhibition may also be due to the plasticity of tumour metabolism, and maybe inhibition of single enzyme can lead to activation of alternate pathways and resistance to treatment (Vander Heiden 2011). However, there may be some situations where glycolytic inhibitors as monotherapy may be effective. Mitochondrial defects and hypoxia have been shown to be associated with resistance to conventional chemotherapy and radiotherapy in cancer cells. Glycolytic inhibitors have been shown to be particularly effective against such cancer cells as mitochondrial oxidative phosphorylation is downregulated either due to a defect in mitochondria or due to lack of oxygen (Pelicano 2006).

Some studies have shown that there may be an underlying defect in the mitochondria of cancer cells. As a result of it, if increased substrates are shunted through the mitochondria of cancer cells, then it will lead to increased ROS generation, oxidative stress and even cell death (Eakin 2007).

Hence altered metabolism in tumour cells provides multiple therapeutic targets such as suppression of glycolysis, promoting oxidative phosphorylation, simultaneous suppression of glycolysis and mitochondria, suppression of glycolysis and promoting oxidative phosphorylation and targeting upstream regulators of metabolic pathways.

1.3.2 Targeting glycolysis

1.3.2.1 GLUT inhibitors

GLUT1 inhibitors

WZB117, a small molecule inhibitor of GLUT1 has been shown to inhibit the growth of human lung cancer cells *in vitro* and in animal models (Qian 2014). Apigenin, a natural flavonoid has also been shown to inhibit GLUT1 and suppress pancreatic cancer cell growth *in vitro* (Qian 2014).

GLUT2 inhibitors

Phloretin a natural flavonoid has been shown to competitively inhibit GLUT2 and suppress tumour growth in breast and bladder cancer cells (Nelson 1993). It has also been shown to induce apoptosis in leukaemia, melanoma and colon cancer cell lines (Tennant 2010, Qian 2014).

GLUT3 inhibitors

A number of conventional chemotherapy agents such as etoposide and Adriamycin have been shown to induce cell death in HeLa cell line by inhibiting GLUT3 expression besides acting at other targets (Qian 2014).

GLUT4 inhibitors

Protease inhibitor ritonavir has been shown to inhibit GLUT4 and suppress the growth of myeloma, lung and breast cancer cells (Qian 2014). Silibinin, a natural flavonoid has also been shown to inhibit GLUT4 and suppress the growth of hepatoma and cervical cancer cells (García-Maceira 2009).

1.3.2.2 Targeting HK

1.3.2.2.1 2-Deoxyglucose

2-Deoxyglucose (2DG) contains a hydrogen group instead of a hydroxyl group at carbon position 2 of the glucose ring. After being phosphorylated by HK to 2-deoxyglucose 6-phosphate, it cannot be further metabolised by GPI and is trapped within the cell. 2DG has been shown to non-competitively inhibit HK and competitively inhibit GPI. 2DG treatment can lead to ATP depletion, cell cycle arrest, growth inhibition and even cell death. Cancer cells in hypoxia or cells with mitochondrial defects are more sensitive to 2DG as oxidative phosphorylation is not active in these cells (Pelicano 2006).

Studies have shown that 2DG can also induce oxidative stress. Increased glycolysis seen in cancer cell leads to increased flux through PPP leading to increased production of NADPH. Similarly, as a result of upregulated glycolysis, increased pyruvate is formed which can also act as a free radical scavenger. However, 2DG after conversion to 2-deoxyglucose 6-phosphate can only undergo the first step of PPP generating one molecule of NADPH per cycle as compared to glucose which generates two molecules of NADPH per cycle. 2DG mediated block in glycolysis also results in decreased pyruvate generation, which also serves to promote oxidative stress (Zhang 2014).

2DG has also been shown to affect protein glycosylation, leading to the accumulation of unfolded and misfolded proteins in the endoplasmic reticulum (ER) (Kang 2006, Kurtoglu 2007). Mannose plays an essential role in N-linked glycosylation which occurs in the lumen of the ER. As a result of structural similarity, 2DG completes with mannose and inhibits N-linked glycosylation. This leads to an accumulation of unfolded protein in the ER which triggers unfolded protein response (UPR). Uncontrolled UPR can promote cell death (Zhang 2014).

2DG mediated ATP depletion can also lead to autophagy induction. 2DG mediated UPR can also play a role in activating autophagy (Zhang 2014).

2DG has shown to be synergistic with radiotherapy and a wide variety of chemotherapy drugs in various cancer both *in vitro* as well as *in vivo*.

- A phase I/II trial of 2DG in combination with radiotherapy in patients with GBM showed that it was well tolerated (Mohanti 1996)
- 2DG has been shown to synergise with Adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancer xenograft models (Maschek 2004)
- 2DG has been shown to synergise with histone deacetylase inhibitor to induce apoptosis in glioma, breast and cervical cancer cell lines (Egler 2008)
- Combination treatment of 2DG and rapamycin analogue CCI-779, has been shown to lead to increased cell killing in lung cancer cell lines in hypoxia (Wangpaichitr 2008)
- Electron transport chain (ETC) blockers such as rotenone and antimycin A have been shown to potentiate 2DG mediated oxidative stress and cell death in colon cancer cells *in vitro* as well as in animal models (Fath 2009)
- 2 DG has been shown to potentiate breast cancer cell killing induced by paclitaxel (Hadzic 2010)
- 2DG has been shown to be safe in patients with cervical, nasopharyngeal, non-small cell lung cancer and advanced castrate-resistant prostate cancer (Stein 2010)
- 2DG when combined with efrapeptins which are known inhibitors of F(1)F(0)-ATPase, led to synergistic growth inhibition in breast cancer cells *in vitro* and *in vivo* (Papathanassiu 2010)
- Combination treatment of 2DG and ABT-737/263 has been shown to lead to synergistic cell killing in leukaemia, small cell lung cancer, cervical, prostate, breast and HCC cell lines (Yamaguchi 2011)
- Dual inhibition of tumour energy pathway by 2DG and metformin has been shown to be effective against human gastric and oesophageal cancer cell lines (Cheong 2011)
- 2DG has also been shown to synergise with etoposide in lymphoma xenograft models (Bénéteau 2012)
- 2-DG can potentiate the cytotoxic effects of cisplatin and radiotherapy in anaplastic thyroid carcinoma (Sandulache 2012)
- 2DG in combination with drugs which target mitochondria such as mito-CP and mito-Q has been shown to lead to breast cancer regression in xenograft models (Cheng 2012)

- 2DG with trastuzumab has been shown to be synergistic in breast cancer cells *in vitro* and *in vivo*. 2DG with prednisolone has also been shown to be synergistic in leukaemia cells *in vitro* (Zhao 2013)
- Combination treatment of 2DG and ATP synthase inhibitor oligomycin has been shown to lead to synergistic cell killing in glioma cells (Kennedy 2013)
- Phase I study of 2DG either alone or in combination with docetaxel, in patients with advanced solid tumours has shown that recommended dose of 2DG in combination with weekly docetaxel was 63 mg/kg/day with tolerable adverse effects (Raez 2013)
- 2DG has been shown to synergise with cisplatin or ABT-737 in neuroblastoma cell lines (Chuang 2013)
- 2DG has been shown to inhibit breast CSC proliferation when used alone, and it showed a synergic effect when used in combination with doxorubicin (Ciavardelli 2014)
- 2DG has been shown to be synergistic with microtubule disruptor 2-methoxyestradiol-3,17-O, O-bis-sulfamate in breast and prostate cancer (Granchi 2014)
- 2DG has been shown to be synergistic with metformin, gemcitabine, doxorubicin and celecoxib in inhibiting pancreatic cancer cell proliferation (Cheng 2014)
- Combination treatment of 2DG with staurosporine or TMZ has been shown to be synergistic in metastatic melanoma cell lines (Zhang 2014)

However, one has to remember that the effectiveness of 2DG depends upon the presence of glucose and high glucose in the microenvironment may negate the effect of 2DG (Maschek 2004, Pelicano 2006, Scatena 2008). 2DG has also been shown to induce the expression of p-glycoprotein coded by multi-drug resistance (MDR) gene and thus may play a role in resistance to chemotherapy in the cancer cell (Ledoux 2003). Low efficacy of monotherapy with 2DG may also be related to its off-target effects like activation of phosphoinositide 3-kinase (PI3K) pathway or induction of pro-survival autophagy (Xi 2011).

1.3.2.2.2 Lonidamine

Lonidamine an indazole 3-carboxylic acid derivative has been shown to inhibit HK bound to mitochondria (Zhao 2011). A number of clinical trials of lonidamine with other anticancer agents in the treatment of glioblastoma, ovary and lung cancer have been performed with some encouraging results (Di Cosimo 2003, Pelicano 2006). However, some other trials with lonidamine had to be terminated because of liver toxicity (Qian 2014).

1.3.2.2.3 3-Bromopyruvate

3-Bromopyruvate (3-BP) is a bromo-halogenated derivative of pyruvate has been shown to inhibit HK II by alkylation of its sulfhydryl group, leading to ATP depletion, BAD dephosphorylation, translocation of BAX to mitochondria and release of cytochrome c resulting in apoptosis (Xu 2005, Madhok 2011). 3-BP has been shown to be effective against liver and breast cancer xenograft models (Ko 2004, Xu 2005).

1.3.2.3 Targeting GPI

siRNA mediated downregulation of GPI has been shown to induce oxidative stress and reduce proliferation in fibrosarcoma cells (Madhok 2011).

1.3.2.4 Targeting PFKFB3

3PO {3-(3-Pyridinyl)-1-(4-pyridinyl)-2-propen-1-one} has been shown to inhibit PFKFB3 which in turn inhibits PFK1 leading to decreased glucose uptake and glycolysis inhibition. 3PO has been shown to inhibit leukaemia, breast and lung cancer cell growth *in vitro* and *in vivo* (Clem 2008). YZ9, a small molecule inhibitor of PFKFB3 has been shown to inhibit the proliferation of lung and cervical cancer cell *in vitro* (Qian 2014).

1.3.2.5 Targeting aldolase

3-deoxy or 3-fluro-D-glucose and 4-deoxy or 4-fluro-D-glucose have been shown to inhibit aldolase and inhibit renal, uterine and lung tumour growth (Pathania 2009).

1.3.2.6 Targeting triosephosphate isomerase

2-Carboxyethylphosphonic acid, N-hydroxy-4-phosphono-butanamide, 2-phosphoglyceric acid and ornidazole have been shown to inhibit triosephosphate isomerase (TPI) and inhibit tumour growth (Pathania 2009).

1.3.2.7 Targeting GAPDH

siRNA mediated downregulation of GAPDH has been shown to sensitise resistant leukemic cells to imatinib and prednisolone (Madhok 2011). Ornidazole, iodoacetate, α- chlorohydrin and koningic acid have also been shown to inhibit GAPDH (Scatena 2008, Pathania 2009). 3-BP was initially thought to inhibit HK II, however it has recently been shown to primarily act by inhibiting GAPDH (Xu 2005, Ganapathy-Kanniappan 2010).

1.3.2.8 Targeting PGM

PGM1 inhibitor PGM1-004A has been shown to inhibit proliferation of human non-small cell lung carcinoma cells (Granchi 2014). MJE3, 3-phosphoglyceric acid and benzene hexacarboxylic acid have also been shown to inhibit PGM (Scatena 2008, Pathania 2009).

1.3.2.9 Targeting enolase

Increased expression of alpha-enolase isoform 1 (ENO1) has been seen in a number of cancers. However, ENO1 is deleted in GBM cells and another isoform of enolase ENO2 is expressed. siRNA mediated downregulation of ENO2 has been shown to inhibit glioma cell growth in cells lacking ENO1 (Granchi 2014). Similarly, siRNA mediated downregulation of ENO1 has been shown to sensitise breast cancer cells to tamoxifen even in cells which were earlier resistant to the drug (Madhok 2011).

1.3.2.10 Targeting PKM2

Studies have shown that either inhibiting or activating PKM2 can lead to the inhibition of tumour growth (Anastasiou 2012).

The small molecule TEPP-46 {thieno[3,2-b] pyrrole[3,2-d] pyridazinone} and DASA-58 {N, N'-diarylsulfonamide have been shown to activate PKM2 leading to inhibition of growth in non-small cell lung cancer xenograft model (Anastasiou 2012). On the other hand, siRNA mediated downregulation of PKM2 has also been shown to induce apoptosis in liver and ovarian cancer cell lines (Goldberg 2012). TLN-232 is a synthetic cyclic heptapeptide which has been shown to target PKM2, and some encouraging results have been obtained in a phase II trial of TLN-232 in patients with refractory metastatic renal cell carcinoma (RCC) (Pathania 2009, Zhao 2011, Porporato 2011).

1.3.2.11 Targeting PDK1

siRNA mediated downregulation of PDK1 has been shown to reduce tumour growth *in vitro* and in animal models of head and neck squamous cell cancer (McFate 2008). Dichloroacetic acid (DCA), an inhibitor of PDK1, is approved for the treatment of lactic acidosis in genetic mitochondrial disease (Stacpoole 2008). DCA has been shown to inhibit glioma cell growth (Duan 2013) as well as sensitise glioma cells to radiotherapy *in vitro* as well as in animal models (Shen 2015). DCA has also been used in patients with glioblastoma and shown to inhibit HIF-1, promote p53 activation and suppress angiogenesis resulting in decreased tumour growth in 3 out of 5 patients (Michelakis 2010). DCA has also been shown to inhibit the growth of cervical, breast, endometrial, non-small cell lung cancer and prostate cancer cells *in vitro* (Michelakis 2008, Qian 2014).

1.3.2.12 Targeting lactate metabolism

Downregulation of LDHA by siRNA has been shown to lead to a rise in NADH/NAD^+ ratio, which in turn leads to glycolysis inhibition. This also promotes increased ROS generation from ETC1 leading to oxidative stress and has been shown to be effective in lymphoma and pancreatic cancer xenograft models (Le 2010). FX11 [3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid] has also been shown to inhibit LDHA and promote cell death in prostate cancer cell lines (Xian 2015). FX11 has also been shown to be synergistic with FK866, another LDHA inhibitor, in lymphoma cell lines (Zhao 2013).

1.3.3 Targeting tumour acidity

Dietary measures which result in increased bicarbonate level in the blood has been shown to inhibit tumour growth and metastasis by counteracting the acidic tumour microenvironment (Porporato 2011).

MCT1 inhibitors have been shown to inhibit the growth of neuroblastoma and lung cancer cells (Jones 2012). Alpha-cyano-4-hydroxycinnamate (CHC) has been shown to suppress MCT1 and inhibit tumour growth. AZD3965, a small molecule inhibitor of MCT1 is under trial for advanced solid tumours and lymphoma (Porporato 2011, Granchi 2014).

1.3.4 Targeting the PPP

Thiamine analogue, oxythiamine has been shown to inhibit transketolase enzyme and reduce tumour growth in lung and colon cancer xenografts (Pelicano 2006, Thomas 2008). 6-Aminonicotinamide (6-AN) has been shown to inhibit G6PD and induce oxidative stress in cancer cells (Pelicano 2006). 6-AN has also been shown to sensitise tumour cells to cisplatin (Budihardjo 1998). Combination of 6-AN and 2DG has been shown to sensitise glioma cells to radiotherapy (Varshney 2005) (Figure 1.5)

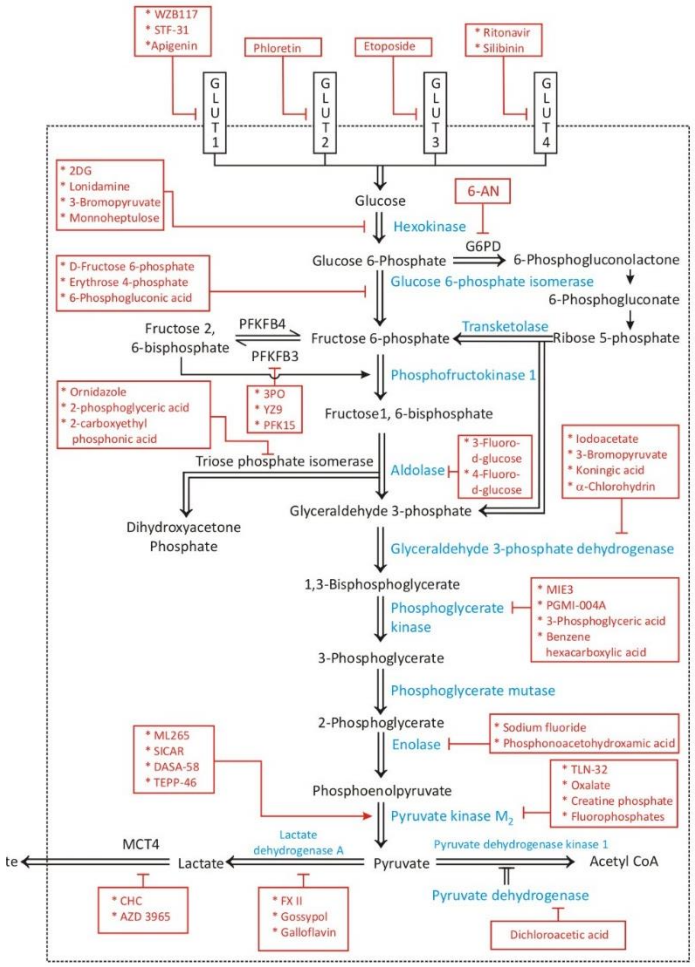


Figure 1.5 Targeting glycolysis for cancer therapy

1.3.5 Targeting nucleic acid metabolism

5-FU mainly acts as a thymidylate synthase inhibitor leading to decreased levels of thymidine in the cell which is essential for DNA replication**.** 5-FU has been used in breast, oesophageal, gastric, pancreatic, anal and skin cancer.

Methotrexate has been shown to competitively inhibit dihydrofolate reductase (DHFR), an enzyme that participates in the synthesis of tetrahydrofolate which is required DNA and RNA synthesis. Either

alone or in combination, methotrexate is used in the treatment of lymphoma, leukaemia, trophoblastic tumour, breast, lung, head & neck and bladder cancer.

1.3.6 Targeting lipid metabolism

C75, a small molecule inhibitor of FAS has been shown to exert significant anti-tumour effects against mesothelioma, breast, prostate and ovarian cancer cell lines (Kuhajda 2006). C75 has also been shown to exert anti-tumour activity in animal models of breast, endometrial and colorectal cancer (Tennant 2010). FAS inhibitor cerulenin has been shown to be synergistic with docetaxel in breast cancer cell lines. FAS inhibitor orlistat has been shown to be synergistic with gemcitabine in pancreatic cancer in vitro (Zhao 2013).

ACL inhibitor SB-204990 has been shown to inhibit fatty acid synthesis and tumour growth both *in vitro* and in animal models (Tennant 2010).

Etomoxir has been shown to inhibit carnitine palmitoyl transferase 1 (CPT1) leading to inhibition of fatty acid import into the mitochondria which in turn leads to decreased ATP generation and has been shown to inhibit the growth of GBM and acute myeloid leukaemia (AML) cells (Galluzzi 2013).

1.3.7 Targeting Amino acid metabolism

L-Asparaginase is used to treat paediatric acute lymphoblastic leukaemia (ALL). Usually, asparagine is not an essential amino acid due to the presence of the enzyme asparagine synthetase in cells. However certain tumours such as paediatric ALL have very little enzyme activity and hence depend upon the uptake of asparagine from the blood. L-asparaginase deaminates asparagine to aspartic acid and thus reduces the availability of asparagine to the tumour cells. L-asparaginase has also been shown to reduce plasma glutamine level (Tennant 2010, Vander Heiden 2011).

1.3.8 Conclusion

In the last decade, rapid advancement has been made in targeting deranged tumour metabolism. However, there are still many questions unanswered and pathways unexplored. The relationship of whole-body metabolism which is often disturbed in cancer patients, to cancer cell metabolism is still unknown (Vander Heiden 2013). Glucose and glutamine provide carbon, nitrogen, hydrogen to a growing cell but do not meet all the needs of a growing cell and other elements such that sulphur and

phosphorus are also needed. Study of pathways regulating these is also important for better targeting of metabolism (Dang 2011). Also, a better understanding of how flux through different pathways are regulated is needed, and more work in measuring metabolism needs to be done so that it is easier to quantify the effect of drugs on tumour metabolism (Vander Heiden 2013).

1.4 Mitochondria in cancer

1.4.1 Introduction

The mitochondrion is a double membrane-bound intracellular structure. It consists of an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM). The intermembrane space is the space between the outer and inner membranes whereas the cristae are formed by infoldings of the inner membrane. The mitochondrial matrix is the space within the inner membrane. Cytochrome c is present in the intermembrane space whereas enzymes of oxidative phosphorylation and ATP synthase are located on the IMM (Bruce 1994). As well as playing a central role in metabolism, mitochondria also play an essential role in regulating apoptosis and redox status of the cell, providing intermediates for anabolic processes, signalling via ROS and calcium homeostasis (Gogvadze 2010, Wallace 2012). Mitochondria contain a double-stranded DNA of 16569 base pairs which code for 37 genes including 13 genes of the ETC (Anderson 1981). mtDNA as compared to nuclear DNA is more prone to oxidative stress as it lacks histones and is closer to the ETC which is a major source of ROS in the cell (Brandon 2006, Bonora 2006). mtDNA is essential for cancer cells and loss of mtDNA has been shown to lead to reduced tumorigenicity, inability of cancer cells to grow in an anchorage-independent fashion and increased sensitivity to cytotoxic drugs (Cavalli 1997, Wallace 2012).

1.4.2 TCA / Krebs cycle

Pyruvate, the end product of glycolysis is decarboxylated by enzyme PDH to form acetyl CoA. Acetyl CoA undergoes aldol condensation with oxaloacetate in the presence of enzyme citrate synthase to form citrate. Citrate then undergoes dehydration and hydration reactions in the presence of aconitase to form isocitrate. Isocitrate then undergoes oxidative decarboxylation in the presence of IDH to form α-Ketoglutarate (α-KG) and NADH. α-KG then undergoes oxidative decarboxylation in the presence of α-KG dehydrogenase to form succinyl CoA and NADH. Succinyl CoA is converted into succinate by substrate-level phosphorylation. Succinate then undergoes oxidation in the presence of succinate dehydrogenase (SDH) to form fumarate and $FADH_2$. Fumarate undergoes hydration reaction in the

presence of enzyme fumarate hydratase (FH) to form malate. Malate undergoes oxidation in the presence of malate dehydrogenase to generate oxaloacetate and completes the cycle.

TCA cycle intermediates play a role in the synthesis of NEAA such as glutamate, aspartate, proline and arginine. Acetyl CoA also plays a vital role in the fatty acid synthesis and through post-translational acetylation reactions, it also plays an important role in signalling and epigenetic modifications (Figure 1.6).

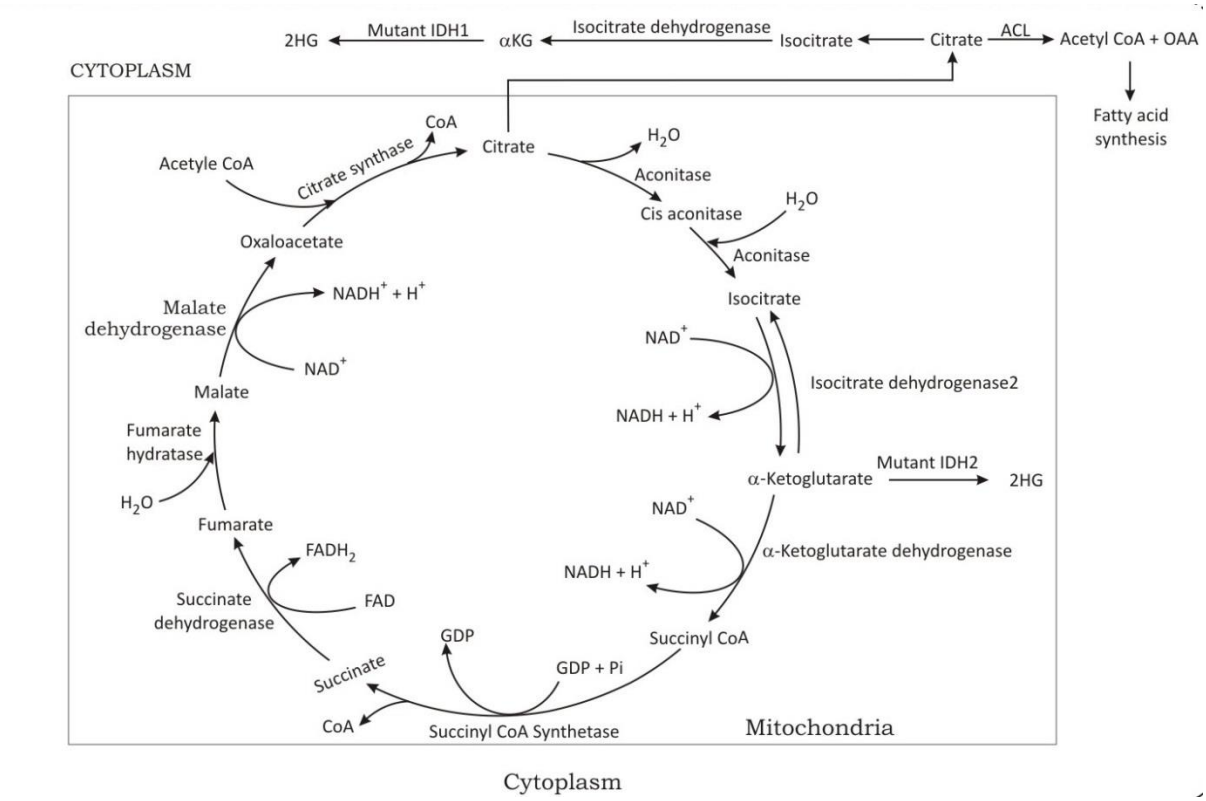


Figure 1.6 TCA or citric acid cycle: Glucose taken up by the cells is converted into pyruvate through glycolysis. Pyruvate is then decarboxylated by enzyme PDH to form acetyl CoA which undergoes aldol condensation with oxaloacetate in the presence of enzyme citrate synthase to form citrate. Citrate then undergoes dehydration and hydration reaction in the presence of aconitase to form isocitrate. Isocitrate then undergoes oxidative decarboxylation in the presence of IDH to form α-KG and NADH. α-KG then undergoes oxidative decarboxylation in the presence of α-KG dehydrogenase to form succinyl CoA and NADH. Succinyl CoA then undergoes substrate level phosphorylation to form succinate and guanosine triphosphate (GTP). Succinate then undergoes oxidation in the presence of SDH to form fumarate and $FADH_2$. Fumarate then undergoes hydration reaction in the presence of enzyme FH to form malate. Malate undergoes oxidation in the presence of malate dehydrogenase to generate oxaloacetate and NADH to complete the cycle. During the TCA cycle, high energy molecule NADH and $FADH_2$ are also generated which participate in ETC to generate ATP. Citrate from TCA cycle of the mitochondria can move into the cytoplasm and can be broken down in acetyl CoA and OAA by ACL. Acetyl CoA is converted into malonyl CoA by ACC, and malonyl CoA can then be acted upon by FAS to generate fatty acids. Cytoplasmic IDH1 and mitochondrial IDH2 convert isocitrate to α-KG. A heterozygous point mutation in IDH1 /IDH2, seen in gliomas, AML and chondromas can lead to a neomorphic enzymatic activity which converts α-KG to 2-hydroxyglutarate (2HG) utilising NADPH.

1.4.3 ETC

NADH is oxidised to NAD^+ by complex I of the ETC and complex II oxidises FADH2 to FAD. The electrons generated are transferred to ubiquinol and then transferred to complex III. Complexes III then transfers the electrons to cytochrome c. Electrons are finally carried to complex IV where molecular oxygen acts as the final electron acceptor and is converted into water. Each complex uses the energy of electron transfer to pump H^+ into the intermembrane space to generate an electrochemical gradient. ATP synthase uses the energy of this electrochemical gradient to synthesise ATP from ADP and inorganic phosphate (Pathania 2009). A small amount of ATP is generated by substrate-level phosphorylation during glycolysis and TCA cycle.

1.4.4 Mutations in TCA cycle enzymes

SDH catalyzes the conversion of succinate to fumarate and also acts as complex II of the ETC. FH catalyzes the conversion of fumarate to malate. SDH and FH mutations result in a buildup of succinate and fumarate in the cell. Germline mutations in genes which codes for SDH can lead to hereditary paragangliomas and pheochromocytomas (Astuti 2001). On the other hand, mutations in genes encoding FH can predispose to uterine and skin leiomyomas and papillary renal cell cancer (Tomlinson 2002).

Cytoplasmic IDH1 and mitochondrial IDH2 convert isocitrate to α-KG. A heterozygous point mutation in IDH1 /IDH2 is seen in gliomas, AML and chondromas and it can lead to a neomorphic enzymatic activity which converts α-KG to 2-hydroxyglutarate (2HG) utilising NADPH (Dang 2009, Ward 2010, Wallace 2012).

SDH/FH/ IDH mutation can affect ten-eleven translocation (TET) family of 5-methylcytosine hydroxylases which can result in an alteration in DNA and histone methylation and lead to epigenetic modulation of gene functions (Figueroa 2010, Xiao 2012). Succinate, fumarate and 2HG also target α-KG dependent enzymes including prolyl hydroxylase which is involved in HIF-1 degradation, resulting in HIF-1 stabilisation (Figueroa 2010, Kurelac 2011).

1.4.5 Anaplerosis

Movement of citrate outside the mitochondria for fatty acid synthesis depletes the mitochondria of oxaloacetate. Glutamine through a process called anaplerosis is converted into glutamate and then α-KG, to contribute to the TCA cycle oxaloacetate (Deberardinis 2007).

1.4.6 Mitochondria and apoptosis

Mitochondria play an essential role in programmed cell death called apoptosis and evasion of apoptosis is a hallmark of cancer cells (Hanahan 2000). Extrinsic apoptosis is mediated by death receptors whereas intrinsic apoptosis is mediated by mitochondria. Intrinsic apoptosis is activated as a result of cellular stresses such as oxidative stress, growth factor deprivation and DNA damage. Mitochondria-mediated intrinsic apoptosis is an important mechanism to ensure that cells with damaged and mutated DNA do not proceed through replication and propagate the defect which can promote tumorigenesis.

Mitochondrial outer membrane permeabilization (MOMP) is considered a critical point in the intrinsic pathway as it irreversibly commits a cell to apoptosis. MOMP leads to the release of cytochrome c and other apoptogenic proteins such as SMAC/Diablo and apoptosis inducing factor (AIF) in the cytosol (Gogvadze 2006).

The Bcl-2 family of proteins plays an important role in regulating MOMP and intrinsic apoptosis. It consists of pro-apoptotic as well as anti-apoptotic proteins and is characterised by the presence of Bcl-2 homology (BH) domains. Bcl-2 family proteins contain 1-4 BH domains. Pro-apoptotic proteins consist of either effector proteins or BH3 only proteins. Effector proteins such as BAX or BAK contain 3 BH domains and induce MOMP. On the other hand, BH3 only proteins promote apoptosis by sequestering anti-apoptotic Bcl-2 proteins and freeing up BAX/BAK (Willis 2005). Anti-apoptotic Bcl-2 proteins consist of 4 BH domains and include Bcl-2, Bcl-w, Bcl-xl and Mcl-1 (Youle 2008, Gogvadze 2010). They interact with BAX or BAK and prevent their oligomerisation (Gogvadze 2010). Presence of either BAX or BAK is essential to initiate intrinsic apoptosis pathway and cells lacking both are resistant to a number of apoptotic stimuli such as staurosporine, etoposide, tunicamycin, thapsigargin, ultraviolet radiation and growth factor deprivation (Wei 2001). BAX is cytosolic in location whereas BAK is located on the OMM. On receiving appropriate apoptotic stimuli, BAX translocate to OMM and BAK undergoes a conformational change (Chipuk 2006). BAX/BAK are then inserted into the OMM , undergo homo/hetero-oligomerization and form pores through which cytochrome c and other apoptotic proteins are released into the cytosol. In the cytosol, cytochrome c interacts with apoptotic protease activating factor 1 (APAF1) and forms a complex called apoptosome which ultimately leads to caspase activation.

1.4.7 Targeting mitochondria for cancer therapy

Reprogrammed metabolism in cancer cells makes cancer cell mitochondria easy to target. Similarly targeting glycolytic pathway can be more effective in cancer cells with a mitochondrial defect or in hypoxia when glycolysis is the primary source of energy (Xu 2005).

1.4.7.1 ABT-737

ABT-737 is a BH3 mimetic which binds to Bcl-2, Bcl-xl and Bcl-w. ABT-737 does not bind to anti-apoptotic protein Mcl-1, and hence cells expressing a high level of Mcl-1 may be resistant to ABT-737 (Oltersdorf 2005).

ABT-737 has been shown to induce apoptotic cell death in glioblastoma cells *in vitro*. Local administration of ABT-737 has also been shown to prolong survival in an intracranial glioma xenograft model (Tagscherer 2008). ABT-737 also synergises with proteasome inhibitor bortezomib to induce apoptosis in malignant human glioma cell lines (Premkumar 2012). Sorafenib has been shown to sensitise glioma cells to ABT-737 by targeting Mcl-1 (Kiprianova 2015). Dinaciclib, a cyclin-dependent kinase inhibitor has been shown to promote proteasomal degradation of Mcl-1 and enhance ABT-737-mediated cell death in malignant human glioma cell lines (Jane 2016).

Navitoclax (ABT-263) is an orally available form of ABT-737 and has been shown to induce tumour regression in xenograft models of small-cell lung cancer and ALL. In xenograft models of aggressive B-cell lymphoma and multiple myeloma, ABT-263 enhanced the efficacy of therapeutic regimens (Tse 2008). ABT-263 has been shown to enhance the sensitivity to metformin and 2DG in paediatric glioma cell lines (Levesley 2013).

1.4.7.2 Gossypol

Gossypol (AT-101), a compound found in cotton plants has been shown to inhibit Bcl-2, Bcl-w, Bcl-xl and Mcl-1. It has also been shown to non-specifically inhibit LDHA (Zheng 2012). Bushunow *et al.* showed that gossypol is well tolerated and has a low, but measurable, response rate in heavily pre-treated, poor prognosis patients with recurrent glioma (1999). Gossypol has also been shown to synergise with TMZ in glioma cell line (Jarzabek 2014). Gossypol has also been shown to enhance radiation-induced autophagy in glioblastoma cells (Keshmiri-Neghab 2014). However, some other results have not been encouraging. Gossypol has been shown to have negligible antitumor activity against anthracycline and taxane-refractory metastatic breast cancer (Van Poznak 2001).

1.4.7.3 Obatoclax

Obatoclax, a small molecule indole bipyrolle drug has been shown to inhibit Bcl-2, Bcl-w, Bcl-xl and Mcl-1. Obatoclax has been shown to synergise with ABT-737 to induce apoptosis in AML cell line and synergistically induced apoptosis in combination with AraC in leukemic cell lines and primary AML cells (Konopleva 2008).

1.4.7.4 Oblimersen

Oblimersen, a phosphorothioated oligonucleotide has been shown to inhibit Bcl-2 biosynthesis, and in a phase III trial, a combination of oblimersen to fludarabine and cyclophosphamide in patients with relapsed or refractory chronic lymphocytic leukaemia (CLL) significantly improved response rate (O'Brien 2007).

1.4.7.5 Heat shock protein 90 inhibitors

Heat shock protein 90 (Hsp90) is present in the mitochondria of cancer cells but not in normal mitochondria. HSP90 forms a complex with tumour necrosis factor (TNF) receptor-associated protein 1 (TRAP1) which interacts with cyclophilin D and controls the mitochondrial permeability transition pore (MPTP) complex (Kang 2007). Membrane permeable form of shepherdin has been shown to disrupt the interaction between HSP90 and anti-apoptotic regulator survivin and lead to MPTP opening and cell death (Plescia 2005).

1.4.7.6 Others

Betulinic acid is a naturally occurring pentacyclic triterpenoid which has been shown to induce ROS overproduction and oxidative stress-mediated MPTP opening and cell death in glioma cell lines (Wick 1999).

Resveratrol, a polyphenolic compound has been shown to inhibit mitochondrial ATP synthesis leading to MOMP and cell death and is under trial in multiple myeloma and colon cancer (Tinhofer 2001, Fulda 2010).

1.5 Reactive oxygen species in cancer

1.5.1 Introduction

ROS are oxygen-containing reactive chemical species. They can be broadly classified as either free radical ROS which carry an unpaired electron such as superoxide anion (O_2^-) and hydroxyl radical ($^{\cdot}OH$) or non-radical ROS such as hydrogen peroxide (H_2O_2) and hydroperoxides (ROOH). ROS are continuously being generated in our body through both enzymatic as well as non-enzymatic reactions and are essential for normal functioning of cells. They play an essential role in promoting growth and differentiation and regulate the activities of multiple signalling pathways. However, as they are also highly reactive in nature, they can also oxidise nucleic acids, lipids and proteins (Trachootham 2009).

Mitochondria are the primary source of ROS. During oxidative phosphorylation, as electrons move through the ETC and a proton gradient is created across the IMM to generate ATP, some of the electrons escape the ETC and react with molecular oxygen to form superoxide anion (O_2^-). This mostly occurs at complex I and III of the ETC, and it is estimated that 0.2%-2.0% of the total oxygen consumed by the mitochondria is converted into superoxide ion (Lenaz 2001, Balaban 2005). ROS formation is an unprogrammed event, but because of reliance of cells on oxidative phosphorylation, it is inevitable. Superoxide ion can further be converted into H_2O_2 either spontaneously or through enzymatic dismutation. H_2O_2 can then either be converted into water by catalase or into highly reactive hydroxyl radical via Fenton or Haber-Weiss reaction (Manda 2009). Other intrinsic sources of ROS include NADPH oxidase and biochemical reactions such as detoxification reaction by cytochrome P450, β oxidation in peroxisomes and prostaglandin synthesis (Figure 1.7). Extrinsic sources of ROS include radiation, chemotherapy/drugs/chemicals, diet, tobacco smoke and pollutants (Trachootham 2009) (Figure 1.8). In normal cells, ROS levels are kept under check as ROS generating mechanisms are balanced out by ROS scavenging mechanisms.

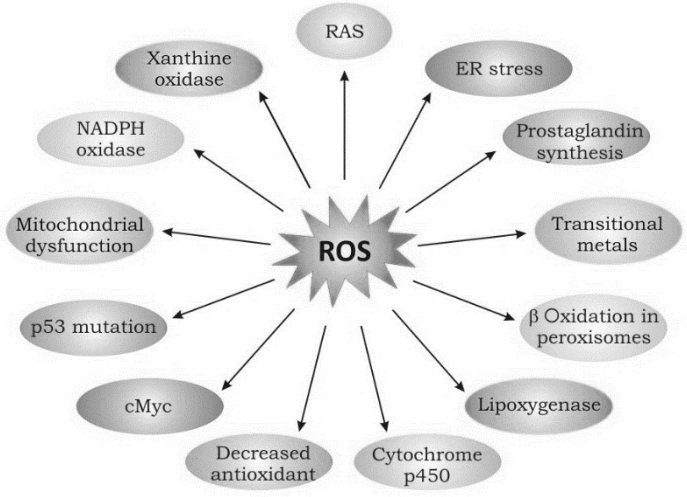


Figure 1.7 Intrinsic sources of ROS: Oxidative phosphorylation in the mitochondria is the primary source of ROS. As cells depend upon oxidative phosphorylation for energy generation, ROS generation is inevitable. Other intrinsic sources of ROS include ER stress, transitional metals, NADPH oxidase and

biochemical reactions such as detoxification reaction by cytochrome p450, β oxidation in peroxisomes and prostaglandin synthesis. Superoxide and H_2O_2 are also generated in peroxisomes via xanthine oxidase. *c-myc* can also alter the expression of a number of target genes such as γ-glutamyl cysteine synthetase, the rate-limiting enzyme catalysing GSH biosynthesis, peroxiredoxin and CYP2C9, a cytochrome P450 isozyme to affect redox balance. Tumour suppressor p53 also acts as a transcription factor to regulate the expression of a number of genes involved in redox balance. p53 also plays a vital role in sensing and removing nuclear and mtDNA damaged by metabolic, oxidative and hypoxic stress thereby prevents genetic instability. Growth factors and cytokines such as such as platelet-derived growth factor, epidermal growth factor (EGF), interleukin-1, TNFα and interferon γ have also been shown to stimulate ROS formation. Chronic inflammatory conditions are also associated with an increased risk of malignancy.ROS levels may be elevated as a result of decreased expression of anti-oxidant enzymes.

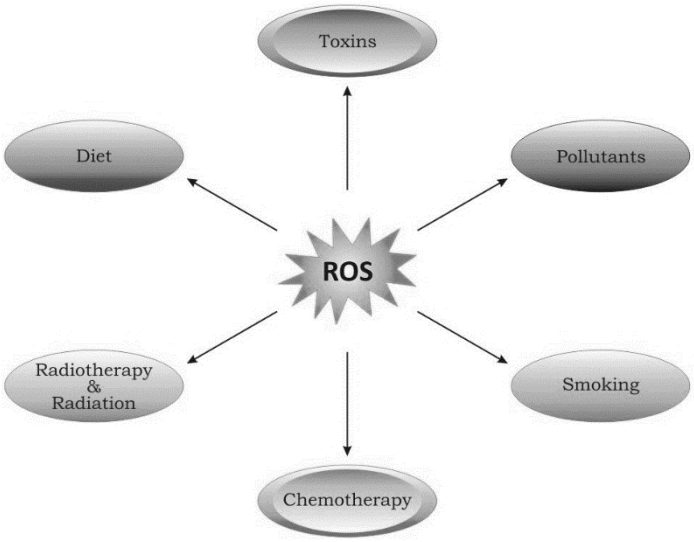


Figure 1.8 Extrinsic sources of ROS

1.5.2 Cellular detoxification from ROS

The glutathione system is one of the major antioxidant defence systems in the body. Glutathione (GSH) can donate reducing equivalents to protein cysteines to maintain them in reduced form, or it can donate the reducing equivalents to ROS and act as a direct scavenger of $O_2^{\cdot-}$, $^{\cdot}OH$ and singlet oxygen.

Superoxide dismutase (SOD) is a metalloenzyme which utilises copper, zinc, manganese and iron as a cofactor and catalyses the dismutation of OH^- to relatively less damaging H_2O_2 and oxygen (Liou 2010).

Thioredoxin acts as an antioxidant by facilitating the reduction of other proteins by cysteine thiol-disulfide exchange. It also plays a role in detoxification of hydroperoxides, singlet oxygen and $^{\cdot}OH$.

Thioredoxin is regenerated by thioredoxin reductase which uses NADPH as an electron donor (Arner 2000, Manda 2009).

1.5.3 Increased oxidative stress in cancer cells

Studies have shown that cancer cells are under increased oxidative stress as compared to their normal counterpart (Toyokuni 1995, Hileman 2001, Trachootam 2009). Evidence for this includes i) increased ROS generation in cancer cells ii) increased level of antioxidants in cancer cells and iii) increased accumulation of ROS mediated reaction products (Pelicano 2004).

1.5.4 Cellular sources of ROS

As oxidative phosphorylation in the mitochondria is the major source of ROS and cells depend upon oxidative phosphorylation for energy generation, ROS generation is inevitable. However, in non-transformed cells, ROS level is kept under check by the antioxidant system in cells. Numerous studies have shown that the redox balance in cancer cells is disturbed and there is oxidative stress in cancer cells, but until now, it has not been possible to describe precisely the exact mechanism for this (Trachootham 2009).

As mitochondria play a major role in ROS generation, a mitochondrial defect in cancer cells as proposed by Warburg may be a cause of oxidative stress. mtDNA mutations have been seen in gliomas as well as several other cancers such as breast, ovarian, gastric, oesophageal, colorectal, hepatic, pancreatic, renal, prostate, thyroid as well as haematological malignancies indicating a role of these mutations in tumour biology (Carew 2002).

Besides mitochondria, superoxide and H_2O_2 are also generated in peroxisomes via xanthine oxidase (Bonekamp 2009). Oncogenes have also been shown to contribute to ROS generation. Studies have shown that *c-myc* can alter the expression of a number of target genes which affect the redox balance in the cell. Tumour suppressor p53 acts as a transcription factor to regulate the expression of a number of genes involved in redox balance (Polyak 1997). p53 also plays a vital role in sensing and removing nuclear and mtDNA damaged by oxidative stress thereby preventing genetic instability (Achanta 2004). Hence p53 mutation, which is seen in many cancers, can also be associated with redox imbalance.

Growth factors and cytokines such as such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), interleukin-1 (IL-1), TNFα and interferon γ have also been shown to stimulate ROS

formation (Liou 2010). Chronic inflammatory conditions such as pancreatitis, ulcerative colitis and chronic hepatitis are also associated with oxidative stress and increased risk of malignancy (Eaden 2001, Berasain 2009).

ROS levels may be elevated as a result of decreased expression of anti-oxidant enzymes. Studies have shown that reduced expression and activity of mitochondrial MnSOD in colorectal and pancreatic cancer can lead to increased superoxide anion level (van Driel 1997, Cullen 2003).

1.5.5 Consequences of oxidative stress in cancer cells

Because of its effect on cell survival and death, ROS has been described as a "double-edged sword" (Glasauer 2014).

ROS mediated signalling pathways are upregulated in a number of cancers and have been shown to play an essential role in transformation, cell cycle progression & proliferation, resistance to cell death, metastasis, angiogenesis, cellular metabolism and maintenance of stemness; the hallmarks of cancer (Storz 2005, Liou 2010, Waris 2006, Trachootham 2009, Glasauer 2014). ROS mediated cancer cell proliferation has been observed in many cancers including lung, liver and breast (Glasauer 2014).

ROS can also affect protein functions through a number of mechanisms including regulation of protein expression, alteration of protein stability and post-translational modification (Pelicano 2004, Klimova and Chandel 2008, Trachootham 2009). As stated earlier O_2^- generated in the mitochondria can damage the mtDNA which is more prone to oxidative damage as compared to nuclear DNA because of its proximity to the ETC as well as lack of histones. O_2^- can then be converted into H_2O_2 which can easily diffuse into the nucleus of the cell and get converted into highly reactive $^{\cdot}OH$ which can then cause DNA strand break, DNA adducts as well as base pair mismatch during replication contributing to tumorigenesis especially in the presence of impaired/defective repair (Wiseman 1996, Cooke 2003).

ROS can also damage lipids in the cell membranes by lipid peroxidation which in turn can cause further damage to the membrane. Lipid peroxidation products like malondialdehyde are also mutagenic (Burcham 1998, Marnett 1999, Hallivwell 1999).

ROS has also been shown to modulate cell cycle by upregulating cyclin B2/D3/E1/E2 to expedite G1/S progression, whereas anti-oxidants have been shown to lower cyclin levels, delay cell cycle progression and inhibit cell proliferation (Felty 2005, Menon 2005, Liou 2010). H_2O_2 can reversibly inactivate PTEN, a negative regulator of Akt (Lee 2002). PTEN inhibition leads to increased ROS generation as a result of decreased expression of anti-oxidant enzymes (Huo 2008). Higher levels of

ROS in cancer cells have also been correlated with anti-cancer drug resistance and poor prognosis. As stated earlier increased oxidative stress in cancer cells has been associated with increased expression of anti-oxidant enzymes, which in turn may confer drug resistance (Pervaiz 2004). ROS via its effect on VEGF, MMP , cytoskeleton, rac-1 and vascular permeability has also been shown to affect cancer cell motility and metastasis. (Liou 2010, Glasauer 2014).

However excessive ROS has also been shown to induce cell death by both intrinsic as well as extrinsic apoptotic pathways (Ozben 2007) and even necrosis (Hampton 1997, Gardner 1997). Kroemer et al. described ROS mediated combined apoptotic and necrotic cell death in the same tissue (1998). Studies have also shown that ROS can activate autophagy-mediated cell death (Scherz-Shouval 2007, Shrivastava 2011) (Figure 1.9 & Figure 1.10).

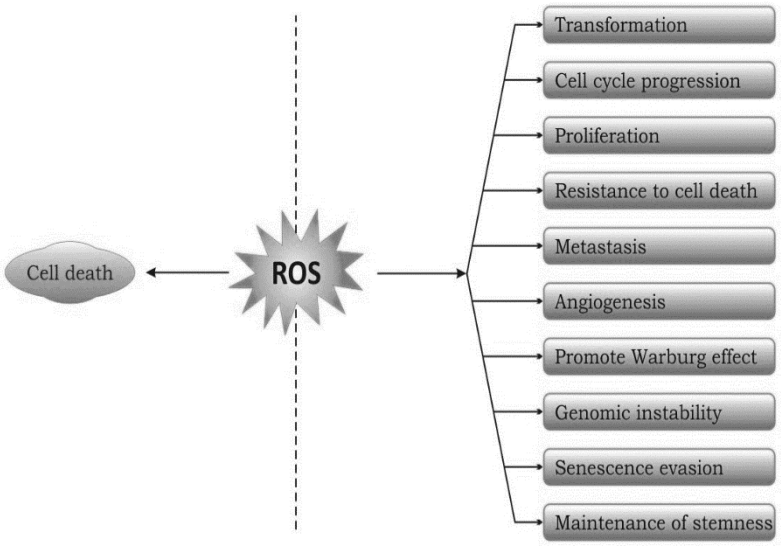


Figure 1.9 Biological effects of ROS: Because of its impact on cell survival and death, ROS has been described as a "double-edged sword". ROS mediated signalling pathways are upregulated in a number of cancers and have been shown to promote hallmarks of cancer. ROS plays an important role in transformation, cell cycle progression & proliferation, resistance to cell death, metastasis, angiogenesis, cellular metabolism and maintenance of stemness; the hallmarks of cancer. A number of transcription factors such as HIF and p53 contain redox-sensitive cysteine residues at their DNA binding site. Oxidation/reduction of these sites can affect the activity of these transcription factors and down-stream protein synthesis. ROS mediated HIF upregulation has been shown to play a role in the Warburg effect. ROS can also oxidise cysteine, tyrosine or methionine amino acid residue of proteins such as RAS or Akt and affect its function. ROS can also activate mitogen-activated protein kinase (MAPK) and promote motility and survival. ROS has been shown to activate anti-oxidant master regulator nuclear factor (erythroid-derived 2)-like 2 (Nrf2) either via activation of Akt or inactivation of kelch-like ECH -associated protein 1 (KEAP1). ROS can also induce DNA strand break, DNA adducts as well as base pair mismatch during replication which can contribute to tumorigenesis. ROS can also oxidise lipids, and lipid peroxidation products like malondialdehyde have been shown to be mutagenic and carcinogenic. ROS has also been shown to modulate cell cycle by upregulating cyclins B2/D3/E1/E2

to expedite G1/S progression. H_2O_2 can also reversibly inactivate PTEN, a negative regulator of Akt. Akt has been shown to promote cell survival by inactivating pro-apoptotic proteins or forkhead box O (FoxO) transcription factor. Akt via mammalian target of rapamycin (mTOR) also promotes cell growth and proliferation. Similarly, oxidative stress can active nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor, which can induce the expression of anti-apoptotic proteins. Higher levels of ROS in cancer cells have also been correlated with anti-cancer drug resistance and poor prognosis. ROS via its effect on VEGF, MMP, cytoskeleton, rac-1 and vascular permeability has also been shown to affect cancer cell motility and metastasis. ROS has also been shown to decrease cell adhesion to extracellular matrix and promote anchorage-independent survival, invasion, migration as well as intravasation. ROS can also promote angiogenesis via HIF-1 stabilisation. However excessive ROS has also been shown to induce cell death.

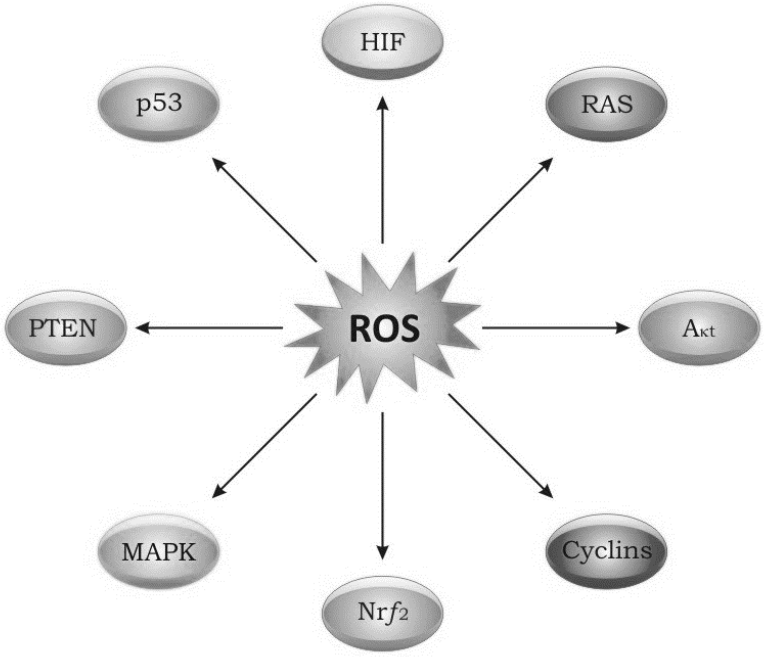


Figure 1.10 ROS mediated regulatory pathways: Transcription factors such as HIF and p53 contain redox-sensitive cysteine residues at their DNA binding site. Oxidation/reduction of these sites can affect the activity of these transcription factors and down-stream protein synthesis. ROS mediated HIF upregulation has been shown to play a role in the Warburg effect. ROS can also oxidise cysteine, tyrosine or methionine amino acid residue of proteins such as RAS or Akt and affect its function. ROS can also activate mitogen-activated protein kinase (MAPK) and promote motility and survival. ROS has been shown to activate anti-oxidant master regulator Nrf2 either via activation of Akt or inactivation of kelch-like ECH -associated protein (KEAP1). ROS has also been shown to modulate cell cycle by upregulating cyclins B2/D3/E1/E2 to expedite G1/S progression. H_2O_2 can also reversibly inactivate PTEN, a negative regulator of Akt.

1.5.6 Targeting ROS

A number of treatment modalities including anti-oxidant treatment, strategies which either increase ROS or decrease anti-oxidant enzymes or combination therapy have been tried for cancer cell killing (Schumaker 2006, Glasauer 2014).

1.5.6.1 Agents which decrease ROS

A number of trials such as Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) Study, Physicians' Health Study I (PHS I), Women's Health Study (WHS), Heart Outcomes Prevention Evaluation–The Ongoing Outcomes (HOPE-TOO) Study and Physicians' Health Study II (PHS II) looking at the effect of anti-oxidants on cancer incidence and cancer related mortality have failed to show any benefit (Hennekens 1996, Rautalahti 1999, Lee 1999, Lonn 2005, Gaziano 2009). One of the reasons for the poor efficacy of anti-oxidants in reducing the cancer incidence and cancer-related mortality could be the fact that these anti-oxidants are not able to target locally produced ROS pools (Glasauer 2014).

1.5.6.2 Agents which increase ROS

Conventional chemotherapy agents such as anthracyclines have been shown to cause an increase in intracellular H_2O_2 and O_2^- levels leading to mitochondrial membrane depolarisation, cytochrome c release and caspase-3 activation which can be inhibited by catalase (Tsang 2003). Doxorubicin has also been shown to generate highly reactive $^{\cdot}OH$ through a Fenton type reaction by chelating intracellular iron (Kotamaraju 2002). ROS has also been implicated at least in part, in the mechanism of action of platinum-based chemotherapy and vinca alkaloids besides radiotherapy and photodynamic therapy mediated cell killing (Ward 1985, Moan 2006, Manda 2009).

1.5.6.3 Agents which decrease antioxidants

GSH in the cell serves as a major source of reducing equivalents for redox modulating enzymes such as peroxidases, peroxiredoxins and thio reductases. Hence treatment which reduces GSH may lead to an increase in oxidative stress and cell death in cancer cells. Isothiocyanates and aziridine derivatives have been shown to conjugate with GSH and lead to cell death by causing GSH depletion (Dvorakova 2000Xu 2001).

Estrogen derivative 2-methoxyestradiol (2-ME) has been shown to inhibit SOD, leading to accumulation of O_2^- in the cell. This leads to damage to the mitochondrial membrane, cytochrome c

release and apoptosis in human leukaemia cells but not in normal lymphocytes (Huang 2000). Phase II trial showed that 2-ME was well tolerated and it showed some anticancer activity in prostate cancer (Sweeney 2005).

PX-12 a small molecule inhibitor of thioredoxin 1 was shown to be effective against advanced solid tumours in a phase I study (Ramanathan 2007).

1.5.6.4 Combination treatment

2-ME has been shown to potentiate other ROS generating agents such as radiotherapy (Zou 2007) and arsenic trioxide in leukaemia and bladder cancer cell lines (Zhou 2003, Kuo 2013). Buthionine sulfoximine (BSO) has been shown to inhibit γ-glutamyl cysteine synthetase and lower the GSH content. It has been shown to sensitise cisplatin and TMZ resistant glioma cells *in vivo* and *in vitro* (Rocha 2014).

1.5.7 Conclusion

Increased oxidative stress has been shown to correlate with tumour aggressiveness and poor prognosis (Patel 2007, Kumar 2008, Jezierska-Drutel 2013). Hence targeting ROS for cancer chemotherapy sounds a promising prospect and some encouraging results have been obtained. However, a better understanding of redox homeostasis is essential to formulate safe and effective chemotherapies based on ROS modulation. Elesclomol which mainly acts by inducing ROS generation was shown to be effective in malignant melanoma patients in a phase II trial (Kirshner 2008). However, when Elesclomol was combined with paclitaxel which also acts by ROS generation in stage IV metastatic melanoma patients, increased mortality was seen (predominantly in patients with high LDH levels) , and the study had to be stopped (O`day 2013).

1.6 5' Adenosine monophosphate-activated protein kinase *pathway in cancer*

1.6.1 Introduction

In non-transformed cells, external signals mediated by growth factors are integrated and coupled with nutrient availability to ensure that cells only proliferate when there is sufficient availability of nutrients (Jones 2005, Shackelford 2009). However, in transformed cells, mutations in signalling pathways can lead to a nutrient uptake in cell-autonomous fashion (Jones 2005).

1.6.2 LKB1-AMPK-TSC-mTOR Pathway

Liver kinase B1 (LKB1) a serine-threonine kinase, is a tumour suppressor gene located on chromosome 19p13 and is the primary upstream kinase of AMPK. 5' Adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimer composed of α catalytic unit and β & γ regulatory unit. When energy status of the cell is optimal, ATP binds to AMPK and inactivates it. However, when there is a fall in ATP level, adenosine monophosphate (AMP) replaces ATP from the γ subunit resulting in allosteric activation. Binding of AMP to γ subunit also exposes threonine 172 on α subunit for phosphorylation by upstream kinase LKB1 (Jones 2005, Kuhajda 2008, Shackelford 2009, Jeon 2012, Russo 2013, Grahame Hardie 2014). Phosphorylation of AMPK can be reversed by phosphatases (Russo 2013), and AMP binding also prevents threonine 172 dephosphorylation by protein phosphatases (Grahame Hardie 2014, Luo 2010). Hypoxia, changes in redox status and pH of the cell can also activate AMPK (Jeon 2012). In certain tissues, calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) can also play a role in phosphorylating threonine 172 and activating AMPK in response to elevated intracellular $Ca2^{+}$ levels independent of ATP/AMP ratio (Hurley 2005, Jeon 2012, Russo 2013, Grahame Hardie 2014). Activated AMPK phosphorylates and activates downstream tuberous sclerosis complex 2(TSC2) which in turn inhibits mammalian target of rapamycin (mTOR) complex 1 (mTORC1) (Jones 2005, Kuhajda 2008). Though TSC2 is the primary negative regulator of mTORC1, cells which lack TSC2 may exhibit mTORC1 inhibition when the ATP levels fall, and this may be the result of LKB1/AMPK mediated direct phosphorylation of raptor subunit of mTORC1 (Shackleford 2009, Luo 2010, Korsse 2013).

1.6.3 Biological effects of AMPK pathway activation

In low energy status, LKB1 phosphorylates and activates AMPK which in turn inhibits Akt. This downregulates glycolysis including PPP resulting in less availability of ribose 5 phosphate and NADPH for biosynthesis reactions (Kuhajda 2008). AMPK also phosphorylates and inactivates ACC thereby inhibiting fatty acid synthesis (Carling 1987, Russo 2013). As a result of ACC inhibition, there is a fall in malonyl CoA level which relieves the inhibition of CPT1, leading to increased FAO. FAO in the mitochondria can also lead to increased NADPH production through malic enzyme and IDH by converting malate and isocitrate to pyruvate and α-KG respectively. Via mTOR inhibition, AMPK negatively regulates enzymes of the glycolytic pathway, sterol regulatory element-binding protein 1 (SREBP1) and HIF (Luo 2010, Russo 2013). AMPK activation also inhibits hepatic gluconeogenesis and

improves insulin sensitivity (Luo 2010, Russo 2013, Grahame Hardie 2014). AMPK has also been shown to phosphorylate and activate UNC-51 like kinase 1 (ULK1), which plays an essential role in initiating autophagy (Russo 2013). AMPK through phosphorylation of histone deacetyltransferase and histone acetyltransferase may also be involved in regulating a wide range of cellular processes (Shackleford 2009, Luo 2010). AMPK through its effect on downstream kinases which are involved in cytoskeleton remodelling may also regulate cell polarity and migration (Shackleford 2009, Fogarty 2010).

p53 acts as a cell cycle checkpoint protein in response to various stress stimuli and induces cell cycle arrest at G1/S or G2/M. AMPK activation leads to phosphorylation of p53 at serine 15 which is essential to mediate AMPK induced p53 dependent cell cycle arrest which persists till energy status of the cell is restored (Jones 2005, Kuhajda 2008, Russo 2013). Hence cells with LKB1/AMPK/TSC mutation will continue to replicate in low energy conditions which may lead to cell death as they cannot undergo cell cycle arrest (Jones 2005, Kuhajda 2008). AMPK has also been shown to inhibit cellular proliferation by regulating cyclin dependent kinase (CDK) inhibitors p21 and p27. AMPK has also been shown to phosphorylate forkhead box O3 (FoxO3) which plays a vital role in regulating metabolism, apoptosis and oxidative stress and is also a target of PI3K/Akt (Chiacchiera 2010) (Figure 1.11).

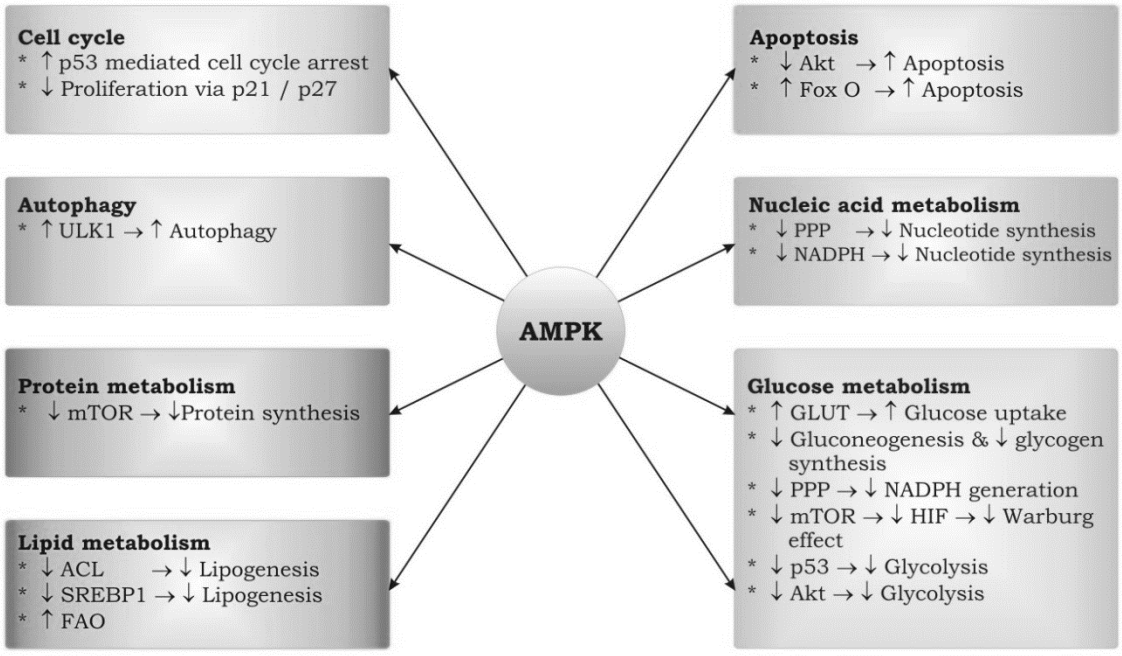


Figure 1.11 Biological effects of AMPK activation: When nutrients are scarce, LKB1 phosphorylates and activates AMPK which in turn inhibits Akt. AMPK down regulates glycolysis including PPP. AMPK also phosphorylates and inactivates ACC thereby inhibiting fatty acid synthesis. As a result of ACC inhibition, there is a fall in malonyl CoA level which relieves the inhibition of CPT1, leading to increased FAO. FAO in the mitochondria can lead to increased NADPH production through malic enzyme and IDH by converting malate and isocitrate to pyruvate and α-KG respectively. Via mTOR inhibition, AMPK

also negatively regulates enzymes of the glycolytic pathway, SREBP1 and HIF. AMPK activation also inhibits hepatic gluconeogenesis and improves insulin sensitivity. AMPK has also been shown to phosphorylate and activate ULK1, which plays an essential role in initiating autophagy/mitophagy. AMPK activation leads to phosphorylation of p53, which is crucial to mediate AMPK induced p53 dependent cell cycle arrest which persists till energy status of the cell is restored. AMPK has also been shown to inhibit cell proliferation by regulating CDK inhibitors p21 and p27. AMPK can also phosphorylate FoxO3 which plays an important role in regulating metabolism, apoptosis and oxidative stress and is also a target of PI3K/Akt.

Thus, AMPK activation promotes oxidative metabolism, rather than aerobic glycolysis seen in tumour cells and immune cells and this can explain the anti-tumour and anti-inflammatory action of AMPK (Grahame Hardie 2014). Decreased AMPK activity promotes tumour growth whereas AMPK activation has been shown to prevent tumour growth. However, as discussed earlier, AMPK can also lead to p53 mediated cell cycle arrest and activate autophagy, both of which are pro-survival (Liang 2007). AMPK has been shown to activate stress kinases and induce apoptosis by activation of the c-Jun-N-terminal kinase (JNK) (Mukherjee 2008). Hence the role of AMPK as an oncogene or tumour suppressor depends upon the degree of AMPK activation as well as the availability of nutrients.

1.6.4 Targeting AMPK pathway

1.6.4.1 Metformin

Metformin, a biguanide derivative is the most widely used drug for type 2 diabetes worldwide. In the 1970s it was approved for the treatment of type 2 diabetes in Europe and in 1995 in the United States (Korsse 2013). Metformin has been shown to reduce blood glucose level by inhibiting hepatic gluconeogenesis which is mediated via the LKB1-AMPK pathway (Rizos 2013). Metformin further lowers blood glucose by delaying the gastrointestinal absorption of glucose. Metformin also inhibits ETC I leading to a rise in AMP level as compared to ATP which activates AMPK. AMPK via TSC inhibits mTOR. AMPK activation also inhibits ACC leading to decreased malonyl CoA formation which lifts the allosteric inhibition on the transport of fatty acid to the mitochondria for oxidation, thus promoting FAO and inhibiting lipogenesis (Fullerton 2013). Metformin via AMPK also inhibits VEGF and hence inhibits angiogenesis (Morales 2015). Metformin also acts as an insulin sensitizer leading to lowered blood insulin and IGF-1 levels (Rizos 2013, Morales 2015) which may play a role in AMPK independent mTOR inhibition (Engelman and Cantley 2010). Obesity is a known risk factor for cancers, and metformin treatment has been shown to lead to weight loss (Rizos 2013). Metformin has also been shown to reduce lipogenesis and ROS formation which may play a role in its anti-cancer effects (Rizos 2013). Hence the effect of metformin in reducing the incidence of cancer in people with diabetes and

reduced mortality from cancer-related causes may be connected to its diverse mechanism of actions rather than limited to its glucose-lowering effect as other anti-diabetic drugs have failed to show similar results (Vander Heiden 2011, Galluzzi 2013).

Type 2 diabetics on metformin have reduced incidence of cancer as compared to general population or diabetics on other glucose-lowering drugs. Also, people with diabetes on metformin have a reduced trend of mortality as compared to other two patient groups (Luo 2010, Franciosi 2013). Data from the UK general practice research database showed that diabetes is associated with an increased risk of breast cancer. Further analysis of 22,621 female type 2 diabetics on metformin showed that long-term use of metformin was associated with reduced risk of developing breast cancer (Bodmer 2010). Other studies have also demonstrated that metformin has a protective effect on breast cancer risk among postmenopausal women with diabetes (Chlebowski 2012). In another meta-analysis of 108,161 patients with type 2 diabetes, it was found that metformin treatment was associated with a significantly lower risk of colorectal cancer (Zhang 2011). Similarly, in a meta-analysis of 1,535,636 patients, it was shown that metformin use was associated with reduced risk of liver, pancreatic, colorectal and breast cancer (Zhang 2013, Zhang 2014). A prospective cohort study of 800,000 individuals showed that type 2 diabetes increases and metformin reduces total, colorectal, liver and pancreatic cancer incidences in the Taiwanese population (Lee 2011). Studies have also shown improved survival in people with diabetes on metformin in breast, ovarian, hepatic, pancreatic and colorectal cancer (Zhang 2013, Zhang 2014, Morales 2015). Metformin use has also been associated with improved progression free survival (PFS) in advanced non-small cell lung cancer (NSCLC) in diabetic patients (Morales 2015). It has also been shown that there is an association of metformin therapy and prolonged PFS in GBM patients with diabetes (Adeberg 2015). Metformin also possesses favourable pharmacokinetic properties. It has 50-60% oral bioavailability, minimal binding to plasma protein, broad tissue distribution, slow absorption, limited drug interaction and rapid urinary excretion (Galluzzi 2013). The incidence of lactic acidosis with phenformin, another biguanide anti-diabetic drug is 40-64/ 100000 patient years whereas it is only 3/100000 patient years with metformin. However, the dose of metformin is restricted by gastrointestinal side effects which may be due to inhibition of ETC and subsequent increase in lactate formation in the gut or liver (Grahame Hardie 2014).

However, the plasma concentration of metformin after a 30mg/kg dose is only 10-40µM whereas studies have shown that the concentration required to activate AMPK *in vitro* is around 1-10mM. Also, in experiments done in *vitro* system, treatment is directly applied to cells, and this does not take into account the influence of other organs which may occur within the body. Similarly, cells in tissue culture are bathed in a supra-physiological level of glucose and medium is supplemented with growth factors

and fetal bovine serum. On the other hand, it may be argued that *in vitro* system does not possess OCT1 which is needed for cellular uptake of metformin and this may explain high metformin requirement *in vitro* experiments (Fogarty 2010).

Metformin has been shown to inhibit the growth of leukaemia, lung, gastric, liver, pancreatic, prostate, endometrial, thyroid, head and neck, cervical, renal and breast cancer cells *in vitro* (Xiao 2012, Bao 2012, Deng 2012, Korsse 2013, Hsu 2015, Plews 2015, Kalogirou 2016). Metformin has also been shown to inhibit the growth of glioma and pancreatic stem cells (Sato 2012, Ning 2016). Other studies have shown that metformin also inhibits tumour growth in animal models of melanoma, prostate, colon, breast, pancreas, ovary, gastric and lung cancer (Ben Sahra 2010, Korsse 2013).

Metformin has been shown to synergise with radiotherapy and chemotherapy drugs or re-sensitise resistant cancer cells.

- Metformin has been shown to enhance the sensitivity of breast, lung and prostate cancer cells to doxorubicin and paclitaxel (Iliopoulos 2011)
- Metformin has been shown to enhance the sensitivity of the xenograft model of ovarian cancer to cisplatin. It has also been shown to reduce the incidence of lung metastasis from ovarian cancer (Rattan 2011)
- Metformin has been shown to enhance the sensitivity of endometrial cancer cells to paclitaxel and cisplatin (Dong 2012)
- Metformin has been shown to kill trastuzumab-refractory breast CSC and re-sensitise xenograft model of breast cancer to trastuzumab (Cufi 2012)
- Metformin has been shown to enhance the sensitivity of breast cancer and CSC to radiotherapy (Song 2012)
- Metformin has been shown to re-sensitise osteosarcoma cell lines to cisplatin through cell cycle modulation (Quattrini 2013)
- Combination treatment of metformin and sorafenib has been shown to induce apoptosis in TMZ resistant glioma stem cells (Aldea 2014)
- Metformin in combination with TMZ and/or irradiation has been shown to induce a synergistic anti-tumour response in glioma cell lines (Sesen 2015).
- Metformin has been shown to re-sensitise pancreatic cancer cell line to gemcitabine (Baron 2015)
- Metformin has been shown to re-sensitise CML to imatinib (Shi 2015)
- Metformin has been shown to be synergistic with TMZ in glioma cells as well as glioma stem cell (Aldea 2011, Yu 2016)

- Metformin has been shown to re-sensitise cisplatin-resistant ovarian cancer cells to the drug by inhibiting oxidative phosphorylation (Matassa 2016)

A number of clinical trials looking at the effect of metformin in cancer patients are ongoing:

- Metformin and chloroquine in IDH 1/2 mutated glioma, intrahepatic cholangiocarcinoma or chondrosarcoma (MACIST) (NCT02496741)
- Combination treatment of metformin, radiation therapy and low carbohydrate diet in patients with recurrent brain tumours (NCT02149459)
- Metformin, neo-adjuvant TMZ and hypo- accelerated radiotherapy followed by adjuvant TMZ in patients with GBM- NCT02780024
- Metformin Hydrochloride and combination chemotherapy in patients with stage III-IV ovarian, fallopian tube or primary peritoneal cancer (NCT02122185)
- Evaluation of metformin on targeting CSC for the prevention of relapse in gynaecologic patients (NCT01579812)
- Metformin and cytarabine for the treatment of relapsed/refractory AML (NCT01849276)
- Advanced lung cancer treatment with metformin and chemo-radiotherapy (ALMERA)- NCT02115464
- Metformin hydrochloride and aspirin in treating patients with hormone-dependent prostate cancer that has progressed after surgery or radiation therapy (PRIMA)- NCT02420652
- Vincristine, dexamethasone, doxorubicin, and PEG-asparaginase (VPLD) and metformin for relapsed childhood ALL (NCT01324180)
- Phase II study of metformin plus paclitaxel/carboplatin/bevacizumab in patients with lung adenocarcinoma (NCT01578551)
- Stereotactic radiosurgery and metformin hydrochloride in treating patients with borderline resectable or locally advanced pancreatic cancer (NCT02153450)
- Megestrol acetate plus metformin to megestrol acetate in patients with atypical endometrial hyperplasia or early stage endometrial adenocarcinoma (NCT01968317)
- Metformin combined with gemcitabine as adjuvant therapy for pancreatic cancer after curative resection (NCT02005419)
- Metformin with or without rapamycin as maintenance therapy after induction chemotherapy in patients with pancreatic cancer (NCT02048384)
- Randomized pilot study to evaluate the effects of a short course of metformin versus no therapy in the period prior to hysterectomy for grade 1-2 adenocarcinoma of the endometrium in obese non-diabetic women (NCT01877564)

- Neoadjuvant metformin in association to chemoradiotherapy for locally advanced rectal cancer (NEOMETRE) (NCT02473094)
- Chemotherapy and radiotherapy with or without metformin hydrochloride in treating patients with stage III NSCLC (NCT02186847)
- Metformin plus modified FOLFOX 6 in metastatic pancreatic cancer (NCT01666730)
- Gemcitabine hydrochloride, paclitaxel, metformin hydrochloride and a standardized dietary supplement in treating patients with pancreatic cancer that cannot be removed by surgery (NCT02336087)
- Metformin plus sorafenib for advanced HCC (NCT02672488)
- Vandetanib in combination with metformin in people with hereditary leiomyomatosis and renal cell cancer or SDH associated kidney cancer or sporadic papillary RCC (NCT02495103)
- Pharmacodynamic study of sirolimus and metformin in patients with advanced lung, liver, breast, kidney cancer and lymphoma (NCT02145559)
- Metformin in combination with radiotherapy vs radiotherapy alone in NSCLC (NCT02285855)
- Phase II study of metformin with erlotinib as second-line therapy of stage IV NSCLC (METAL) is ongoing (Fasano 2015)
- Phase II study to evaluate the activity and safety of everolimus in combination with octreotide LAR and metformin in patients with advanced pancreatic neuroendocrine tumours (MetNET-1) is ongoing (Pusceddu 2014)
- Phase II randomised trial of neoadjuvant metformin plus letrozole vs placebo plus letrozole for estrogen receptor-positive postmenopausal breast cancer (METEOR) is also ongoing (Kim 2014)
- Combination of metformin to neoadjuvant radiotherapy and chemotherapy in the treatment of locally advanced rectal cancer (METCAP) - NCT02437656
- Bicalutamide with or without metformin for biochemical recurrence in overweight or obese prostate cancer patients (BIMET-1)- NCT02614859
- Temsirolimus in combination with metformin in patients with advanced cancers - NCT01529593
- Metformin and simvastatin in bladder cancer - NCT02360618
- Enzalutamide and metformin hydrochloride in treating patients with hormone-resistant prostate cancer - NCT02339168
- A pre-surgical trial of the combination of metformin and atorvastatin in newly diagnosed operable breast cancer - NCT01980823
- Metformin in children with relapsed or refractory solid tumours - NCT01528046

- Metformin and carbohydrate restriction with platinum-based chemotherapy in stage IIIB/IV non-squamous non-small cell lung cancer (NS-NSCLC) (METRO)- NCT02019979
- Safety and efficacy of cyclophosphamide, metformin and olaparib in endometrial cancer patients (ENDOLA)- NCT02755844
- Study of metformin with carboplatin/paclitaxel chemotherapy in patients with advanced ovarian cancer (OVMET)- NCT02312661
- Study of doxorubicin, docetaxel, trastuzumab and metformin in operable and locally advanced HER2 positive breast cancer (met-HEReMYTA)- NCT02488564
- Impact of the addition of metformin to abiraterone in metastatic prostate cancer patients (MetAb-Pro)- NCT01677897

1.6.4.2 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside

5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) is taken up by the cells and phosphorylated to mono-phosphorylated form ZMP. ZMP mimics AMP and binds to γ subunit of AMPK and activates AMPK without affecting ATP/AMP ratio in the cell (Russo 2013). AICAR has been shown to inhibit the growth of glioblastoma cells and xenografts expressing the activated EGFR mutant (EGFRvIII) (Guo 2009).

1.6.4.3 Salicylate

Salicylate has also been shown to activate AMPK. Aspirin which is a prodrug of salicylate has been shown to be associated with reduced incidence of death from prostate, lung and colon cancer (O`Brien 2015). Salicylate has been shown to inhibit the growth of prostate and lung cancer cell lines, and these effects are synergistic with metformin (O`Brien 2015).

1.6.4.4 Thiazolidinediones

Thiazolidinediones such as rosiglitazone and pioglitazone at least in part exert anti-diabetic effect via AMPK activation. Like metformin, they are thought to inhibit ETC I leading to fall in ATP and rise in AMP level which in turn activates AMPK (Fogarty 2010). Thiazolidinediones use is associated with reduced incidence of lung cancer, and it has been shown to inhibit the growth of lung, breast and colon cancer cells (Blanquicett 2008).

1.6.4.5 Others

A number of natural compounds such as quercetin, resveratrol, epigallocatechin gallate (EGCG) and berberine have been shown to activate AMK, and it is thought that this is as a result of inhibition of ETC 1 of the mitochondria and resultant fall in ATP/AMP ratio (Fogarty 2010, Russo 2013).

1.7 Hypoxia and cancer

1.7.1 Introduction

Hypoxia is defined as fall in oxygen level below a critical threshold which affects the normal functioning of cells, tissues or organs (Höckel 2001). When the partial pressure of oxygen falls below 10 mm of Hg, cells begin to show ATP depletion though most mitochondria can perform oxidative phosphorylation up to a partial pressure of 0.5mm of Hg of oxygen (Höckel 2001).

As a tumour proliferates, delivery of oxygen to tumour cells cannot keep pace with the oxygen requirement of the cells, leading to hypoxia. Though new vessels are formed during tumour growth, they are usually disorganised and leaky and thus fail to relieve hypoxia (Weljie 2011). Also, as a tumour grows, cells further away from the vessels have less access to oxygen which can also contribute to tumour hypoxia (Semenza 2000, Semenza 2009, Semenza 2010).

1.7.2 HIF

HIF is the major transcription factor activated as a result of tumour hypoxia and it regulates multiple genes to promote tumour angiogenesis, metastasis and survival besides regulating metabolism. HIF is known to target about 1-2% of the whole genome (Brahimi-Horn 2007). HIF-1 transcription factor consists of oxygen sensitive HIF-1α subunit which heterodimerizes with constitutively expressed HIF-1β. This complex then binds to hypoxia responsive elements (HRE) in the promoter region of target genes (Denko 2008). HIF-1α is hydroxylated by oxygen-dependent prolyl hydroxylases using α-KG derived from the TCA cycle. Hydroxylated HIF-1α is then recognised by von Hippel–Lindau (VHL) gene product for proteasomal degradation (Semenza 2003, Semenza 2009, Semenza 2010). Hence in normoxia, HIF-1α is continuously being synthesised and degraded (Kim 2006). However, in hypoxia prolyl hydroxylases are inhibited, which leads to HIF-1α stabilisation. HIF-1α can also be stabilised in normoxia through a number of different mechanisms. Mutation in the VHL gene, which is needed for recognition of hydroxylated HIF-1α and subsequent proteasomal degradation can stabilise HIF-1α (Kaelin 2002). Oncogenes such as Src, PI3K and H-Ras have been shown to stabilise HIF-1 in normoxia

(Chen 2001, Semenza 2003, Semenza 2010). Mutations in TCA cycle enzymes such as SDH and FH have also been associated with HIF-1α stabilisation in normoxia (Selak 2005, Isaacs 2005).

1.7.3 Effects of HIF induction

1.7.3.1 Hypoxia and metabolism

HIF-1 has been shown to upregulate GLUTs and multiples enzymes of the glycolytic pathway (Denko 2008). HIF-1 also stimulates PDK1 which leads to decreased mitochondrial respiration and reduced oxygen utilisation for energy production which may be relevant in the hypoxic tumour environment. By inhibiting oxidative phosphorylation, HIF-1 also leads to decreased ROS generation and reduced oxidative stress (Denko 2008). Hypoxia can also lead to fall in ATP level and rise in AMP level which can activate AMPK. This, in turn, can inhibit the mTOR, the master regulator of cellular metabolism. Hypoxia can also inhibit mTOR via AMPK independent method via regulated in development and DNA damage responses 1 (REDD1) (Pouysségur 2006).

1.7.3.2 Hypoxia and angiogenesis

Hypoxia promotes tumour cell survival by upregulating angiogenesis. HIF-1 has been shown to upregulate VEGF, the master regulator of angiogenesis and MMP which helps in neovascularisation and sprouting of newly formed blood vessels (Muñoz-Nájar 2006, Krock 2011, Muz 2015).

1.7.3.3 Hypoxia and metastasis

Hypoxia influences a number of steps in the metastatic process including invasion, migration, extravasation, formation of pre-metastatic niche, as well as growth and survival at a distant site (Zhang 2008, Azab 2012, Jing 2013, Muz 2015).

1.7.3.4 Hypoxia and p53

Via p53 regulation, HIF plays a vital role in metabolism, cell cycle control and apoptosis. In basal conditions, p53 is kept at very low levels by murine double minute 2 (MDM2) mediated ubiquitination and subsequent proteasomal degradation. It is thought that HIF-1 may suppress MDM2 mediated p53 ubiquitination.

1.7.3.5 Hypoxia and cell death

HIF-1 upregulates transforming growth factor β (TGFβ) which in turn activates downstream activators such as snail, slug and twist which antagonise p53 mediated apoptosis and promote resistance to chemotherapy and radiotherapy (Kurrey 2009). HIF has been shown to upregulate BH3 only protein BNIP3 which may contribute to regulation of apoptosis by HIF (Guo 2001, Brahimi-Horn 2007). However severe hypoxia can lead to p53 mediated cell death. Hypoxia can also activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which plays an essential role in DNA transcription, cytokine production and cell survival (Höckel 2001) (Figure 1.12 & Figure 1.13)

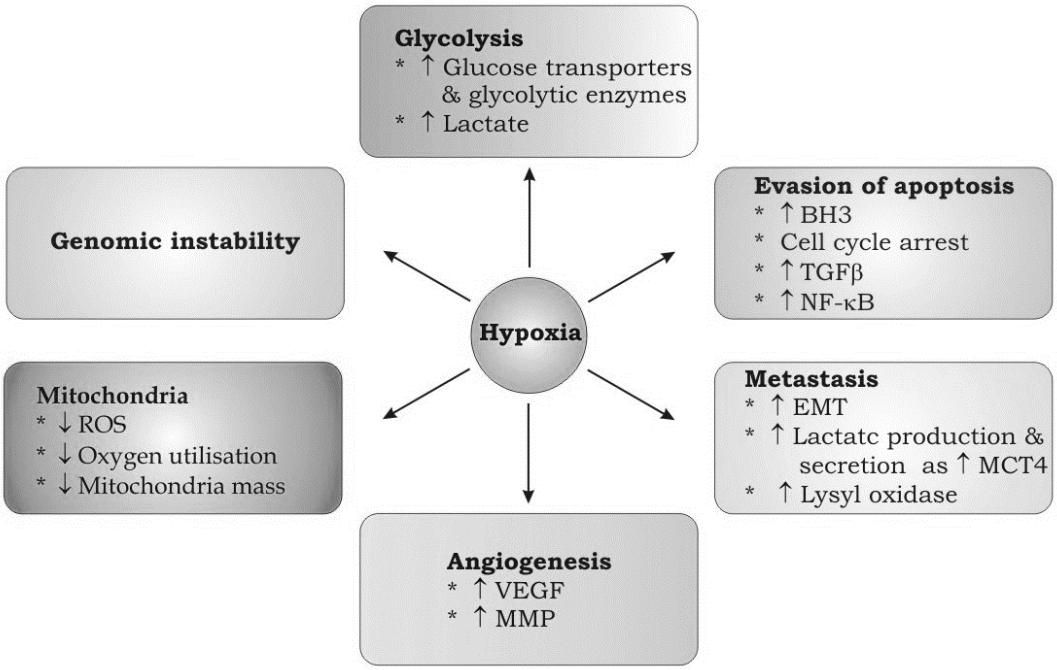


Figure 1.12 Biological effects of hypoxia: HIF is the major transcription factor activated as a result of tumour hypoxia, and it regulates multiple genes to promote tumour angiogenesis, metastasis and survival besides regulating metabolism. It has been shown to upregulate GLUTs and multiples enzymes of the glycolytic pathway. HIF-1 also stimulates PDK1 which in turn inhibits PDH, an enzyme which catalyses the conversion of pyruvate into acetyl CoA. This leads to decreased oxygen utilisation for energy production and reduced oxidative stress. Hypoxia can also inhibit mTOR, the master regulator of cellular metabolism. HIF-1 has been shown to upregulate VEGF and MMP which promote angiogenesis. HIF-1 has also been shown to promote epithelial mesenchymal transition (EMT). Lysyl oxidase (LOX) secreted by hypoxic tumour cells helps in creating a pre-metastatic niche. HIF-1 also upregulates MCT4 which plays a vital role in transporting lactate out of the cell leading to an acidic microenvironment. HIF-1 upregulates TGFβ which in turn activates downstream activators such as snail, slug and twist which antagonise p53 mediated apoptosis and promote chemo and radio-resistance. HIF has been shown to upregulate BH3 only protein BNIP3 which may contribute to regulation of apoptosis by HIF. Hypoxia can also activate NF-κB which plays an essential role in DNA

transcription, cytokine production and cell survival. However severe hypoxia can lead to p53 mediated cell death.

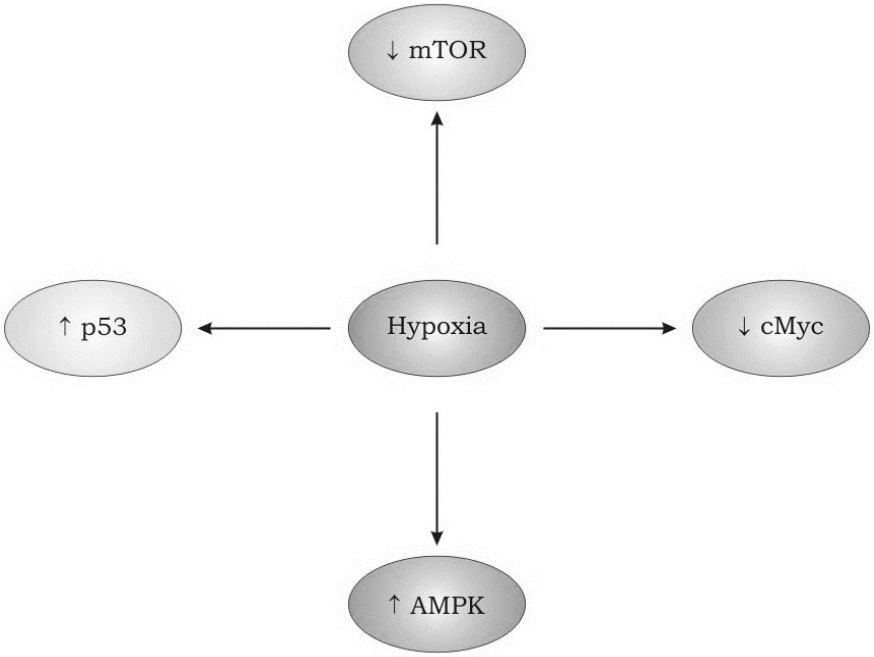


Figure 1.13 Hypoxia mediated regulatory pathways: Hypoxia can lead to fall in ATP level and rise in AMP level which can activate AMPK. This, in turn, can inhibit mTOR. Hypoxia can also inhibit mTOR via AMPK independent method via REDD1. Via p53 regulation HIF plays an important role in metabolism, cell cycle control and apoptosis. In basal conditions, p53 is kept at very low levels by MDM2 mediated ubiquitination and subsequent proteasomal degradation. It is thought that HIF-1α may suppress MDM2 mediated p53 ubiquitination. By interfering with myc-MAX interaction, hypoxia can inactivate *myc*.

1.7.4 Hypoxia as a prognostic marker

HIF-1 expression correlates with aggressiveness of a tumour and poor survival (Semenza[a] 2010, Muz 2015). A number of studies have shown that hypoxic tumours carry a poorer prognosis with an increased incidence of metastasis and recurrence (Höckel 2001). Downregulation of HIF-1α has been shown to sensitise U251 glioma cells to TMZ treatment (Tang 2016). Knockdown of HIF-1α in glioma cells has been shown to reduce migration *in vitro* and invasion *in vivo* and impair their ability to form tumour spheres (Méndez 2010). Hypoxia also plays a role in resistance to chemotherapy and radiotherapy. Hypoxia has been shown to upregulate glycolysis, lactate and p53, all of which play a role in resistance to treatment (Brahimi-Horn 2007, Muz 2015). Radiotherapy depends upon the presence of oxygen to generate free radicals which damage the DNA. In hypoxia, lack of oxygen results in less free radical production and less sensitivity to radiotherapy. (Höckel 2001, Brahimi-Horn 2007, Weljie 2011, Muz 2015). Chemotherapy agents such as bleomycin also require free radical for effective

cell killing whereas low pH of the extracellular environment in hypoxia can lead to decreased uptake of drugs such as doxorubicin by the cell (Kizaka-Kondoh 2003).

1.7.5 Targeting hypoxia for cancer therapy

HIF-1 is an attractive target as it has low expression in normal cells (Denko 2008). However, delivery of drugs to the hypoxic area of a tumour may be an issue. Another issue in targeting hypoxia is that all cells within a tumour may not be hypoxic; hence monotherapy may not be a solution.

1.7.5.1 Bioreductive drugs

Bioreductive drugs are chemicals which can be acted upon by enzymes in the body to a reduced toxic form. Tirapazamine (TPZ) and AQ4N belong to this category. In hypoxia, TPZ is reduced to a radical, which induces DNA damage. Similarly, AQ4N is reduced to AQ4 in hypoxia which induces DNA damage.

Phase II study of TPZ and radiation therapy for glioblastoma showed that survival in patients treated with radiation and TPZ was equivalent to the control population. Patients treated with radiation and TPZ (159 mg/m^2) had a longer survival when compared with the historical controls however this was not statistically significant (Del Rowe 2000). Phase I study of the prodrug AQ4N in patients with advanced malignancies showed that AQ4N was well tolerated when administered weekly on a 3-of-4-week schedule (Papadopoulos 2008).

1.7.5.2 HSP90 inhibitors

Hsp90 plays a vital role in stabilisation of HIF-1α in hypoxia. Geldanamycin and its analogue 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) have been shown to bind to Hsp90 and interfere with HIF-1α stabilisation. Geldanamycin has been shown to induce mitotic catastrophe and subsequent apoptosis in human glioma cells in a caspase-dependent manner (Nomura 2004). 17-AAG has been shown to inhibit the growth of glioma cells *in vitro* and *in vivo* (Newcomb 2007). 17AEP-GA has been shown to be a potent inhibitor of GBM cell proliferation, survival, migration and invasion *in vitro* (Miekus 2012).

1.7.5.3 Topoisomerase inhibitors

Topoisomerase are enzymes which wind and unwind DNA and hence play an important role in protein synthesis. Topotecan has been shown to form a complex with DNA/topoisomerase 1 complex and

induce DNA strand breaks (Mooring 2011). A phase I/II study of oral topotecan in children with recurrent or progressive high-grade glioma showed that topotecan (median dose 0.9 mg/m^2 per day) was well tolerated and somewhat effective in children with recurrent high-grade gliomas (Wagner 2004). Addition of topotecan to concurrent chemoradiotherapy in patients with GBM has been shown to be feasible with acceptable toxicity levels. Patients also benefited from an improvement in their symptoms and even patients with unfavourable prognosis factors had a higher median survival in comparison to similar groups of patients who were treated with radiotherapy only (Klautke 2006). Concurrent topotecan and radiation as primary treatment of GBM showed a 16% increase in 6 month PFS for patients receiving topotecan with radiation as compared with patients having radiotherapy alone (Grabenbauer 2009). U.S. Food and Drug Administration (FDA) has approved topotecan for relapsed small cell lung cancer (O`Brian 2006). In June 2006, FDA approved topotecan in combination with cisplatin for the treatment of stage IVB recurrent or persistent cervical cancer which was not amenable to curative treatment with surgery and/or radiotherapy. Topotecan has also been approved by FDA as a single agent for the treatment of patients with metastatic carcinoma of the ovary after disease progression on or after initial or subsequent chemotherapy (Madhok 2011).

1.7.5.4 2-ME

2-ME has been shown to downregulate HIF-1 at the post-transcriptional level and inhibit HIF-1 induced VEGF transcription. 2-ME has been shown to induce glioma cell death *in vitro* (Braeuninger 2005). It has also been shown to induce a dose-dependent inhibition of brain tumour growth in xenograft models (Kang 2006). 2-ME has also been shown to enhance the sensitivity of glioma cell lines to radiotherapy (Zou 2007).

1.7.5.5 Bortezomib

Bortezomib has been shown to inhibit the expression of HIF target genes VEGF & erythropoietin and also inhibit the recruitment of p300 coactivator (Shin 2008). Bortezomib has been approved for the treatment of multiple myeloma. Phase I trial of bortezomib (1.3 mg/m^2) and concurrent TMZ and radiotherapy for CNS malignancies showed that the combination was safe and well tolerated (Kubicek 2009). Phase 1 clinical trial of bortezomib in adults with recurrent malignant glioma has shown some clinical activity (Phuphanich 2010). Another phase I study showed that bortezomib and whole brain irradiation for brain metastasis were well tolerated at one month follow up, though greater demyelination in hippocampus-associated white matter structures was seen in this group (Lao 2013).

1.7.5.6 Bevacizumab

Bevacizumab is a monoclonal antibody against VEGF, a HIF-1 target gene. It has been approved for use as a first-line treatment in metastatic colorectal cancer along with standard chemotherapy. It has also been approved for use as a first-line treatment in advanced non-squamous non-small cell lung cancer in combination with carboplatin/paclitaxel. A phase II study of bevacizumab in combination with TMZ in patients with recurrent GBM showed that the combination was well tolerated (Sepúlveda 2015). Phase II trial of upfront bevacizumab, irinotecan, and TMZ for unresectable GBM showed that the combination was well tolerated and can lead to a radiographic response in unresectable and/or sub totally resected GBM (Peters 2015). A trial combining bevacizumab and temsirolimus in paediatric patients with refractory CNS tumours showed that the combination was well-tolerated and resulted in stable disease of at least four months (partial response in three out of six paediatric patients) (Piha-Paul 2014). A phase II trial of radiation, TMZ, erlotinib, and bevacizumab for initial treatment of GBM showed that that the combination of bevacizumab, erlotinib, TMZ, and radiotherapy was well tolerated and led to improved PFS but did not reach the primary endpoint of improved overall survival (OS) (Clarke 2014). However, in some studies, though bevacizumab led to reduced tumour growth, the tumours were more invasive with more metastatic potential (Mountzios 2014, Muz 2015).

1.7.6 Conclusion

Hypoxia is an attractive target for cancer therapeutics as it plays a vital role in cancer biology and regulates multiple genes to promote tumour angiogenesis, metastasis and survival besides regulating metabolism.

1.8 PI3K-Akt-mTOR pathway and cancer

1.8.1 Introduction

Serine/threonine kinase Akt belongs to AGC kinase family and is composed of 3 conserved domains; N-terminal pH domain, central kinase catalytic domain and a C-terminal extension which contains the regulatory hydrophobic motif (Porta 2014). Akt plays an essential role in regulating metabolism, cell growth, proliferation, angiogenesis and metastasis (Testa 2001). PI3K/Akt pathway has also been shown to affect response to treatment as it modulates cell survival pathways (Fresno Vara 2004).

mTOR is a serine/threonine kinase and belongs to the PI3K related kinase family. It acts through 2 distinct complexes called mTORC1 and mTORC2. mTORC1 is composed of mTOR, raptor, mLST8 and

PRAS40. It is highly sensitive to rapamycin. mTORC2 is composed of mTOR, Rictor, mLST8 and sin1 and it is not sensitive to rapamycin (Laplante 2012, Kaeberlein 2013, Porta 2014). Both are essential for cell survival, and loss of either raptor or rictor results in decreased cell viability (Kaeberlein 2013). Besides cancer, mTOR dysregulation has also been shown to play a role in obesity, type2 diabetes and neurodegenerative diseases (Laplante 2012, Kaeberlein 2013). It is thought that mTOR hyperactivation can lead to translation of some specific messenger RNA (mRNA) which plays an important role in tumorigenesis by modulating metabolism, angiogenesis, metastasis, cell growth, proliferation and survival (Laplante 2012).

1.8.2 Regulation of PI3K-Akt pathway

PI3K belong to lipid kinase family which can phosphorylate the hydroxyl group on the 3rd position of the inositol ring of phosphatidylinositol (Fresno Vara2004). Class 1 PI3K are heterodimers consisting of a catalytic unit (p110) and a regulatory unit (p85). Three isoforms (α, β and γ) of catalytic subunit p110 are described. Class 1 PI3K is subdivided into 1A PI3K which is activated by receptor protein tyrosine kinase (RPTK) and class 1B which is activated by receptor coupled with G-proteins (Fresno Vara 2004). Activated class 1 PI3K acts on phosphatidylinositol 4,5-bisphosphate (PIP2) to generate second messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Testa 2001, Vivanco 2002, Fresno Vara2004).

Akt can be activated by various mechanisms including mutations in the Akt gene itself. PTEN dephosphorylates PIP3 to PIP2 and prevents Akt activation. PTEN mutation, seen in cancers such as GBM, prostate and endometrial cancer can lead to Akt activation (Fresno Vara 2004). Activation of PI3K, overexpression of growth factors such as EGFR in GBM and ErbB2 in breast cancer can also activate Akt (Altomare 2005).

Anaplastic large cell lymphoma, GBM, small cell lung carcinoma, gastric carcinoma, multiple myeloma, ovarian thyroid and pancreatic cancer show frequent Akt activation (Altomare 2005). Akt hyperactivation has also been correlated with resistance to chemotherapy in a number of cancer cell lines such as non-small cell lung cancer, ovarian and pancreatic cancer (Fresno Vara 2004, Altomare 2005).

1.8.3 Biological effects of Akt activation

Akt phosphorylates and inactivates TSC leading to mTOR activation thus upregulating protein and lipid biosynthesis including HIF levels. Via HIF-1, Akt contributes to the Warburg effect (Manning 2007). Akt upregulates GLUT4 translocation to the cell membrane (Manning 2007). Akt also promotes the association of HK II to mitochondria for more effective phosphorylation of glucose to glucose 6-phosphate (Manning 2007). Akt has been shown to support aerobic glycolysis even in non-transformed cells when overexpressed. Akt has also been shown to activate ACL which plays a vital role in fatty acid biosynthesis (Manning 2007).

Akt has been shown to exhibit anti-apoptotic activity by preventing the release of cytochrome c from the mitochondria. Akt also promotes binding of HK II to the mitochondria and increases mitochondrial membrane integrity and inhibits apoptosis (Majewski 2004). It has also been shown to phosphorylate and inactivate pro-apoptotic BAD and procaspase-9. Glycogen synthase kinase 3β (GSK3β) has been shown to inhibit Mcl-1 and Akt by inhibiting GSK3β upregulates anti-apoptotic Mcl-1 (Manning 2007).

Akt by phosphorylating and inactivating GSK also promotes glycogen synthesis which plays a vital role in tumour growth. GSK3β plays an important role in the proteasomal degradation of G1 cyclins and SREBP1, and hence GSK3β inactivation also leads to increased stability of these cyclins and promotes lipid biosynthesis (Zhou 2001, Testa 2001, Vivanco 2002, Liang 2002, Manning 2007). Akt has also been shown to be involved in the activation of endothelial nitric oxide synthase which has been shown to play an essential role in angiogenesis and vascular tone. (Testa 2001, Manning 2007). Akt also promotes invasion and metastasis by upregulating MMP expression and epithelial mesenchymal transition (EMT) (Testa 2001, Altomare 2005, Manning 2007) (Figure 1.14).

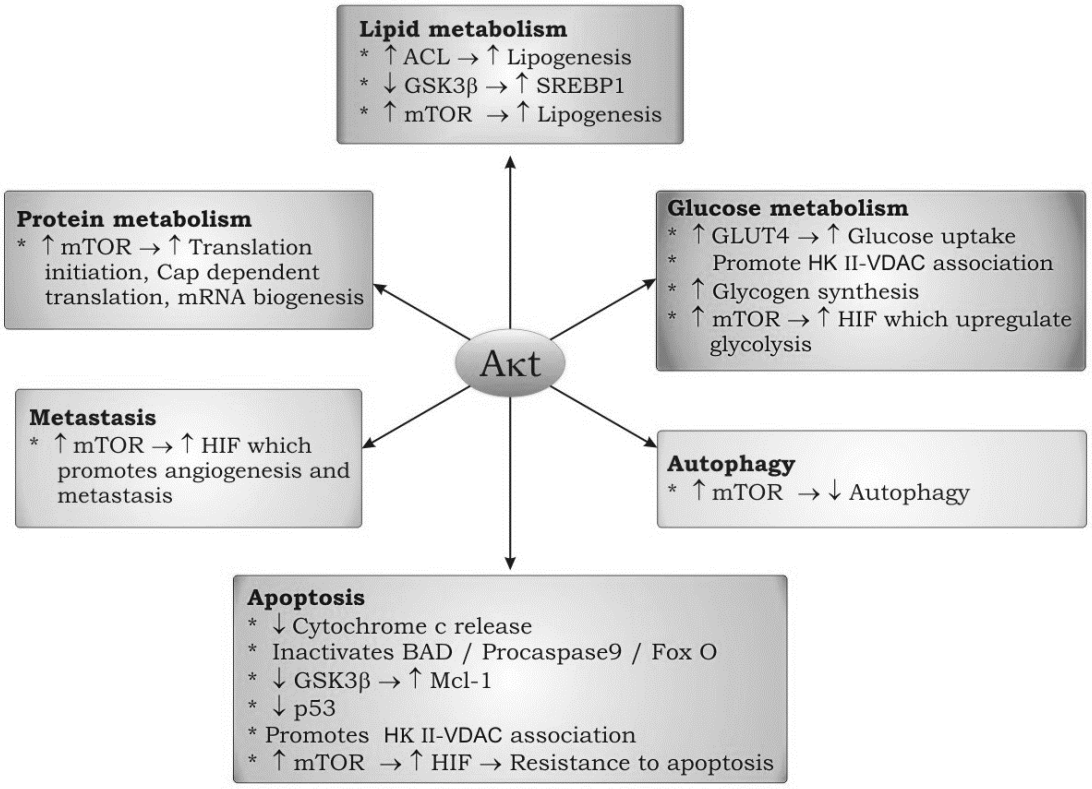


Figure 1.14 Biological effects of Akt activation: Akt has been shown to phosphorylate and inactivate TSC leading to mTOR activation thus upregulating protein and lipid biosynthesis including HIF levels. Via HIF-1, Akt contributes to the Warburg effect and promotes angiogenesis and metastasis. Akt via mTOR also inhibits autophagy. Akt also upregulates glucose uptake by the cell as it increases GLUT4 translocation to the cell membrane. Akt has been shown to exhibit anti-apoptotic activity by preventing the release of cytochrome c from mitochondria. It also promotes binding of HKII to the mitochondria and increases mitochondrial membrane integrity and inhibits apoptosis. Akt has also been shown to phosphorylate and inactivate pro-apoptotic BAD and procaspase-9. Akt also inactivates forkhead transcription factors, which induces expression of pro-apoptotic factors such as BIM and Fas ligand. Akt has also been shown to phosphorylate and activate cAMP response element-binding protein (CREB) and IκB kinase, which upregulates NF-κB which in turn results in transcription of anti-apoptotic genes. Via MDM2 phosphorylation, Akt has also been shown to inhibit expression of p53 regulated genes involved in cell cycle arrest and apoptosis. GSK3β has been shown to inhibit Mcl-1 and Akt by inhibiting GSK3β upregulates anti-apoptotic Mcl-1. Akt promotes glycogen synthesis which plays an essential role in tumour growth by phosphorylating and inactivating GSK3β. GSK3β has been shown to play an important role in the proteasomal degradation of G1 cyclins, and SREBP1 and hence GSK3β inhibition leads to increased stability of these cyclins and promotes lipid biosynthesis. Akt has also been shown to activate ACL which plays an essential role in fatty acid biosynthesis.

1.8.4 Regulation of mTOR pathway

mTORC1 receives input from insulin, IGF-1, energy status, oxygen and amino acid availability, oxidative stress, change in pH and various other stresses a cell is exposed to, to regulate cellular processes such as metabolism, autophagy, cell growth and survival (Laplante 2012). TSC, a heterodimer consisting of TSC1 or hamartin) and TSC2 or tuberin is an upstream regulator of mTORC1. They function as GTPase

activating protein (GAP) for Ras homolog enriched in brain (Rheb). Rheb when bound to guanosine-5'-triphosphate (GTP), can interact with mTORC1 and activate it. TSC1 and TSC2 convert GTP bound to Rheb into guanosine diphosphate (GDP) and inactivate Rheb which prevents mTORC1 activation (Inoki 2003, Manning 2007).

Protein kinase B or Akt has been shown to phosphorylate TSC2 which results in its inactivation and destabilisation. This disrupts the interaction of TSC2 with TCS1 (Inoki 2002) leading to mTORC1 activation. Akt has also been shown to activate mTORC1 directing without the involvement of TSC1/TSC2 (Laplante 2012).

Extracellular signal regulated kinase 1/2 (Erk 1/2) has also been shown to induce post-translational inactivation of TSC2 which disrupts TSC1 -TSC2 interaction and activates mTORC1 (Ma 2005). Wnt pathway, which plays an important role in cell growth, proliferation, differentiation and migration has also been shown to activate mTORC1 via GSK3β inhibition. GSK3β has been shown to phosphorylate and activate TSC2 a negative regulator of mTORC1 (Inoki 2006). Pro-inflammatory cytokines such as TNFα and IkB kinase can also inactivate TSC1/2 and in turn activate mTORC1 (Laplante 2012). Amino acids such as leucine and arginine have also been shown to play an important role in mTORC1 activation by promoting translocation of mTORC1 from the cytosol to the lysosomal surface where it binds to Rheb for activation (Laplante 2012).

AMPK is activated in response to a fall in ATP/AMP ratio, hypoxia, changes in redox and pH status of the cell. AMPK, in turn, phosphorylates TSC2 and increases its GAP activity towards Rheb to inhibit mTORC1 (Inoki[a] 2003, Shackleford 2009). AMPK has also been shown to phosphorylate raptor directly leading to binding of 14-3-3 protein and inhibition of mTORC1 and this can explain the reason why some TSC deficient cells are still responsive to energy stresses (Gwinn 2008). DNA damage response can also inhibit mTOR. PTEN which plays an essential role in DNA damage response and DNA repair is a negative regulator of the PI3K/Akt pathway which is an activator of mTORC1 (Ming 2012). Hypoxia has also been shown to inhibit mTOR independent of AMPK. REDD1, a HIF-1 target gene has been shown to activate TSC2 which in turn inhibits mTORC1 (Brugarolas 2004). p53 has been shown to inhibit mTOR via AMPK upregulation. Loss of p53 which is a common event in many cancers can lead to mTOR hyperactivation (Feng 2005, Akeno 2015) (Figure 1.15)

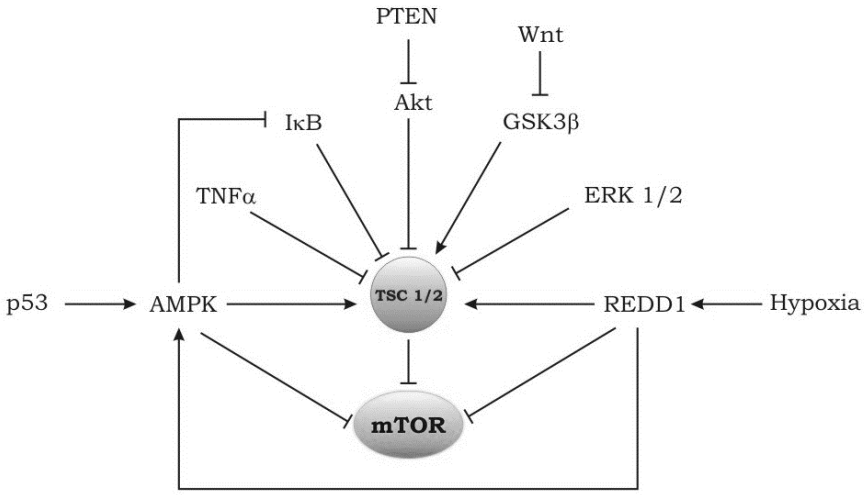


Figure 1.15 mTOR regulatory pathways: mTORC1 receives input from insulin, insulin-like growth factor and various stresses a cell is exposed to, to regulate cellular processes such as metabolism, autophagy, cell survival and growth. TSC is an upstream negative regulator of mTORC1. Akt has been shown to phosphorylate TSC2 which results in its inactivation and destabilisation. This disrupts the interaction of TSC2 with TCS1 which leads to mTORC1 activation. ERK 1/2 has also been shown to induce post-translational inactivation of TSC 2 which disrupts TSC1-TSC2 interaction and activates mTORC1. Wnt pathway has also been shown to activate mTORC1 via GSK3β inhibition. GSK3β has been shown to phosphorylate and activate TSC2 a negative regulator of mTORC1. Pro-inflammatory cytokines such as TNF and IkB kinase can also inactivate TSC1/2 and in turn activate mTORC1. AMPK has been shown to phosphorylate TSC2 and increases its GAP activity towards Rheb to inhibit mTORC1. AMPK has also been shown to phosphorylate raptor directly leading to binding of 14-3-3 protein and inhibition of mTORC1 and this can explain the reason why some TSC deficient cells are still responsive to energy stresses. Hypoxia via REDD1 has also been shown to inhibit mTOR independent of AMPK. PTEN which plays an essential role in DNA damage response and DNA repair is a negative regulator of PI3K/Akt pathway which is an activator of mTORC1. p53 has also been shown to inhibit mTOR via AMPK upregulation. Loss of p53 which is a common event in many cancers can lead to mTOR hyperactivation.

1.8.5 Biological effects of mTOR activation

mTORC1 plays a vital role in protein synthesis. mTORC1 phosphorylates translational regulators eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1). Phosphorylation of 4E-BP1 prevents its binding to cap-binding protein eIF4E and thus allows eIF4E to form complex with eIF4F which is necessary for cap-dependent translation. Phosphorylation of S6K1 leads to its activation. Ribosomal protein 6 which is a component of the 40s ribosome is a substrate for S6K1 and hence mTORC1 via S6K1 promotes mRNA biogenesis and translation initiation (Laplante 2012, Magnuson 2012).

mTORC1 also plays an essential role in lipid metabolism. SREBP 1/2 transcription factor controls the expression of a number of genes involved in cholesterol, fatty acid, triglyceride and phospholipid

biosynthesis. mTORC1 has been shown to upregulate SREBP1/2. mTORC1 has also been shown to upregulate peroxisome proliferation-activated receptor gamma (PPAR-γ) which is the master regular of adipogenesis and promotes fatty acid uptake, synthesis, esterification and storage in adipose cells (Laplante 2012).

mTOR also activates the transcription and translation of HIF-1 contributing to the Warburg effect. ULK1 is a serine-threonine kinase which is required to initiate autophagy. mTORC1 has been shown to phosphorylate and inhibit ULK1 (Jung 2011).

mTORC2 does not respond to variations in nutrient availability. However, growth factors such as insulin through PI3K promote mTORC2 ribosomal binding leading to mTORC2 activation (Zinzalla 2011), whereas mTORC2 has been shown to phosphorylate Akt which promotes full Akt activation (Vivanco 2002, Masui 2014). mTORC2 has also been shown to regulate *c-myc* expression by inactivating class IIa histone deacetylases which in turn inactivates forkhead box proteins FoxO1 and FoxO3. This prevents *c-myc* mRNA translation inhibition. mTORC2 also plays an essential role in lipid metabolism via Akt dependent and independent pathways (Masui 2014) (Figure 1.16).

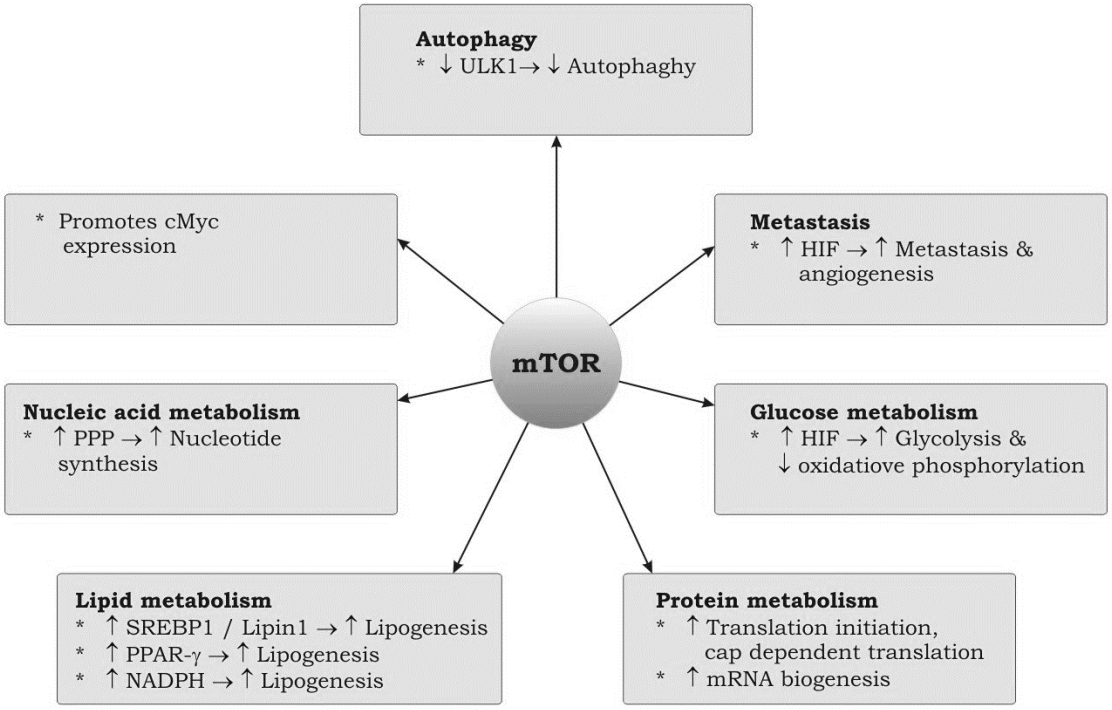


Figure 1.16 Biological effects of mTOR activation: mTORC1 plays an important role in protein synthesis. mTORC1 mediated phosphorylation and inhibition of 4E-BP1 promotes cap-dependent translation. mTORC1 via phosphorylation of S6K1 promotes mRNA biogenesis and translation initiation. mTORC1 also plays an essential role in lipid metabolism. SREBP 1/2 transcription factor, the master regulator of lipid synthesis has been shown to be upregulated by mTORC1. mTORC1 has also been shown to upregulate PPAR-γ which is the master regular of adipogenesis and promotes fatty acid uptake, synthesis, esterification and storage in adipose cells. mTOR also activates the transcription and

translation of HIF-1 and contributes to the Warburg effect, promoting growth, proliferation, metastasis, angiogenesis and survival. mORC1 has been shown to phosphorylate and inhibit ULK1 to downregulate autophagy. mTORC2 has been shown to phosphorylate Akt which promotes full Akt activation. mTORC2 also controls *myc* expression.

1.8.6 Targeting PI3K-Akt-mTOR pathway

1.8.6.1 ErbB1 inhibitors

The ErbB family of proteins consists of four receptor tyrosine kinases. ErbB1 is also called EGFR. Overexpression of EGFR in GBM has been shown to activate Akt (Altomare 2005).

Small molecule tyrosine kinase inhibitors competitively inhibit binding of ATP to the receptor which prevents autophosphorylation and kinase activation (Fresno Vara 2004). In November 2005, the FDA approved erlotinib in combination with gemcitabine for the treatment of locally advanced, unresectable, or metastatic pancreatic cancer. FDA has also approved erlotinib for the treatment of locally advanced or metastatic NSCLC which has failed at least one prior chemotherapy regimen. In July 2015, FDA approved gefitinib as a first-line treatment for NSCLC. Gefitinib either alone or in combination with TMZ or irinotecan have been trialled in GBM patients (Li 2016). Erlotinib either alone or in combination with sorafenib or bevacizumab or TMZ/radiation, or TMZ/carmustine or bevacizumab/TMZ has completed trials in glioma patients (Li 2016).

Monoclonal EGFR antibody cetuximab was approved by the FDA in March 2006 for use in combination with radiation therapy for treating head and neck squamous cell carcinoma (HNSCC) or as a single agent in patients who have had prior platinum-based therapy. Panitumumab was initially approved in September 2006 for EGFR expressing metastatic colorectal cancer with disease progression on or following fluoropyrimidine, oxaliplatin and irinotecan containing regimens. However, in July 2009, the FDA updated the labels of panitumumab and cetuximab for the treatment of metastatic colorectal cancer to include information about KRAS mutations as they have little or no effect in tumours with KRAS mutation.

1.8.6.2 ErbB2 inhibitor.

ErbB2 can form a heterodimer with ErbB3 and upregulate PI3K/Akt pathway (Fresno Vara 2004). Trastuzumab, a monoclonal antibody against ErbB2 has been approved for use in breast cancer patients with ErbB2 overexpression.

1.8.6.3 Akt inhibitor

Akt inhibitor miltefosine (6% topical solution) in phase III showed that it increases the time to treatment failure in cutaneous metastasis from breast cancer (Porta 2014). Akt inhibitors such as MK2206, RX-0201, PBI-05204, GSK690693 are currently undergoing trials (Porta 2014).

1.8.6.4 Pan-class 1 PI3K inhibitors

Pan-class 1 PI3K inhibitors such as GDC-0941, BKM120 and PX866 are undergoing trials for melanoma, non-Hodgkin`s lymphoma, HNSCC, breast, colorectal, endometrial and pancreatic cancer (Porta 2014). BKM120 in combination with surgery or bevacizumab or TMZ/radiation is under trial in glioma patients (Li 2016). PX-866 has also undergone trials in GBM patients (Li 2016).

1.8.6.5 Isoform-specific PI3K inhibitors

2nd generation Isoform-specific PI3Kα inhibitor GDC-0032 and PI3Kβ inhibitor GSK2636771 are undergoing trials for different solid tumours (Porta 2014). Isoform-specific PI3Kδ inhibitor CAL-101 and IPI-145 are also undergoing trials in various haematological malignancies (Porta 2014).

1.8.6.6 Targeting mTOR pathway

Presence of multiple signalling pathways regulating mTOR and being regulated by mTOR and the presence of multiple feedback loops is one of the main reasons for the limited efficacy of rapalogs in clinical practice. Combined mTORC1/2 inhibitors have shown more efficacy than rapalogs which only inhibits mTORC1.

Rapamycin, a macrolide produced by *Streptomyces hygroscopius* was first isolated from the soil in 1975 and showed anti-fungal and immunosuppressant effects (Laplante 2012, Kaeberlein 2013, Porta 2014). Rapamycin (sirolimus) has been shown to inhibit cancer cell growth in breast, lung, prostate, pancreas and kidney cell lines. It has also been shown to inhibit tumour growth in xenograft model of prostate, pancreas, kidney and breast cancers. Sirolimus has also been shown to synergise with gemcitabine and radiotherapy (Korsse 2013). However, due to unfavourable pharmacokinetics, its development has been hampered (Laplante 2012, Kaeberlein 2013, Porta 2014).

Following successful phase III trials, temsirolimus has been approved for the treatment of mantle cell lymphoma (Porta 2014). Following RECORD-1, a phase III double-blind randomised placebo-controlled trial everolimus has been approved for the treatment of advanced RCC after treatment failure with sunitinib and/or sorafenib (Porta 2014). Following RADIANT-1 a phase II trial and RADIANT-2/ RADIANT-3 phase III trials, everolimus has been approved for pancreatic neuroendocrine tumours (Porta 2014). BOLERO-2, a Phase III trial showed that everolimus and aromatase inhibitor exemestane lead to improved PFS as compared to exemestane alone in postmenopausal hormone receptor-positive advanced breast cancer patients (Porta 2014). Phase III of ridaforolimus showed improved PFS in advanced sarcoma patients as compared to placebo (Porta 2014). Following EXIST-1, a double-blinded placebo-controlled phase III trial, everolimus was approved by FDA for treatment of subependymal giant cell astrocytoma (SEGA) in patients with TSC who could not have surgery (Porta 2014). EXIST-2, a double-blinded placebo-controlled phase III trial showed that everolimus leads to a significant reduction in the size of renal angiomyolipoma associated with TSC or sporadic lymphangioleiomyomatosis (Porta 2014).

Sirolimus either alone or in combination with erlotinib has completed trial in GBM patients (Li 2016). Everolimus either alone or in combination with gefitinib or sorafenib or TMZ/radiation or bevacizumab/TMZ/radiation is under trial in GBM patients (Li 2016). Temsirolimus either alone or in combination with docetaxel or doxorubicin or TMZ/radiation or erlotinib or perifosine or bevacizumab is also under trial in GBM patients (Li 2016). Phase I study of sirolimus and vandetanib, an EGFR and VEGF receptor inhibitor in adults with recurrent glioblastoma showed that the combination is well tolerated (Chheda 2015). Another study showed that combination of temsirolimus and bevacizumab was well-tolerated and resulted in stable disease of at least four months (partial response) in three out of six paediatrics patients with chemo-refractory CNS tumours (Piha-Paul 2014). Phase II study of concurrent radiation therapy, TMZ and bevacizumab followed by bevacizumab/everolimus as the first-line treatment for patients with glioblastoma showed that the combination was safe and efficacious (Hainsworth 2012). Similarly, phase 1 study of daily everolimus combined with both concurrent radiation and TMZ followed by adjuvant TMZ showed that the combination was well tolerated with an acceptable toxicity profile (Chinnaiyan 2013). However, phase II trial of everolimus, TMZ and radiotherapy in patients with newly diagnosed GBM did not translate into an appreciable survival benefit compared with historical controls treated with conventional therapy (Ma 2015).

1.8.6.7 3rd Generation dual PI3K/mTOR inhibitors

mTOR inhibition can lead to feedback upregulation of PI3K/Akt pathway. Hence targeting both mTOR and PI3K/Akt pathways is more beneficial than just targeting mTOR. NVP-BEZ235 has been shown to bind to ATP binding cleft of mTOR and PI3k and inhibit both. It is undergoing trial in advanced breast, endometrial, renal and other solid tumours (Korsee 2013). NVP-BEZ235 has been shown to induce G1 cell cycle arrest and apoptosis in glioma cell lines. Combination treatment of TMZ and NVP-BEZ235 has been shown to synergistically inhibit glioma cell growth and induced apoptosis. In a xenograft model, a combination treatment of TMZ and NVP-BEZ235 has been shown to significantly reduce tumour growth rate and prolonged median survival of tumour bearing mice (Yu 2015). NVP-BEZ235 has also been shown to enhance the radio-sensitivity of glioma cells as well as glioma stem cells (Wang 2013, Gil del Alcazar 2014) (Figure 1.17).

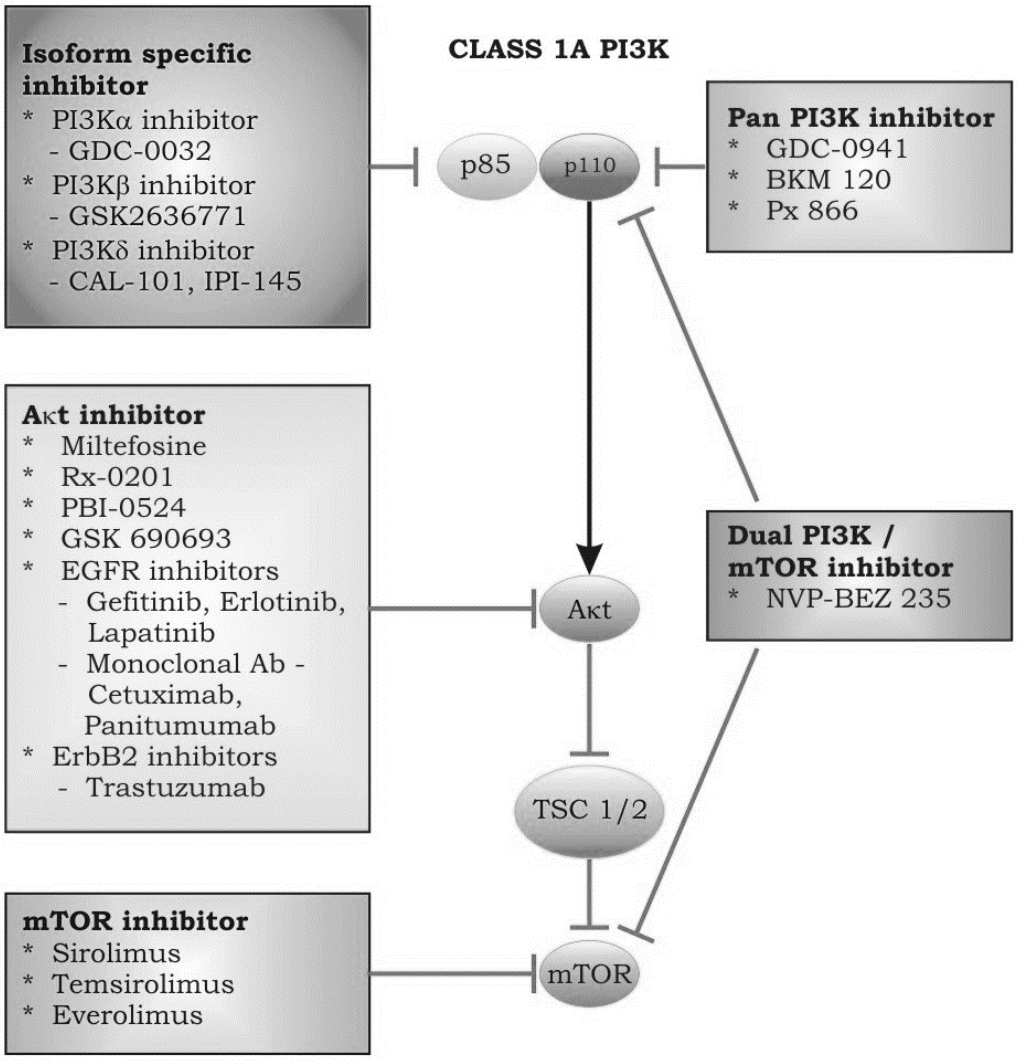


Figure 1.17 Targeting of PI3K/Akt/mTOR pathway: Overexpression of EGFR in GBM has been shown to activate Akt. Small molecule tyrosine kinase inhibitor gefitinib has been approved by the FDA as a first-line treatment for NSCLC. FDA has also approved erlotinib in combination with gemcitabine for the treatment of locally advanced, unresectable or metastatic pancreatic cancer. Erlotinib has also been

approved for the treatment of locally advanced or metastatic non-small cell lung cancer that has failed at least one prior chemotherapy regimen. Phase III trials with monoclonal EGFR antibody panitumumab and cetuximab have shown encouraging results. Cetuximab was approved by the FDA in March 2006 for use in combination with radiation therapy for treating HNSCC as a single agent in patients who have had prior platinum-based therapy. Panitumumab was EGFR expressing KRAS wild-type metastatic colorectal cancer with disease progression on or following fluoropyrimidine, oxaliplatin and irinotecan containing regimens. Trastuzumab, a monoclonal antibody against ErbB2 has been approved for use in breast cancer patients with ErbB2 overexpression. Akt inhibitor miltefosine (6% topical solution) in phase III showed that it increases the time to treatment failure in cutaneous metastasis from breast cancer patients. Akt inhibitors such as MK2206, RX-0201, PBI-05204, GSK690693 are undergoing trials. Pan-class 1 PI3K inhibitors such as GDC-0941, BKM120 and PX866 are undergoing trials for melanoma, non-Hodgkin lymphoma, squamous cell carcinoma of head and neck, breast, colorectal, endometrial and pancreatic cancer. BKM120 in combination with surgery or bevacizumab or TMZ/radiation is also under trial in glioma patients. PX-866 has also undergone trial in glioblastoma patients. 2nd generation Isoform-specific PI3Kα inhibitor GDC-0032 and PI3Kβ inhibitor GSK2636771 are undergoing trials for different solid tumours. Isoform-specific PI3Kδ inhibitor CAL-101 and IPI-145 are also undergoing trials in various haematological malignancies. mTOR inhibitor temsirolimus has been approved for the treatment of mantle cell lymphoma. Everolimus has been approved for the treatment of advanced RCC after treatment failure with sunitinib and/or sorafenib. Everolimus has also been approved for pancreatic neuroendocrine tumours. Everolimus has also been approved by FDA for treatment of SEGA in patients with TSC who could not have surgery. NVP-BEZ235 has been shown to bind to ATP binding cleft of mTOR and PI3k and inhibit both. It is undergoing trial in advanced breast, endometrial, renal and other solid tumours. It has also been shown to induce G1 cell cycle arrest and apoptosis in glioma cell lines. Combination treatment of TMZ and NVP-BEZ235 has been shown to synergistically inhibit glioma cell growth and induced apoptosis. In a xenograft model, a combination treatment with TMZ and NVP-BEZ235 has been shown to significantly reduce tumour growth rate and prolonged median survival of tumour bearing mice. NVP-BEZ235 has also been shown to enhance radio-sensitivity of glioma cells as well as glioma stem cells. Phase 1 trial of NVP-BEZ235 has been successfully completed which showed stable disease response in some patients with melanoma, colon, endometrial and pancreatic cancer.

1.9 Endoplasmic reticulum stress and cancer

1.9.1 Introduction

ER plays a vital role in folding and post-translational modification of proteins (Healy 2009). Glucose deprivation, hypoxia, oxidative stress, aberrant calcium metabolism, viral infection and expression of mutant proteins can alter the protein folding capacity of ER and induce ER stress (Healy 2009, Chakrabarti 2011). If unfolded or misfolded proteins continue to accumulate in the ER, then it induces the UPR.

Growing tumours are frequently exposed to fluctuations in glucose and oxygen levels, which can be detrimental for growth, proliferation and survival. UPR may act as an adaptive mechanism to provide

growth and survival advantage to the proliferating tumour cells (Healy 2009, Backer 2011, Li 2012). Besides cancer, UPR has also been implicated in many other pathologies such as diabetes, myocardial ischaemia, atherosclerosis, Parkinson's disease and Alzheimer's (Ogata 2006, Chakrabarti 2011).

1.9.2 Glucose regulatory protein 78

Glucose regulatory protein 78 (GRP78) also known as immunoglobulin heavy chain binding protein (BiP) belongs to HSP70 family and is the best-characterised ER chaperone protein (Healy 2009, Li 2012). GRP78 represents the pro-survival arm of UPR. GRP78 has been shown to be upregulated in primary glioma samples and most glioma cell lines whereas it is undetectable in healthy brain tissue (Pyrko 2007, Lee 2007). The GRP78 expression has also been shown to correlate with proliferation rate of glioma cell lines and knockdown of GRP78 has been shown to retard the growth of tumour cells (Pyrko 2007, Lee 2007). In gliomas, overexpression of GRP78 has been thought to contribute to resistance to TMZ, etoposide, cisplatin and radiotherapy (Backer 2011), whereas knockdown of GRP78 has been shown to overcome drug resistance (Virrey 2008).

GRP 78 plays a vital role in protein folding & assembly and proteasomal degradation of misfolded proteins. It also functions as Ca^{2+} binding protein in the ER and thus prevents efflux of Ca^{2+} into the cytosol (Pyrko 2007, Chakrabarti 2011, Li 2012). Under non-stressed conditions, GRP78 is bound to transmembrane ER stress sensors protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), preventing their activation. However, under conditions of ER stress, GRP78 binds to the unfolded protein in the ER, thus freeing up stress sensors which lead to activation of multiple transcription programs (Pyrko 2007, Healy 2009, Backer 2011, Chakrabarti 2011, Li 2012).

Studies have shown that GRP78 promotes genomic instability, tumour cell proliferation, angiogenesis, metastasis, evasion of apoptosis and resistance to therapy (Healy 2009, Li 2012). GRP78 can promote genome instability and mutations through inhibition of p53 activity (Li 2012). GRP78 has also been shown to be involved in the regulation of cancer-associated inflammatory cytokines such as interleukin 6, interleukin 10 and TGFβ (Li 2012). GRP78 can interact with ER localised caspase 7 and prevent activation of pro-apoptotic Bcl-2 proteins such as BAX and in turn inhibit release of cytochrome c from the mitochondria (Healy 2009, Chakrabarti 2011, Li 2012). Upregulated GRP78 can also form complex with Bcl-2 interacting killer (BIK) which leads to reduced Bcl-2 sequestration. GRP78 has been shown to activate p38, Erk 1/2, PI3K and NF-κB signalling cascade which can promote cell survival (Li 2012). GRP78 is also expressed on the surface of endothelial cells, and GRP878 knockdown

has been shown to reduce tumour angiogenesis (Dong 2008, Li 2012). GRP78 has also been shown to protect cancer cells from immune surveillance (Park 2007, Li 2012).

Though UPR is primarily a pro-survival response, in the event of prolonged or severe ER stress, UPR can also initiate cell death program (Healy 2009). CCAAT/enhancer-binding protein homologous protein (CHOP) represents the pro-apoptotic arm of UPR and has been shown to sensitise cells to ER stress-induced apoptosis through upregulation of Bim, downregulation of bcl-2 and activation of ER oxidase (Pyrko 2007). CHOP has also been shown to upregulate TNF-related apoptosis-inducing ligand (TRAIL) receptors and promote extrinsic pathway of apoptosis. CHOP can also activate JNK (Healy 2009) which in turn can phosphorylate Bcl-2 leading to disassociation of Bcl-2 from beclin 1 and thus activating autophagy (Chakrabarti 2011). There is also cross-talk between ER and the mitochondria during apoptosis. During ER stress, there is a release of Ca^{2+} from the ER into the cytosol which can be then taken up by the mitochondria leading to activation of mitochondrial apoptosis (Chakrabarti 2011).

PERK is a type I transmembrane protein composed of an ER luminal stress sensor and a cytosolic protein kinase domain. Upon disassociation from GRP78, PERK homodimerizes and becomes autophosphorylated generating activated PERK (Healy 2009). It phosphorylates eIF2α to inhibit cap-dependent protein translation which leads to global attenuation of translation initiation which in turn reduces the influx of proteins into the ER to decrease protein folding load (Chakrabarti 2011). However, cap-independent protein translation continues thus allowing synthesis of critical proteins which mediate the UPR itself. This includes transcription factor ATF 4 which induces ER chaperones, proteins involved in regulation of amino acid metabolism and resistance to oxidative stress, all of which promote cell survival (Healy 2009). However, ATF4 also promotes CHOP expression. So, PERK activation is initially protective, but in case of prolonged ER stress, it can promote cell death (Healy 2009, Chakrabarti 2011).

ATF6 activation leads to a pro-survival transcriptional program (Chakrabarti 2011). Upon disassociation from GRP78, ATF6 translocates to the Golgi apparatus, where it is cleaved by specific proteases 1 and 2 (SP-1 and SP-2). Active ATF6 then translocates to the nucleus where it induces transcription of genes including GRP78, GRP94, protein disulphide isomerase and XBP1. These protein functions to counteract ER stress and hence have a pro-survival role (Healy 2009, Chakrabarti 2011).

IRE1 is a 100 kDa type 1 ER transmembrane protein which acts as both serine-threonine kinase as well as endoribonuclease (Healy 2009, Chakrabarti 2011). IRE1 initiates a program with both pro-survival and pro-apoptotic components (Chakrabarti 2011). Upon disassociation from GRP78, IRE1 undergoes homo-oligomerisation and autophosphorylation to generate activated IRE1 (Healy 2009, Chakrabarti

2011). Endoribonuclease activity results in unconventional splicing of XBP1 mRNA to form a potent transcription factor which codes for a diverse set of genes including ER chaperones (Healy 2009, Chakrabarti 2011). Activated IRE1 also plays a role in JNK, p38 MAPK, NF-κB and ERK activation. IRE1 has also been shown to directly interact with BAX/BAK (Chakrabarti 2011) (Figure 1.18).

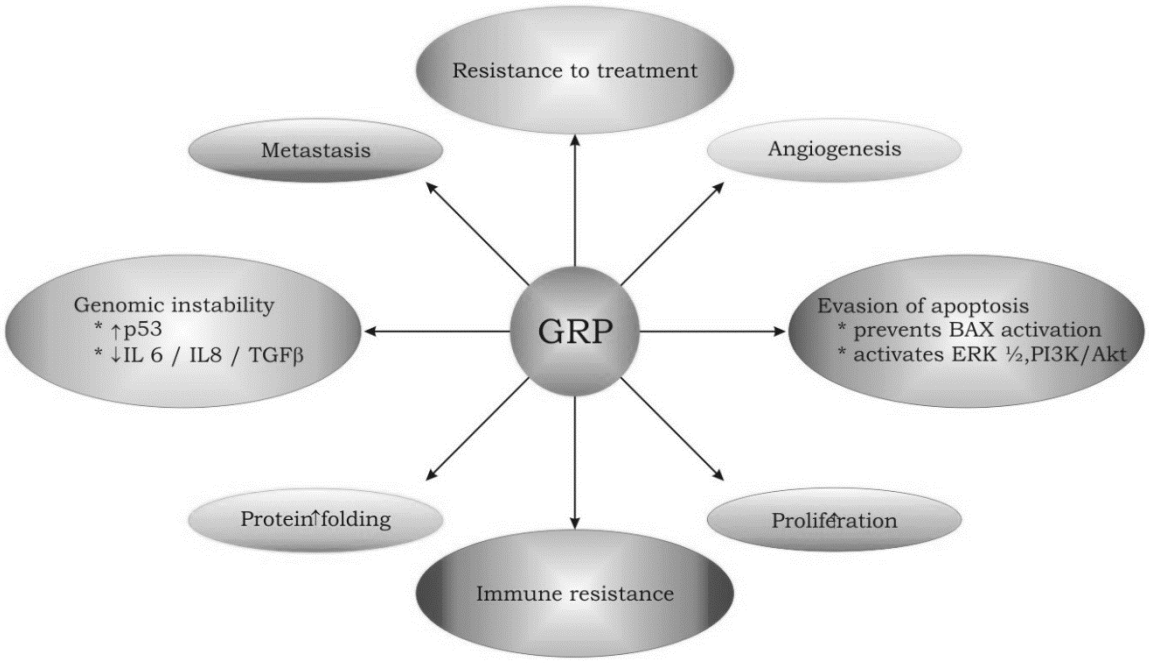


Figure 1.18 Biological effects of GRP: GRP78 plays an important role in protein folding & assembly and proteasomal degradation of misfolded proteins. It also functions as Ca^{2+} binding protein in the ER and thus prevents efflux of Ca^{2+} into the cytosol. GRP78 represents the pro-survival arm of UPR. It also promotes genomic instability, tumour cell proliferation, angiogenesis, metastasis, evasion of apoptosis and resistance to therapy.

1.9.3 Targeting ER stress

Untransformed cells are usually not subjected to ER stress whereas proliferating tumour cells are exposed to fluctuations in glucose and oxygen availability and frequently exhibit ER stress and UPR induction. Thus, a therapeutic window may exist to target tumour cells (Healy 2009). Naturally occurring compounds such as genistein present in soy, EGCG present in green tea, salicylic acid from plants and versipelostatin isolated from *Streptomyces versipellis* have been shown to inhibit GRP78 which is the pro-survival arm of UPR. Proteasome inhibitor such as bortezomib is thought to cause accumulation of misfolded proteins which can lead to ER stress and cell death. HSP90 inhibitors such

as 17-AAG and 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) have been shown to activate all the three branches of the UPR. Brefeldin A, an inhibitor of protein transfer from the ER to the Golgi apparatus has also been shown to induce ER stress and trigger apoptosis in many cancer cell lines. Protease inhibitor nelfinavir and atazanavir have been shown to induce cell death in glioma cells due to induction of UPR (Healy 2009).

1.10 Modes of cell death

1.10.1 Introduction

Cell death can be classified according to morphological characteristics (apoptosis, necrosis or autophagy), enzymes involved (with or without involvement of nuclease, caspase, calpain or cathepsin), immunological features (immunogenic or non-immunogenic) or functional characteristics (physiological or pathological, programmed or accidental) (Kroemer 2009, Duprez 2009).

1.10.2. Apoptosis

1.10.2.1 Introduction

The term apoptosis was first used by Kerr, Wyllie and Currie in 1972 (Kerr 1972) and it is the most widely studied form of cell death (Ma 2015).

Morphologically apoptosis is characterised by rounding up of the cell, cell shrinkage (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), retraction of pseudopods, plasma membrane blebbing and finally formation of apoptotic bodies (Elmore2007, Kroemer 2009, Duprez 2009, Ma 2015). Apoptotic bodies consist of cytoplasm with tightly packed organelle with or without a nuclear fragment, which is then recognised and engulfed by surrounding cells and phagocytes (Elmore 2007 Duprez 2009, Ma 2015) (Figure 1.19). As the integrity of the plasma membrane is maintained during the process of apoptosis, apoptotic cell death does not elicit an immune reaction or induce inflammation (Elmore 2007, Duprez 2009).

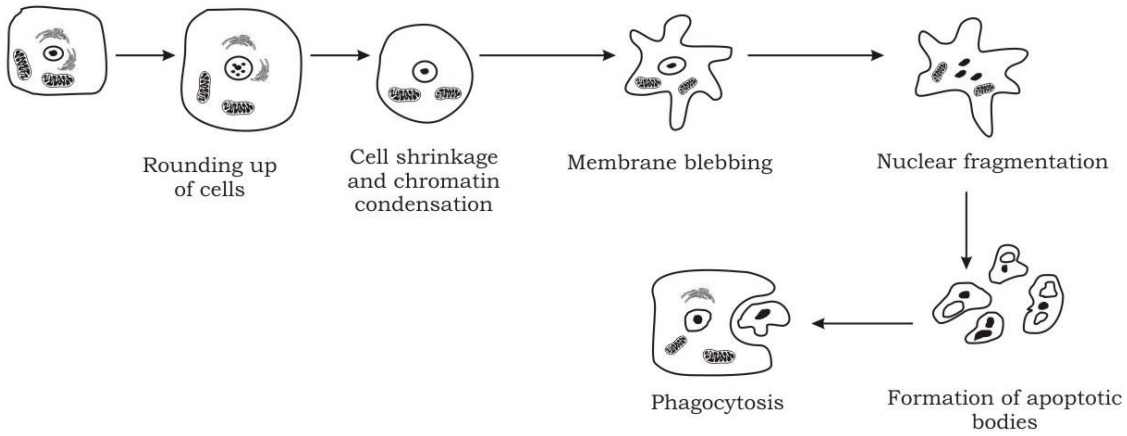


Figure 1.19 Mechanism of apoptosis: Apoptosis is characterised by rounding up of the cell, pyknosis, chromatin condensation, plasma membrane blebbing, karyorrhexis, retraction of pseudopods, and finally formation of apoptotic bodies. Apoptotic bodies are then recognised and engulfed by surrounding cells and phagocytes. As the integrity of the plasma membrane is maintained during the process of apoptosis, apoptotic cell death does not elicit an immune reaction or induce inflammation.

Biochemically apoptosis is characterised by involvement of caspases, internucleosomal DNA fragmentation, degradation of cytoskeletal and nuclear proteins, protein cross-linking, externalisation of phosphatidylserine, changes in mitochondrial permeability and participation of Bcl-2 family member proteins (Elmore 2007, Ma 2015). Caspases are cysteinyl-aspartate specific proteases which can be further subdivided into initiator caspases such as caspase 2,8,9 and 10 and executioner caspases such as caspase 3,6 and 7. Initiator caspases are present as inactive monomers and are activated as a result of oligomerisation and autoproteolysis (Duprez 2009, Ma 2015).

Two distinct pathways of apoptosis have been described i) extrinsic pathway ii) intrinsic pathway.

The extrinsic pathway is activated by binding of death ligands such as TNF superfamily or TRAIL with their corresponding cell surface death receptors such as TNF receptor 1 or TRAIL receptor 1 and 2. This leads to the formation of death inducing signalling complex (DISC) which then leads to auto-catalytic activation of procaspase 8 which in turn then activates the executioner caspases (Elmore 2007, MacFarlane 2012, Ma 2015). Besides, caspase 8 can also cleave BH3 only protein Bid to activate the mitochondrial pathway of apoptosis and serve to amplify death receptor-induced cell death (Duprez 2009, MacFarlane 2012) (Figure 1.20).

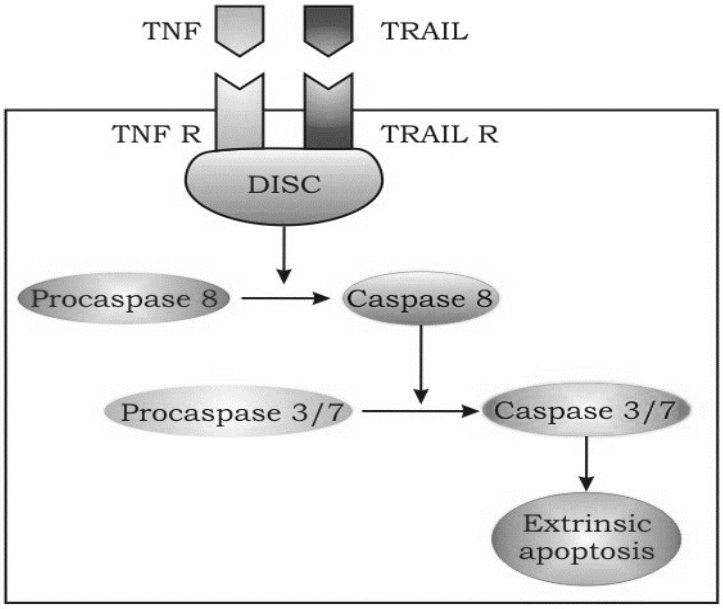


Figure 1.20 Extrinsic apoptosis: Extrinsic apoptosis pathway is activated by binding of death ligands such as TNF superfamily or TRAIL with their corresponding cell surface death receptors such as TNF receptor 1 or TRAIL receptor 1 and 2. This leads to the formation of DISC which then leads to autocatalytic activation of procaspase 8 which in turn then activate the executioner caspases 3 and 7.

The intrinsic pathway is activated by a number of stimuli such as DNA damage, cytotoxic insults, growth factor withdrawal and oxidative stress and acts through mitochondria which are controlled by Bcl-2 family of proteins (Duprez 2009, Ma 2015). In non-stressed conditions, anti-apoptotic Bcl-2 proteins prevent pro-apoptotic Bcl-2 proteins from causing mitochondrial damage and thus help to maintain mitochondrial integrity (Duprez 2009). When cells are stressed, BH3 only proteins are activated which antagonise anti-apoptotic Bcl-2 proteins and relieve the inhibition of pro-apoptotic Bcl-2 proteins BAX and BAK. This can lead to BAX/BAK oligomerisation which can lead to the opening of MPTP, loss of mitochondrial transmembrane potential and MOMP. MOMP results in the release of cytochrome c into the cytosol which binds to APAF1 and caspase 9 to form apoptosome which then activates executioner caspases to propagate intrinsic pathway of apoptosis (Shi 2006, Elmore 2007, Duprez 2009, Park 2012, Ma 2015). During this process, other pro-apoptotic proteins such as Smac/Diablo, AIF, endonuclease G and caspase-activated DNase (CAD) are also released from the mitochondria which contribute to cell death (Elmore 2007, Duprez 2009) (Figure 1.21).

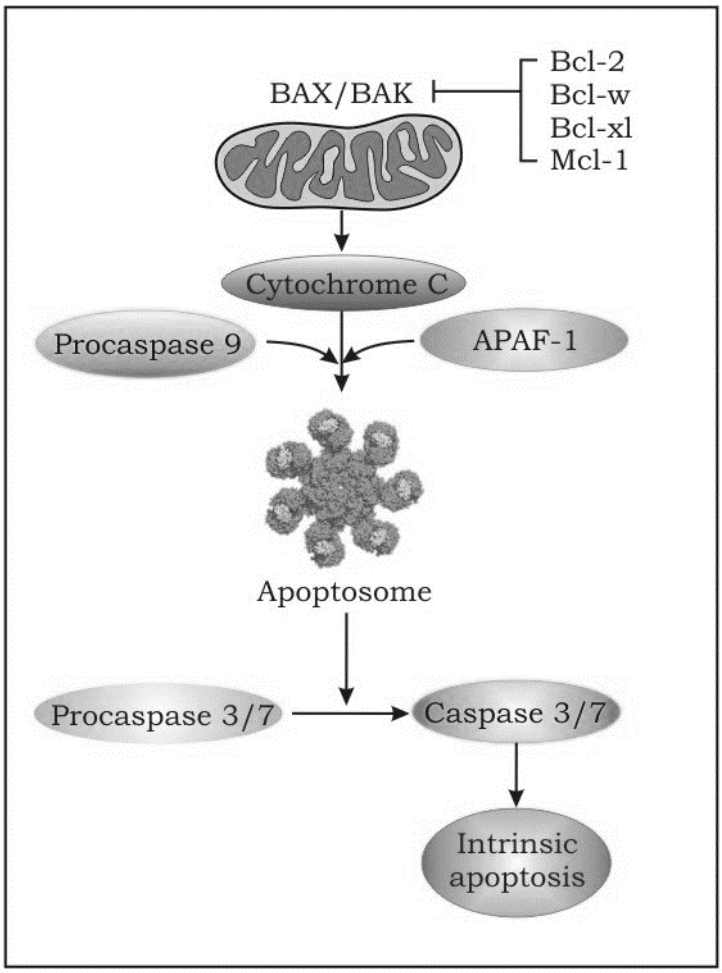


Figure 1.21 Intrinsic apoptosis: In non-stressed conditions, anti-apoptotic Bcl-2 proteins prevent pro-apoptotic Bcl-2 proteins from causing mitochondrial damage and thus help to maintain mitochondrial integrity. When cells are stressed, BH3 only proteins are activated which antagonise anti-apoptotic bcl-2 proteins which in turn relieve the inhibition of pro-apoptotic Bcl-2 proteins BAX/BAK. This can lead to BAX/BAK oligomerisation which can lead to the opening of MPTP, loss of mitochondrial transmembrane potential and MOMP. MOMP results in the release of cytochrome c into the cytosol which binds to APAF1 and caspase 9 to form apoptosome which then activates executioner caspases to propagate intrinsic pathway of apoptosis

Apoptosis has been shown to occur during normal development and ageing (Elmore 2007). Apoptotic cell death plays an important role in the development of the CNS and the immune system. During adulthood, apoptosis plays an important role in wound healing, post weaning mammary gland involution, post-ovulatory regression of uterus and in terminating an immune response by getting rid of activated immune cells (Elmore 2007, Duprez 2009).

1.10.2.2 Apoptosis assay

A cytomorphological assay such as transmission electron microscopy is considered the gold standard to confirm apoptosis. Other methods include detection of DNA fragmentation such as DNA ladder or Terminal dUTP Nick End -Labelling (TUNEL) method, detection of caspases and cleaved substrates, assay to detect membrane alterations such as FITC-labelled Annexin V and mitochondria-based assays such as assay for Bcl-2 member proteins, laser scanning confocal microscopy and cytochrome c assay.

1.10.2.3 Caspase inhibitors

ZVAD-fmk is used in experiments as a pan-caspase inhibitor. However, it does not act on all caspases with equal efficiency, and it may also have off-target effects as it can inhibit calpains and cathepsins at high concentrations. In certain circumstances, caspase inhibition by ZVAD-fmk has been shown to shift apoptotic cell death to necrotic or autophagic cell death or even a mixed cell death morphology (Kroemer 2009).

1.10.3 Autophagy

Autophagy is a genetically programmed and evolutionary conserved catabolic pathway which allows eukaryotes to degrade and recycle obsolete organelles and proteins (Duprez 2009, Ma 2015). It can be activated by intracellular stress such as accumulation of damaged organelles as well as extracellular stimuli such as nutrient starvation, hypoxia and high temperature (Ma 2015).

Autophagy is characterised by sequestration of degenerating cytoplasmic organelles or cytosol within a double-membrane vesicle called autophagosome. Autophagosome then fuses with the lysosome to form autophagolysosome. Inside the autophagolysosome, both inner membrane of the autophagosome, as well as its luminal contents, is degraded by acidic lysosomal hydrolases (Kroemer 2009, Ma 2015) (Figure 1.22). Beclin-1 plays an important role in autophagic vacuole formation whereas anti-apoptotic Bcl-2 family members repress autophagy by binding to beclin-1. During nutrient deprivation, Bcl-2 is phosphorylated which releases beclin-1 and stimulates autophagy (Duprez 2009).

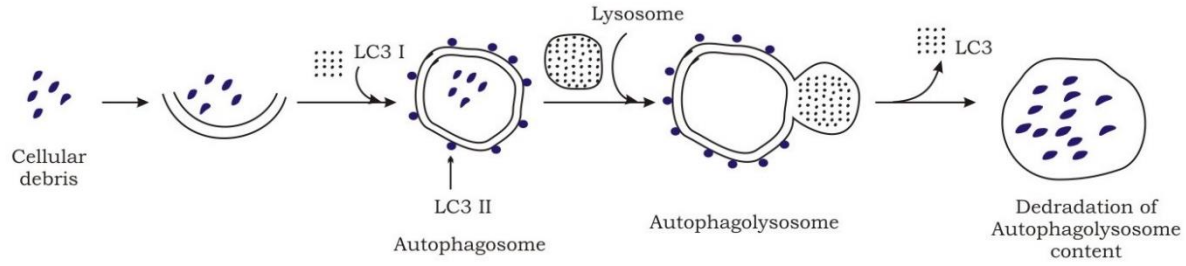


Figure 1.22 Mechanism of autophagy: - Autophagy is characterised by sequestration of degenerating cellular debris, cytoplasmic organelles or cytosol within a double-membrane vesicle called autophagosome. Autophagosome then fuses with the lysosome to form autophagolysosome. Inside the autophagolysosome, both inner membrane of the autophagosome, as well as its luminal contents, is degraded by acidic lysosomal hydrolases. Autophagosome formation requires the participation of cytoplasmic protein LC3 which undergoes lipidation by phosphatidylethanolamine and is the recruited to the autophagosome membrane.

However, there is controversy regarding the exact role of autophagy; some regard it as pro-survival whereas others regard it as a form of cell death. Studies have shown that knockdown of autophagy related genes (Atg) results in enhanced cell death and thus points to a pro-survival role of autophagy whereas other studies have shown that autophagy may participate in cell death as overexpression of Atg 1 kinase is sufficient to kill fat and salivary gland cells (Kroemer 2009).

Morphologically autophagy is characterised by autophagic vacuolization of the cytoplasm, absence of chromatin condensation with little or no association with phagocytes (Kroemer 2009).

Biochemically autophagy is characterised by an accumulation of LC3-II protein. Autophagy is regulated by autophagy-related genes which are required to activate signalling pathways which trigger the formation of autophagosomes. Autophagosome formation requires the participation of cytoplasmic protein LC3 (Atg8) which undergoes lipidation by phosphatidylethanolamine and is the recruited to the autophagosome membrane. Accumulation of lipidated LC3 protein (LC3-II) is used as a marker of autophagy (Duprez 2009, Ma 2015).

Studies have shown that autophagy has a role in a number of important cellular process such as growth, differentiation, immune response as well as ischaemia/reperfusion injury (Duprez 2009).

1.10.4 Necrosis

Necrotic cell death can be activated by a number of stimuli such as extreme physical, chemical, oxidative, osmotic or thermal stress, ischaemia, radiation or toxins (Ma 2015).

Morphologically necrosis is characterised by a gain in cell volume (oncosis), formation of cytoplasmic vacuoles and blebs, swelling of organelles, plasma membrane rupture and subsequent release of cytoplasmic contents into the extracellular space which triggers an inflammatory and autoimmune reaction (Elmore 2007, Kroemer 2009, Duprez 2009, Ma 2015). Necrosis is often accompanied by chromatin condensation and irregular DNA degradation pattern (Ma 2015) (Figure 1.23).

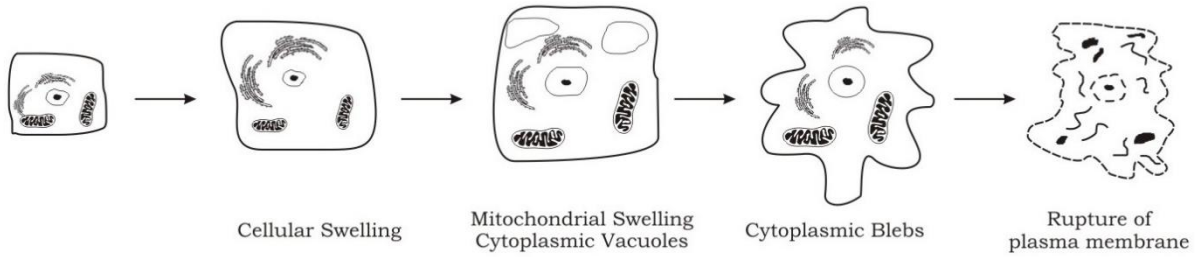


Figure 1.23 Mechanism of necrosis: Necrosis is characterised by a gain in cell volume (oncosis), formation of cytoplasmic vacuoles and blebs, swelling of organelles, plasma membrane rupture and subsequent release of cytoplasmic contents into the extracellular space which triggers an inflammatory and autoimmune reaction.

Biochemically, at present, there are no changes which can be used to define necrosis, and necrotic cell death is identified by the absence of apoptotic or autophagic markers (Kroemer 2009).

Earlier necrosis was regarded as a passive, ATP independent chaotic form of cell death (Okada 2004). However, there is emerging evidence that necrotic cell death may be finely regulated by signal transduction pathways (Kroemer 2009, Duprez 2009).

During necrosis, there is an increase in intracellular calcium level which can affect the ability of mitochondria to generate ATP; which is the molecular hallmark of necrosis. Increased intracellular calcium level can also activate calpains which in turn can disrupt lysosomal membranes. These changes lead to disruption of cell membrane and release of cellular contents into the extracellular space (Ma 2015).

Activation of death ligands associated with extrinsic apoptosis in conditions that are unfavourable for apoptosis such as when pro-apoptotic enzymes are absent or limited leads to a form of cell death which is defined as necroptosis (Duprez 2009, Ma 2015). Necroptosis or programmed necrosis has been shown to play a role in stroke, myocardial infarction and chemotherapy-induced cell death (Ma 2015). Biochemically necroptosis is defined as a form of cell death which is dependent on a serine-threonine kinase activator called RIP1 (Kroemer 2009, Ma 2015).

Necrotic cell death is seen during the longitudinal growth of bones in young animals, ovulation and is also found in intestinal epithelial cells in adults (Duprez 2009, Ma 2015).

1.10.5 ER mediated apoptosis

Nutrient deprivation or oxidative stress have been shown to activate caspase 4 located within the ER (Daniel 2000, Kim 2006, Heath-Engel 2008). There is downregulation of a number of anti-apoptotic proteins and upregulation of pro-apoptotic proteins and release of Ca^{2+} from the ER into the cytoplasm. Caspase 4, in turn, activates caspase 3 to initiate ER-mediated apoptosis. Ca^{2+} may also be taken up by mitochondria to cause initiate intrinsic pathway of apoptosis (Heath-Engel 2008, Rosati 2010).

1.10.6 Crosstalk between apoptosis-autophagy-necrosis

Components of the extrinsic apoptosis pathway have been shown to induce autophagy. Apoptosis and autophagy also share a number of molecules such as beclin and Bcl-2 which facilitates cross talk (Jagannathan 2015). Similarly, at low doses, many noxious stimuli such as heat, hypoxia, radiation and chemotherapy drugs can induce apoptosis whereas higher doses can result in necrosis (Elmore 2007).

As a result, type of cell death may be context dependent on cell type, the developmental stage of tissue, growth conditions as well as intensity and duration of treatment (Elmore 2007, Jagannathan 2015).

1.11 Glutamine and cancer

1.11.1 Introduction

Glutamine is the most abundant amino acid in the plasma (Bergström 1974). It is classified as a NEAA as mammals can synthesise glutamine in most tissues. However, some cells during rapid growth or other stresses have been shown to consume glutamine at a much higher rate as compared to other amino acids and in such circumstances, glutamine can become conditionally essential (Eagle 1955, Lacey 1990, DeBerardinis 2010).

1.11.2 Glutamine biosynthesis

Glutamine synthetase catalyses the condensation of glutamate and ammonia to form glutamine.

Glutamate + NH3+ ATP ---------> Glutamine + ADP + phosphate

1.11.3 Role of glutamine in intermediary metabolism

Glutamine plays a vital role in mitochondrial anaplerosis, and it is one of the primary reasons that glutamine is consumed in large quantities by proliferating cells (Deberardinis 2010, Daye 2012). During purine/pyrimidine and hexosamine biosynthesis glutamine donates its amide group and is converted into glutamate. Similarly, glutaminase (GLS) can release the amide group of glutamine as free ammonia and convert it into glutamate. Glutamate can then be converted into α-KG by either glutamate dehydrogenase in the mitochondria or by transaminases in the mitochondria as well as the cytosol to generate NEAA in the process (Hensley 2013).

Rapidly dividing cancer cells need to synthesise nucleotides which are the building blocks for DNA and RNA biosynthesis. Glutamine is the obligate nitrogen donor for many enzymatic reactions during purine and pyrimidine synthesis. During these reactions, glutamine donates its amide group and is converted into glutamate (Wise 2010). Importance of glutamine in nucleotide biosynthesis and proliferation is highlighted by the fact that K-ras transformed fibroblasts when cultured in glutamine starved media, show decreased proliferation and abortive S phase entrance which can be rescued by adding deoxyribonucleotides (Gaglio 2009). Glutamate generated can also act as a primary nitrogen donor via transaminase reactions to generate NEAA such as alanine, serine, aspartate and ornithine (Wise 2010, Hensley 2013).

Glutamine also plays a vital role in the redox balance of the cell. On the one hand, glutamine metabolism through the mitochondria has been shown to generate ROS whereas, on the other hand, glutamine is involved in GSH and NADPH synthesis which play an important role in cellular anti-oxidant defence (Wise 2010, Weinberg 2010, Daye 2012, Lyssiotis 2013). GSH is a tripeptide and consists of glutamate, cysteine and glycine (Wu 2004). Glutamine can lose amide group and generate glutamate either by the action of GLS or during nucleotide/hexosamine biosynthesis. Glutamate, in turn, can play a role in cystine uptake by the cell via X_c^- antiporter (Sato 1999, Lo 2008). GSH can reduce disulfide bonds of cytoplasmic proteins to cysteines by serving as an electron donor and has been shown to act as a direct scavenger of superoxide anion, singlet oxygen and hydroxyl radical. In the process, GSH is converted to its oxidised form, glutathione disulfide (GSSG). Glutathione reductase then uses NADPH as an electron donor to reduce GSSG to GSH , which can then again be used for

redox reaction (Pompella 2003) (Figure 1.24). In *myc* driven cells, it has been shown that glutamine carbon is preferentially used for GSH synthesis as compared to carbon from glucose (Le 2012).

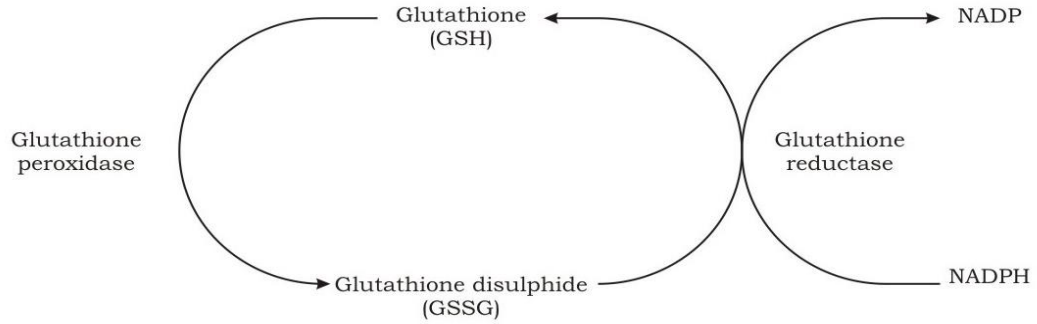


Figure 1.24 GSH anti-oxidant system: GSH is a tripeptide, and it consists of glutamate, cysteine and glycine. GSH can reduce disulfide bonds of cytoplasmic proteins to cysteines by serving as an electron donor and has been shown to act as a direct scavenger of superoxide anion, singlet oxygen and hydroxyl radical. In the process, GSH is converted to its oxidised form GSSG. Glutathione reductase then uses NADPH as an electron donor to reduce GSSG to GSH , which can then again be used for the redox reaction.

1.11.4 Role of glutamine in signal transduction

Nicklin *et al*. in 2009 showed that a proportion of glutamine taken up by the cell through SLC1A5 gene coded glutamine importer underwent rapid efflux through glutamine transporter coded by SLC7A5 gene in exchange for branched chain amino acids such as leucine which in turn activated mTORC1 independently of its upstream kinase (2009). Similarly, ROS generated via glutamine metabolism in the mitochondria can affect various signalling pathways and plays an important role in transformation, cell cycle progression & proliferation, resistance to cell death, metastasis, angiogenesis, cellular metabolism and maintenance of stemness; which are the hallmarks of cancer (Storz 2005, Waris 2006, Trachootham 2009, Liou 2010, Weinberg 2010, Daye 2012, Glasauer 2014).

1.11.5 ***Regulation of glutamine metabolism***

Myc activation/amplification is one of the most common oncogenic events. *Myc* driven tumours can be addicted to glutamine and glutamine deprivation can lead to apoptotic cell death (Wise 2008, Yuneva 2007, Wise 2010, Gao 2009). *Myc* as a transcription factor has been shown to upregulate glutamine transporters encoded by genes SLC1A5 and SLC38A5 (Fuchs 2005, Wise 2008, Wise 2010, Ren 2015). *Myc* has also been shown to upregulate GLS level through suppression of its negative regulator micro RNA 23a/b (Gao 2009). GLS which catalyses the breakdown of glutamine to glutamate

(Glutamine + H2O → Glutamate + NH3) exists in four isoforms which are encoded by GLS 1 and GLS 2 genes (Botman 2014). Tumour suppressor p53 has been shown to induce the expression of GLS 2 leading to increased oxidative phosphorylation and GSH production (Hu 2010) (Figure 1.25 & Figure 1.26).

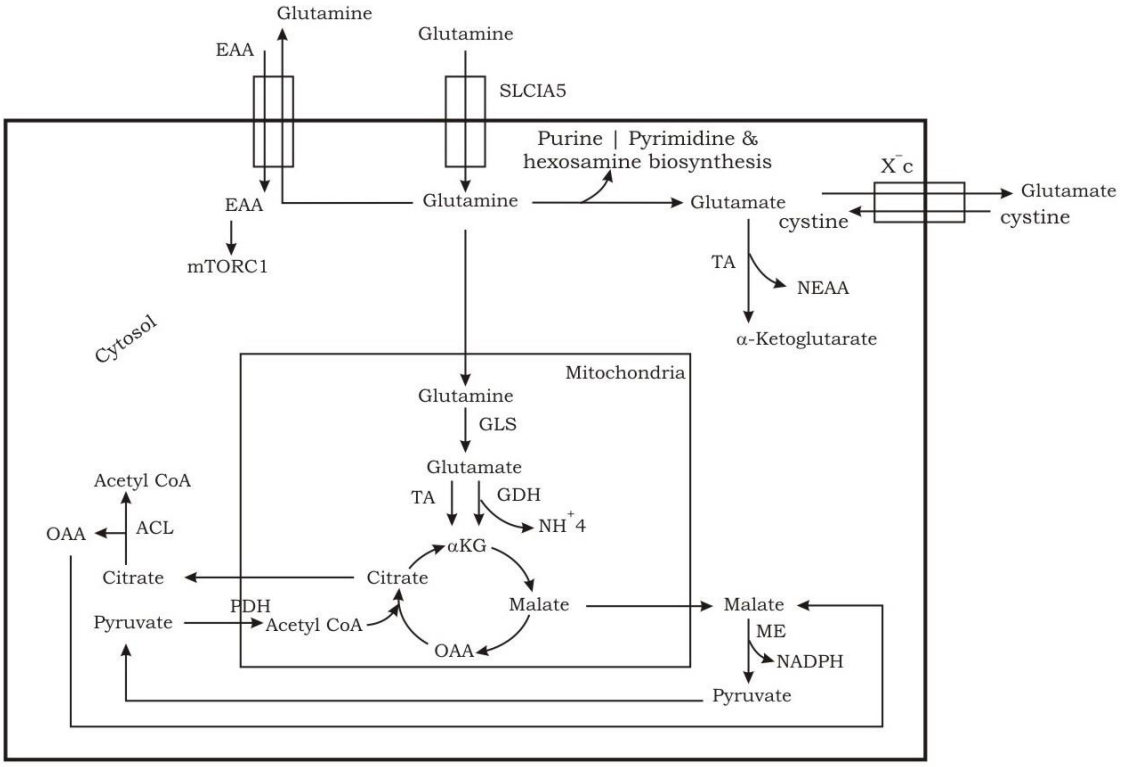


Figure 1.25 Glutamine/Glutamate metabolism: Glutamine is taken up by the cells through SLC1A5 gene coded glutamine importer. Part of this glutamine can undergo efflux through glutamine transporter coded by SLC7A5 gene in exchange for branched chain amino acids such as leucine which in turn leads to mTORC1 activation independent of its upstream kinase. During purine/pyrimidine and hexosamine biosynthesis glutamine donates its amide group and is converted into glutamate. Similarly, GLS can release amide group of glutamine as free ammonia and convert it into glutamate. Glutamate can then be converted into α-KG by either glutamate dehydrogenase in the mitochondria or by transaminases in the mitochondria as well as the cytosol to generate NEAA in the process. GSH is a tripeptide and consists of glutamate, cysteine and glycine. Glutamine can lose amide group and generate glutamate either by the action of GLS or during nucleotide/hexosamine biosynthesis. Glutamate, in turn, can play a role in cystine uptake by the cell via X c- antiporter.

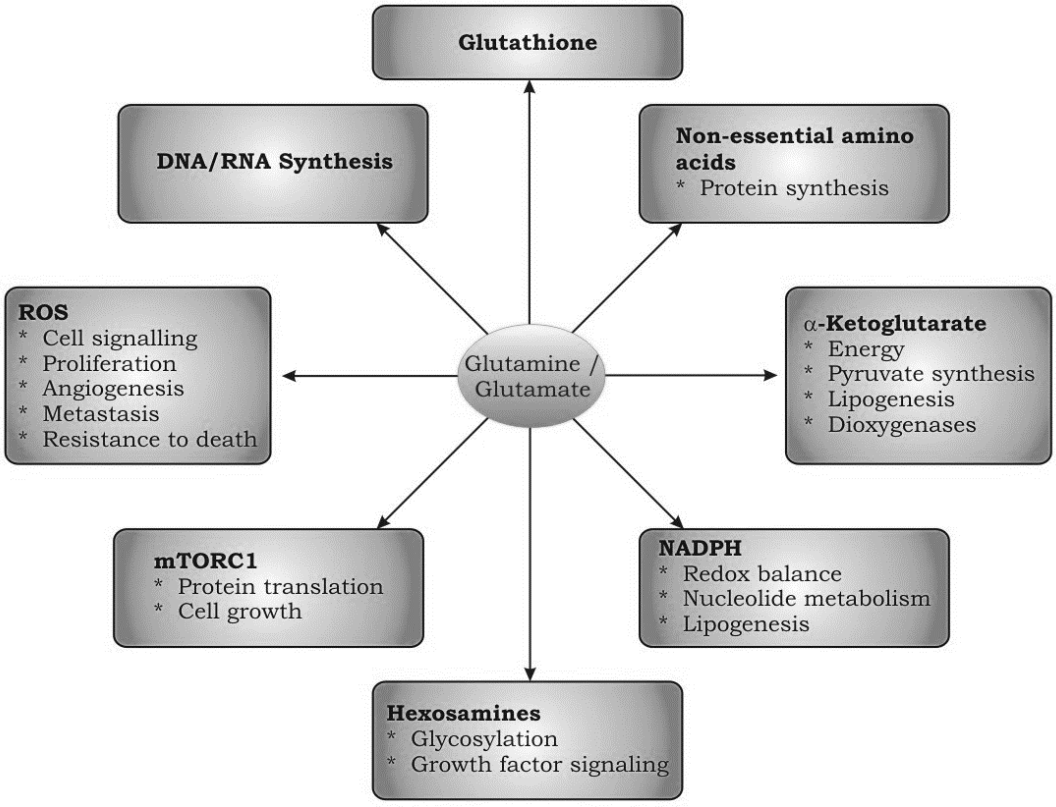


Figure 1.26 Biological effects of glutamine/glutamate: Glutamine plays an important role in mitochondrial anaplerosis. During purine/pyrimidine and hexosamine biosynthesis glutamine donates its amide group and is converted into glutamate. Similarly, GLS can release amide group of glutamine as free ammonia and convert it into glutamate. Glutamate can then be converted into α-KG by either glutamate dehydrogenase in the mitochondria or by transaminases in the mitochondria as well as the cytosol to generate NEAA in the process. αKG generated in the mitochondria can undergo a number of fates. It can undergo oxidative phosphorylation and generate energy or via TCA cycle, α-KG can also be converted into malate, which can undergo oxidative decarboxylation by the malic enzyme to form pyruvate, NADPH and carbon dioxide (CO_2). NAPDH plays an important role in redox balance, nucleotide metabolism and lipid biosynthesis. Similarly, through the TCA cycle, glutamine-derived α-KG can also be converted into OAA which can combine with acetyl CoA to form citrate. Citrate can then be exported out of the mitochondria and broken down into acetyl CoA and OAA by ACL. Acetyl CoA thus generated in the cytoplasm can be utilised for lipid biosynthesis and acetylation reactions. Glutamine derived α-KG can also directly participate in lipid biosynthesis by undergoing reductive carboxylation to generate isocitrate which can then convert into citrate. α-KG can also act as a substrate for dioxygenases such as prolyl hydroxylases and histone demethylases to modify proteins and DNA. Glutamine is the obligate nitrogen donor for many enzymatic reactions during purine and pyrimidine synthesis. Glutamine also plays an important role in the redox balance of the cell. On the one hand, glutamine metabolism through the mitochondria has been shown to generate ROS whereas on the other hand glutamine is involved in GSHand NADPH synthesis which play an important role in the cellular antioxidant defence. Glutamine has also been shown to play a role in mTORC1 activation. Similarly, ROS generated via glutamine metabolism in the mitochondria can affect various signalling pathways and play an important role in transformation, cell cycle progression & proliferation, resistance to cell death, metastasis, angiogenesis, cellular metabolism and maintenance of stemness; which are the hallmarks of cancer. Glutamine derived nitrogen is a component of hexosamines and

hexosamines have been shown to glycosylate growth factor receptors and promote their cell surface localisation besides playing an important role in glycosylation reactions to modify proteins and lipids.

1.11.6 Glutamine/Glucose connection

There are conflicting reports about glucose and glutamine metabolism interconnections. Some studies have shown that glucose and glutamine metabolism may have a regulatory effect on each other as glucose deprivation can lead to a marked reduction in glutamine uptake by the cell. Glucose is used for hexosamine biosynthesis which in turn is used for cell surface receptor glycosylation, and hence glucose can regulate growth factor-dependent cellular uptake of glutamine (Wellen 2010). Similarly, glutamine has been shown to inhibit thioredoxin interacting protein (TXNIP), a negative regulator of glucose uptake and hence promote Warburg effect by enhancing glucose uptake and aerobic glycolysis (Kaadige 2009). Some other studies have also shown that glucose and glutamine metabolism can complement each other. Glucose deprivation in glioblastoma cell line led to rapid increase in glutamate dehydrogenase whereas silencing of GLS led to an increased conversion of glucose-derived pyruvate to OAA via pyruvate carboxylase to contribute to mitochondrial metabolism (Yang 2009, Cheng 2010). However, downregulation of glycolysis by Akt inhibition had no effect on glutamine metabolism in *myc* amplified glioblastoma cell line (Wise 2008).

However, one has to remember that not all cancers are *myc* driven and hence all of them may not be sensitive to glutamine deprivation. Similarly, cancer cells have remarkable metabolic flexibility and may activate /upregulate alternative pathways to generate glutamine or mitochondrial intermediates in conditions when glutamine is scarce (Maher 2012, Hensley 2013).

1.11.7 Targeting Glutamine metabolism

1.11.7.1 Glutamine analogues

6-Diazo-5-oxo-L-norleucine (L-DON), azaserine and acivicin have significant activity as glutamine analogues and they can interrupt nucleotide biosynthesis. However, they are currently not in clinical use because of associated toxicity (Ahluwalia 1990, Ovejera 1979).

1.11.7.2 Glutamine uptake inhibitor

Myc has been shown to upregulate glutamine transporters (Hassanein 2013, Ren 2015, Wise 2010, Fuchs 2005). Gamma-l-glutamyl-p-nitroanilide (GPNA), an inhibitor of glutamine transporter coded by

gene SLC1A5, has been shown to decrease glutamine consumption, inhibit cell growth, induced autophagy and apoptosis in a subgroup of non-small cell lung cancer cell lines that overexpress SLC1A5 gene (Hassanein 2013, Hassanein 2015).

1.11.7.3 GLS Inhibition

Studies have shown that GLS is upregulated in a number of tumours and its activity correlates with tumour growth *in vivo* (Knox 1969, Gao 2009). Downregulation of GLS by siRNA has also been shown to inhibit Ehrlich ascites tumour cell growth (Lobo 2000). Similarly, knockdown of GLS expression, as well as its inhibition by bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide (BPTES), has been shown to slow the growth of glioblastoma cells expressing mutant IDH1 when compared with those expressing wild-type IDH1 (Seltzer 2010).

1.11.7.4 Transaminase inhibitor

Glutamine is converted into glutamate which is converted into α-KG by either transaminase or glutamate dehydrogenase to participate in mitochondrial anaplerosis. Amino-oxyacetic acid (AOA) inhibits aminotransferases non-specifically, and it has been shown to be cytostatic in a xenograft model of breast cancer (Thornburg 2008) and cytotoxic in *myc* amplified glioblastoma cell line (Wise 2008). EGCG a green tea polyphenol has diverse biological effects including inhibition of glutamate dehydrogenase and has been shown to kill glutamine addicted cells during glycolysis inhibition (Li 2006, Yang 2009).

1.11.7.5 Inhibition of glutamine-dependent mTOR activation

GPNA an inhibitor of SLC1A5 gene coded glutamine transporter and 2-aminobicyclo-(2,2,1) heptanecarboxylic acid (BCH) an inhibitor of SLC7A5 coded glutamine transporter have been shown to inhibit glutamine-dependent mTOR activation (Nicklin 2009).

1.11.7.6 Others

A number of other drugs already in use such as L-asparaginase which is in use for the treatment of paediatric ALL and phenylbutyrate, FDA approved drug for the treatment of hyperammonaemia in patients with acute liver failure or congenital urea cycle disorder have also been shown to lower glutamine level in the blood (Wu 1978, Thibault 1995, Wise 2010).

1.11.8 Conclusion

Glutamine, the most abundant amino acid in the cell plays a vital role in cancer cell biology. In recent years, there has been a rapid expansion in knowledge about glutamine metabolism which has led to

the successful targeting of glutamine metabolism in cancer cell lines and animal models. However, there are still a number of hurdles before glutamine metabolism can be successfully targeted in human cancers without systemic toxicity.

1.12 Summary

Glioblastoma is a highly aggressive and infiltrating tumour, and with advances in radiotherapy, chemotherapy and surgical technique, there has been some improvement in patient survival, but a cure is still not possible.

The “Warburg effect” described by Otto Warburg in the 1920s showed that the tumour cells are addicted to glucose. Upregulated glycolysis not only provides intermediates for biosynthesis but also plays a vital role in transformation, cell cycle progression, proliferation, resistance to cell death, metastasis, angiogenesis and maintenance of stemness; the hallmarks of cancer. Epidemiological studies have also shown a relationship between altered metabolism and cancer. Diabetes is associated with an increased risk of bladder, breast, colorectal, endometrial, liver, pancreatic and renal cancer. Studies have also shown that diabetes is associated with increased mortality in patients with breast and colon cancer as compared to non-diabetic patients with these cancers.

In this chapter, we have discussed metabolism in non-transformed and tumour cells and the strategies which have been used to target deranged tumour metabolism. In our experiments, we have looked at the effect of glucose deprivation on glioma cell viability and the effect of different treatment conditions on glioma cell viability in the absence of glucose. Increased pyruvate produced as a result of upregulated aerobic glycolysis can serve multiple purposes in the tumour cells. In our study, we have looked at the effect of pyruvate on glucose deprivation-induced cell death. In this chapter, we have also discussed that mitochondria, besides playing a central role in metabolism, also play an essential role in regulating apoptosis and redox status of the cell, providing intermediates for anabolic processes, signalling via ROS and calcium homeostasis. In our study, we have used hypoxia and metformin to target mitochondria. ROS mediated signalling pathways are upregulated in a number of cancers and have been shown to play an essential role in transformation, cell cycle progression & proliferation, resistance to cell death, metastasis, angiogenesis, cellular metabolism and maintenance of stemness. We have looked at the sources of ROS in our experimental conditions and used free radical scavenger N-acetylcysteine (NAC) to target ROS . LKB1-AMPK-TSC-mTOR and PI3K-Akt-mTOR pathways have been shown to play an essential role in tumour metabolism, cell proliferation and death. In our experiments, we have used metformin and AICAR to

target these pathways. Similarly, hypoxia plays a vital role in tumour metabolism, angiogenesis, metastasis and cell death and in our study, we have looked at the effect of hypoxia on glioma cell viability as well as the effect of hypoxia on glioma cells in different experimental conditions. We have also studied the effect of glucose deprivation and hypoxia on glioma cell migration. In this chapter, we have discussed that ER chaperone protein GRP78 promotes genomic instability, tumour cell proliferation, angiogenesis, metastasis, evasion of apoptosis and resistance to therapy. In our experiments, we have looked at the expression of GRP78 in glioma cells and the effect of different experimental conditions on GRP78 expression. In this chapter, we have also discussed various modes of cell death, and in our study, we have tried to characterise cell death in the glioma cells in response to different treatment conditions. Glutamine plays an important role in intermediary metabolism and glutamine/glucose connection is important for tumour metabolism. In our study, we looked at the effect of glutamine withdrawal on glioma cell viability in different treatment conditions. Finally, in our study, we have used 2DG and metformin combination to simultaneously target the glycolysis and the TCA cycle in glioma cells and characterised cell death.

Chapter 2

2.1 Materials

2.1.1 Chemicals

Reagent	***Solubility***	***Supplier***
AICAR	Distilled water	Sigma Aldrich
Ammonium chloride	Distilled water	Sigma Aldrich
Ammonium persulphate (APS)	Distilled water	Fisher Scientific
2-DG	Distilled water	Sigma Aldrich
Etoposide	Dimethyl sulphoxide (DMSO)	Sigma Aldrich
Hydrogen peroxide	Water	Sigma Aldrich
IM-54	DMSO	Sigma Aldrich
Lithium chloride	Distilled water	Sigma Aldrich
Metformin	Distilled water	Sigma Aldrich
3-Methyladenine	Distilled water	Sigma Aldrich
N-Acetylcysteine	Distilled water	Sigma Aldrich
Staurosporine	DMSO	Sigma Aldrich
STO-609	DMSO	Sigma Aldrich
TMZ	DMSO	Sigma Aldrich
ZVAD-fmk	DMSO	Calbiochem

2.1.2 Materials and medium

Reagents and materials	***Supplier***
Corning culture flasks	Sigma Aldrich
Cryotube	Sigma Aldrich
Dimethyl sulfoxide	Merck
Dulbecco's modified eagle's medium (DMEM)	Gibco
Ethanol	Fisher Scientific
FACS tube	BD Biosciences
Fetal calf serum	Bio Sera Lab
Glycine	Fisher Scientific
Haemocytometer	Weber Scientific
Hank's Balanced Salt Solution	Gibco
Hydrochloric acid	Fisher Scientific
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma Aldrich
Nitrocellulose membrane	GE Healthcare Life Sciences
Odyssey marker	LI-COR
Phosphate-buffered saline tablet	Thermo Scientific
Plastic universal container	Fisher Scientific
Polypropylene tube	BD Biosciences
Propidium iodide	Sigma Aldrich
Sodium Dodecyl Sulfate (SDS)	Fisher Scientific
Sodium hydroxide (NaOH)	Fisher Scientific

TEMED	Thermo Fisher Scientific
Tissue culture flasks	Sigma Aldrich
Tris-Base	Fisher Scientific
Type 1 collagen	BD Bioscience
Trypan Blue	Sigma Aldrich
Trypsin-EDTA	Sigma Aldrich
Virkon	Du pont
Western blot gel cassette	Invitrogen

2.1.3 Reagents

10X Running Buffer: - Tris-Base 29g, Glycine 144g, SDS 10g, made up to 1L with distilled water

25X Transfer Buffer: - Tris-Base 18.2g, Glycine 90.0g, made up to 500mls with distilled water

1X transfer Buffer: - 25X Transfer Buffer 40mls, Methanol 200mls, Distilled water 760mls

4% Stacking Gel: - 2M Tris-HCl pH 6.8- 0.63ml, 20% SDS-0.1ml, Protogel-1.33ml, Distilled water-7.94ml, APS-30µl, TEMED-6µl

Running Gel

Stock	6%	7.5%	10%	12.5%	15%
2M Tris-HCl pH 8.8	1.87ml	1.87ml	2.8ml	1.87ml	1.87ml
10% SDS	100µl	100µl	150µl	100µl	100µl
Protogel	2.0ml	2.5ml	5ml	4.16ml	4.95ml
Distilled Water	6.03ml	5.50ml	7ml	3.87ml	3.08ml
10% APS	30µl	30µl	60µl	30µl	30µl
TEMED	6µl	6µl	12µl	6µl	6µl

2.1.4 Primary antibodies

Primary antibody	*Concentration*	*Supplier*
β-Actin	1:2000	Sigma Aldrich
AMPK	1:1000	Cell Signaling technology
Caspase 3	1:1000	Cell Signaling technology
GRP78	1:1000	Thermofisher
LC3	1:2500	Abcam
pAMPK	1:1000	Cell Signaling technology
Poly (ADP-ribose) polymerase (PARP)	1:1000	Cell Signaling technology

2.1.5 Secondary antibodies

Secondary antibody	*Concentration*	*Supplier*
IR Dye goat anti-rabbit	1:5000	LI-COR
IR Dye goat anti-mouse	1:5000	Li-COR

2.2 Methods

2.2.1 Stock solutions and media

All chemicals and reagents were molecular biology grade. Phosphate buffered saline (PBS) was prepared from tablets in double glass-distilled water and sterilised by autoclaving for 15 minutes at 121°C. Cell lines were grown in DMEM containing 2milli molar (mM) L- Glutamine which was enriched with 10% fetal calf serum (FCS).

2.2.2 Tissue culture, cryopreservation, passaging and harvesting of cells

The following cell lines were used for *in vitro* studies: -

Cell Line	*Growth Medium*
U87MG (Human Glioblastoma)	DMEM + 10% FCS
LN229 (Human Glioblastoma)	DMEM + 10% FCS
U251MG (Human Glioblastoma)	DMEM + 10% FCS
Human fibroblast cells	DMEM + 10% FCS

Glioma cell lines were procured from American Type Culture Collection (ATCC®). Human fibroblast cells were provided by Dr Francesco Del Galdo (Leeds Institute of Molecular Medicine, Leeds; UK). Tissue culture was performed under strict aseptic conditions using NuAire class II microbiological safety cabinets. Tissue culture gowns and nitrile gloves were used for personal protection and infection prevention. The cabinets were cleaned with 2% virkon and 75% ethanol before and after the procedure. Plastic culture flasks of various sizes (25, 75 and 150cm^2) were used for routine maintenance of cell lines. A humidified Sanyo incubator (37˚C with 5% CO_2) was used for growing

cells. A Galaxy R CO_2 incubator (Scientific Laboratory Supplies Limited, UK) was used to maintain cells in hypoxia. Cells were routinely tested for Mycoplasma by our group by polymerase chain reaction (PCR) assay and found to be free of Mycoplasma.

Cells were centrifuged and cell pellets were suspended in freezing medium {DMEM (with 10% FCS) + 10% DMSO}. 1 ml aliquots were placed in 1.2 ml cryotubes and placed in the -80˚C freezer overnight (N Algene™ Cryo 1˚C freezing container) and the next day transferred to liquid nitrogen for long-term storage. For cell recovery, a single aliquot was rapidly thawed in a 37°C water bath. Following this, the aliquot was mixed with 10 ml of FCS enriched DMEM media which was centrifuged (Sanyo Harrier 15/80 centrifuge) at 1500 rpm for 5 minutes to remove the DMSO. The cell pellet was re-suspended in fresh media and plated in tissue culture flasks. Cells were harvested when they were near confluent in the culture flasks. The media was discarded; adherent cells were washed with PBS and then harvested using trypsin-EDTA (10X stock solution diluted 1:10 in Hanks Buffered Salt Solution). 50ml polypropylene tubes and 25 ml sterile plastic "universal" containers were used in harvesting and cell counting. Cells were viewed under an Olympus microscope (x20).

2.2.3 Cell counting

Cells were harvested as described above. 20µL of cell-containing media was mixed with 20µL of 0.1% of trypan blue. It was then loaded on a standard haemocytometer and cells were counted in 16 small chambers under the microscope. The number of cells counted was doubled to account for 1:1 dilution with trypan blue and multiplied by 10^4 to give the number of cells/ml.

2.2.4 Glucose withdrawal

Cells were harvested and counted. 75000 cells in 1ml media (DMEM + 10% FCS) were seeded in 24 well plate (Corning) and left overnight in the incubator. After 24 hours, the media was discarded, wells were washed gently with PBS and 1ml of appropriate media was added. For glucose withdrawal experiments the following media were used: -

- glucose-free DMEM containing 2mM L-Glutamine enriched with 10% FCS.

-glucose and pyruvate-free DMEM containing 2mM L-Glutamine enriched with 10% FCS.

-glucose and pyruvate-free DMEM containing 2mM L-Glutamine enriched with 2% dialysed FCS(Bio sera lab; UK).

-glucose and pyruvate-free DMEM lacking L- glutamine enriched with 2% dialysed FCS.

2.2.5 FACS analysis

Cells were harvested and counted. 75000 cells in 1ml media (DMEM + 10% FCS) were seeded in 24 well plates and left overnight in a humidified incubator. After 24 hours, the media was discarded, wells were washed gently with PBS and 1ml of media (with or without glucose) was added. All experiments were carried out in triplicate. At appropriate time points, the media from the wells were collected in respective FACS tubes. The wells were washed with PBS which was also collected. The adherent cells in the well were then trypsinised and collected in the same FACS tube. 2ml of FACS buffer {1% (v/v) FCS;0.1%(w/v) sodium azide in sterile PBS, filter sterilised using a 0.2µm filter, stored at 4°C} was added to each FACS tube and centrifuged at 1500 rpm at 4°C in a Sorvall RT6000B refrigerated centrifuge for 5 minutes. The supernatant was then discarded, and 300µL of FACS buffer and 5µL of propidium iodide was added to each tube. The tubes were gently vortexed to break up the pellet (Vortex-2 genie) and then analysed using a FACS Calibre machine (Becton Dickinson) in conjunction with BD cellquest pro software (version 0.4.af7b) operating on an Apple Macintosh computer (Mac OS X version 10.4.11).

2.2.6 Migration assay

Cells were harvested and counted. $7.5X10^4$ cells in 1ml media (DMEM + 10% FCS) was used. 20 µl of cell-containing media was placed onto the lid of 100 mm dish (Falcon) which was inverted over the dish containing 10 ml of DMEM. Hanging drops cultures were incubated for 48 hours after which the spheroids were harvested using a pipette.

Cell spheres were then put in 500µl of rat tail collagen-1 which was neutralised with 1 M sodium hydroxide in 5 × DMEM. After polymerisation at 37 °C, the collagen was overlaid with 500µl of appropriate DMEM.

2.2.8 Western blot

APS was freshly made (100mg in 1ml of distilled water). Running gel was made as per protocol. APS and TEMED were added last. Running gel was poured into Invitrogen disposable gel cassette and distilled water was overlaid. Once the gel was set, water was removed by tipping the gel cassette.

4% stacking gel was then made as per protocol, and it was laid over running gel, and a comb was placed in the stacking gel to create chambers for the protein lysate. Running buffer was prepared as per protocol, and 80 ml of 10X running buffer was diluted into 720 ml of distilled water to create 1X running buffer. Once the gel was set, the comb was removed and wells were washed. Cassettes were assembled in the tank and clamps were placed. 200ml of 1X running buffer was added to the inner tank in between the two cassettes. 2 µl of Odyssey marker was heated to 95˚ for 5 minutes, quenched on ice, spin down and loaded onto gels. Samples were then loaded. 600 ml of running buffer was added to the outer tank. The sample was run at 120 V for 50-60 minutes. Immunoblot transfer was then done. For this sponges and filter paper were pre-soaked in 1X transfer buffer. Nitrocellulose was cut into 6 X 8.5 cm size and placed in transfer buffer. Cassettes were cracked opened. Gels were removed. Stacking gel and foot end of gel were cut and removed. Sponges, filter paper, gel and nitrocellulose were assembled in cathode core of blot apparatus. Air bubbles were removed by rolling a pipette over each layer. Anode was placed on top and sandwiched together tightly. It was placed in Novex tank holder and clamped. The inner chamber was filled with 1X transfer buffer and the outer chamber was filled with distilled water. The sample was run at 25V for 1-2 hours. The liquid was removed, and then the nitrocellulose was removed. Nitrocellulose was washed with PBS for 5 minutes. For LI-COR visualisation, nitrocellulose was placed in 50 ml falcon, and 4 ml of Odyssey blocking buffer was added. It was placed on a roller apparatus overnight at 4˚C. Next day Odyssey blocking buffer was diluted with PBS/0.1 % Tween in 1:1 ratio for primary antibody dilution. Blots were incubated with primary antibody overnight at 4˚C. The membrane was given a quick wash with PBS/Tween and three more washes with PBS/Tween for 15 minutes each. The secondary antibody was diluted in the remaining volume of 1:1 LI-COR blocking buffer and PBS Tween. The membrane was incubated with secondary antibody for 1 hour while being protected from light. The membrane was given a quick wash with PBS/Tween and 3 more wash with PBS/Tween for 15 minutes each and then scanned using LI-COR machine.

2.2.8 Statistical analysis

Data was collected in Microsoft excel® and statistical significance was determined using unpaired student`s t-test. “*” denotes a statistical significance of $p<0.05$ and “**” denotes a statistical significance of $p<0.01$.

Aims and objectives

As discussed earlier, upregulated glycolysis in tumour cells promote transformation, proliferation, cell cycle progression, resistance to cell death, metastasis, angiogenesis, cellular metabolism and maintenance of stemness; the hallmarks of cancer. In this study, our aim was to target deranged tumour metabolism.

Specific aims of the project were

1) To look at the effect of glucose withdrawal on glioma cell viability and characterise glucose withdrawal-induced cell death.
2) To look at the effect of glucose withdrawal in combination with metformin on glioma cell viability and characterise cell death.
3) To look at the effect of combination treatment of metformin and 2DG on glioma cell viability and characterise cell death.

Chapter 3

3.1 Introduction

In this chapter, we have looked at the effect of glucose withdrawal on glioma cell viability. As discussed earlier, cancer cells exhibit the Warburg effect and continue to use glycolysis in the presence of oxygen, consume much more glucose and produce more lactate than their normal counterpart. As upregulated glycolysis not only generates intermediates for biosynthesis but provides cancer cells with a number of advantages for proliferation, growth and survival, we decided to investigate the effect of glucose withdrawal on glioma cells *in vitro*. Growth medium and FCS used for cell culture contain glucose and pyruvate, both of which can be used for glycolysis, so we looked at the effect of complete glucose withdrawal on glioma cell viability by using pyruvate free medium and dialysed serum. ROS have been shown to play a vital role in cancer biology and glucose deprivation-induced cell death. So, we investigated the effect of free radical scavenger NAC in glucose-deprived glioma cells. We then looked at the effect of adding pyruvate to the media lacking glucose as pyruvate can act as a free radical scavenger besides being used as a substrate to generate energy via the TCA cycle. We also looked at the effect of glucose withdrawal on glioma cell viability in the presence of pyruvate and FCS. We then investigated the impact of 2%, and 10% dialysed FCS on glioma cell viability. We were using dialysed FCS instead of normal FCS for complete glucose withdrawal from the media. In cell culture, 10% FCS is generally used. Initially, we had used 10% dialysed FCS, but because of cost implications, we switched to 2% FCS. We did experiments utilising 2%, and 10% dialysed FCS to find out if a lower concentration of dialysed FCS had an additive effect on glioma cell viability. We also compared glioma cell viability in normal and dialysed FCS in the presence and absence of glucose from the growth media. H_2O_2 has been shown to induce oxidative stress, so we decided to investigate the effect of H_2O_2 on glioma cell viability. We then looked at the effect of hypoxia and glutamine withdrawal on glioma cell viability. We also examined the mechanism of glucose deprivation-induced glioma cell death. We then cultured glioma cells which survived glucose deprivation and subjected them to glucose deprivation again to see if they belonged to a subset of glioma cells which were inherently resistance to glucose deprivation-induced cell death. Going further, we looked at the change in morphology of glioma cells with glucose deprivation and the effect of glucose deprivation and hypoxia on glioma cell migration. Finally, we looked at the expression of AMPK and GRP78 which are markers of energetic stress and endoplasmic stress respectively in glioma cells subjected to glucose deprivation.

3.2 Results

3.2.1 Glucose deprivation induces glioma cell death *in vitro*

To test the effect of glucose deprivation on glioma cell viability U87MG, LN229 and U251MG cell lines were used. Studies have shown that glucose deprivation can induce death in cancer cells *in vitro*. It was hypothesised that our cell lines like other cancer cell lines, will have deranged metabolism with a high rate of glycolysis and abnormal respiration and hence glucose deprivation should induce cell death. To test this, a propidium iodide assay was performed at different time points after complete glucose withdrawal in the presence of 2% dialysed FCS as discussed in materials and methods section 2.2.4 and 2.2.5. Dialysed FCS was used instead of normal FCS to remove any glucose present in the serum, which could have influenced the results. In U87MG, though cell death was seen at 8-hour time point, it was statistically not significant ($p=0.2673$) but at 16, 24, 40-and 48-hour time points, significant cell death was seen with glucose withdrawal in U87MG glioma cells ($p=0.006$, 0.0001, 0.0004 and 0.0004 respectively). In LN229 cells, again cell death seen at 8-hour time point was statistically not significant ($p=0.8565$) but at 16, 24, 40-and 48-hour time points, significant cell death was seen with glucose withdrawal ($p=0.0205$, 0.0008, 0.0104 and 0.0002 respectively). Similarly, in U251MG cells, cell death seen at 8-hour time point was statistically not significant ($p=0.9979$) but at 16, 24, 40-and 48-hour time point, significant cell death was seen with glucose withdrawal in U251MG glioma cells ($p<0.001$ at 16-hour time point and $p<0.0001$ at 24, 40-and 48-hour time points). So, in all three cell lines, glucose deprivation, induced cell death. However, the extent of cell death seen was cell line dependent. U251MG was most sensitive whereas LN229 was most resistant to glucose withdrawal-induced cell death. Also, cell death in response to glucose withdrawal was induced at an earlier time point in U251MG cells as compared to the other two cell lines.

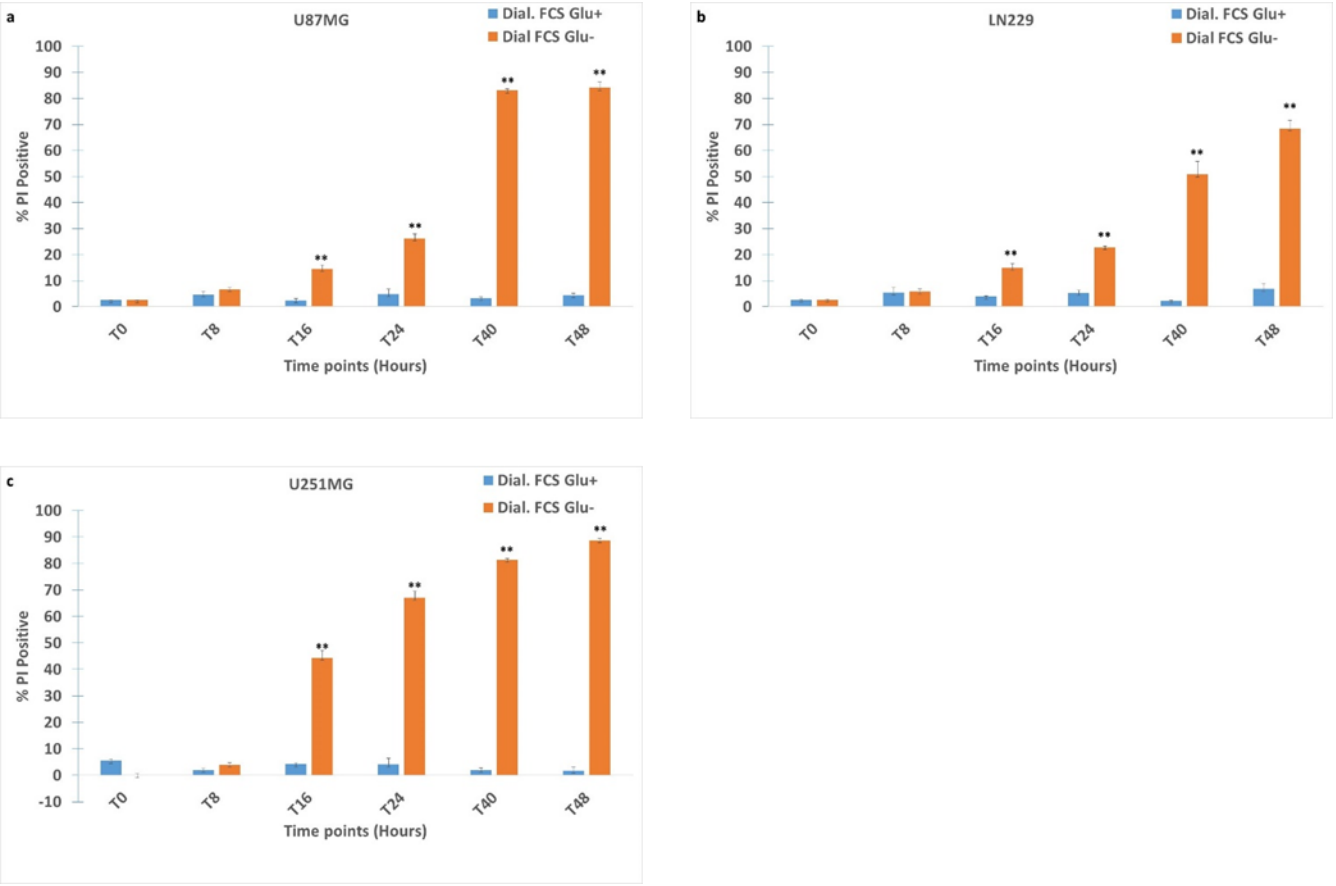


Figure 3.1 a, b, c: Glucose deprivation induces cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. For glucose withdrawal, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS was added to the wells. At appropriate time points, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicate, and error bars represent standard error of the mean. In all three cell lines, glucose deprivation-induced significant cell death at 16, 24, 40-and 48-hour time points.

3.2.2 NAC suppresses glucose deprivation-induced cell death in a concentration-dependent manner

To test the effect of NAC a free radical scavenger on glucose withdrawal-induced cell death, U87MG, LN229 and U251MG cell lines were used. It was hypothesised that if cell death following glucose withdrawal were ROS mediated, then it would be effectively rescued/ suppressed by free radical scavenger NAC. To test this, a propidium iodide assay was performed after 24 hours of complete glucose withdrawal in the presence or absence of NAC as discussed in materials and methods section 2.2.4 and 2.2.5. In U87MG, LN229 and U251MG glioma cell lines, glucose withdrawal-induced cell death was suppressed by NAC (p=0.0002, 0.0047 and 0.0006 respectively). However, the extent of

suppression of cell death varied from cell line to cell line, which may be related to the amount of ROS generated.

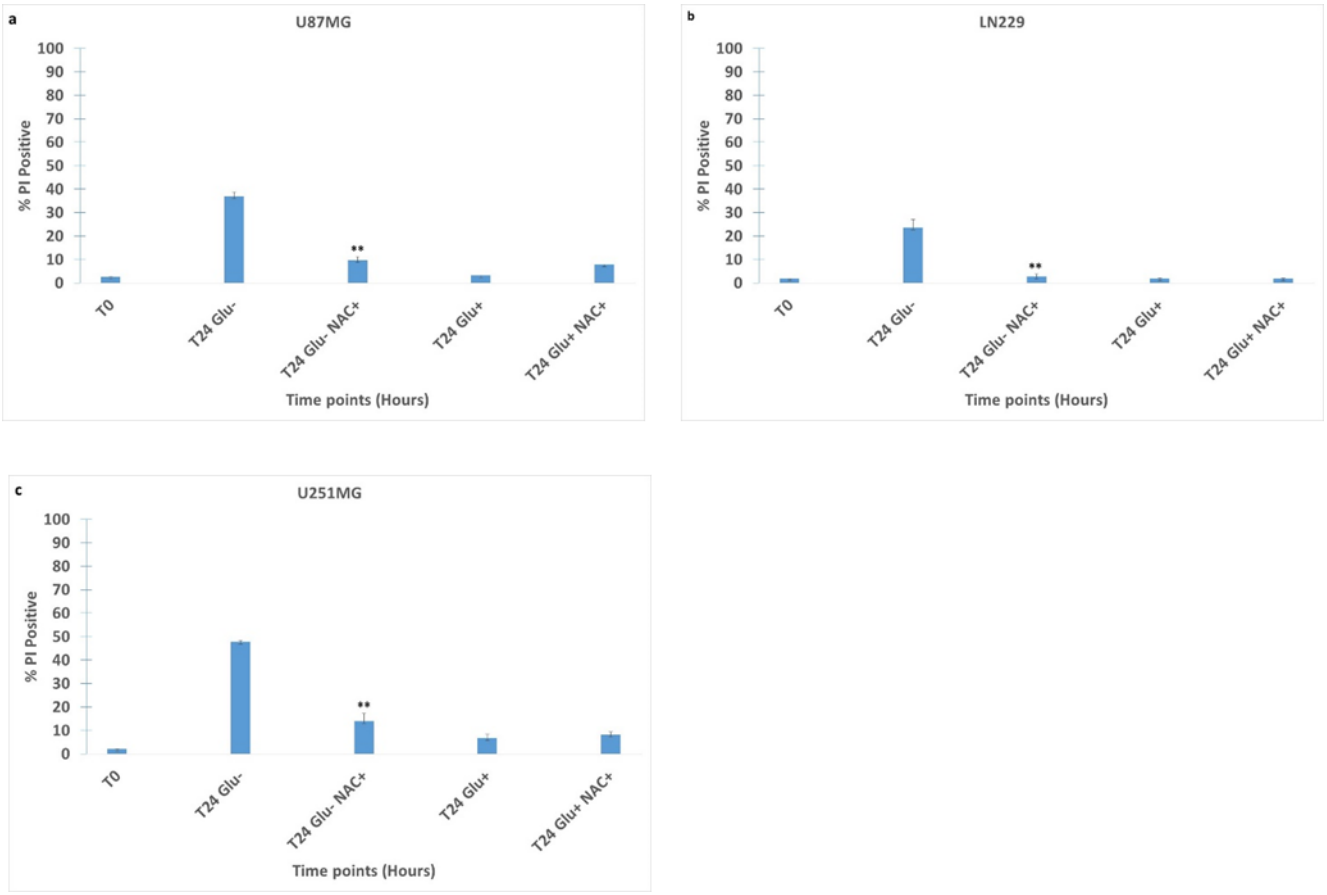


Figure 3.2 a, b, c: The free radical scavenger NAC suppresses glucose deprivation-induced cell death in glioma cell lines. U87, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. For glucose withdrawal and effect of NAC, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS, with or without 1 mM NAC, was added to the wells. After 24 hours, suspension and adherent cells were collected in appropriate tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. In U87MG, LN229 and U251MG glioma cell lines, glucose withdrawal-induced cell death was suppressed by NAC (p=0.0002, 0.0047 and 0.0006 respectively).

3.2.3 NAC fails to suppress glucose deprivation-induced cell death at longer time points

To test the effect of NAC on prolonged glucose deprivation-induced cell death, U87MG and LN229 cell lines were used. We have shown earlier that NAC can suppress glucose withdrawal-induced cell death (Figure 4.2). It was hypothesised that prolonged glucose deprivation might lead to excessive ROS generation and oxidative stress as a result of which NAC may not be able to suppress glucose withdrawal-induced cell death at later time points. To test this, a propidium iodide assay was

performed after 60 hours of glucose deprivation. In both U87MG and LN229 cell lines, NAC could not significantly suppress glucose withdrawal-induced cell death to the level which was seen at an earlier time point (p=0.1471 and 0.1547 respectively). U251MG was not used in the experiment as nearly all the cells were dead after 48 hours.

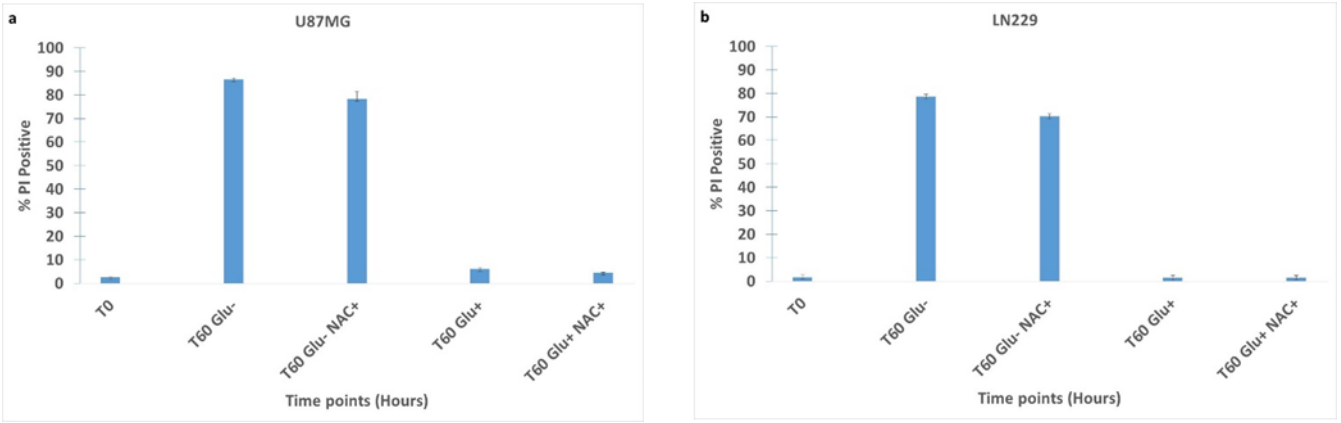


Figure 3.3 a, b: The free radical scavenger NAC is unable to suppress glucose deprivation-induced cell death in glioma cell lines at longer time points. U87MG and LN229 cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested and cell viability was determined by a propidium iodide assay to serve as a baseline. For glucose withdrawal and effect of NAC, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS, with or without 1 mM NAC, was added to the wells. After 60 hours, suspension and adherent cells were collected in appropriate tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. In both U87MG and LN229 cell lines, NAC could not significantly suppress glucose withdrawal-induced cell death to the level which was seen at an earlier time point (p=0.1471 and 0.1547 respectively).

3.2.4 Pyruvate suppresses glucose deprivation-induced glioma cell death

To test the effect of pyruvate on glucose withdrawal-induced cell death, U87MG and LN229 cell lines were used. Pyruvate is a known ROS scavenger, and it can also be converted into acetyl CoA and metabolised via mitochondrial oxidative phosphorylation to generate ATP. It was hypothesised that pyruvate being a ROS scavenger should be able to suppress glucose withdrawal-induced cell death. As pyruvate can act as a source of ATP, it may also help to tide over a period of energetic stress induced by the absence of glucose. To test this, a propidium iodide assay was performed at different time points after withdrawal of media which lacked glucose but contained methyl pyruvate (110mg/L). In U87MG cell line, pyruvate was able to suppress glucose withdrawal-induced cell death at all times points (p=0.0016, 0.0011, 0.0032 and 0.0001 for time point 8, 16, 24 and 48 hours respectively). Similarly, in LN229 glioma cell line, pyruvate was able to suppress glucose withdrawal-induced cell

death at all times points (p=0.0416, 0.0088, 0.0021 and 0.0007 for time point 8, 16, 24 and 48 hours respectively). However, the extent of suppression of cell death was different in U87MG and LN229 cell lines, which may be related to oxidative stress generated in the cell lines as well as the dependence of the cell lines on glycolysis to meet their bioenergetic and biosynthetic needs.

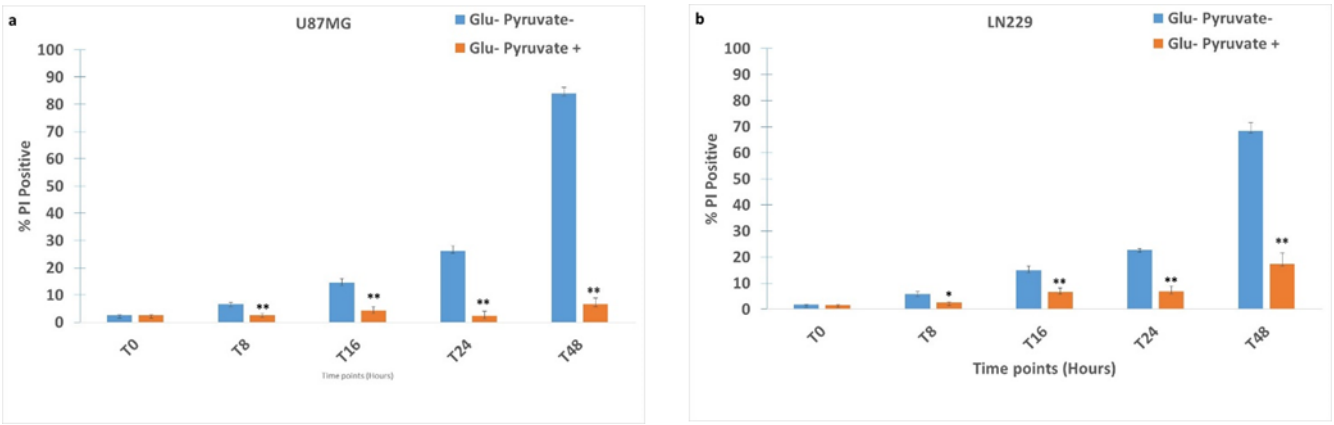


Figure 3.4 a, b: Pyruvate suppresses glucose deprivation-induced cell death in glioma cell lines. U87MG and LN229 cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. For glucose withdrawal, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose, with or without pyruvate supplemented with 2% dialysed FCS was added to the wells. At appropriate time points, suspension and adherent cells were collected in appropriate tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. In U87MG and LN229 glioma cell lines, pyruvate was able to suppress glucose withdrawal-induced cell death at all times points.

3.2.5 Glucose deprivation has no effect on glioma cell viability in the presence of pyruvate and FCS

We hypothesised that when glioma cells are treated with varying concentration of glucose, in the presence of pyruvate and FCS, it will have no significant effect on glioma cell viability as pyruvate and glucose present in media and FCS can be utilised by cancer cells to meet their energetic needs as well as to produce NADPH. To test this, a propidium iodide assay was performed at different time points in cells treated with varying concentration of glucose in the presence of pyruvate and 10% FCS as discussed in materials and methods section 2.2.4 and 2.2.5. In U8MG cell line, no significant cell death was seen at any time points (p=0.2189, 0.1184, 0.1115, 0.458, 0.1843, 0.1464, 0.833 for time point 2, 4, 8, 16, 24, 48 and 72 hours respectively). Similarly, in LN229 glioma cell line, no significant cell death was seen at any time points (p=0.2944, 0.1378, 0.3273, 0.8473, 0.0812, 0.0578 and 0.0633 for time point 2, 4, 8, 16, 24, 48 and 72 hours respectively).

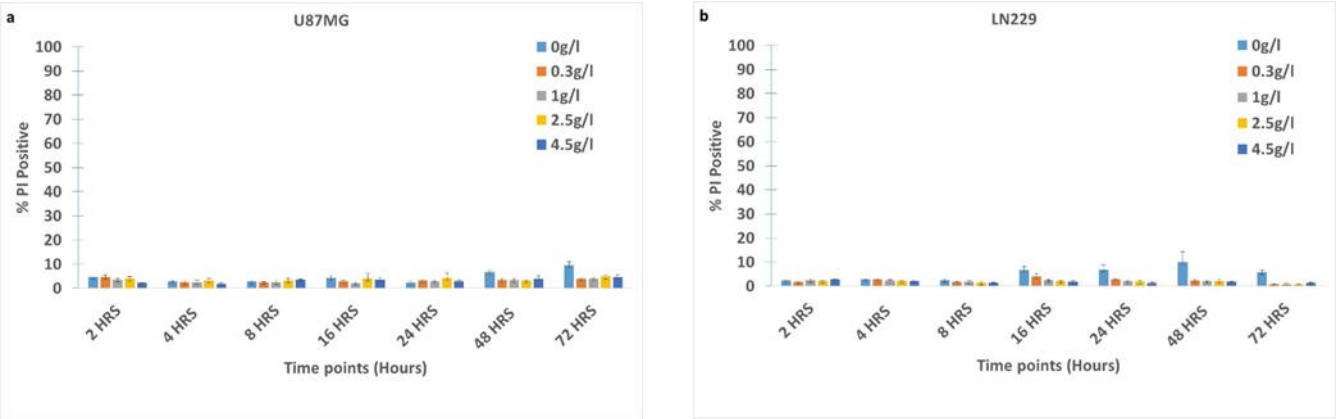


Figure 3.5 a, b: Glucose deprivation does not affect glioma cell line viability in the presence of pyruvate and FCS. U87MG and LN229 cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was discarded, and wells were gently washed with PBS. Then DMEM (lacking glucose but containing pyruvate) containing 0g/l, 0.3g/l, 1g/l, 2.5g/l and 4.5g/l glucose supplemented with 10% FCS was added to appropriate wells. At different time points, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicate, and error bars represent standard error of the mean. Glioma cells treated with varying concentration of glucose, in the presence of pyruvate and FCS had no significant effect on cell viability.

3.2.6 Glucose present in FCS suppresses glucose withdrawal-induced cell death in glioma cells

To test the effect of glucose present in FCS on glucose withdrawal-induced cell death, U87MG, LN229 and U251MG cell lines were used. It was hypothesised that glucose present in FCS might act as a source of glucose during the experiment and contribute to rescue of cancer cells from glucose deprivation-induced cell death whereas dialysed FCS, which contains a negligible amount of glucose, may not be able to rescue cancer cells from death. To test this, a propidium iodide assay was performed at different time points after withdrawal of media which lacked glucose but was supplemented with normal or dialysed FCS. In U87MG cell line, though more cell death was seen in cells treated with dialysed FCS as compared to those treated with FCS at 8-hour time point, it was statistically not significant ($p=0.152$). However, at other time points, dialysed FCS led to significantly more cell death as compared to FCS ($p= 0.0127$, 0.0025, <0.0001 and <0.0001 for 16, 24, 40-and 48-hour time points respectively). Similarly, in LN229 cell line, though more cell death was in cells treated with dialysed FCS as compared to those treated with FCS at 8-hour time point, it was statistically not significant ($p=0.9754$). However, at other time points, dialysed FCS led to significantly more cell death as compared to FCS ($p= 0.0278$, 0.0057, 0.0113 and 0.0003 for 16, 24, 40-and 48-hour time points respectively). In U251MG, dialysed FCS led to significantly more cell death as compared to media

which contained normal FCS at 8, 16, 24, 40-and 48-hour time points (p=0.0444, 0.0006, 0.0026, 0.0016 and 0.0022 respectively).

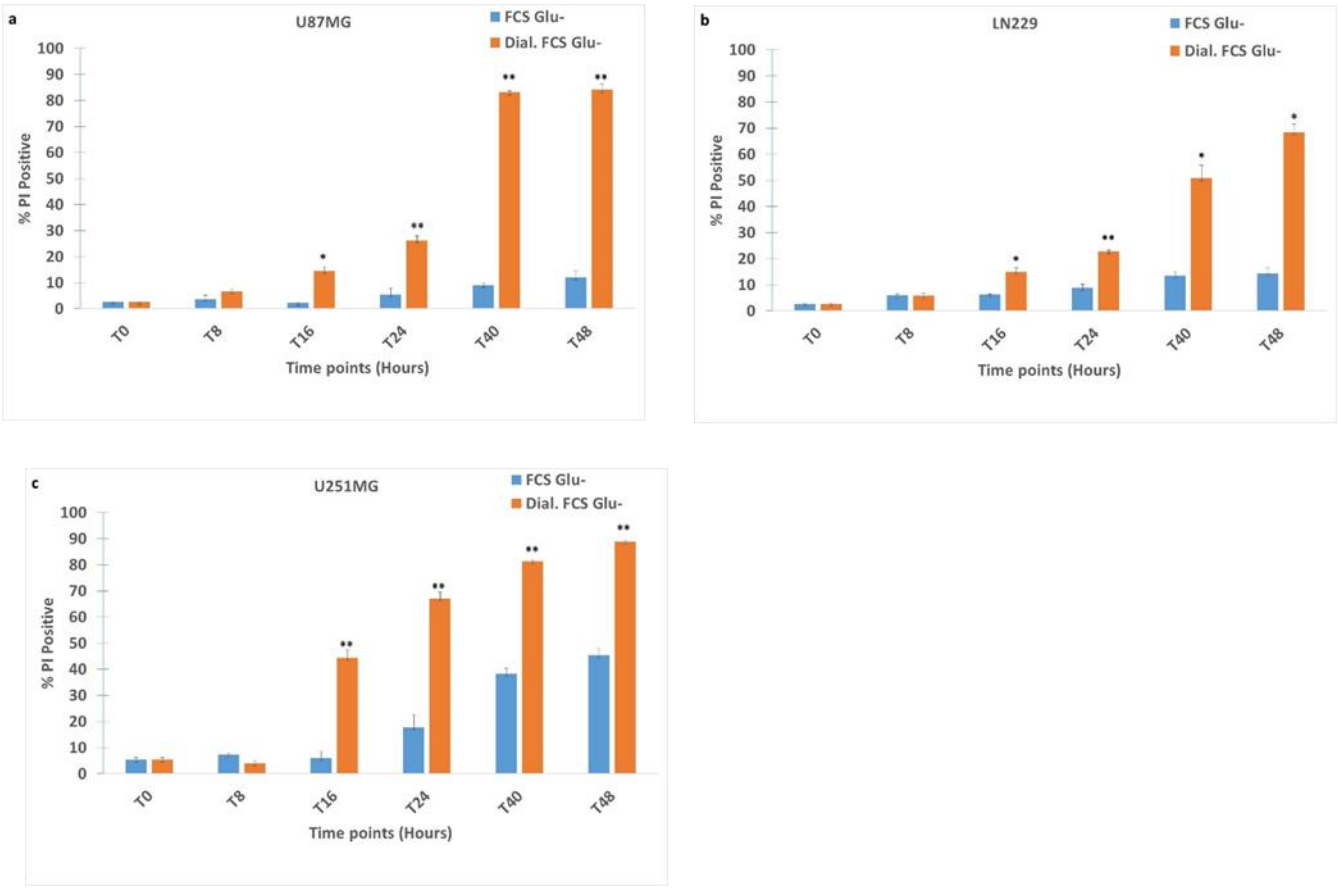


Figure 3.6 a, b, c: Dialysed FCS as compared to normal FCS led to significant cell death in glioma cell lines when subjected to glucose withdrawal. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. For glucose withdrawal, media was removed from wells, wells were washed gently with PBS and then DMEM lacking glucose and pyruvate supplemented with 2% dialysed FCS or 2% FCS was added to the wells. At appropriate time points, suspension and adherent cells were collected. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. Dialysed FCS led to significantly more cell death as compared to FCS in all three glioma cell lines at 16, 24, 40-and 48-hour time points.

3.2.7 Dialysed FCS does not affect glioma cell viability in the presence of glucose

We had already shown that dialysed FCS led to marked glioma cell death in glucose deprivation conditions (Figure 4.6). However, we hypothesised that in the presence of glucose, dialysed FCS as compared to FCS should not affect glioma cell growth adversely, if the effect was due to glucose present in the dialysed serum only. To test this, a propidium iodide assay was performed at a 24-hour time point, after adding media which contained either dialysed FCS or FCS. In U87MG cell line, dialysed

FCS had no significant effect on the viability (p=0.565, 0.7131, 0.2419, 0.5311 and 0.9027 for 8, 16, 24, 40-and 48-hour time points respectively). Similarly, in LN229 cell line, dialysed FCS had no significant effect on the viability (p= 0.2147, 0.2099, 0.4502 and 0.4488 and 0.06504 for 8, 16, 24, 40-and 48-hour time points respectively). Also, in U251MG cell line, dialysed FCS had no significant effect on the viability (p=0.7766, 0.0667, 0.9363, 0.1455 and 0.2264 for 8, 16, 24, 40-and 48-hour time points respectively).

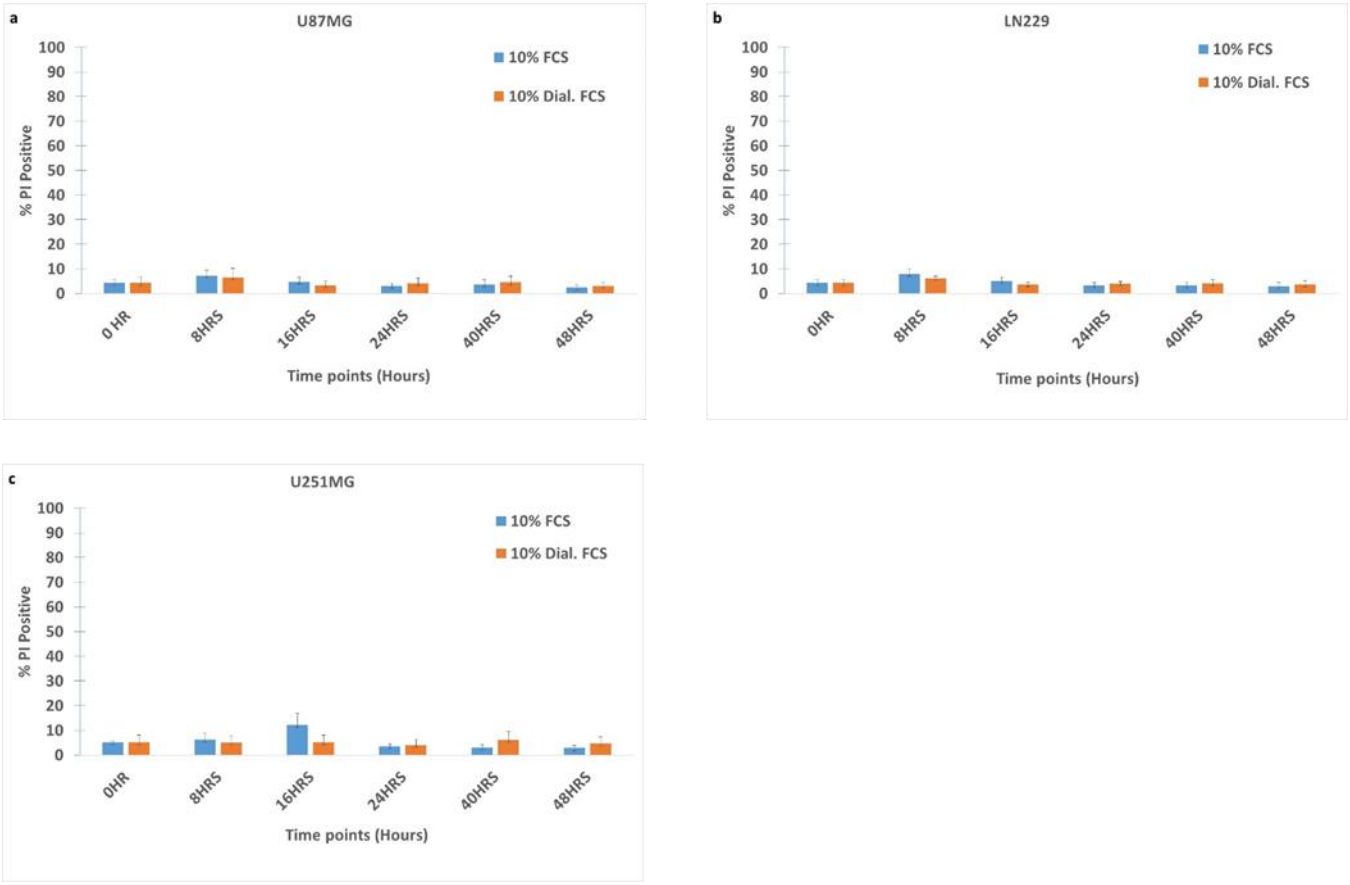


Figure 3.7 a, b, c: Dialysed FCS does not affect the viability of glioma cell lines in the presence of glucose. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. Media was removed from wells, wells were washed gently with PBS, and then DMEM (containing glucose and pyruvate), supplemented with either 2% dialysed FCS or 2% FCS, was added to the wells. At appropriate time points, suspension and adherent cells were collected. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. Dialysed FCS had no effect on glioma cell viability in the presence of glucose.

3.2.8 2% and 10% dialysed FCS have a similar effect on glucose deprivation-induced glioma cell death

We have already shown that dialysed FCS led to marked cell death in glucose deprivation condition as compared to normal FCS (Figure 4.6). To maintain cells in culture, 10% FCS is generally used. However,

because of cost implications, we used 2% dialysed FCS instead of 10% dialysed FCS for glucose withdrawal experiments. In view of this, we tested whether 2% dialysed FCS instead of 10% dialysed FCS had any additional effect on cell viability. To test this, a propidium iodide assay was performed at a 24-hour time point after adding media (lacking glucose and pyruvate) supplemented with 2% FCS or 10% FCS or 2% dialysed FCS or 10% dialysed FCS. There was no significant difference in death induced by 2%, and 10% dialysed FCS in U87MG and U251MG glioma cell lines (p=0.4317 and 0.1282 respectively).

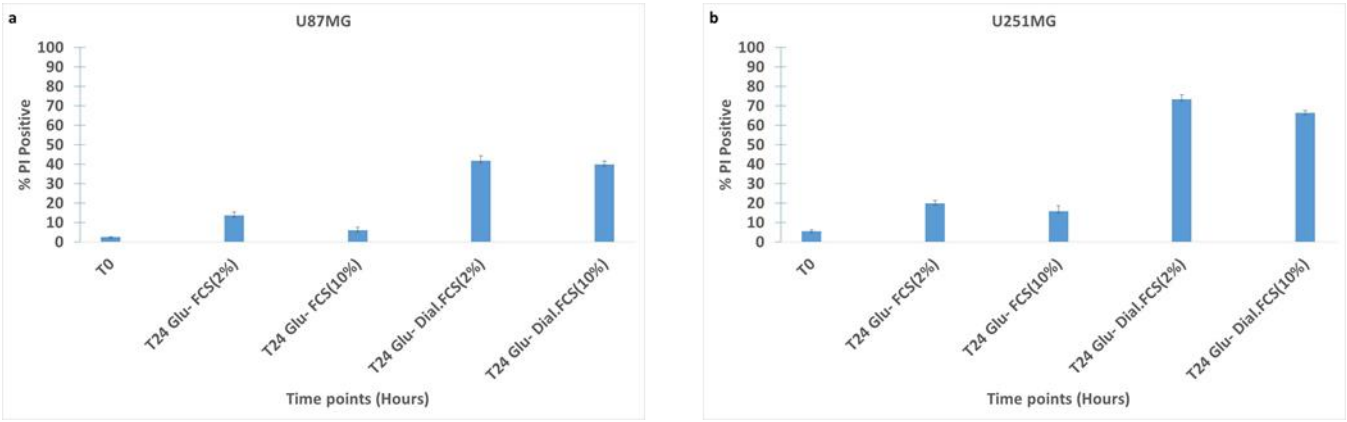


Figure 3.8 a, b: Effect of 2% dialysed FCS on glucose withdrawal-induced cell death is similar to 10% dialysed FCS. U87MG and U251MG glioma cell were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. Media was removed from wells, wells were washed gently with PBS and then DMEM (lacking glucose and pyruvate) supplemented with 2% FCS or 10% FCS or 2% dialysed FCS, or 10% dialysed FCS were added to the wells. After 24 hours, suspension and adherent cells were collected. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. There was no significant difference in death induced by 2%, and 10% dialysed FCS in U87MG and U251MG glioma cell lines (p=0.4317 and 0.1282 respectively).

3.2.9 H_2O_2 induces dose-dependent cell death in glioma cells

We have earlier shown that glucose deprivation-induced cell death in glioma cell lines is mediated by oxidative stress and it is suppressed by the free radical scavenger NAC (Figure 4.2). We hypothesised that glioma cells would be sensitive to other agents such as H_2O_2 which induces oxidative stress. To test this, a propidium iodide assay was performed after 24 hours of complete glucose withdrawal. 0.1mM, 0.5mM, 1.0mM and 2mM H_2O_2 was added in appropriate wells to induce oxidative stress. In all three glioma cell lines, U87MG, LN229 and U251MG, though cell death was seen with 0.1mM and 0.5mM H_2O_2, it was statistically not significant (p=0.1851, 0.1671 and 0.7734 respectively for 0.1mM H_2O_2 and p=0.16, 0.167 and 0.594 for 0.5mM H_2O_2 respectively). However, in all three cell lines,

U87MG, LN229 and U251MG, 1 and 2mM H_2O_2 treatment induced statistically significant cell death, reconfirming the fact that glioma cell lines were sensitive to oxidative stress ($p<0.05$).

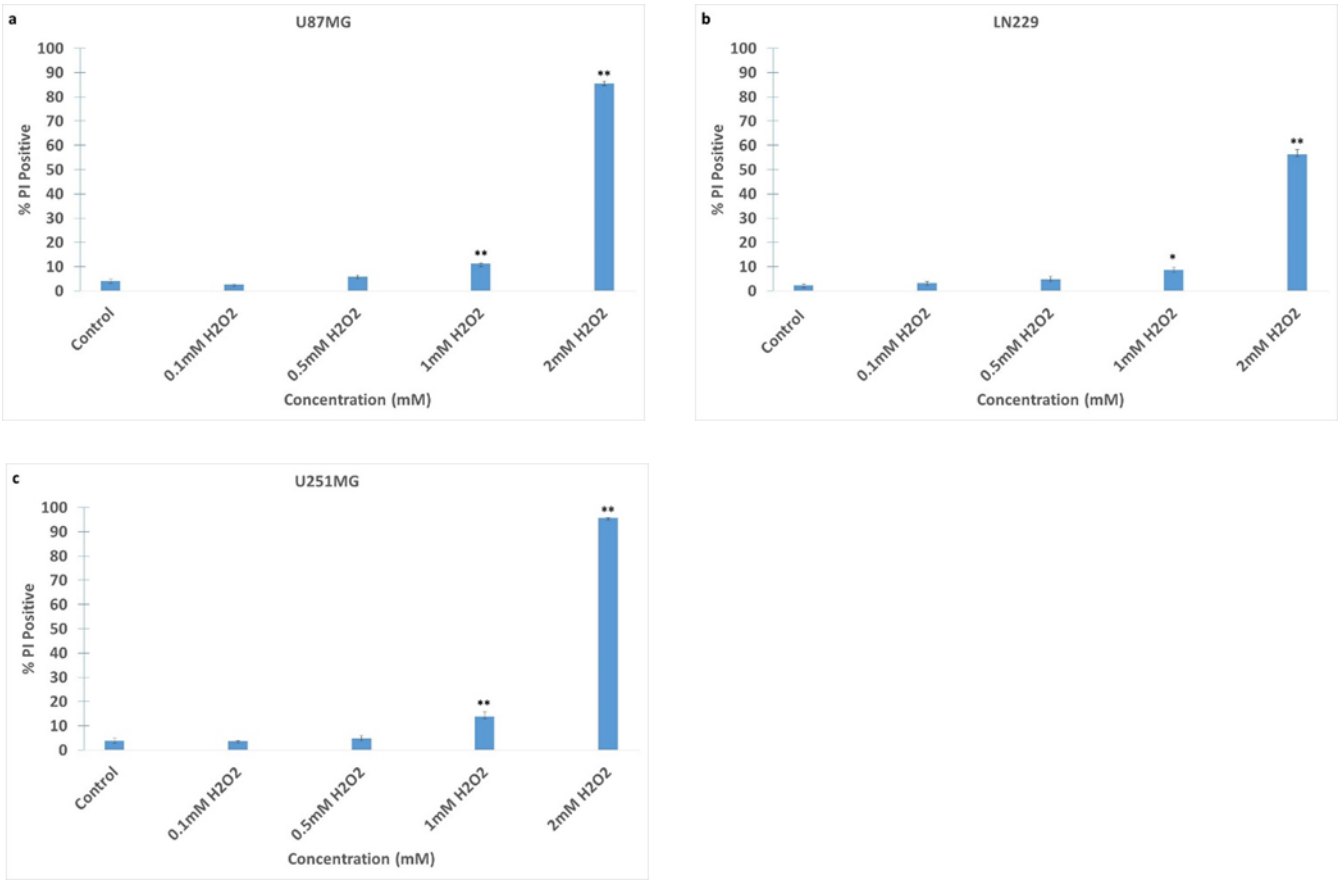


Figure 3.9 a, b, c: H_2O_2 treatment led to a dose-dependent increase in cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was discarded and fresh media containing 0.1mM, 0.5mM, 1.0mM and 2mM H_2O_2 was added in appropriate wells. Cells cultured in DMEM media was used as a control. After 24 hours, suspension and adherent cells were collected in appropriate tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. In all three cell lines, U87MG, LN229 and U251MG, 1 and 2mM H_2O_2 treatment induced statistically significant cell death, reconfirming the fact that glioma cell lines were sensitive to oxidative stress ($p<0.05$).

3.2.10 Untransformed human fibroblasts are resistant to glucose deprivation-induced cell death

To test the effect of glucose withdrawal on untransformed cells, human fibroblast cells were used. It was hypothesised that, as fibroblasts were untransformed cells, glycolysis and TCA cycle should not be deranged and these cells would not be completely dependent on glucose to meet their bioenergetic and biosynthetic needs. To test this, a propidium iodide assay was performed at a 24-hour time point after withdrawal of media which lacked glucose. Glucose withdrawal had no significant effect on the viability of fibroblast cells ($p=0.3888$). During glucose deprivation, due to the

presence of intact functioning mitochondria, the cells may have oxidised fatty acids and amino acids present in the media as an alternate source of energy. Also, as they have intact mitochondria, mitochondrial metabolism would not have resulted in increased ROS generation to affect cell viability.

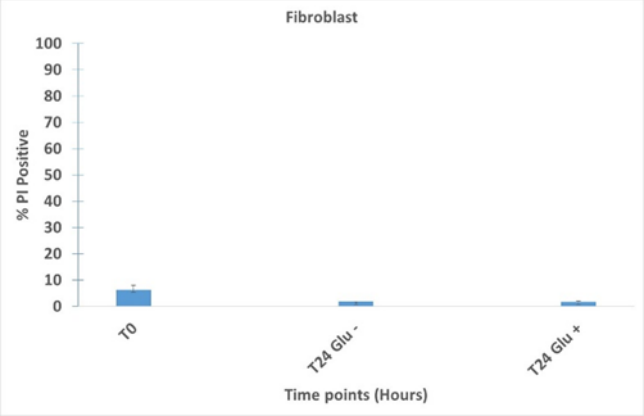


Figure 3.10 Untransformed human fibroblast cells are resistant to glucose deprivation-induced cell death. Cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. For glucose withdrawal, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate supplemented with 2% dialysed FCS was added to the wells. After 24 hours, suspension and adherent cells were collected. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. Glucose withdrawal had no significant effect on the viability of fibroblast cells (p=0.3888).

3.2.11 Hypoxia suppresses glucose deprivation-induced cell death in glioma cells

To test the effect of hypoxia on glucose withdrawal-induced cell death, U87MG, LN229 and U251MG cell lines were used. We have already shown that glucose withdrawal-induced cell death is ROS mediated (Figure 4.2). It was hypothesised that glucose withdrawal-induced cell death was dependent on mitochondrial metabolism for ROS generation and as mitochondrial oxidative phosphorylation is suppressed in hypoxia, hypoxia should result in suppression of glucose deprivation-induced cell death. To test this, a propidium iodide assay was performed at different time points after withdrawal of glucose in hypoxic and normoxic conditions. U87MG cells showed significantly less cell death in hypoxia at 24, 40-and 48-hour time points in response to glucose withdrawal as compared to normoxia (p=0.0435, 0.0031 and 0.0001 respectively). Similarly, LN229 cells showed significantly less cell death in hypoxia at 24, 40-and 48-hour time points in response to glucose withdrawal as compared to normoxia (p=0.0013, 0.0027 and 0.0006 respectively). Interestingly U251MG, which was the most sensitive cell line to glucose withdrawal in normoxia, showed significantly more cell death at 24, 40- and 48-hour time points (p=0.0414, 0.0037 and 0.0057 respectively).

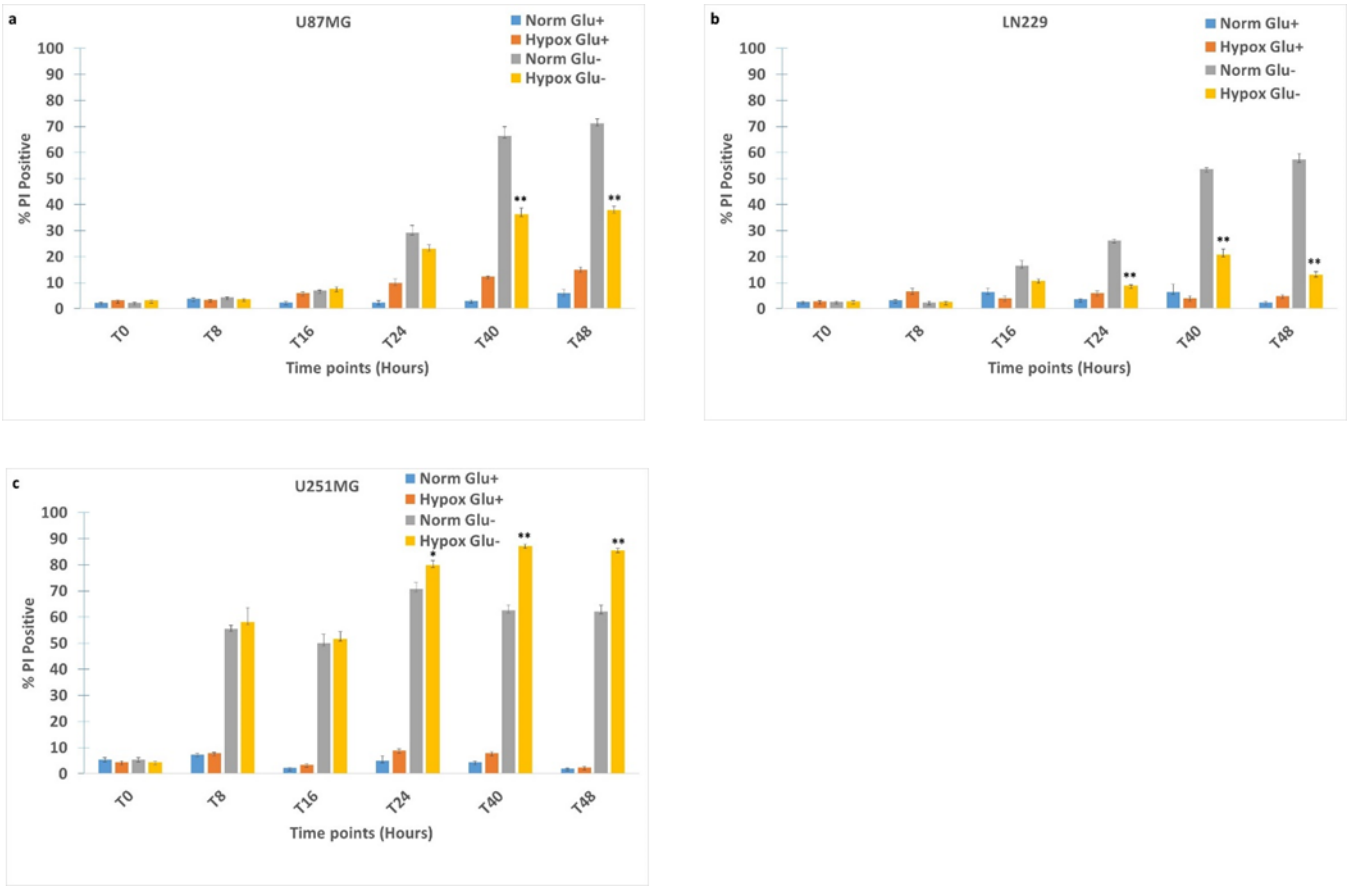


Figure 3.11 a, b, c: Hypoxia suppresses glucose withdrawal-induced cell death in U87MG and LN229 glioma cells lines whereas it potentiates glucose withdrawal-induced cell death in U251MG glioma cells line. Cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS and placed in normoxic and hypoxic incubators. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. For glucose withdrawal, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate supplemented with 2% dialysed FCS was added to the wells, and the plates were replaced in hypoxic and normoxic incubators. At appropriate time points, suspension and adherent cells were collected. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. U87MG cells showed significantly less cell death in hypoxia at 24, 40-and 48-hour time points in response to glucose withdrawal as compared to normoxia (p=0.0435, 0.0031 and 0.0001 respectively). Similarly, LN229 cells showed significantly less cell death in hypoxia at 24, 40-and 48-hour time points in response to glucose withdrawal as compared to normoxia (p=0.0013, 0.0027 and 0.0006 respectively). Interestingly U251MG, which was the most sensitive cell line to glucose withdrawal in normoxia, showed significantly more cell death at 24, 40-and 48-hour time points (p=0.0414, 0.0037 and 0.0057 respectively).

3.2.12 Glutamine withdrawal does not potentiate glucose deprivation-induced cell death in glioma cells

To test the effect of glutamine withdrawal on glucose deprivation-induced cell death U87MG, LN229 and U251MG glioma cell lines were used. It was hypothesised that as the cell lines were not *myc* amplified glutamine would not play an important role in meeting the energy requirements or reducing

the capacity of the cells. Hence cell death seen as a result of glucose deprivation represents the effect of inhibition of glycolysis on the cancer cells without any artefact created by glutamine rescue. To test this, a propidium iodide assay was performed at a 24-hour time point after withdrawal of glucose and glutamine. Glutamine withdrawal had no significant effect on glucose withdrawal-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.2606, 0.7732 and 0.2276 respectively).

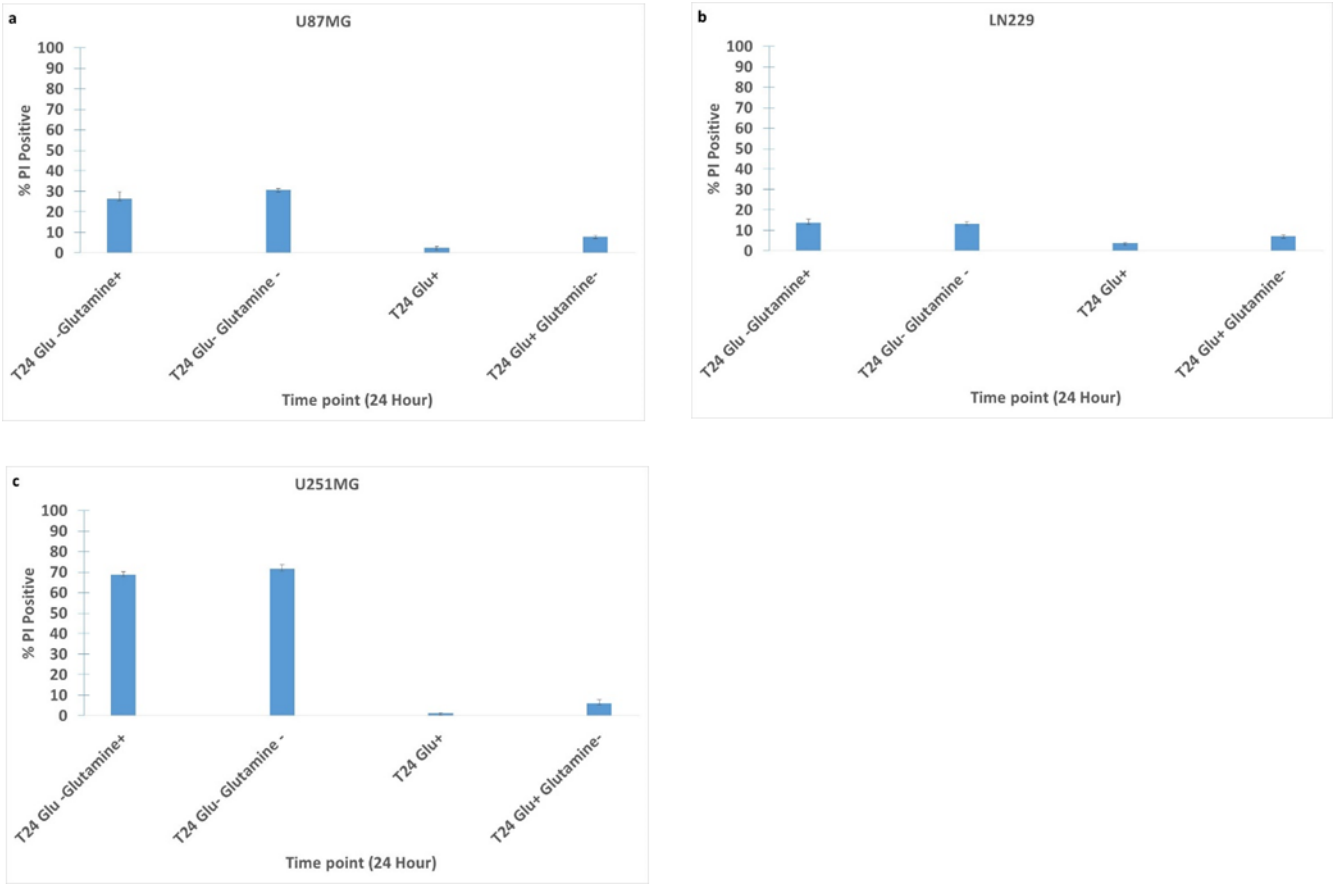


Figure 3.12 a, b, c: Glutamine withdrawal does not potentiate glucose withdrawal-induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate (supplemented with 2% dialysed FCS) with or without 2mM L-glutamine was added to the wells. After 24 hours, suspension and adherent cells were collected. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. Glutamine withdrawal had no significant effect on glucose withdrawal-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.2606, 0.7732 and 0.2276 respectively).

3.2.13 Glucose deprivation-induced cell death in glioma cells is caspase-independent

To investigate the mechanism of glucose deprivation-induced cell death, U87MG, LN229 and U251MG cell lines were used. It was hypothesised that pan-caspase inhibitor ZVAD-fmk may rescue or suppress glucose withdrawal-induced cell death, as apoptosis has been shown to play a role in glucose

deprivation-induced cell death in some cancer cell lines. To test this, a propidium iodide assay was performed at a 24-hour time point after withdrawal of glucose (with and without caspase inhibitor). Interestingly, caspase inhibitor ZVAD-fmk had no significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.4988, 0.1453 and 0.6028 respectively).

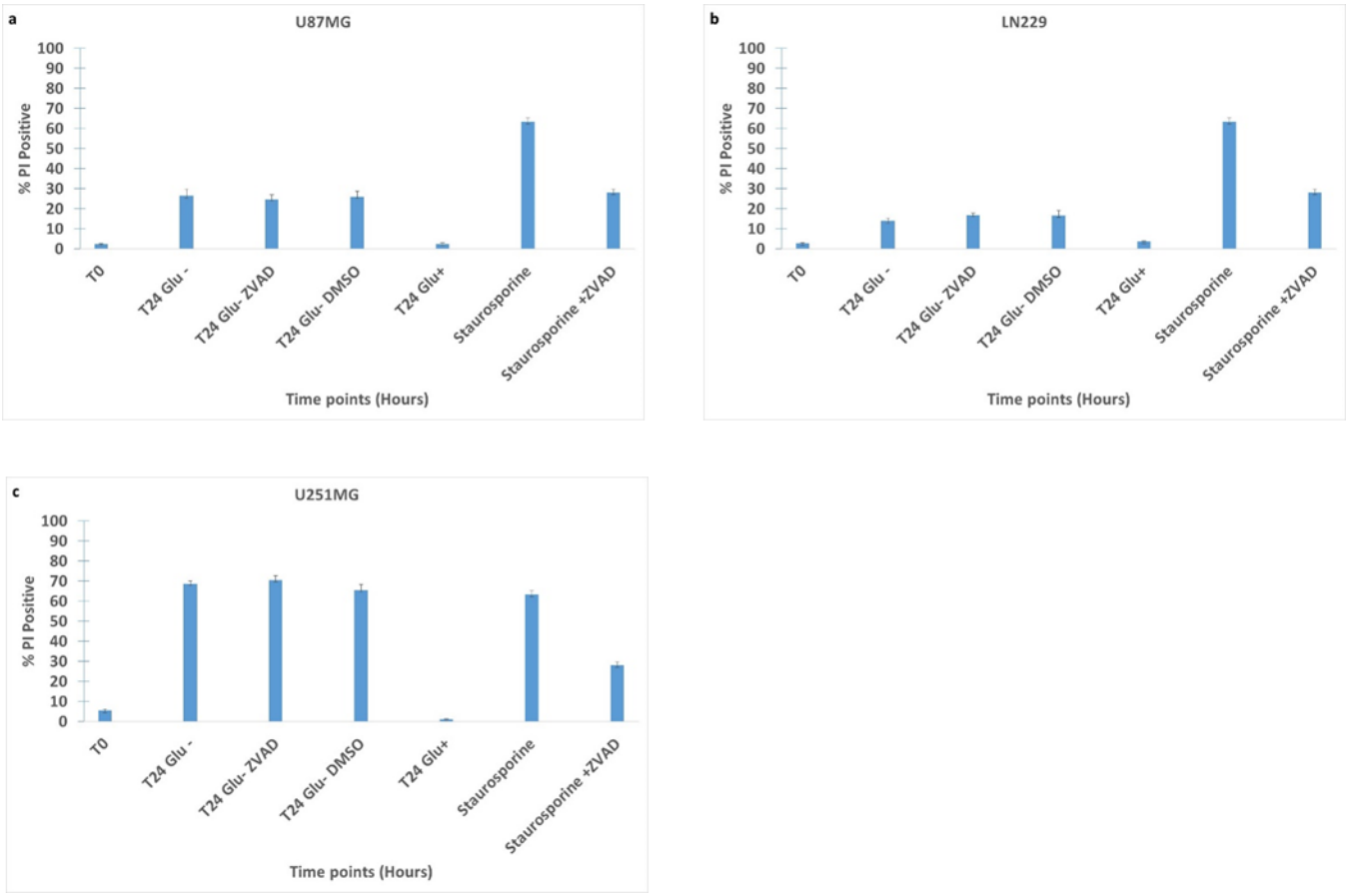


Figure 3.13 a, b, c: The pan-caspase inhibitor ZVAD-fmk does not rescue glucose withdrawal-induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. Cells were pre-incubated with 50 microM (µM) ZVAD-fmk to allow the ZVAD-fmk to be present inside the cells at the time when glucose was withdrawn. After 1 hour, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate (supplemented with 2% dialysed FCS) were added to the wells. 50 µM ZVAD-fmk was added to appropriate wells. DMSO was also added in appropriate wells as the control since ZVAD-fmk was dissolved in DMSO. Also, 1 µM Staurosporine, a known inducer of apoptosis was used as a control for ZVAD-fmk. After 24 hours, the supernatant was collected into marked FACS tubes. Adherent cells were trypsinised and collected in appropriate tubes too. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. Caspase inhibitor ZVAD-fmk had no significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.4988, 0.1453 and 0.6028 respectively).

3.2.14 Glucose deprivation-induced cell death in glioma cells is not mediated via autophagy

To test the effect of autophagy inhibition on glucose deprivation-induced cell death, U87MG, LN229 and U251MG cell lines were used. It was hypothesised that 3-methyladenine (3-MA) and ammonium chloride (NH_4Cl) which are known to inhibit autophagy might rescue or suppress glucose withdrawal-induced cell death, as autophagy has been shown to play a role in glucose deprivation-induced cell death in some cancer cell lines. To test this, a propidium iodide assay was performed at a 24-hour time point after withdrawal of glucose (with or without 3-MA or NH_4Cl). 3-MA had no any significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.4360, 0.4728 and 0.5666 respectively). Similarly, NH_4Cl had no any significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.2372, 0.8831 and 0.6877 respectively).

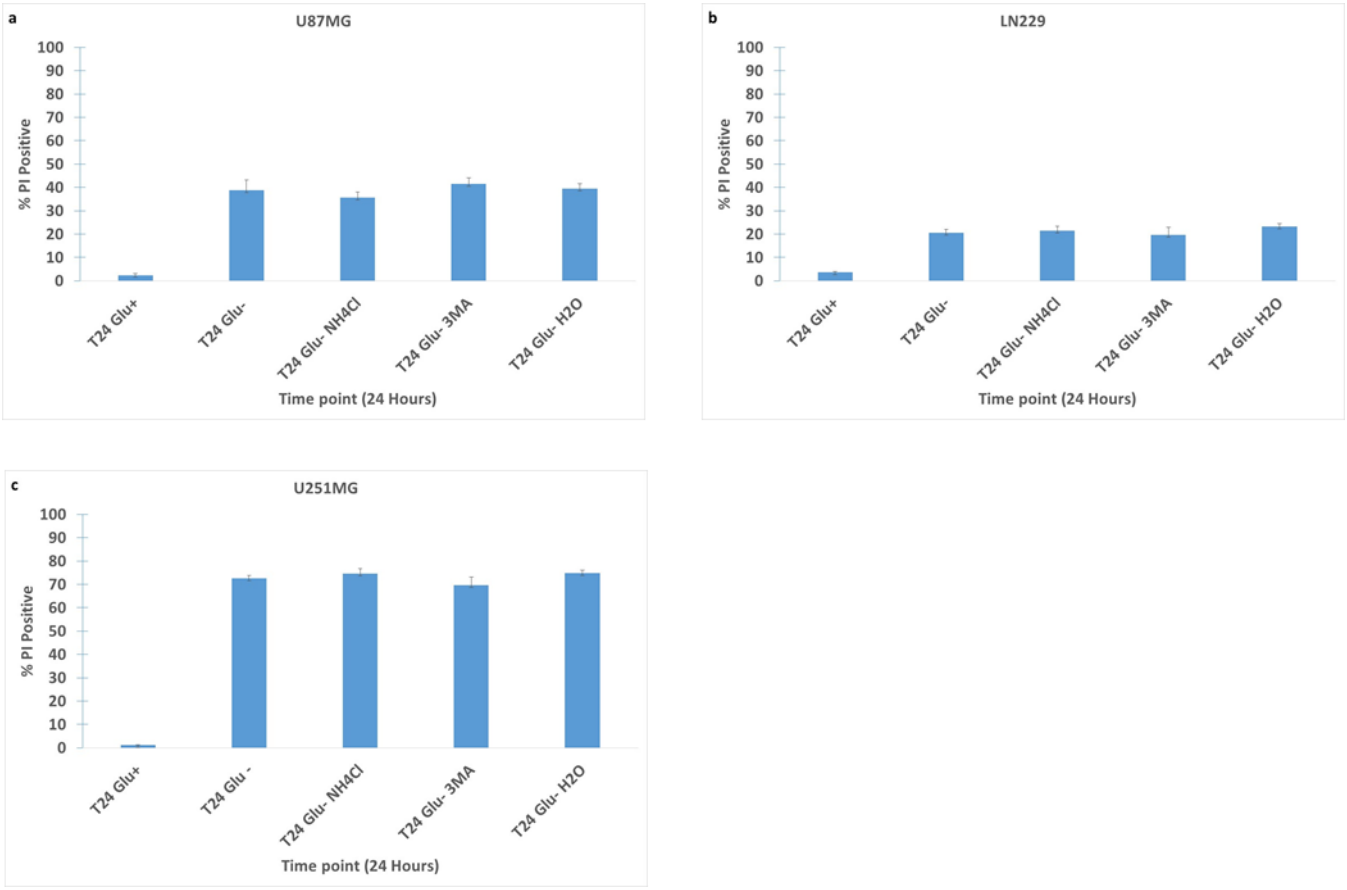


Figure 3.14 a, b, c: Autophagy inhibitors 3-MA or NH_4Cl do not rescue glucose withdrawal-induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 23 hours, cells were pre-incubated with 5mM 3-MA or 20mM NH_4Cl to allow them to be present inside the cells at the time when glucose was withdrawn. After 1 hour, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate (supplemented with 2% dialysed FCS) were added to the wells. 5mM 3-MA or 20mM NH_4Cl were added to appropriate wells. Water was added in appropriate wells as a control since 3-MA and NH_4Cl were dissolved in water. After 24 hours, the supernatant was collected into marked FACS tubes. Adherent cells were trypsinised and collected in appropriate tubes too. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and

error bars represent standard error of the mean. 3-MA or NH_4Cl had no any significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines.

3.2.15 Glucose deprivation-induced cell death in glioma cells is not mediated via necrosis

To test the effect of necrosis inhibition on glucose deprivation-induced cell death, U87MG, LN229 and U251MG cell lines were used. It was hypothesised that IM-54, a selective inhibitor of necrosis might rescue or suppress glucose withdrawal-induced cell death, as necrosis has been shown to play a role in glucose deprivation-induced cell death in some cancer cell lines. To test this, a propidium iodide assay was performed at a 24-hour time point after withdrawal of glucose (with or without IM-54). Interestingly, IM-54 had no significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cells (p=0.1456, 0.7477 and 0.3748 respectively).

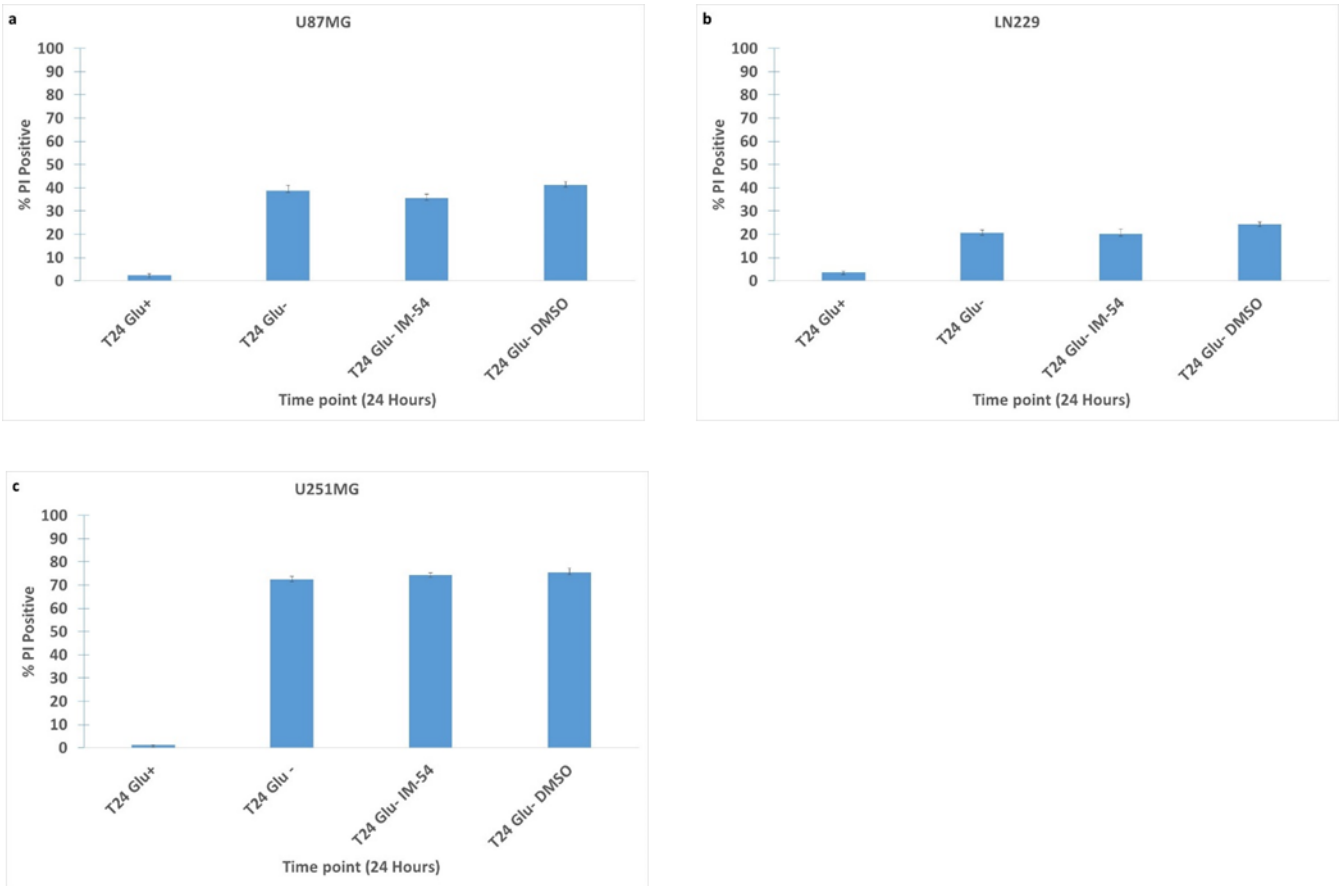


Figure 3.15 a, b, c: Necrosis inhibitor IM-54 does not rescue glucose withdrawal-induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 23 hours, cells were pre-incubated with 3µM IM-54 to allow it to be present inside the cells at the time when glucose was withdrawn. After 1 hour, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate (supplemented with 2% dialysed FCS) were added to the wells. 3µM IM-54 was also added to appropriate wells. DMSO was added in appropriate wells as a control since IM-54 was dissolved in DMSO. After 24 hours, the supernatant was collected into marked FACS tubes. Adherent cells were

trypsinised and collected in appropriate tubes too. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. IM-54 had no significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cells (p=0.1456, 0.7477 and 0.3748 respectively).

3.2.16 Cell lines derived from glucose-deprived cells are also sensitive to glucose deprivation-induced cell death

We have earlier shown that glucose deprivation-induced cell death in glioma cell lines (Figure 4.1). We hypothesised that cell population which survived glucose deprivation-induced cell death must be resistant subset and hence cell lines derived from these cells will be more resistant to glucose deprivation-induced cell death. To test this, a propidium iodide assay was performed at a 24-hour time point after complete glucose withdrawal in U87MG, LN229 and U251MG cell lines which were derived from cells previously subjected to glucose deprivation. We found that U87MG, LN229 and U251MG glioma cell lines were still sensitive to glucose deprivation-induced cell death (p=0.0011, 0.0043 and 0.0009 respectively).

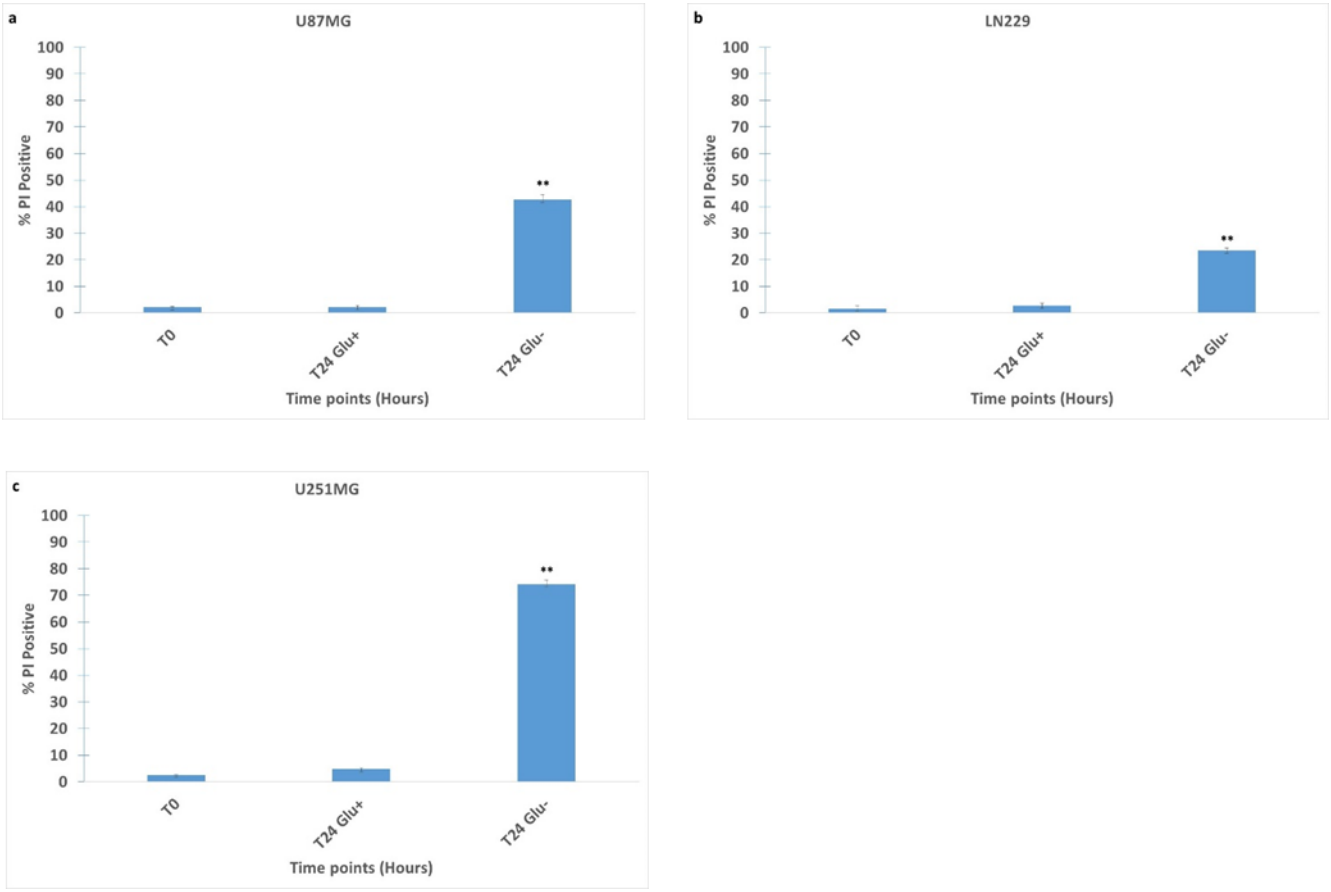


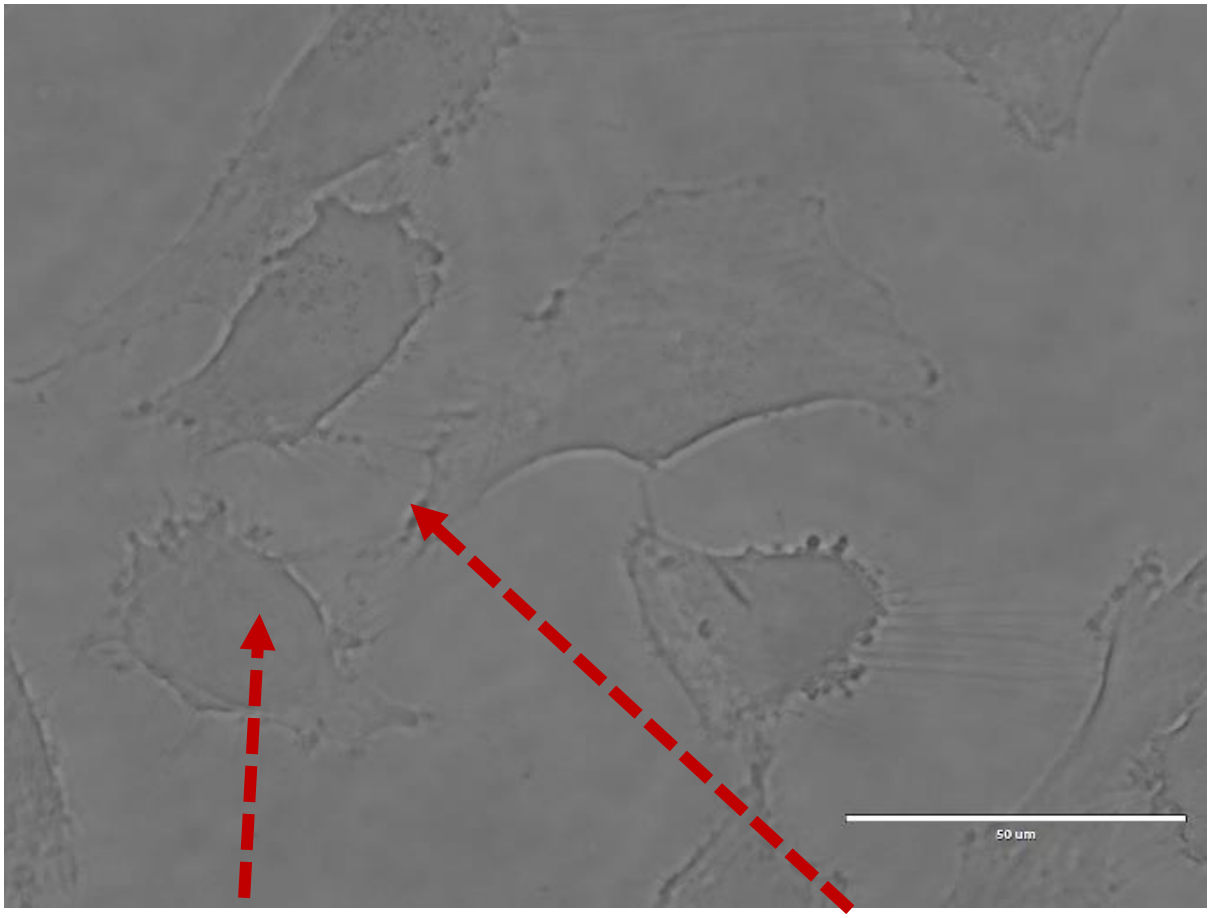
Figure 3.16 a, b, c: Cells derived from glucose-deprived cells are also sensitive to glucose deprivation-induced cell death. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was discarded, wells were washed with PBS

and DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS was added to the wells. After 24 hours, adherent cells were harvested, washed with PBS and cultured again in DMEM medium. When cells were confluent, they were harvested again and seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. For glucose withdrawal, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS was added to the wells. After 24 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicate, and error bars represent standard error of the mean. U87MG, LN229 and U251MG cell lines derived from cells previously subjected to glucose deprivation were also sensitive to glucose deprivation-induced cell death (p=0.0011, 0.0043 and 0.0009 respectively).

3.2.17 Change in morphology of glioma cells with glucose withdrawal and NAC treatment

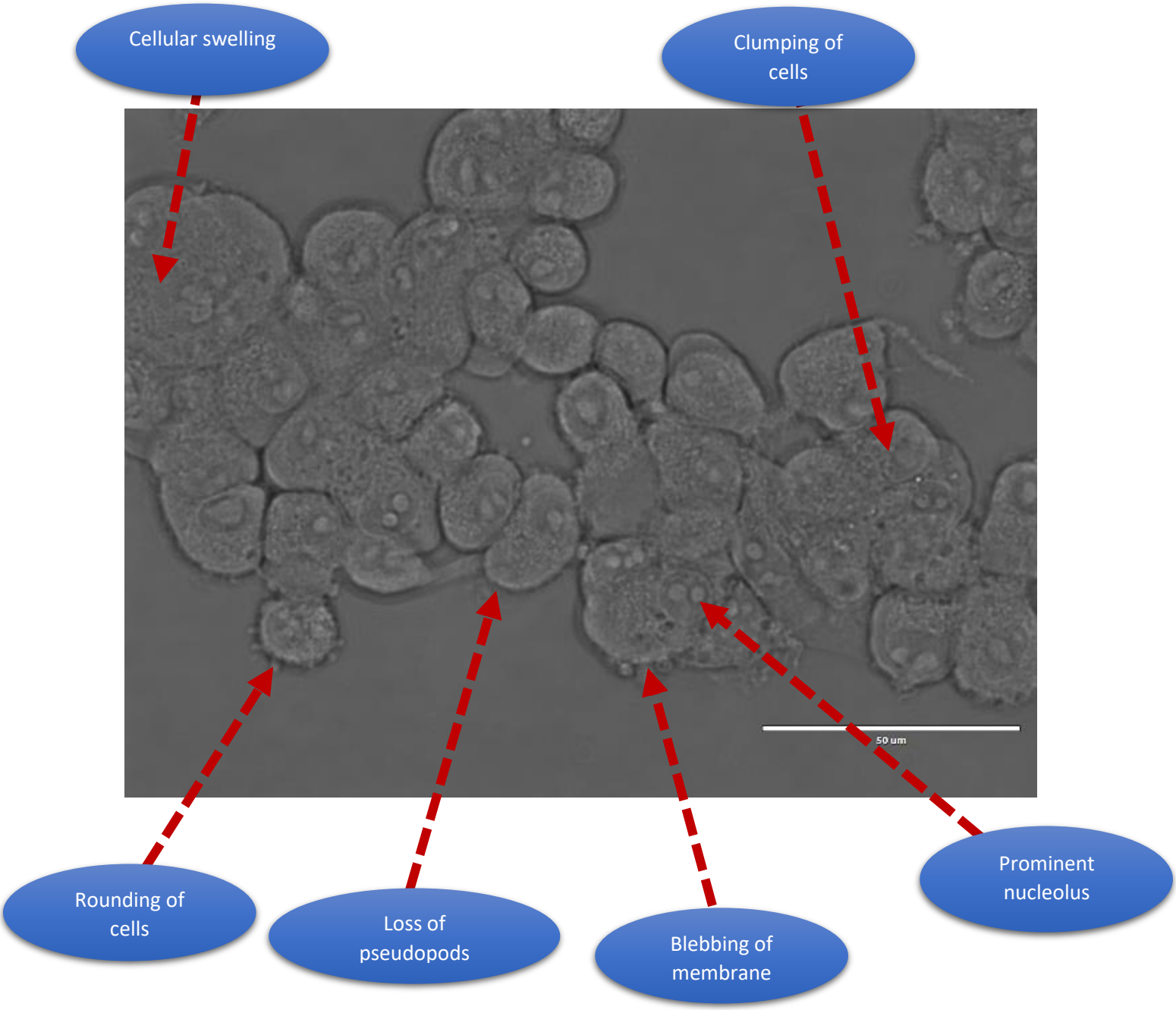
To study the effect of glucose deprivation on cell morphology, U87MG cells were grown in glucose-containing media and images were taken using a microscope. Subsequently, cells were subjected to glucose deprivation with or without free radical scavenger NAC and images were again taken. When cells were deprived of glucose, cells got detached from the corning flask, and some features of apoptosis such as rounding up of the cell and plasma membrane blebbing were seen. Similarly, some feature of necrosis such as the formation of cytoplasmic vacuoles was also seen. With NAC treatment though cell death was suppressed, morphology did not return to normal.

a



Cell growing as monolayer

Cell membrane processes (pseudopods)


Cellular swelling
Clumping of cells
50 um
Rounding of cells
Loss of pseudopods
Blebbing of membrane
Prominent nucleolus

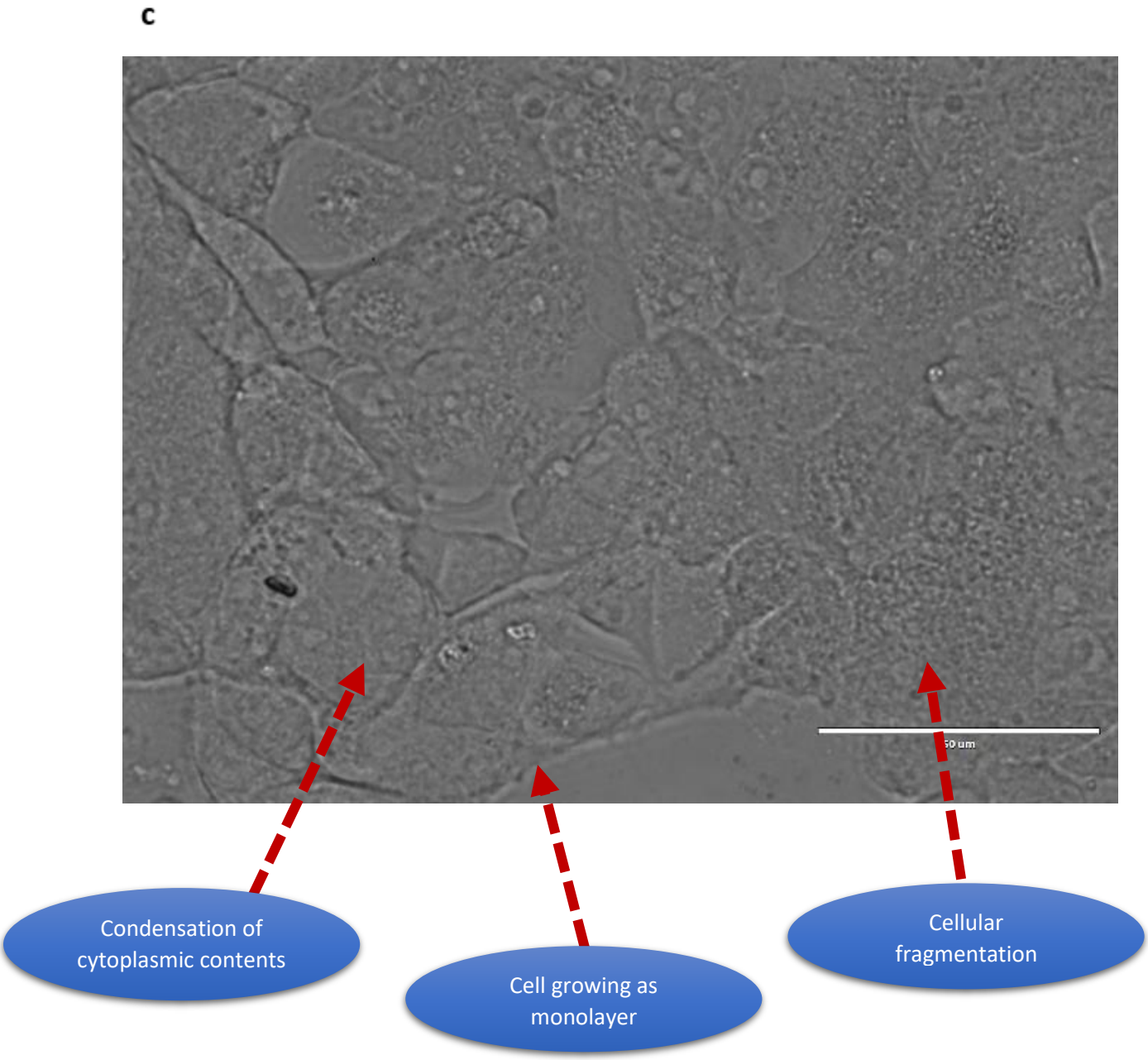
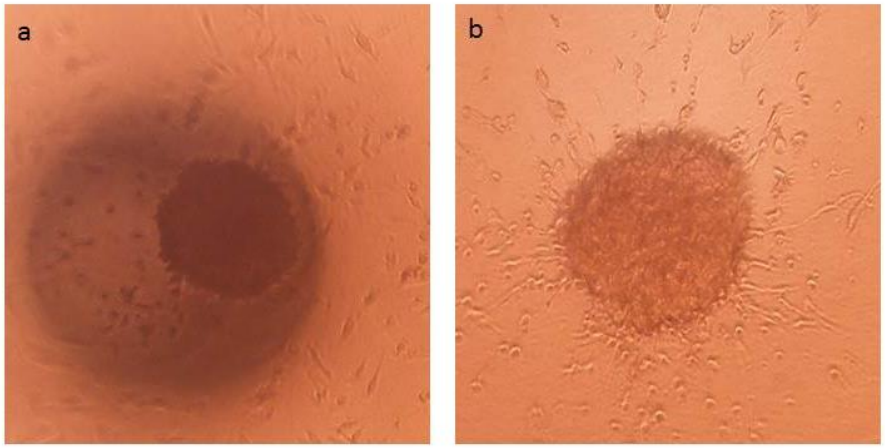


Figure 3.17 a, b, c: Change in glioma cell morphology with glucose deprivation. U87MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS and after 24-hour images were taken with the help of a microscope (a). The media was then discarded, wells were washed with PBS and DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS, with or without 1mM NAC was added to the well. Images were taken 24-hours post glucose withdrawal with (b) or without NAC (c).

3.2.18 Glucose deprivation promotes glioma cell migration

We performed a spheroid migration assay on glucose deprived glioma cells and showed that glucose deprivation promotes glioma cell migration. This is in agreement with previously published work of

my supervisor who showed that that glucose deprivation promotes glioma cell migration through micro-RNA 451 mediated regulation of the LKB1/AMPK pathway (Godlewski 2010). Migration index was calculated as described by Cockle et al (Cockle 2015). Migration index of glioma spheroid grown in glucose deprived condition was significantly higher than that of the control (p=0.0032).



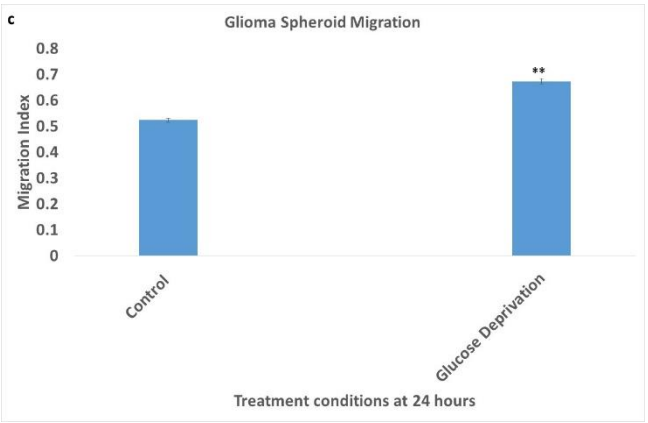
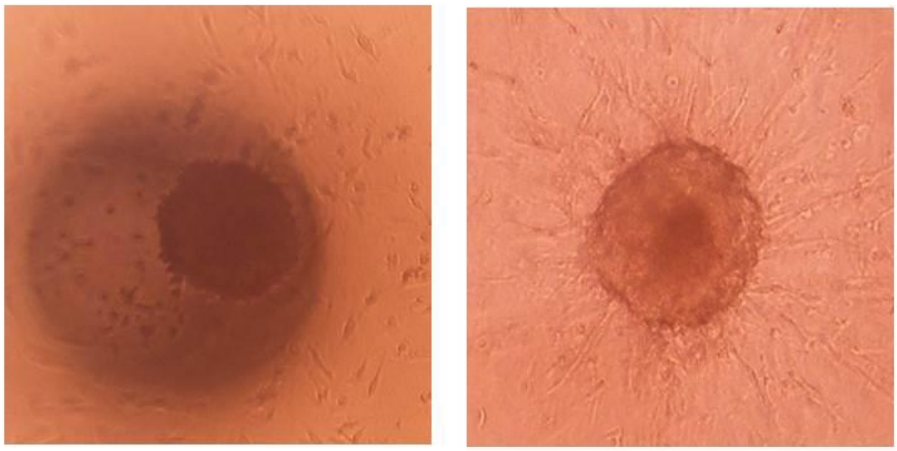


Figure 3.18 a, b: Glucose deprivation promotes glioma cell migration. U87MG cells were harvested and counted. $7.5X10^4$ cells in 1ml media (DMEM + 10% FCS) was used. 20 µl of the cell containing media was placed onto the lid of 100 mm dish (Falcon) which was inverted over the dish containing 10 ml of DMEM. Hanging drops cultures were incubated for 48 hours after which the spheroids were harvested using a pipette. Cell spheres were then put in 500µl of rat tail collagen-1 which was neutralised with 1 M sodium hydroxide in 5 × DMEM. After polymerisation at 37 °C, the collagen was overlaid with either (a) 500µl of glucose containing DMEM or (b) 500µl of DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS. The Falcon was placed in an incubator for 24 hours, and images were taken using a microscope. Figure 3.18 c: Glioma spheroid migration index in control and glucose deprived condition. Migration index of glioma spheroid grown in glucose deprived condition was significantly higher than that of the control (p=0.0032).

3.2.19 Hypoxia promotes glioma cell migration

We performed glioma cell migration in normoxia and hypoxia and showed that hypoxia like glucose withdrawal promotes glioma cell migration. Migration index of glioma spheroid grown in hypoxia was significantly higher than that of the control (p=0.0047).



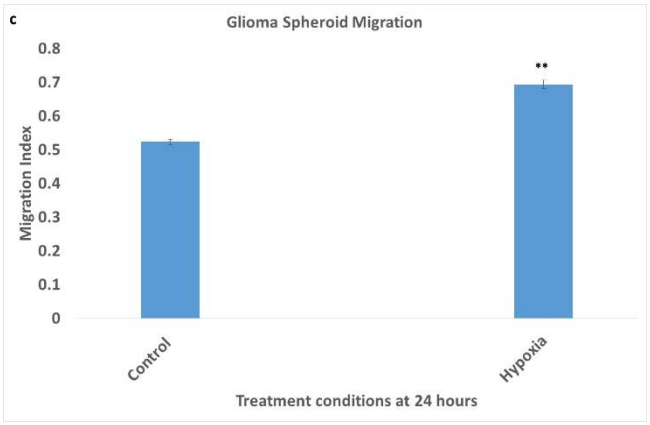
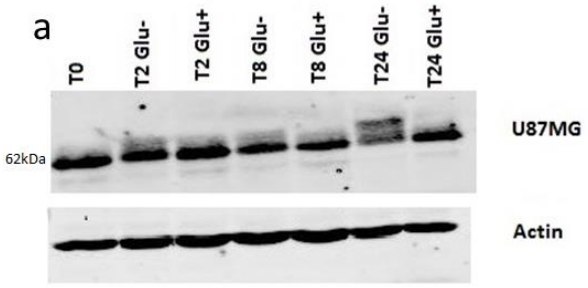


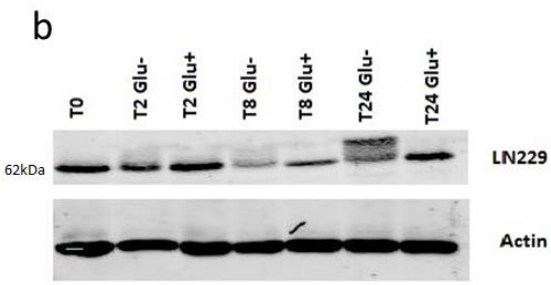
Figure 3.19 a, b: Hypoxia promotes glioma cell migration. U87MG cells were harvested and counted. 7.5X10^4 cells in 1ml media (DMEM + 10% FCS) was used. 20 µl of the cell containing media was placed onto the lid of 100 mm dish (Falcon) which was inverted over the dish containing 10 ml of DMEM. Hanging drops cultures were incubated for 48 hours after which the spheroids were harvested using a pipette. Cell spheres were then put in 500µl of rat tail collagen-1 which was neutralised with 1 M sodium hydroxide in 5 × DMEM. After polymerisation at 37 °C, the collagen was overlaid with 500µl of glucose-containing DMEM. This was placed in either (a) normoxic or (b) hypoxic incubator for 24 hours and images were taken using a microscope. Figure 3.19 c: Glioma spheroid migration index in normoxia and hypoxia. Migration index of glioma spheroid grown in hypoxia was significantly higher than that of the control in normoxia (p=0.0047).

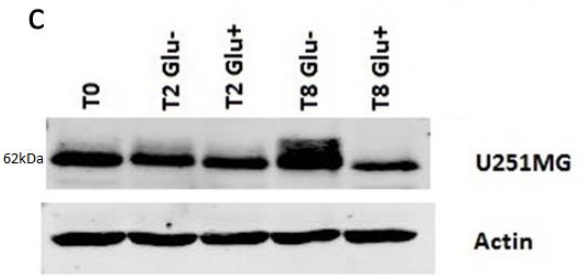
3.2.20 Glucose deprivation leads to energetic stress and upregulates pAMPK expression

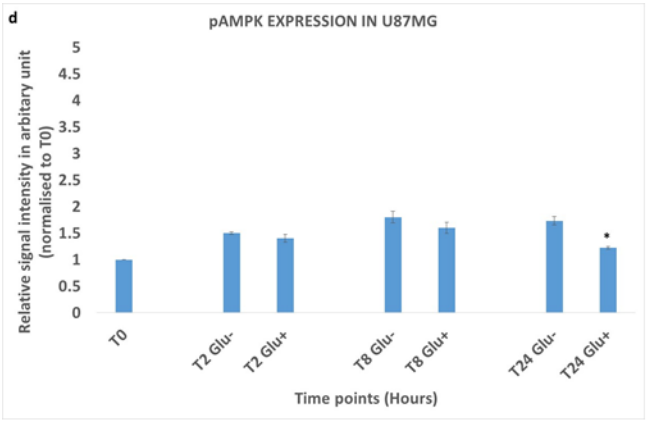
Cancer cells depend upon upregulated glycolysis to meet their energetic and biosynthetic needs as well as to generate reducing equivalents. We hypothesised that glucose deprivation will disrupt glycolysis and lead to energetic stress. pAMPK is an energy sensor of the cell and upregulated in

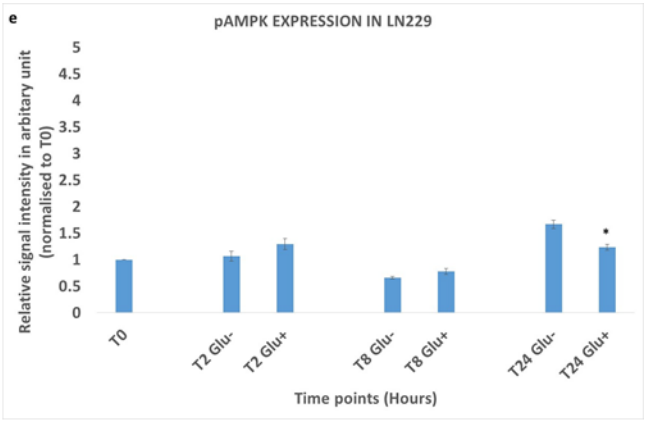
response to ATP depletion. To test this, western blot analysis was performed after subjecting glioma cells to glucose deprivation. U87MG and LN229 were deprived of glucose for 24 hours. However, U251MG were deprived of glucose for only 8 hours as not enough protein lysate was available for analysis at later time points. We showed that in U87MG, LN229 and U251MG glioma cell lines, pAMPK was upregulated in response to glucose deprivation at 24-hour time points (p=0.042, 0.0486 and 0.0008 respectively). pAMPK was found to be upregulated at earlier time points also, however, it was statistically not significant (p>0.05).











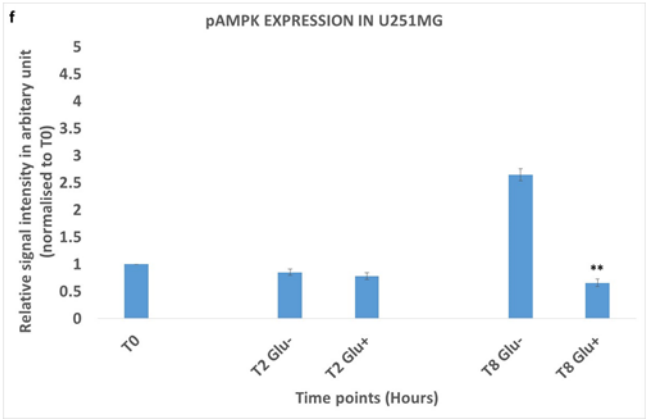
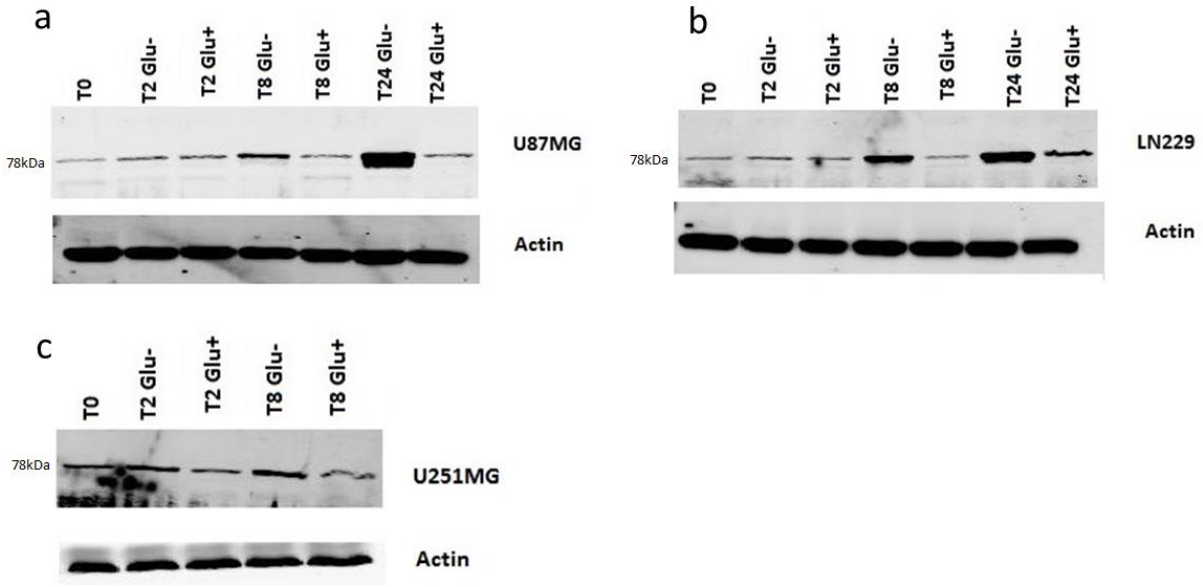


Figure 3.20 a, b, c: Glucose deprivation leads to energetic stress and upregulates pAMPK expression. 1X10 ^{5} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS was added to the wells. At appropriate time points, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against pAMPK (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 3.20 d, e, f: Relative signal intensity of pAMPK expression in arbitrary unit (normalised to T0) in U87MG, LN229 and U251MG glioma cell lines. In U87MG, LN229 and U251MG glioma cell lines, pAMPK was upregulated in response to glucose deprivation at 24-hour time points (p=0.042, 0.0486 and 0.0008 respectively). pAMPK was found to be upregulated at earlier time points also, however, it was statistically not significant (p>0.05).

3.2.21 Glucose deprivation leads to ER stress and upregulates ER chaperone GRP78

Glucose deprivation has been shown to induce UPR as it interferes with N-linked protein glycosylation. So, we hypothesised that glucose deprivation would induce ER stress in glioma cell lines and lead to GRP78 upregulation. To test this, western blot analysis was performed after subjecting glioma cells to glucose deprivation. U87MG and LN229 were deprived of glucose for 24 hours. However, U251MG were deprived of glucose for only 8 hours as not enough protein lysate was available for analysis at later time points. We showed that in U87MG glioma cell line, glucose deprivation led to ER stress and upregulation of ER chaperone GRP78 at 8-and 24-hour time points (p=0.0025 and 0.0005 respectively). Similarly, in LN229 glioma cell line, glucose deprivation led to ER stress and upregulation of ER chaperone GRP78 at 8-and 24-hour time points (p=0.0013 and 0.0017 respectively). In U251MG glioma cell line, glucose deprivation led to ER stress and upregulation of ER chaperone GRP78 at 8-hour time point (p=0.0007).



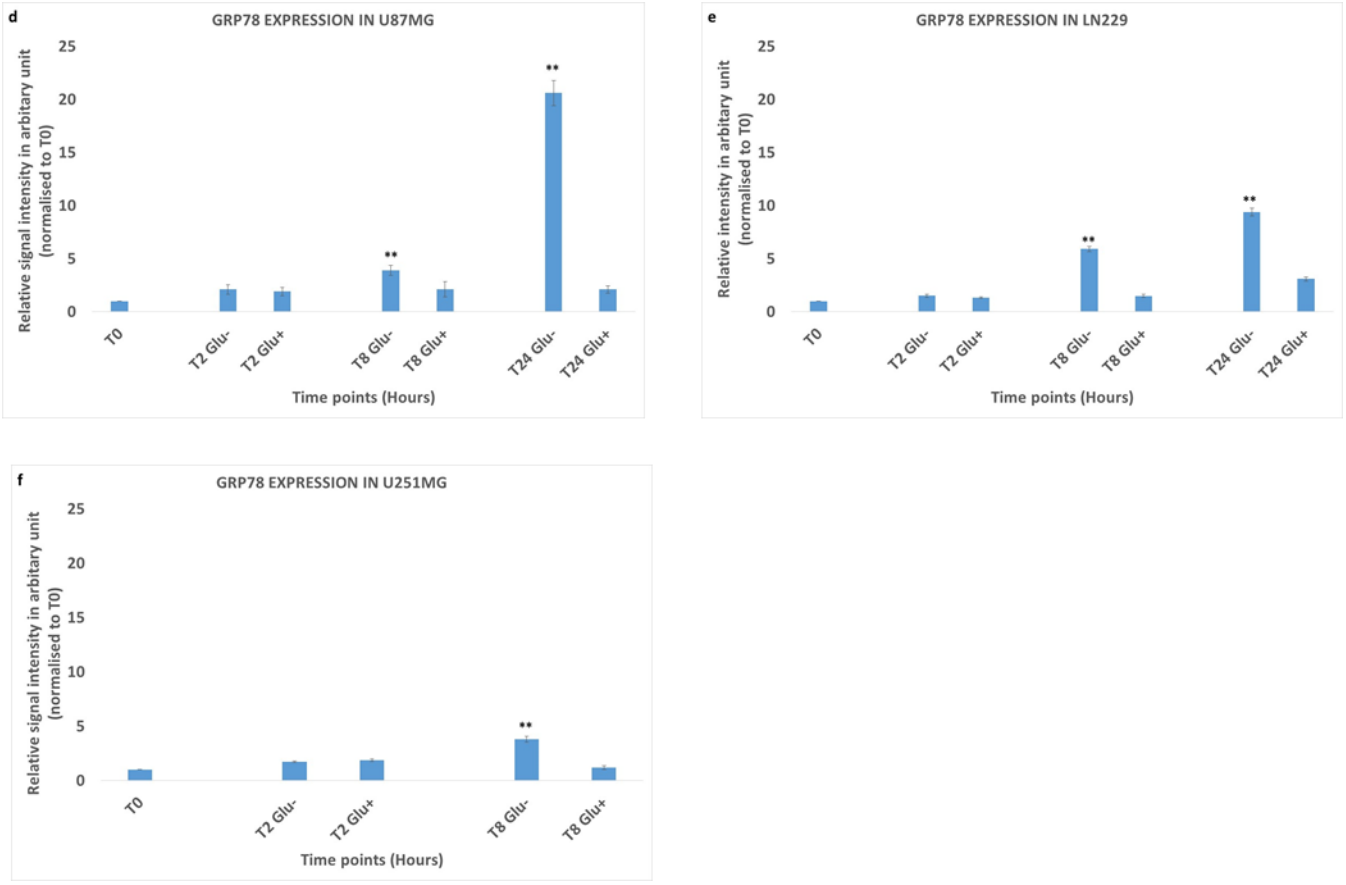
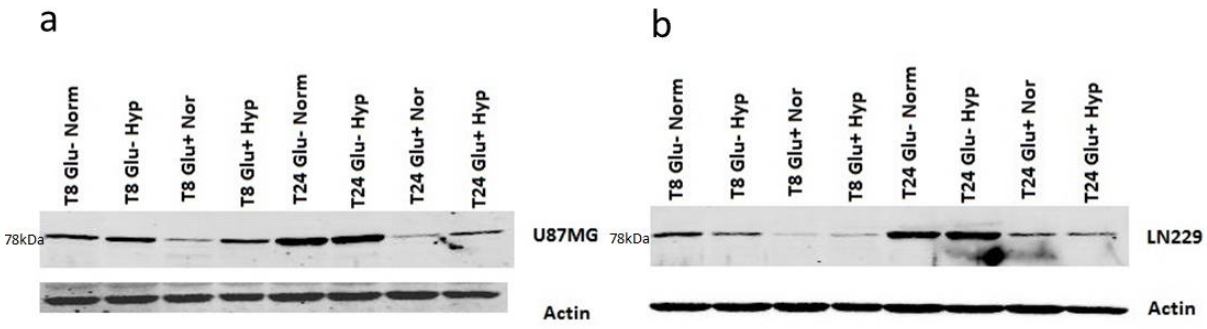


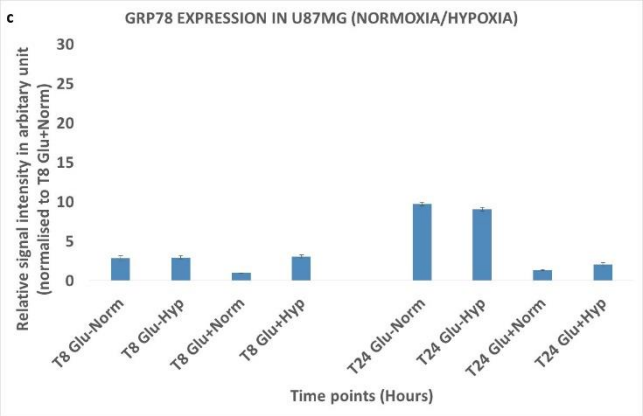
Figure 3.21 a, b, c: Glucose deprivation leads to ER stress and upregulates ER chaperone GRP78. 1X10 ^{5} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS was added to the wells. At appropriate time points, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against GRP78 (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 3.21 d, e, f: Relative signal intensity of GRP78 expression in arbitrary unit (normalised to T0) in U87MG, LN229 and U251MG glioma cell lines. In U87MG glioma cell line, glucose deprivation led to ER stress and upregulation of ER chaperone GRP78 at 8-and 24-hour time points (p=0.0025 and 0.0005 respectively). Similarly, in LN229 glioma cell line, glucose deprivation led to ER stress and upregulation of ER chaperone GRP78 at 8-and 24-hour time points (p=0.0013 and 0.0017 respectively). In U251MG glioma cell line also, glucose deprivation led to ER stress and upregulation of ER chaperone GRP78 at 8-hour time point (p=0.0007).

3.2.22 Hypoxia does not affect glucose deprivation-mediated GRP78 upregulation

We have earlier shown that hypoxia suppresses glucose deprivation-induced cell death in U87MG and LNN229 cell lines whereas it potentiates glucose deprivation-induced cell death in U251MG cell line (Figure 4.11). GRP78 is a pro-survival arm of UPR. We hypothesised that hypoxia would lead to GRP78

upregulation in U87MG and LN229 cell line. To test this, western blot analysis was performed after subjecting glioma cells to glucose deprivation in normoxia and hypoxia. In U87MG glioma cell line, hypoxia had no effect on glucose deprivation-induced GRP78 expression as compared to normoxia at 8-or 24-hour time points (p=0.8489 and 0.3138 respectively). Similarly, in LN229 glioma cell line, hypoxia had no effect on glucose deprivation-induced GRP78 expression as compared to normoxia at 8-or 24-hour time points (p=0.8008 and 0.82 respectively).





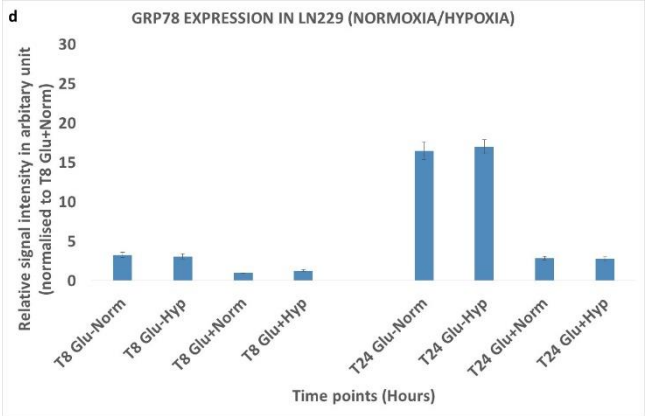


Figure 3.22 a, b: Hypoxia does not affect glucose deprivation-mediated GRP78 upregulation. 1X10 ^{5} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight in normoxia and hypoxic incubator. Following morning, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS was added to the wells. Dishes were again placed in a normoxic and hypoxic incubator. At appropriate time points, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against GRP78 (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 3.22 c, d: Relative signal intensity of GRP78 expression in arbitrary unit (normalised to T8 Glu+Norm) in U87MG and LN229 glioma cell lines. In U87MG glioma cell line, hypoxia had no

effect on glucose deprivation-induced GRP78 expression as compared to normoxia at 8-or 24-hour time points (p=0.8489 and 0.3138 respectively). Similarly, in LN229 glioma cell line, hypoxia had no effect on glucose deprivation-induced GRP78 expression as compared to normoxia at 8-or 24-hour time points (p=0.8008 and 0.82 respectively).

3.3 Discussion

Our experiments to investigate the effect of complete withdrawal of glucose on glioma cell viability provide a proof of concept that deranged tumour metabolism can be successfully targeted. It is impossible to get rid of all the glucose *in vivo;* however, these experiments show that addiction of tumour cells to glucose can be exploited to cause selective tumour cell killing. The difference in glucose uptake of cancer cells and untransformed cells has already formed the basis of diagnostic imaging such as FDG-PET. Through these experiments, we have shown that cancer cells as compared to their normal counterpart are more dependent on upregulated glycolysis for their growth, proliferation and survival and hence more sensitive to glucose deprivation-induced cell death.

The results showed that complete glucose deprivation leads to cell death in all three glioma cell lines (p=0.0001, 0.0008 and <0.0001 for U87MG, LN229 and U251Mg cell lines at 24-hour time point respectively). When growth medium or serum containing glucose was used, then comparatively less cell death was seen and that too at a longer time point (p=0.1843 and 0.0812 for U87MG and LN229 at 24-hour time point respectively). These findings are in agreement with other authors. Jones *et al.* also noted that complete glucose deprivation was essential to elicit significant cell death during glucose withdrawal experiments in transformed fibroblasts (2005). Coloff *et al*. showed that as little as 0.018gm/L of glucose was sufficient to prevent glucose deprivation-induced cell death (2011). Izuishi *et al.* during their work on liver, pancreas, colon and gastric cancer cells observed that cancer cells can survive glucose deprivation longer in the presence of serum as they can use protein present in serum as a source of amino acids which can be subsequently used to generate energy (2000). Similarly, Mathews *et al*. during their work on the HeLa cell line noted that the presence of pyruvate during glucose and glutamine withdrawal could contribute to TCA cycle and rescue cell death. They also showed that when media lacked glucose, glutamine and pyruvate there was higher cell death as compared to in media which lacked glucose and glutamine but contained pyruvate or when media contained low glucose and low glutamine but lacked pyruvate (2014). Our results also showed that methyl pyruvate was able to partially rescue glucose withdrawal-induced cell death (p=0.0032 and 0.0021 for U87MG and LN229 for 24-hour time point respectively). Pyruvate besides being a source of energy via the TCA cycle is also a known scavenger of free radicals. Spitz *et al*. were also able to rescue breast cancer cells from glucose withdrawal-induced cell death by using methyl pyruvate

(2000). Similarly, Buzzai *et al*. showed that methyl pyruvate was able to rescue glucose deprivation-induced cell death in glioma cell lines (2005). Yang *et al*. were also able to rescue glioma cells from glucose deprivation-induced cell death by using methyl pyruvate (2009). We have also shown that when cells surviving glucose deprivation were re-cultured in glucose-containing medium and subjected to glucose deprivation again, it did not result in less cell death ($p=0.0011$, 0.0043 and 0.0009 for U87MG, LN229 and U251MG respectively). This shows that glioma cells which survived initial glucose deprivation were not a resistant subset of cells else their daughter cells would not have been prone to glucose deprivation-mediated cell death.

Among the three adult glioma cell lines we used, U251MG was most sensitive to glucose withdrawal-induced cell death whereas LN229 was least sensitive. Variation in susceptibility to glucose withdrawal-induced cell death among different cell lines of same cancer has also been reported by other authors too (Izuishi 2000, Elstrom 2004, Buzzai 2005, Priebe 2011, Graham 2012). According to them, the susceptibility of cancer cells to glucose withdrawal-induced death is directly related to pAkt expression in the cells. When Akt is active, it promotes movement of citrate from TCA cycle of the mitochondria into the cytosol where it is broken down in acetyl CoA and OAA by ACL. Akt has been shown to phosphorylate and activates ACL. During fatty acid synthesis, this acetyl CoA is converted into malonyl CoA by ACC. Malonyl CoA is then acted upon by FAS to form fatty acids during which NADPH is consumed. Malonyl CoA has been shown to inhibit CPT1 which is responsible for the transport of fatty acids into the mitochondria. Fatty acid transported into the mitochondria can either be used to generate energy or it can be diverted into NADPH producing reactions. Hence activated Akt on the one hand by promoting fatty acid synthesis depletes cell of NADPH and on the other hand by elevating malonyl CoA level inhibits transport of fatty acid into the mitochondria which could have been used to generate energy and NADPH (Kuhajda 2008, Coloff 2011, Carracedo 2013). Priebe *et al*. showed that in ovarian cancer, the cell line with the highest resistance to glucose withdrawal-induced cell death had very little pAkt expression and when Akt was activated in this cell line {myristoylated Akt (myrAkt)}, it led to increased sensitivity to glucose deprivation-induced cell death (2011). Buzzai *et al.* also showed that LN229 glioma cells lacked constitutively active Akt and it was resistant to glucose deprivation which is in agreement with our results. He also showed that LN18 glioma cell line had constitutively active Akt and it was sensitive to glucose deprivation-induced cell death. When Akt was activated in LN229 (myrAkt), it showed increased cell death with glucose withdrawal and knockdown of Akt in LN229 myrAkt again made it resistant to glucose withdrawal-induced cell death (2005). Elstorm *et al*. showed that LN229 as compared to LN18 consumed less glucose and was less sensitive to glucose withdrawal-induced cell death. They also showed that activation of Akt in LN229 led to increased glucose consumption and increased sensitivity to glucose withdrawal-induced cell

death (2004). Graham *et al*. also showed that U87MG glioma cell line which contains constitutively active Akt was more sensitive to glucose withdrawal cell death as compared to LN229 and when Akt expression was introduced in LN229, it also became sensitive to glucose withdrawal-induced cell death (2012). All three of our cell lines were purchased from ATCC®, and mutation data were available for all the three cell lines. U87MG and U251MG were PTEN mutant which is a negative regulator of Akt, and hence these two cell lines had active Akt, which would explain their sensitivity to glucose withdrawal-induced cell death. On the other hand, LN229 had wild-type PTEN which will lead to inactivation of Akt, and this can explain relative resistance of LN229 to glucose withdrawal-induced cell death. Also, p53 mutation status of U87MG and U251MG were compared. U251 is p53 mutant whereas U87MG has wild-type p53. p53 acts as a cell cycle checkpoint protein in response to various stress stimuli and induce cell cycle arrest. Glucose deprivation has been shown to lead to p53 mediated cell cycle arrest (Jones 2005). However, p53 mutant U251 cells may have been unable to undergo cell cycle arrest when subjected to glucose deprivation and had undergone death, which can explain why more cell death was seen in p53 mutant U251MG cells as compared to U87MG which contains wild-type p53.

Our results also showed that glucose deprivation-induced cell death was suppressed by NAC ($p=0.0002$, 0.0047 and 0.0006 for U87MG, LN229 and U251MG respectively). NAC is a known anti-oxidant which cannot be metabolised in the body to generate energy. This shows that though glucose deprivation besides causing oxidative stress may have also led to ATP depletion and energetic stress, it is oxidative stress which is responsible for cell death. Our findings are in agreement with others. Jelluma *et al*. showed that NAC was able to rescue glioma cells from glucose deprivation-induced cell death (2006). Hong *et al* also showed that though with glucose deprivation, ATP level decreased but it was not completely depleted at 18 hours and knockdown of nicotinamide phosphoribosyltransferase (NAMPT), which is a rate-limiting enzyme involved in NAD^+ biosynthesis, did not lead to further ATP depletion but caused increased cell death pointing to the fact that oxidative stress rather than ATP depletion was cause of cell death in breast cancer cell lines (2016). They also showed that during glucose deprivation NAMPT via NAD^+ could replenish NADPH store. A number of studies have shown that during glucose deprivation, oxidative phosphorylation is upregulated to generate energy which leads to oxidative stress as a result of increased ROS generated in the mitochondria and decreased NADPH and pyruvate being generated via glycolysis (Blackburn 1999, Ahmad 2005, Jelluma 2006, Hong 2016). Besides, synthesis of GSH which participates in anti-oxidant defence is an active process and requires ATP, so ATP depletion and energetic stress as a result of glucose deprivation can further downregulate GSH biosynthesis and potentiate oxidative stress (Shi 2006). Studies have shown that with glucose deprivation there is a decrease in NADPH level and

steady-state increase in the level of H_2O_2, superoxide, GSH precursor γ-glutamylcysteine and oxidised GSH leading to oxidative stress (Blackburn 1999, Spitz 2000, Ahmad 2005, Mathews 2014). Ahmad *et al*. showed that overexpression of anti-oxidant catalase or SOD offered protection against glucose deprivation-induced cell death (2005). Similarly, Aykin-Burns *et al*. showed that in colon and breast cancer cells, glucose deprivation led to NADPH depletion and cell death and overexpression of catalase or SOD partially rescued glucose deprivation-induced cell death (2009). We have also shown that with prolonged glucose deprivation, NAC was unable to protect cells from death which may be due to the fact that cells were overwhelmed by the oxidative stress ($p=0.1471$ and 0.1547 for U87MG and LN229 respectively). Our data also show that H_2O_2 was able to induce death in glioma cells ($p<0.05$). H_2O_2 is known to induce oxidative stress (Nogueira-Pedro 2013), and this further confirms the susceptibility of glioma cells to oxidative stress.

We have also shown that complete glucose deprivation in normal human fibroblasts did not lead to any significant cell death ($p=0.3888$). Cancer cells are known to be more sensitive to glucose deprivation than their normal counterparts (Choi 2013) and Spitz *et al*. also noted that normal fibroblasts are resistant to glucose withdrawal-induced cell death (2000). As normal human fibroblasts are non-transformed cells, they are not known to harbour defects in their mitochondria, so during glucose deprivation, they may be able to upregulate mitochondrial oxidative phosphorylation to meet their energy demands without potentiating oxidative stress. Normal proliferating cells also do not exhibit activation of oncogenes or loss of tumour suppressor genes (Dang 2011), and they possess cell cycle checkpoints (Vander Heiden 2011) which may be another reason that such cells are resistant to glucose deprivation-induced cell death.

Our data also show that hypoxia did not result in glioma cell death when glucose was present. This is in agreement with the findings of Steinbach *et al*. who also did not see any cell death in glioma cells when glucose was present in hypoxia (2013). We have also shown that in hypoxia with glucose deprivation, less cell death was seen in U87MG and LN229 cell lines as compared to cell death with glucose deprivation in normoxia ($p=0.0435$ and 0.0013 respectively at 24-hour time point). However, in U251MG cell line more cell death was seen with glucose deprivation in hypoxia than in normoxia ($p=0.0414$ at 24-hour time). Spitz *et al*. also saw less death with glucose deprivation in hypoxia (2000). During glucose deprivation, mitochondrial oxidative phosphorylation is upregulated to generate energy from the fatty acid and amino acids and mitochondria is the main site for ROS generation. Hence mitochondria play an important role in glucose deprivation-induced ROS generation and oxidative stress and this may be the reason that hypoxia leads to less cell death in glucose deprivation condition as hypoxia suppresses mitochondria (Spitz 2000). Hypoxia via HIF upregulates PDK1 which in turn inhibits PDH and leads to the decreased entry of substrate into the mitochondria. This can also

lead to decreased ROS generation and reduced oxidative stress (Denko 2008). Hypoxia can also activate NF-κB which plays an important role in anti-oxidant defence (Höckel 2001). Besides, hypoxia has also been shown to antagonise p53 mediated apoptosis (Kurrey 2009) which may also explain less cell death seen in hypoxia. We have also demonstrated that hypoxia promotes glioma cell migration which is in agreement with others. Knockdown of HIF-1α in glioma cells has been shown to reduce migration *in vitro* and invasion *in vivo* and impair the ability of cancer cells to form tumour spheres (Méndez 2010).

Glutamine plays an important role in energy generation, redox balance and biosynthesis (DeBerardinis 2007, DeBerardinis 2010, Wise 2010, Weinberg 2010, Daye 2012, Lyssiotis 2013), however, we did not see any synergistic effect of glutamine withdrawal on glucose deprivation-induced glioma cell death ($p=0.2606$, 0.7732 and 0.2276 for U87MG, LN229 and U251MG respectively). Our findings are in agreement Graham *et al*. who showed that glutamine failed to rescue glucose deprivation-induced cell death in glioma cell lines (2012). On the other hand, Lee *et al*. showed that glutamine was able to rescue glucose deprivation-induced cell death in breast cancer cells (1997). Similarly, Spitz *et al* showed that glutamine can rescue glucose withdrawal-induced cell death in breast cancer cells (2000). This shows that there are conflicting reports about glucose and glutamine metabolism interconnections. Some studies have shown that glucose and glutamine metabolism may have a regulatory effect on each other as glucose deprivation can lead to a marked reduction in glutamine uptake by the cell (Wellen 2010). On the other hand, glutamine has been shown to inhibit TXNIP a negative regulator of glucose uptake and hence promote the Warburg effect by enhancing glucose uptake and aerobic glycolysis (Kaadige 2009). Some other studies have also shown that glucose and glutamine metabolism can complement each other. Glucose deprivation in glioblastoma cell line has been shown to lead to rapid increase in glutamate dehydrogenase whereas silencing of GLS led to an increased conversion of glucose-derived pyruvate to oxaloacetate via pyruvate carboxylase to contribute to mitochondrial metabolism (Yang 2009, Cheng 2010). However, one has to remember that not all cancers are *myc* driven and hence all of them may not be sensitive to glutamine deprivation. Similarly, cancer cells may be metabolic flexible and may activate or upregulate alternative pathways to generate glutamine (Maher 2012, Hensley 2013).

We have also shown that glucose deprivation-induced cell death was not as a result of apoptosis, necrosis or autophagy. ZVAD-fmk a pan-caspase inhibitor failed to rescue glioma cells from glucose deprivation-induced cell death ($p=0.4988$, 0.1453 and 0.6028 for U87MG, LN229 and U251MG respectively). Similarly, IM-54 (1-Methyl-3-(1-methyl-1H-indol-3-yl)-4-(pentylamino)-1H-pyrrole-2,5-dione, 2-(1H-Indol-3-yl)-3-pentylamino-maleimide), a selective inhibitor of necrosis induced by oxidative stress failed to rescue glioma cells from glucose deprivation-induced cell death ($p=0.1456$,

0.7477 and 0.3748 for U87MG, LN229 and U251MG respectively). Also, autophagy inhibitors 3-methyladenine (p=0.4360, 0.4728 and 0.5666 for U87MG, LN229 and U251MG respectively) and ammonium chloride (p=0.2372, 0.8831 and 0.6877 for U87MG, LN229 and U251MG respectively) failed to rescue glucose deprivation-induced cell death. León-Annicchiarico *et al*. also noted that glucose deprivation led to a form of cell death which did not resemble apoptosis, autophagy, necroptosis or ferroptosis. They also mention that in their experimental conditions, ATP levels were maintained until the end and so they ruled out necrosis as a form of cell death too as necrosis is usually associated with ATP loss. However, unlike us, in their experimental conditions, cell death was not rescued by NAC (2015). Other authors have also shown that glucose deprivation can lead to multiple different modes of cell death. Jelluma *et al*. showed that glucose deprivation-induced apoptotic cell death in U251MG glioma cell line (2006) where glucose deprivation has been shown to induce necrotic cell death in hepatoma cells (Suzuki 2003). Caro-Maldonado *et al*. showed that in BAX/BAK deficient cell, glucose deprivation-induced an atypical form of apoptosis which was death receptor-independent and caspase 8 mediated (2010). Glucose deprivation has also been shown to lead to mitotic catastrophe and cell death (Isono 2014). However, one has to appreciate that type of cell death depends upon a number of factors, and it may be context dependent upon cell type, the developmental stage of tissue, growth conditions as well as intensity and duration of treatment (Elmore 2007, Jagannathan 2015). At low doses, a number of noxious stimuli such as heat, hypoxia, radiation and chemotherapy drugs can induce apoptosis whereas same stimuli at higher doses can result in necrosis (Elmore 2007). A low dose of 3-BP has been shown to lead to moderate ATP depletion and apoptosis whereas a higher concentration of 3-BP can lead to massive ATP depletion and necrosis (Xu 2005). Similarly, hypoxia has been shown to induce apoptotic cell death in most cancer cells, but in glioblastoma cells, it induces necrotic cell death with minimal apoptosis (Steinbach 2003). Also, the mode of cell death can depend upon cell line used as well as the duration of treatment. Hypoxia followed by 24-hour normoxia has been shown to lead to necrotic cell death with minimal apoptosis in LN229 glioma cells whereas in U87MG glioma cells, initially early apoptotic changes were seen though at later time points necrotic cell death similar to LN229 was seen (Steinbach 2003). One also has to remember that apoptosis and certain form of necrosis are active processes requiring ATP and severe ATP depletion during glucose deprivation may make cell resistant to such forms of cell death (Steinbach 2003). The form of cell death may also depend upon the rate of ATP depletion. Del Nagro *et al*. showed that slower rate of ATP depletion could lead to activation of caspase 3 and cells may show apoptosis or autophagy whereas rapid ATP depletion does not lead to caspase 3 activation and cells do not show apoptosis or autophagy. They identified oncosis as a major form of cell death when ATP level fell more than 20-fold (2014). Oncotic cell death is a specific form of cell death which was

initially described in cardiomyocytes following profound myocardial ischaemia. Oxygen and glucose deprivation have also been shown to induce oncotic cell death *in vitro*. ER stress in combination with cellular swelling followed by cell detachment and death suggest oncosis as the cause of death. Oncotic cell death can be differentiated from apoptosis by time-lapse microscopy (Bikas 2015). In our experiments, one of the reasons why it was difficult to characterise cell death may have been due to the fact that cell death resulted from an interplay between glucose deprivation-induced ROS induction and oxidative stress, glucose deprivation-mediated ATP depletion and glucose deprivation-induced ER stress and UPR. Besides, oxidative stress can also induce ER stress and ER stress can lead to leakage of Ca^{2+} into the cytosol to activate AMPK via CAMKK2 (Xi 2013).

We have also shown that on the one hand glucose deprivation leads to glioma cell death but on the other hand it promotes glioma cell migration ($p=0.0032$). This is in agreement with previous findings of my supervisor who showed that glucose withdrawal promotes glioma cell migration. They showed that glucose deprivation leads to miR-451 downregulation which in turn allowed activation of LKB1 and its downstream substrate AMPK. They proposed that miR-451 mediated regulation of LKB1 played a key role in the response of glioma cells to glucose deprivation by regulating the balance of glioma cell proliferation, migration, and survival (Godlewski 2010).

Our data also show that hypoxia promotes glioma cell migration ($p=0.0047$). Hypoxia has also been shown to upregulate MMP which help in sprouting of newly formed blood vessels (Muñoz-Nájar 2006, Muz 2015). Hypoxia also influences a number of steps in the metastatic process including invasion, migration, extravasation, formation of pre-metastatic niche, as well as growth and survival at a distant site. HIF-1 has been shown to promote EMT (Rankin 2016). HIF-1 has also been shown to activate other genes involved in invasion and metastasis such as c-met proto-oncogene and autocrine motility factor (Brahimi-Horn 2007) which help in tumour cell migration.

These experiments also demonstrated that in media containing glucose, there was no pAMPK activation whereas glucose deprivation led to energetic stress and pAMPK upregulation in all three glioma cell lines ($p=0.042$, 0.0486 and 0.0008 for U87MG, LN229 and U251MG respectively). This is in agreement with other authors. Bikas *et al*. also noted that there was no pAMPK upregulation in high glucose condition whereas, in low glucose, there was increased phosphorylation of AMPK in thyroid cancer cell lines (2015). When energy status of a cell is optimal, ATP binds to AMPK and inactivates it. However, when there is a fall in ATP level, AMP replaces ATP from the γ subunit resulting in allosteric activation of AMPK. Binding of AMP to γ subunit also exposes threonine 172 on α subunit of AMPK for phosphorylation by upstream kinase LKB1 (Jones 2005, Kuhajda 2008, Shackelford 2009, Jeon 2012, Russo 2013, Grahame Hardie 2014). During energetic stress, pAMPK downregulates glycolysis, inhibits

anabolic processes such as protein synthesis & lipogenesis and at the same time promotes p53 mediated cell cycle arrest and upregulates FAO to conserve energy (Jones 2005, Russo 2013, Grahame Hardie 2014). Lee *et al*. also showed that glucose deprivation leads to 4-fold ATP depletion within 2 hours in breast cancer cell line (1997). Besides energetic stress, ROS can also activate AMPK (Wu 2013), and it may be that in our experiments both energetic stress, as well as ROS, mediated oxidative stress was responsible for AMPK upregulation. We also noted that glucose deprivation-induced pAMPK upregulation did not correlate with cell death and pAMPK was unable to protect cells from glucose deprivation-induced cell death. We saw pAMPK upregulation in all three cell lines, but there was relatively more cell death in U251MG and U87MG cell lines as compared to LN229 cell line. Buzzai *et al*. also saw AMPK activation in both LN229 and LN18 cell line and noted that in LN18 cell line, pAMPK upregulation failed to protect it from glucose deprivation-induced cell death which may be due to the fact that that the cell line had active Akt which prevented it from upregulating FAO (2005). Our data also showed that glucose deprivation led to ER stress and induced UPR leading to upregulation of ER chaperone GRP78 ($p=0.0005$, 0.0017 and 0.0007 for U87MG, LN229 and U251MG respectively). Our findings are in agreement with others who have also shown that glucose deprivation leads to UPR induction and increased GRP78 expression (Park 2007, Matsuo 2012). Glucose deprivation has been shown to induce UPR as it interferes with N-linked protein glycosylation (Xu 2005). However, ROS has also been shown to induce ER stress (Kuznetsov 2011), and it may be that in our experiments glucose deprivation-mediated alteration in N-linked protein glycosylation, as well as ROS, mediated oxidative stress was responsible for induction of ER stress and GRP78 upregulation. GRP78 expression was independent of p53 and PTEN status of the three glioma cell lines which is in agreement with other authors (Pyrko 2007).

However, despite the successful targeting of glioma cells by glucose deprivation shown here, one has to remember that the field of metabolism research is filled with controversies and contradictions. Steinbach et al. noted that glucose deprivation in hypoxia resulted in increased cell death which they attributed to ATP depletion as a result of the inhibition of glycolysis as well as oxidative phosphorylation (2003). Wu *et al*. showed that nutrient starvation led to apoptotic cell death and NAC led to increased apoptosis in nutrient-deprived cells (2013). Unlike our experiments, both Ahmad *et al.* (2005) and Jelluma *et al*. (2006) showed that glucose deprivation did not result in any significant fall in ATP level and pAMPK was not elevated as a result of glucose deprivation (Jelluma 2006). Jelluma *et al*. also showed that Akt played no role in glucose deprivation-induced cell death in glioma cells and PTEN knockdown in normal astrocytes did not sensitise them to glucose deprivation-induced cell death (2006).

There is also a persisting controversy regarding the existence and role of mitochondrial defects in cancer cells. Inhibition of oxidative phosphorylation has been shown to upregulate glycolysis but when glycolysis is inhibited, some cancer cells fail to upregulate oxidative phosphorylation, which may point to the fact that there may be an underlying defect in the mitochondria of the cancer cells (Wu 2007). On the other hand, some studies have shown that Warburg effect may not be a feature of all cancer cells and even in glycolytic cancer cells, oxidative phosphorylation may not be completely redundant (Moreno-Sanchez 2007, Cairns 201). Studies have also shown that oxidative phosphorylation may be normal in tumour cells with no underlying defect in the mitochondria of the cancer cells (Eakin 2007). So, it may be that, rather than being impaired, mitochondrial function may be reprogrammed to meet the biosynthetic needs of rapidly proliferating cancer cells (Weinhouse 1976, Fantin 2006, Moreno-Sánchez 2007, Ward 2012) and such cells can utilise fatty acids, lactate and amino acids as fuel for their energetic needs (Cairns 2011).

There is also conflicting evidence that the Warburg effect offers resistance against cell death as TMZ resistant glioma cells have been shown to have decreased glucose consumption and lactate production which are the hallmarks of the Warburg effect. Oliva *et al.* suggested that increased efficiency of mitochondrial coupling resulted in reduced ROS generation and oxidative stress as a result of TMZ treatment (2011). Similarly, CSC have been shown to exhibit an increased reliance on oxidative phosphorylation as compared to aerobic glycolysis. These CSC have reduced ROS generation and show increased resistance to radiotherapy and chemotherapy (Vlashi 2011). Tumour may also exhibit reverse Warburg effect. (Sonveaux 2008).

3.4 Future work

Our experiments on glucose deprivation-induced glioma cell death have provided interesting results and paved the way for further studies. Future work should look at cell cycle and ATP assay in response to glucose deprivation. It would also be interesting to look at mechanism leading to more cell death in U251MG in response to glucose deprivation in hypoxia when the other two cell lines showed less cell death in response to glucose deprivation in hypoxia. Future work can also involve outlining pathways which promote glioma spheroid migration in hypoxia.

Chapter 4

4.1 Introduction

In this chapter, we have looked at the effect of metformin on glucose deprivation-induced glioma cell death. During our work on glucose deprivation-induced glioma cell death earlier, we have shown that glucose deprivation led to ER stress and UPR which in turn leads to upregulation of ER chaperoneGRP78 (Figure 4.21). GRP78 is the pro-survival arm of UPR and downregulation of GRP 78 has been shown to lead to cell death. Park *et al* showed that glucose deprivation led to GRP78 upregulation and macrocyclic compound versipelostatin downregulated GRP78 leading to massive cell death in fibrosarcoma, colon and stomach cancer cells (2004). Park *et al*. also showed that verrucosidin isolated from Penicillium could down-regulate GRP78 and potentiate glucose deprivation-induced cell death in fibrosarcoma and colon cancer cells (2007). Similarly, Saito *et al*. showed that metformin could down-regulate GRP78 and potentiate glucose deprivation-induced cell death in fibrosarcoma, colon, stomach and renal cancer cells (2009). So, we decided to investigate the effect of metformin on glucose deprivation-induced glioma cell death. As the anti-proliferate effect of metformin is highly dependent upon its concentration in the extracellular microenvironment (Bikas 2015), we decide to use low (1mM) as well as high (8mM) concentration of metformin. AICAR like metformin has been shown to upregulate pAMPK, so we also looked at the effect of AICAR on glucose deprivation-induced glioma cell viability. 2DG has been shown to inhibit HK and GPI and lead to ATP depletion (Pelicano 2006). However, 2DG has also been shown to affect protein glycosylation, which may lead to accumulation of unfolded proteins in the ER and GRP78 upregulation (Park 2004, Kang 2006, Kurtoglu 2007, Saito 2009). Priebe *et al* showed that 2DG and glucose deprivation led to synergistic cell death which points to the fact that 2DG may induce cell death independent of its effect on glycolysis (2011). So, we decided to investigate the effect of 2DG on glucose deprivation-induced glioma cell death. We also investigated the mechanism of metformin and glucose deprivation-induced cell death. In our experiments of glucose deprivation in hypoxia, we have shown that in hypoxia, less cell death was seen in U87MG and LN229 cells as compared to in normoxia (Figure 4.11) most likely as a result of mitochondrial inhibition in hypoxia. Metformin has been shown to inhibit ETC I, so we decided to investigate the effect of glucose deprivation and metformin on glioma cell viability in hypoxia. Lithium has been shown to be neuroprotective in cultured cells and animal models (Luo 2010). Also, anti-apoptotic Mcl-1 has been shown to play an important role in glucose deprivation-mediated cell death (Coloff 2011), and GSK3β has also been shown to inhibit mTOR and down-regulate Mcl-1 (Manning 2007). So, we decided to investigate the effect of inhibition of GSK3β on glioma cell viability by using lithium chloride which is a known inhibitor of GSK3β. During our work on glucose deprivation, we have

shown that glucose deprivation leads to pAMPK upregulation (Figure 4.20). Besides energetic stress, ROS via oxidative stress-mediated CAMKK2 pathway can also upregulate pAMPK. So, we decided to use STO-609 which inhibits CAMKK2 and investigate its effect on glucose deprivation-mediated glioma cell viability. Finally, we looked at the effect of metformin and glucose deprivation of cell morphology, pAMPK and GRP78 expression.

4.2 Results

4.2.1 Metformin does not affect glioma cell viability in the presence of glucose

Metformin has been shown to inhibit ETC I, induce energetic stress and upregulate pAMPK. We hypothesised that metformin would not have any effect on glioma cell viability in the presence of glucose as cancer cells depend upon upregulated glycolysis to meet their bioenergetic and biosynthetic needs. To test this, a propidium iodide assay was performed at different time points in the presence of 1 or 8mM metformin as discussed in materials and methods section 2.2.4 and 2.2.5. Metformin had no significant effect on U87MG, LN229 and U251 glioma cell viability in the presence of glucose at 24-hour time point ($p=0.6537$, 0.1831 and 0.514 respectively). However, at 8-hour time point statistically significant cell death was seen in U87MG and U251 cell lines with metformin treatment ($p=0.0077$ and 0.0075 respectively). Similarly, at 12-hour time point, statistically significant cell death was seen in LN229 cell line with 8mM metformin ($p=0.0267$) and in U251MG cell line with 1mM metformin ($p=0.0351$). Further study may be needed to look at the effect of metformin on glioma cell lines in the presence of glucose at shorter time points.

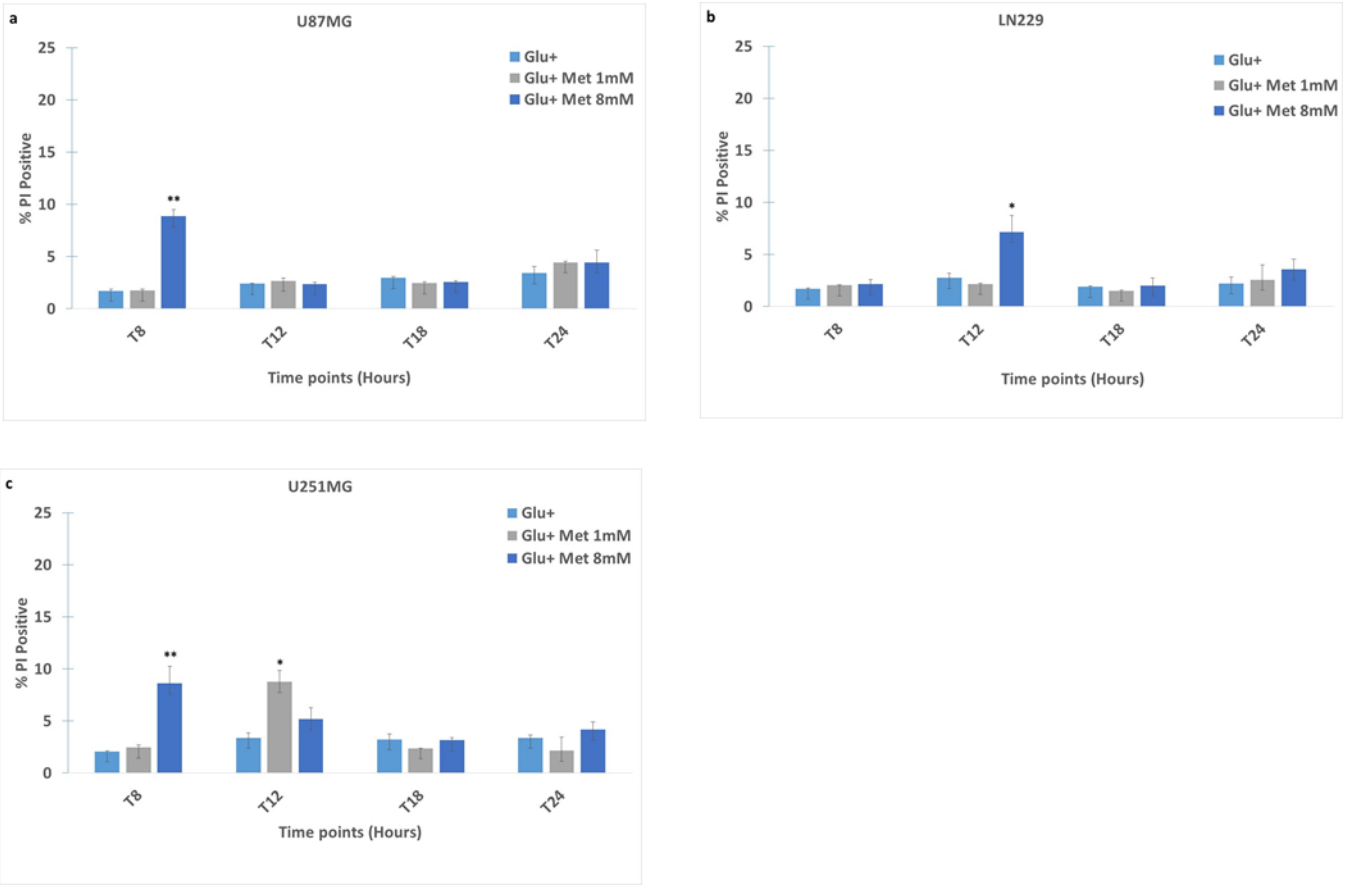


Figure 4.1 a, b, c: Metformin does not affect glioma cell viability in the presence of glucose. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested and cell viability was determined by a propidium iodide assay to serve as a baseline. Media was removed from wells and wells were washed gently with PBS. DMEM supplemented with 2% dialysed FCS and containing 1 or 8mM metformin was added to appropriate wells. At different time points, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. Metformin had no significant effect on U87MG, LN229 and U251 glioma cell viability in the presence of glucose at 24-hour time point (p=0.6537, 0.1831 and 0.514 respectively). However, at 8-hour time point statistically significant cell death was seen in U87MG and U251 cell lines (p=0.0077 and 0.0075 respectively). Similarly, at 12-hour time point, statistically significant cell death was seen in LN229 cell line with 8mM metformin (p=0.0267) and in U251MG cell line with 1mM metformin (p=0.0351).

4.2.2 Metformin potentiates glucose withdrawal-induced cell death in glioma cell lines in a dose-dependent manner

Metformin has been shown to inhibit ETC I, induce energetic stress and up-regulate pAMPK. It has also been shown to downregulate GRP78 in some cancer cell lines to potentiate cell death (Saito 2009). We have previously demonstrated that glucose deprivation leads to GRP78 upregulation (Figure 4.21). We hypothesised that metformin would potentiate glucose withdrawal-induced cell death in U87MG, LN229 and U251MG glioma cell lines. To test this, a propidium iodide assay was performed at different

time points after complete glucose withdrawal in the presence 1 or 8mM metformin. In U87MG glioma cell line, 1mm metformin potentiated glucose deprivation-induced cell death at all time points; however, it was statistically significant at 24-hour time point only (p=0.0069). 2mM metformin significantly potentiated glucose deprivation-induced cell death in U87MG glioma cell line at all time points (p=0.0004, 0.0019 0.0031 and 0.0067 for time points 8, 12, 18 and 24 hours respectively). In LN229 cell line, 1mm Metformin potentiated glucose deprivation-induced cell death at all time points; however, it was statistically significant at only 24-hour time point (p=0.0101). Similarly, 2mM metformin potentiated glucose deprivation-induced cell death at all time points; however, it was statistically significant at only 24-hour time point (p=0.001). 1mM metformin significantly potentiated glucose deprivation-induced cell death in U251MG glioma cell line at all time points (p=0.019, 0.017, 0.0063 and 0.0318 for time points 8, 12, 18 and 24 hours respectively. Similarly, 2mM metformin significantly potentiated glucose deprivation-induced cell death in U251MG glioma cell line at all time points (p=0.0031, 0.0041, 0.0005 and 0.0017 for time points 8, 12, 18 and 24 hours respectively).

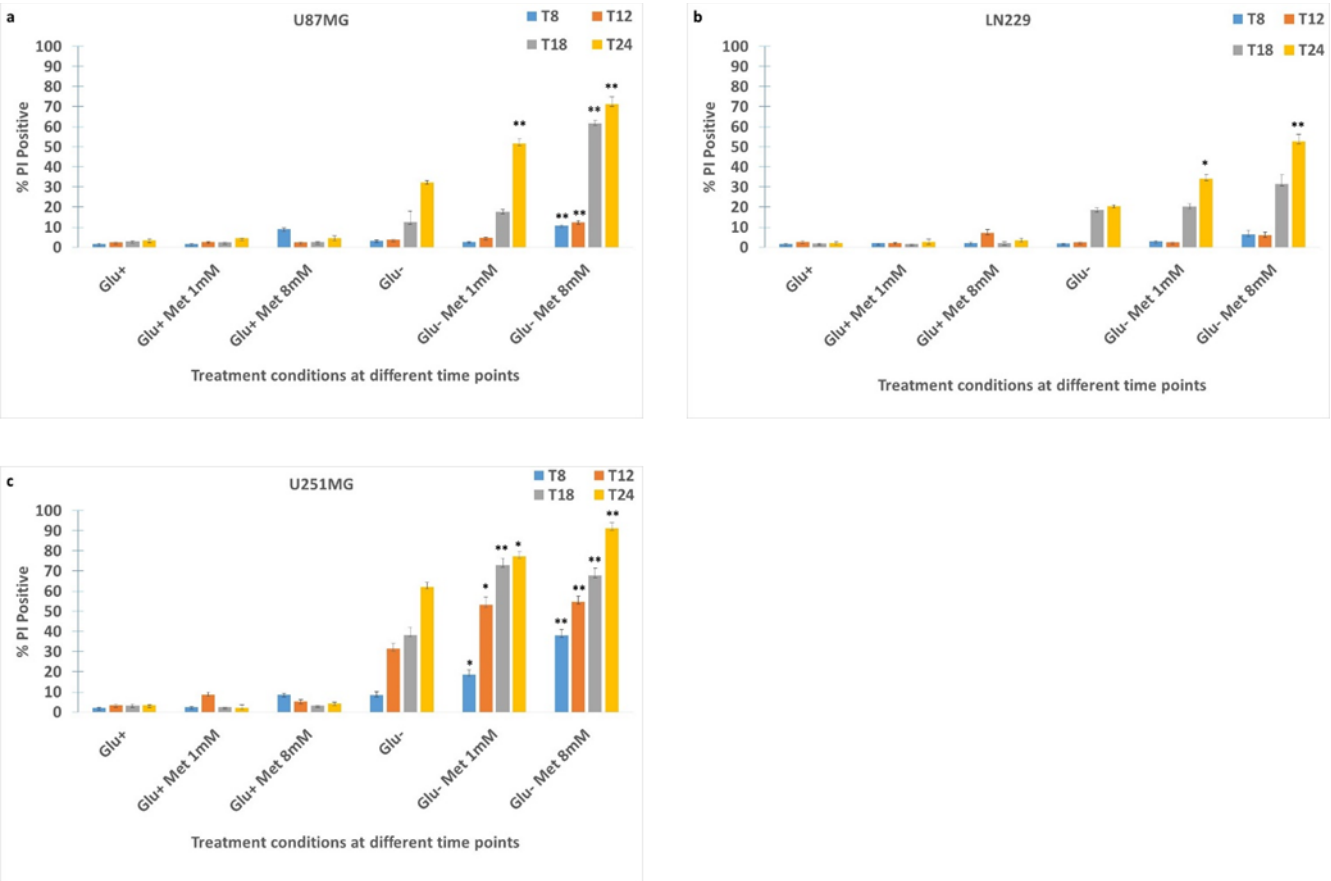


Figure 4.2 a, b, c: Metformin potentiates glucose deprivation induces cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested and cell viability was determined by a propidium iodide assay to serve as a baseline. Media was removed from appropriate wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS and containing 1 or 8mM metformin was added to appropriate wells. At different time points,

suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicate, and error bars represent standard error of the mean. Metformin significantly potentiated glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines at 24-hour time point.

4.2.3 AICAR does not affect glioma cell viability in the presence of glucose

AICAR like metformin has also been shown to upregulate pAMPK. We hypothesised that AICAR would not affect glioma cell viability in the presence of glucose. To test this, a propidium iodide assay was performed after treating glioma cells with 1 mM AICAR for 24 hours as discussed in materials and methods section 2.2.4 and 2.2.5. AICAR had no significant effect on U87MG, LN229 and U251 glioma cell viability in the presence of glucose (p=0.0673, 0.5059 and 0.4775 respectively).

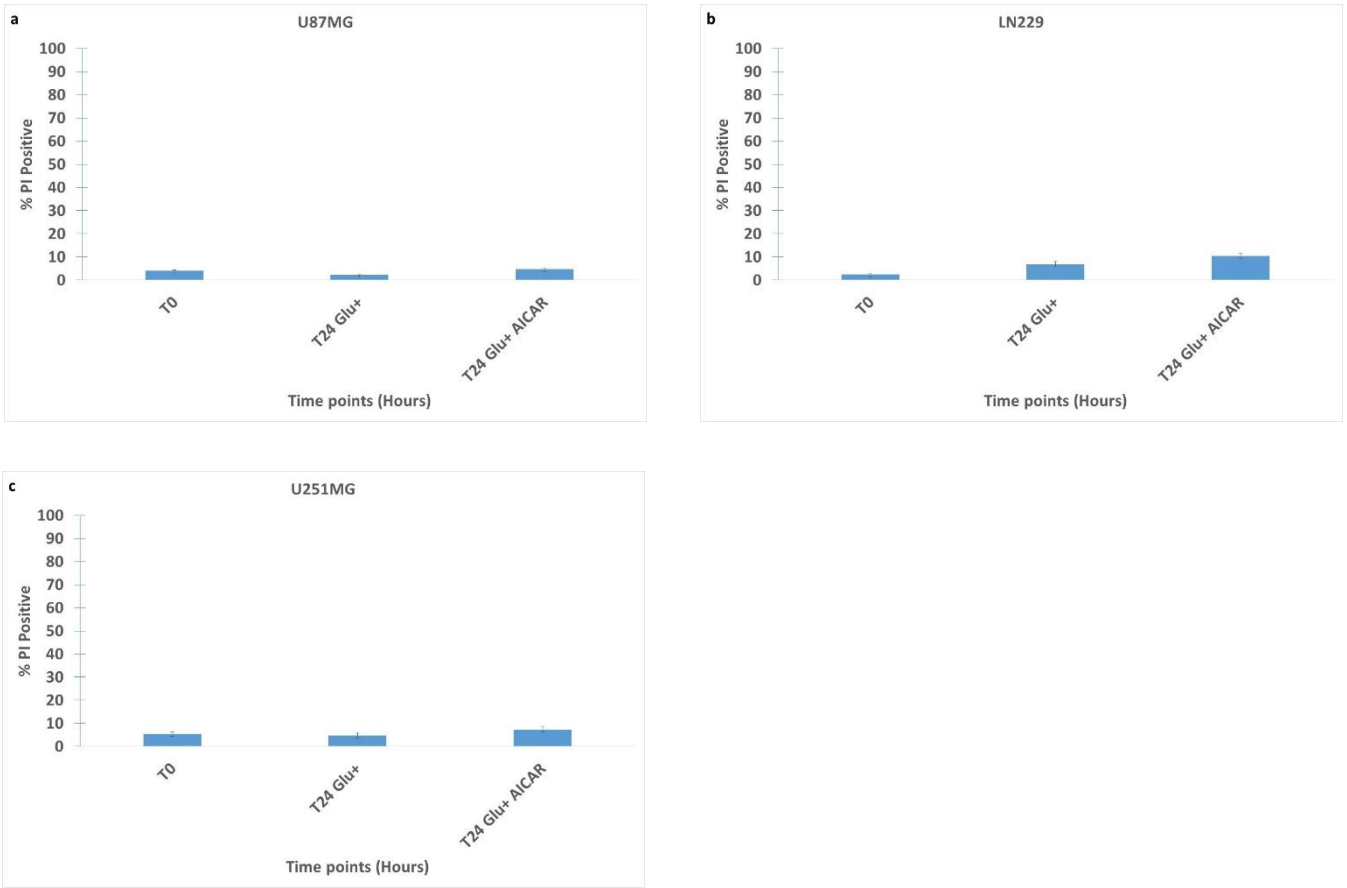


Figure 4.3 a, b, c: AICAR does not affect glioma cell viability in the presence of glucose. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. Media was removed from wells and wells were washed gently with PBS. DMEM supplemented with 2% dialysed FCS and containing 1 mM AICAR was added to appropriate wells. At

24 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. AICAR had no significant effect on U87MG, LN229 and U251 glioma cell viability in the presence of glucose (p=0.0673, 0.5059 and 0.4775 respectively).

4.2.4 AICAR potentiates glucose withdrawal-induced cell death in glioma cell lines

We hypothesised that AICAR like metformin will potentiate glucose withdrawal-induced cell death in U87MG, LN229 and U251MG glioma cell lines. To test this, a propidium iodide assay was performed at different time points after complete glucose withdrawal in the presence 1 mM AICAR. AICAR significantly potentiated glucose deprivation-induced cell death in U87MG, LN229 and U251 glioma cell lines (p=0.0003, 0.0273 and 0.0003 respectively).

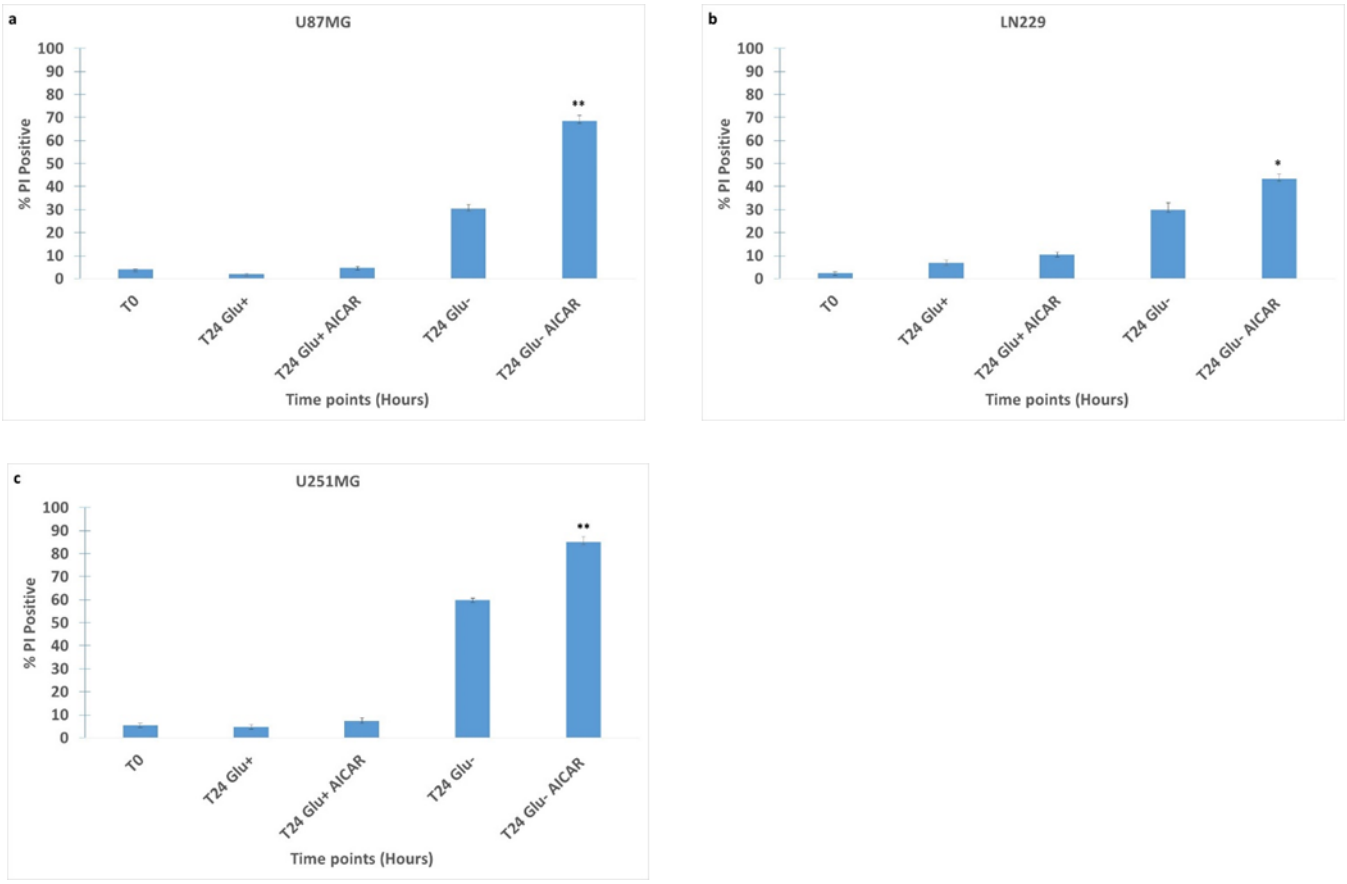


Figure 4.4 a, b, c: AICAR potentiates glucose deprivation induces cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. Media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS and containing 1 mM AICAR was added to appropriate wells. At 24 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicate, and error bars represent standard error of the mean. AICAR significantly potentiated

glucose deprivation-induced cell death in U87MG, LN229 and U251 glioma cell lines (p=0.0003, 0.0273 and 0.0003 respectively).

4.2.5 2DG does not affect glioma cell viability in the presence of glucose

Cancer cells exhibit upregulated glycolysis and 2DG has been shown to inhibit glycolysis. However, cancer cells have also been shown to be metabolically flexible. We hypothesised that 2DG would not affect glioma cell viability in the presence of glucose. To test this, a propidium iodide assay was performed after treating glioma cells with 10mM 2DG for 24 hours as discussed in materials and methods section 2.2.4 and 2.2.5. 2DG had no significant effect on U87MG, LN229 and U251 glioma cell viability in the presence of glucose (p=0.1735, 0.131 and 0.1578 respectively).

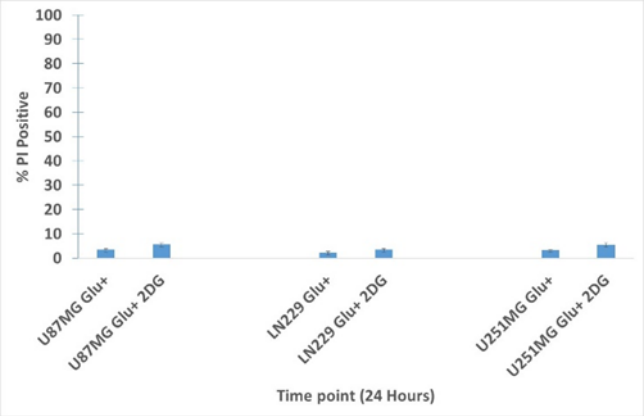


Figure 4.5 2DG does not affect glioma cell viability in the presence of glucose. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells and wells were washed gently with PBS. DMEM supplemented with 2% dialysed FCS and containing 10 mM 2DG was added to appropriate wells. After 24 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. 2DG had no significant effect on U87MG, LN229 and U251 glioma cell viability in the presence of glucose (p=0.1735, 0.131 and 0.1578 respectively).

4.2.6 2DG suppressed glucose deprivation-induced cell death in glioma cell lines.

Cancer cells exhibit upregulated glycolysis and 2DG has been shown to inhibit glycolysis. We hypothesised that 2DG would potentiate glucose deprivation-induced cell death in U87MG, LN229 and U251 glioma cell lines. To test this, a propidium iodide assay was performed at different time points after complete glucose withdrawal in the presence of 10mM 2DG. Interestingly 2DG significantly suppressed glucose deprivation-induced cell death in U87MG, LN229 and U251 glioma cell lines (p=0.0022, 0.0004 and 0.0015 respectively).

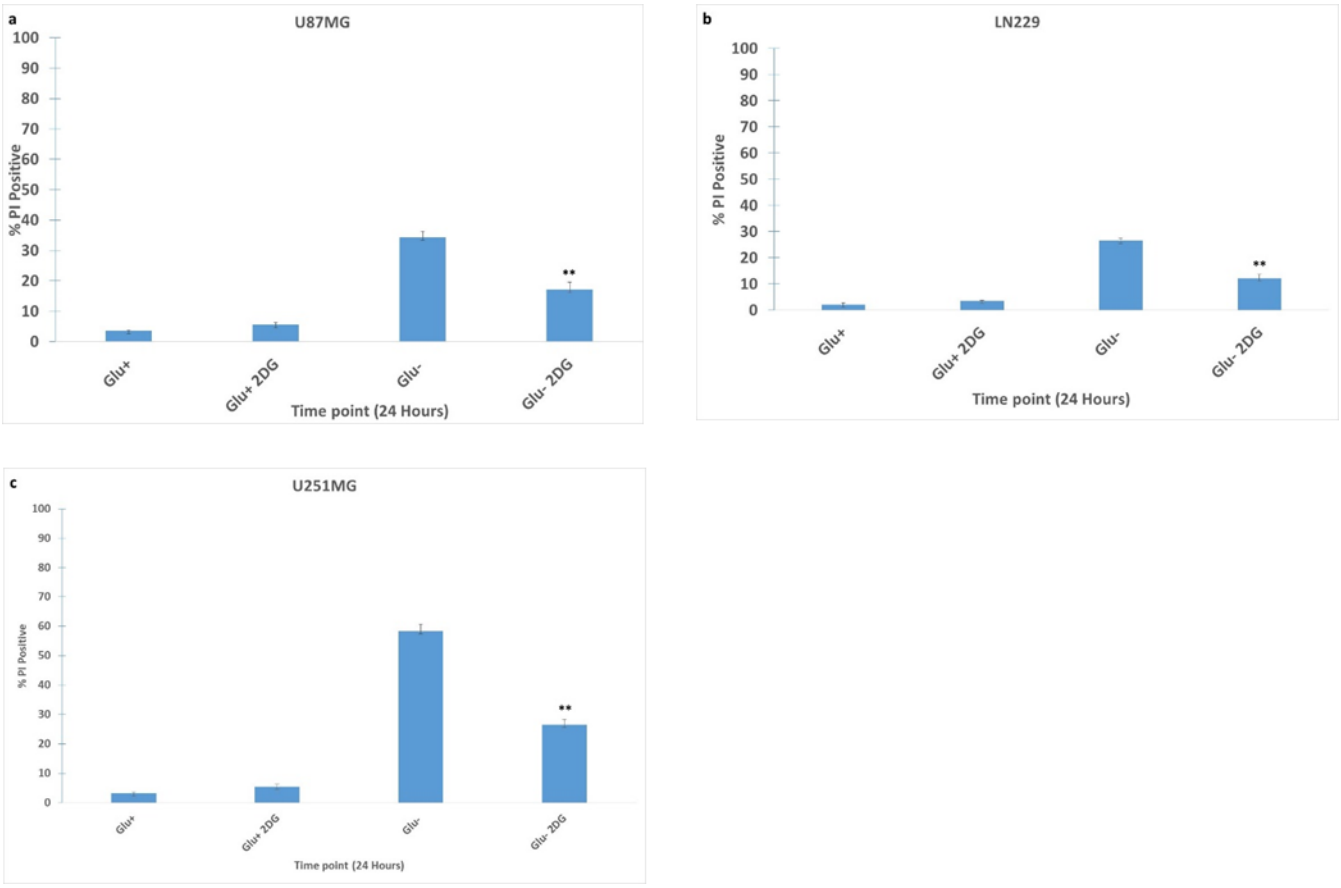


Figure 4.6 a, b, c: 2DG suppresses glucose deprivation induces cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from appropriate wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS and containing 10mM 2DG was added to appropriate wells. After 24 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicate, and error bars represent standard error of the mean. 2DG significantly suppressed glucose deprivation-induced cell death in U87MG, LN229 and U251 glioma cell lines (p=0.0022, 0.0004 and 0.0015 respectively).

4.2.7 Cell death induced by a combination of metformin and glucose deprivation is caspase-independent

To investigate the mechanism of metformin and glucose deprivation-induced cell death, U87MG, LN229 and U251MG cells were used. We hypothesised that cell death was not mediated by apoptosis. To test this, propidium iodide assay was performed at a 24-hour time point after glucose withdrawal in the presence of metformin (with and without caspase inhibitor). Caspase inhibitor ZVAD-fmk had no significant effect on cell death in U87MG, LN229 and U251 glioma cell lines (p=0.5996, 0.3344 and 0.3396 respectively).

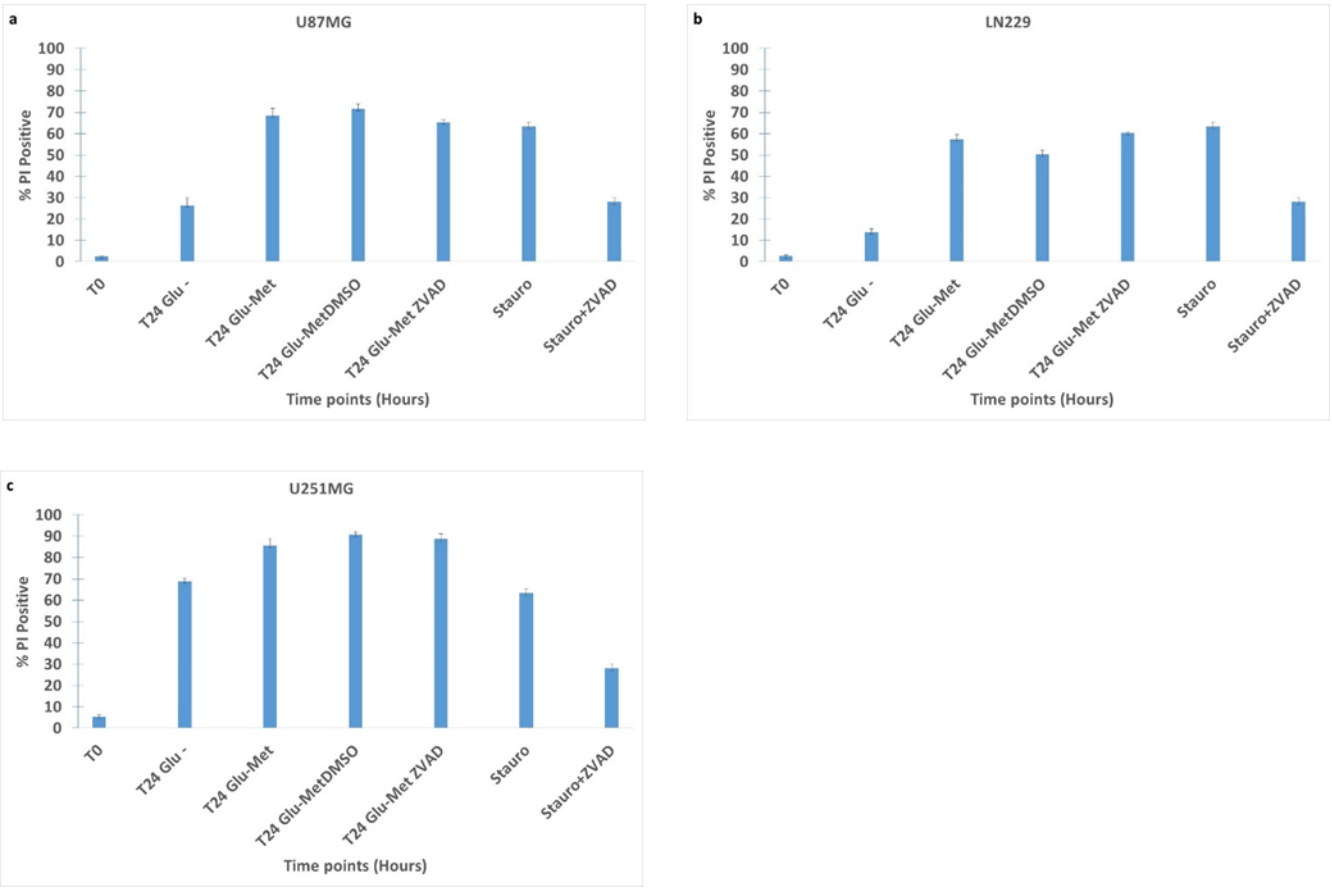


Figure 4.7 a, b, c: The pan-caspase inhibitor ZVAD-fmk does not rescue metformin and glucose withdrawal combination induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested, and cell viability was determined by a propidium iodide assay to serve as a baseline. Cells were pre-incubated with 50 microM (µM) ZVAD-fmk to allow the ZVAD-fmk to be present inside the cells at the time when glucose was withdrawn, and metformin was added. After 1 hour, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate (supplemented with 2% dialysed FCS) were added to the wells along with 8mM metformin. 50 µM ZVAD-fmk was added to appropriate wells. DMSO was also added in appropriate wells as the control since ZVAD-fmk was dissolved in DMSO. Also, 1 µM Staurosporine, a known inducer of apoptosis was used as a control for ZVAD-fmk. After 24 hours, the supernatant was collected into marked FACS tubes. Adherent cells were trypsinised and collected in appropriate tubes too. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. Caspase inhibitor ZVAD-fmk had no significant effect on cell death in U87MG, LN229 and U251 glioma cell lines (p=0.5996, 0.3344 and 0.3396 respectively).

4.2.8 Cell death induced by metformin and glucose deprivation combination is not mediated via autophagy

To test the effect of autophagy inhibition on metformin and glucose deprivation-induced cell death, U87MG, LN229 and U251MG cells were used. A propidium iodide assay was performed at a 24-hour time point after glucose withdrawal in the presence of metformin (with or without 3-MA or NH_4Cl).

3-MA had no significant effect on metformin and glucose deprivation-induced cell death in U87MG, LN229 and U251 glioma cell lines (p=0.6371, 0.4025 and 0.1747 respectively). Similarly, NH_4Cl had no significant effect on metformin and glucose deprivation-induced cell death in U87MG, LN229 and U251 glioma cell lines (p=0.329, 0.7580 and 0.9027 respectively).

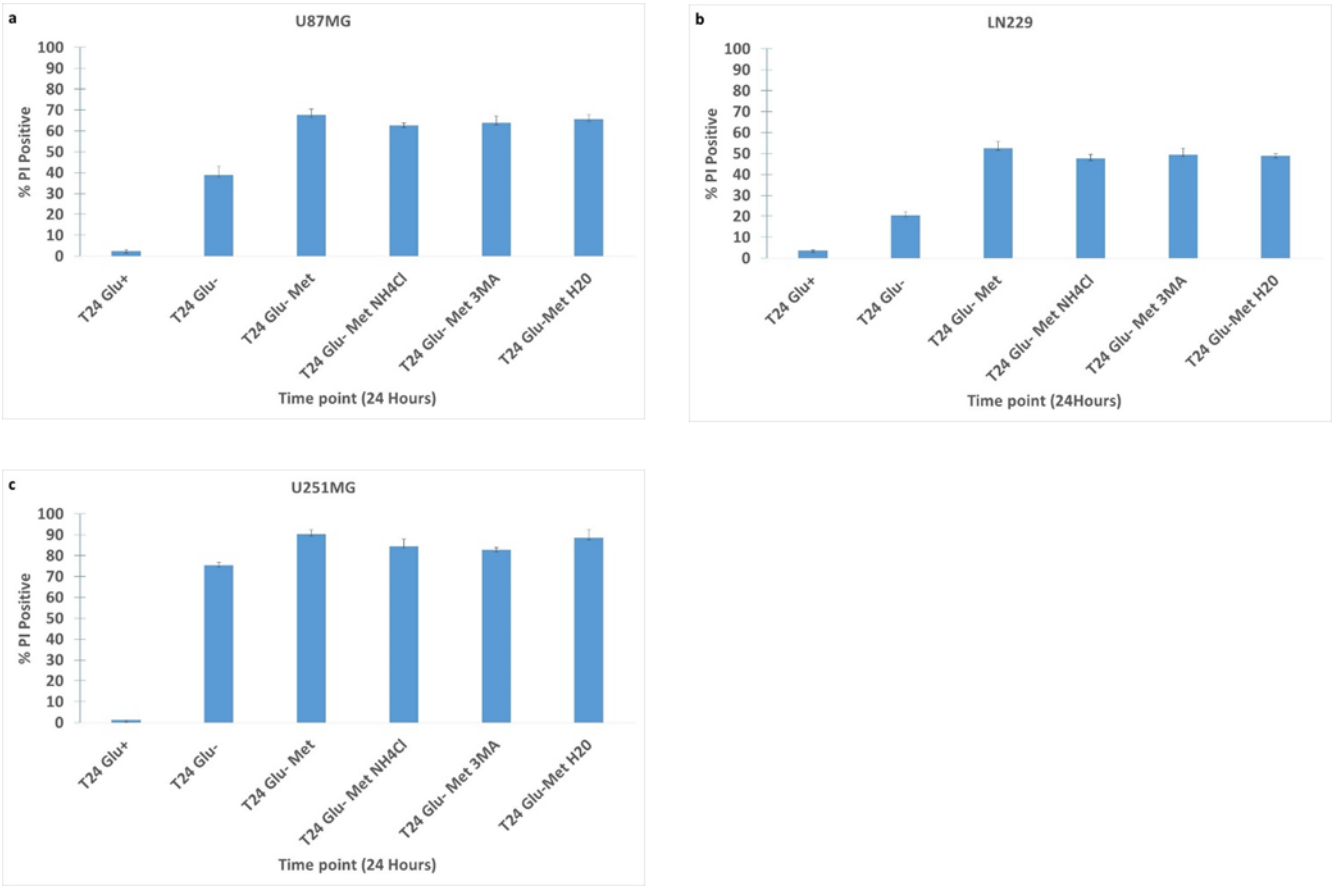


Figure 4.8 a, b, c: Autophagy inhibitors 3-MA or NH_4Cl do not rescue metformin and glucose withdrawal-induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 23 hours, cells were pre-incubated with 5mM 3-MA or 20mM NH_4Cl to allow them to be present inside the cells at the time when glucose was withdrawn. After 1 hour, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate (supplemented with 2% dialysed FCS) and containing 8mM metformin were added to the wells. 5mM 3-MA or 20mM NH_4Cl were also added to appropriate wells. Water was added in appropriate wells as a control since 3-MA and NH_4Cl were dissolved in water. After 24 hours, the supernatant was collected into marked FACS tubes. Adherent cells were trypsinised and collected in appropriate tubes too. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. 3-MA or NH_4Cl had no significant effect on metformin and glucose deprivation-induced cell death in U87MG, LN229 and U251 glioma cell lines.

4.2.9 Cell death induced by metformin and glucose deprivation combination is not mediated via necrosis

To test the effect of necrosis inhibition on metformin and glucose deprivation-induced cell death, U87MG, LN229 and U251MG cells were used. A propidium iodide assay was performed at a 24-hour time point after glucose withdrawal in the presence of metformin, with or without IM-54 which is a selective inhibitor of necrosis. IM-54 had no significant effect on metformin and glucose deprivation-induced cell death on U87MG, LN229 and U251MG glioma cell lines (p=0.3124, 0.6317 and 0.9027 respectively).

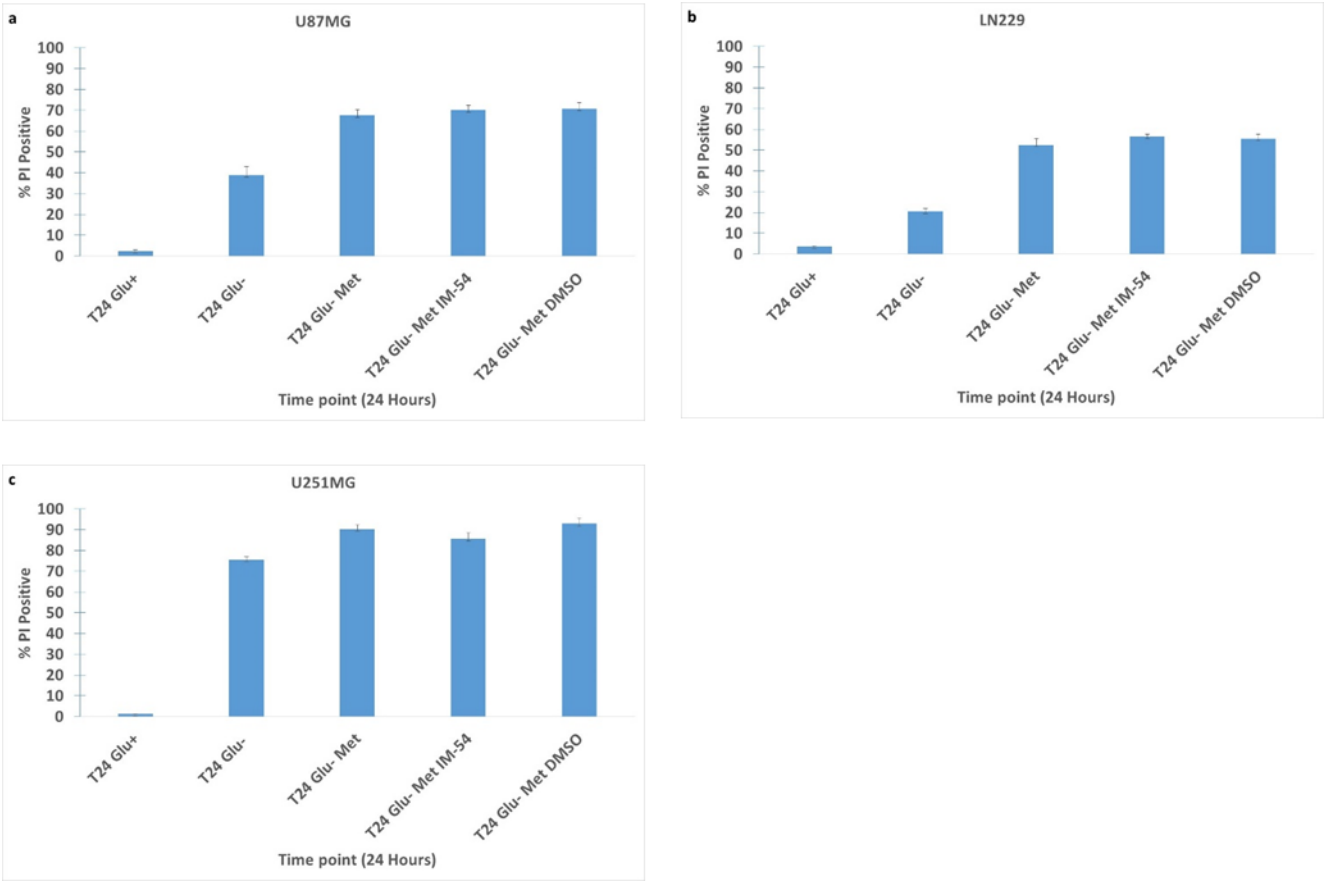


Figure 4.9 a, b, c: Necrosis inhibitor IM-54 does not rescue metformin and glucose withdrawal-induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 23 hours, cells were pre-incubated with 3µM IM-54 to allow it to be present inside the cells at the time when glucose was withdrawn. After 1 hour, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate (supplemented with 2% dialysed FCS) and containing 8mM metformin were added to the wells. 3µM IM-54 was also added to appropriate wells. DMSO was added in appropriate wells as the control since IM-54 was dissolved in water. After 24 hours, the supernatant was collected into marked FACS tubes. Adherent cells were trypsinised and collected in appropriate tubes too. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. IM-54 had no significant effect on metformin and glucose deprivation-induced cell death on U87MG, LN229 and U251MG glioma cell lines (p=0.3124, 0.6317 and 0.9027 respectively).

4.2.10 Cell death induced by metformin and glucose deprivation combination is not mediated by oxidative stress

We have earlier shown that glucose deprivation-induced cell death is mediated by oxidative stress and suppressed by free radical scavenger NAC (Figure 4.2). To test the effect of NAC on metformin and glucose withdrawal-induced cell death, U87MG, LN229 and U251MG cells were used. A propidium iodide assay was performed at a 24-hour time point after glucose withdrawal in the presence of metformin, with or without 1 or 2mM NAC. NAC was able to suppress metformin and glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines a dose-dependent manner, but the degree of suppression was less than that seen with glucose deprivation alone (p=0.644, 0.3414 and 0.0671 respectively). This showed that metformin and glucose deprivation-induced cell death is not mediated by oxidative stress.

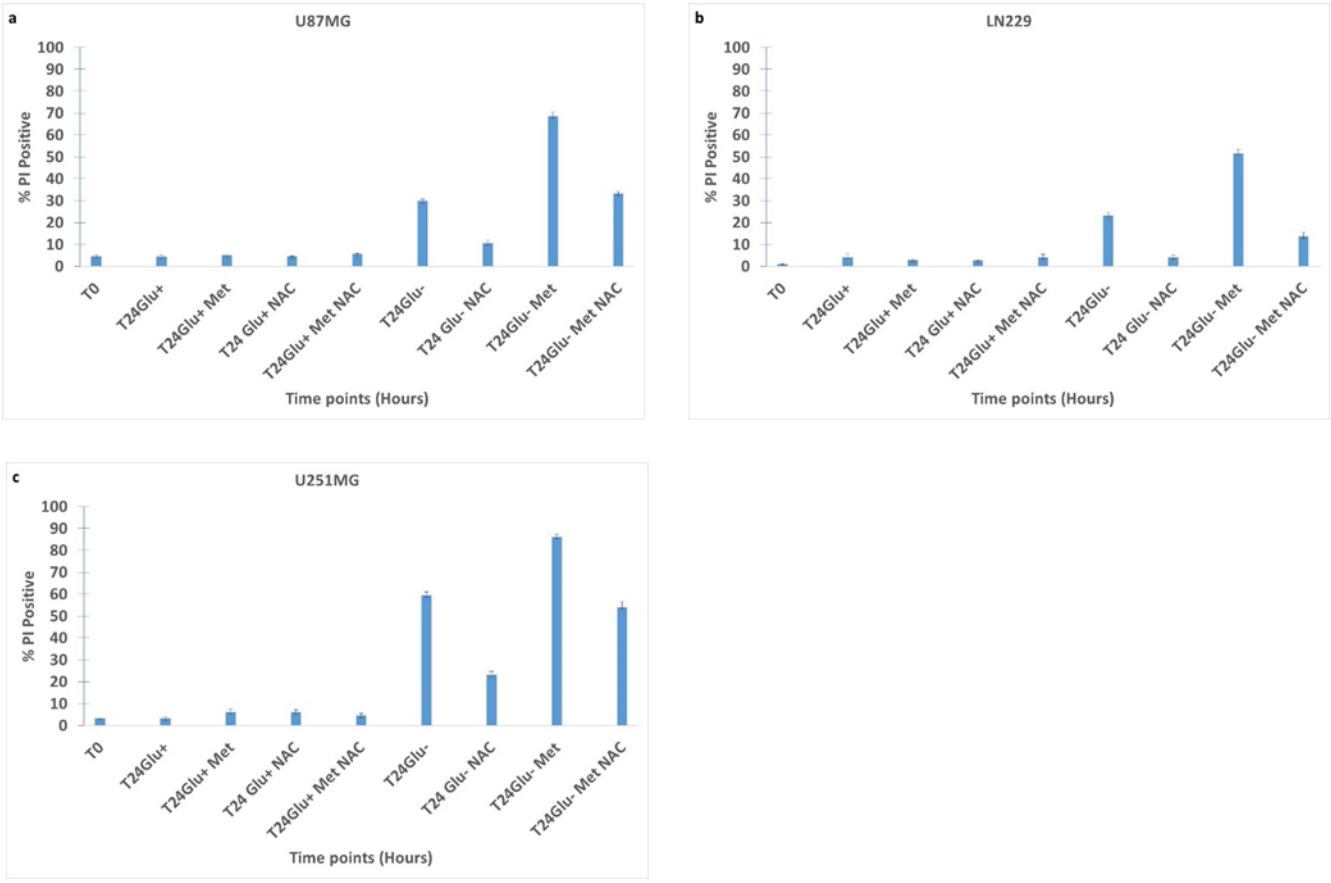


Figure 4.10 a, b, c: The free radical scavenger NAC does not rescue metformin and glucose withdrawal-induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested and cell viability was determined by propidium iodide assay to serve as a baseline. Media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate (supplemented with 2% dialysed FCS) and containing 8mM metformin were added to the wells. 1 and 2mM NAC was also added to appropriate wells. After 24 hours, the supernatant was collected into marked FACS tubes. Adherent cells were trypsinised and collected in appropriate

tubes too. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. NAC had no significant effect on metformin and glucose deprivation-induced cell death.

4.2.11 NAC did not have a significant effect on death induced by metformin and glucose deprivation combination at longer time points

To test the effect of NAC on cell lines exposed to metformin and glucose deprivation for prolonged duration, U87MG and LN229 cells were used. We have earlier shown that NAC can suppress glucose withdrawal-induced cell death (Figure 4.2) whereas it was unable to suppress metformin and glucose deprivation-induced cell death to the same extent as glucose deprivation alone (Figure 5.10). It was hypothesised that NAC might not be able to suppress cell death as a result of prolonged exposure to metformin and glucose deprivation. To test this, a propidium iodide assay was performed after 60 hours of metformin and glucose deprivation. In both U87MG and LN229 cells, NAC could not significantly suppress cell death to the level which was seen at an earlier time point (p=0.4868 and 0.1858 respectively). U251MG was not used in the experiment as nearly all the cells were dead after 48 hours.

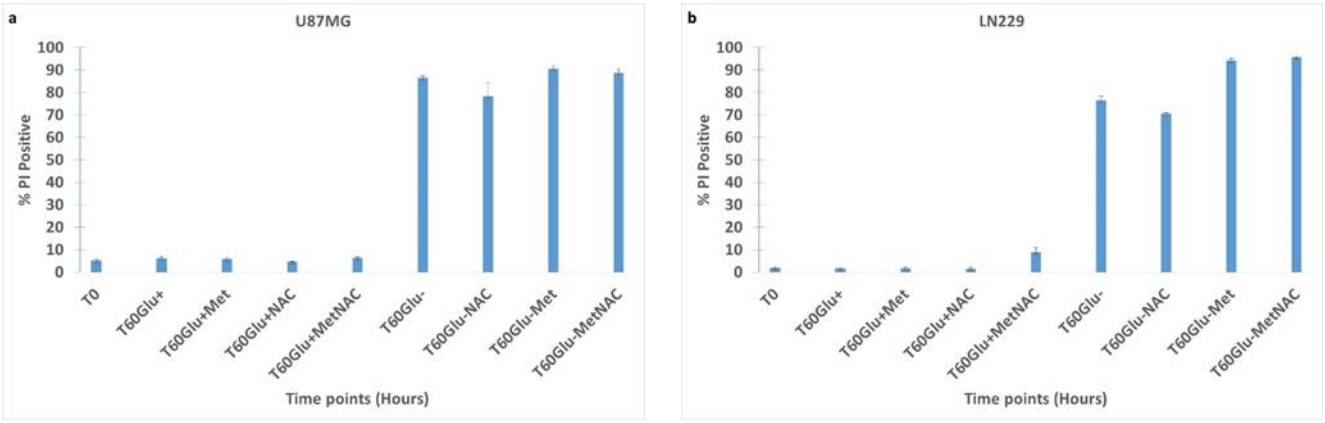


Figure 4.11 a, b: The free radical scavenger NAC is unable to suppress metformin and glucose deprivation-induced cell death in glioma cell lines at longer time points. U87MG and LN229 cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, T0 cells were harvested and cell viability was determined by propidium iodide assay to serve as a baseline. For metformin/glucose withdrawal and effect of NAC, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS and 8mM metformin with or without 1 mM NAC, was added to appropriate wells. After 60 hours, suspension and adherent cells were collected in appropriate tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. NAC had no significant effect on death induced by metformin and glucose deprivation combination at longer time points.

4.2.12 Hypoxia does not affect metformin and glucose deprivation-induced cell death.

We have earlier shown that hypoxia suppressed glucose deprivation-induced cell death is U87MG and LN229 cell lines (Figure 4.11). We have also shown that glucose deprivation-induced cell death is mediated via oxidative stress (Figure 4.2). Metformin has been shown to inhibit mitochondrial ETC I so we hypothesised that metformin and glucose deprivation-induced cell death would also be suppressed by hypoxia. To test this, propidium iodide assay was performed at different time points after treating cells with metformin and glucose withdrawal in hypoxic and normoxic conditions. Interestingly hypoxia had no effect on metformin and glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.5838, 0.0825 and 0.0882 respectively).

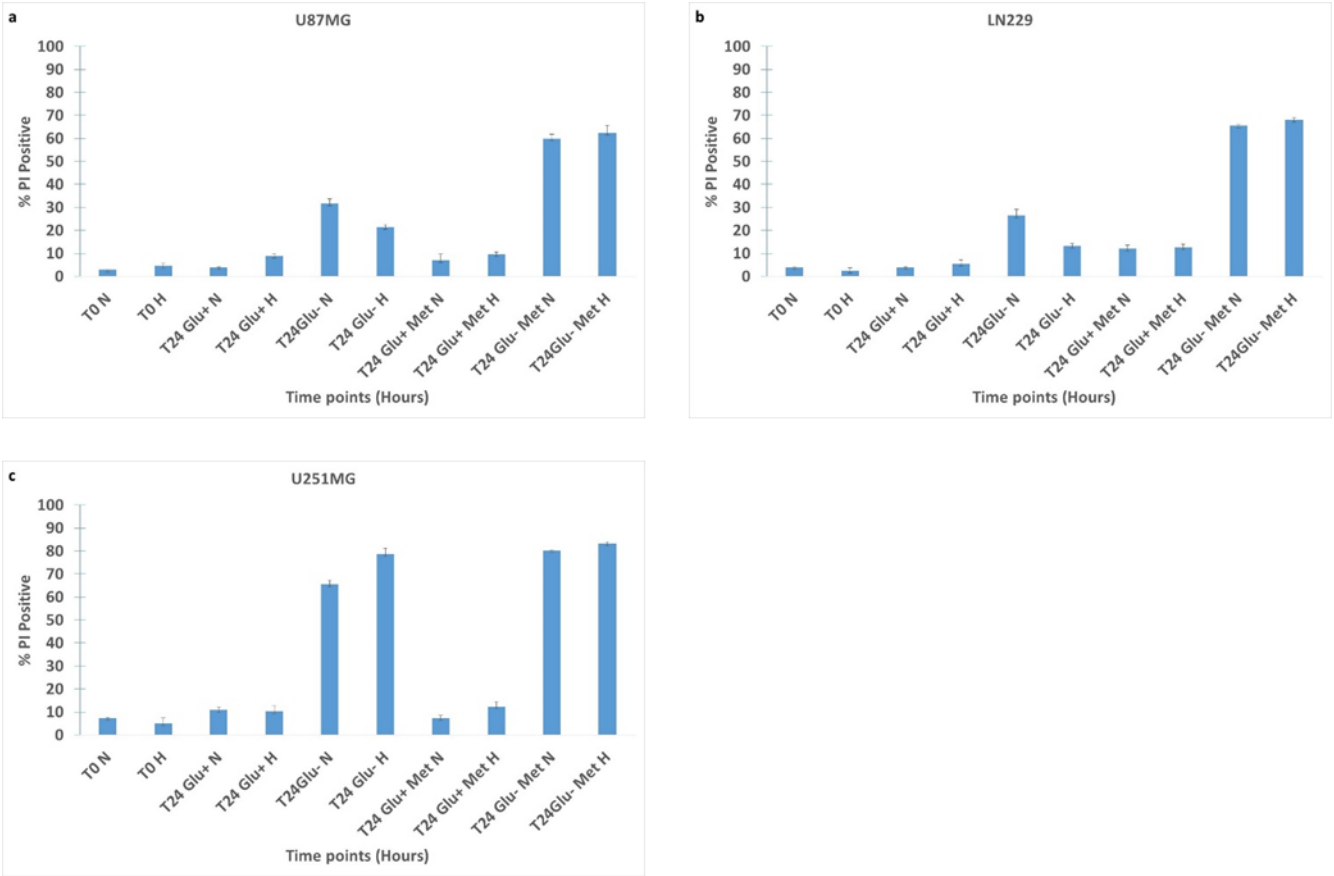


Figure 4.12 a, b, c: Hypoxia does not suppress metformin and glucose withdrawal-induced cell death. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS and placed in normoxic and hypoxic incubators. After 24 hours, T0 cells were harvested and cell viability was determined by propidium iodide assay to serve as a baseline. For metformin/glucose withdrawal, media was removed from wells, wells were washed gently with PBS, and then DMEM lacking glucose and pyruvate supplemented with 2% dialysed FCS and 8mM metformin was added to the wells, and the plates were replaced in hypoxic and normoxic incubators. At appropriate time points, suspension and adherent cells were collected. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. Hypoxia had no effect on metformin and glucose deprivation-

induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.5838, 0.0825 and 0.0882 respectively).

4.2.13 Lithium chloride does not affect glucose deprivation-induced cell death

To test the effect of lithium on glucose deprivation-induced cell death, a propidium iodide assay was performed at 24 hours after complete glucose withdrawal in the presence 5 or 20mM lithium chloride. Lithium chloride had no significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.1537, 0.2288 and 0.9754 respectively for 5mM lithium and p=0.32, 0.9134 and 0.3596 respectively for 20mM lithium).

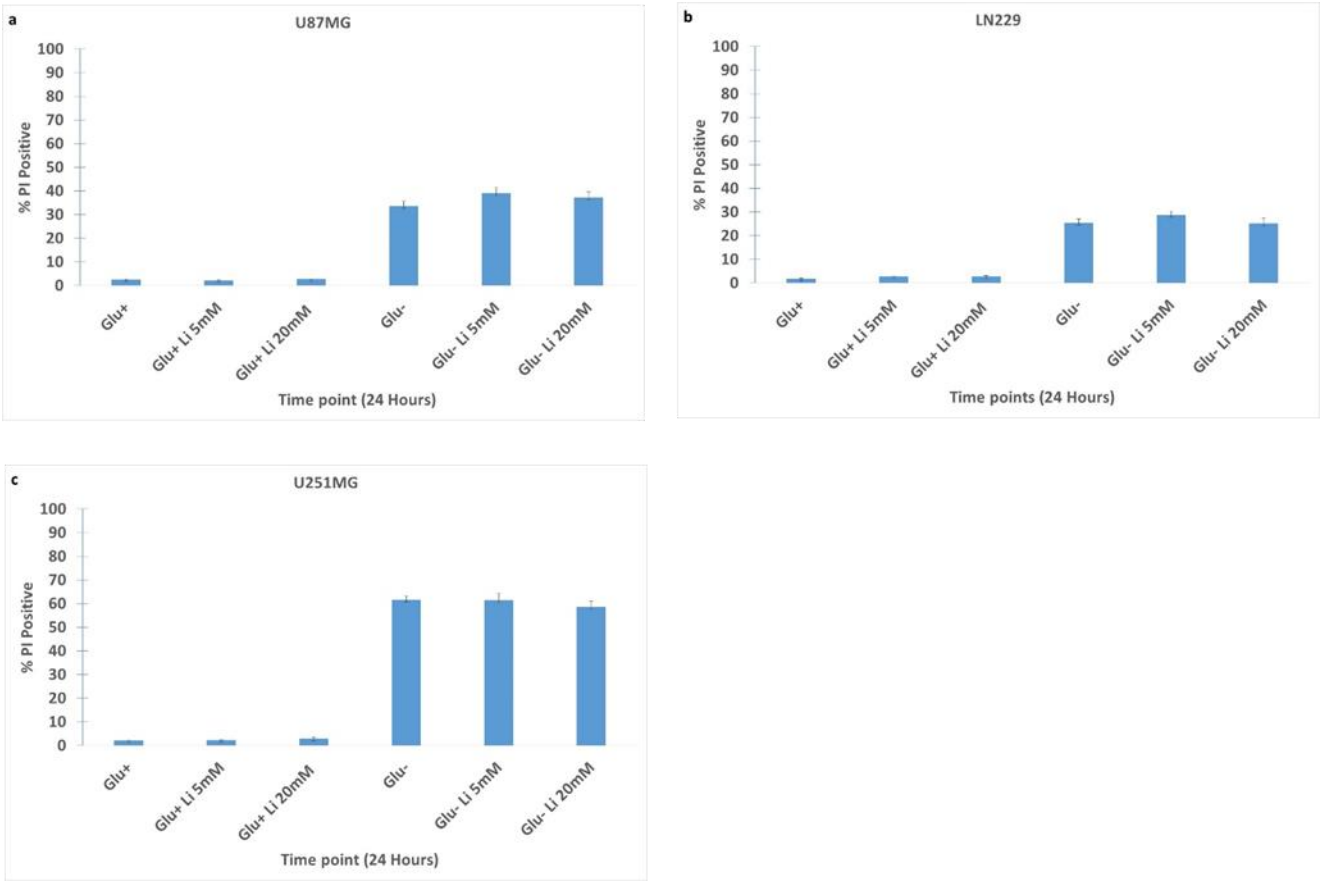


Figure 4.13 a, b, c: Lithium chloride does not affect glucose deprivation-induced glioma cell death. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells and wells were washed gently with PBS. DMEM supplemented with 2% dialysed FCS and containing 5 or 20mM lithium chloride was added to appropriate wells. After 24 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. 5 or 20mM lithium chloride had no significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines.

4.2.14 STO-609 does not affect glucose deprivation-induced cell death

We have earlier shown that glucose deprivation leads to pAMPK upregulation (Figure 4.20). pAMPK upregulation occurs as a result of energetic stress. However, pAMPK can also be activated via CaMKK. To investigate the role of CaMKK in glucose deprivation-induced glioma cell death, a propidium iodide assay was performed at 24 hours after complete glucose withdrawal in the presence of 2.5µM or 10µM STO-609. STO-609 is a cell-permeable, selective inhibitor of CAMKK2. STO-609 did not have any significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines (p=0.2879, 0.4141 and 0.5666 respectively for 2.5µM STO-609 and p=0.0689, 0.1897 and 0.7047 respectively for 10µM STO-609).

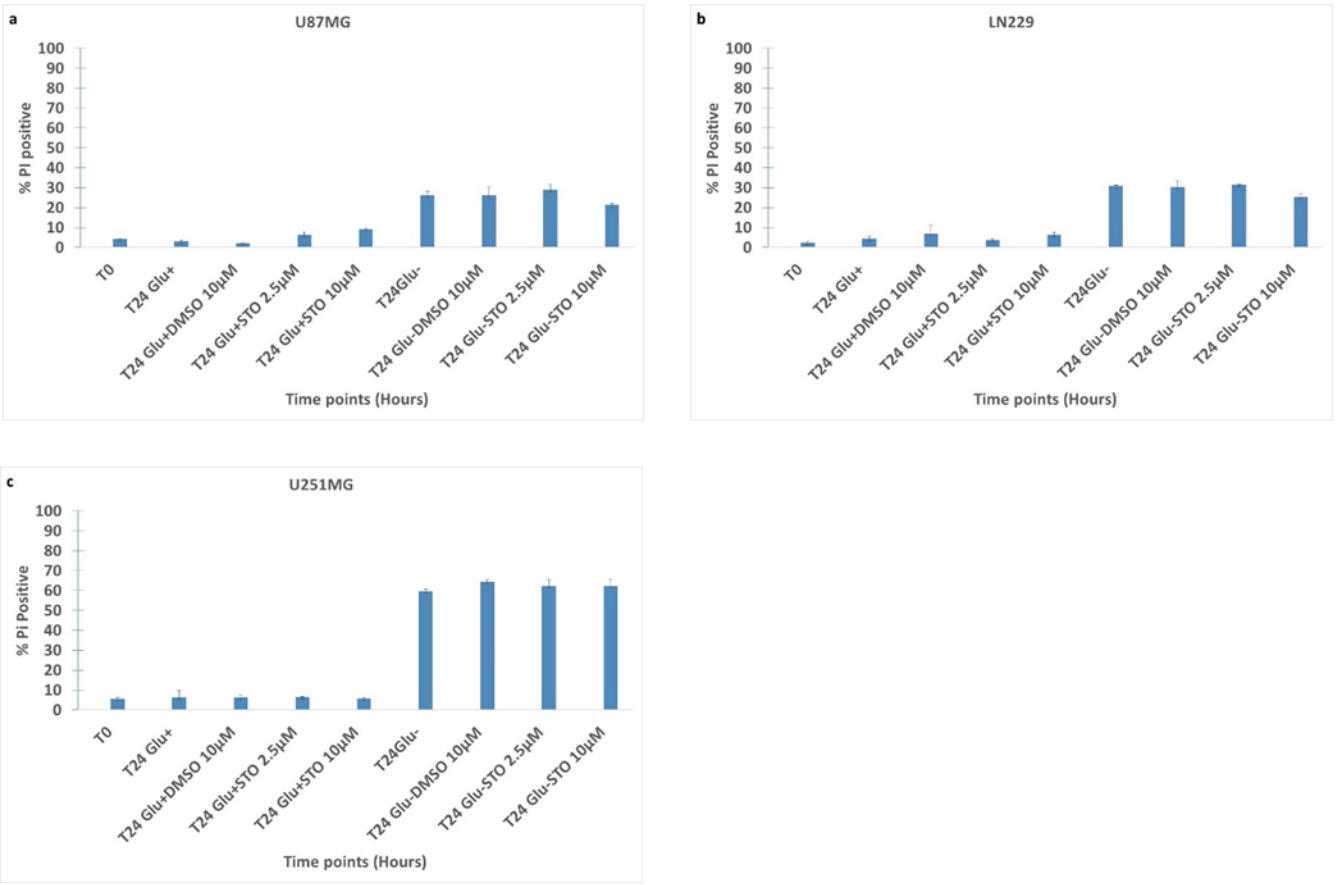
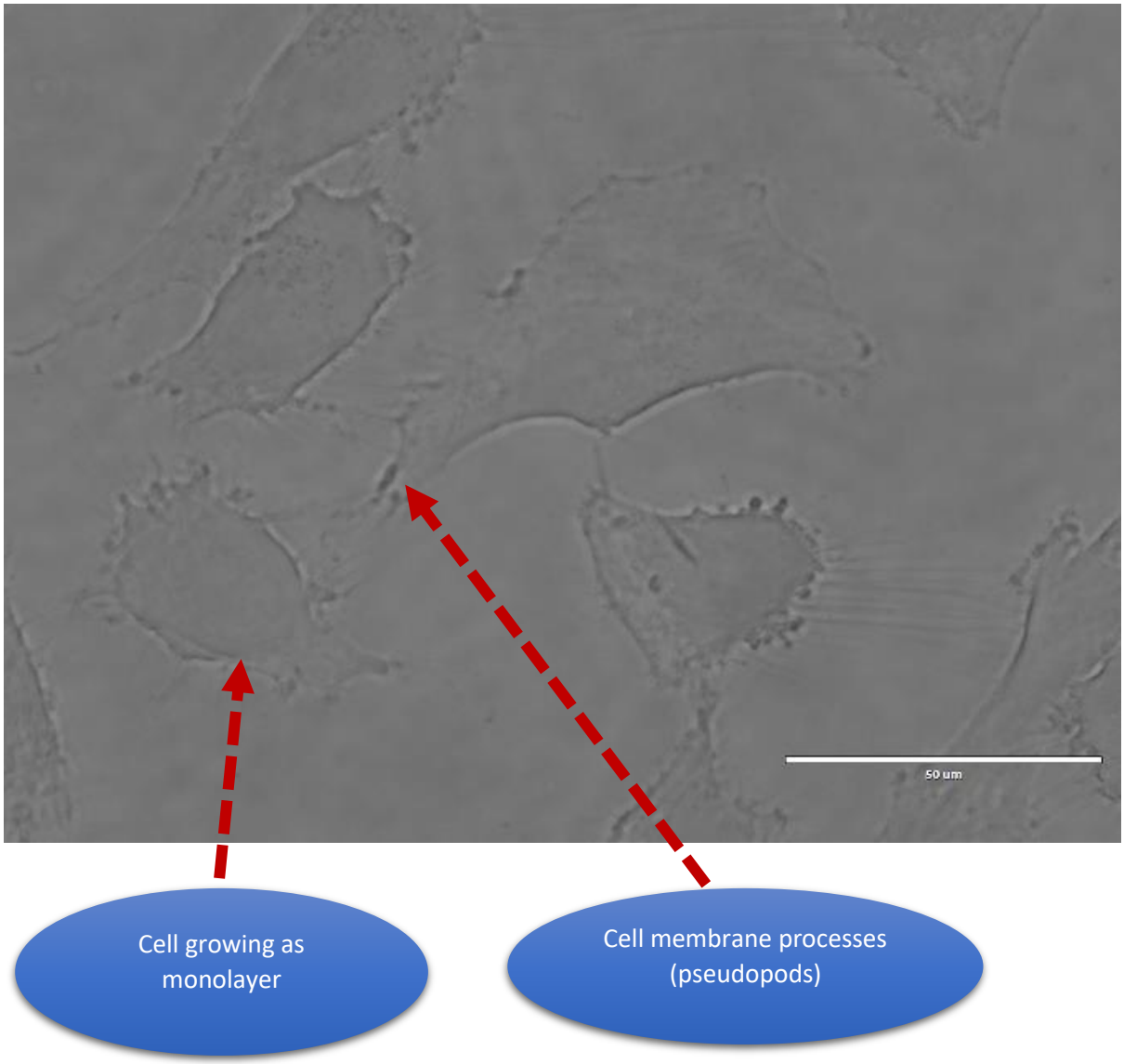


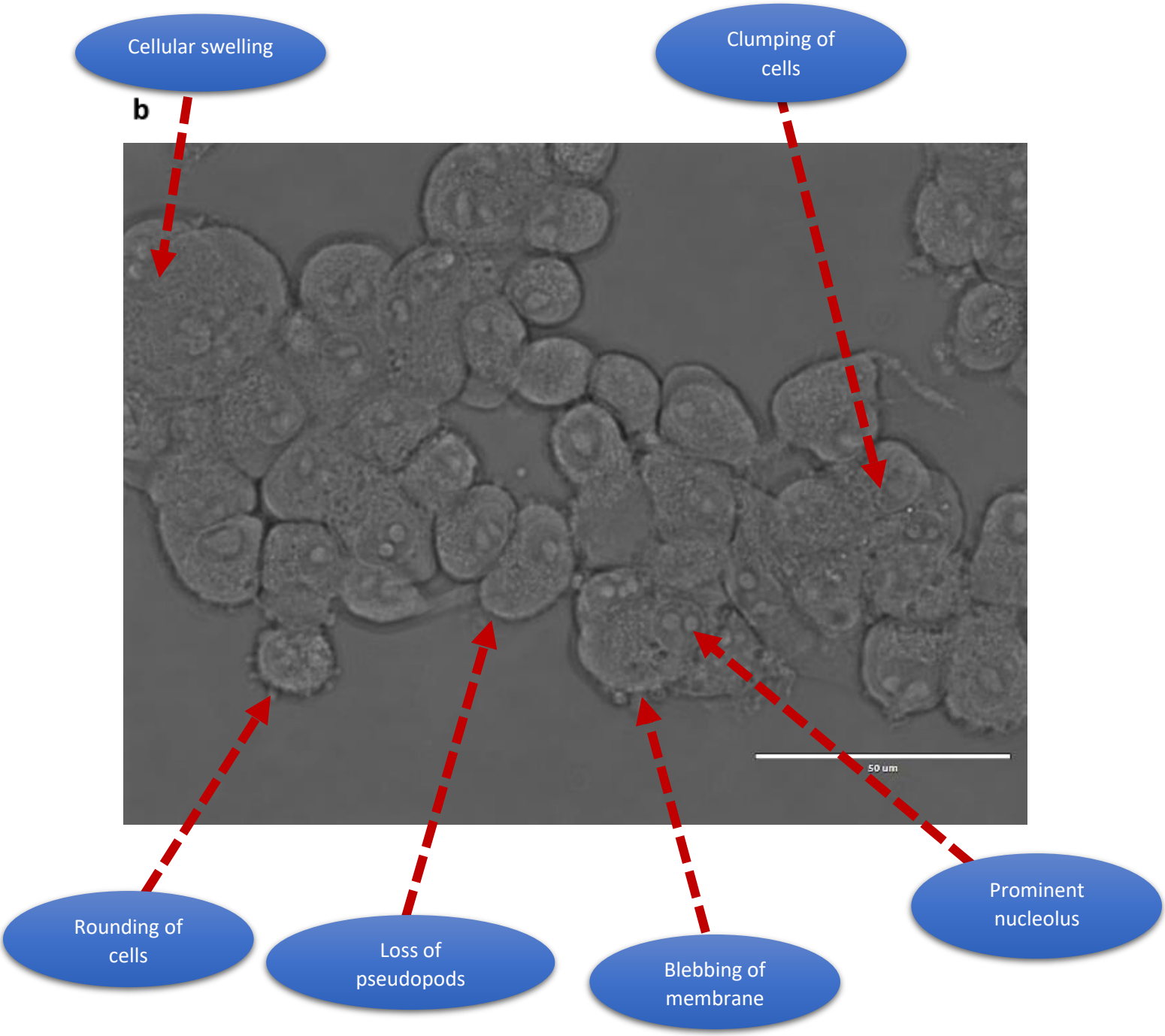
Figure 4.14 a, b, c: STO-609 does not affect glucose deprivation-induced glioma cell death. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells and wells were washed gently with PBS. DMEM supplemented with 2% dialysed FCS and containing 2.5µM, or 10µM of STO-609 was added to appropriate wells. After 24 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. 2.5µM or 10µM STO-609 did not have any significant effect on glucose deprivation-induced cell death in U87MG, LN229 and U251MG glioma cell lines.

4.2.15 Change in morphology of glioma cells with metformin and glucose deprivation treatment.

To study the effect of glucose deprivation and metformin on cell morphology, U87MG cells were grown in glucose-containing media and images were taken using a microscope. Subsequently, cells were subjected to glucose deprivation with or without metformin for 24 hours and then images were taken using a microscope. When deprived of glucose, cells got detached from the corning flask and some features of apoptosis such as rounding up of the cell and plasma membrane blebbing were seen. Similarly, some feature of necrosis such as the formation of cytoplasmic vacuoles was also seen. With metformin and glucose deprivation, there was further change in the morphology of cells, but it did not resemble any described form of cell death.

a




Cellular swelling
Clumping of cells
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Rounding of cells
Loss of pseudopods
Blebbing of membrane
Prominent nucleolus

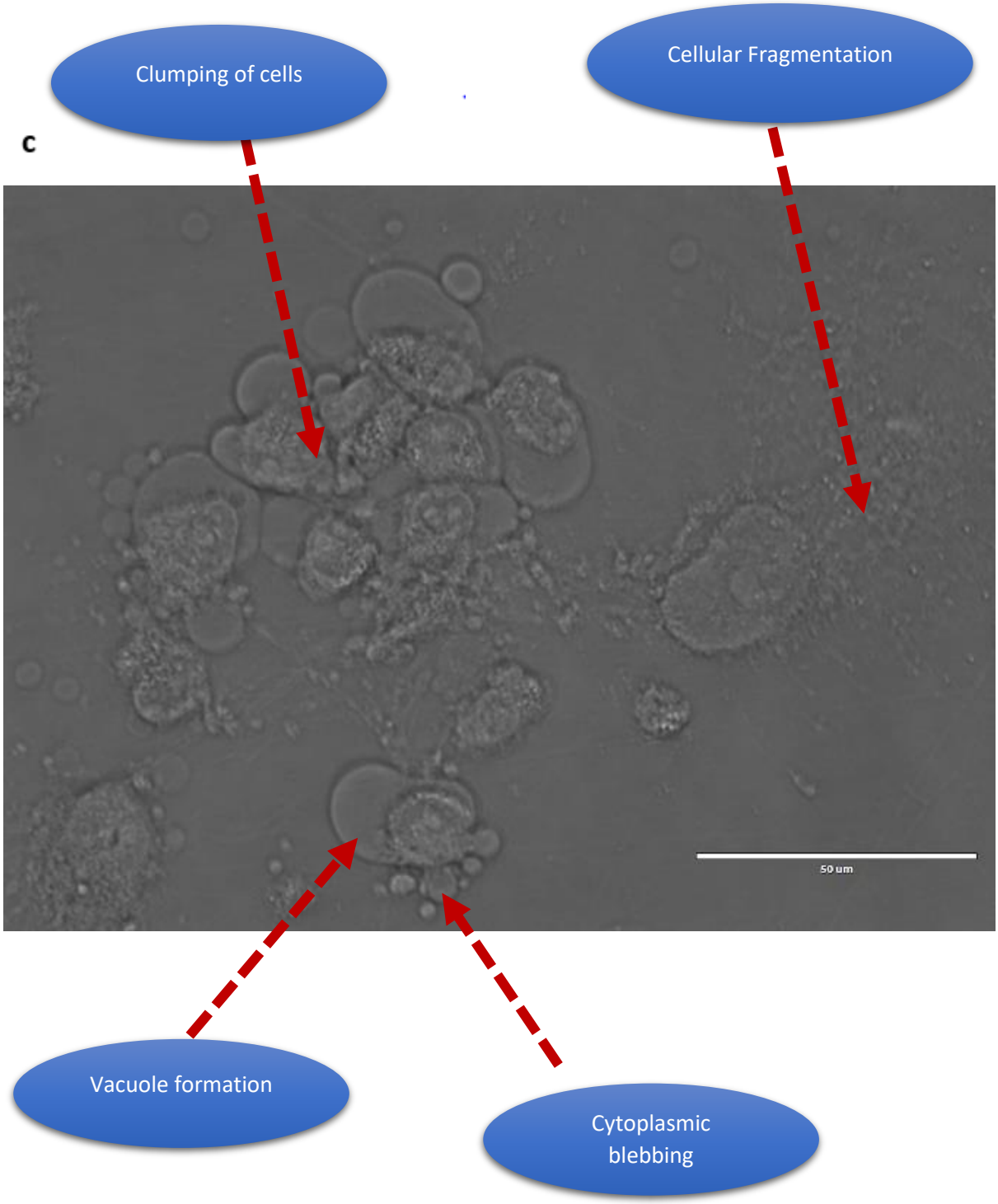


Figure 4.15 a, b, c: Change in glioma cell morphology with glucose deprivation and metformin treatment. U87MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS and after 24-hour images were taken with the help of a microscope (a). The media was then discarded, wells were washed with PBS and DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS, with or without 8mM metformin was added to the well. Images were taken 24-hours post glucose withdrawal with (b) or without metformin (c).

4.2.16 Metformin promotes glucose deprivation-induced energetic stress and up-regulates pAMPK expression

We have earlier shown that cancer cells depend upon unregulated glycolysis to meet their energetic and biosynthetic needs as well as to generate reducing equivalents. Glucose deprivation led to energetic stress and pAMPK upregulation (Figure 4.20). Metformin has been shown to inhibit ETC I and lead to energetic stress. We hypothesised that metformin would promote glucose deprivation-induced energetic stress and upregulate pAMPK expression. To test this, western blot analysis was performed after treating glucose deprived glioma cells with metformin. Metformin treatment led to a significant increase in glucose deprivation-induced pAMPK upregulation at 24-hour time point (p=0.0003). Though metformin treatment led to an increase in glucose deprivation-induced pAMPK upregulation at 8-hour time point too, it was statistically not significant (p=0.1624).

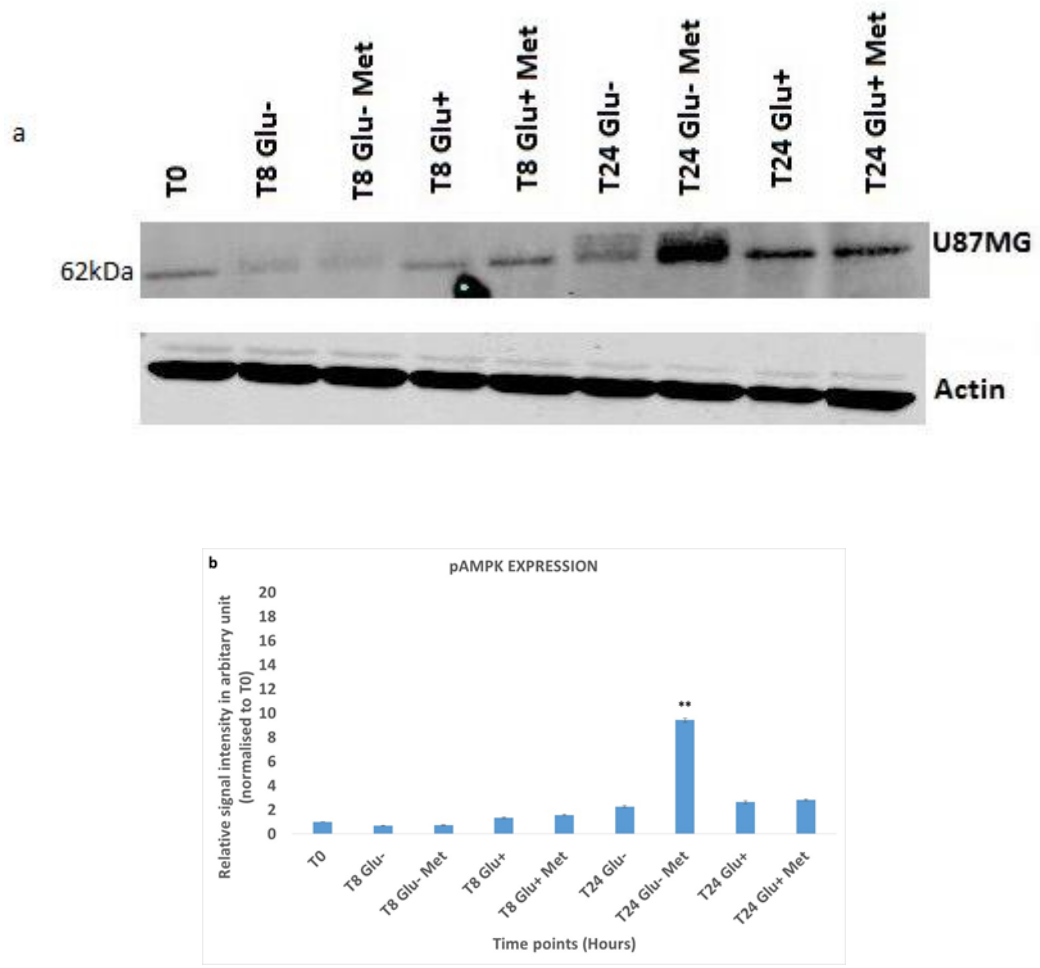


Figure 4.16 a: Metformin promotes glucose deprivation-induced energetic stress and up-regulates pAMPK expression. 1X10 ^{5} cells /ml of U87MG cells were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2% dialysed FCS was added to the wells. 8mM metformin was also added in appropriate wells. At appropriate time points, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE,

electroblotted to nitrocellulose membrane and probed with the antibody against pAMPK (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 4.16 b: Relative signal intensity of pAMPK expression in arbitrary unit (normalised to T0) in U87MG glioma cell line. Metformin treatment led to a significant increase in glucose deprivation-induced pAMPK upregulation at 24-hour time point (p=0.0003). Metformin treatment also led to an increase in glucose deprivation-induced pAMPK upregulation at 8-hour time point, however, it was statistically not significant (p=0.1624).

4.2.17 Metformin downregulates glucose deprivation-mediated GRP78 upregulation

We have shown that glucose deprivation induces UPR and upregulates ER chaperone protein GRP78 (Figure 4.21). We hypothesised that in glioma cells, metformin would downregulate glucose deprivation-induced GRP78 upregulation. To test this, western blot analysis was performed after treating glucose deprived glioma cells with metformin. We showed that metformin treatment led to down-regulation of glucose deprivation-induced GRP78 upregulation at both 8-and 24-hour time points (p=0.026 and 0.0021 respectively).

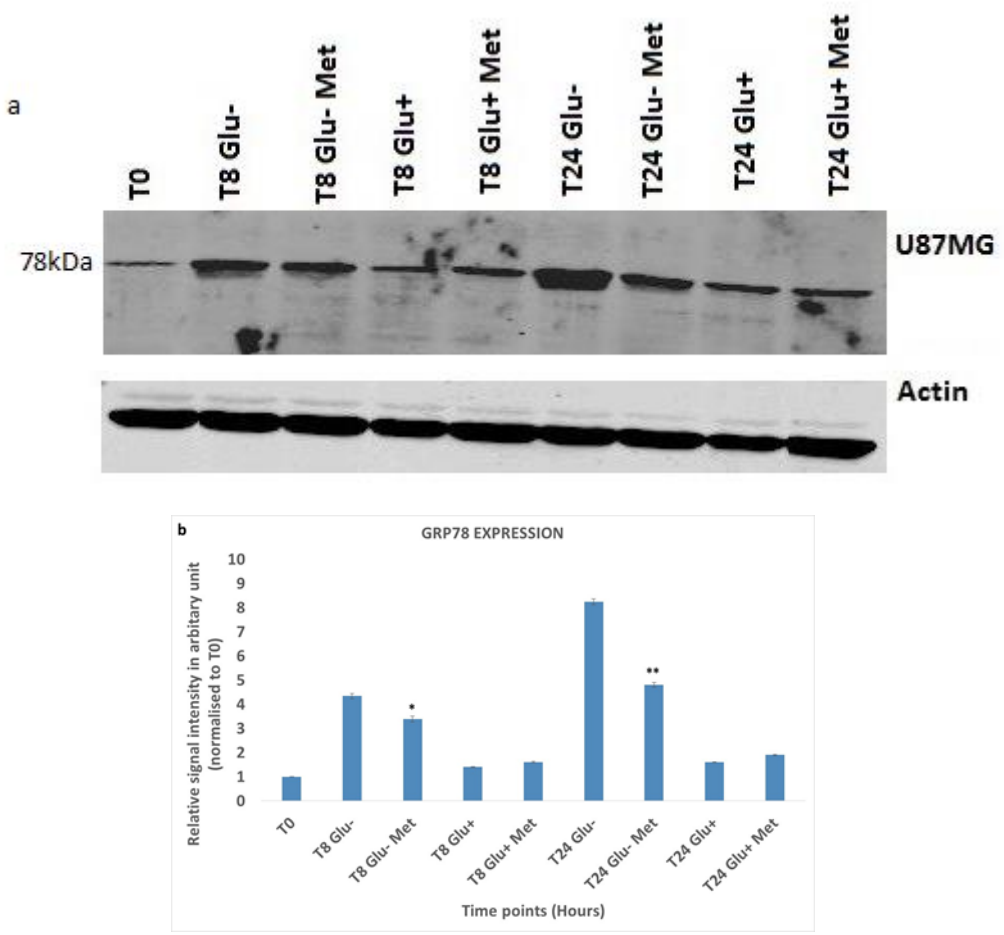


Figure 4.17 a: Metformin downregulates glucose deprivation-mediated GRP78 upregulation. 1X10 5 cells /ml of U87MG cells were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then DMEM (lacking glucose and pyruvate) supplemented with 2%

dialysed FCS was added to the wells. 8mM metformin was also added in appropriate wells. At different time points, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against GRP78 (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 4.17 b: Relative signal intensity of GRP78 expression in arbitrary unit (normalised to T0) in U87MG glioma cell lines. Metformin treatment led to down-regulation of glucose deprivation-induced GRP78 upregulation at both 8-and 24-hour time points (p=0.026 and 0.0021 respectively).

4.3 Discussion

In these experiments, we have shown that though metformin had no significant effect on U87MG, LN229 and U251 glioma cell viability in the presence of glucose at 24-hour time point (p=0.6537, 0.1831 and 0.514 respectively). However, at 8-hour time point statistically significant cell death was seen in U87MG and U251 cell lines (p=0.0077 and 0.0075 respectively). Similarly, at 12-hour time point, statistically significant cell death was seen in LN229 cell line with 8mM metformin (p=0.0267) and in U251MG cell line with 1mM metformin (p=0.0351). However, when metformin was added to glucose-deprived glioma cell lines, it led to marked glioma cell death (p=0.0067, 0.001 and 0.0017 for U87MG, LN229 and U251MG glioma cell line at 24-hour time point respectively). Our findings were similar to other authors. Ahmad *et al*. showed that glucose deprivation-induced oxidative stress and cell death in transformed fibroblasts, which was potentiated by ETC I blocker rotenone and ETC III blocker antimycin A (2005). Yu *et al*. showed that in low glucose condition metformin treatment led to marked cell death in oesophageal cancer cells (2016). Similarly, Narise *et al*. also showed that biguanide phenformin was highly toxic to colon cancer cells in glucose-deprived medium (2014). Haga *et al*. showed that p53 wild-type, as well as p53 mutant glioma, fibrosarcoma, breast, pancreas, stomach, hepatocellular and colorectal cancer cells, were sensitive to glucose deprivation and metformin-induced cell death (2010). However, Buzzai *et al*. showed that glucose deprivation and metformin treatment resulted in less death in p53 wild-type cells as compared to p53 mutant cells (2005). In our experiments, cell death was not related to p53 status as we saw less cell death in LN229 cells as compared to U251MG cells though both were p53 mutant. One of the reasons for this could be the fact that U251MG is PTEN mutant and hence contained constitutively active Akt. Our data also show that like metformin, AMPK mimic AICAR also potentiates glucose deprivation-induced cell death (p=0.0003, 0.0273 and 0.0003 for U87MG, LN229 and U251MG glioma cell line at 24-hour time point respectively), whereas it had no effect on glioma cell viability in the presence of glucose (p=0.0673, 0.5059 and 0.4775 for U87MG, LN229 and U251MG glioma cell line at 24-hour time point respectively).

Glucose via the PPP can generate two molecules of NADPH whereas 2DG via PPP can only generate one molecule of NADPH. So 2DG can promote oxidative stress as compared to growth conditions when glucose is present (Xi 2013, Hong 2016). 2DG induced cell death has been shown to be partially rescued by overexpression of SOD or catalase indicating that 2DG can induce oxidative stress-mediated cell death. Study has also shown that 2DG mediated radio-sensitization is inhibited by anti-oxidant NAC. It has also been shown that 2DG mediated cytotoxicity is enhanced by BSO which is an inhibitor of GSH biosynthesis. 2DG has also been shown to enhance the cytotoxicity of cisplatin and adriamycin which are thought to act by exerting oxidative stress (Aykin-Burns 2009). However, our data show that 2DG rescued cells from glucose deprivation-induced cell death (p=0.0022, 0.0004 and 0.0015 for U87MG, LN229 and U251MG glioma cell line respectively). We have also earlier shown that glucose deprivation-mediated cell death is as a result of oxidative stress and it is rescued by anti-oxidant NAC (Figure 4.2). Studies have shown that 2DG can be partially utilised in PPP to generate 1 molecule of NADPH leading to a decline in ROS levels when added to media lacking glucose (Xi 2013, Hong 2016). Hence in the presence of glucose, 2DG leads to oxidative stress whereas, in glucose-deprived media, 2DG can downregulate oxidative stress.

Our data also show that that the free radical scavenger NAC was only partially able to rescue death in glucose-deprived metformin-treated glioma cells (p=0.644, 0.3414 and 0.0671 for U87MG, LN229 and U251MG glioma cell line respectively). We also saw marked AMPK activation when metformin was added to glucose-deprived medium (p=0.0003) along with down-regulation of GRP78 (p=0.0021). So, it may be that oxidative stress, ER stress, as well as energetic stress, played a role in glucose deprivation and metformin-induced cell death. We have also shown that glucose deprivation and metformin combination induced cell death was caspase-independent and not inhibited by pan-caspase inhibitor ZVAD-fmk (p=0.5996, 0.3344 and 0.3396 for U98MG, LN229 and U251MG respectively). Bikas *et al*. also showed that glucose deprivation and metformin-induced cell death in thyroid cancer cells was caspase-independent (2015). They showed that glucose deprivation and metformin led to oncotic cell death. We have also demonstrated that metformin and glucose deprivation-induced cell death was not mediated via autophagy or necrosis as autophagy inhibitor 3-methyladenine (p=0.6371, 0.4025 and 0.1747 for U87MG, LN229 and U251MG respectively) and ammonium chloride (p=0.329, 0.7580 and 0.9027 for U87MG, LN229 and U251MG respectively) and necrosis inhibitor IM-54 (p=0.3124, 0.6317 and 0.9027 for U87MG, LN229 and U251MG respectively) had no significant effect on glioma cell viability.

Going further, we have shown that CaMKK inhibitor STO-609 had no effect on glucose deprivation-induced cell death indicating the fact that ROS mediated AMPK activation may not have a role in glucose deprivation-induced AMPK activation (p=0.2879, 0.4141 and 0.5666 for 2.5µM STO-609 and

p=0.0689, 0.1897 and 0.7047 for 10μM STO-609 in U87MG, LN229 and U251MG glioma cell lines respectively). This is in agreement with other authors. Xi *et al*. did not see any effect of STO-609 on glucose deprivation-induced AMPK activation (2013).

Our data also show that when metformin was added to glucose-deprived glioma cells, it led to energetic stress and marked AMPK activation (p=0.0003). This is in agreement with other authors. Bikas *et al*. showed that glucose deprivation and metformin led to a loss of mitochondrial membrane potential and ATP depletion (2015). AMPK has also been shown to inactivate ERK 1/2 pathway. ERK 1/2 inhibits apoptosis and promotes cell survival. So, metformin mediated AMPK upregulation may inactive ERK1/2 and promote cell death (Zordoky 2014). Park *et al*. also showed that glucose withdrawal and metformin lead to increased pAMPK level and cell death in hepatocellular cancer cell line (2015).

We have also shown that metformin downregulated glucose deprivation-induced GRP78 overexpression (p=0.0021). mTORC1 phosphorylates translational regulators 4E-BP1 which is essential for cap-dependent translation (Laplante 2012, Matsuo 2012). Biguanides have been shown to cause hypophosphorylation of 4E-BP1 which leads to hyperactivation of 4E-BP1. 4E-BP1 then binds to eIF4E and inhibits translation initiation. Matsuo *et al*. showed that biguanide metformin or phenformin in the presence of glucose deprivation led to hypophosphorylation of 4E-BP1 which in turn inhibited translation initiation and global protein synthesis leading to inhibition of UPR and decreased GRP78 expression (2012). eIF4E is an oncoprotein which is overexpressed in a number of cancers, and it may be important for tumour cell growth and survival. So, the anti-tumour effect of biguanide could be as a result of UPR downregulation as well as the fact that it decreases eIF4E level by causing hyperactivation of 4E-BP1. Biguanide mediated hyperactivation of 4E-BP1 is thought to occur as a result of mTOR inhibition which can be via AMPK independent mechanism in cell lines which lack LKB1 (Matsuo 2012). Metformin has been shown to inhibit mTORC1 via Rag GTPase in the absence of AMPK. Similarly, metformin can inhibit mTOR by enhancing the association of PRAS40 with raptor in an AMPK independent manner (Bikas 2015). Saito *et al*. also showed that metformin disrupts UPR by suppressing the formation of a spliced form of XBP1 mRNA (2009). GRP78 activates pro-survival PI3K/Akt pathway and metformin has been shown to suppress this activation (Jagannathan 2015). Metformin can also suppress GRP78 induced autophagy to promote cell death. Park *et al*. showed that when metformin was added to glucose-deprived HCC cells, it induced apoptosis and inhibited autophagy. Metformin treatment led to a fall in the level of autophagy-related proteins namely Atg3, Atg5, Atg7, Atg12, LC3II and beclin1 in glucose-deprived HCC cells (2015).

Through these experiments, we have shown that metformin treatment led to marked cell death when media was deprived of glucose. Though non-transformed proliferating cells like cancer cells can show aerobic glycolysis, glucose concentration in tumour cells has been shown to be 10-40 times lower than in normal tissues thus providing an opportunity for selective targeting of cancer cells (Menendez 2012). As metformin induces cell death in low glucose condition, hence a combination of metformin and anti-angiogenesis treatment can also work. Similarly, calorie restriction and metformin treatment may also work (Menendez 2012). Combination treatment of metformin, radiation therapy and low-carbohydrate diet in patients with recurrent brain tumours is currently undergoing trial (NCT02149459). Also, metformin at a concentration as low as 0.01mmol/L has been shown to inhibit stem cells. Hence, combination treatment involving metformin can also work on cell population which are resistant to most chemotherapy agent (Menendez 2012).

The maximum safe dose of metformin in 60 kg man is 2550 mg/day. Menendez *et al*. showed that metformin is synthetically lethal with glucose withdrawal in cancer cells and in this study, they used 250mg/kg/day of metformin in mice which is equivalent to 1200mg/day in 60 kg man (2012). This points to the fact that the concentration of metformin used in the lab is clinically achievable.

4.4 Future work

Through these experiments, we have shown that metformin potentiates glucose deprivation-induced cell death. This can form the basis of further studies into the cell cycle and ATP assay in glioma cells treated with metformin and glucose deprivation. It will also be interesting to look at NADPH and lactate levels in glucose-deprived cells in the presence of metformin.

Chapter 5

5.1 Introduction

GRP78 is the pro-survival arm of UPR. However, during sustained ER stress, the pro-apoptotic arm of UPR can activate IRE1, CHOP, caspases and apoptotic signalling kinase-1 and its downstream target JNK which can lead to cell death (Leclerc 2013). Transcription factor CHOP also sensitises cells to ER stress-induced apoptosis through down-regulation of anti-apoptotic Bcl-2 and activation of GADD34 and ER01α, an ER oxidase (Pyrko 2007). Even in the absence of any drug treatment, knockdown of GRP78 has been shown to result in marked upregulation of CHOP indicating that GRP78 keeps the level of pro-apoptotic CHOP in check (Pyrko 2007). 2DG treatment has been to shown to lead to ER stress (Kang 2006, Kurtoglu 2007). As a result of structural similarity with mannose, 2DG can be incorporated into lipid-linked oligosaccharide chains. However, unlike mannose, 2DG cannot accommodate the required complex branching structure of lipid-linked oligosaccharide molecules resulting in premature termination of synthesis of these molecules leading to accumulation of misfolded/unfolded protein and initiating UPR (Desalvo 2012). Park *et al*. showed that 2DG treatment led to GRP78 upregulation and macrocyclic compound versipelostatin down-regulated GRP78 leading to massive cell death in colon cancer, fibrosarcoma and stomach cancer cells (2004). Park *et al.* also showed that 2DG mediated GRP78 upregulation can be down-regulated by verrucosidin isolated from Penicillium and this potentiated glucose deprivation-induced cell death in fibrosarcoma and colon cancer cells (2007). 2DG has also been shown to upregulate GRP78 and CHOP in pancreatic, melanoma and breast cancer cell lines (Xi 2011). Saito *et al*. showed that metformin could also downregulate GRP78 in 2DG treated cells and induce cell death in fibrosarcoma, colon, stomach and renal cancer cells (2009). Leclerc *et al*. showed that metformin not only downregulates GRP78, it also induces stress in ER lumen by upregulating CHOP/IRE1α/ATF6 in ALL cells, preventing cancer cells from effectively engaging the UPR (2013). Because of these encouraging results of the synergistic action of metformin and 2DG treatment on cell death in other cancer cell lines, we decided to investigate the effect of metformin and 2DG on glioma cell death. AICAR like metformin has been shown to upregulate pAMPK, so we also examined the effect of combination treatment of AICAR and 2DG on glioma cell viability. Going further we investigated the mechanism of cell death seen with metformin and 2DG treatment. We also looked at the effect of metformin and 2DG treatment on energetic stress, oxidative stress and autophagy. Green tea extract EGCG or siRNA mediated downregulation of GRP78 has been shown to potentiate TMZ mediated apoptosis in glioblastoma cells (Pyrko 2007). As metformin, has been shown to downregulate GRP78, we also decide to investigate the effect of combination treatment of metformin and TMZ on glioma cell viability. Studies have also demonstrated

that UPR plays a role in resistance to etoposide (Mann 2012) so we also investigated the effect of metformin and etoposide on glioma cell viability.

5.2 Results

5.2.1 Combined metformin and 2DG treatment induced glioma cell death in *vitro*

To test the effect of metformin and 2DG treatment on glioma cell viability U87MG, LN229 and U251MG cells were used. A Propidium iodide assay was performed at 24-and 96-hour time points. In all three cell lines, U87MG, LN229 and U251MG, combined metformin and 2DG treatment led to significant cell death at 96-hour time point (p=0.002, 0.0063 and 0.0007 respectively). Though cell death was seen in all three cell lines at 24-hour time point, it was statistically not significant (p=0.0939, 0.4389 and 0.0739 respectively).

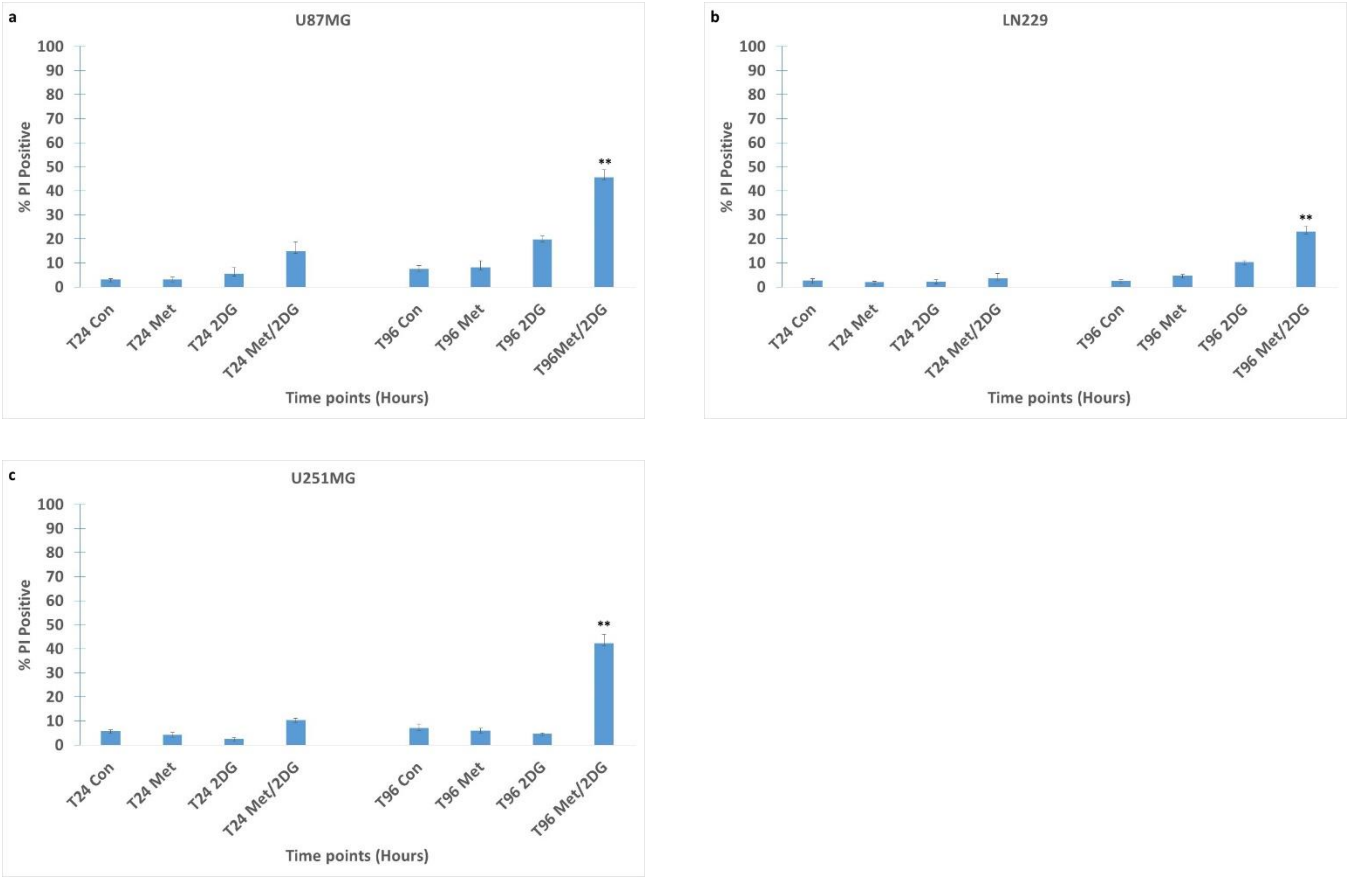


Figure 5.1 a, b, c: Metformin and 2DG treatment induce cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells, wells were washed gently with PBS, and then DMEM with 8mm metformin and 10mM 2DG were added to appropriate wells. At 24 and 96 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard

error of the mean. In all three cell lines, U87MG, LN229 and U251MG, combined metformin and 2DG treatment led to significant cell death at 96-hour time point (p=0.002, 0.0063 and 0.0007 respectively).

5.2.2 Combined AICAR and 2DG treatment induced glioma cell death in *vitro*

To test the effect of AICAR and 2DG treatment on glioma cell viability U87MG, LN229 and U251MG cells were used. A Propidium iodide assay was performed at 24-and 96-hour time points. In all three cell lines, U87MG, LN229 and U251MG, combined AICAR and 2DG treatment led to significant cell death at 96-hour time point (p=0.0021, 0.0371 and 0.0089 respectively). Though cell death was seen in all three cell lines at 24-hour time point, it was statistically not significant (p= 0.0875, 0.07567 and 0.7127 respectively).

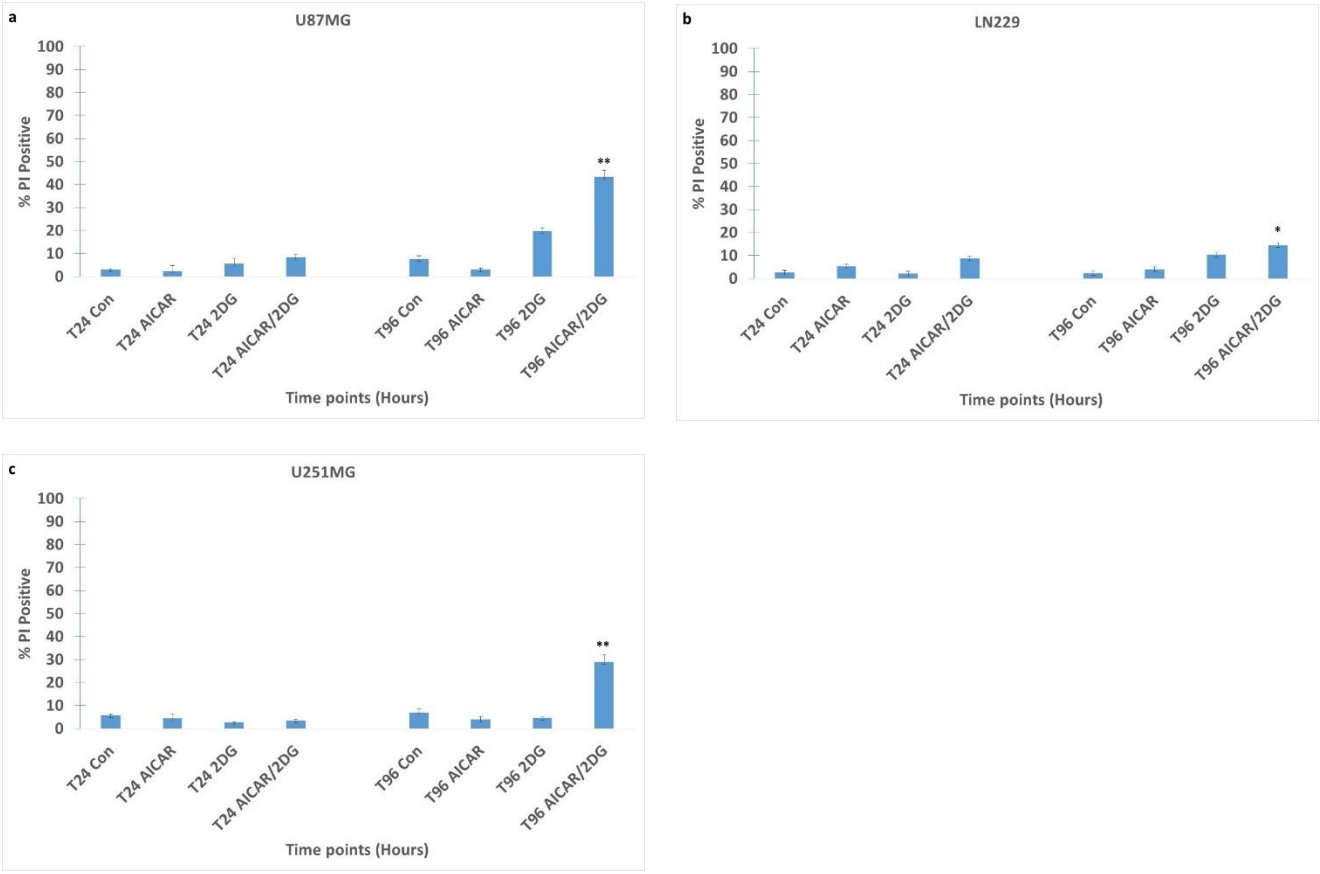


Figure 5.2 a, b, c: AICAR and 2DG treatment induce cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells, wells were washed gently with PBS, and then DMEM with 1mM AICAR and 10mM 2DG were added to appropriate wells. At 24 and 96 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. In all three cell lines, U87MG, LN229 and U251MG, combined AICAR and 2DG treatment led to significant cell death at 96-hour time point (p=0.0021, 0.0371 and 0.0089 respectively).

5.2.3 Cell death induced by metformin and 2DG combination is not mediated via oxidative stress

To test the effect of free radical scavenger NAC on metformin and 2DG induced cell death, U87MG, LN229 and U251MG cell lines were used. A Propidium iodide assay was performed at a 96-hour time point after treating cells with metformin and 2DG with or without NAC. NAC had no significant effect on metformin and 2DG combination induced glioma cell death in U87MG, LN229 and U251MG cell lines at 96-hour time point (p=0.5359, 0.8074 and 0.4107 respectively). This showed that metformin and 2DG induced cell death was not mediated by oxidative stress.

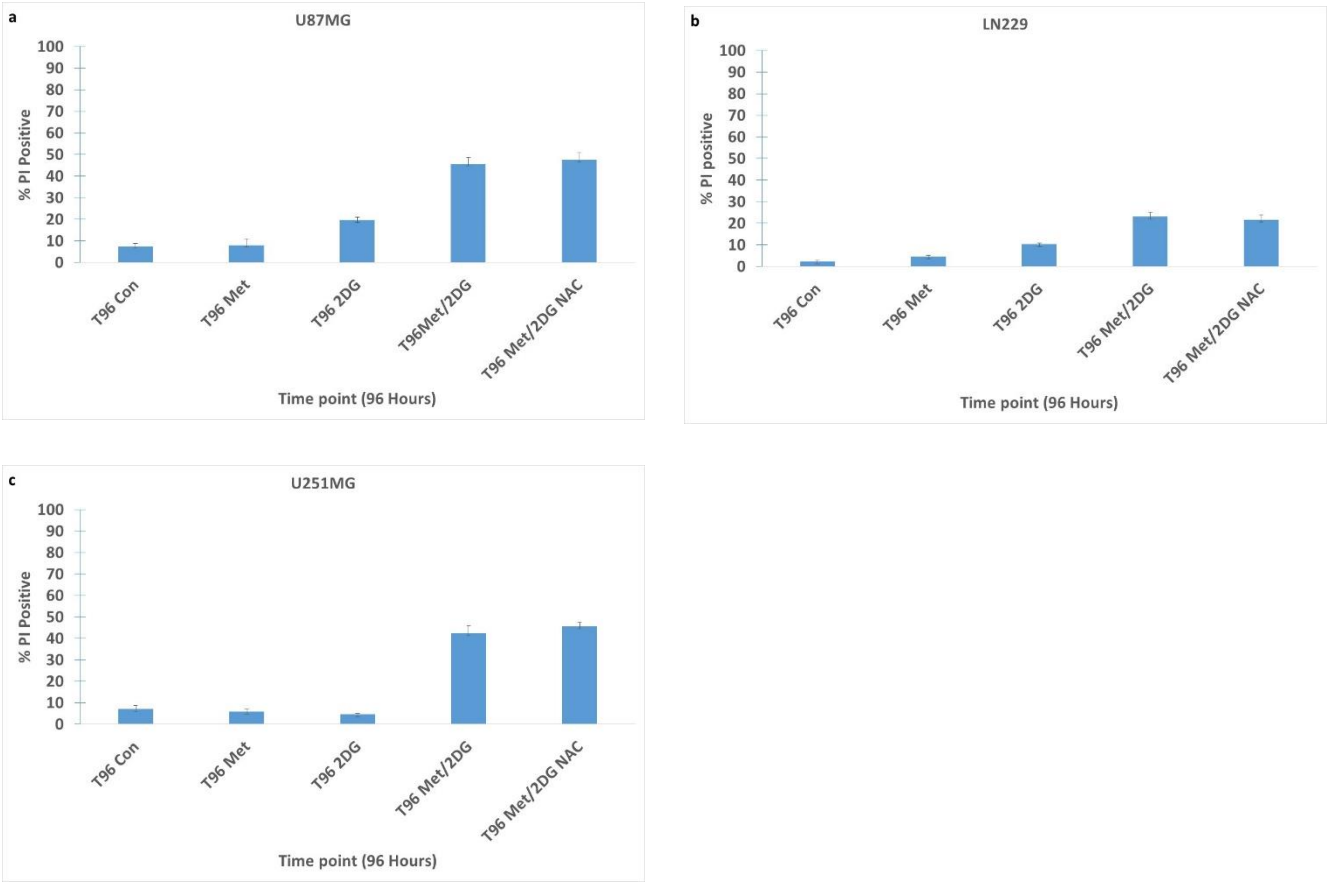


Figure 5.3 a, b, c: Free radical scavenger NAC does not rescue metformin and 2DG induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells and wells were washed gently with PBS. DMEM with 8mM metformin and 10mM 2DG with or without 1mM NAC were added at appropriate time points. After 96 hours, the supernatant was collected into marked FACS tubes. Adherent cells were trypsinised and collected in appropriate tubes too. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. NAC had no significant effect on metformin and 2DG combination induced glioma cell death in U87MG, LN229 and U251MG cell lines at 96-hour time point (p=0.5359, 0.8074 and 0.4107 respectively).

5.2.4 Cell death induced by metformin and 2DG combination is caspase-independent

To test the role of apoptosis in metformin and 2DG induced cell death, U87MG, LN229 and U251MG cell lines were used. A Propidium iodide assay was performed at 96-hour time point after treating cells with metformin and 2DG with or without pan-caspase inhibitor ZVAD-fmk. ZVAD-fmk had no significant effect on metformin and 2DG combination induced glioma cell death in U87MG, LN229 and U251MG cell lines at 96-hour time point (p=0.61, 0.274 and 0.3478 respectively). This showed that metformin and 2DG induced cell death was caspase-independent.

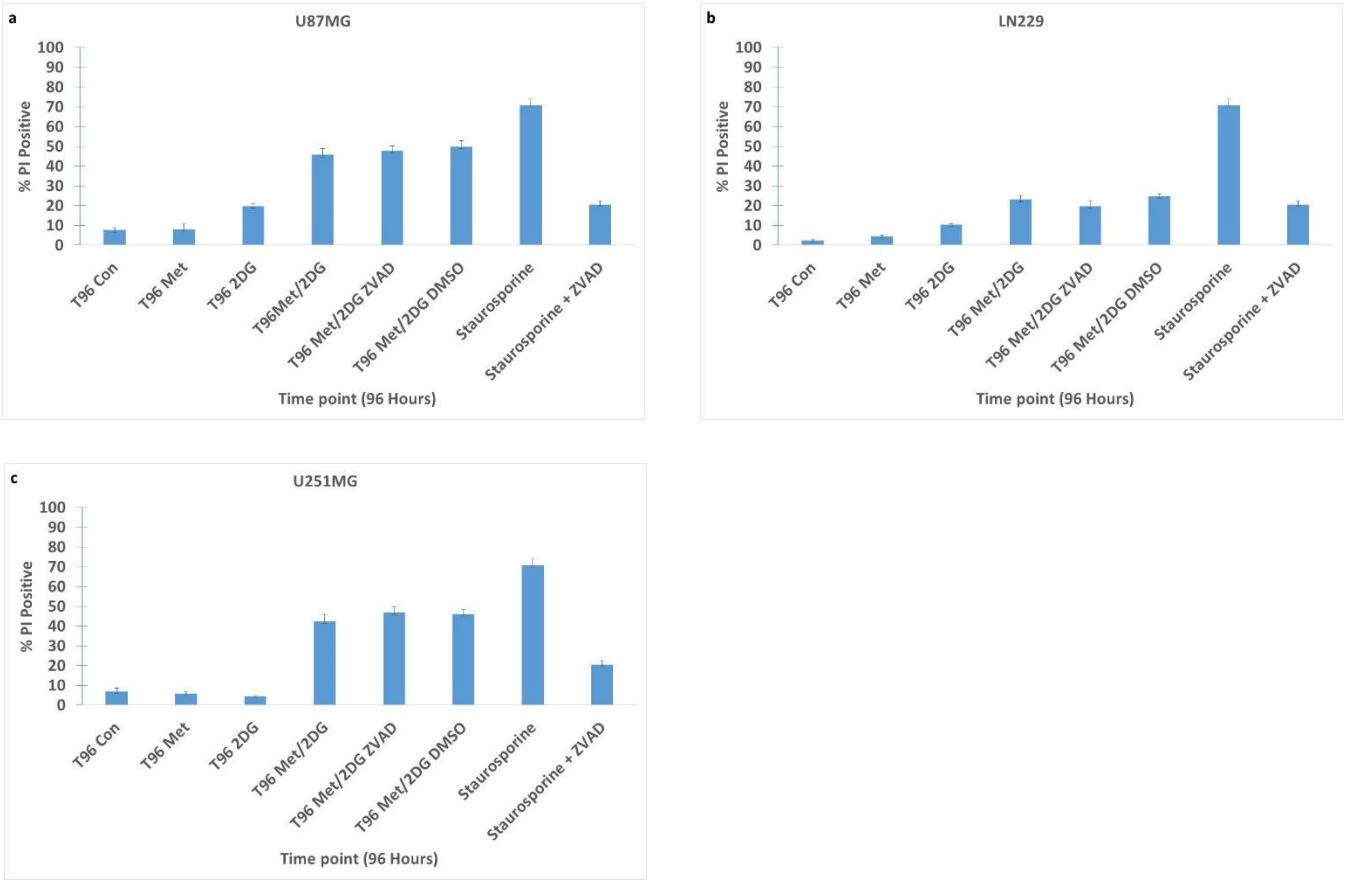
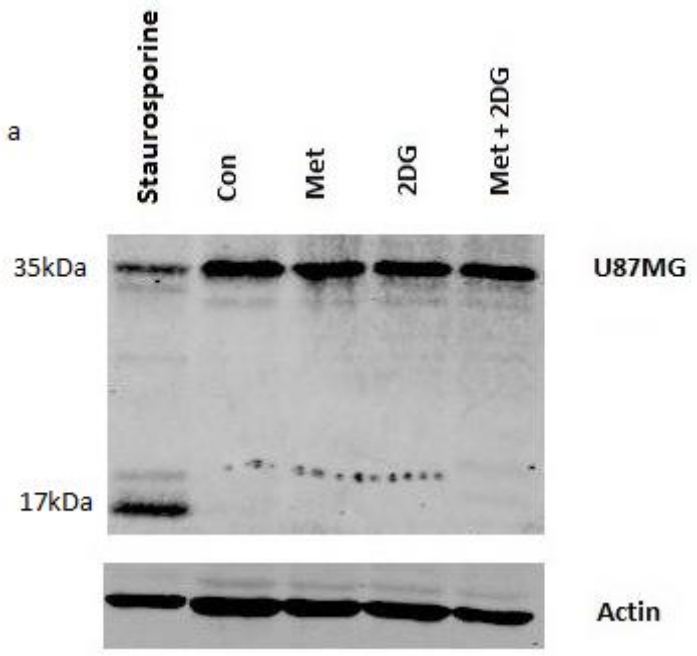


Figure 5.4 a, b, c: Pan-caspase inhibitor ZVAD-fmk does not rescue metformin and 2DG induced cell death glioma cell lines. in U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 23 hours, cells were pre-incubated with 50 µM ZVAD-fmk to allow the ZVAD-fmk to be present inside the cells at the time when metformin and 2DG treatment was added. After 1 hour, media was removed from wells, wells were washed gently with PBS and then DMEM containing 8mM metformin and 10mM 2DG with or without 50 µM ZVAD-fmk was added to appropriate wells. DMSO was also added in appropriate wells as a control since ZVAD-fmk was dissolved in DMSO. Also, 1 µM Staurosporine, a known inducer of apoptosis was used as a control for ZVAD-fmk. After 96 hours, the supernatant was collected into marked FACS tubes. Adherent cells were trypsinised and collected in appropriate tubes too. Cell viability was assessed using a propidium iodide

assay. All experiments were done in triplicates, and error bars represent standard error of the mean. ZVAD-fmk had no significant effect on metformin and 2DG combination induced glioma cell death in U87MG, LN229 and U251MG cell lines at 96-hour time point (p=0.61, 0.274 and 0.3478 respectively).

5.2.5 Metformin and 2DG induced cell death in glioma cells does not lead to caspase 3 cleavage

We have earlier shown that metformin and 2DG induced cell death is not inhibited by pan-caspase inhibitor ZVAD-fmk (Figure 5.4). So, we hypothesised that metformin and 2DG combination treatment would not lead to caspase 3 cleavage into an active form. To test this, western blot analysis was performed after treating U87MG glioma cells with metformin and 2DG for 96 hours. We showed that metformin and 2DG combination treatment had no effect on expression of the cleaved active form of caspase 3 whereas staurosporine, a known inducer of apoptosis upregulated cleaved caspase 3 expression (p=0.0015).



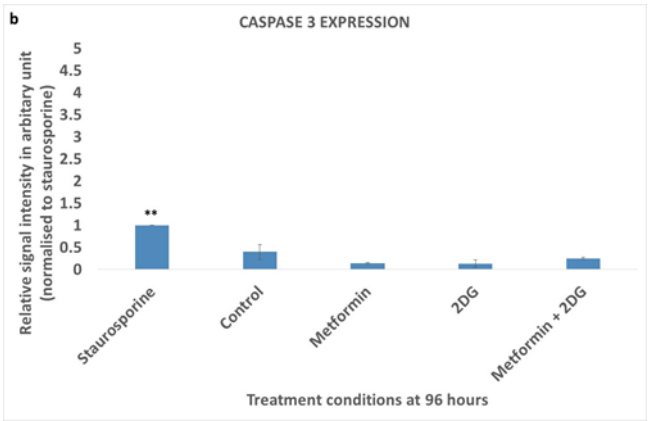
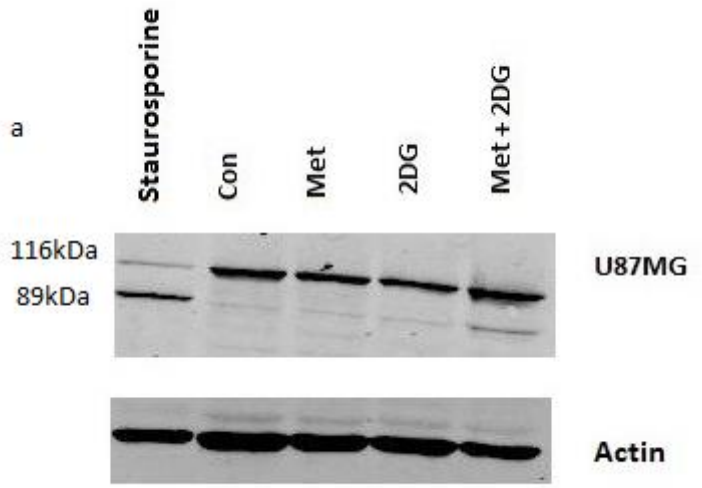


Figure 5.5 a: Metformin and 2DG induced cell death in glioma cells does not lead to caspase 3 cleavage into an active form. 7.5X10 ^{4} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then medium containing 8mM metformin, 10mM 2DG or combination of 8mM metformin and 10mM 2DG was added. Cells grown in normal DMEM was used as a control. After 96 hours, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against caspase 3. Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 5.5 b: Relative signal intensity of caspase 3 expression in arbitrary unit (normalised to staurosporine). Metformin and 2DG combination treatment had no effect on expression of the cleaved active form of caspase 3 whereas staurosporine, a known inducer of apoptosis, upregulated cleaved caspase 3 expression (p=0.0015).

5.2.6 Metformin and 2DG induced cell death in glioma cells does not lead to PARP cleavage

PARP is a family of proteins mainly involved in DNA repair and programmed cell death. We have earlier shown that metformin and 2DG induced cell death is not inhibited by pan-caspase inhibitor ZVAD-fmk (Figure 5.4) and does not lead increased expression of the cleaved active form of caspase 3 (Figure 5.5). So, we hypothesised that metformin and 2DG combination treatment would have no effect on PARP cleavage. To test this, western blot analysis was performed after treating U87MG glioma cells with metformin and 2DG for 96 hours. Interestingly we did see some PARP cleavage with metformin and 2DG treatment though cell death was not inhibited by ZVAD-fmk. Staurosporine, a known inducer of apoptosis led to marked upregulation of cleaved active form of PARP (p=0.0017).



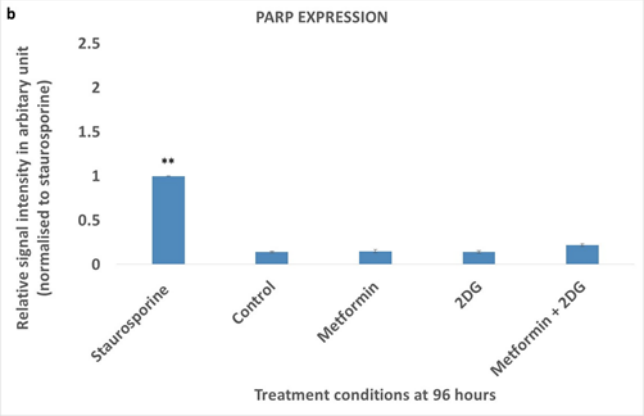
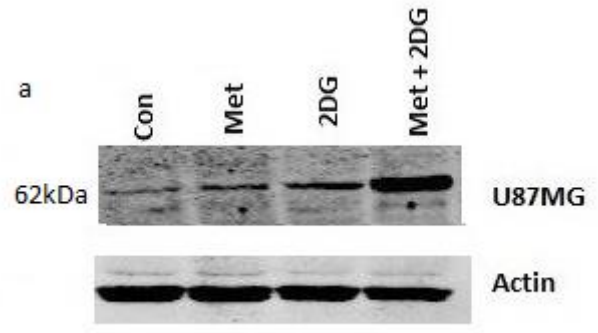


Figure 5.6 a: Metformin and 2DG induced cell death in glioma cells does not lead to PARP cleavage. 7.5X10 ^{4} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS and then medium containing 8mM metformin, 10mM 2DG or combination of 8mM metformin and 10mM 2DG was added. Cells grown in normal DMEM was used as a control. After 96 hours, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against PARP (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 5.6 b: Relative signal intensity of PARP expression in arbitrary unit (normalised to staurosporine). As compared to staurosporine, metformin and 2DG combination did not lead to significant PARP cleavage (p=0.0017).

5.2.7 Metformin promotes 2DG induced energetic stress and up-regulates pAMPK expression

We have earlier shown that cancer cells depend upon unregulated glycolysis to meet their energetic and biosynthetic needs as well as to generate reducing equivalents. We have also shown that glucose deprivation led to energetic stress and pAMPK upregulation (Figure 3.20). Metformin has been shown to inhibit ETC I and lead to energetic stress. 2DG has also been shown to inhibit glycolysis and lead to energetic stress. So, we hypothesised that metformin and 2DG treatment will lead to synergistic upregulation of pAMPK expression. To test this, western blot analysis was performed after treating U87MG glioma cells with metformin and 2DG for 96 hours. We showed that metformin and 2DG combination led to marked to pAMPK upregulation as compared to the control (p=0.0055).



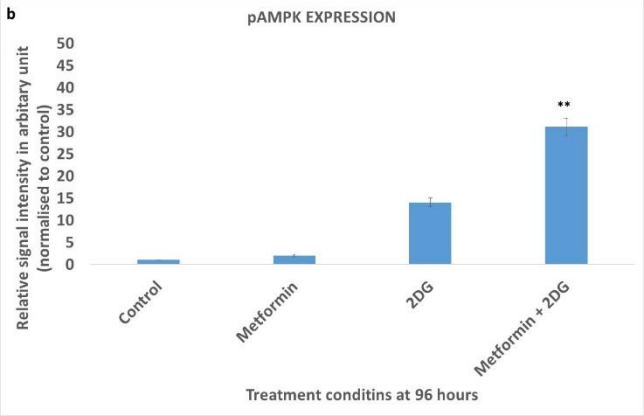
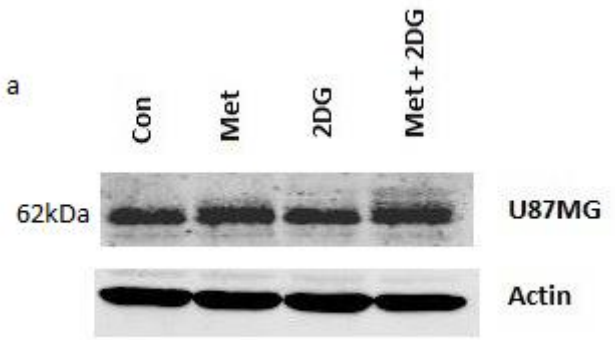


Figure 5.7 a: Metformin promotes 2DG induced energetic stress and up-regulates pAMPK expression. 7.5X10 ^{4} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then medium containing 8mM metformin, 10mM 2DG or combination of 8mM metformin and 10mM 2DG was added. Cells grown in normal DMEM was used as a control. After 96 hours, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against pAMPK (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 5.7 b: Relative signal intensity of pAMPK expression in arbitrary unit (normalised to control). Metformin and 2DG combination led to marked to pAMPK upregulation as compared to the control (p=0.0055).

5.2.8 Metformin and 2DG combination does not affect total AMPK level

We have earlier shown that metformin and 2DG combination led to energetic stress and marked pAMPK upregulation (Figure 5.7). To test the effect of metformin and 2DG on total AMPK level, western blot analysis was performed after treating U87MG glioma cells with metformin and 2DG for 96 hours. We showed that the combination of metformin and 2DG had no significant effect on total AMPK level as compared to the control (p=0.1217).



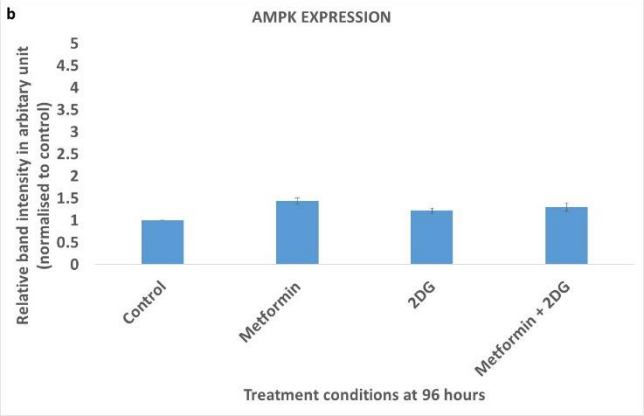
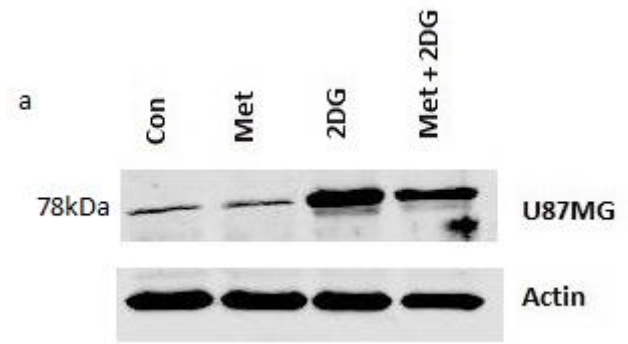


Figure 5.8 a: Metformin and 2DG combination does not affect total AMPK level. 7.5X10 ^{4} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then medium containing 8mM metformin, 10mM 2DG or combination of 8mM metformin and 10mM 2DG was added. Cells grown in normal DMEM was used as a control. After 96 hours, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against AMPK (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 5.8 b: Relative signal intensity of AMPK expression in arbitrary unit (normalised to control). The combination of metformin and 2DG had no significant effect on total AMPK level as compared to the control (p=0.1217).

5.2.9 Metformin down regulates 2DG mediated GRP78 upregulation

We have shown that glucose deprivation induces UPR and upregulates ER chaperone protein GRP78 (Figure 3.21). We hypothesised that metformin will down-regulate 2DG induced GRP78 upregulation. To test this, western blot analysis was performed after treating U87MG glioma cells with metformin and 2DG for 96 hours. We showed that 2DG mediated GRP78 expression was downregulated by metformin (p=0.0003).



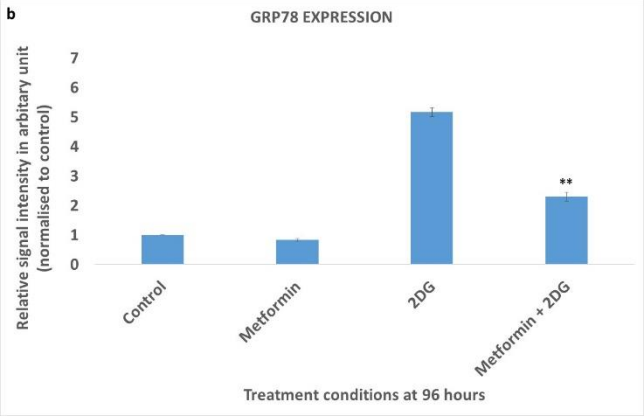


Figure 5.9 a: Metformin downregulates 2DG mediated GRP78 upregulation. 7.5X10 ^{4} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then medium containing 8mM metformin, 10mM 2DG or combination of 8mM metformin and 10mM 2DG was added. Cells grown in normal DMEM was used as a control. After 96 hours, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against GRP78 (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 5.9 b: Relative signal intensity of GRP78 expression (normalised to control). 2DG mediated GRP78 expression was downregulated by metformin (p=0.0003).

5.2.10 Metformin downregulates 2DG induced autophagy

We have earlier shown that metformin and 2DG led to marked pAMPK upregulation (Figure 5.7) and GRP78 downregulation (Figure 5.8). Metformin has also been shown to suppress GRP78 induced autophagy and promote cell death. Park showed that when metformin was added to glucose-deprived HCC cell lines, it induced apoptosis and inhibited autophagy (2015). 2DG like glucose deprivation has been shown to induce autophagy. So, we hypothesised that 2DG would also induce autophagy in glioma cell line and metformin can suppress 2DG mediated autophagy to promote cell death. To test this, western blot analysis was performed after treating U87MG glioma cells with metformin and 2DG for 96 hours. We showed that 2DG induced autophagy in glioma cells as it led to LC3 II upregulation. However, in the presence of metformin, 2DG induced autophagy was suppressed as evident by fall in LC3 II level (p=0.0156).

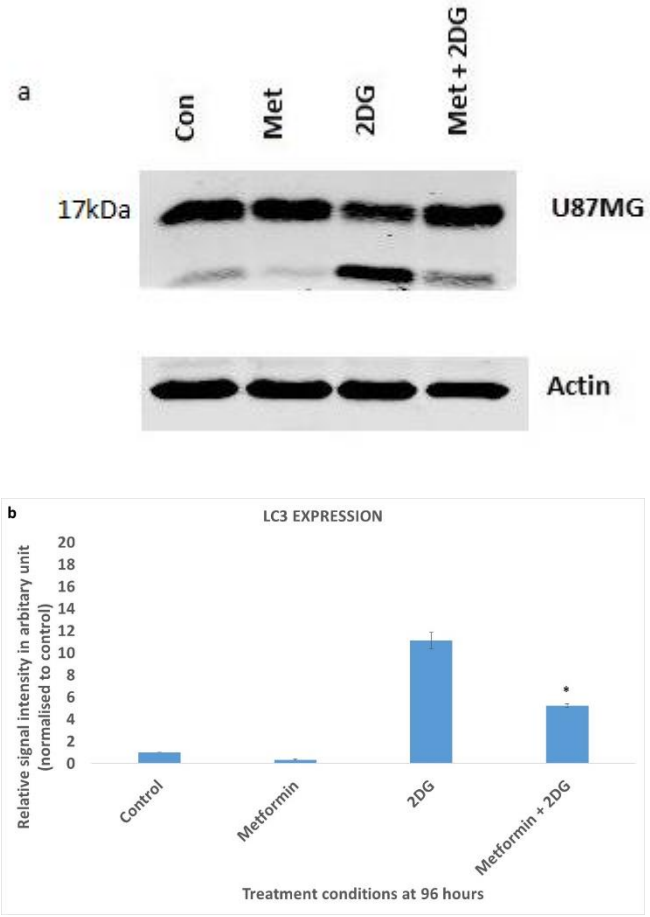


Figure 5.10 a: Metformin downregulates 2DG induced autophagy. 7.5X10 ^{4} cells /ml were seeded in DMEM media supplemented with 10% FCS and allowed to adhere overnight prior to treatment. Following morning, media was removed from wells, wells were washed gently with PBS, and then medium containing 8mM metformin, 10mM 2DG or combination of 8mM metformin and 10mM 2DG was added. Cells grown in normal DMEM was used as a control. After 96 hours, suspension and adherent cells were collected and lysed in RIPA buffer. An equal amount of protein was separated by SDS-PAGE, electroblotted to nitrocellulose membrane and probed with the antibody against LC3 II (1:1000). Antibody binding was detected using the Odyssey system. Equal lane loading was confirmed using a monoclonal antibody against β-actin (1:2000). Figure 5.10 b: Relative signal intensity of LC3 II expression (normalised to control). In the presence of metformin, 2DG induced autophagy was suppressed as evident by fall in LC3 II level (p=0.0156).

5.2.11 Metformin potentiates TMZ induced glioma cell death

Current standard care of treatment for patients diagnosed with glioblastoma includes chemotherapy agent TMZ. To investigate the effect of metformin on TMZ induced glioma cell death, a propidium iodide assay was performed at 24 and 96-hour time points. In all the three cell lines, U87MG, LN229 and U251MG, metformin potentiated TMZ induced glioma cell death at 96-hour time point (p=0.0122, 0.0492 and 0.0112 respectively). Though cell death was seen in all the three cell lines, U87MG, LN229

and U251MG at 24-hour time point, it was statistically significant in U251MG only (p=0.4179, 0.9773 and 0.0414 respectively).

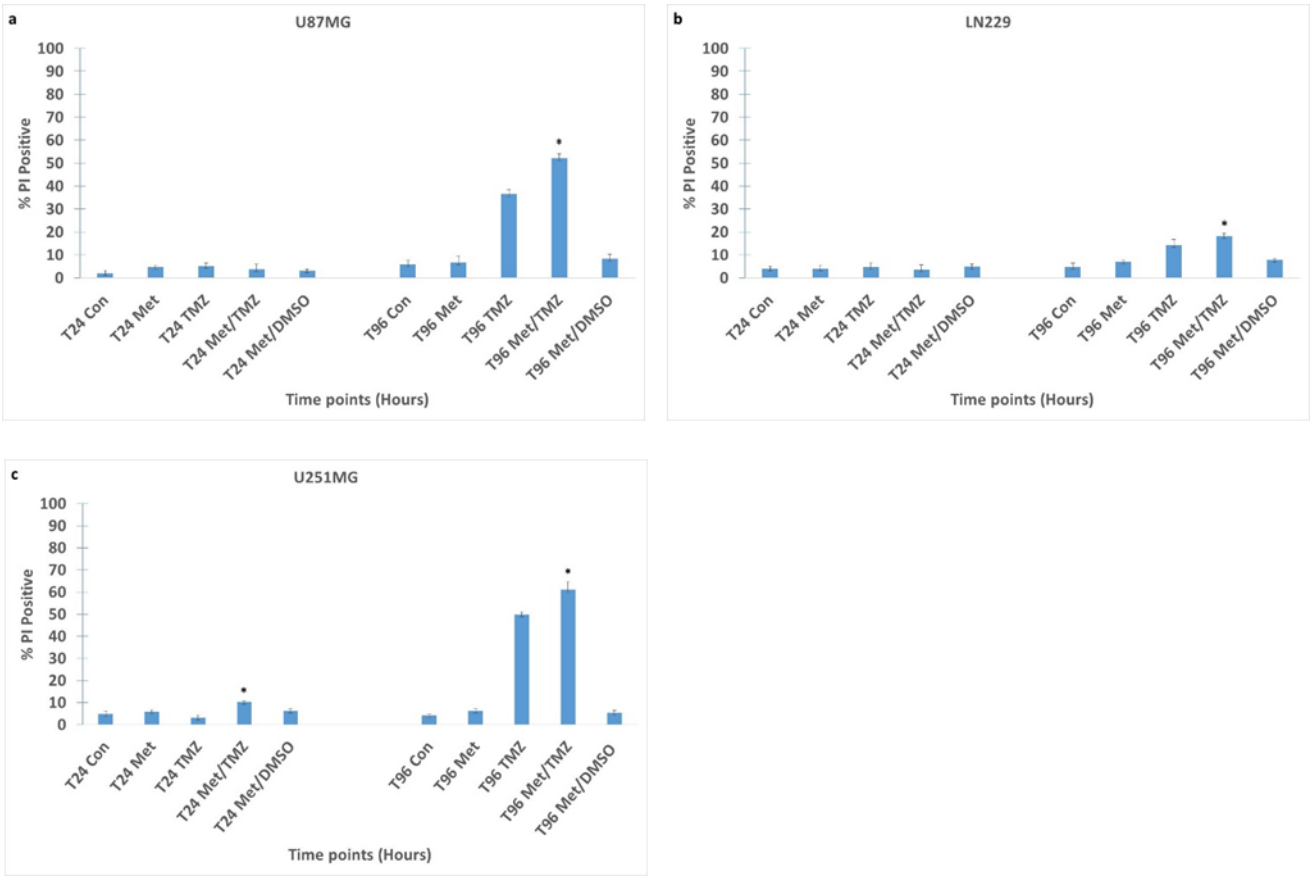


Figure 5.11 a, b, c: Metformin potentiates TMZ induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells, wells were washed gently with PBS, and then DMEM with 8mm metformin and 50µM TMZ were added to appropriate wells. At 24 and 96 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. In all three cell lines, U87MG, LN229 and U251MG, metformin potentiated TMZ induced glioma cell death at 96-hour time point (p=0.0122, 0.0492 and 0.0112 respectively). Though cell death was seen in all three cell lines, U87MG, LN229 and U251MG at 24-hour time point, it was statistically significant in U251MG cell line only (p=0.4179, 0.9773 and 0.0414 respectively).

5.2.12 Metformin has no effect on etoposide-induced glioma cell death

Etoposide has been shown to induce glioma cell death. To investigate the effect of metformin on etoposide-induced glioma cell death, a propidium iodide assay was performed at 24-and 96-hour time points. In all the three cell lines, U87MG, LN229 and U251MG, metformin had no significant effect on etoposide-induced glioma cell death at 96-hour time point (p=0.2954, 0.5363 and 0.7332 respectively). Cell death was also seen in all the three cell lines at 24-hour time point, but again it was statistically not significant (p=0.2554, 0.0913 and 0.2235 respectively).

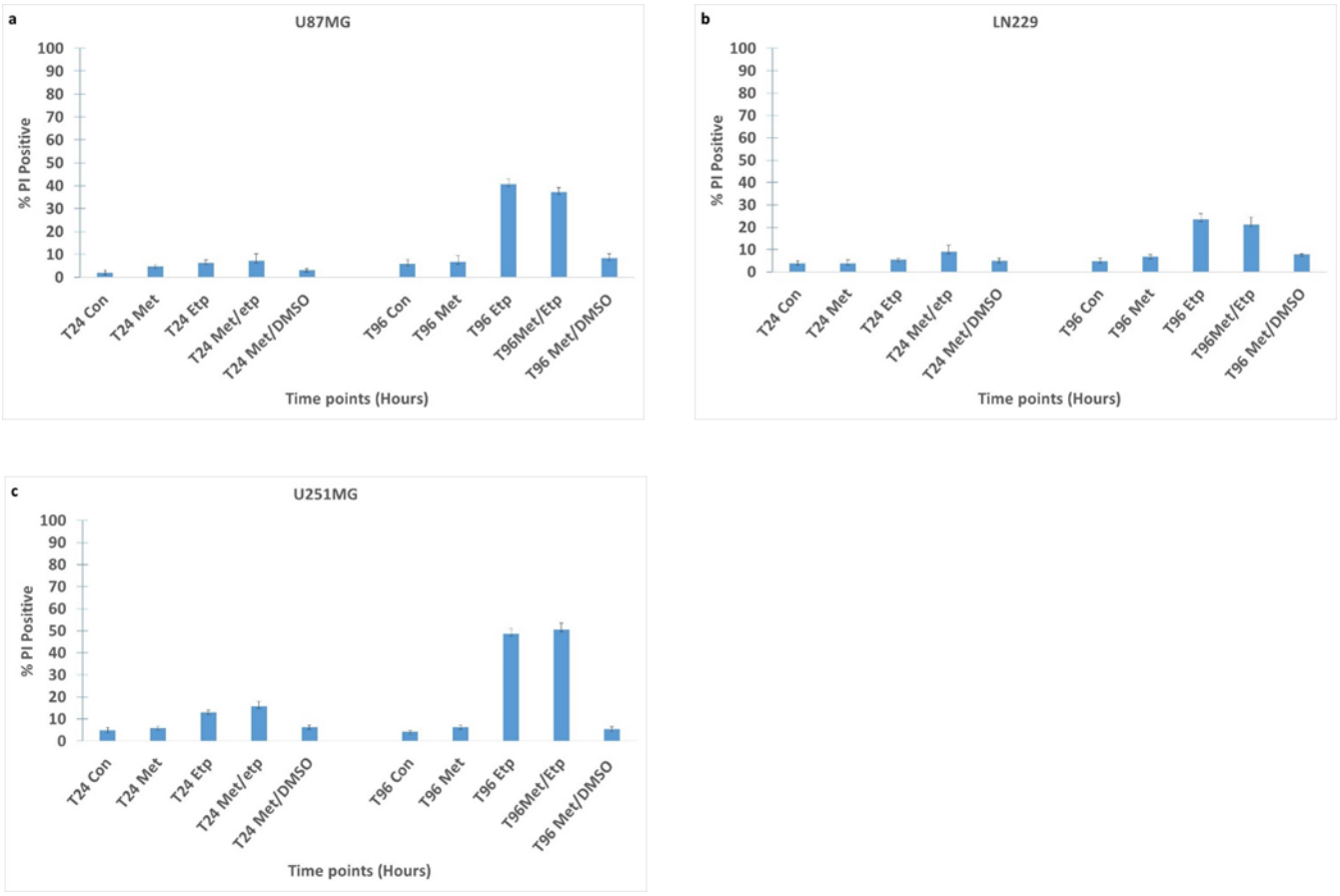


Figure 5.12 a, b, c: Metformin has no effect on etoposide-induced cell death in glioma cell lines. U87MG, LN229 and U251MG cells were seeded at 7.5 X 10^4 cells/ml in DMEM media supplemented with 10% FCS. After 24 hours, media was removed from wells, wells were washed gently with PBS, and then DMEM with 8mm metformin and 50µM etoposide were added to appropriate wells. At 24 and 96 hours, suspension and adherent cells were collected into marked FACS tubes. Cell viability was assessed using a propidium iodide assay. All experiments were done in triplicates, and error bars represent standard error of the mean. In U87MG, LN229 and U251MG glioma cell lines, metformin had no significant effect on etoposide-induced cell death at 96-hour time point (p=0.2954, 0.5363 and 0.7332 respectively). Cell death was also seen in all the three cell lines at 24-hour time point, but again it was statistically not significant (p=0.2554, 0.0913 and 0.2235 respectively).

5.3 Discussion

Studies have shown that when cancer cells are unable to generate ATP through oxidative phosphorylation, they upregulate glucose uptake and glycolysis to generate energy (Xu 2005). Studies have also shown that cells with mitochondrial defects are more sensitive to glycolytic inhibitors as they depend on glycolysis for ATP generation (Xu 2005). In our experiments, we used metformin which is an inhibitor of the ETC I and has been shown to decrease ATP generation. However, in the presence of metformin, cells can upregulate glucose uptake and glycolysis to generate ATP for growth, proliferation and survival (Bikas 2015). As a result of this, we did not see any significant cell death with metformin monotherapy. However, when we used a combination treatment of metformin and 2DG,

cells would have been unable to upregulate glycolysis and oxidative phosphorylation which resulted in cell death (p=0.002, 0.0063 and 0.0007 for U87MG, LN229 and U251MG cell lines at 96-hour time point respectively). Our data also show that AICAR which like metformin upregulates pAMPK when used in combination with 2DG, led to synergistic cell death in glioma cells (p=0.0021, 0.0371 and 0.0089 for U87MG, LN229 and U251MG cell lines at 96-hour time point respectively). We have also shown that metformin and 2DG mediated cell death was caspase-independent (p=0.61, 0.274 and 0.3478 for U87MG, LN229 and U251MG cell lines at 96-hour time point respectively). We did see some PARP cleavage, but cell death was not rescued by pan-caspase inhibitor ZVAD-fmk. Similarly, we have also shown that metformin and 2DG induced cell death was not as a result of oxidative stress as NAC failed to rescue cell death (p=0.5359, 0.8074 and 0.4107 for U87MG, LN229 and U251MG cell lines at 96-hour time point respectively). We have demonstrated that metformin and 2DG combination leads to energetic stress and pAMPK upregulation in glioma cells (p=0.0055). Our data also show that metformin and 2DG combination led to down-regulation of pro-survival GRP78 as well as autophagy leading to significant cell death in glioma cells (p=0.0156). Finally we have shown that though metformin was synergistic with TMZ (p=0.0122, 0.0492 and 0.0112 for U87MG, LN229 and U251MG cell lines at 96-hour time point respectively), it did not potentiate etoposide-induced cell death in glioma cells (p=0.2954, 0.5363 and 0.7332 for U87MG, LN229 and U251MG cell lines at 96-hour time point respectively).

Metformin and 2DG induced cell death in my experiments were not mediated by apoptosis which is in agreement with results published by our group in paediatric glioma cell lines (Levesley 2013). However, unlike us, Ben Sahra *el al*. saw apoptotic cell death in prostate cancer cells were treated with metformin and 2DG combination (2010). Bikas *et al*. saw caspase 3, and PARP activation with metformin and 2DG treated thyroid cancer cells (2015). Saito *et al*. also saw apoptotic cell death in fibrosarcoma, colon, stomach and renal cancer cells with metformin and 2DG treatment (2009). However, Liu et al. showed that combination treatment of fenofibrate which is an ETC I blocker like metformin and 2DG lead to necrotic as well as apoptotic cell death in osteosarcoma, breast cancer and melanoma cell lines (2016).

We saw cell death with metformin and 2DG in p53 wild-type as well as p53 mutant cell lines. This is in agreement with Fan *et al*. who saw cell death in wild as well as mutant p53 lung cancer cell lines with a combination treatment of 2DG and berberine an ETC I blocker like metformin (2013). However, Ben Sahra *et al*. showed that p53 mutant prostate cancer cells were resistant to metformin and 2DG combination treatment (2010).

Our findings are in agreement with other authors. Cheong *et al*. saw ATP depletion, AMPK activation and cell death with metformin and 2DG treatment in gastric and oesophageal cancer cell lines (2011). Similarly, Bikas *et al*. saw marked upregulation of pAMPK and cell death with metformin and 2DG treatment in thyroid cancer cell lines (2015). Ben Sahra *et al*. showed that metformin and 2DG combination resulted in >90% reduction in ATP level, marked AMPK upregulation, mTOR inhibition and cell death in p53 dependent fashion in prostate cancer cells whereas metformin or 2DG alone only resulted in 60% reduction in ATP level (2010).

Like our data (Figure 5.9), Saito *et al*. also showed that metformin can down-regulate GRP78 in 2DG treated cells and induced cell death in fibrosarcoma, colon, stomach and RCC cells. They also showed that metformin suppressed 2DG induced ATF4 expression and formation of a spliced form of XBP1 mRNA formation, leading to a fall in GRP78 level (2009). Matsuo *et al*. also showed combination treatment with metformin and 2DG resulted in GRP78 downregulation and cell death (2012). They also showed that biguanides treatment causes hypophosphorylation and hence hyperactivation of 4E-BP1 leading to inhibition of translation initiation and down-regulation of global protein synthesis including GRP78 (2012). Haga *et al.* also showed that with metformin and 2DG combination treatment, ATF4 activation did not occur which resulted in GRP78 down-regulation and cell death (2010). Fan *et al*. showed that combination treatment with berberine, an ETC I blocker like metformin and 2DG resulted in marked ATP depletion, AMPK activation and disruption of UPR leading to significant cell death in lung cancer cells (2013). Berberine was shown to inhibit 2DG mediated splicing of XBP1 (Fan 2013). Similarly, Lin *et al*. showed that dasatinib treatment in HNSCC cells led to ER stress and GRP78 upregulation. However, when metformin was used in combination with dasatinib, there was downregulation of GRP78 along with increased phosphorylation of AMPK and eIF2α. eIF2α has been shown to upregulate CHOP, the pro-apoptotic arm of UPR. So, a combination of a decrease in GRP78 and increase in CHOP led to significant cell death (2014). Liu *et al*. showed that combination treatment of fenofibrate which is an ETC I blocker like metformin and 2DG lead to energetic stress, AMPK activation, down-regulation of GRP78 and mTOR leading to synergistic cancer cell killing in osteosarcoma, breast cancer and melanoma cell lines. They showed that though combination treatment with 2DG and fenofibrate led to decreased expression of GRP78 and CHOP, there was increased phosphorylation of eIF2α which is an indicator of ER stress and upregulation of pAMPK which is an indicator of energetic stress, leading to synergistic cell death. The authors showed that both ER stress, as well as energetic stress, was responsible for cell death as mannose was only able to partially rescue 2DG and fenofibrate mediated cell death and similarly, glucose only partially rescued death in 2DG and fenofibrate-treated cells (2016).

Studies have shown that ER stress is a potent inducer of autophagy ((Xi 2011, Xi 2013, Shen 2014). UPR has been shown to induce autophagy through transcription activation of LC3 via ATF4 and of Atg5 via CHOP (Shen 2014). According to Ding *et al*. transcriptional up-regulation of Atg12, PERK, ATF6, and eIF2α may also play a role in ER stress-mediated induction of autophagy (Ding 2007). ER, stress-induced autophagy is protective in nature. Our data show that 2DG mediated autophagy in glioma cells was suppressed by metformin, as demonstrated by fall in LC3II level, leading to significant cell death. Inhibition of 2DG mediated autophagy by 3-methyladenine (3-MA) has also been shown to lead to increased cell death in melanoma, pancreatic and breast cancer cells (Xi 2011). Similarly, ER stress inhibitor tauroursodeoxycholate has been shown to reduce the level of GRP78, and LC3II in bufalin treated glioma cells, leading to significant cell death (Shen 2014). Kandala *et al*. also showed that down-regulation of GRP78 resulted in inhibition of autophagy in ovarian cancer cells (2012). Similarly, Ding *et al*. also showed that suppression of autophagy by 3-MA or siRNA against Atg6/beclin1 or Atg8/LC3II led to increased ER stress and increased cell death (2007). Liu *et al*. showed that combination treatment of fenofibrate which is an ETC I blocker like metformin and 2DG lead to inhibition of autophagy and synergistic cancer cell killing in osteosarcoma, breast cancer and melanoma cell lines (2016). Ben Sahra *et al*. also showed that metformin inhibits 2DG mediated autophagy and results in downregulation of beclin 1 in prostate cancer cells (2010). Choi *et al*. also showed that combination treatment of tephrosin, a retinoid and 2DG lead to marked ATP depletion, upregulation of pAMPK, down-regulation of mTOR and inhibition of autophagy resulting colon, lung and cervical cancer cell death (2011). However, they argued that autophagy was not being inhibited by pAMPK/mTOR pathway as pAMPK upregulation and mTOR down-regulation would generally upregulate autophagy. We also saw marked pAMPK upregulation with metformin and 2DG combination treatment along with inhibition of autophagy. So, it may be that in our experiments too, autophagy down-regulation was not mediated by pAMPK and metformin inhibits autophagy through a pathway other than mTOR. Park et al. showed that when metformin was added to glucose-deprived HCC cell lines, it induced apoptosis and inhibited autophagy (Park 2015). Metformin treatment led to a fall in the level of autophagy-related proteins namely Atg3, Atg5, Atg7, Atg12, LC3II and beclin1 in glucose-deprived hepatocellular cancer cells and compound C, an AMPK inhibitor blocked metformin mediated decrease in LC3II and beclin 1 which indicated that metformin inhibits autophagy in AMPK dependent manner (2015). Also, instead of downregulation of autophagy, Cheong *et al*. saw sustained ATP depletion, autophagy induction and significant cell death in metformin and 2DG treated gastric and oesophageal cancer cell lines (2011). It may be that effect of metformin on autophagy is cell line specific. Bikas et also showed that in BCPAP thyroid cancer cell line LC3II was detected in both high and low glucose condition and metformin had no effect on autophagy. BCPAP cell line shows BRAF

mutation and BRAF mutation has been shown to be associated with mTOR inhibition and autophagy induction. On the other hand, in FTC133 thyroid cancer cell, metformin in the presence of high glucose had no effect on LC3II expression whereas metformin in the presence of glucose deprivation led to autophagy induction (2015).

AMP mimic AICAR like metformin has been shown to activate AMPK and inhibit mTOR (Kuznetsov 2011). We showed that combination treatment of 2DG and AICAR also led to death in glioma cell lines. The most likely mechanism would have been AICAR and 2DG mediated synergistic increase in pAMPK, leading to down-regulation of GRP78 and cell death. However, combination treatment of 2DG and AICAR caused less cell death that metformin and 2DG and it may be due to the fact that though AICAR causes AMPK upregulation but it does not induce ATP depletion. However, Cheong *et al*. showed that though 2DG and metformin led to significant cell death in gastric and oesophageal cancer cell lines, the combination treatment of 2DG and AICAR did not lead to cell death. They argued that 2DG and metformin combination induced cell death occurred independently of AMPK (2011).

Use of glycolytic inhibitors as monotherapy has resulted in very few positive results, and hence dual targeting of the mitochondrial and the glycolytic pathway can be a promising chemotherapeutic strategy (Cheng 2012). However, one has to remember that the effectiveness of 2DG depends upon the presence of glucose and high glucose in the microenvironment may negate the effect of 2DG (Maschek 2004, Pelicano 2006, Scatena 2008). Mechanism of action of 2DG may depend upon the concentration of 2DG used. Studies have shown that at a concentration of less than 4mmol/L, 2DG induces cell death due to inhibition of N-linked glycosylation. Whereas at 8mmol/L, 2DG may act by inhibiting N-linked glycosylation as well as by inhibiting ATP generation (De Salvo 2012). Effect of 2DG on pAMPK may also be cell line specific. In BCPAP thyroid cancer cell, 2DG has been shown to have no effect on pAMPK expression either in low or high glucose conditions. Whereas, in low glucose condition, 2DG treatment lead to further down-regulation of p-PS6, a marker of mTOR activity. On the other hand, in FTC133 thyroid cancer cell line, 2DG treatment led to increased pAMPK expression, both in high as well as low glucose condition along with downregulation of p-PS6 (Bikas 2015).

Researchers have also pointed to the fact that plasma concentration of metformin after a 30mg/kg dose is only 10-40µM whereas most studies *in vitro* have used metformin concentration in the range of 1-10mM. We used metformin concentration of 8mM. Also, for experiments done in *vitro*, treatment is directly applied to cells, and this does not take into account the influence of other organs which may occur within the body. However, a high requirement of metformin in tissue culture may be due to the fact that cells are bathed in a supra-physiological level of glucose and growth medium is supplemented with growth factors and fetal bovine serum. Also, *in vitro* system does not possess OCT1, needed for

cellular uptake of metformin which may also explain high metformin requirement in *in vitro* experiments (Fogarty 2010). Bikas *et al*. showed that 24-hour pre-treatment with 2DG led to apoptosis in thyroid cancer cells at 48 hours with a clinically achievable dose of 25µM of metformin (2015).

There may also be concern about the effect of dual inhibition of glycolysis and oxidative phosphorylation in normal cells. However, it has been shown that combination treatment of berberine an ETC I blocker like metformin and 2DG did not result in significant cell death in normal human lung fibroblast cells (Fan 2013). Similarly, Haga *et al*. showed that though 2DG did lead to GRP78 induction in non-transformed cell and ETC blocker suppressed it, but the combination treatment did not result in increased cell death in non-transformed cells (2010).

Also, the effect of AMPK on ER stress is study specific. A number of studies like ours have shown that AMPK activation leads to increased ER stress along with a decrease in GRP78 expression which in turn promotes cell death. However, on the other hand, AMPK has also been shown to inhibit ER stress and protect cardiomyocyte against hypoxic injury (Terai 2005). Similarly, Kim *et al*. showed that AMPK activation inhibits ER stress in renal fibrosis (2015). It may be that the effect of AMPK on ER stress is cell-dependent and AMPK induces ER stress in cancer cells whereas it inhibits ER stress in normal cells (Lin 2014).

However, one has to be cautious in using AMPK activators as some studies have shown that AMPK may have a role in promoting tumours. AMPK has been shown to be essential for glioma cell survival and spheroid migration in low glucose condition (Godlewski 2010). Similarly, AMPK activation has been shown to be important for pancreatic and prostate cancer cell growth and downregulation of AMPK in pancreatic cancer cells has been shown to impair their ability to grow in an anchorage-independent manner and form tumours *in vivo* and impair their ability to survive in low glucose condition (Kato 2002, Jeon 2012). It has also been shown that CAMKK2 expression is highly elevated in prostate cancer cells and that AMPK is important for prostate cancer cell growth, survival and migration (Jeon 2012). AMPK may help the cancer cells to survive low energy conditions as it has been shown that cancer cells lacking LKB1 are more sensitive to energy stress-mediated apoptosis (Shaw 2004). Studies have also shown that during glucose deprivation AMPK activation promotes cell survival by inactivating mTOR, inhibiting anabolic processes and promoting catabolic processes to maintain ATP levels in the cells. During glucose deprivation, AMPK activation also leads to increased glucose intake and it upregulates FAO. Increased glucose uptake can be directed towards PPP to generate NADPH. Similarly, FAO besides generating energy can also generate NADPH which can counteract oxidative stress and protect cancer cells. AMPK also prevents fatty acid synthesis thereby conserving NADPH (Jeon 2012).

5.4 Future work

Metformin is the most widely prescribed drug for type 2 diabetes. 2DG has already in use in humans in diagnostic radiology. TMZ is part of standard chemotherapy for patients with glioma. Our results have shown that metformin and 2DG combination leads to significant cell death in glioma cell lines. We have also shown that metformin potentiates TMZ mediated glioma cell death. These experiments may pave the way for future preclinical work to see if combination treatment can suppress glioma cell growth *in vivo.*

Conclusion

In the 1920s, Otto Warburg and his colleagues showed that normal liver tissue exhibited the "Pasteur effect," which is inhibition of fermentation in the presence of oxygen, whereas tumour tissue continued to produce lactate even in the presence of oxygen (aerobic glycolysis). Warburg showed that tumour tissue consumed ten-fold more glucose than accounted for by respiration and twice as much lactate as produced by normal tissue. This phenomenon was described as the "Warburg effect". Upregulated glycolysis has not only been shown to provide intermediates for biosynthesis, but studies have also demonstrated that it plays a vital role in transformation, cell cycle progression, proliferation, resistance to cell death, metastasis, angiogenesis and maintenance of stemness; the hallmarks of cancer.

Our study has shown that the glucose addiction of glioma cells can be successfully targeted. In chapter three, we have demonstrated that glucose deprivation led to glioma cell death *in vitro* in U87MG, LN229 and U251MG cell lines, which was rescued by free radical scavenger NAC, indicating that the cell death was mediated by ROS. Pyruvate, a known ROS scavenger and a source of energy via its conversion to acetyl CoA was also able to suppress glucose deprivation-induced glioma cell death. We have also shown that glucose deprivation had no effect on glioma cell viability in the presence of pyruvate and FCS. Our study also showed that untransformed human fibroblasts were resistant to glucose deprivation-induced cell death. The study showed that glucose withdrawal-induced cell death was dependent on mitochondrial metabolism for ROS generation and as mitochondrial oxidative phosphorylation is suppressed in hypoxia, hypoxia resulted in suppression of glucose deprivation-induced cell death. We have shown that glucose deprivation-induced glioma cell death was not mediated by apoptosis, autophagy or necrosis and glutamine withdrawal did not potentiate glucose deprivation-induced cell death in glioma cells. We have also shown that glucose deprivation and hypoxia promoted glioma cell migration. Our study also showed that glucose deprivation-induced energetic and ER stress in glioma cells and led to upregulation of pAMPK and GRP78 respectively.

In chapter four, we have shown that metformin and AICAR have no effect on glioma cell viability in the presence of glucose. However, metformin and AICAR potentiated glucose withdrawal-induced cell death in glioma cell lines in a dose-dependent manner. On the other hand, though 2DG had no effect on glioma cell viability in the presence of glucose, it suppressed glucose deprivation-induced death in glioma cell lines. Our study also showed that cell death induced by a combination of metformin and glucose deprivation was not mediated by oxidative stress, apoptosis, autophagy or necrosis. We have demonstrated that hypoxia had no effect on metformin and glucose deprivation-induced cell death. We have also shown lithium chloride and STO-609 had no effect on glucose deprivation-induced cell

death. Our study showed that on the one hand, metformin promoted glucose deprivation-induced energetic stress and up-regulated pAMPK expression, whereas, on the other hand, metformin downregulated glucose deprivation-mediated UPR and GRP78 expression leading to significant glioma cell death.

In chapter five, we have shown that combination treatment of metformin and 2DG or AICAR and 2DG induced glioma cell death *in vitro*. We have also shown that cell death induced by metformin and 2DG combination was not mediated via oxidative stress or apoptosis. Our study showed that on the one hand, metformin promoted 2DG induced energetic stress and up-regulated pAMPK expression, whereas, on the other hand, metformin downregulated 2DG induced UPR and autophagy leading to significant glioma cell death. Finally, we have also shown that metformin potentiated TMZ induced glioma cell death *in vitro.*

Our experiments on glucose deprivation-induced glioma cell death have provided interesting results and paved the way for further studies. Future work should look at cell cycle and ATP assay in response to glucose deprivation, metformin treatment in the absence of glucose and metformin and 2DG treated cells. It would also be interesting to look at mechanism leading to more cell death in U251MG in response to glucose deprivation in hypoxia when the other two cell lines showed less cell death in response to glucose deprivation in hypoxia. Future work can involve outlining pathways which promote glioma spheroid migration in hypoxia. It would be interesting to look at pathways which connect fatty acid, glucose and glutamine metabolism in glioma cells. Quantifying oxidative stress and pH change in the media will also provide us with valuable information. Similarly, further study of the PPP in glioma cells will add to our understanding of tumour metabolism. Oncotic cell death is a specific form of cell death which was initially described in cardiomyocytes following profound myocardial ischaemia. ER stress in combination with cellular swelling followed by cell detachment and death are the hallmarks of oncotic cell death. Future work can involve study of oncotic cell death markers in glucose deprivation.

TMZ is part of standard treatment in patients with glioblastoma, and we have shown that metformin potentiates TMZ induced glioma cell death *in vitro.* It is our sincere hope that this study may pave the way for future preclinical work to see if combination treatment of metformin and 2DG or metformin and TMZ can suppress glioma cell growth *in vivo.*

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