INVESTIGATING THE ANTI-CANCER ACTIVITY OF NOVEL PHENOTHIAZINES IN GLIOBLASTOMA

by

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DECLARATION

I declare that this work "***Investigating the anti-cancer activity of novel phenothiazines in glioblastoma***" is my original work and that all the sources that I have used or cited have been indicated and acknowledged by means of complete references, and that this document has not been submitted for degree purposes at any other academic institution.

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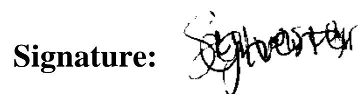
Signature:  **Date: 12th June, 2018**

DEDICATION

This Thesis is dedicated to God Almighty for bringing me this far, to my parents Mr. & Mrs. Dick Omoruyi for their prayers and to my adorable wife, Aisosa for her love and inestimable support towards bringing this phase to a conclusion.

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As we express our gratitude, we must never forget that the highest appreciation is not to utter words, but to live by them- John F. Kennedy

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ABBREVIATIONS

ABC	ATP binding cassette
ABCB1	ATP-binding cassette transporter protein
ACNU	1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl) 3-nitrosourea hydrochloride
AIC	5-aminoimidazole-4-carboxamide
AIF	Apoptosis inducing factor
AJs	Adherens junctions
Akt	Protein kinase B
AMPK	Adenosine monophosphate kinase
Apaf-1	Apoptotic protease-activating factor-1
As_2O_3	Arsenic trioxide
Atg	Autophagy-related genes
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM- and Rad3-Related
AVO	Acidic vesicular organelles
BAD	Bcl-2-associated death promoter
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated X protein
BBB	Blood-brain barrier
BCA	Bicinchoninic acid assay
Bcl2	B-cell lymphoma protein 2
BCL-A1	BCL2-related protein A1
BCL-B	Bcl-B protein
BCL-W	BCL-W protein
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BCSC	Brain cancer stem cells
BH3	Bcl-2 homology 3
BID	BH3 interacting-domain death agonist
BIK	Bcl-2-interacting killer
BIM	Bcl-2 homology 3-only protein
BMF	Bcl-2-modifying factor
Bmi1	B-lymphoma Mo-MLV insertion region 1
BMP	Bone morphogenetic protein
BOK	Bcl-2 related ovarian killer)
Ca^{2+}	Calcium ion
CaM	Calmodulin
CANSA	Cancer Association of South Africa
CARDs	Caspase recruitment domains
Caspases	Cysteine-dependent aspartate-directed proteases
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CDC2	Cell division cycle2

CDK1	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase Inhibitor 2A
CHI3L1	Chitinase-3-like protein 1 gene
Chk2	Checkpoint kinase 2
c-Myc	Cancer myelocytomatosis
CNS	Central Nervous System
CO_2	Carbon dioxide
OS	Overall survival
CSC	Cancer stem cells
CSF	Cerebrospinal fluid
CT	Computated tomography
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DBD	DNA-binding domain
DISC	Death signalling complex
DMEM	Dulbeco modified eagle's medium
DMSO	Dimethyl sulfoxide
DN	Dominant-negative
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DRD2	Dopamine receptors D2
DSBs	Double-strand breaks
E2F	E2 factor
ECL	Electro chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	Epidermal growth factor receptor variant 3
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAAD	Fas-Associated Death Domain
FACS	Fluorescence-activated cell sorting
FASL	Fas ligand
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate (FITC)
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GABRA1	Gamma-aminobutyric acid receptor subunit alpha-1 gene
GBM	Gioblastoma multiforme
GFAP	Glial fibrillary acidic protein
HCl	Hydrochloric acid
HER	Human epidermal growth factor receptor
H-Ras	Harvey rat sarcoma viral oncogene homolog

Hsp27	Heat shock protein 27
IC_{50}	Concentration required to kill 50% of the cells
IDH	Isocitrate dehydrogenase
IGF1	Insulin-like growth factor-1
IgG	Immunoglobulin G
JNK	c-Jun NH_2-terminal kinase
KLF4	Kruppel-like factor 4
L1CAM	L1 cell adhesion molecule
LC3	Light chain 3
LIF	Leukemia inhibitory factor
LKB1	Liver kinase B1
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MCL1	Myeloid leukemia cell differentiation protein
MDM2	Murine double minute 2
MDM4	Murine double minute 4
MDR	Multiple drug resistance
MeCCNU	Methyl-chlorethyl-cyclohexyl-nitroso-urea
MGMT	O6-methylguanine methyltransferase
MITC	Methyl-triazeno-imidazole-carboxamide
MMAC1	Mutated in multiple advanced cancer1
MOMP	Mitochondria outer membrane permeability
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRP1	Multidrug resistance-associated protein 1
mTOR	Mammalian target of Rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide
Mutp53	Mutant p53
NEFL	Neurofilament light polypeptide gene
NF1	Neurofibromin 1
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSC	Neural stem cells
AML	Acute myeloid leukaemia
Olig2	Oligodendrocyte transcription factor
OSCC	Oral squamous cell carcinoma to promote cell death
p14ARF	Alternate reading frame
p38MAPK	p38 Mitogen-activated protein kinase
p62/SQSTM 1	p62/sequestosome1
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBS/T	Phosphate buffered saline with tween
PCD	Programmed cell death
pCDC2	Phosphorylated cell division control 2

pChk2	phosphorylated Checkpoint kinase 2
PDGF	Platelet derived growth factor receptor
PDGFR	Platelet derived growth factor receptor
PDGFRA	Platelet-derived growth factor receptor alpha gene
PDX	Patient-derived xenografts
pERK1/2	Phosphorylated extracellular signal-regulated kinase
Pgp	P-glycoprotein
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIKK	Phosphatidylinositol-3-kinase-like kinase family
PIP2	Phosphatidylinositol-4, 5-bisphosphate
PIP3	Phosphatidy-linositol-3, 4,5-triphosphate
pJNK	Phosphorylated c-Jun NH2-terminal kinase
PKC	Protein Kinase C
PTEN	Phosphatase and tensin homology
PTZs	Phenothiazines
PUMA	p53 upregulated modulator of apoptosis
Rb	Retinoblastoma gene
ROS	Reactive oxygen species
SALL4	Sal-like protein 4
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
SEM	Standard Error of Means
SLC12A5	Potassium-chloride transporter member 5 gene
SMAC	Second mitochondria-derived activator of caspases
SOX 2	Sex-determining region Y-box 2
STAT3	Signal transducer and activator of transcription 3
SYT1	Synaptotagmin-1 gene
TBS/T	Tris buffered saline with tween
TGF-β	Transforming growth factor beta
TJs	Tight junctions
TMZ	Temozolomide
TNF	Tumour necrosis factor
TNF-α,	Tumour necrosis factor alpha
TP I	Topoisomerase inhibitors 1
Tp53	Tumour Suppressor p53
TRAIL	TNF-related apoptosis-inducing ligand
TSC1/2	Tuberous sclerosis proteins 1 and 2
ULK1	Unc-51 like autophagy activating kinase
VEGF	Vascular endothelial growth factor
Vps34	Vacuolar sorting protein 34
WHO	World Health Organization
wtp53	wild-type p53
γH2AX	Phosphorylated histone 2Ax

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RESEARCH OUTPUT DURING THE PERIOD

The compounds DS00326 and DS00329 have been filed for patenting hence a delay in the publication of written manuscripts from this thesis. However, during the period of study, I contributed in the following published articles and made conference presentation:

1. Denya I, Malan SF, Enogieru AB, **Omoruyi SI**, Ekpo OE, Kapp E, Zindo FT, Joubert J (2018). Design, synthesis and evaluation of indole derivatives as multifunctional agents against Alzheimer's disease. *MedChemComm.*, **9**: 357-370
2. Kapp E, Visser H, Sampson SL, Malan SF, Streicher EM, Foka GB, Warner DF, **Omoruyi SI**, Enogieru AB, Ekpo OE, Zindo FT (2017). Versatility of 7-substituted coumarin molecules as antimycobacterial agents, neuronal enzyme inhibitors and neuroprotective agents. *Molecules*, **22**(10):1644.
3. **Omoruyi SI**, Jardine A, Ekpo O, Prince S (2017). The *in vitro* anticancer activity of novel phenothiazines in brain cancer cells. European Association for Cancer Research (EACR), American Association for Cancer Research (AACR) and Italian Cancer Society (SIC) Special Conference on the Challenges of Optimising Immuno and Targeted Therapies: From Cancer Biology to the Clinic. 24-27th June 2017, Florence, Italy, abstract 277.
4. Akinrinmade OA, **Omoruyi SI**, Dietrich D, Ekpo OE (2016). Long-term consumption of Fermented Rooibos Herbal Tea offers neuroprotection against ischemic brain injury in rats. *Acta Neurobiologiae Experimentalis;* **77**: 94-105

ABSTRACT

Glioblastoma multiforme (GBM) remains the most malignant of all primary adult brain tumours. It is a highly invasive and vascularized neoplasm with limited treatment options and very low survival rate. GBM tumours are heterogeneous in nature with cellular hierarchy and at the apex of this hierarchy are the glioblastoma stem cells, known to promote tumourigenesis and resistance to chemotherapeutic agents and tumour recurrence. Currently, the standard care for GBM involves surgical resection, radiation, and chemotherapy treatment with temozolomide. Unfortunately, median survival after treatment is still daunting and tumour relapse is very frequent. Indeed, patients with recurrent glioblastoma have less than a year survival. To address this, novel therapies need to be developed with the early introduction of promising agents into clinical trials and subsequent approval for use. Importantly, for these novel therapies to be approved for GBM, they need to be safe, effective as well as being able to penetrate the blood-brain barrier (BBB). Due to the high cost and process time for the development of new drugs, existing approved drugs are currently being repurposed for new indications and this is gaining significance in clinical pharmacology as it allows rapid delivery of useful drugs from bench to bedside. Drugs of the antipsychotic class are well known to cross the BBB due to their neuroleptic action. To this end, the aim of this study was to identify and characterize the anti-cancer activities of novel phenothiazine-derivatives belonging to the antipsychotic class of drugs in glioblastoma. To achieve this, several novel phenothiazine-derivatives were initially screened for possible anti-cancer activity in the U87 and U251 malignant GBM cells. Two lead compounds, DS00326 and DS00329, were identified and their anti-cancer activities were determined in U87 and U251 cells as well as in primary patient-derived xenograft (PDX) glioblastoma cultures. DS00326 and DS00329 significantly inhibited glioblastoma cell viability, with minimal effects

observed in the non-cancerous FG0 fibroblasts. The IC_{50} values of DS00326 and DS00329 for U251, U87 and PDX cells ranged from 1.61 to 12.53μM. Flow cytometry analyses showed that DS00326 and DS00329 treatment led to an increase in the G1 population of cells. Additionally, DS00326 and DS00329 induced double-strand DNA breaks, which lead to activation of the canonical DNA damage response pathway. Furthermore, DS00326 and DS00329 induced apoptosis as shown by morphological markers, flow cytometry with annexin V-FITC/propidium iodide staining, as well as western blotting with an antibody to detect levels of cleaved PARP. Interestingly, treatment with DS00326 and DS00329 also induced autophagy as evident by the increase of acidic vesicular organelles in cells following staining with acridine orange as well as an increase in levels of the autophagy marker LC3-II. Autophagy was seen as a pro-death pathway in the U87 and U251 cells as inhibition of autophagy led to a reversal of cytotoxicity and consequently increased cell survival. Moreover, it was demonstrated that DS00326 and DS00329 inhibited the PI3/Akt pathway while modulating the mitogen-activated protein kinases p38, ERK1/2 and JNK signalling pathways. Importantly DS00326 and DS00329 displayed anti-cancer stem cell activities by the inhibition of neurosphere formation and regulation of stem cell markers SOX2 and GFAP in PDX cells. Together, the findings from this study suggest that DS00326 and DS00329 may be effective in the treatment of glioblastoma and provide a strong rationale for further clinical studies exploiting phenothiazines and their derivatives as treatments for glioblastoma.

Keywords: Drug repurposing, Phenothiazines, Chemotherapy, Anti-cancer agents, Brain tumours, Glioblastoma, DNA damage, Apoptosis and Autophagy

CHAPTER ONE

INTRODUCTION

1.1 Background

Brain tumours are a mixed group of primary and metastatic neoplasms that show varying degrees of malignancy. Though malignant lesions were thought to be relatively uncommon, their occurrence has increased lately in industrialized and developed nations (Ostrom et al., 2015). Primary malignant brain tumours have drawn wide attention not only because of their poor prognosis, but also for their direct impact on neurologic function, psychological health, and quality of life (Lin et al., 2017). Brain tumours account for 85% to 90% of all primary central nervous system (CNS) tumours (Mehta et al., 2011). Globally, the overall incidence of malignant brain tumours was reported to be about 5.57/100000 individuals with adults mostly affected (Leece et al., 2017). In the United States, it is estimated that 23, 000 new cases and 16,700 new deaths from brain tumours and other nervous system tumours will be reported in 2017 (American Cancer Society, 2017). In South Africa, brain cancer is ranked the eighteenth most common cancer in men and one of the most challenging cancers to treat (Bradshaw et al., 2003). It has been reported that the lifetime risk of developing a central nervous system tumour was 1 in 849 for men, and 1 in 1,611 for women (Norman et al., 2006). According to the South Africa National Cancer Registry of 2013 (the last available), brain cancers were combined with central nervous system cancers and it was estimated that 206 cases were diagnosed for men and 140 for women (Cancer Association of South Africa, CANSA 2017).

Furthermore, of all brain tumours, gliomas, which emanate from the glial cells of the brain contribute a significant proportion. The most frequent and aggressive glioma is the

glioblastoma multiformes (GBM) (World Health Organization [WHO] grade IV) which are highly vascularized and invasive neoplasms with limited treatment options (Ohgaki, 2009, Mellinghoff and Gilbertson, 2017). The standard treatment consisting of surgery plus radiotherapy, followed by chemotherapy (Elliott et al., 1996, Giese et al., 2003, Smith and Jenkins, 2000). However, due to the diffuse invasiveness of malignant GBMs, they are nearly impossible to resect completely. Despite treatment, the median survival of GBM patients is about 12 to 36 months, a prognosis that warrants intensive investigation of new therapeutic options capable of improving the burdens posed by these tumours (Krex et al., 2007, Mellinghoff and Gilbertson, 2017).

An approach to this involves repurposing of already approved and existing drugs for new indications which is known as "drug repurposing". This is predicated on the fact that the safety, pharmacological properties, dosage and potential toxicity of approved drugs and many abandoned compounds have already been tested in humans (Ashburn and Thor, 2004). Thus, getting such drugs to the market will be much easier as some of the expensive drug discovery processes will be circumvented. The importance of drug repositioning is also underpinned by the fact that similar molecular pathways may contribute to different diseases (Oprea et al., 2011). Notable amongst the family of drugs with potential repurposing properties are the antipsychotic drugs of the phenothiazine family.

Phenothiazines (PTZs) are an important class of three-ring heterocyclic compounds widely used in medicinal chemistry as they exhibit a wide range of biological effects. Together with their main neuroleptic action, they have been reported to possess other biological activities including; antihistaminic, antitussive, antiemetic, antibacterial, and antioxidant properties (Gupta, 1988, Morak-Młodawska et al., 2010). PTZs have also

been reported to play a role in ameliorating cancer developmental processes. PTZs and their related compounds have long been reported to possess anti-cancer activities in a variety of cancer cell lines (Nagy et al., 1996, Wuonola et al., 1998, Gil-Ad et al., 2004, Zhelev et al., 2004, Pluta et al., 2010). A previous study showed that PTZs induced protein phosphatase 2A (PP2A)-mediated apoptosis in T-cell acute lymphoblastic leukaemia (Gutierrez et al., 2014b). Recently, newly synthesized PTZ derivatives have also been shown to possess anti-cancer activity either via induction of apoptotic and/or autophagic cell death in various cancer cells (Wu et al., 2016, Brem et al., 2017, Ghorab et al., 2017, Ma et al., 2017).

The anti-cancer potential of PTZs appear to be mediated via anti-calmodulin (CaM) activity, inhibition of Protein Kinase C (PKC) and P-glycoprotein transport function and multi-drug resistance (Jaszczyszyn, 2006). The mechanisms of these activities have been linked to the chemical structure of compounds from the phenothiazine family (Nowak et al., 2007). Structural modifications that increase the lipophilicity of PTZs also increase their effect on physiological processes. Additionally, it has been found that both types of substituents in position 2 of the PTZ ring and the length of the alkyl bridge connecting the ring system with the amino group play some role in the reverting activity (Michalak et al., 2006). Thus, the chemical structure of PTZ provides a valuable molecular template for the development of agents able to interact with a wide variety of biological processes.

1.2 Problem statement

Cancer is a multifaceted disease comprised of complex genetic and epigenetic aberrations that disrupt the normal balance of cellular life and death. Apoptosis (programmed cell death), which represents one of the most important forms of cell death in multicellular organisms, is typically dysregulated in human cancers. On the molecular level, deregulation of several pathways can trigger apoptosis. One of such pathways is the

downregulation or inactivation of p53, a tumour suppressor gene, which results in inhibition of apoptosis and in turn, enhanced tumour growth and development (Bauer and Helfand, 2006, Gasco et al., 2002, Morton et al., 2010). Dysfunction of the apoptotic pathway is also linked to cancer-treatment resistance, as most conventional chemotherapies, as well as radiotherapy, rely on their ability to induce apoptotic cell death in cancer cells. Hence, investigation of anti-apoptotic agents is of paramount importance.

Brain tumours remain one of the most difficult cancers to treat and the risk factors are largely unknown, but early exposure to ionizing radiation and certain chemicals such as; acrylamide, gasoline and gasoline vapour have been considered to play a role (Mehlman, 1991, Fisher et al., 2007, Klaunig, 2008). Furthermore, the prognosis of glioblastoma is poor, possibly as a result of the delay in diagnosis combined with the poor efficacy of some currently available therapies (Louis et al., 2007, Wen and Kesari, 2008). Treatment of brain tumours is quite challenging as the brain is protected by a selective barrier referred to as the blood-brain barrier (BBB) which hinders the passage of some possible anti-cancer agents due to their molecular properties (Laquintana et al., 2009, Bhowmik et al., 2015, Weidle et al., 2015). This barrier allows the easy passage of water, gases (oxygen and carbon dioxide) and lipid-soluble substances like alcohol. Again, it moderately allows the passage of electrolytes but it is almost impossible for non-lipid soluble large organic molecules and plasma proteins to go through (Bates, 1985, Lawther et al., 2011). The BBB is formed by the endothelial cells of the brain which is characterized by the presence of junctional complexes, which include tight junctions and adherens junctions at the inter-endothelial spaces. These complexes restrict the permeability of substances across the endothelium (Ballabh et al., 2004, Hawkins and Davis, 2005). For instance, the anti-cancer agent paclitaxel which shows good *in vitro*

activity in glioblastoma, however, fails *in vivo* as it is poorly permeable to BBB (Fellner et al., 2002).

Furthermore, drug resistance in glioblastoma which is associated with the ABC transporters necessary for the removal of drugs and other substances from cells also poses a challenge (Gottesman et al., 2002). Tumour resistance to drugs mediated by such transporters is called multiple drug resistance (MDR) which can be acquired or intrinsic (Goldie, 2001, Stavrovskaya and Stromskaya, 2008). More so, systemic toxicity from some chemotherapeutic drugs like the platinum metal complexes and other approved drugs also poses a challenge to treatment of brain tumours (Lee, 2016, Grossman et al., 2003).

Additionally, the brain in similarity to other organs with well-marked out cellular hierarchies during organogenesis (e.g., breast, skin, and colon), presents with tumours which are heterogeneous and contains a hierarchical distribution of cells (Reya et al., 2001). Notable of such cells are the cancer stem cells otherwise referred to as the tumour-initiating cells or glioma-initiating cells in glioblastoma with self-renewing ability (Heddleston et al., 2009, Cho et al., 2013). These cancer stem cells are known to promote tumour progression as well as resistance to chemotherapeutic agents (Lathia et al., 2015). Hence, the identification of new experimental strategies involving novel anti-cancer agents or drugs capable of killing resistant cells is therefore urgently needed in cancer research.

1.3 Significance of study

Owing to the poor prognosis of brain tumours and low survival rates, there is therefore, an urgent need to develop more efficacious drugs selectively toxic to cancer cells, capable of crossing the BBB and reversing drug resistance. Drugs of the phenothiazine class of

antipsychotics have been widely known to play a role in the reversal of multidrug resistance in cancer via their ability to inhibit the efflux pump P-glycoprotein (Molnár et al., 1998, Michalak et al., 2006, Spengler et al., 2014, Mishra et al., 2017). Additionally, as neuroleptic drugs, they effectively cross BBB to elicit their activities (Crivori et al., 2000, Korth et al., 2001, Michalak et al., 2006, Zhang et al., 2008). Considering the properties attributed to PTZs and their derivatives, this present study will focus on repurposing novel phenothiazine-derivatives as potential anti-cancer agents in glioblastoma. More so, it is hopeful that these investigations of new therapeutic options will lead to the introduction of promising anti-cancer agents into clinical trials.

1.4 Aims and objectives of study

1.4.1 Aims

1. The first aim of this study is to identify and characterise the anti-cancer activity of remodelled novel PTZ compound(s) in glioblastoma cancer cell lines (U87 and U251).
2. To evaluate the anti-cancer activities of the identified PTZ-derivatives in primary patient-derived xenograft (PDX) glioblastoma cultures (T10 and T91).

1.4.2 Objectives

To achieve the aims above, the following specific objectives were carried out:

1. To evaluate the cytotoxic effects of PTZ compounds in the glioblastoma cell lines and only promising compounds with high toxic effects on glioblastoma cells but less effects on non-cancerous human fibroblasts were further investigated.
2. To explore the long-term effects of the lead compound(s) on cancer cell survival and proliferation.

3. To determine the impact of PTZ compound(s) on cell cycle progression and their ability to induce DNA damage response.
4. To investigate if the mode of cell death induced by the lead compound(s) is via apoptosis or autophagy.
5. To determine the signalling pathway(s) mediating cell death induced by PTZ compound(s).
6. To determine the anti-cancer stem cell activity of the lead compound(s) in PDX glioblastoma cultures.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cancer

Cancer comprises a group of over hundred diseases which occur as a result of the abnormal and rapid proliferation of cells and as such should not be confused with a single disease. In living organisms, normal cells are constantly under the influence of molecular signals which dictate whether they are to undergo division, replication or death (Hejmadi, 2009). However, in cancer conditions, cancer cells are quite independent and autonomous of these signals, thus resulting in their uncontrolled growth and might finally invade other nearby normal tissues or go into circulation to invade distant tissues, a process termed metastasis. Cancer cells show a lot of differences from normal cells, usually with respect to growth, cell-to-cell interactions, gene expression, morphology and membrane properties (Lodish et al., 2000).

2.1.1 Hallmarks of cancer

In the course of understanding cancer in general and not individual cancers, it is imperative to identify traits that are similar or common amongst all cancer types and this leads to the term “Hallmarks of Cancer” (**Figure 2.1**). Hanahan and Weinberg in 2000 came up with this concept and they described it to constitute of six biological capabilities acquired during the complex progression of normal cells to tumour cells in humans. These hallmarks constitute the bedrock of understanding cancer characteristics which could in turn inform development of possible chemotherapeutic agents (Hanahan and Weinberg, 2000, Fouad and Aanei, 2017). The six hallmarks are summarized below;

1. Acquisition of self-sufficiency in growth signals, leading to unchecked growth.
2. Uncontrolled growth and proliferation as a result of loss of sensitivity to anti-growth signals

3. Evading apoptosis, so as to promote cell prolifertion even in presence of genetic errors and external anti-growth signals.
4. Inability to drive cells to a state of senescence, hence encouraging endless replication (immortality).
5. Presence of sustained angiogenesis, allowing the tumour to grow beyond the limitations of passive nutrient diffusion.
6. Acquisition of ability to invade neighbouring tissues, the defining property of invasive carcinoma.

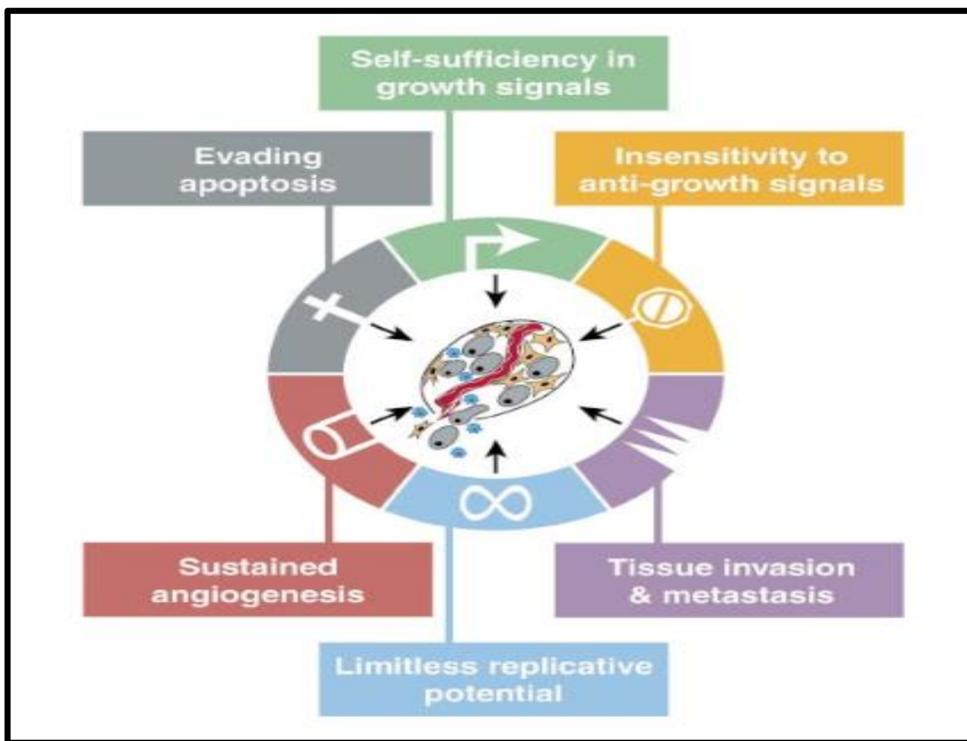


Figure 2.1. Acquired capabilities of cancer (Hanahan and Weinberg, 2000)

Furthermore, in 2011, Hanahan and Weinberg revisited the six original themes under the concept of the hallmarks of cancer since the previous six traits described in 2000 could no longer accommodate the complex dynamics taking place in tumour cells. In their revised concept, the interaction of tumour cells with the "tumour microenvironment" was given consideration in the progression of normal cells to malignant cells as well as the

multi-steps involved in tumourigenesis will hardly happen without the presence of two enabling factors (**Figure 2.2**) identified to include:

1. Loss of genetic stability and ability to repair genetic errors, leading to an increased mutation rate thus amplifying all the other capabilities.
2. Inflammation caused by cells of the immune system and most times promote tumourigenesis (Hanahan and Weinberg, 2011).

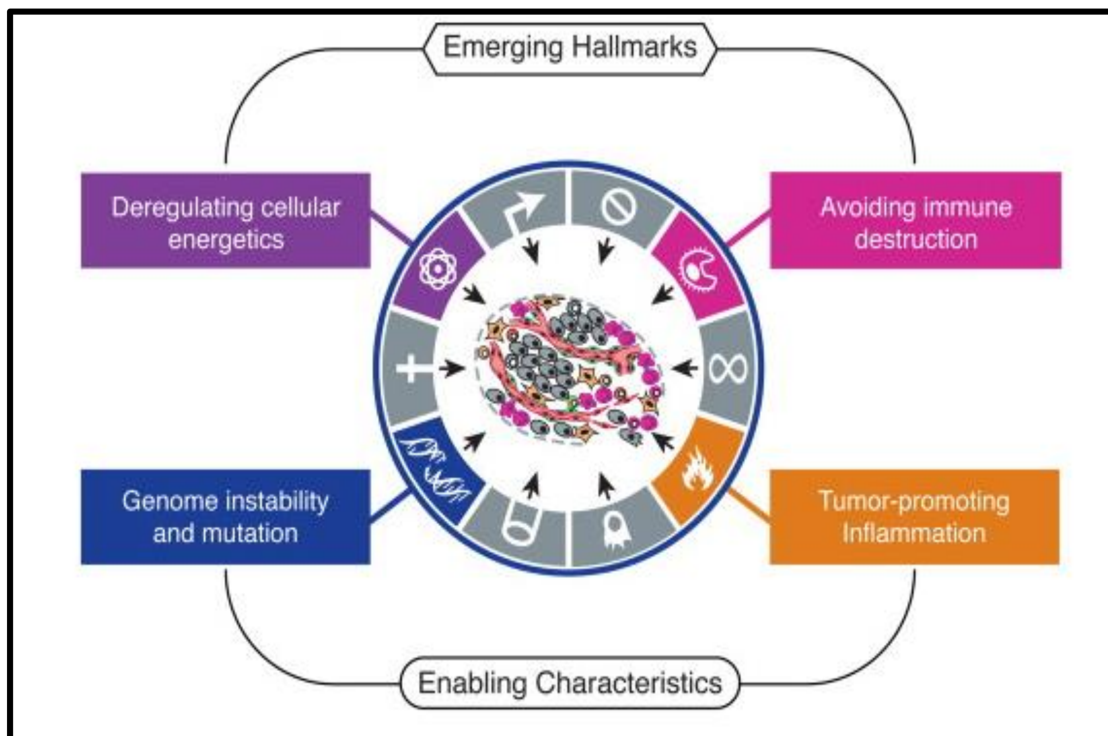


Figure 2.2. Emerging hallmarks and enabling characteristics (Hanahan and Weinberg, 2011)

It is expected that with more research into different aspects of cancer pathogenesis and progression, new components will be added to the existing hallmarks of cancer (Colotta et al., 2009, Luo et al., 2009, Negrini et al., 2010, Fouad and Aanei, 2017). Some of these include; reordering cellular energy and metabolism towards promoting continuous cell proliferation and preventing cancer cells from being destroyed by cells of the immune system (**Figure 2.2**).

2.1.2 Biology of cancer and carcinogenesis

Carcinogenesis is the process by which cancer is formed or the transformation of normal cells to cancer cells, usually through the mutation of certain genes that play key roles in the regulation of cell growth and differentiation (Croce, 2008). This process leads to genetic mutations induced by physical or chemical agents and can basically be divided into three different stages; initiation, promotion, and progression (**Figure 2.3**) (Hennings et al., 1993). Initiation involves an irreversible mutation in a single gene; promotion involves an increase in cell proliferation of initiated cells and thus leads to an increase in population of such cells. While the final stage which is progression involves the accumulation of more genetic mutations that lead to the acquisition of the malignant or invasive phenotype (Pitot, 1993). In other words, cancer usually occurs when a normal cell accumulates genetic and/or epigenetic changes caused by the activation or amplification of oncogenes and/or the mutation or loss of tumour suppressor function, thus giving cells the ability to proliferate indefinitely (Hanahan and Weinberg, 2000). Some factors including specific alterations leading to the transformation of the normal cell, the cell of origin and tumour micro-environment are considered critical when considering tumour cell establishment, characterization, progression and therapeutic resistance (Pecorino, 2012). Tumours frequently consist of cytogenetically different clones which came from the original transformed cells as a result of gene alterations and this heterogenic nature of tumours plays a role in their differences in clinical behaviour as well as responses to the treatment of tumours of the same diagnostic type (Croce, 2008).

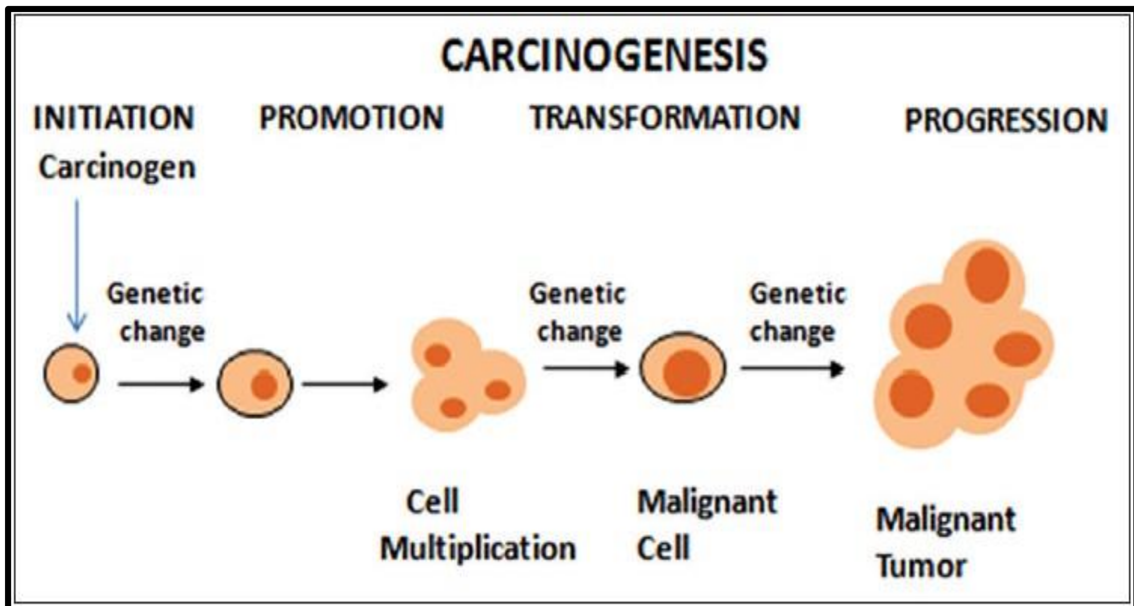


Figure 2.3. Different stages of carcinogenesis (Kaur et al., 2014)

2.1.3 Proto-oncogenes

Proto-oncogenes represent a group of genes that stimulate normal cells to progress into cancerous cells when mutation(s) occur (Weinstein and Joe, 2006). Mutations in proto-oncogenes are typically dominant in nature, and the mutated version of a proto-oncogene could give rise to an oncogene. Many proto-oncogenes play an important role during embryogenesis, because they are often involved in stimulating cell growth and proliferation as well as tissue repair and homeostasis during development (Chial, 2008a). Considering their role in normal development, their removal from the body can equally not inhibit cancer as they only trigger excessive growth signal when mutation occurs. It has been documented that conversion of proto-oncogenes to oncogenes involves no less than 3 mechanisms (Pierotti et al., 2003). These mechanisms include;

- Through point mutations in a proto-oncogene which permanently leads to the activation of proteins that normally would go from active to inactive states and vice-versa. An example of this form of mutation is the Ras protein family that promotes cell proliferation. Their function could be turned on

and off depending on the form of nucleotide (di-phosphate or tri-phosphate) to which they are bound. However, when mutation occurs, the Ras proto-oncogene switches to an oncogene as it becomes permanently active and regardless of the signals the cell receive, it continues to promote excessive proliferation (Chow, 2010).

- Through chromosomal translocation which occurs as result of the irregular reattachment of a broken chromosome thus leading to either a formation of a fusion protein or alteration in protein expression (Lodish et al., 2000).
- Through gene amplification which is as a result of the expansion number of copies of a gene within the genome of a cell. In cancer, the existence of multiple copies of a proto-oncogene may exist in the cell and this will result in overexpression the encoded protein (Pierotti et al., 2003, Chow, 2010).

2.1.4 Oncogenes

Oncogenes are converted products of normal genes called proto-oncogenes that control cell growth and replication (Bishop, 1983, Croce, 2008). These cellular genes, change their structure, control and regulate cell division and function under the action of mutagenic factors or viruses. However, upon alteration to become oncogens, there may be an increased activity of the abnormal protein products thus contributing to tumour growth (Croce, 2008). As a result of oncogenes, cells acquire the ability to divide rapidly and in some cases, cells already programmed for death (apoptosis) can be rescued and for this reason, oncogenes are becoming targets for drugs (Chow, 2010). One of the earliest oncogenes to be identified in cancer studies is the Ras oncogene and it was reported that mutations in proto-oncogenes of the Ras family (H-Ras, N-Ras and K-Ras) are often found in over 30% of all tumours in humans and could be more in specific tumours like melanomas (Bos, 1989, Hobbs et al., 2016).

2.1.5 Tumour suppressor genes

Tumoour suppressor genes function most times by preventing cells from undergoing excessive and improper cell growth and division (Cooper, 2000). They also act by promoting cells to senescence cells or undergo apoptosis in order to maintain balance and may be involved in DNA repair processes (Chial, 2008b). A loss in their function can result to excessive and uncontrollable growth of cells which underpins cancer and they differ from oncogenes which depend on the activation of proto-oncogenes (Lam and Schmidt, 2012). It has been reported that loss in function of tumour suppressor genes may even be more crucial than proto-oncogene to oncogene activation in the progression and development of several human cancers (Weinberg, 2013). The first tumour suppressor gene to be identified was the retinoblastoma (Rb) gene whose loss of function leads to retinoblastoma (Du and Pogoriler, 2006). Another tumour suppressor gene that has been found mutated in many human cancer is the p53 tumour suppressor gene (Lodish et al., 2000, Surget et al., 2014).

2.1.5.1 The p53 tumour suppressor

The p53 tumour suppressor is a transcription factor encoded by the human gene TP53 and its mutation or methylation has been implicated in many cancers as it plays a role in carcinogenesis (Levine and Oren, 2009). The TP53 gene has been reported to be the most commonly mutated gene with its mutations reported in about 50% of all cancers (Vogelstein et al., 2000, Kandoth et al., 2013). This mutation can be at varying degrees ranging from 10% in haematopoietic cancers to nearly 100% in high grade ovarian cancers (Hainaut and Hollstein, 1999, Peller and Rotter, 2003, Ahmed et al., 2010). The p53 mutation has also been shown to be one of the drivers of gliomagenesis alongside Isocitrate dehydrogenase 1 (IDH1) mutation (Liu et al., 2012, Wang et al., 2015, Rahman et al., 2018).

The p53 protein plays a major role mainly in the regulation of many signalling pathways in carcinogenesis and acts as a sensor of DNA damage and other cellular/metabolic stress including changes in reactive oxygen species (ROS) levels and hypoxia (Hong et al., 2014, Vogelstein et al., 2000). These stressors activate p53 protein in post-translational manner which could be either phosphorylation, acetylation or methylation to rapidly increase the protein levels while stabilizing it (Brooks and Gu, 2006). Following activation, p53 binds to its target genes (p21, Bcl-2-associated X protein (BAX) and p53 upregulated modulator of apoptosis (PUMA)) to selectively regulate the transcriptional expression of these genes and the products of these genes usually play a role in various cellular processes ranging from cell cycle arrest, apoptosis, DNA repair, antioxidant function as well as senescence (Feng and Levine, 2010, Hong et al., 2014). This interaction with p53 and its target genes contributes to its tumour suppressive role.

Furthermore, the p53 status of any cancer type could determine the malignancy of the cancer and a loss in its function is usually a requirement for tumour initiation and progression (Muller and Vousden, 2014). This role of p53 has been demonstrated in Li–Fraumeni syndrome patients with germline mutations of p53 as these patients were more susceptible to cancers (breast cancers, osteosarcoma soft tissue sarcomas, leukemias, CNS tumors amongst others) (Malkin et al., 1990, Bougeard et al., 2008, Guidi and Giunti, 2017). Again, experimental animals with p53 knockdown were reported to develop tumours (Jacks et al., 1994). Furthermore, overexpression and/or amplification of different p53 negative regulators, such as; murine double minute 2 (MDM2), murine double minute 4 (MDM4) and leukemia inhibitory factor (LIF) was shown to attenuate p53 functions thus promoting tumour progression (Wade et al., 2013, Yu et al., 2014).

While the importance of the wild-type p53 (wtp53) has been elucidated, inactivation of p53 could occur mainly through point mutations and sometimes through small deletions/insertions in the gene (van Oijen and Slootweg, 2000). However, mutation in p53 which ideally should be a loss in function of the wtp53 does not necessarily turn out to be so as in some cases of single base-pair amino acid substitution in the full length of the protein could stabilize the protein leading to its accumulation in tumour cells (Oren and Rotter, 2010, Muller and Vousden, 2014). This phenomenon is known as a gain in tumour-promoting function through a dominant-negative (DN-which then regulates the remaining wtp53) usually at the DNA-binding domain (DBD), which codes for the region required for wtp53 to bind to its target genes (Freed-Pastor and Prives, 2012, Oren and Rotter, 2010). Due to this loss in function, transforming cells with mutant TP53 gene can give rise to genomic instability, make cells evade apoptosis and aid progression into malignancies (**Figure 2.4**) (Merkel et al., 2017). Owing to the major importance of p53, it has been an interest of several researchers and could serve as a target for chemotherapy and targeted therapy (Duffy et al., 2017).

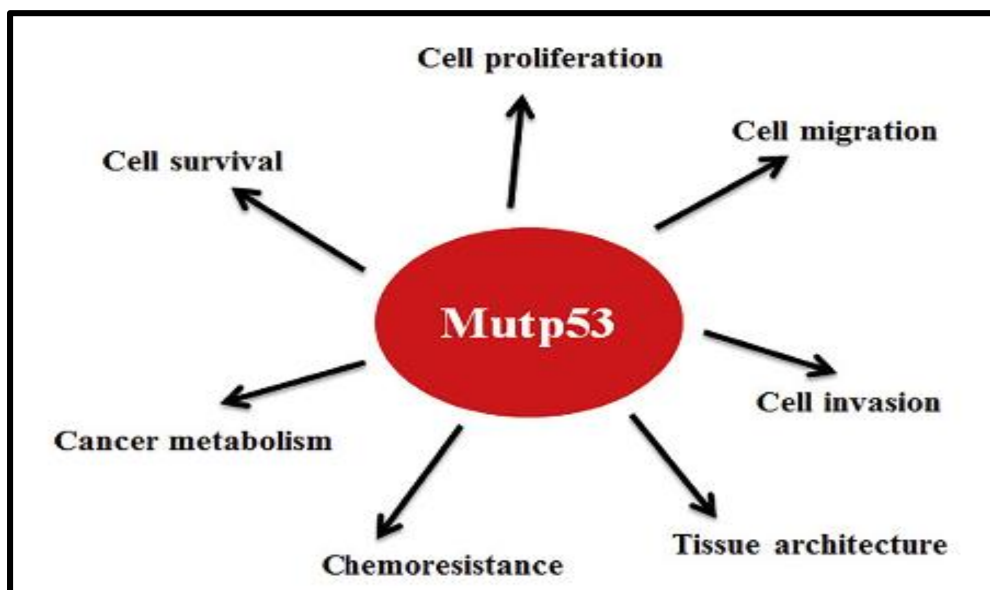


Figure 2.4. Effects of p53 mutation (Yue et al., 2017)

The remaining section of this chapter will focus on central nervous system (CNS) and CNS cancers with particular attention to brain tumours (glioblastoma multiforme). This section also looks at chemotherapy for brain tumours as well as the concept of drug repurposing for cancer treatment with focus on phenothiazines and their mechanisms of action in inducing cell death.

2.3 Central nervous system

The central nervous system (CNS) is the part of the nervous system which comprises of the brain and spinal cord responsible for the integration of information and coordination of bodily activities (Dalley et al., 2010). The brain is enclosed in the skull and protected by the cranium, while the spinal cord continues caudally from the brain and exists through the foramen magnum while being protected by the vertebrae (Nieuwenhuys et al., 2007). The brain and spinal cord are covered with a three-layered membrane or connective tissue called the meninges which serve as a support system.

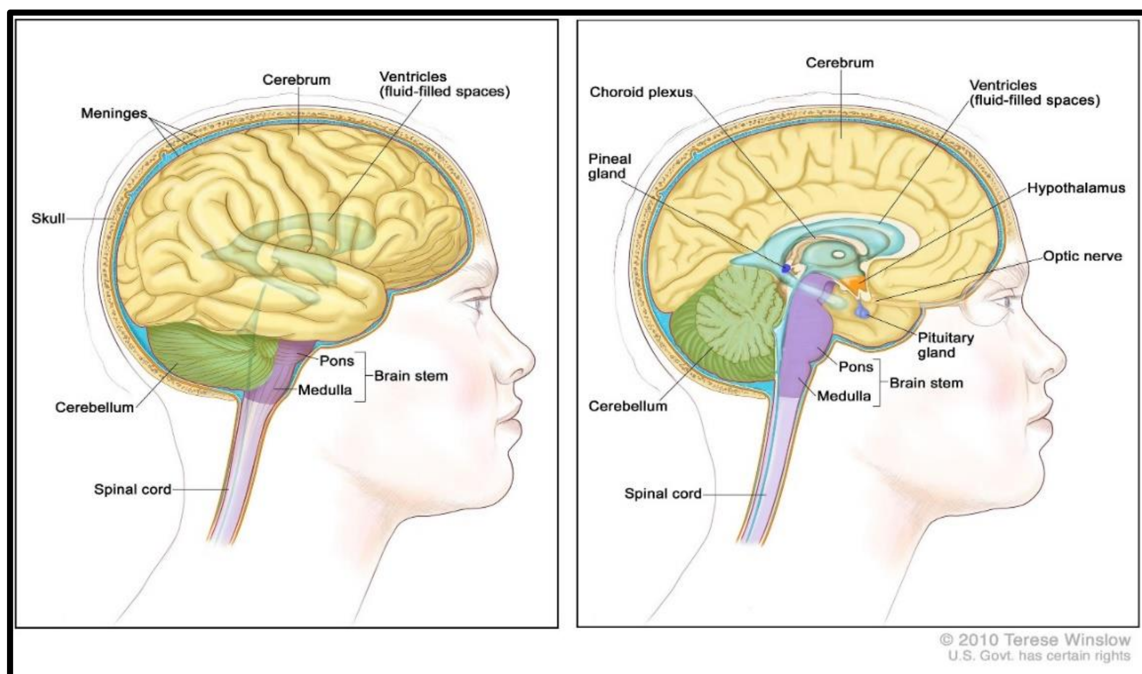


Figure 2.5. Anatomy of the brain ©https://www.cancer.gov/

Within the central nervous system lies hollow cavities which link the cavities of the brain (cerebral ventricles) and continue with the central canal of the spinal cord (**Figure 2.5**). The ventricles are lined with specialized epithelial cells called the choroid plexus which are responsible for the production of cerebrospinal fluid which helps to cushion and protect the brain and spinal cord from injury. The central nervous system is also made up of grey and white matter which can be differentiated by colour and location. The white matter consists of axons and oligodendrocytes, while the grey matter consists of neurons and unmyelinated fibres (Fields, 2008, Ransom et al., 2011).

Furthermore, present in the CNS are neurons and glial cells also known as the supporting cells of the CNS. While neurons are responsible for neurotransmission, glial cells majorly offer support to the neurons. Though the primary functions of glial cells are still not completely elucidated, there is evidence which suggests that these cells are responsible for coordinating the neuronal environment as well as playing roles in the repair processes within the CNS. Most importantly during development, glial cells provide scaffolds for outgrowing axons and migrating neurons (Agulhon et al., 2008, Brodal, 2004). Glial cells have also been reported to produce short electric currents capable of producing calcium signals, which could influence coordination of synaptic activity (Volterra and Meldolesi, 2005, Gourine et al., 2010). Further, glial cell activation and inflammation have been reported to play a role in neurodegenerative diseases such as Parkinson’s disease, multiple sclerosis and Alzheimer’s disease (Lemke, 2001).

Glial cells are divided into three main categories with respect to their structure and function and these include astrocytes, oligodendrocytes and microglia cells. Astrocytes are star-shaped glial cells with many processes and play a role in the formation of BBB and maintenance of extracellular ion balance, while oligodendrocytes have fewer and

shorter processes, and majorly produce myelin sheath for axon insulation. The microglia on the other hand are the smallest of the glial cells and are macrophagic in function (Brodal, 2004). In addition to the three main types of glial cells are other specialized cells which include the Schwann cells that are found only in the peripheral nervous system and form myelin sheaths that influence axonal thickness. Also present are ependyma cells which are epithelial cylindrical cells that line the ventricular surfaces and function mainly for the production, transport and absorption of cerebrospinal fluid (CSF). Other support cells include the Muller cells in the vertebrate retina and the Bergman cells residing in the cerebellum as well as pituicytes that resides in the posterior of the pituitary gland, where they participate in the control of secretory events (Fischer and Bongini, 2010, Rosso and Mienville, 2009).

2.3.1 CNS cancers

CNS tumours are formed by abnormal growth of cells within the CNS and account for one of the most difficult malignancies to treat (Mellinghoff and Gilbertson, 2017). Malignant brain, CNS or intracranial tumours account for about 1.8% of cancer cases diagnosed in adults worldwide in 2012 amounting to 256,213 cases (Ferlay et al., 2012). CNS tumours can either be located within the cranium (intracranial) or outside the cranium (extracranial). Since over 80% of CNS tumours are usually brain tumours, the following section will focus on brain tumours.

2.3.2 Brain tumours

Brain tumours are defined as the growth of abnormal cells in the tissues of the brain and can either be benign (noncancerous) or malignant (cancerous). In contrast to normal cells, cancerous cells result from uncontrolled growth of cells which can stimulate cells to grow into adjacent tissue. Although benign tumours are not cancerous, they could sometimes be large, put weight on adjacent healthy structures, thus potentially affecting the function

of these normal tissues. Brain tumours could either be primary or secondary and gliomas (tumours of the glial cells) account for the most common brain tumours (Behin et al., 2003). Other types of brain tumours are shown in (**Figure 2.6**).

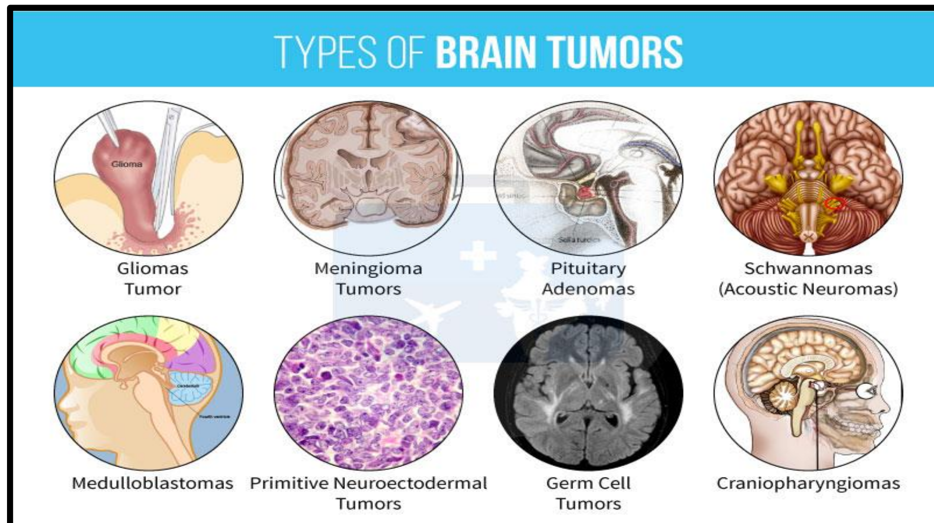


Figure 2.6. Types of brain tumours ©https://www.quora.com/

2.3.2.1 Risk factors of brain tumours

The causes of brain and other CNS tumours are not well understood (Peter et al., 1995) but increasing age has been documented to be a known strong risk factor though some tumour types have also been diagnosed in younger individuals (McKinney, 2004). Sex also contribute to the tumour types as invasive brain and CNS tumours are more common in males while most non-invasive tumours are known to be common in females (Candolfi et al., 2007). Accordingly, gliomas which are usually malignant are more common in males; while meningiomas are more common in females and are usually benign (Mehta et al., 2011).

More so, early exposure to certain chemicals have been suggested to play a role in the etiology of brain tumours. One study reported that children who develop brain tumours are likely to have been exposed to higher than average amounts of pesticides and could possibly have been born with a reduced ability to detoxify these chemicals (Nielsen et

al., 2010). Parental occupational exposure to pesticide could also be a risk factor for brain tumours in both children and young adults (Van Maele-Fabry et al., 2013). In women, protracted or high maternal exposure to beauty-products such as hair dyes and glues could increase the average risk for brain tumours (Efird et al., 2005, Spinelli et al., 2010).

Exposure to electromagnetic radiation has also been documented as a risk factor for brain tumours (Levitt and Lai, 2010, Kesari et al., 2011, Yakymenko et al., 2011). It was reported that protracted cell phone usage may be associated with the development of an ipsilateral brain tumour (Khurana et al., 2009). Mobile phone radiation was also reported to activate the hsp27/p38MAPK stress pathway in human endothelial cells which could be a molecular mechanism for cancer initiation and disruption of the BBB permeability (Leszczynski et al., 2002). It was also reported that mobile phone radiation causes brain tumours and should be classified as a probable human carcinogen (2A) (Morgan et al., 2015).

Genetic predisposition has been noticed in about 5-10 % of glioma cases and some genetic alterations associated with increased risk of gliomas inculde retinoblastoma (RB), Li-Fraumeni syndrome, neurofibromatosis 1 and 2 amongst others (Fisher et al., 2007, Bondy et al., 2008, Ohgaki, 2009).

2.3.2.2 Symptoms

Brain tumour symptoms are usually observed in the late stage of disease and one of the earliest symptoms are those signs emanating from increased intracranial pressure such as; nausea and vomiting, headache, and blurred vision. Other symptoms may include seizures, weakness or numbness of a side or part of the body, mood changes, thinking or improper general state of well-being as well as tension headaches. In addition, one third of patients experience at least one epileptic seizure (Louis et al., 2007). Patients

diagnosed with glioblastoma also show changes in the quality of life as well as some neurobehavioural deficit including memory loss (Klein et al., 2001, Preusser et al., 2011).

2.4 Gliomas

Gliomas are tumours that arise from the glial cells of the brain or spinal cord with the most common site being the brain (Mamelak and Jacoby, 2007). Gliomas are identified with respect to the specific cell type they share histological chracteristics with, but not necessarily where they originate from (**Figure 2.7**). Glioma types include ependymomas from ependymal cells, astrocytomas from astrocytes (glioblastoma multiforme is the most common astrocytoma), oligodendrogliomas from oligodendrocytes (Rao, 2003). Sometimes a mixed glioma which contains more than one cell type could occur and an example is oligoastrocytoma.

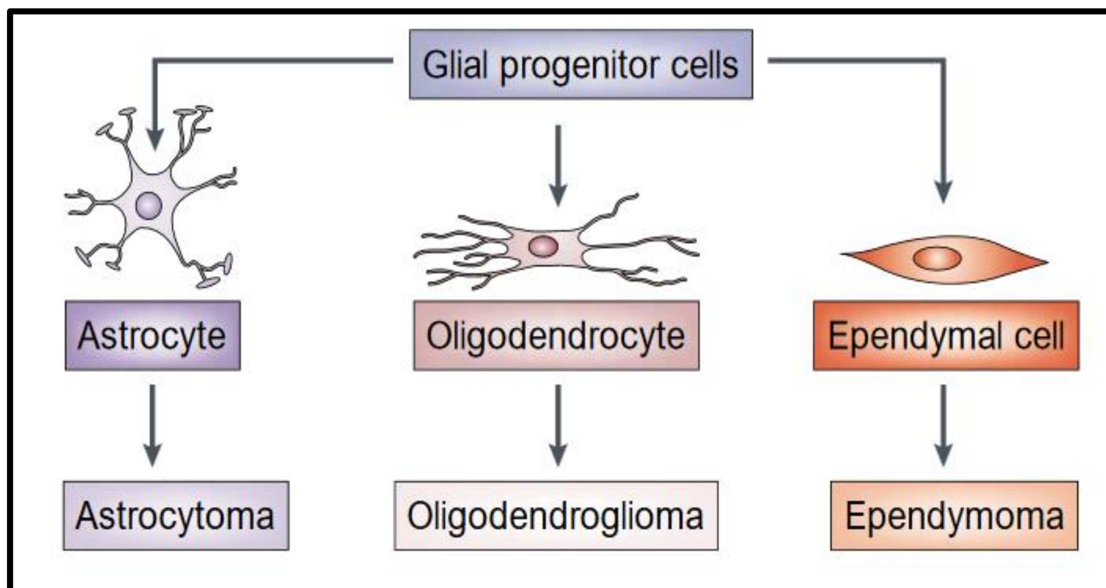


Figure 2.7. Glial cells and their distinct tumour type (Rao, 2003).

Gliomas are further categorized according to their grade, which is determined by pathologic evaluation of the tumour. The most common grading system in use, is the World Health Organization (WHO) grading system which categorizes gliomas into four

grades from I with best prognosis and least advanced to grade IV with worst prognosis and most advanced. **Table 2.1** below summarizes the WHO classification of gliomas.

Table 2.1. The World Health Organization (WHO) grading system for astrocytomas

Grade	Example	Criteria
I	Pilocytic astrocytoma, myxopapillary ependymoma/subendymoma	Low proliferating, discrete, non-invasive tumour (low grade)
II	Diffuse astrocytoma, papillary, cellular and clear cell ependymoma	Modest proliferating, partly invasive tumour (low grade)
III	Anaplastic astrocytoma and anaplastic ependymoma	Fast proliferating, invasive tumour (intermediate to high grade)
IV	Glioblastoma multiforme, hghly malignant glioma-like pineoblastoma and medulloblastoma	Rapidly proliferating, highly invasive tumour (high grade)

2.4.1 Glioblastoma multiforme

Glioblastoma multiforme is a malignant brain tumour and represents one of the most dreaded types of cancer due to their poor prognosis as well as their direct effect on the on quality of life and cognitive function of patients (Omuro and DeAngelis, 2013). Of all malignant brain tumours, glioblastoma multiforme (GBM) account for about 60-75% of astrocytic tumours and due to their high intratumoural heterogeneity, they are extremely complex and difficult to treat (Furnari et al., 2007, Parsons et al., 2008, Inda et al., 2014). Median survival is usually less than one year from the time of diagnosis and most patients die within two years, although cases of "long-term survivors", who might have been given the wrong histological diagnosis at first exist (Stupp et al., 2005). Unless there was

a progression of the tumour from a low grade astrocytoma, in more than 50% of patients, the clinical history is less than 3 months.

2.4.2 Gliomagenesis

Gliomagenesis is the biological processes involved in the transformation of a normal cell into a neoplastic one, usually driven by genetic alteration of these cells (El-Aneed, 2004, Idbaih et al., 2008). Glioblastomas can be of two subtypes; primary or "de novo" and secondary glioblastomas (Stupp et al., 2005, Louis et al., 2007, Zheng et al., 2008). Primary glioblastoma which mostly in adults (average age of 60 years) and account for about 90% of cases and occur in lesions where a low grade astrocytoma has not been previously reported and they progress rapidly (Mischel et al., 2003, Shai et al., 2003). Secondary glioblastomas, on the other hand, are more common in younger adults (average age of 45 years) and represent a progression of malignancy from a low grade tumour such as diffuse astrocytoma (WHO grade II) to glioblastoma multiforme (WHO grade IV) (Furnari et al., 2007). Usually, more than 70% of WHO grade II gliomas progress into WHO grade III/IV diseases within a period of 5 to 10 years of diagnosis and they represent only about 5% of glioblastomas (Furnari et al., 2007, Louis et al., 2007, Parsons et al., 2008).

Phenotypically, primary and secondary glioblastoma cannot be distinguished but genetically, they display distinct abnormalities, suggesting that gliomagenesis may occur through different pathways (**Figure 2.8**). Primary glioblastoma in adults is usually associated with overexpression and mutation of epidermal growth factor receptor (EGFR), deletion of the p16 gene, loss of heterozygosity (LOH) of chromosome 10q as well as mutation of the phosphatase and tensin homology (PTEN) gene (Wrensch et al., 2002, Ohgaki and Kleihues, 2007). Secondary glioblastoma is associated with abnormalities in the pathway regulating the tumour suppressor RB, mutations in the p53

gene and LOH of chromosome 10q (Ohgaki and Kleihues, 2007). In all, these genetic alterations in both primary and secondary glioblastoma multiforme, triggers activation of cascade of signalling pathways leading to uncontrollable proliferation of affected cells.

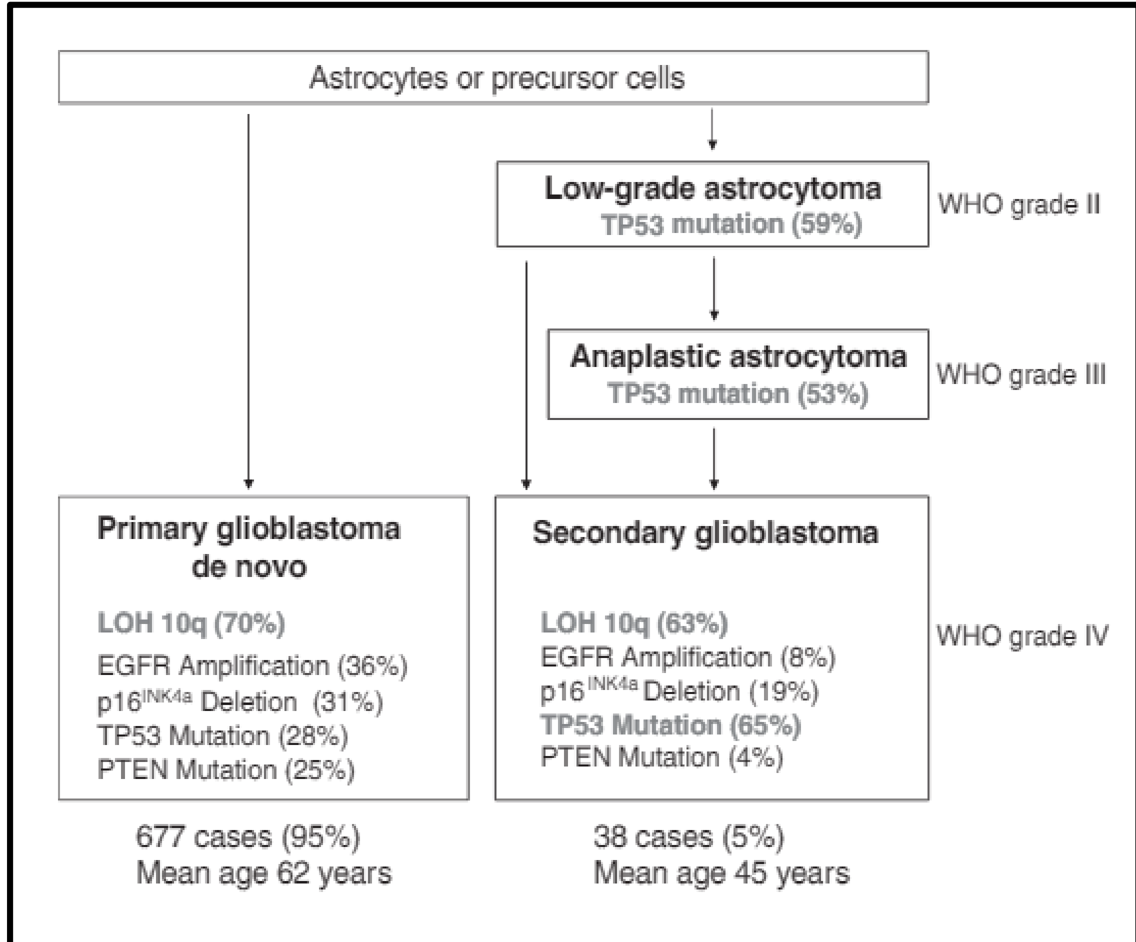


Figure 2.8. Genetic pathways leading to primary and secondary glioblastomas at population level (Ohgaki et al., 2004a)

2.4.3 Genetic and signalling pathways in glioblastoma

Genetic changes play a role in various steps leading to cancer formation and they include; uncontrolled regulation of cell proliferation, apoptosis, migration and cell-to-cell communications. Research into these genetic abnormalities has enhanced the knowledge of the complex nature of tumours, thus allowing identification of unique genetic profiles associated with different glioblastoma subtypes (Crespo et al., 2015). This can also help

improved treatment of patients by categorizing patients into the various subtypes as well as aid personalized medicine.

2.4.3.1 Loss of heterozygosity

Loss of heterozygosity (LOH) on chromosome 10 is a common event in both primary and secondary glioblastomas and it is the most regular genetic variation in glioblastomas, present in about 60–80% of cases (Ichimura et al., 1998, Fults et al., 1998, Ohgaki et al., 2004a). Despite majority of glioblastomas seem to have lost an entire copy of chromosome 10, studies have reported three commonly deleted loci of the chromosome, which include; 10p14-p15, 10q23–24, and 10q25-qter, thus suggesting the presence of several tumour suppressor genes in the tumours (Rasheed et al., 1995, Karlbom et al., 1993, Fults et al., 1998, Nakamura et al., 2001a). Importantly, primary glioblastomas show LOH at all informative markers, thus implying a complete loss of the entire chromosome 10. However, secondary glioblastomas with LOH show a partial or complete loss of chromosome 10q but no loss of 10p is evident (Fujisawa et al., 2000).

The presence of similar histologic features between primary and secondary glioblastomas suggest a similar genetic alteration and consequently phenotype since the lesions are as a result of alterations. The LOH 10q25-qter is linked with the histological transition from low-grade or anaplastic astrocytoma to glioblastoma phenotypes and is frequently deleted distal from PTEN at 10q25-qter in primary and secondary glioblastomas (Fujisawa et al., 1999, Ohgaki et al., 2004b, Ohgaki and Kleihues, 2005). This implies that transition to highly malignant glioblastoma phenotype is linked to a loss of a putative tumour-suppressor gene on 10q25-qter.

2.4.3.2 TP53/MDM2/p14^{ARF} pathway

The TP53 pathway which is mutated in many cancers and controls the cell cycle, plays a critical role in the development of secondary glioblastomas. TP53 mutations are the first

detectable genetic alteration in two-thirds of precursor low-grade diffuse astrocytomas. This frequency is similar to that in anaplastic astrocytomas and secondary glioblastomas derived from low grade tumours (Watanabe et al., 1996, Watanabe et al., 1997, Ohgaki et al., 2004b). TP53 is frequently mutated in 65% of secondary glioblastomas and in less than 30% of primary glioblastomas.

Furthermore, amplification of the murine double minute 2 (MDM2), a negative regulator of TP53 is present in less than 10% of primary glioblastomas with TP53 mutations (Reifenberger et al., 1993, Biernat et al., 1997, Uhrinova et al., 2005). The p14ARF, also known as alternate reading frame (ARF) gene, encodes for a cell cycle regulator that sometimes form a stable complex with MDM2 in cases of sustained mitogenic signals from MYC and Ras proteins, leading to the induction of p53-dependent cell growth arrest and apoptosis and autophagy (Abida and Gu, 2008). Loss of p14ARF expression is frequently observed in about 76% glioblastomas and this correlates with homozygous deletion or promoter methylation of the p14ARF gene. Promoter methylation of p14ARF was however noticed to be more reoccurring in secondary than primary glioblastomas, but no significant difference was observed in the overall frequency of p14ARF alterations (Nakamura et al., 2001a).

2.4.3.3 EGFR/PTEN/Akt/mTOR pathway

The EGFR/PTEN/Akt/mTOR pathway is a critical signalling pathway that plays a role in the development of primary glioblastomas (Kita et al., 2007). Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor which is over-amplified in about 40% of primary glioblastomas but rarely seen in secondary glioblastomas (Watanabe et al., 1996, Ohgaki et al., 2004b, Kapoor and O'Rourke, 2003). In cases of over-amplification of EGFR, EGFR overexpression was also noticed and this is more common in primary glioblastomas (>60%) compared to secondary glioblastomas (<10%)

(Watanabe et al., 1996). Aside amplification and overexpression, EGFR amplicons can also be mutated and the most common form of this is the variant 3 (EGFRvIII), with deleted exons which is constitutively active thus leading to transforming effects and mitogenic activities (Huang et al., 1997).

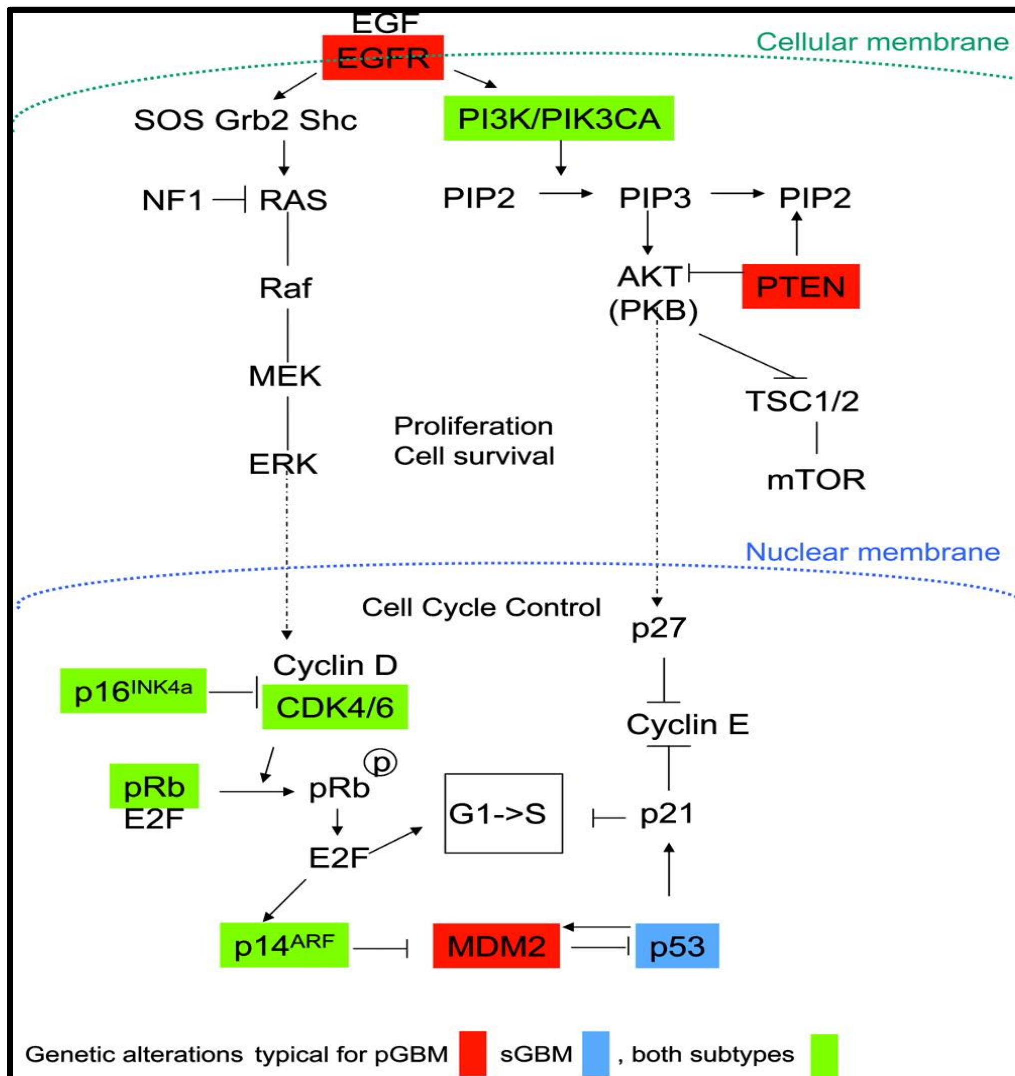


Figure 2.9. Major signalling pathways involved in the pathogenesis of glioblastomas (Ohgaki and Kleihues, 2007)**.**

Upon activation of EGFR either by binding to a growth factor (epidermal growth factor, transforming growth factor-alpha) or constitutive activation, p27 is down regulated and phosphatidylinositol 3-kinase (PI3K) is recruited to the cell membrane thus leading to the phosphorylation of phosphatidylinositol-4, 5-bisphosphate (PIP2) to

Phosphatidylinositol-3, 4,5-triphosphate (PIP3) (**Figure 2.9**) (Narita et al., 2002, Mellinghoff et al., 2005). This cascade leads to the activation of downstream targets such as protein kinase B (Akt) and mammalian target of Rapamycin (mTOR) which promote cell proliferation and survival as well as inhibit apoptotic pathways (Kita et al., 2007).

The PTEN gene which is mutated in about 15 to 40% of glioblastomas (almost exclusive to primary glioblastomas) is a negative regulator of the PI3AKT pathway and known to inhibit proliferation and cell survival, hence it is classified as a tumour suppressor (Dahia, 2000, Knobbe et al., 2002, Mellinghoff et al., 2005).

2.4.3.4 p16INK4a/RB1 pathway

The p16INK4a/RB1 pathway tends to be a major pathway in both primary and secondary glioblastomas with deletions frequently seen in primary (31%) than in secondary (19%) glioblastomas (Nakamura et al., 2001a, Ohgaki et al., 2004b). The retinoblastoma 1 (RB1) gene encodes a protein that regulates cell cycle progression from G1 to S phase via the phosphorylation of RB1 by CDK4/cyclin D1 complex. This phosphorylation induces the transcription factor E2F as well as its target genes to control G1 to S phase transition (**Figure 2.9**) (Sherr and Roberts, 1999). The promoter methylation linked with transcription silencing of RB1 gene occurs in 43% of secondary glioblastoma compared to 4% of primary glioblastoma (Nakamura et al., 2001c).

Furthermore, a combination of genome-wide expression profiling and clinical evaluation has also revealed that glioblastomas can be classified into four subtypes, which are; proneural, neural, classical and mesenchymal (Verhaak et al., 2010, Morokoff et al., 2015).

1. **Classical:** Tumours of this subtype show common genetic alterations exhibited by glioblastomas and they include amplification of chromosome 7 and loss of chromosome 10 as well as EGFR amplification and CDKN2A deletion.
2. **Mesenchymal:** This subtype shows focal hemizygous deletions of a region at 17q11.2, containing the gene NF1 (neurofibromin 1 or neurofibromatosis-related protein NF-1 gene) and more pronounced necrosis as well as inflammation, with expression of genes involved in wound healing and NF-KB signalling. They also show high expression of CHI3L1 (chitinase-3-like protein 1 gene) and MET (c-Met or hepatocyte growth factor receptor gene) (Phillips et al., 2006).
3. **Proneural:** Tumours of this subtype are more common in younger patients and frequently show mutation in the TP53 and the IDH1 (isocitrate dehydrogenase 1) gene as well as overexpression and mutation of PDGFRA (platelet-derived growth factor receptor alpha gene). These mutations are usually typical of secondary glioblastomas as opposed to primary glioblastomas (Kleihues and Ohgaki, 1999).
4. **Neural:** This subtype is characterised by the expression of various gene types that are also typical of the normal brain nerve cells and show low mutations on the other genes earlier mentioned. These genes include GABRA1 (gamma-aminobutyric acid receptor subunit alpha-1 gene), NEFL (neurofilament light polypeptide gene), SLC12A5 (potassium-chloride transporter member 5 gene) as well as SYT1 (synaptotagmin-1 gene) and patients of this subtype are usually older in age (Verhaak et al., 2010).

2.4.4 Pathophysiology of glioblastoma

Lesions of glioblastoma are usually large at the time of diagnosis and sometimes occupy most part of a brain lobe. These lesions are frequently located in the subcortical white

matter of the cerebral hemispheres and tumour infiltration sometimes may progress into the adjacent cortex of the brain (**Figure 2.10**) (Louis et al., 2007). The tumour mass of glioblastoma multiforme is always poorly delineated with a high degree of regional heterogeneity. It is usually differentiated into a peripheral and hypercellular zone with rapidly proliferating cancer cells and a necrotic core consisting of dead tissue in the central area of the tumour which contribute up to 80 % of the total tumour mass (Persano et al., 2011, Patel and Hathout, 2017).

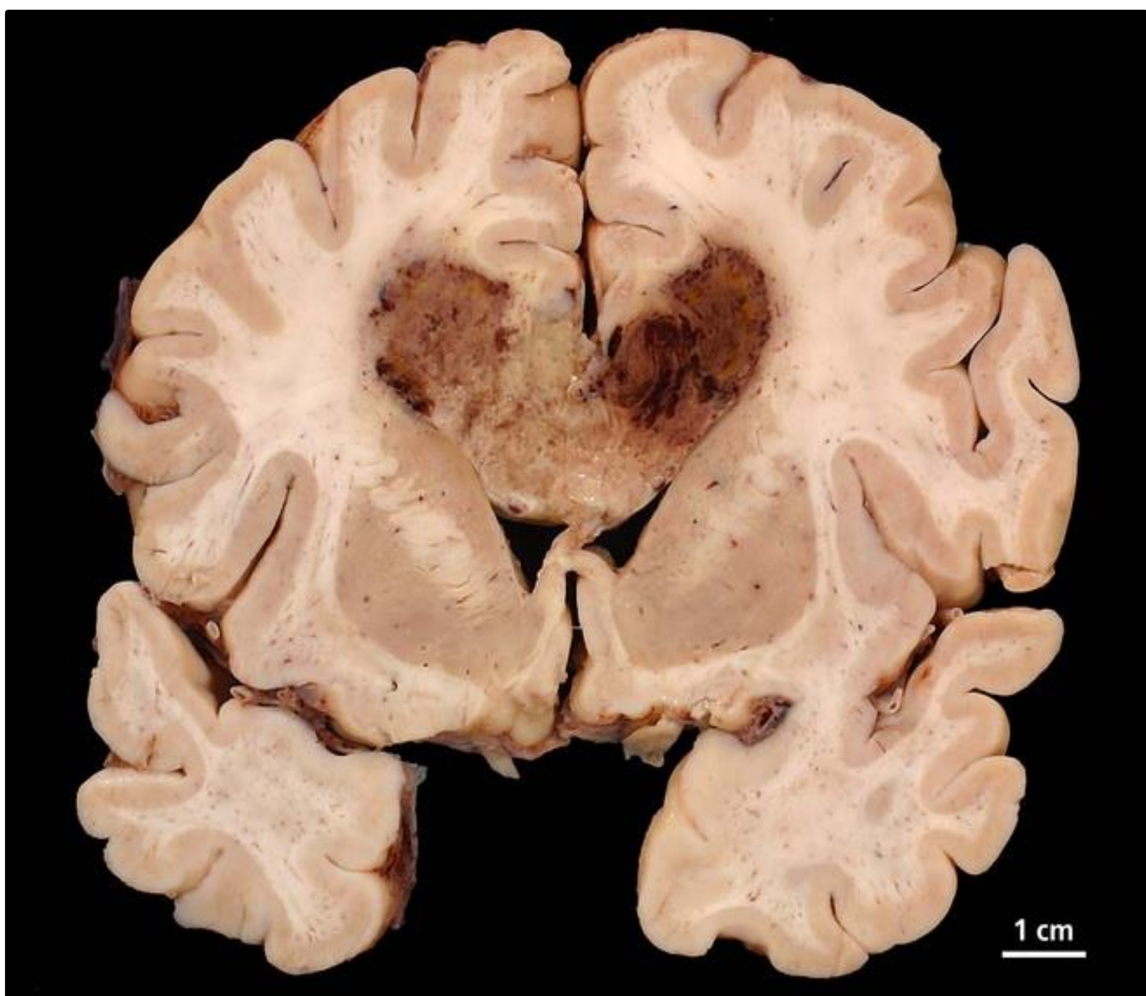


Figure 2.10. Brain section of a 69 year old male diagnosed with glioblastoma

(http://www.webpathology.com/image.asp?case=738&n=2)

Histopathologically, glioblastoma multiforme lesions typically exhibit a peripheral zone of cellular hyperplasia harbouring cancer cells with atypical nuclei, cellular

pleomorphisms, increased mitotic activity, and poor stages of differentiation (Moore and Kim, 2010). Another typical feature of glioblastoma multiforme is the presence of areas with vascular hyperplasia as well as palisading cells around the area of necrosis in the tumour tissue (Rong et al., 2006b). The rapid invasion and infiltration of the surrounding brain parenchyma, majorly along myelinated brain structures like the corpus callosum or within perivascular spaces is often reported to be associated with glioblastoma (Brat et al., 2002, Rong et al., 2006a). Infiltrating tumour cells are scattered within the surrounding of the brain tissue and this contrast could enhance the tumour border during high resolution scans. These cells are sometimes linked to tumour recurrence since they escape surgical resection and high-dose radiotherapy of primary tumours mass. Though glioblastoma multiforme possess high infiltrative ability, distant metastases are rarely found, both within and outside the CNS as they do not infiltrate the subarachnoid space nor the lumen of vessels (Louis et al., 2007).

2.4.5 Cancer stem cells in glioblastoma

After the formation of the neural tube in CNS development, it is lined with neuroepithelial cells which are proliferative in nature and produce most of the cells that make up the future CNS (Nowakowski and Hayes, 1999). These neuroepithelial cells line the ventricular and subventricular zones of the CNS and are thought to be multipotent neural stem cells (NSCs) thus serving as precursors to various cell types including neurons, glial cells and ependymal cells (Doetsch et al., 1999, Johansson et al., 1999). In the adult brain, a remnant of the subventricular zone is noticeable and its cells continue to proliferate and give rise to neurons and glial cells, a process termed “adult neurogenesis”.

There has been increasing evidence supporting the idea that malignant tumours are initiated and maintained by a population of tumour cells which share similar biological

characteristics as normal adult stem cells (Lim et al., 2007, Lathia et al., 2011). The theory of the cancer stem cell was initially shown on acute myeloid leukaemia (AML) and since then, solid tumours including glioblastomas have been shown to possess cancer stem-like cells (Lapidot et al., 1994). This theory postulates that cancer stem cells (CSCs) are characterized by their ability to self-renew and differentiate into specialized cell types that could represent the bulk of the original tumour (Magee et al., 2012). However, the mechanism propagating these CSCs to create heterogenic cell populations is still not clearly elucidated. To support this theory of CSC, brain cancer stem cells (BCSCs) or glioma stem cells (GSCs) have been shown to possess NSC characteristics as they are capable of maintaining their own population through self-renewal as well as give rise to cells of the CNS such as neurons, oligodendrocytes and astrocytes. The GSCs also express different NSC markers such as CD133 and Nestin. Aside from their NSC-like characteristics, GSCs formed xenograft tumours which were similar to the parent tumour when these cells were inoculated into immune-compromised mice (Singh et al., 2004, Emmenegger and Wechsler-Reya, 2008, Varghese et al., 2008).

Furthermore, GSCs have been reported to negatively affect treatment of glioblastoma multiforme as they promote resistance to chemotherapy, largely because chemotherapeutic drugs are cytotoxic and attacking rapidly proliferating cells whereas BCSCs are slowly dividing or sometimes quiescent (Stupp and Hegi, 2007, Dirks, 2008). The presence of many CD133 positive cells in glioblastoma negatively affects chemotherapy as these cells promote drug resistance genes, aid DNA repair and prevent apoptosis (Liu et al., 2006). GSC markers could either be of embryonic origin or from neural progenitor cells and they include: CD133, nestin, NANOG, SALL4, STAT3, SOX2, c-Myc, Olig2, Bmi1, CD44, L1CAM, and KLF4 (Schmitz et al., 2007, Li et al., 2009, Olmez et al., 2015, Zhang et al., 2015). Considering the role of GSCs in tumour

initiation, progression and therapy resistance, they contribute to tumour relapse and high mortality rate from glioblastomas and could serve as potential drug targets for glioblastoma treatment (Bradshaw et al., 2016).

2.4.6 Diagnosis and treatment options for glioblastoma

Magnetic resonance imaging (MRI) or computated tomography (CT) scans are the major tool employed for the diagnosis of malignant brain tumours. MRI helps to define important areas for proper surgical planning while the use of diffuse-weighted imaging, diffusion tensor imaging, and perfusion imaging can help ascertain the relative cerebral blood volume, identify important adjacent structures as well as monitor responses to therapy (Young, 2007).

The treatment of glioblastomas remains a difficult and complex one as contemporary treatments are not curative (Preusser et al., 2011). The standard treatment for glioblastoma involves surgery followed by radiotherapy and concomitant and adjuvant chemotherapy with temozolomide (TMZ) (Furnari et al., 2007, Sathornsumetee et al., 2007, Lara-Velazquez et al., 2017). However for older patients (≥70 years old), less invasive therapy consisting of radiotherapy and chemotherapy is usually employed (Glantz et al., 2003, Roa et al., 2004, Keime-Guibert et al., 2007). Application of surgical techniques can help reduce total tumour size and symptoms of tumours, but it fails to completely remove all infiltrative tumour cells as some of these cells are located in adjacent brain parenchyma. Although surgical techniques have improved, survival of patients who went through surgery alone remains poor (Lara-Velazquez et al., 2017). It has also been reported that radiation therapy when combined with surgery or surgery in addition to chemotherapy prolongs survival in glioblastoma patients compared to surgery alone (Parisi et al., 2015). For instance, survival has been shown to increase from 3-4

months to 7-12 months when radiotherapy was administered in addition to surgery (Stupp et al., 2005, Huang et al., 2017).

Chemotherapy is becoming increasingly important in the treatment of GBM and its significance and challenges will be further discussed in the next section.

2.5 Chemotherapy for glioblastomas

The oldest and most traditional group of cancer chemotherapy are the alkylating agents which act via the addition of an alkyl group to the DNA molecule and in turn, prevent replication, proliferation and eventually induces cell death. These alkylating agents can be categorized into various groups which include the classical alkylators (nitrosourea, nitrogen mustards, alkyl sulfonates), alkylating-like agents (platinum complexes), and nonclassical alkylators (procarbazine, altretamine, dacarbazine, and TMZ) (Nabors et al., 2016). Other classes of drugs include the microtubule inhibitors, topoisomerase inhibitors, vascular endothelial growth factor inhibitors, growth factor receptor inhibitors as well as signalling pathway inhibitors (Tan et al., 2018). This section reviews existing chemotherapeutic agents for glioblastoma and the current treatments.

2.5.1 Nitrosoureas

The nitrosoureas, predominantly Carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea; BCNU) and Lomustine (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea CCNU) are DNA alkylating agents used in chemotherapy for glioblastoma (Nabors et al., 2016). Nitrosoureas were the first drugs that showed activity against gliomas and are highly lipophilic and are thus able to penetrate the BBB (Fewer et al., 1972, Wilson et al., 1976). Nitrosoureas are no longer used as first line treatment of primary brain tumours but for second line use when there is a tumour reccurrence (Taal et al., 2014). BCNU is given via intravenous infusion and its metabolism takes place in the liver, while CCNU is administered orally and absorbed from the gastrointestinal tract within 3 hours post-

administration. Gliadel is a polymer formulation of BCNU approved by the FDA in 2002 and is now being used for local therapy as a wafer (Gutenberg et al., 2013, Sai et al., 2014). Other nitrousurea derivatives such as 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl) 3-nitrosourea hydrochloride (ACNU, nimustine) and methyl-chlorethyl-cyclohexyl-nitrosourea (MeCCNU, semustine), have also been used, though semustine is less frequently used (Imbesi et al., 2006, Wang et al., 2014).

2.5.2 Platinum analogues (Cisplatin and Carboplatin)

Cisplatin and carboplatin are the most common platinum complexes used for glioblastoma treatment. Cisplatin is the oldest and more toxic while the newer complex carboplatin is less toxic and shows improved anti-cancer activity. Platinum analogues are water-soluble and poorly penetrate the BBB and their mechanism of action is via DNA damage as they form cross-links that displace the chloride ligand in the double helix of the DNA, thus triggering apoptosis (Fuertes et al., 2003). Cisplatin has been used alongside other drugs and as a salvage therapy in cases of glioblastoma reoccurrence (Capdevila et al., 2014, Roci et al., 2014). However, its known side effects include alopecia, nephrotoxicity, hearing loss and myeloid suppression (Schacht et al., 2012). CNS and peripheral neurotoxicity as well as stroke have also been reported as side effects (Grisold et al., 2009, Grisold et al., 2012).

2.5.3 Microtubule inhibitors (Paclitaxel, Docetaxel and Vinca alkaloids)

The microtubule inhibitors are a class of compounds that inhibit the functions of microtubules which take part in cellular processes involving transport, cell shape, migration, and mitosis. Microtubules are important role players in mitosis thus making them targets for cancer therapy (Perez, 2009). Microtbule agents are divided into two classes which are the microtubule stabilizing agents and the microtubule destabilizing agents. The taxanes comprising of paclitaxel isolated from the bark of the Western yew

tree (*Taxus brevifolia*) and docetaxel, a semisynthetic analogue are examples of microtubule stabilizing drugs. This group of compounds poorly penetrate the BBB and carry out their action by stabilizing microtubules, leading to cell cycle arrest (Abal et al., 2003). On the other hand, microtubule destabilizing drugs include vinblastine and vincristine also known as vinca alkaloids and were first isolated from the *Vinca rosea* (periwinkle) plant and are lipophilic in nature with relative penetration through the blood brain barrier (Boyle et al., 2004, Perez, 2009). These compounds bind to microtubules and eventually lead to cell death (Cairncross et al., 2013). The taxanes show haematological toxicities and sometimes peripheral neuropathy which are also side effects of the vinca alkaloids (Michaud et al., 2000).

2.5.4 Topoisomerase inhibitors

Topoisomerase inhibitors react with topomerase enzymes thus disrupting DNA replication and eventually leading to cell death. They could be divided into the TP I inhibitors (topotecan and irinotecan- isolated from the *Camptotheca acuminata* tree) and TP II inhibitors which are podophyllotoxin alkaloids found in the American mayapple (etoposide and teniposide).

Topotecan is a small water-soluble small molecule camptothecin analogue which readily penetrates the BBB and binds directly to topoisomerase I (Newton et al., 1999). It was earlier demonstrated that topotecan showed efficacy against CNS tumours which was enhanced when used in combination with alkylators such as BCNU (Coggins et al., 1998). Topotecan has also been used in combination with radiation therapy in a phase II clinical trial for glioblastoma treatment, however no statistically significant survival was observed (Fisher et al., 2002). Irinotecan is used in the treatment of solid tumours including gliomas and recurrent glioblastoma (Zhang et al., 2012). It has also been used in combination with other chemotherapeutic agents like bevacizumab, rapamycin,

irinotecan, sunitinib, and TMZ in phase II clinical studies (Hofland et al., 2014, Mesti et al., 2015, Nonnenmacher et al., 2015).

Etoposide, a topoisomerase II inhibitor, selectively targets dividing tumour cells and is highly lipophilic but due to its large size, crosses the BBB with difficulty. It was reported to have a minimal effect as a single agent in glioblastoma patients (Fulton et al., 1996, Chamberlain, 1997). Toxicity of this drug has also been reported to include haematological, gastrointestinal toxicity and hypersensitivity (Hartmann and Lipp, 2006). In addition, toxicity to the cardiovascular system has also been reported to be a long-term effect of treatment with some Topoisomerase II inhibitors (Seiter, 2005).

2.5.5 Targeted therapy

Targeted therapy involves the use of antibodies and small molecule inhibitors to target cancer cells. These inhibitors either target growth factors or signalling pathways such as PI3K/Akt/mTOR and Ras/Raf/MAPK involved in tumour progression, metastasis and angiogenesis.The secretion of vascular endothelial growth factor (VEGF) by tumour cells leads to proliferation of normally quiescent endothelial cells and the formation of new capillaries. Angiogenesis has been implicated as a promoter of the cancer phenotype (Linkous and Yazlovitskaya, 2011). Bevacizumab is a chemotherapeutic agent that targets angiogenesis by inhibiting VEGF in the blood, and in turn, reduces the binding of VEGF to its receptors and inhibits tumour cell invasion (Lu et al., 2012). However, this drug is known to cause hypertension and fatigue as side effects like other chemotherapeutic drugs (Pavlidis and Pavlidis, 2013). Other VEGF inhibitors for glioblastoma treatment include; thalidomide, lenalidomide and cannabinoids (Warren et al., 2011, Giglio et al., 2012, Rocha et al., 2014).

Inhibitors of growth factor receptors are also used in combination with other drugs. Cell surface growth factor receptors which exist in primary brain cancers, include the epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and c-MET (Thorne et al., 2016, Miekus, 2017, Roskoski, 2018). EGFR which is mutated or amplified in a majority of malignant gliomas is known to promote initiation and progression of glioblastoma (Azuaje et al., 2015, Liu et al., 2015, Westphal et al., 2017). The ErbB receptor family of tyrosine kinases comprises four members: EGFR (ErbB1/HER1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4) which can be targeted with EGFR tyrosine kinase inhibitors. Selective and reversible inhibitors of the EGFR tyrosine kinase domain are Gefitinib (Iressa) and erlotinib (Tarceva) while irreversible inhibitors are lapatinib and afatinib (Voelzke et al., 2008, Thiessen et al., 2010, Karavasilis et al., 2013).

2.5.6 Temozolomide as current chemotherapy for glioblastoma

Temozolmide (Temodal®; TMZ) is an oral DNA alkylating agent and is the current chemotherapeutic agent for treatment of glioblastoma (Chinot et al., 2004). Early studies into this drug focused on its use for recurrent glioblastoma (Yung et al., 2000). However, over the last decade TMZ has been considered the choice chemotherapeutic agent alongside other forms of therapy for glioblastoma (Stupp et al., 2005). The standard regimen for patients is 150 or 200 mg/m^2/day for five consecutive days, to be repeated every four weeks (Rai et al., 2016). In one clinical study, TMZ treatment was described to be palliative rather than curative, as it only increased overall survival by just 2.5 months when compared to radiation alone. The median overall survival in glioblastoma is only about 14.6 months and a great percentage of patients have little or no benefit following TMZ treatment (Chamberlain, 2010).

2.5.6.1 Mechanism of action of TMZ

TMZ is administered orally and its absorption is not affected by food as it has little interaction with food (Baker et al., 1999). It is readily absorbed with 100% oral bioavailability within one to two hours of administration and the CSF concentration can be as high as 30 to 40% of the plasma concentration (Ostermann et al., 2004). Once in circulation, TMZ is rapidly hydrolysed into its active metabolite, methyl-triazeno-imidazole-carboxamide (MITC). This is in turn, broken down to an inactive metabolite, 5-aminoimidazole-4-carboxamide (AIC) on the side and the final active end product, the methyldiazonium cation which then alkylates DNA by transferring a methyl group to the nucleotide (**Figure 2.11**). Alkylation most times occur at the N^7 position of guanine and N^3 position of adenine (Newlands, Stevens et al. 1997). Only 5% of all DNA alkylation happens at the O^6 position of guanine but methylation on this region is chiefly responsible for the anti-cancer activity of TMZ mainly via apoptosis and autophagy induction (Roos et al., 2007).

Cytotoxicity of TMZ is cell-cycle dependent, thus it is more potent in rapidly dividing cancer cells rather than quiescent cells (Verbeek et al., 2008). Following methylation of the O^6 position of guanine, there is a mispair of the O^6-methylguanine with thymine instead of cytosine during DNA replication. This mismatch is recognized by the mismatch repair system that attempts to repair it by removing the thymidine of the daughter strand, thus leaving the parental strand not repaired (Lee, 2016). In the same light, the O^6-methylguanine left in the parental strand pairs up with another thymidine during the gap filling, thus failing to repair and hence DNA double strand breakage occurs (Verbeek et al., 2008). This process activates apoptotic pathways and eventually cell death.

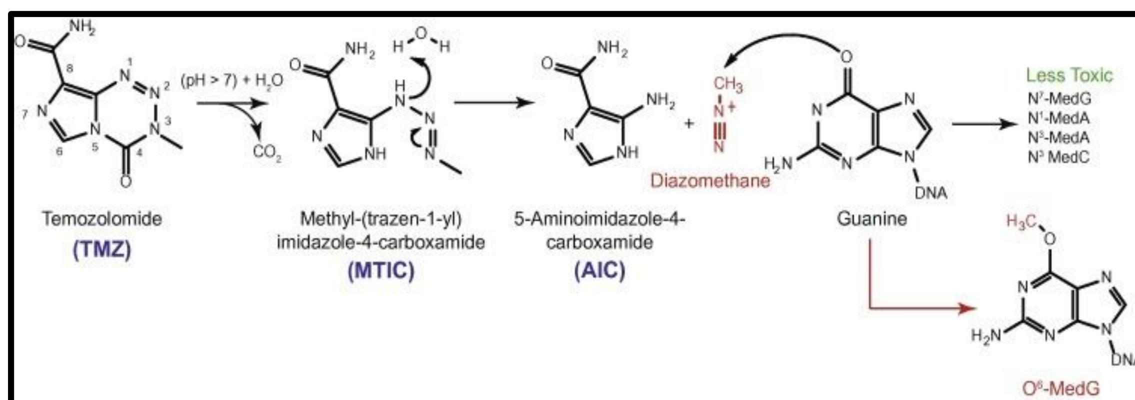


Figure 2.11. Mechanism of action of TMZ (Rai et al., 2016)

2.5.6.2 Treatment challenges of TMZ

2.5.6.2.1 Side effects of TMZ

As with most chemotherapeutic agents, gastrointestinal toxicities including vomiting and nausea are the main forms of toxicity following TMZ treatment and these could be easily managed with standard antiemetics. Haematological toxicities (myelosuppression) in the form of thrombocytopenia and neutropenia have also been reported and are usually more serious (Trinh et al., 2009). Lymphopenia is of increasing concern as it primarily affects CD4 count and may impact up to 60% of patients and rare cases of aplastic anaemia have also been reported (Su et al., 2004, Oh et al., 2010). In a study with 300 patients to ascertain the toxicity profile of TMZ, bone marrow toxicity was reported to be the most predominant of all toxicities amongst patients (Bae et al., 2014). Long-term usage and in combination with other drugs, may lead to other more specific problems which may arise (Hau et al., 2007, Jiang et al., 2014).

2.5.6.2.1 Chemoresistance of TMZ

Although TMZ therapy seems efficacious, resistance to TMZ often occurs due to intrinsic or acquired mechanisms, causing treatment failure and recurrence. An important mechanism involved in the chemoresistance of glioblastoma is the methylation status of the O^6-methylguanine methyltransferase (MGMT) gene. The O^6-Methylguanine-DNA

methyltransferase is a DNA repair protein that can remove alkyl adducts from the O^6 position of guanine in DNA (Belanich et al., 1996). MGMT has been implicated in playing a critical role in chemoresistance from tumours exposed to alkylating agents (Pegg and Byers, 1992). Repair of O^6-alkylguanine adducts by tumour cells has been implicated in drug resistance because it reduces the cytotoxicity of alkylating chemotherapeutic agents (Belanich et al., 1996).

In several human cancers in including glioblastoma, MGMT was observed to be commonly downregulated through promoter region methylation or hypermethylation (Esteller and Herman, 2004). Loss of MGMT expression as a result of methylation of promoter CpG islands was more frequent (75%) in secondary glioblastomas than in primary glioblastomas (36%) (Watts et al., 1997, Nakamura et al., 2001b). It was previously showed that hypermethylation of specific regions of the MGMT promoter was associated with silencing of the gene, hence improving TMZ-sensitivity in the tumour (Qian and Brent, 1997, Esteller et al., 1999). MGMT promoter methylation status in glioma stem-like cells has equally been shown to correlate to TMZ sensitivity under differentiation-promoting conditions (Villalva et al., 2012). On the other hand, hypomethylation of MGMT leads to upregulation of MGMT expression and enhanced chemoresistance to TMZ and other alkylating agents (Esteller et al., 2000).

2.5.7 The blood-brain barrier (BBB) as a challenge to treatment

The BBB is a highly selective semipermeable membrane that serves as a barrier between circulating blood and extracellular fluid in the CNS, thus protecting the brain from toxins (Woodworth et al., 2014). It is formed by the endothelial cells of the brain in association with astrocytes. The BBB may prevent entry of therapeutic molecules from blood to brain as it only allows passage of low molecular weight lipophilic agents by passive diffusion (Kumar et al., 2007). Presence of efflux pumps, such as P-glycoprotein aids in exocytosis,

thus removing drugs from the CNS and resulting in low accumulation of drugs (Tews et al., 2000). In glioblastoma, the BBB is sometimes disrupted at the tumour sites thus allowing passive accumulation of chemotherapeutic agents around the area of the disruption (Bhowmik et al., 2015). Consequently, the degree of BBB disruption varies from one region of the tumour to another, therefore passive diffusion via the disrupted BBB might be minimal. Since the BBB is intact around the infiltrative tumour area, the invasive and infiltrating tumour cells may not be efficiently reached by passive diffusion of chemotherapeutic agents (Wen and Kesari, 2008, Noell et al., 2011).

Despite the progress made in the therapeutic management of glioblastoma multiforme, median overall survival (OS) is approximately 15 months, with a 2-year survival rate of 8% to 26% (Sanai and Berger, 2008, Wick et al., 2011). This might be attributed largely to the late diagnosis of the disease and the inability of available therapy to effectively eradicate all glioblastoma cells. Hence new chemotherapeutic options with reduced side effects and high BBB-crossing efficiencies are needed for glioblastoma multiforme to improve patient survival. The next section of this chapter focuses on drug repurposing.

2.6 Drug repurposing

The process involved in the development of a new drug begins with the discovery of new hits or therapeutic targets including enzymes, receptors and genes (Mohs and Greig, 2017). These targets are further optimized by medicinal chemistry approaches to identify potential novel drug candidates which then go through thorough preclinical testing in cell and animal based studies. Following success in the testing stage, the drug is then recruited into the clinical trials in human subjects. This process involves a lot of time as it typically takes about 10-17 years or sometimes even longer (**Figure 2.12**) (Pantziarka et al., 2014). The cost involved in drug development is also enormous as an average of 2 billion US dollars is spent to bring a new drug from the lab to market (Paul et al., 2010, Swinney

and Anthony, 2011). Therefore, efforts to accelerate the drug discovery process have been the focus of many research groups.

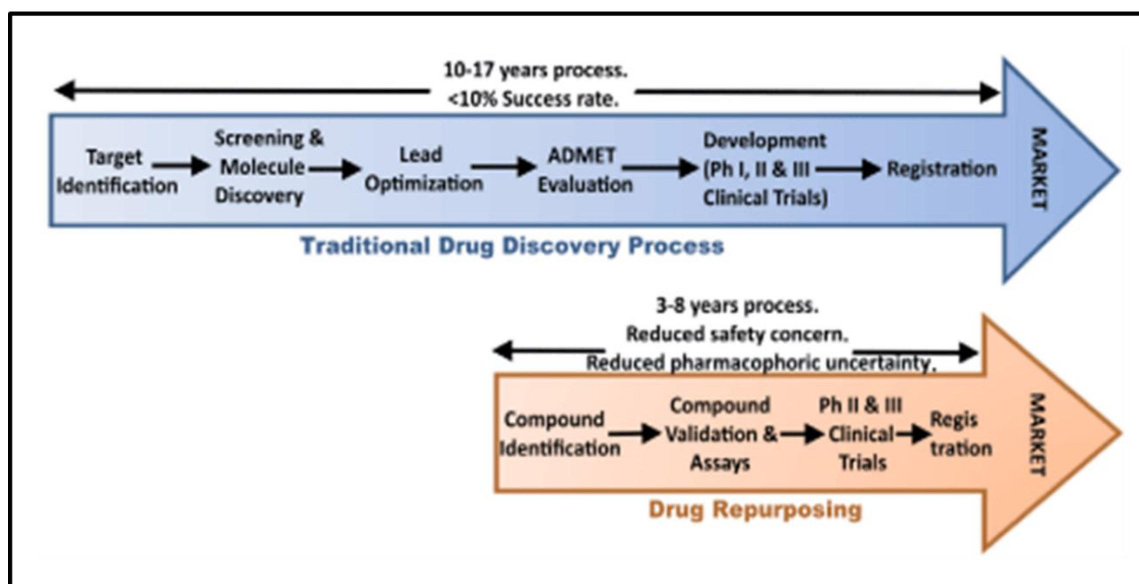


Figure 2.12. Time-line of drug development (Chakraborty and Trivedi, 2015)

Generally, drugs considered to be efficacious in Phase III trials are approved by the Food and Drug Administration (FDA) but most drugs fail to get FDA approval due to lack of efficacy mainly in phase II trials (Petsko, 2010). A decline in success rate for phase II clinical trials has been attributed to a miss in target for which the drug was intended for (Petsko, 2010, Arrowsmith, 2011). Considering that numerous diseases share similar molecular origin, it could be postulated that about 90% of approved drugs do have secondary indications and can thus be used for treating other disease conditions (Gelijns et al., 1998).

Drug repurposing (also referred to as drug repositioning, re-profiling, drug re-tasking or therapeutic switching) is the identification of new therapeutic indications for known drugs (Ashburn and Thor, 2004). The two terms 'repositioning' and 'repurposing' have been used interchangeably, but they both have their individual meanings which slightly differ. "Drug repurposing" applies to the use of existing drugs for new diseases, without

any chemical modification, while "drug repositioning" on the other hand refers to the chemical modification of known drugs for new indications and this could involve a change or substitution of functional groups or addition of more functional groups. Both drug repositioning or repurposing allow pre-existing drugs to be used for new indications which were originally not part of their targets. These drugs can either be approved and marketed compounds routinely used in a clinical setting, or drugs that have been archived for not succeeding in clinical trials, or discontinued for other reasons (**Figure 2.13**). In short, drug repositioning was defined as renewing failed drugs and expanding successful ones (Barratt and Frail, 2012).

Drug repurposing could be on-target or off-target in approach. When the target or pathway is similar with the original indication for the drug, it is termed on-target and this accounts for about 80% of repurposed drugs (Swinney and Anthony, 2011, Eder et al., 2014). On the other hand, the off-target repurposing is more novel and has been used when the pathway or target is different from the original indication (Barratt and Frail, 2012, Gupta et al., 2013, Sukkar, 2014). Drug repurposing comes with its own benefit as exploring existing pharmacopeia decreases the time and financial cost required to drive a drug through the difficult process to market. Since the safety, efficacy as well as toxicity of repurposed drugs are already known, it decreases the burden and safety testing that researchers and companies are required to undertake. More so, additional uses for existing drugs would also boost the company's profile thus improving the overall profit base (Ashburn and Thor, 2004).

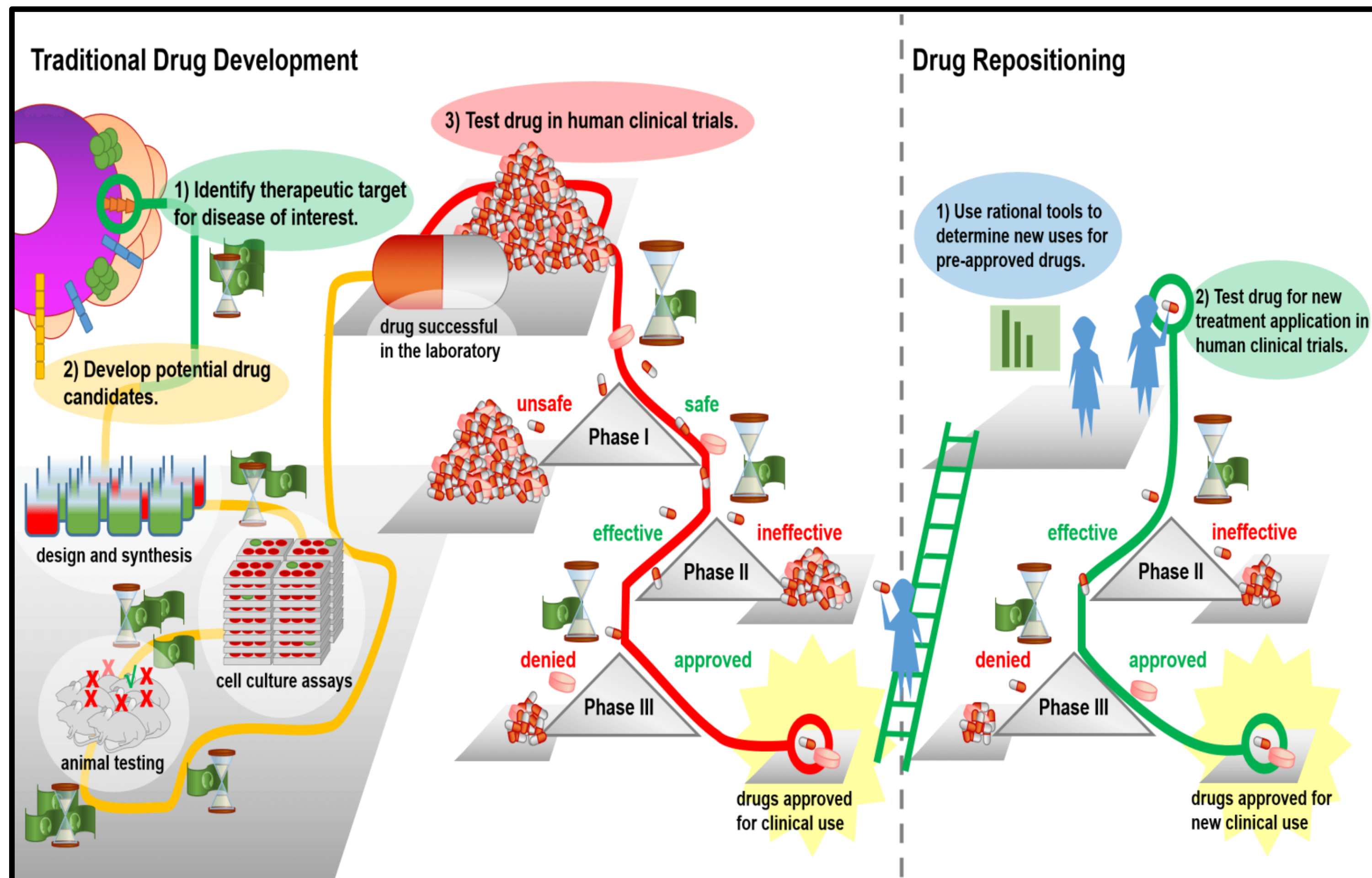


Figure 2.13. Comparisons between drug repositioning and traditional drug development ©https://www.creative-biolabs.com/drug-discovery/

Drug repurposing processes can be largely divided into two; the first process is based on experimental screening approaches as done for new drugs and the second process is the *in silico* process which uses previously generated data to identify potential new drug targets (Cha et al., 2018).

2.6.1 Repurposed drugs for cancer

The development of anti-cancer drugs is particularly long and expensive, which validates the repurposing of drugs as a promising avenue for new treatments (Gupta et al., 2013). Several strategies have been used to effectively identify and introduce current non-cancer drugs for use in cancer-related treatment (Rotella, 2012). This might be attributed to low efficiency of conventional drug discovery due to decreased number of approved compounds and the high number of failures in Phase I trials of potentially novel anti-cancer agents (Hay et al., 2014).

Some of the most common older drugs that have been repurposed for cancer are shown in the table (**Table 2.2**) below. These are drugs that were initially used for other disease conditions and include; antibiotic, antiarthritic, antitussive, sedative, immune-suppressant, analgesic, antidiabetic, muscle relaxant, antiepileptic, cardioprotective amongst others (Gupta et al., 2013). These drugs can be categorized into two groups, with the first being drugs that received approval for other uses but now used for cancer chemotherapy after their biological activities have been better elucidated (eg thalidomide, metformin, statins amongst others). The second category is made up of approved drugs that were randomly chosen to ascertain their particularity for specific targets and they include nitroxoline and noscapine (Gupta et al., 2013).

Table 2.2. Non cancer drugs and their new indication for cancer (Gupta et al., 2013)

Drug	Original indication (mechanism)	New anti-cancer indication (mechanism)
Thalidomide	Antiemetic in pregnancy	Multiple myeloma
Aspirin	Analgesic, antipyretic	Colorectal cancer
Valproic acid	Antiepileptic	Leukemia, solid tumors
Celecoxib	Osteoarthritis, rheumatoid arthritis	Colorectal cancer, lung cancer
Statins	Myocardial infarction	Prostate cancer, leukemia
Metformin	Diabetes mellitus	Breast, adenocarcinoma, prostate, colorectal
Rapamycin	Immunosuppressant	Colorectal cancer, lymphoma, leukemia
Methotrexate	Acute leukemia	Osteosarcoma, breast cancer, Hodgkin lymphoma
Zoledronic acid	Anti-bone resorption	Multiple myeloma, prostate cancer, breast cancer
Leflunomide	Rheumatoid arthritis	Prostate cancer
Wortmannin	Antifungal	Leukemia
Minocycline	Acne	Ovarian cancer, glioma
Vesnarinone	Cardioprotective	Oral cancer, leukemia, lymphoma
Thiocolchicoside	Muscle relaxant	Leukemia, multiple myeloma
Nitroxoline	Antibiotic	Bladder, breast cancer
Noscapine	Antitussive, antimalarial, analgesic	Multiple cancer types

More recently, some previously known drugs for other indications and disease conditions are currently in clinical trials where they are been repurposed for the treatment of glioblastoma. In particular, metformin, mebendazole, disulfiram, chloroquine and chlorpromazine belonging to the metabolic, antihelminthic, nervous system, antiparasitic and antipsychotic class of drugs respectively have been reported to be at various stages in clinical trials (Abbruzzese et al., 2017, Basso et al., 2018, Tan et al., 2018). Owing to achievements of drug repurposing, the approach has continued to gain recognition in the pharmaceutical/drug development process as more FDA approved drugs are currently being explored for new indications (Patwardhan and Chaguturu, 2016). Importantly, the

repurposing of antipsychotic drugs or drugs associated with the nervous system for glioblastoma presents an advantage as such drugs readily cross the BBB which is a well-known challenge in the treatment of glioblastoma.

2.7 Phenothiazines

PTZs are a very versatile and important family of drugs with an array of biological activities. PTZs are basic compounds in pharmacology and since their discovery, they have been exploited for new pharmacological entities (Varga et al., 2017). These heterocyclic dopamine receptor antagonists are mainly used as antipsychotics and sometimes as antiemetics to relieve vomiting arising from surgery or chemotherapy in cancer patients (Axelrod, 1997, Shen, 1999). They are most times referred to as typical antipsychotics (first generation antipsychotics) of which chlorpromazine was the first to be developed and holds an important place in the history of psychiatry. However, the use of PTZs as antipsychotics has been overtaken by second generation antipsychotics, like clozapine and risperidone which have superior safety profiles and specifically target the dopamine pathway (Shen, 1999). Importantly, PTZs still remain in use today as chlorpromazine and fluphenazine continues to make the list of essential medicines as drawn by the WHO (Ohlow and Moosmann, 2011).

PTZs mediate their antipsychotic effects by inhibiting the interaction of dopamine and its dopamine receptor type 2 (D2) (Feinberg and Snyder, 1975). PTZs could also act as antagonists against other neurological transmitter receptors such as the serotonin (5HT2A), muscarinic (M1), adrenergic (α1) and histamine (H1) receptors, but perform less on these targets when compared to their interaction with dopamine receptors (Jaszczyszyn et al., 2012). Common side effects of the original PTZs include drowsiness and sedation but new generations of PTZs have been able to improve on these effects, but development of tolerance may arise over long-term usage.

2.7.1 Structure of phenothiazines

Structurally, PTZs are tricyclic compounds containing a sulphur and a nitrogen with the structural formula S(C6H4)2 NH (**Figure 2.14**) (Jaszczyszyn et al., 2012). PTZs can be divided in to three subgroups based on the substitution on the nitrogen residue; the aliphatic compounds (bearing acyclic groups), the piperidines (bearing piperidine-derived groups), and the piperazines (bearing piperazine-derived substituents). More so, examples of commonly used PTZs include; chlorpromazine, triflupromazine, trifluoperazine, fluphenazine, thioridazine, mesoridazine, perphenazine, prochlorperazine and promazine (**Figure 2.14**).

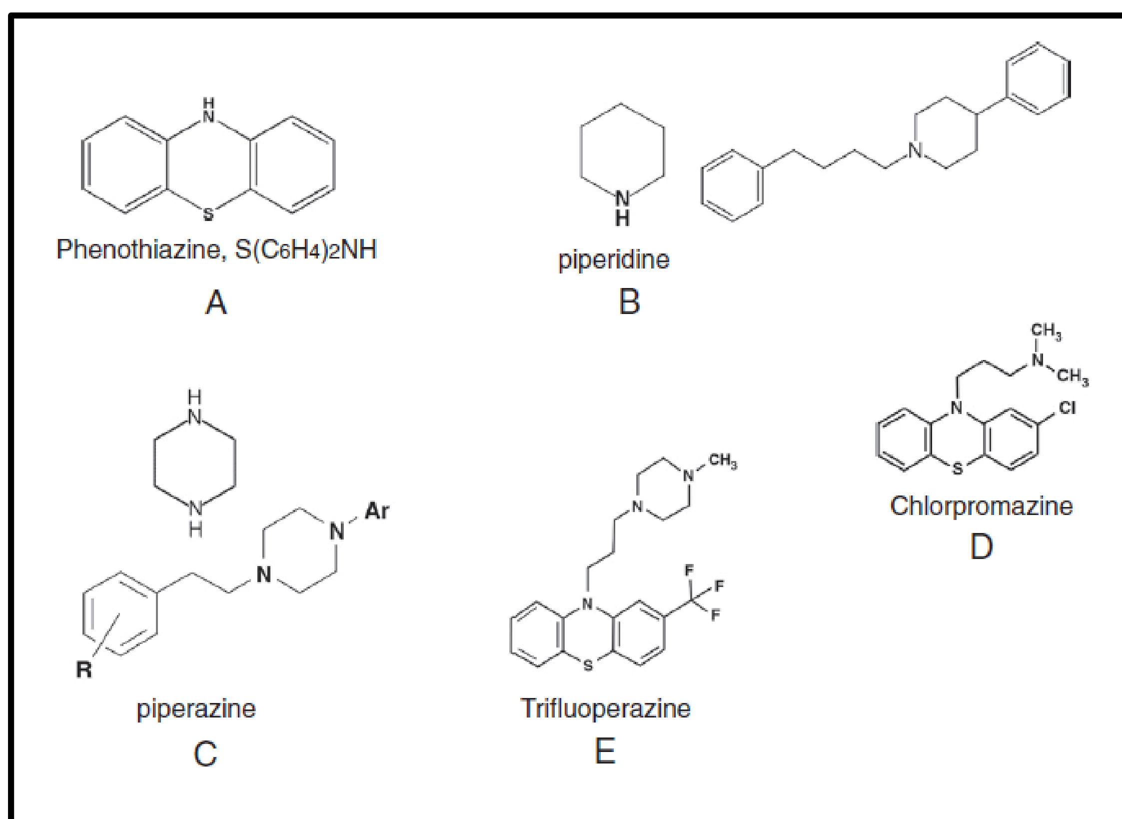


Figure 2.14. Structure of phenothiazines (A) Parent phenothiazine ring **(B)** piperidine **(C)** piperazine **(D)** chlorpromazine **(E)** trifluoperazine (Jaszczyszyn et al., 2012).

Different substitutions on the core ring confer specificity to different cellular targets and the involvement of different molecular orbitals has been implicated in the expression of

various biological activities exhibited by PTZs (Molnar et al., 1993). The aliphatic side-chains confer the least hydrophobicity, and therefore are not as effective in crossing cellular membranes. Their anti-plasmodial activity has been linked to “Cl” or “CF3” substitution at the 2nd carbon position and can equally be altered by the length of the side chain and hydrophobicity. Again, triflupromazine which has a methyl-thio substituent and fluorine moiety at carbon 10 and 2 respectively exhibits marked antibacterial activity (Sudeshna and Parimal, 2010). Studies have also indicated that the most useful PTZs (chlorpromazine and trifluoperazine) have substituents at carbons 2 and 10 and are chiefly responsible for their psychotropic actions and influence potency of the drugs respectively (Ford et al., 1989). It can thus be reported that the nature of substitutions has a significant influence on the pharmacological actions of the drugs which maybe through the enhancement of receptor specificity (Horn and Snyder, 1971). The parental PTZ core ring structure also show some biological activities including antioxidant, antihelminthic, and antiseptic activities (Mitchell, 2006). PTZs are continuously being used for development of novel therapeutic options as they have a long established history in the clinics and are also well tolerated. The anti-tumour activities of phenothiazines have previously been reported (Morak-Młodawska et al., 2009, Sudeshna and Parimal, 2010).

2.7.2 Phenothiazines and cancer

PTZs are already involved in clinical management of cancer as they are prescribed for management of nausea and emesis arising from chemotherapy in advanced stages of cancer (Morgan Jr et al., 2001). As antiemetics, PTZs help to ensure that patients receive full dosage of therapy as they prevent reflux of oral medicines and also help in relaxation of the patients through their sedative actions (Fortner et al., 1985). Most importantly, studies have also reported on reduced cancer incidence amongst patients suffering from schizophrenia with a possible link to phenothiazines or antipsychotic drugs (Barak et al.,

2005, Dalton et al., 2006). Aside from their antiemetic and sedative roles, PTZs also display antiproliferative activity in cancer cells. Pre-clinical and clinical studies have shown that several drugs of the PTZ family have antiproliferative and antineoplastic properties in diverse human cancer cell lines; these include non-small cell lung, breast, melanoma, lymphoblastoma and many other types of cancers (Ikediobi et al., 2008, Argyriou et al., 2009, Wu et al., 2016).

2.7.3 Mechanisms of action of PTZs in cancer

2.7.3.1 Modulation of calcium activities

One of the main activities of PTZs in eukaryotic cells is the prevention of Ca^{2+} from binding to its transport protein calmodulin (CaM) (Motohashi, 1991). CaM has been described as multifunctional and widespread protein which binds four calcium cations (Sakai and Krishna, 1999). CaM is a critical protein that regulates several biochemical process including; proliferation, metabolism, inflammation, and cell death dependent on calcium and thus plays an important role in cell physiology. The inhibition of CaM activity by PTZs, could lead to the inhibition of cell proliferation as well as a reduction in activity of several CaM-dependent enzymes in other intracellular biochemical pathways (Weiss et al., 1982). Trifluoperazine, and chlorpromazine as inhibitors of CaM showed a decrease in cell proliferation and clonogenecity in human and murine leukemic cells (Hait et al., 1985). Due to the alteration of Ca^{2+}–calmodulin complex, trifluoperazine has been reported to activate autophagic degradation (Høyer-Hansen et al., 2007, Zhang et al., 2007, Tafani et al., 2008). Although, PTZs have also shown inhibitory activities on protein kinase C which is not dependent on CaM (Aftab et al., 1991, Rho et al., 2011).

2.7.3.2 Inhibition of p-glycoprotein and reversal of multidrug resistance

Resistance of cancer cells to multiple chemotherapeutic agents is still a common and unresolved problem in the management of cancer (Gillet and Gottesman, 2010). This phenomenon is referred to as multidrug resistance (MDR) and can arise *de novo* or after exposure of cancer cells to a single drug (Housman et al., 2014). The best known mechanism associated with this is the overexpression of certain ATP binding cassette (ABC) transporters, majorly p-glycoprotein (Pgp) as these are thought to enhance drug resistance by facilitating drug efflux in human tumours (Robert et al., 2010). ABC transporters are members of a superfamily of trans-membrane proteins that depends on ATP hydrolysis to move various substances, including nutrients and xenobiotics (drugs inclusive) across biological membranes (Sharom, 2008). Considering the role of efflux pumps in drug resistance, it could be posited that efflux inhibitors may improve chemotherapy. It has also been reported that many calcium channel blockers (e.g. verapamil) and Ca^{2+}/CaM antagonists enhanced Pgp-mediated transport of vincristine and doxorubicin (Tsuruo et al., 1982). The effect of verapamil and promethazine on MDR reversal has also been investigated in doxorubicin resistant MCF-7 cells and a significant decrease in both MDR1 and MRP1 expression levels were reported after promethazine and verapamil treatment (Dönmez et al., 2011). Trifluoperazine has also been reported to overcome drug resistance in lung cancer as well as inhibiting cancer stem cells (Yeh et al., 2012). In another study, novel PTZ-derivatives were shown to possess MDR reversing activity on Pgp (ABCB1)-related resistance of mouse T-lymphoma cells (Spengler et al., 2014). PTZs were also reported to improved plasma retention of doxorubicin and etoposide in patients receiving chemotherapy and this was thought to be via regulation of Pgp (Raschko et al., 2000).

2.7.3.3 Induction of reactive oxygen species

Reactive oxygen species (ROS) are important signalling molecules that modulate cellular responses and could perform dual roles in cancer cells. ROS affects genomic stability to enhance proliferation, survival, angiogenesis, and metastasis of cancer cells (Sabharwal and Schumacker, 2014). Growth factors and cytokines also mediate their numerous biological activities in cancer cells by ROS production (Woo et al., 2000, Kim et al., 2010). However, the role of ROS in cancer is dual as ROS generation has also been reported to induce cell death in cancer cells (Nogueira et al., 2008, Xie et al., 2011). Trifluoperazine was reported to increase oxidative stress as well as elicit apoptosis associated with lysosomal expansion and intense vacuolation in human lung cancer cells (Zong et al., 2011b). Thioridazine, a PTZ has been reported to sensitize renal carcinoma Caki cells to TRAIL-induced apoptosis via reactive oxygen species-mediated inhibition of Akt signalling as well as modulation of other signalling pathways (Min et al., 2014b).

2.7.3.4 Inhibition of Phosphatidylinositol 3-kinase (PI3K)/Akt signalling

The PI3K/Akt pathway is activated by multiple signalling entities, including PDGF, EGF and insulin-like growth factor-1(IGF1) and is constitutively active in numerous cancers where it promotes cell proliferation and survival (Altomare and Testa, 2005). Interestingly, it has been reported in several studies that PTZs might be promoting their antitumour activity via negative regulation of this pathway. Thioridazine was reported to inhibit phosphorylation of Akt in ovarian, cervical and endometrial cancer cells on both Thr-308 and Ser-473 which are necessary for the activation of Akt by upstream kinases (Rho et al., 2011, Kang et al., 2012, Park et al., 2014). Thioridazine also inhibited angiogenesis and tumour growth by targeting the VEGFR-2/PI3K/mTOR pathway in ovarian cancer xenografts as well as inhibiting Akt in renal carcinoma Caki cells (Min et al., 2014a, Park et al., 2014). Phenothiazines were reported to have induced PP2A-

mediated apoptosis and induce autophagy by inhibiting Akt phosphorylation in T cell acute lymphoblastic leukemia and oral cancer cells (Gutierrez et al., 2014a, Wu et al., 2016). Chlorpromazine has equally been reported to induce autophagic cell death in U87 glioblastoma cells by inhibiting Akt/mTOR pathway (Shin et al., 2013). Altogether, these data indicate that PTZs may impact PI-3K/Akt/mTOR signalling on multiple levels.

2.7.3.5 Inhibition of anti-cancer stem cell activities

Another mechanism mediating the anti-cancer activity of PTZs is their ability to inhibit cancer stem cells. Thioridazine, was reported to selectively induce differentiation in cancer stem cells by negatively regulating the dopamine receptors D2 (DRD2) expressed in most tumour stem cells. It also showed affinity to neoplastic cells and inhibited human somatic CSCs capable of initiating leukemic disease *in vivo* (Sachlos et al., 2012). More recently, thioridazine treatment significantly inhibited lung cancer xenografts in mice as well as impaired growth of lung cancer stem-like cells both *in vitro* and *in vivo* (Shen et al., 2017).

2.7.3.6 Regulation of DNA damage response

PTZs have been reported to impact on DNA damage response by inhibiting DNA-dependent kinase activities, thus delaying the DNA repair process (Eriksson et al., 2001, Gangopadhyay et al., 2007). It has also been reported that trifluoperazine inhibited the repair of double strand DNA breaks in human cancer cells and also delayed the ability of γH2AX resolution in lung cancer cells with DNA-damage (Gangopadhyay et al., 2007, Zong et al., 2011a). Furthermore, PTZs act as inhibitors of Tousled-like kinases (which participate in DNA repair chromatin assembly) and affected double-strand DNA breaks repair and also potentiated tumour death (Ronald et al., 2013). More recently, a novel PTZ was shown to induce DNA damage via generation of ROS in the Ca922 oral cancer cells (Wu et al., 2016).

2.8 DNA damage response pathway

Mammalian cells are frequently faced with the challenge of damaged DNA which can either arise from replication stress or from other sources including exposure to chemotherapeutic agents, UV light, reactive oxygen species generated during cell metabolism or inflammation (Goldstein and Kastan, 2015). In response to this DNA lesions, the DNA damage response (DDR) which involves a cascade of events is triggered (**Figure 2.15**). The DDR could lead to a halt in the cell cycle to repair damaged DNA, however if cells fail to repair damaged DNA, cells may undergo senescence or proceed to programmed cell death (Kastan and Bartek, 2004). DNA lesions could either involve a single-strand or double-strand of the DNA, where double-strand DNA breaks (DSBs) are known to be more lethal (Goldstein and Kastan, 2015).

Following DNA damage, members of the phosphoinositide-3-kinase-related protein kinase (PIKK) including ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-Related), and DNA-PK (DNA-dependent protein kinase) are usually recruited to the site of damage (Zou, 2007, Lempiäinen and Halazonetis, 2009). In particular, ATM and DNA-PKs mainly respond following DSBs while the ATR is activated following single-strand breaks (Stokes et al., 2007). However, ATM can also activate other downstream targets of the ATR pathway and some anti-cancer agents have been shown to activate both pathways, thus indicating that both pathways are critical for the DDR (Liang et al., 2009).

In response to DSBs, ATM is recruited to the sites of damage, leading to phosphorylation of histone H2AX (γH2AX), which results in the accumulation of more ATM complexes to increase their activity (Celeste et al., 2002). Upon the increase in ATM activity above a certain threshold, ATM phosphorylates the checkpoint kinases (Chk2) at threonine 68

to activate it (Buscemi et al., 2004). Activation of Chk2 continues to spread the DDR signalling leading to phosphorylation of its substrates p53 and CDC25 which could trigger an arrest of the cell cycle (Lukas et al., 2003). The phosphorylation of p53 leads to its activation and stabilization and p53 which in turn transcriptionally activates the p21 cyclin-dependent kinase inhibitor to induce a cell cycle arrest.

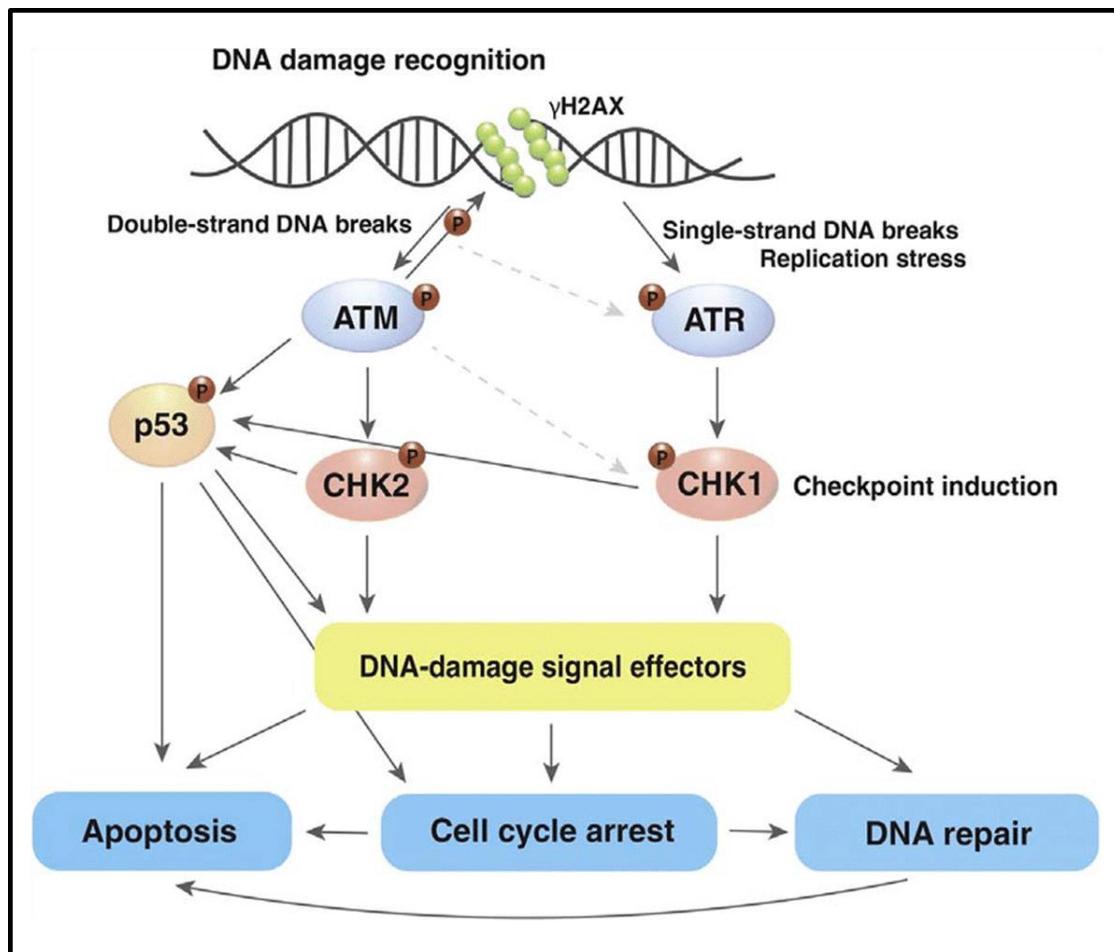


Figure 2.15. DNA damage response pathway (Tšuiko et al., 2018)

2.9 Programmed cell death

When cells are no longer needed, they commit suicide via the activation of intracellular death programmes and this process is referred to as programmed cell death (PCD) (Ouyang et al., 2012). PCD could either be referring to apoptosis (type I), autophagy (type II) or programmed necrosis (type III) and in some cases, the three forms of PCD may jointly decide the endpoint of cells of malignant cancers (Clarke, 2002, Gozuacik

and Kimchi, 2007, Laubenbacher et al., 2009). Apoptosis and programmed necrosis definitely culminate in cell death while autophagy can play dual roles, either as pro-death or pro-survival and all pathways can be morphologically distinguished (Bialik et al., 2010). Disruption of PCD has been implicated in cancer and therefore understanding the signalling pathways involved in PCD could aid in the development of novel anti-cancer agents (Sun and Peng, 2009, Ouyang et al., 2012). Majority of anti-cancer agents have been reported to elicit their anti-cancer efficacy mainly through apoptosis but lately autophagy and necrosis have been implicated as other modes of cell death (Pommier et al., 2004, Lopez and Tait, 2015, Dillon and Green, 2016). For the purpose of this study, this section will be restricted to apoptosis and autophagy which are the most common forms of cell death.

2.9.1 Apoptosis

Apoptosis is a form of programmed cell death (the earliest identified) that is essential for normal cell functioning and the term was derived from the Greek language referring to the "dropping off" of leaves from a tree (Hotchkiss et al., 2009). Apoptosis involves a series of well-orchestrated events in which cells are programmed to die after they receive specific stimuli and plays a vital role in growth control (Cope and Tomei, 1991). Apoptosis is implicated in both physiological and pathological processes (Danial and Korsmeyer, 2004). It is very critical for the survival of multicellular organisms as it continuously gets rid of contaminated or infected cells that may hamper normal cell function (Labbe and Saleh, 2008). A notable physiological role of apoptosis is the removal of inter-digital cells during limb development, clearance of non-functional nerve cells, and activated lymphocytes (Nagata, 2018). Apoptosis also plays a role in the eradication of unwanted cells that are rapidly proliferating or cells with damaged DNA (Dillon and Green, 2016). Dysregulation of apoptosis is associated with carcinogenesis

as cancer cells usually evade apoptosis and present with damaged DNA and uncontrolled proliferation (Brown and Attardi, 2005). Thus apoptosis is critical for cancer treatment which explains why many anti-cancer therapies are developed to target apoptotic pathways (Wong, 2011, Hongmei, 2012).

Morphologically, cellular changes associated with early apoptosis can be observed using the light microscope and these include cell shrinkage (reduced cell size and tightly packed cytoplasmic organelles) and pyknosis (condensed chromatin) (Häcker, 2000). Biochemically, cells undergoing apoptosis show varying degrees of biochemical alteration including; cleavage of proteins, DNA fragmentation, as well as protein cross-linking (Hengartner, 2000).

2.9.1.1 Mechanism of apoptosis

The mechanisms involved in apoptosis are characterized by a cascade of complex signalling events involving of a number of cysteine aspartyl-specific proteases (caspases) which are sophisticated and energy dependent (Nagata, 2018). The two major apoptotic pathways and include the extrinsic pathway (which involves binding of death ligands to death receptors) as well as the intrinsic pathway (mitochondrial pathway) (**Figure 2.16)** (Parrish et al., 2013). However, it has now been established that both pathways are inter-linked as molecules from one pathway can influence the other (Igney and Krammer, 2002). However, both pathways terminate to activate a series of caspases and converge in activating caspase 3 and 7 (executioner caspases) which eventually result in the cleavage of various cellular substrates including Poly (ADP-ribose) polymerase (PARP) (Slee et al., 2001). The resulting dead cells are taken up by phagocytic cells through a process known as efferocytosis (from the latin word “efferre” meaning “to take to the grave” or “to bury”) (Henson, 2003).

2.9.1.1.1 Extrinsic pathway

The extrinsic apoptotic pathway is receptor-mediated and is also referred to as the death signalling pathway as it is activated by death factors of the TNF family (FasL, Fas ligand; TNF-α, tumour necrosis factorα; TRAIL, TNF-related apoptosis-inducing ligand) (Nagata, 1997, Strasser et al., 2009). The death receptors comprise of a cytoplasmic component which is called the death domain and is involved in their interactions with the appropriate ligand. The binding of death receptors to their corresponding ligands in turn alters the conformation of their intracellular domains thus leading to their activation (Fulda and Debatin, 2006). Following activation, death receptors recruit apoptotic proteins which in turn leads to the formation of the death signalling complex (DISC) which activates the initiator caspases involved in the extrinsic pathway (caspase 8) (Mannick et al., 1999, Ouyang et al., 2012). For example, when there is a stimulus, Fas combines with FasL which leads an alteration in conformation to enhance its interaction with the FADD (Fas-Associated Death Domain) which binds to pro-caspase 8. In turn, the complex formed activates the pro-caspase-8, which culminates in the triggering of pro-caspase-3, the perquisite executing enzyme for apoptosis (Nagata, 2018). Aside the direct induction of apoptosis via activation of caspases, the extrinsic pathway signalling can indirectly amplify death signals and also trigger the intrinsic/mitochondrial pathway apoptosis (Wu et al., 2001)

2.9.1.1.2 Intrinsic pathway

The intrinsic apoptotic pathway is mediated by the mitochondria and is the most deregulated death pathway in cancer and usually a target of most anti-cancer therapies (Lopez and Tait, 2015). It is triggered by a plethora of signals including DNA damage, hypoxia, endoplasmic reticulum (ER) stress and growth factor deprivation (Labi and Erlacher, 2016). The intrinsic pathway is activated following increased outer

mitochondria membrane permeability (MOMP) which in turn, triggers the release of two sequestered groups of pro-apoptotic proteins from the intermembrane space to the cytosol (Saelens et al., 2004). The first group of sequestered proteins includes cytochrome c, SMAC/DIABLO while the second group includes the apoptotic protease-activating factor-1 (Apaf-1) (Garrido et al., 2006, Putcha et al., 2002). Cytochrome c binds Apaf-1 to stimulate (d)ATP-dependent oligomerization of Apaf-1 into a caspase-activating complex known as the Apaf-1 apoptosome (Hill et al., 2004, Wu and Bratton, 2013). Consequently, apoptosome recruits the initiator procasapase-9 through the caspase recruitment domains (CARDs) to activate caspase-9 (Kurokawa and Kornbluth, 2009). Activation of caspase-9 triggers the activation of the executioner caspase 3 which finally leads to cleavage and degradation of cytoplasmic structural proteins and chromosomal DNA (Benn and Woolf, 2004). Apart from the caspase-dependent pathway, MOMP also releases other proteins such as apoptosis inducing factor (AIF) and endonuclease G which are not caspase dependent but leads to DNA fragmentation and thus cell death (Li et al., 2001, Penninger and Kroemer, 2003).

The intrinsic apoptotic pathway is tightly regulated by opposing actions of the members of the B-cell lymphoma protein 2 (Bcl2) family of proteins which are divided into 3 categories; the pro-apoptotic proteins, pro-survival or anti-apoptotic and the regulatory or sensory proteins (Koff et al., 2015). The anti-apoptotic proteins comprises of the BCL-2, BCL-XL, BCL-W, MCL1, BCL-B and A1 which act by inhibiting the pro-apoptotic proteins (BAX, BAK and BOK) and in turn containing cytochrome c within the mitochondria (Wei et al., 2001). Furthermore, the regulatory proteins consist of the BH3-only proteins (BAD, BIK, BID, HrK, BIM, BMF, NOXA and PUMA), responsible for opposing and downregulating the anti-apoptotic proteins thus leading to the activation of the pro-apoptotic proteins and subsequent release of cytochrome c to the cytosol (Youle

and Strasser, 2008). Another negative regulator of this pathway is the inhibitor of apoptosis (IAPs) protein family which directly inhibits caspases via several modes of action (LaCasse et al., 2008).

However, there seems to be a cross-talk between the extrinsic and intrinsic apoptotic pathway (**Figure 2.16)** as it has been suggested that the mitochondria and Fas pathways may be mediated by the caspase-8 cleavage of Bid (Li et al., 1998, Degli Esposti, 2002, Igney and Krammer, 2002).

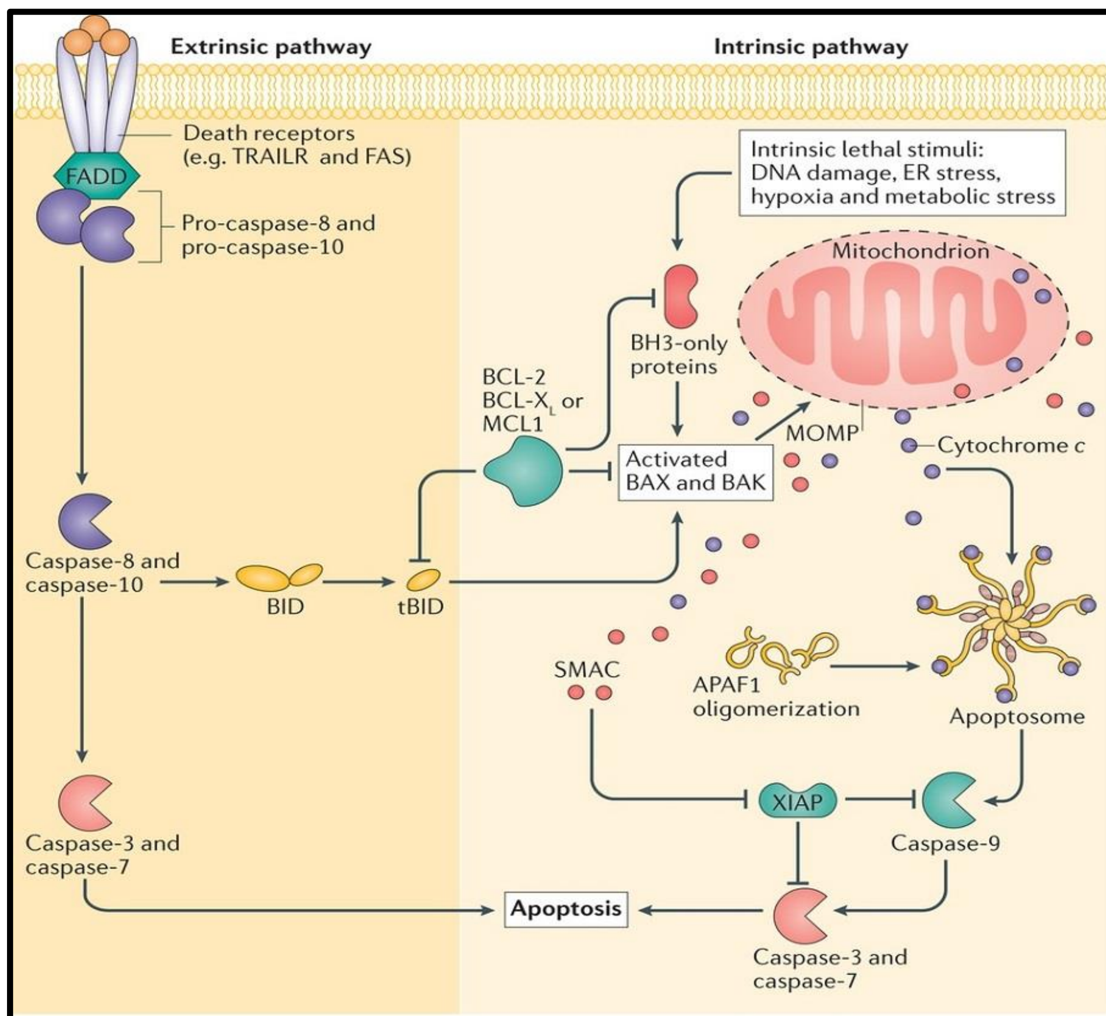


Figure 2.16. Mechanism of apoptosis ©https://biologydictionary.net/apoptosis/

2.9.2 Autophagy

The term autophagy was coined from two Greek words, "auto" which means "oneself" and "phagy" which means to eat (Levine and Kroemer, 2008). It is a conserved intracellular degradation pathway that involves the trafficking of cytoplasmic organelles, infectious agents and protein aggregates to the lysosomes through double-membrane vesicles called autophagosomes (Mizushima and Klionsky, 2007, Bento et al., 2016). Autophagy plays a complex tissue specific role in normal body structures and contributes to pathogenicity in various conditions including aging, liver and neurodegenerative diseases (Levine and Kroemer, 2008, Mizushima and Komatsu, 2011).

Autophagy plays a dual role in cancer pathophysiology as it can promote tumour survival on the one hand or tumour suppression on the other. In the early stages of cancer, autophagy is thought to prevent cancer progression, but upon establishment of cancer, increased autophagic flux enhances tumour cell survival and proliferation (White, 2012, Amaravadi et al., 2016). The loss of a single allele of the *Beclin-1* autophagy gene was found in about 40 to 75% of human breast, prostate, and ovarian cancers, thus giving an indication of the tumour suppressive role of autophagy in these cancers (Qu et al., 2003). Autophagy also enhances resistance of cells to adverse conditions (starvation) thus promoting survival (Degenhardt et al., 2006). Hypoxia in the necrotic core of tumours and high metabolic demand associated with rapid cell proliferation, is associated with stress in the tumour and is known to induce autophagy (Mazure and Pouyssegur, 2010). Inhibition of pro-survival autophagy by genetic or chemotherapeutic agents was shown to drive apoptosis in tumour cells (Amaravadi et al., 2007, Carew et al., 2007).

Conversely, autophagy induction has been implicated in the modulation of cell death but this is largely dependent on the cell and the extent of autophagy. Prolonged autophagy has been shown to induce tumour cell death (Yu et al., 2004). Several preclinical studies

have shown that autophagy mediates cell death in cancer depending on the nature of the cell. A novel small molecule (STF-62247) was also reported to induce autophagic cell death in VHL-deficient renal cell carcinoma cells (Turcotte et al., 2008). More recent studies have also reported that arsenic trioxide, radiation and other anti-cancer agents induced autophagic cell death in many cancer cells including glioblastoma (Chiu et al., 2016, Yu et al., 2016, Mahase et al., 2017, Wu et al., 2018).

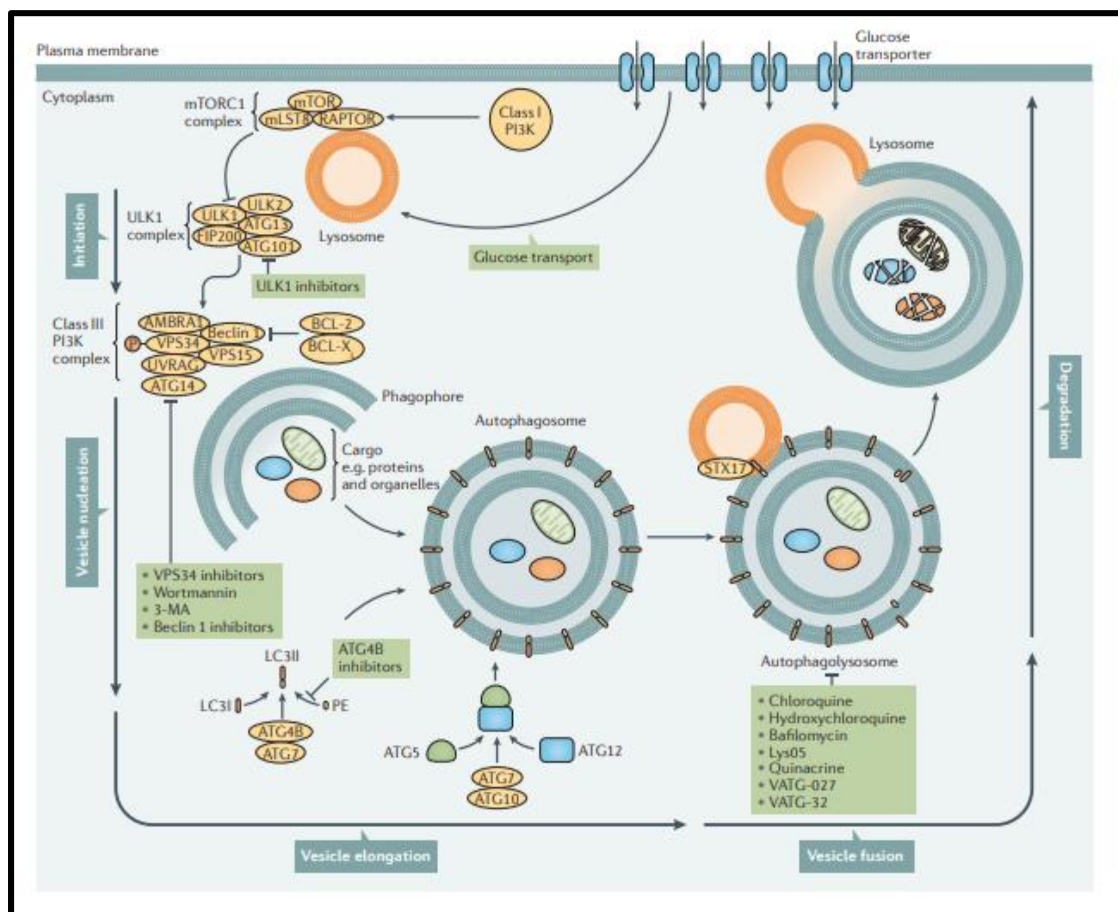


Figure 2.17. Autophagic pathway (Levy et al., 2017)

2.9.2.1 Regulation of autophagy

The stages involved in autophagosome formation include initiation, nucleation of the autophagosome, expansion and elongation of the autophagosome membrane as well as closure and fusion with the lysosome, and the degradation of intravesicular products (**Figure 2.17**). These processes are regulated by the autophagy-related genes (Atg)

(Mizushima et al., 2011). Autophagy initiation is controlled by the ULK1 (also known as ATG1) kinase complex which comprises of ULK1, Atg13, and Atg17, and their interaction with mTOR complex 1, a downstream target of the PI3K pathway (Klionsky, 2007, Jung et al., 2009, Mizushima, 2010). Inhibition of mTORC1 leads to the formation of autophagosomes and this happens in conjunction with the formation of a complex between the vacuolar sorting protein 34 (Vps34), a class III PI3K and Beclin-1(Pattingre et al., 2008). Upon formation of the Beclin-1/Vps34 complex, PtIns3P is secreted for the recruitment of other autophagy genes important for autophagosome formation (Pattingre et al., 2008). Expansion of autophagosome membrane is enabled by the Atg5-Atg12 complex in conjunction with Atg16 (Matsushita et al., 2007). Following this, members of the LC3 and GABARAP protein families are conjugated to the lipid phosphatidylethanolamine (PE) and recruited to the membrane. Furthermore, the Atg4 and ATG7, conjugates LC3-I and PE to form membrane-bound, lipidated form, LC3-II which serves as a marker for autophagosome formation (Klionsky et al., 2016). The final stage of the autophagy process is the fusion of autophagosomes and lysosomes to form autolysososmes. This stage is characterized by the binding of LC3-II to the adaptor protein p62/sequestosome1 (SQSTM 1), which is involved in the cargo of proteins to the proteasome for degradation. (Pankiv et al., 2007). Usually, p62/SQSTM 1 is degraded during autophagy but it accumulates when there is impairment of fusion of autophagosomes to lysosomes and can be used as a measure of autophagic flux (Mathew et al., 2009, Klionsky et al., 2016).

A critical regulator of autophagy is mTOR which activates downstream of the PI3/Akt pathway (a pro-survival pathway that is active in many cancer types) (Pattingre et al., 2008, Scherz-Shouval and Elazar, 2011). The loss in function of tumour suppressors (LKB1, PML, PTEN, and TSC1/2) or gain-of-function mutations in tyrosine kinases

receptor can also activate mTOR (Semenza, 2010). Furthermore, adenosine monophosphate kinase (AMPK) activated by a decline in intracellular energy (metabolic sensor) also results in the inhibition of mTOR, thus initiating autophagy (Jung et al., 2009, Li et al., 2013). AMPK has also been found to phosphorylate ULK1 directly, an interaction necessary for cell survival during starvation as well as maintenance of mitochondrial homeostasis (Egan et al., 2011). The p38, ERK and JNK which are members of the mitogen activated protein kinase (MAPK) family have also been implicated in the regulation of autophagy (Wagner and Nebreda, 2009, Sui et al., 2014, Zhou et al., 2015, Mukherjee et al., 2017).

Most of the steps in the autophagy pathway are potential drug targets and provides a means though which autophagy can be positively or negatively influenced (Levy et al., 2017). Pharmacological inhibitors of autophagy can either affect the pathway at the early or late stage, with early stage inhibition targeting class III PI3K (Vps34) and interfering with its recruitment to the membranes. Inhibitors include 3-methyadenine (3MA), wortmannin, and LY294002. Conversely, inhibitors of the late stage which act by preventing the fusion of autophagosomes to lysosomes include the antimalarial drugs chloroquine (CQ) and hydroxychloroquine (HCQ) as well as bafilomycin A1, and monensin (Shacka et al., 2006).

2.9.3 Cross-talk between apoptosis and autophagy

There exists a cross-talk between autophagy and apoptosis owing to the fact that both pathways sometimes share similar mediators and regulators (Maiuri et al., 2007). It has been shown that numerous anti-cancer agents induce both apoptosis and autophagy thus suggesting a link (Kroemer and Levine, 2008). Other studies have shown that p62/SQSTM 1 (autophagy) interacts with caspase-8 (extrinsic apoptotic pathway) to enable its aggregation and activation leading to enhanced TRAIL-mediated apoptosis

(Jin et al., 2009). Members of the caspase and calpain family also provide evidence to suggest a link between apoptosis and autophagy as they cleave Beclin-1, Atg4 and Atg5 proteins, thus negatively regulating autophagy (**Figure 2.18**) (Luo and Rubinsztein, 2010, Li et al., 2011).

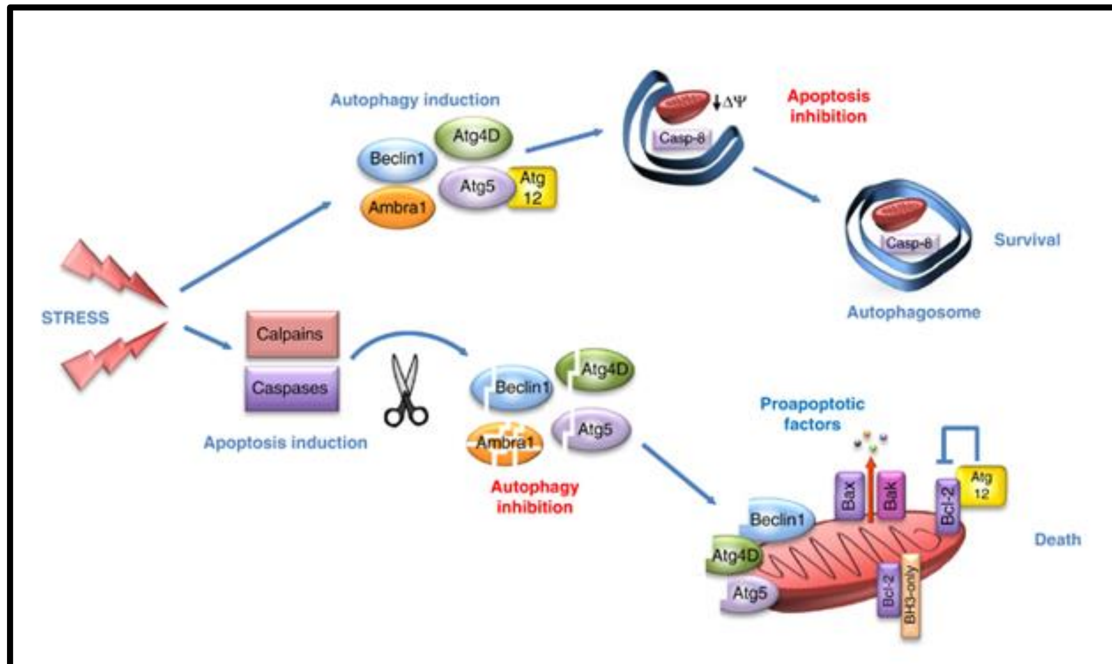


Figure 2.18. Cross-talk between apoptosis and autophagy (Fimia et al., 2013)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Cell lines and culture conditions

3.1.1 Human malignant glioma cell lines U251, U87 and non-cancer cell line, FG0 fibroblasts

The human malignant glioma cell lines U251, U87 (glioblastoma multiforme, World Health Organization grade IV) and normal fibroblast cell line FG0 were grown in a monolayer using GlutaMAX™ Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies Corporation, Paisley, UK) and 1% 100 U/mL penicillin and 100 µg/mL of streptomycin (Lonza Group Ltd., Verviers, Belgium). Cells were maintained at 37°C, in a humidified atmosphere consisting of 5% CO_2 and 95% air. Cell growth media was changed routinely after two to three days and sub-culturing of cells were performed with a solution of 0.25% trypsin EDTA (Lonza Group Ltd., Verviers, Belgium) when cells achieved 80% confluency. Mycoplasma tests were routinely conducted and experiments were conducted on cells devoid of mycoplasma.

3.1.2 Primary cell culture and reagents

The work on primary glioblastoma cell cultures was done in collaboration with, Associate Professor Petra Hamerlik, principal investigator at the Brain Tumour Biology Group, Danish Cancer Society Research Centre (DCRC), Copenhagen, Denmark. Primary glioblastoma cell cultures T10 and T91 were established following approval by Danish Ethical Committee/Danish Data Protection Agency (2006-41-6979/KF- 01-327718) and duly signed informed consents were obtained from each patient 24 hours prior to surgery (Staberg et al., 2018). Cells were grown in complete Neurobasal®-A Medium (Minus Phenol Red), supplemented with 10 mL B27 minus vitamin A (50X),

12587010, 5 ml Glutamax, (100X), 35050-038 (all manufactured by Invitrogen, Taastrup, Denmark), 20 ng/ml EGF recombinant, 1mg, 236-EG-01M (R&D systems), 20 ng/mL FGF recombinant, 1mg, 4114-TC-01M (R&D systems) as well as penicillin (50 U/mL), and streptomycin (50 µg/mL) (Invitrogen, Taastrup, Denmark). For T10 cells, cells were grown as spheres in 15 cm dishes while the T91 cells grew as fairly adherent monolayers and maintained in a humidified chamber as mentioned previously for the malignant glioblastoma cells. For sub-culturing, cells were dissociated using the cell dissociation reagent TrypLE Express (1X) (Invitrogen, Taastrup, Denmark) prior to every experiment to obtain single cells.

3.2 Mycoplasma tests

In order to detect mycoplasma infection, U87 and U251 cells were cultured in antibiotic-free medium on a coverslip in a 35 mm dish for 24 hours. A 3:1 ratio of methanol and acetic acid solution was then used to fix the cells for 10 seconds after which cover slips were washed with distilled water to remove excess fixative. Coverslips were left to air dry at room temperature and cells were then stained with a 0.5 µg/mL Hoechst 33258 (Sigma-Aldrich, USA) for 10 seconds. Cells were washed again with distilled water to remove excess staining solution and mounted on a slide using a mounting fluid (**Appendix 7.1**). Cells were viewed using the DAPI channel in a fluorescence microscope (Zeiss Axioskop 2 MOT Fluorescence Microscope, Germany).

3.3 Treatments

3.3.1 Treatment with phenothiazine derivatives

Several remodelled PTZ derivatives were synthesized by our collaborator, Dr Anwar Jardine of the Chemistry Department, University of Cape Town, with the aim of assessing their anti-cancer activities. The required quantity of compounds to make stock solutions of 10 mM were weighed and dissolved in appropriate volumes of Dimethyl

sulfoxide, DMSO (Sigma-Aldrich, USA). Aliquots of 10 µL of compounds were placed in Eppendorf tubes and stored at -20°C for use within one month to prevent repeated freeze and thaw cycles. On the day of treatment, a single Eppendorf tube of each compound was taken out of the freezer and diluted in appropriate volumes of medium to get the final concentrations to be used for treatment. A vehicle control containing the same amount of DMSO (solvent used to dissolve PTZs) as the highest concentration of PTZ treatment was used to treat the cells. After cytotoxicity screening of the compounds, 4 compounds displayed cytotoxicity under 30 µM and the two most active compounds, DS00326 and DS00329 were further characterized.

3.3.2 Treatment with autophagy inhibitors

In order to inhibit autophagy, cells were treated with 10 nM of bafilomycin and 1µM wortmannin (Sigma Aldrich, USA) for 1 hour before treatment with PTZs. Cells were allowed to incubate for 48 hours whereafter 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide (MTT) assays were performed to determine cell viability. For western blotting experiments to investigate autophagy flux, cells were treated with 200 nM of bafilomycin for the last 2 hours before termination of the experiment (Del Bello et al., 2013).

3.4 Cell viability assay

The MTT assay, a colorimetric assay for assessing the metabolic activity of cells, *in vitro* was used to measure cell viability. MTT is a yellow tetrazolium salt which is reduced to purple formazan in living cells. The MTT cell proliferation assay kit (Roche, USA) was used following the manufacturer's protocol. Briefly, U251 and U87 cells were seeded in sterile 96-well cell culture plates at a density of 4000 cells per well and allowed to settle overnight. The expended medium was subsequently substituted with fresh growth medium comprising of increasing concentrations of the various PTZ-derivatives

and vehicle (DMSO) treated cells served as control. All treatments including the vehicle control lasted for 48 hours and were done in quadruplicate. After treatments, cells were incubated with 10 µL of the MTT solution (5 mg/mL) for 4 hours at 37°C, and the resultant formazan was solubilized using 100 µL of the solubilizing buffer (10% SDS in 0.01 M HCl). Plates were then left overnight at 37°C and absorbance was read at 570 nm using a microplate reader (BMG Labtech Omega® POLARStar). The percentage cell viability was calculated using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated well}}{\text{Absorbance of untreated well}} \text{ X } 100$$

For the primary patient-derived xenograft glioblastoma cells, T10 and T91, cells were trypsinized into single cells and seeded at a density of 3000 cells per well in a 96-well plate at a volume of 50 µL for each well. Cells were allowed to settle overnight after which they were incubated for 48 hours at 37°C with 50 µL of increasing drug concentrations (0 to 15 µM) of DS00326 and DS00329, giving a final volume of 100 µL/well. After 48 hours, 100 µL of CellTiter-Glo® luminescent cell viability assay (Promega, Madison, USA) reagent was added to each well and allowed to incubate for 20 minutes at 37°C. Luminescence was read as stated in the manufacturer’s protocol using the VICTOR Multilabel Plate Reader (PerkinElmer, Inc.) and cell viability was calculated.

The concentration required to kill 50% of the cells (IC_{50}) was determined through a survival curve using GraphPad Prism6 software (GraphPad software, San Diego, CA, USA) from three experimental repeats conducted in quadruplicates.

3.5 Selectivity index

In order to determine the selectivity of DS00326 and DS00329 to cancer cells, their effect on cell viability in normal cells (non-cancerous) was ascertained. The FG0 normal fibroblasts were cultured and seeded at a density of 3500 cells per well in a 96-well plate and allowed to settle overnight. Following that, cells were treated with increasing concentrations of DS00326 and DS00329 used to treat the glioblastoma cells for 48 hours. After treatment, MTT assay was performed as stated above to determine cell viability. Selectivity index was calculated with the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated well}}{\text{Absorbance of untreated well}} \text{ X } 100$$

3.6 Clonogenic assay

The clonogenic assay is an *in vitro* assay that serves as a veritable tool to determine if an anti-cancer agent can inhibit survival in tumour cells after treatment. It also ascertains the reproductive capacity of cells to form colonies when cells are plated in very low densities, a colony is regarded as an aggregate of 50 cells (Franken et al., 2006). This experiment was conducted to investigate the impact of DS00326 and DS00329 on the survival of both U87 and U251 glioblastoma cells. Briefly, cells were seeded in 6 cm dishes at a density of 1.641 X 10^5 cells/dish (density at which cells attained 60-70% confluence) and allowed to settle for 24 hours, after which they were treated with the vehicle or two different concentrations (half the IC_{50} and IC_{50}) of DS00326 and DS00329 for 24 hours. Following treatment, cells were trypsinized, resuspended in medium and replated in 35 mm dishes at a low density of 500 cells/dish, followed by incubation at 37°C and routine replacement of the growth media every 3 to 4 days to allow for colony formation. Dishes were monitored to observe formed colonies were stained any time between 10 to 21 days with the vehicle-treated cells as indicators.

For staining, cells were washed 3 times with 1X PBS to remove excess media, fixed for 15 minutes in methanol: acetic acid (3:1) and washed 3 times with 1 X PBS (**Appendix 7.2**) to remove excess fixative. Thereafter, cells were stained for 15 minutes with 0.5% crystal violet (Sigma-Aldrich, USA) in 100% methanol and washed again with 1X PBS to remove excess stain followed by gently immersing dishes in distilled water. Images of dishes were taken with a digital camera and the area covered by colonies was calculated using colony area plugin on ImageJ software (Guzmán et al., 2014). Percentage of areas covered by colonies was then calculated for each treatment concentration relative to the vehicle control treated cells and data sets were analysed using GraphPad Prism version 6.

3.7 Cell morphology

In order to ascertain morphological changes associated with the treatments, U87 and U251 cells were plated on 6 cm dishes and left overnight at 37°C to achieve a confluency of 60 - 70%. After treatments, expended media were replaced and cells were treated with the vehicle or IC_{50} of DS00326 (6.11 μM - U87; 4.51 μM - U251) as well as DS00329 (9.29 μM - U87; 5.12 μM - U251) for 48 hours. Morphological changes following treatment were monitored using an inverted light microscope (Olympus, USA) and photographs taken with a Zeiss Axiocam (Germany) camera.

3.8 Flow cytometry

3.8.1 Cell cycle analysis

The cell cycle profile was analysed in both U87 and U251 cells by labelling cells with a DNA staining dye and in this case propidium iodide (PI) was used to measure the DNA content of cells in their respective phases of the cell cycle (G0/G1, S and G2/M). Briefly, cells were treated with either the vehicle or IC_{50} of DS00326 or DS00329 for 24 and 48 hours. After treatment, cells were trypsinized and washed twice in 1X PBS before

centrifuging at 1500 rpm for 5 minutes. Pellets were resuspended in 2 mL of cold 1X PBS and 8 mL of ice-cold 70% ethanol was added to the cells for fixation and cells were stored in -20˚C. On the day of FACS analysis, cells were taken out of storage and centrifuged at 1500 rpm at room temperature and pellets were resuspended in 1X PBS and centrifuged again. The resultant pellets were stained with 0.5 mL of FxCycle™ PI/RNase (Molecular Probes, Life Technologies, UK) for 30 minutes. Acquisition of cell cycle was done using the FACSCalibur flow cytometer (Beckman Coulter, USA) and data was analysed using the Cell Quest Pro version 5.2.1.

For the primary PDX glioblastoma cultures, T10 and T91 cells were plated in 6 cm dishes at a suitable density and allowed to settle overnight, after which cells were then treated with either vehicle or medium containing compounds DS00326 (5.06 µM - T10; 1.60 µM - T91) and DS00329 (12.53 µM - T10; 5.4 µM - T91) for 48 hours. Cells were dissociated after treatment and placed in 15mL conical tubes, followed by centrifugation to remove the dissociation reagent and washing with 1XPBS before re-centrifugation. The supernatant thereafter was discarded and cells were resuspended in 300 µL 1X PBS before fixing in 700 µL ice cold 70% methanol and stored at -20˚C until FACS acquisition. On the day of acquisition, cells were centrifuged to remove fixative and were washed with 1XPBS before staining with 300 µL FACS solution (10 µg/mL of PI, 0.1% (v/v) Triton X-100, 10 µg/mL and 100 µg/mL of DNase-free RNase A in PBS) for 1 hour. After staining, cell cycle profiles of cells were acquired using the FACSVerse Flow Cytometer (BD Biosciences, USA).

3.8.2 Quantification of apoptosis

The Annexin V/PI assay staining kit (Molecular Probes, Life Technologies, UK) was used to measure apoptosis. This assay is based on the fact that apoptotic cells have exposed phosphatidylserine molecules (Fadok et al., 1992) and thus bind Annexin V,

while necrotic cells have compromised membranes and thus take up propidium iodide (Vermes et al., 1995). Four different populations of cells are easily distinguished: unlabelled (viable) cells, cells bound to Annexin V-FITC only (early apoptotic), cells stained with PI (necrotic) and cells that stain positive for both Annexin V-FITC and PI (late apoptotic cells).

Briefly, cells were plated at an appropriate density and allowed to attach for 24 hours before treatment with either the vehicle or IC_{50} of DS00326 or DS00329 for 24 and 48 hours. After treatment, cells were trypsinized, pelleted by centrifugation at 1500 rpm for 5 minutes, resuspended and centrifuged twice in ice-cold PBS, and further processed as per manufacturer's protocol. Briefly, pellets were resuspended in 1X Annexin binding at a density of 1.0 X 10^5 cells/mL in 100 µL and incubated with Annexin V-FITC/PI for 15 minutes at room temperature. Thereafter, 400 µL of 1X Annexin binding buffer was added to each sample, and analysed with Beckman Counter FACSCalibur flow cytometer (Beckman, USA) using the BD CellQuestTM Flow Cytometry Software (BD Biosciences, USA).

3.9 Fluorescent microscopy

3.9.1 Acridine orange staining for acidic vesicular organelles (AVOs)

Formation of acidic vesicular organelles (AVOs), a morphological feature of cells undergoing autophagy was investigated for by acridine orange staining (Levine and Yuan, 2005). Acridine orange is a weak base that accumulates in acidic compartment of cells and it gives a bright orange fluorescence in the cytoplasm and this can be observed by fluorescent microscopy. The fluorescent intensity is proportional to the degree of acidity in the cells. In order to investigate the formation of AVOs in U87 and U251, cells were plated on coverslips in 35 mm dishes and allowed to attach for 24 hours. Thereafter, cells were treated with the vehicle or IC_{50} of DS00326 or DS00329 for 24 and 48 hours.

After treatment, cells were stained with 1 μg/mL acridine orange in PBS for 15 minutes in the dark and then mounted on a glass slide for visualization on the confocal microscope (Zeiss LSM 510 Meta with NLO, Software: ZEN 2015, Lasers: Argon 488 green, solid-state laser: 561 nm Red, Germany).

3.9.2 Immunofluorescence for γH2AX

To investigate γH2AX foci formation as indication of DNA damage following treatments, cells were plated in coverslips. Briefly, overslips to be used were put into appropriate dishes and washed twice with 70% ethanol and twice with 1X PBS. Coverslips were coated with geltrex (50 mL PBS + 80 µL geltrex) and incubated overnight at 37°C. The next day, coverslips were moved to 35 mm dishes to be used for experiments. Cells were seeded at a density of 1.5 $X10^5$ and allowed to settle overnight at 37°C before treatment with either IC_{50} of DS00326 or DS00329 for 48 hours. After treatment, cells were fixed in 4% paraformaldehyde for 10 minutes and then washed thrice afterwards with PBS to remove excess fixative. Cells were then permeabilized with 80 µL 0.25% Triton X- 100 in PBS for 10 minutes at room temperature and washed thrice after with 1X PBS. Following this step, cells were incubated in γH2AX primary antibody (1:2000 in DMEM supplemented with 10% FBS) and left overnight at 4°C. After primary antibody incubation, cells were washed thrice and incubated with secondary antibody (AlexaFluro-conjugated 488) in PBS for 1 hour and washed thrice again afterwards before staining with 80 µL of DAPI (1:5000 in PBS) for 8 minutes in the dark. Washing was repeated thrice and coverslips mounted on glass slides with mounting medium. Glass slides were allowed to dry for about 2 hours in the dark at room temperature before storing at -20°C.

3.10 Western blotting

In order to determine the protein expression in cells following treatment of U87 and U251 cells as well as T10 and T91 PDX glioblastoma cells with DS00326 and DS00329, western blotting was conducted.

3.10.1 Harvest of protein

U87 and U251 cells were seeded in 6 cm dishes and allowed to settle for 24 hours after which they were treated with either vehicle or IC_{50} of DS00326 or DS00329 for 24 and 48 hours. On the day of harvest, growth medium containing floating cells in the dishes were collected in 15 mL tubes, cells were washed and trypsinized with 750 µL trypsin/EDTA and centrifuged at 2000 rpm for 3 minutes. The resultant pellets were resuspended in 500 µL of ice-cold 1X PBS and transferred to 1 mL centrifuge tubes. The samples were centrifuged again for 1 minute on a bench top centrifuge and washed with 500 µL 1X PBS. Finally, a volume of 2X boiling blue (**Appendix 7.3**) was used to resuspend the pellets and boiled for 10 minutes at 100°C on a dry heating block. After heating, samples were allowed to cool down and were stored at -20°C until when needed. For PDX glioblastoma cells, T91 and T10 cells were treated with either vehicle or IC_{50} of DS00326 or DS00329 for 48 hours and for apoptosis investigation cells were treated for both 24 and 48 hours. Cells were plated in 6 cm dishes and allowed to settle overnight before they were treated. After treatment, cells were harvested in 15 mL falcon tubes and centrifuged at 1200 rpm for 4 minutes to remove medium. After centrifugation, cells were resuspended in 1 mL 1X PBS before transferring to Eppendorf tubes and centrifuged again at 1200 rpm for 4 minutes. PBS was removed and pellets stored at -80°C for subsequent lysate preparation (**Appendix 7.4**). Following lysate preparation, the BCA assay was conducted using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) to determine the protein concentration of samples (**Appendix 7.5**). Protein samples were

prepared by calculating the volume of protein lysates, water and 4X loading dye (**Appendix 7.6**) required to give 20 μg of protein which was loaded in gels for all experiments

3.10.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels ranging from 8-15% (**Appendix 7.7**) were used to resolve proteins depending on their molecular weights. Equal volumes of protein samples were loaded on the gels in addition to a column loaded with the western blot page ruler pre-stained protein ladder to ensure the accuracy of protein sizes (Fermentas, USA). Gel electrophoresis was performed at room temperature and gels were run at 100 volts in an electrophoresis tank (Bio-Rad) containing running buffer (**Appendix 7.8**) until adequate separation of proteins was achieved.

3.10.3 Transfer of gels

After running gels, proteins were then transferred onto a hybond electrochemi-luminiscence (ECL) nitrocellulose membrane (Amersham Bioscience, USA). Gels, sponges, and Whatmann filter paper were equilibrated in pre-chilled transfer buffer (**Appendix 7.9**) for approximately 5 minutes. Transfer apparatus was assembled by placing the sponge on the black side of transfer cassette followed by Whatman paper, the gel, cellulose membrane, Whatman paper and lastly the sponge at the second end of the cassette. This sandwiching was done carefully so as to avoid bubbles which might compromise the transfer of proteins. The cassette was placed into an electrophoresis tank containing transfer buffer with the black side of the cassette correspondingly placed on the black side of the tank. Proteins were electro-blotted onto the membrane at 100 volts for 90 minutes. Upon completion of the transfer, ECL nitrocellulose membranes were

stained with 1X ponceau staining solution to visualized transferred proteins from gel to membrane.

For primary PDX glioblastoma cells, the Trans-Blot® Turbo™ Blotting System (BioRad,USA) was used for protein transfer. Briefly, cold 1X transfer buffer was poured into two BioRad plastic containers and ECL nitrocellulose membrane (BioRad, USA) was placed in one of the containers while the other had the transfer stacks. Following this, the sandwich was prepared by putting one stack of transfer filters on the bottom of the transfer chamber, followed by the membrane, the gel and another stack of filters on the top and the transfer chamber was locked. Proteins were transferred using the 1mini gel- High MW programme on the machine which runs for 10 minutes. After transfer, membranes were ponceau-stained and images were taken using the colorimetric programme on the ChemiDoc Imaging Systems (BioRad, USA).

3.10.3 Antibody incubation and western blot detection

After ponceau staining, membranes were cut according to the molecular weight of the proteins of interest and then blocked in 5% non-fat milk in either 1X PBS/T or 1X TBS/T (PBS or TBS containing 0.1% Tween 20) for 1 hour before incubation with their respective primary antibodies in the same blocking buffer with milk overnight on a shaker at 4°C. After incubation with primary antibodies, membranes were washed twice for 5 minutes and twice again for 10 minutes either in PBS/T or TBS/T (PBS or TBS containing 0.1 % Tween 20). After washing, membranes were incubated for 1 hour in secondary antibody; either goat anti-mouse or goat anti-rabbit IgG peroxidase-conjugated secondary antibodies (1:5000 or 1:10000) (Bio-Rad, Hercules, CA, USA). After incubation, membranes were washed again as stated above and left in the last washing buffer before detection. Detection was done in the dark room and proteins contained on membranes were visualized on an autorad (Kodak Films) using the

enhanced chemiluminescence western blot substrate (pierce, USA). For PDX glioblastoma cells, proteins were visualized using the ChemiDoc Imaging Systems (BioRad, USA). **Table 3.1** below shows a list of antibodies with their respective conditions used for western blotting. p38 and Tubulin antibodies were used as loading control because these proteins are strongly and ubiquitously expressed in our samples without changes in level of expression following treatment.

3.11 Data analysis

Data generated were statistically analysed using Graph Pad Prism Version 6 and were expressed as means ± SEM (Standard Error of Means) computed from three independent experiments. A significance of $P \leq 0.05$ is considered for all comparisons in this study.

Table 3.1. Antibodies and conditions for western blotting

Primary antibody	Catalogue No. and manufacturer	Blocking Buffer	Wash buffer	Dilution	Secondary antibody
Histone H2A.X (rabbit polyclonal)	Cell Signaling(#2577)	5% milk in TBS/T	TBS/T	1:1000	G∝R (1:5000)
p53 (Mouse monoclonal)	Santa Cruz(Sc-126)	5% milk in PBS/T	PBS/T	1:500	G ∝M (1:5000)
PARP-1 (rabbit polyclonal)	Santa Cruz (Sc-7150)	5% milk in PBS/T	PBS/T	1:500	G∝R (1:5000)
p21 (rabbit polyclonal)	Santa Cruz (Sc-397)	5% milk in PBS/T	PBS/T	1:500	G∝R (1:5000)
p38 (rabbit polyclonal)	Sigma (M0800)	5% milk in PBS/T	PBS/T	1:5000	G∝R (1:5000)
Phospho-P38 (rabbit polyclonal)	Cell Signaling (#9211)	5% milk in TBS/T	TBS/T	1:1000	G∝R (1:5000)
LC3β (rabbit polyclonal)	Cell signalling (#2775)	5% milk in TBS/T	TBS/T	1:1000	G∝R (1:5000)
pChk2 (rabbit polyclonal)	Cell signalling (#2661)	5% milk in TBS/T	TBS/T	1:1000	G∝R (1:5000)
Cyclin A (rabbit polyclonal)	Santa Cruz (sc-751)	5% milk in PBS/T	PBS/T	1:1000	G∝R (1:5000)
Cyclin B (mouse monoclonal)	Santa Cruz (sc-752)	5% milk in PBS/T	PBS/T	1:1000	G ∝M (1:5000)
CDK2	Cell signalling (#2546)	5% milk in PBS/T	PBS/T	1:1000	G∝R (1:5000)
pAkt (rabbit polyclonal)	Cell signalling (#9271)	5% milk in TBS/T	TBS/T	1:1000	G∝R (1:5000)

pERK1/2 (rabbit polyclonal)	Cell signalling (#4370)	5% milk in TBS/T	TBS/T	1:1000	GαR (1:5000)
ERK1/2 (rabbit monoclonal)	Cell signalling (#9107)	5% milk in PBS/T	PBS/T	1:1000	G ∝M (1:10000)
JNK (rabbit polyclonal)	Cell signalling (#9252)	5% milk in PBS/T	PBS/T	1:1000	GαR (1:10000)
pJNK (rabbit polyclonal)	Cell signalling (#9251)	5% milk in PBS/T	PBS/T	1:1000	GαR (1:10000)
Sox2 (rabbit monoclonal)	R&D systems (Mab2018)	5% milk in PBS/T	PBS/T	1:300	GαR (1:10000)
GFAP (rabbit polyclonal)	Dako (Z0334)	5% milk in PBS/T	PBS/T	1:10000	GαR (1:10000)
pcdc2 (rabbit polyclonal)	Cell signalling (#9111)	5% milk in PBS/T	PBS/T	1:1000	GαR (1:10000)
ATM (rabbit monoclonal)	Cell Signaling (#2873)	5% milk in PBS/T	PBS/T	1:1000	GαR (1:10000)
pATM (rabbit monoclonal)	Genetex (GTX61739)	5% milk in PBS/T	PBS/T	1:5000	G∝R (1:10000)
Tubulin (mouse monoclonal)	Sigma (T9026)	5% milk in PBS/T	PBS/T	1:10000	G ∝M (1:10000)

CHAPTER FOUR

THE ANTI-CANCER ACTIVITY OF DS00326 AND DS00329 IN U87 AND U251 MALIGNANT GLIOBLASTOMA CELLS

4.1 Screening of a group of phenothiazine derivatives in malignant U87 and U251 glioblastoma cells

A panel of remodelled phenothiazines comprised of 32 phenothiazine-derivatives were all initially screened for their cytotoxic effects in U251 and U87 human malignant glioblastoma cells (WHO grade IV). To this end, the cells were treated with a concentration range (0 to 30 μM) of the compounds or vehicle for 48 hours and the MTT cell viability assay was performed. The concentration that inhibits cell growth by 50% (IC_{50}) was calculated from sigmoidal plots using GraphPad Prism version 6 and four compounds were found to exhibit cytotoxic activity (**Table 4.1**). DS00326 and DS00329 were selected for further characterisation because they were most cytotoxic in both glioblastoma cell lines tested and their chemical structures are shown in **Figure 4.1.**

Table 4.1. IC_{50} of values of phenothiazines on U251 and U87 glioblastoma cells for 48 hours

Serial No	Compounds with Library Name	IC_{50} (µM)	
		U251	U87
1	DS00341	16.67	11.57
2	DS00325	8.06	26.41
3	DS00326	4.51	6.11
4	DS00329	5.12	9.29

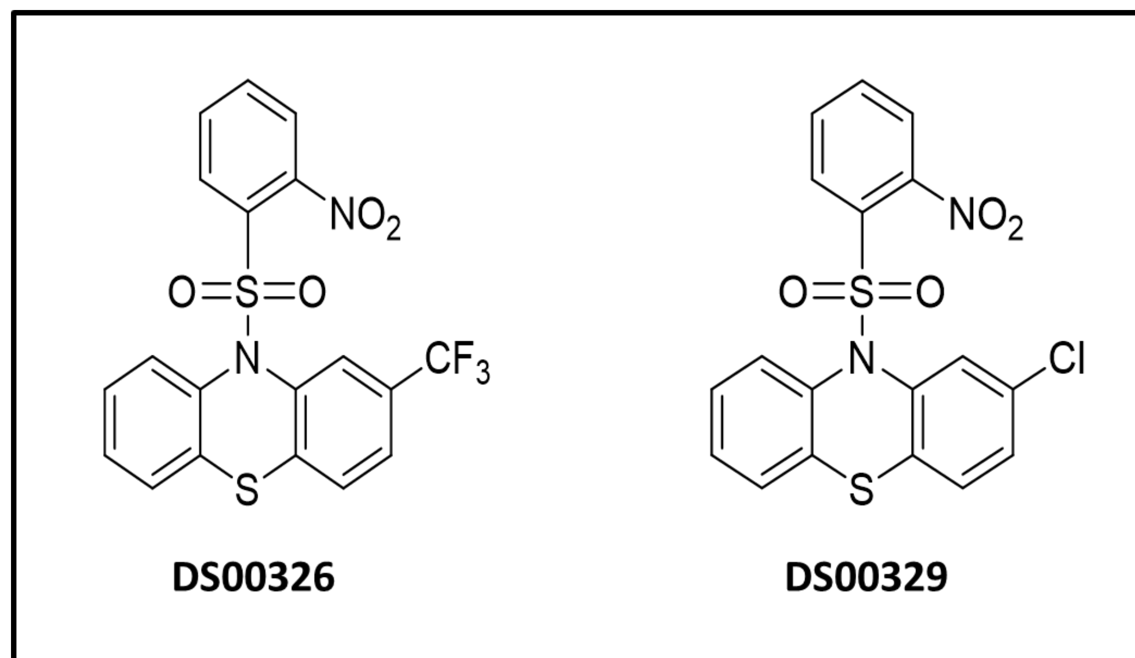
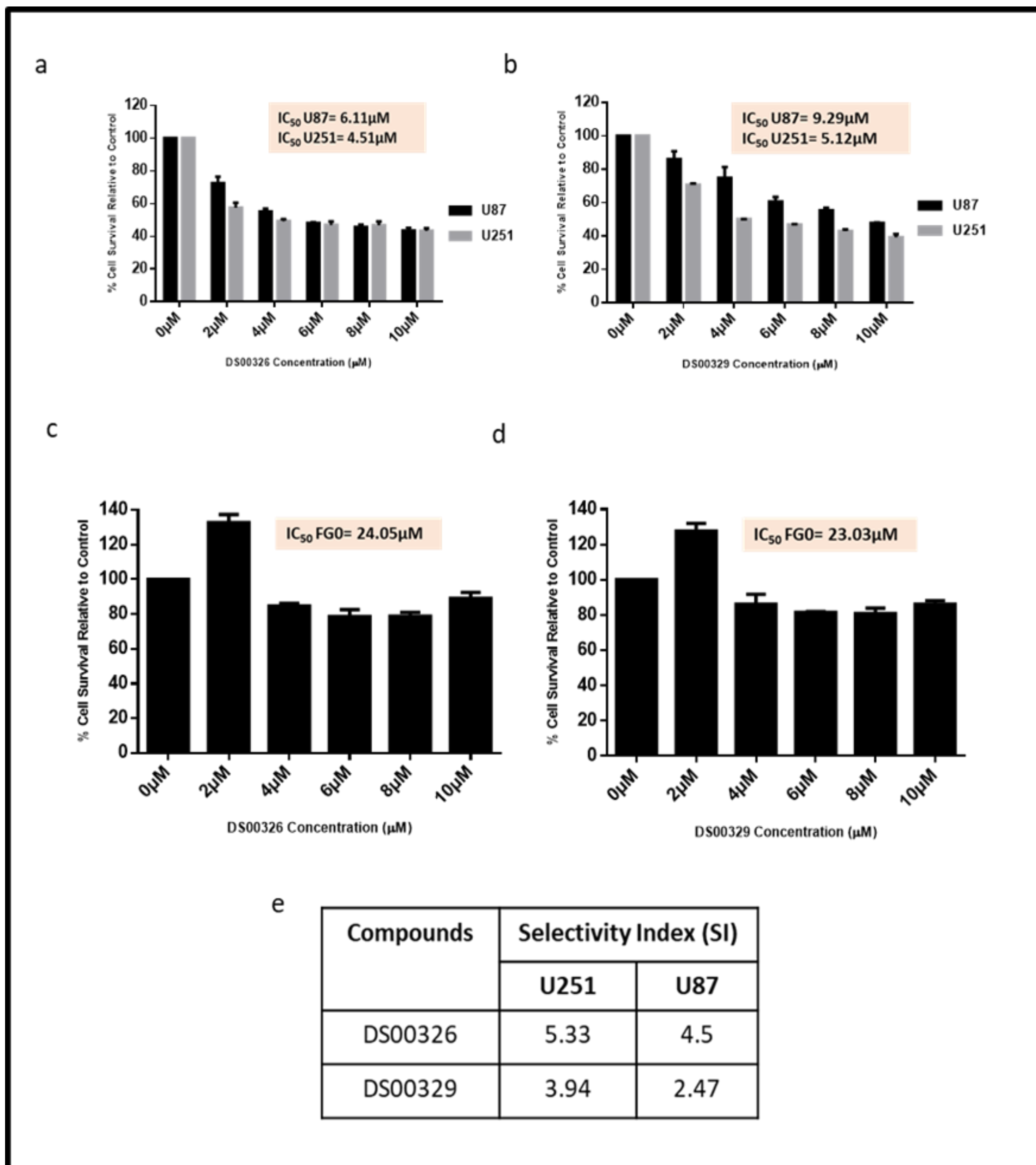


Figure 4.1. Chemical structures of phenothiazines, DS00326 and DS00329.

4.2 DS00326 and DS00329 inhibit cell viability in U87 and U251 malignant glioblastoma cells

To determine the anti-cancer activity of the phenothiazine derivatives DS00326 and DS00329 in glioblastoma cells, U87 and U251 cells were treated with lower concentrations (0 to 10 μM) than previously tested of either DS00326 or DS00329 for 48 hours and MTT assays were performed. Results show that DS00326 and DS00329 inhibited cell viability in both cell lines tested (**Figures 4.2a and 4.2b**). Of the two compounds, DS00326 was more active as it displayed lower IC_{50} values for both cell lines. Compared to the U87 cells, the U251 cells were more sensitive to both compounds because lower IC_{50} values were obtained for them. Indeed, the IC_{50} values for DS00326 and DS00329 were 4.51 μM and 5.12 μM respectively in the U251 cells compared to 6.11 μM and 9.29 μM in the U87 cells.

In order to determine the selectivity of DS00326 and DS00329 for glioblastoma cells, the FG0 non-cancerous fibroblasts were also treated with DS00326 or DS00329 (0 to 10 μM) and MTT assays were performed. **Figures 4.2c and 4.2d** show that the IC_{50} values for DS00326 and DS00329 in FG0 cells were approximately 24.05 μM and 23.03 μM respectively. When the selectivity index (IC_{50} in FG0/IC_{50} for glioblastoma cells) was calculated the values obtained for DS00326 and DS00329 were 5.33 and 4.5 respectively in the U251 cells and 3.94 and 2.47 in the U87 cells (**Figure 4.2e**). DS00326 thus showed a greater selectivity for the glioblastoma cells. Together these results demonstrate that the remodelled phenothiazines DS00326 and DS00329 selectively inhibit cell viability in malignant glioblastoma cells.



Compounds	Selectivity Index (SI)	
	U251	U87
DS00326	5.33	4.5
DS00329	3.94	2.47

Figure 4.2 DS00326 and DS00329 is selective for glioblastoma cells. Percentage cell viability (as measured by MTT assays) in U87 and U251 cell lines exposed for 48 hours to vehicle or increasing concentrations (0-10 µM) of DS00326 **(a)** and DS00329 **(b)**. **(c)** Effect of DS00326 and **(d)** DS00329 on FG0 cells. **(e)** Selectivity index for compounds on cells relative to FG0 cells. Results show bars with the mean percentage ± SEM of data of at least three experiments performed in quadruplicate. The concentration of DS00326 and DS00329 that killed 50% of the cells (IC_{50}) was calculated using GraphPad Prism Version 6.

4.3 DS00326 and DS00329 inhibit cell survival of malignant glioblastoma cells

To determine the impact of DS00326 and DS00329 on the long-term survival of U87 and U251 glioblastoma cells, a clonogenic assay was performed. This *in vitro* assay assesses the ability of single cells to divide and form colonies (a colony is defined to consist of at least 50 cells) after a short exposure to cytotoxic agents or radiation (Franken et al., 2006). Briefly, cells were plated in 60 mm dishes and were exposed to ½IC_{50} and IC_{50} of either DS00326 (U87: 3.05 μM and 6.11 μM; U251:2.26 μM and 4.51 μM) or DS00329 (U87: 4.65 μM and 9.29 μM; U251: 2.61 μM and 5.12 μM) or vehicle for 24 hours. After treatment, cells were re-plated at low densities (500 cells/35 mm dish) and allowed to grow over a maximum period of fourteen days. Medium was changed every four days and vehicle treated cells were used as indicators to know when to terminate experiments. **Figures 4.3a and 4.3b** show that, compared to the vehicle, ½IC_{50} and IC_{50} of both DS00326 and DS00329 severely reduced the ability of U87 and U251 cells to form colonies. **Figures 4.3c and 4.3d** show the results obtained when the colony areas were quantified using the Image J software and those obtained for the drug treated cells were expressed relative to the vehicle treated cells which were set to 100%. At ½IC_{50} DS00326, percentage colony area was 1.93% and 8.2% for U87 and U251 cells respectively, and at the $IC_{50,}$ U87 cells were unable to form any colonies and 1.26% was obtained for U251 (**Figure 4.3c**). DS00329 on the other hand showed 40.33% and 25.56% colony area at ½IC_{50} for U87 and U251 respectively, and no colonies were obtained for U87 and 4.51% was obtained for U251 at the IC_{50} of this compound (**Figure 4.3d**). Taken together, these results show that DS00326 and DS00329 inhibit colony formation and thus survival of U87 and U251 glioblastoma cells.

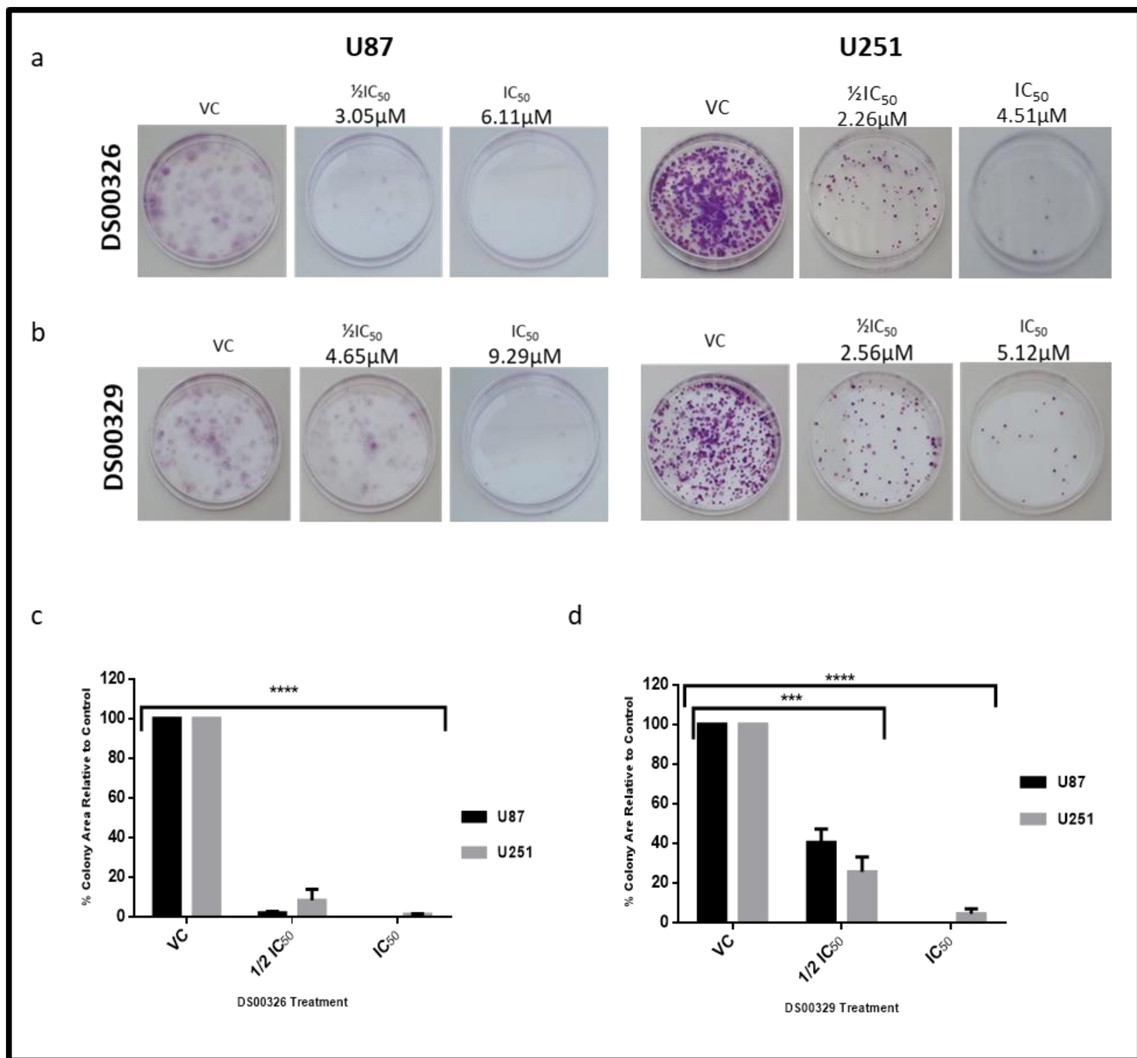


Figure 4.3 DS00326 and DS00329 inhibit survival of glioblastoma cells. Clonogenic assays were conducted for U87 and U251 glioblastoma cells following exposure for 24 hours to vehicle (VC), DS00326 or DS00329 at indicated concentrations. The cells were re-plated at a low density of 500 cells per 35mm dish and allowed to form colonies, stained with 0.5% crystal violet and images were taken. **(a, b)** Colony formation for U87 and U251 cells treated with DS00326 **(a)** and DS00329 **(b)**. Colonies were quantified using the colony area plugin on Image J software and percentage colony area were calculated for both ½IC_{50} and IC_{50} concentrations of **(c)** DS00326 and **(d)** DS00329 using the formula **(Colony area of treated cells/Colony area of control) x 100**.

4.4 DS00326 and DS00329 induce a G1 arrest in U87 and U251 malignant glioblastoma cells

To determine the mechanism by which DS00326 and DS00329 induced cell death in U87 and U251 cells, their impact on the cell cycle profile was ascertained by flow cytometry. U87 and U251 cells were treated with IC_{50} of DS00326 and DS00329 for 24 and 48 hours, after which cells were fixed and stained with propidium iodide to determine DNA content and thus cell cycle phase. **Figure 4.4** shows that following DS00326 and DS00329 treatment of U87 cells, there was a significant increase in the number of G1 phase cells with a corresponding decrease in S phase cells at both time points tested. **Figure 4.5** shows the results obtained for the U251 cells treated with vehicle, DS00326 or DS00329. It is important to note that the U251 cells showed a profile typical of polyploidy cells i.e. they showed two cycling populations of cells (**Figure 4.5a**) which has previously been reported for long-term passage of U251 cells (Torsvik et al., 2014). The results shown in the pie-charts are thus the sum of the number of cells in each phase of the cycle for the two populations. Compared to the vehicle treated cells, there was a progressive increase in G1 cells which was accompanied by a decrease in the number of cells in S and G2/M. Taken together, these results show that DS00326 and DS00329 treatments lead to malignant glioblastoma cells arresting in the G1 phase of the cell cycle. This is consistent with previous studies which reported that a phenothiazine, thioridazine, induces a G1 cell cycle arrest in cervical and endometrial cancer cells (Kang et al., 2012).

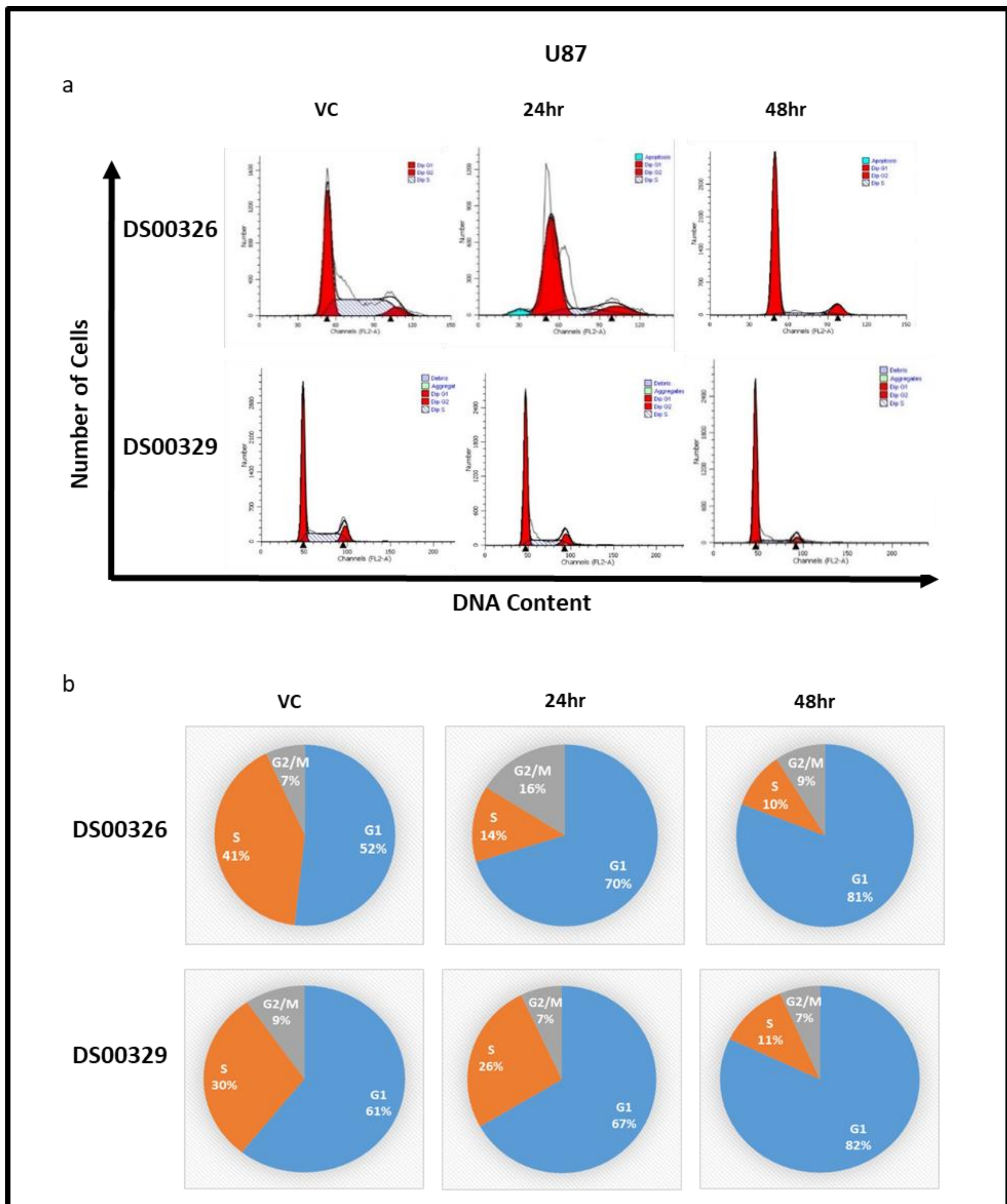


Figure 4.4. DS00326 and DS00329 induce a Gl cell cycle arrest in U87 cells. (a) Cell cycle profile of U87 cells exposed to either vehicle (VC) or DS00326 or DS00329 for 24 and 48 hours as determined by propidium iodide staining using flow cytometry. The number of cells in each phase of the cell cycle was expressed as a percentage of the total number of cells analysed. **(b)** Pie-chart showing the percentage of cells in the G1, S and G2/M phases of the cell cycle. Chart shows the average of three independent experiments.

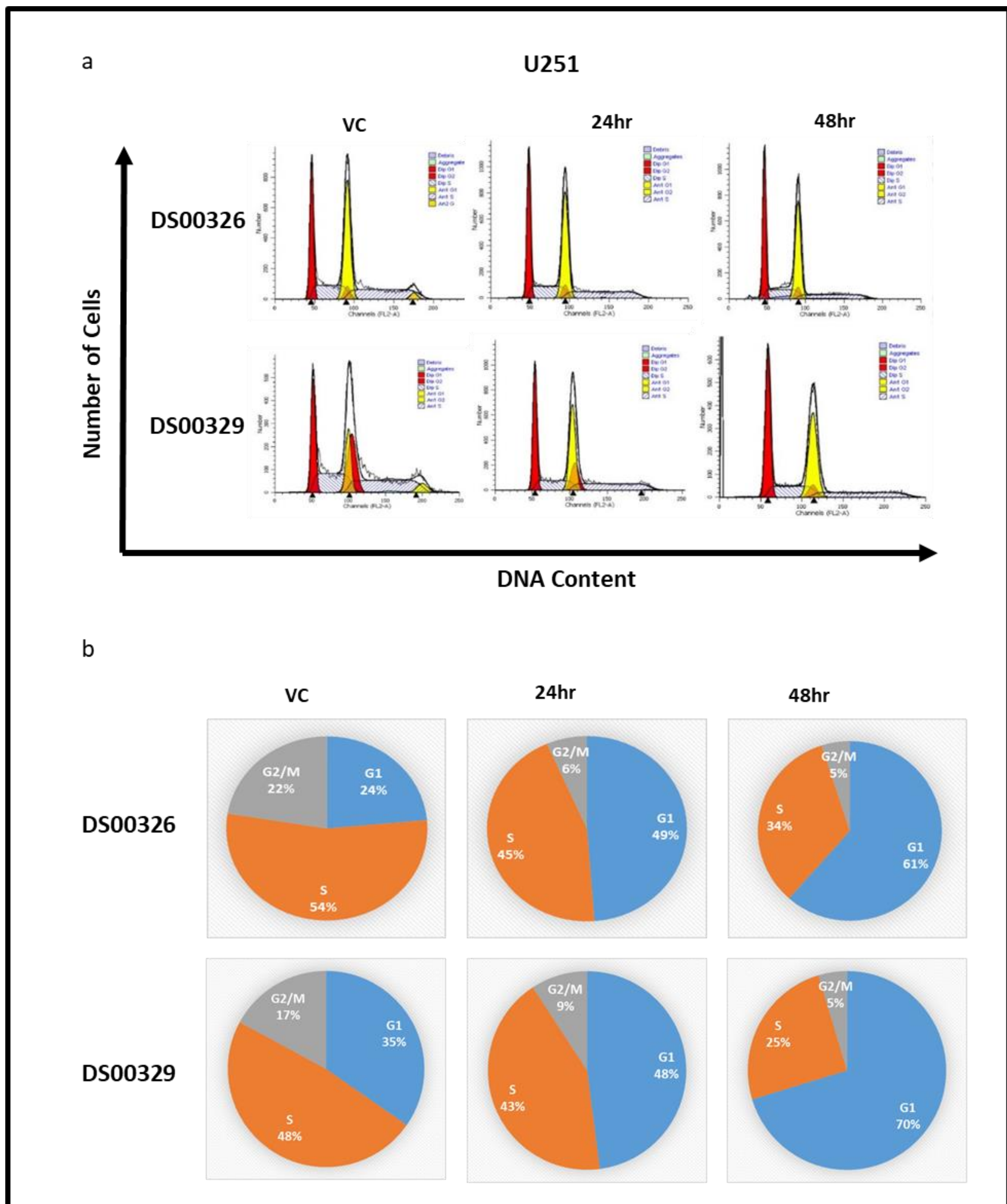


Figure 4.5. DS00326 and DS00329 induce a Gl cell cycle arrest in U251 cells. (**a**) Cell cycle profile of polyploidy U251 cells exposed to either vehicle (VC) or DS00326 or DS00329 for 24 and 48 hours as determined by propidium iodide staining using flow cytometry. The number of cells in each phase of the cell cycle was expressed as a percentage of the total number of cells. **(b)** Pie-chart showing the percentage of cells in the G1, S and G2/M phases of the cell cycle. Chart shows the average of three independent experiments.

4.5 DS00326 and DS00329 induce a DNA damage response and modulate the expression of cell cycle regulatory proteins in malignant glioblastoma cells

Exposure of cells to radiation or chemotherapeutic agents induce DNA damage including a break in one or both strands of the DNA. An early response to double stranded DNA damage is the phosphorylation of histone 2AX on serine 139 and this variant is called γH2AX (Rogakou et al., 1998). Following activation of γH2AX, the checkpoint kinase (Chk2) is activated by phosphorylation on threonine 68 by ATM followed by Chk2 phosphorylating several substrates including p53 which leads to the stabilization and activation of this tumour suppressor (Takai et al., 2002). Once activated p53 transcriptionally activates a number of cell cycle genes including the cyclin dependent kinase inhibitor, p21 which triggers cell cycle checkpoints. This results from p21 inhibiting especially cyclin/cyclin-dependent kinase2 (CDK2) complexes (Abbas and Dutta, 2009). CDK2 binds to cyclin E to promote transition from G1 to S while it binds to cyclin A to promote entry into the S and G2 phases. The cyclinB/CDK1 complex however is required for transition into M phase.

To ascertain whether the cytotoxicity induced by DS00326 and DS0039 in glioblastoma cells involve DNA damage, U87 and U251 cells were treated with these compounds for 24 and 48 hours and western blotting was performed with antibodies to γH2AX and phosphorylated (active) Chk2 (pChk2). Results show that both DS00326 and DS00329 induce double stranded DNA damage as indicated by an increase in γH2AX levels (**Figure 4.6**). This was accompanied by an increase in levels of pChk2, in the U87 and U251 cells treated with DS00326. While this was also the case for the U87 cells treated with DS00329, pChk2 levels did not increase in U251 cells treated with this compound. We next determined if elevated pChk2 levels lead to changes in levels of cell cycle proteins. Results show a marked increase in p53 and p21 levels in the U87 cells treated

with both DS00326 and DS0039. Interestingly, even though the U251 cells have mutated non-functional p53 (Brázdová et al., 2009, Luan et al., 2010), the levels of p53 increased in U251 cells treated with both DS00326 and DS00329 for 48 hours which may imply that these drugs lead to an increase in p53 mRNA levels and thus protein (**Figure 4.6**). Furthermore, p21 levels increased in U251 cells treated with DS00326 and DS0039 which suggest that p21 is upregulated in a p53-independent manner in these cells. However, it is worth noting that mutant p53 in U251 cells has been reported to (1) exert a regulatory effect on target gene transcription by participating in the organization of chromatin into functional domains and (2) retain residual transcriptional activity (Menendez et al., 2006, Brazdova et al., 2009).

To confirm that DS00326 and DS00329 induce a G1 cell cycle arrest in U87 and U251 cells, the levels of CDK2 as well as cyclin A and cyclin B were next determined. The rationale was that a G1 arrest should be accompanied by a decrease in these proteins. Indeed, **Figure 4.6** shows that the induction of p21 in both cell lines was accompanied by a downregulation of CDK2, cyclin A and cyclin B. This also corroborates the FACs analyses (**Figures 4.4 and 4.5**) which show that DS00326 and DS00329 induce a G1 arrest at the expense of the S and G2/M phases. Together these results suggest that DS00326 and DS00329 induce DNA damage and a G1 cell cycle arrest which may be p21 dependent.

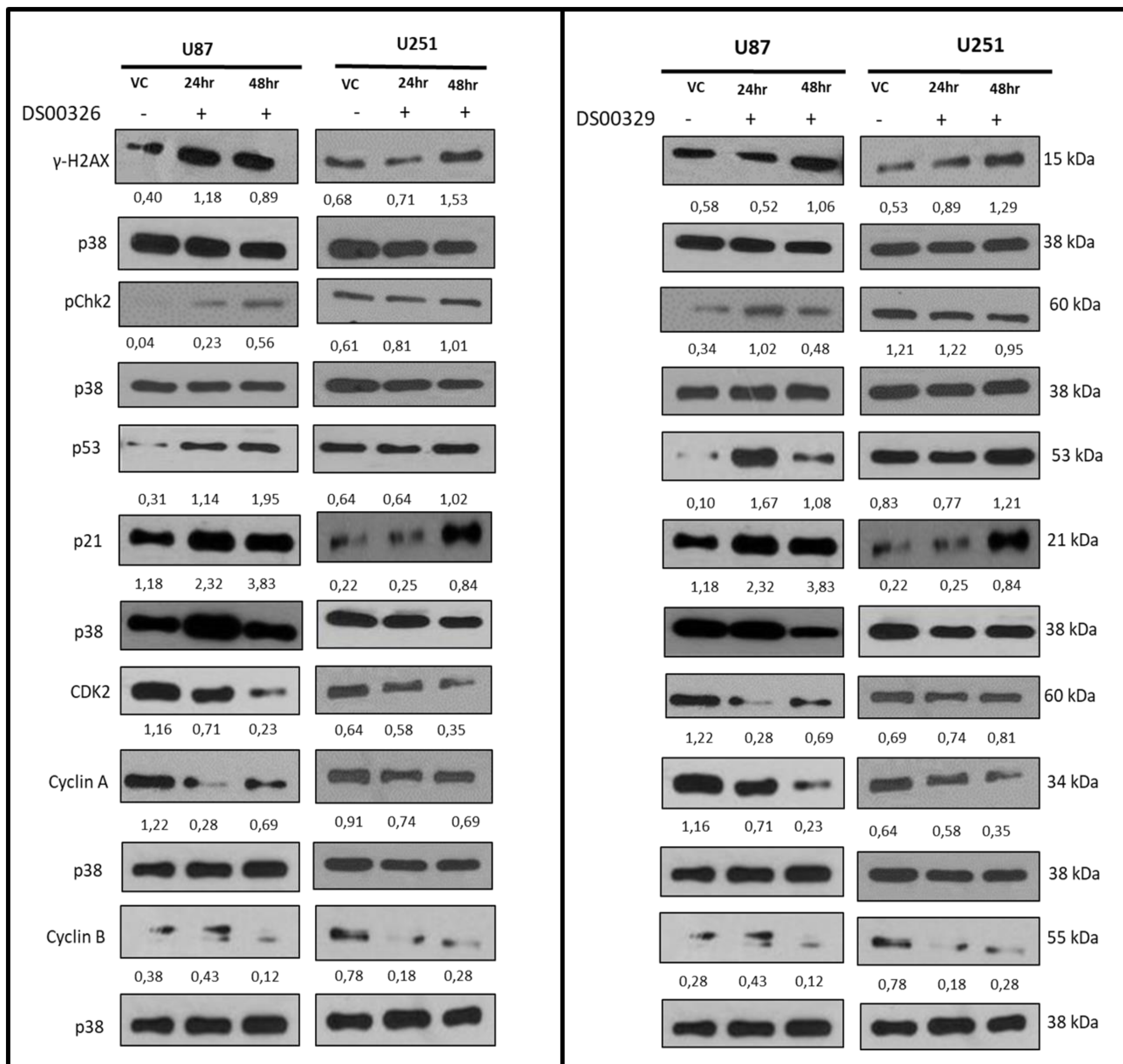


Figure 4.6. DS00326 and DS00329 induce a DNA damage response and regulate key cell cycle proteins in U87 and U251 cells. Cells were treated with either vehicle (VC) or DS00326 or DS00329 for 24 and 48 hours and proteins were extracted and analysed by western blotting with antibodies to γH2AX, pCHK2, p53, p21, CDK2, Cyclin A, Cyclin B and p38 (loading control). Densitometric values (numbers under each representative blot) show the ratio of γH2AX, pCHK2, p53, p21, CDK2, Cyclin A, Cyclin B to p38.

4.6 DS00326 and DS00329 induce apoptosis in malignant glioblastoma cells

To investigate whether DS00326 and DS00329 induce apoptosis, the U87 and U251 cells were exposed to these compounds or vehicle for 48 hours and visualized by light microscopy. **Figure 4.7a and 4.8a** show that both DS00326 and DS00329 induce disruption of the cell membrane (membrane blebbing) as well as cell shrinkage which are early morphological changes characteristic of apoptosis.

To confirm that DS00326 and DS00329 induce apoptosis at a molecular level, levels of poly (ADP-ribose) polymerase (PARP) were investigated in U251 and U87 cells following treatment with the compounds. PARP has a molecular weight of 116 kDa and during apoptosis it is cleaved into a sub-fragment of 89 kDa by caspases (Simbulan-Rosenthal et al., 1998). **Figures 4.7b and 4.8b** show that treatment of U251 and U87 cells with DS00326 and DS00329 lead to increased PARP cleavage as evident by increased levels of the 89 kDa fragment, especially after 48 hours.

To quantify the apoptosis induced by DS00326 and DS00329, FACS analyses were performed on U251 and U87 cells treated for 24 and 48 hours with the compounds and stained with annexin V-FITC/propidium iodide. Annexin V is a cellular protein that binds to phosphatidylserine on the exterior part of the plasma membrane when cells undergo apoptosis (Crowley et al., 2016). This assay quantifies live viable cells (left bottom quadrant), apoptotic cells (right bottom and right top quadrants) as well as necrotic cells (left top quadrant) as indicated in scatter plots shown in **Figures 4.9a and 4.10a**. The results show that DS00326 (**Figure 4.9)** and DS00329 (**Figure 4.10)** treatment lead to a significant increase in the percentage of U87 and U251 cells undergoing apoptosis and necrosis (only for the U87 cells treated with DS00326) especially at 48 hours. Indeed,

the percentage of U87 apoptotic cells increased from 8.44% to 9.97% and 21.05% when treated with vehicle and DS00326 for 24 hours and 48 hours respectively (**Figure 4.9b**).

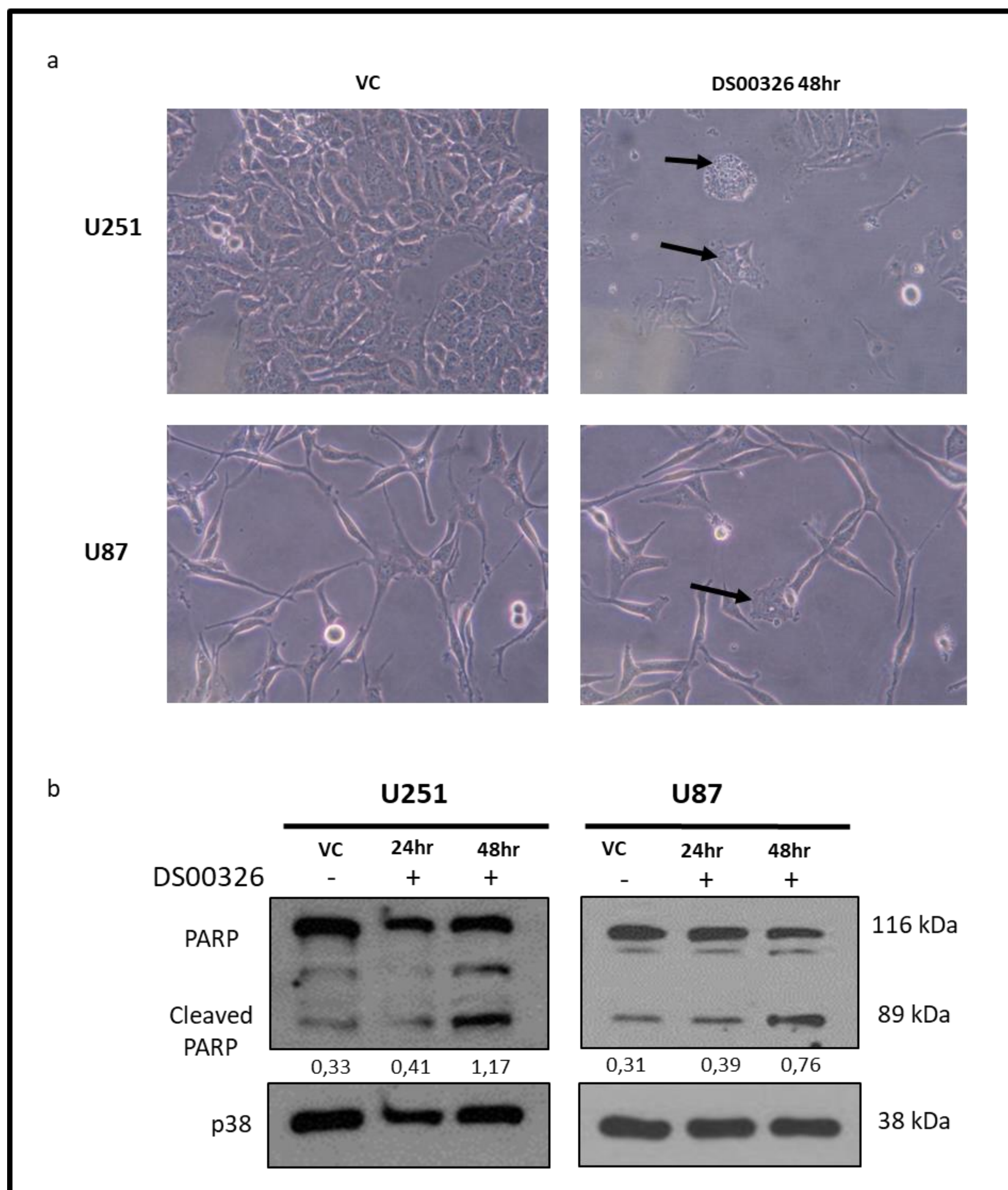


Figure 4.7. DS00326 induces apoptosis in glioblastoma cells. (a) Light microscopy images (400X) of U251 and U87 cells treated with either vehicle (VC) or DS00326 showing morphological features of membrane blebbing and cell shrinkage **(black arrows)**. **(b)** Representative blot of analysis of protein extracts from vehicle (VC) or DS00326 treated U251 and U87 cells for 24 and 48 hours with 8% SDS-PAGE and western blotting using antibody against PARP. P38 was used as a loading control and densitometric values (numbers under each blot) show ratio of cleaved PARP (89 kDa) to that of p38.

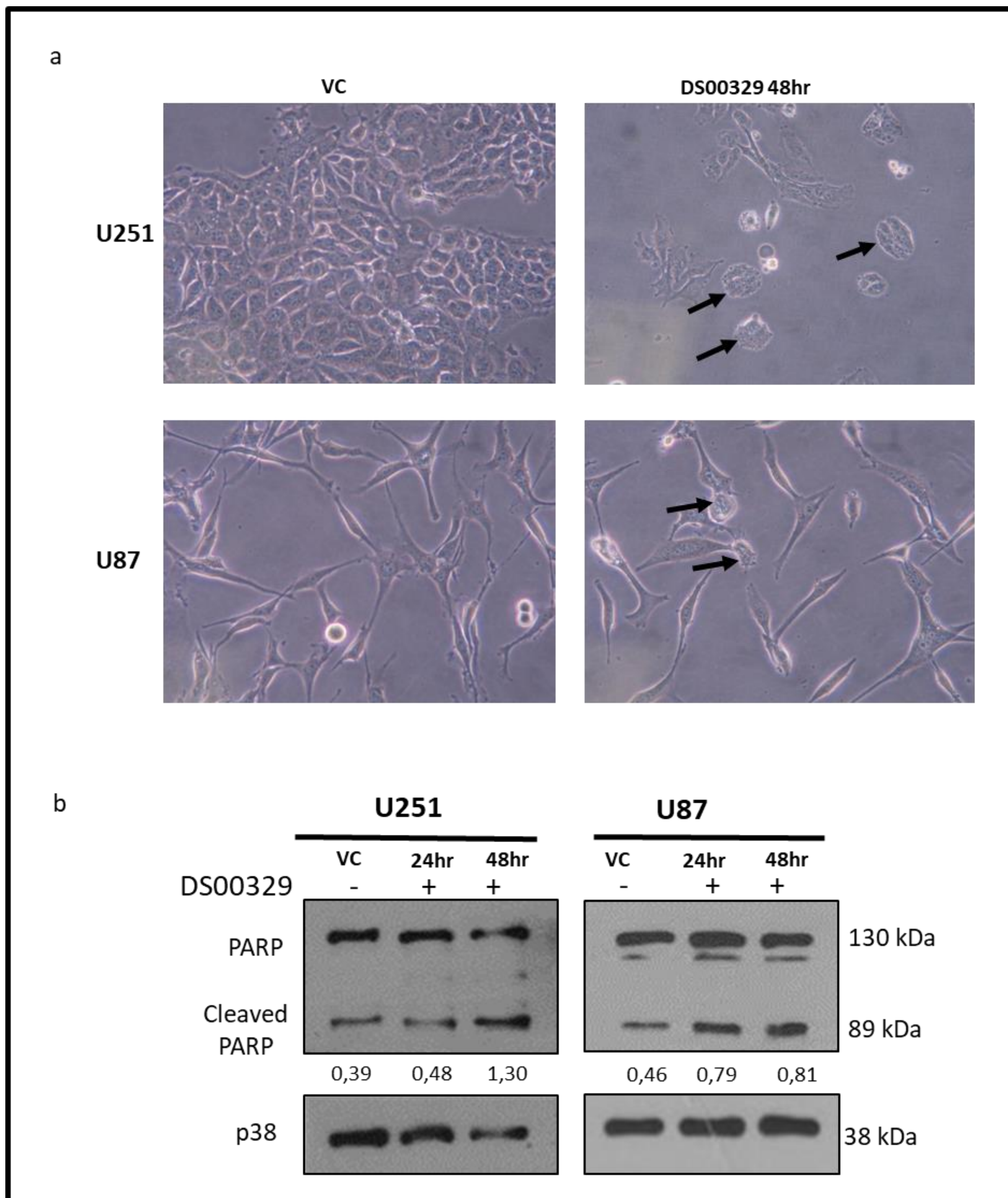


Figure 4.8. DS00329 induces apoptosis in glioblastoma cells. (a) Light microscopy images (400X) of U251 and U87 cells treated with either vehicle (VC) or DS00329 showing morphological features of membrane blebbing and cell shrinkage **(black arrows)**. **(b)** Representative blot of analysis of protein extracts from vehicle (VC) or DS00329 treated U251 and U87 cells for 24 and 48 hours with 8% SDS-PAGE and western blotting using antibody against PARP. P38 was used as a loading control and densitometric values (numbers under each blot) show ratio of cleaved PARP (89 kDa) to that of p38.

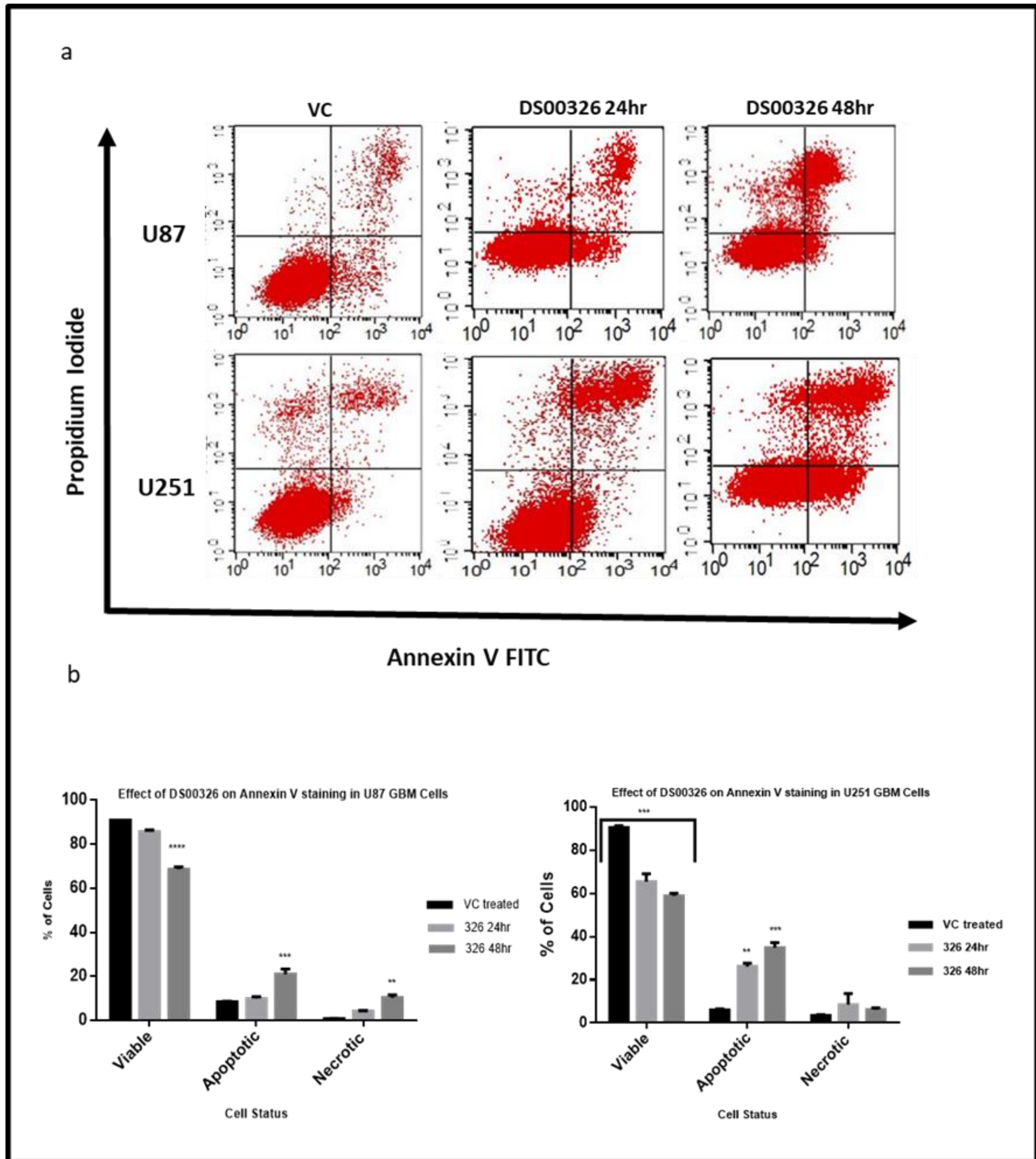


Figure 4.9. DS00326 induces apoptosis as demonstrated by annexin V/PI staining. **(a)** U87 and U251 cells were treated with either vehicle (VC) or DS00326 for 24 and 48 hours and flow cytometry was used to analyse the cells after staining with annexin V – FITC/ propidium iodide. The results show induction of apoptosis in both U87 and U251 treated cells compared with control. Lower left (LL) quadrant represents live cells, upper left (UL) quadrant represents necrotic cells, lower right (LR) quadrant represents early apoptotic cells and upper right (UR) quadrant represents late apoptotic cells. **(b)** Graph showing percentages of live necrotic and apoptotic cells (a total of cells in the lower right and upper right quadrants). Data show averages of repeated experiments and each bar represent means ± SEM and significance ($P \leq 0.05$) indicated by * was tested between treated cells and vehicle control.

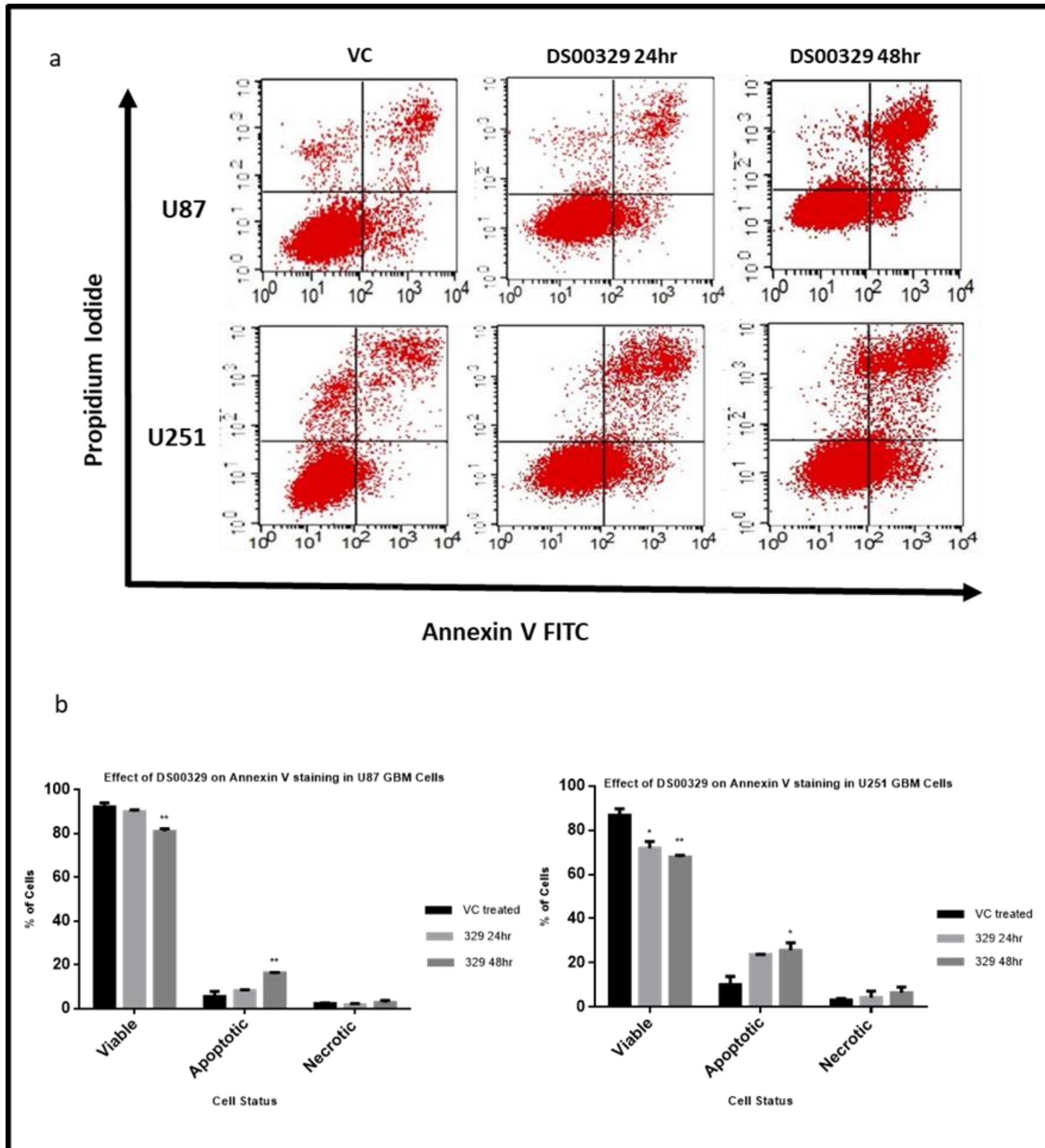


Figure 4.10. DS00329 induces apoptosis as demonstrated by annexin V/PI staining. **(a)** U87 and U251 cells were treated with either vehicle (VC) or DS00329 for 24 and 48 hours and flow cytometry was used to analyse the cells after staining with annexin V – FITC/ propidium iodide. The results show induction of apoptosis in both U87 and U251 treated cells compared to control. Lower left (LL) quadrant represents live cells, upper left (UL) quadrant represents necrotic cells, lower right (LR) quadrant represents early apoptotic cells and upper right (UR) quadrant represents late apoptotic cells. **(b)** Graph showing percentages of live necrotic and apoptotic cells (a total of cells in the lower right and upper right quadrants). Data show averages of repeated experiments and each bar represent means ± SEM and significance ($P \leq 0.05$) indicated by * was tested between treated cells and vehicle control.

Similarly, the percentage of U251 apoptotic cells increased from 6.05% to 26.16% and 34.90% for vehicle and DS00326 treatment for 24 and 48 hours respectively (**Figure 4.9b**). When the U87 cells were treated with vehicle and DS00329 for 24 hours and 48 hours, the percentage of apoptotic cells increased from 5.58% to 8.24% and 16.21% respectively (**Figure 4.10b**) and the U251 cells treated with vehicle and DS00329 for 24 hours and 48 hours show 10.13% to 23.73% and 27.72% apoptotic cells respectively (**Figure 4.10b**).

Taken together, the above experiments demonstrate that DS00326 and DS00329 induce apoptosis in U87 and U251 glioblastoma cell lines.

4.7 DS00326 and DS00329 induce autophagy in glioblastoma cells and inhibit the Akt pathway

Several anti-cancer agents including phenothiazines have been reported to induce autophagy in cancer cells (Zong et al., 2011a, Aliwaini et al., 2013, Wu et al., 2016). Autophagy is a process through which organelles and proteins are sequestered into autophagic vesicles (autophagosomes) which then fuses with the lysosomal acidic components of the cells forming autophagolysosomes (Mathew et al., 2007). To investigate whether DS00326 and DS00329 induce autophagy in U87 and U251 cells, the cells were treated with the compounds for 24 and 48 hours and stained with acridine orange to identify acidic vesicular organelles (AVO), a hallmark of autophagy (Paglin et al., 2001). The results indeed show that after treatment with both compounds there was an increase in AVO, as visible by the emission of bright orange fluorescence, within the U87 and U251 cells and this was more prominent at the 48-hour time point (**Figures 4.11a and 4.12a**). During autophagy, the microtubule-associated protein light chain 3 (LC3-I) is cleaved and conjugated to phosphatidylethanolamine to form LC3-II, which is recruited to the membranes of autophagosomes (Tanida et al., 2004). Western blot

analyses confirmed that U87 and U251 glioblastoma cells treated with DS00326 and DS00329 show a time dependent increase in levels of LC3-II (**Figure 4.11b and 4.12b**).

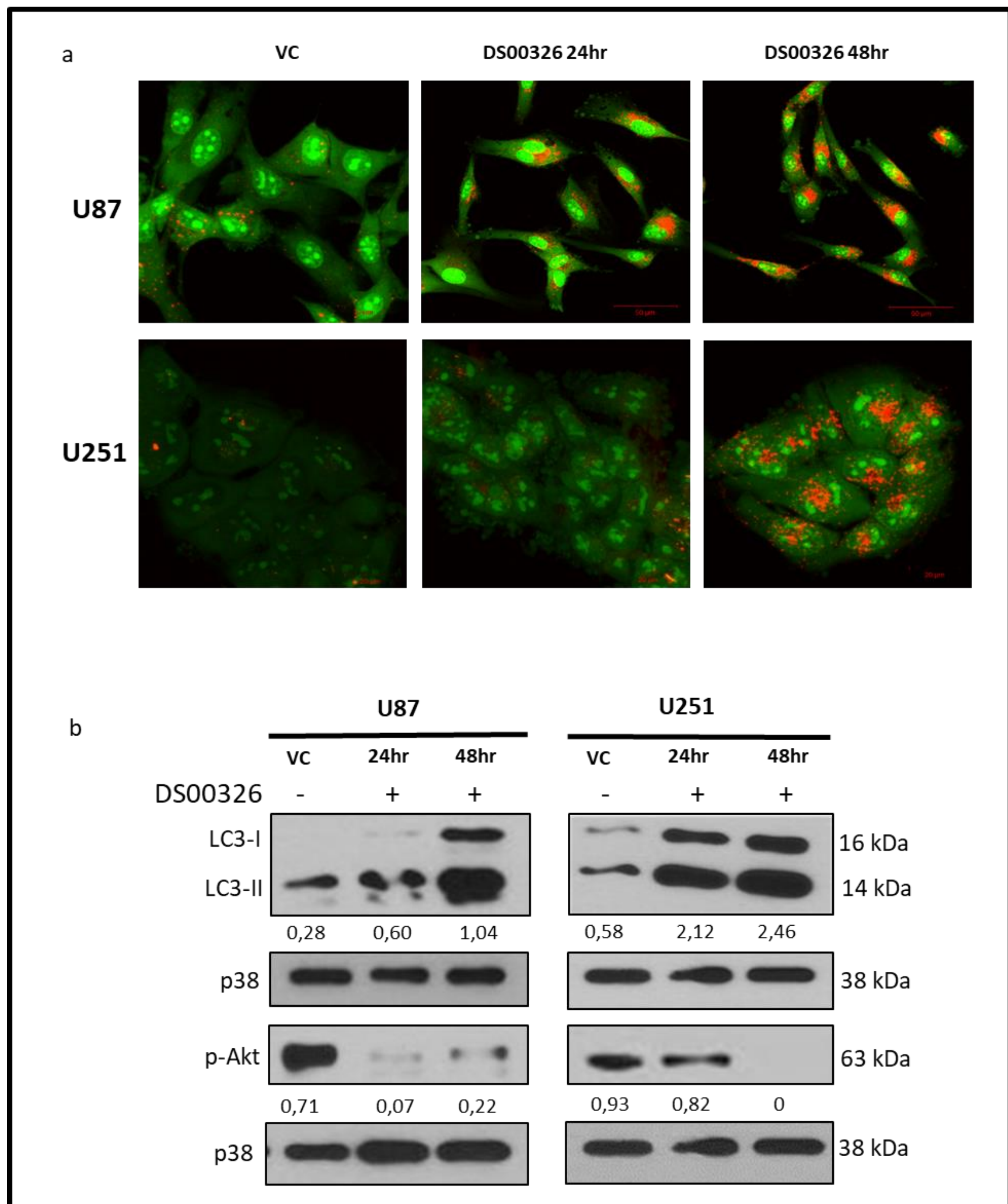


Figure 4.11. DS00326 induces autophagy and inhibits the Akt pathway. **(a)** U87 and U251 cells were treated with either vehicle (VC) or DS00326 for 24 and 48 hours and acridine orange staining for AVO as indication for autophagy. Increased acidic components when visualized under the confocal microscope are depicted by the increased orange fluorescence in the treated cells when compared with control. **(b)** Western blot analysis of protein extracts from vehicle (VC) or DS00326 treated U251 and U87 cells for 24 and 48 hours with antibodies to LC3, pAkt and p38 (loading control). Densitometric values (numbers under each blot) show the ratio of LC3-II (14kDa) and pAkt to that of p38.

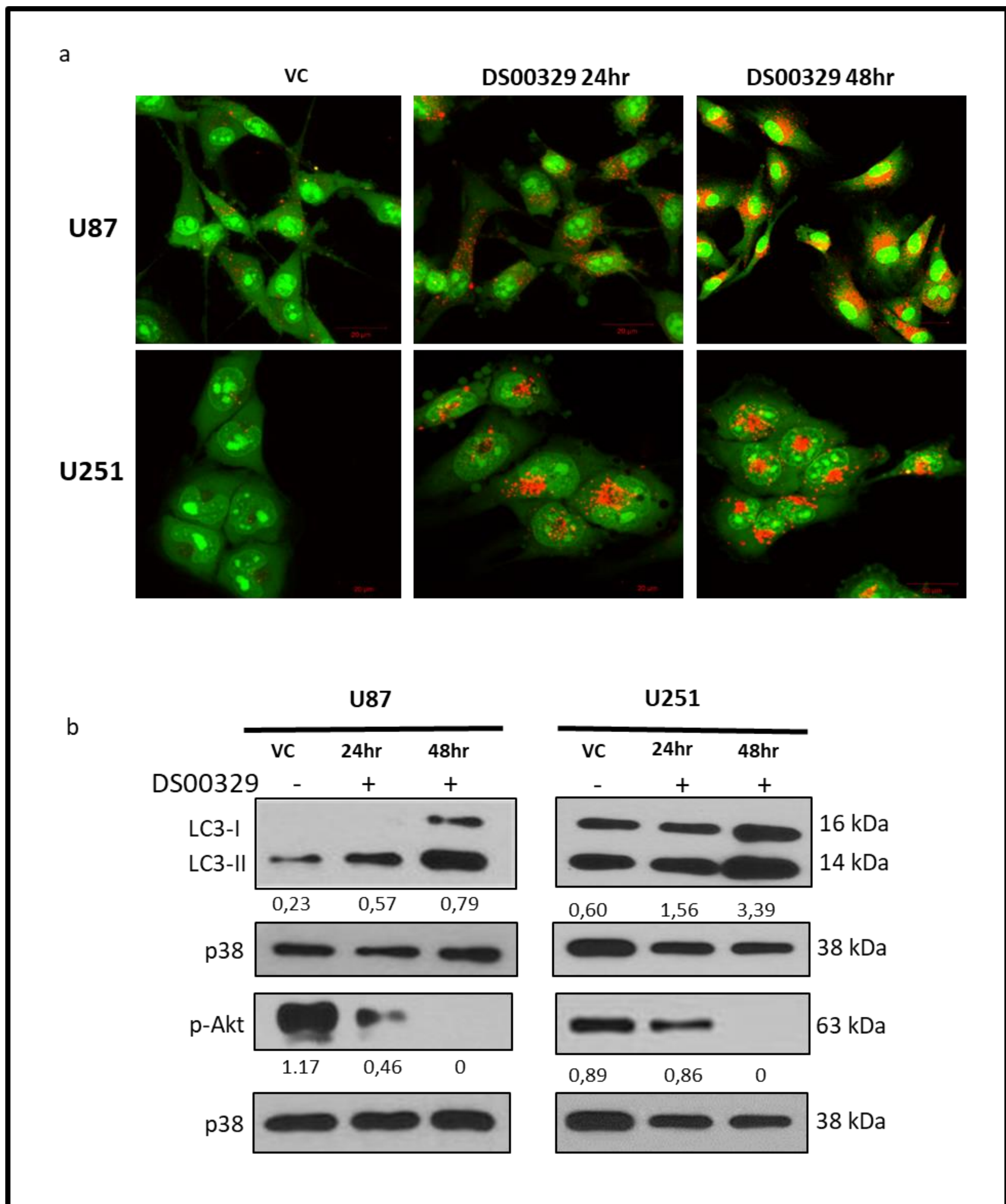


Figure 4.12. DS00329 induces autophagy and inhibits the Akt pathway. **(a)** U87 and U251 cells were treated with either vehicle (VC) or DS00329 for 24 and 48 hours and acridine orange staining for AVO as indication for autophagy. Increased acidic components when visualized under the confocal microscope are depicted by the increased orange fluorescence in the treated cells when compared with control**. (b)** Western blot analysis of protein extracts from vehicle (VC) or DS00326 treated U251 and U87 cells for 24 and 48 hours with antibodies to LC3, pAkt and p38 (loading control). Densitometric values (numbers under each blot) show the ratio of LC3-II (14kDa) and pAkt to that of p38.

Depending on the stimulus, autophagy can function as either a pro-survival or a pro-death pathway. Indeed, whereas some anti-cancer drugs induce autophagic cell death, others induce autophagy which leads to tumour cell survival and consequently drug resistance. The Akt pathway is constitutively active in many cancers where it promotes tumour survival and the inhibition of the Akt/mTORC1 pathway is a trigger to promote autophagic cell death. The impact of DS00326 and DS00329 on levels of phosphorylated Akt (pAkt) was therefore next investigated by western blotting. The results show that there was a marked reduction in pAkt levels at 24 and 48 hours of treatment (**Figure 4.11b and 4.12b**). Together these results suggest that the autophagy induced by DS00326 and DS00329 in U87 and U251 cells was probably pro-cell death.

4.8 Autophagy induced by DS00326 and DS00329 is a pro-death pathway

To confirm that DS00326 and DS00329 induce autophagic cell death, U87 and U251 cells were pre-treated with wortmannin and bafilomycin A1, chemical inhibitors of early and late stage autophagy respectively, followed by treatment with the compounds and cell viability was measured using the MTT assay (Yamamoto et al., 1998). The results show that blocking autophagy induced by DS00326 lead to a significant increase in cell viability of U87 cells and while not significant, this trend could also be seen in U251 cells (**Figure 4.13**). Similarly, inhibiting autophagy induced by DS00329 lead to a significant increase in cell viability of U87 and U251 cells (**Figure 4.14**). These results confirm that DS00326 and DS0039 induced autophagy in glioblastoma cells is pro-death.

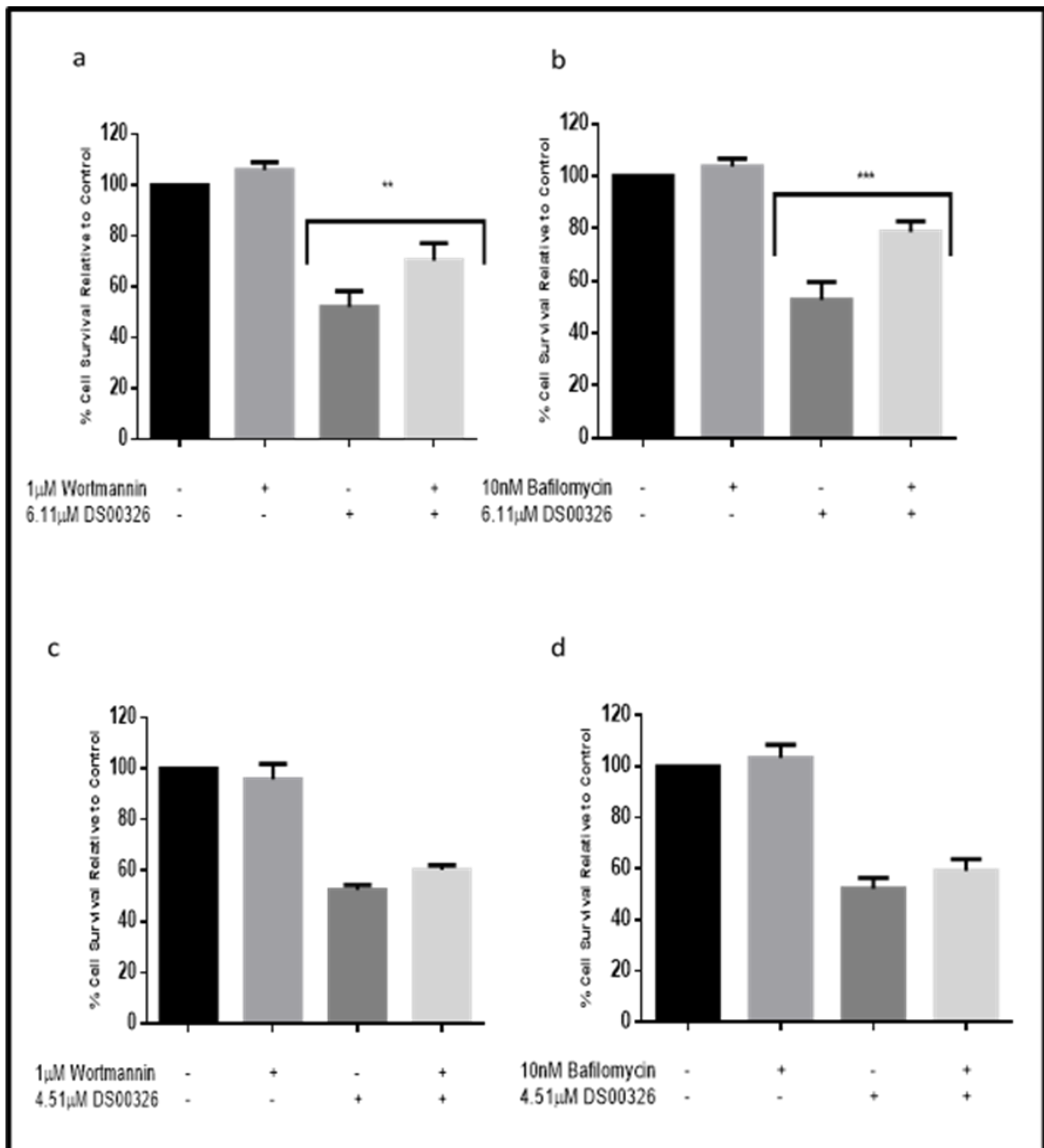


Figure 4.13. Inhibition of autophagy inhibited DS00326 induced cell death in glioblastoma cells. U87 and U251 were plated on 96 well plates and treated with either vehicle, inhibitors of autophagy (wortmannin and bafilomycin) or DS00326 for 48 hours and MTT assay was performed after treatment. Percentage cell survival of U87 cells treated with DS00326 following inhibition with wortmannin **(a)** and bafilomycin **(b)**. Percentage cell survival of U251 cells treated with DS00326 following inhibition with wortmannin **(c)** and bafilomycin **(d)**. Results show bars with the mean percentage ± SEM of data of at least three experiments performed in quadruplicate and significance ($P \leq 0.05$) indicated by * was tested between cells treated with DS00326 and the corresponding inhibitor.

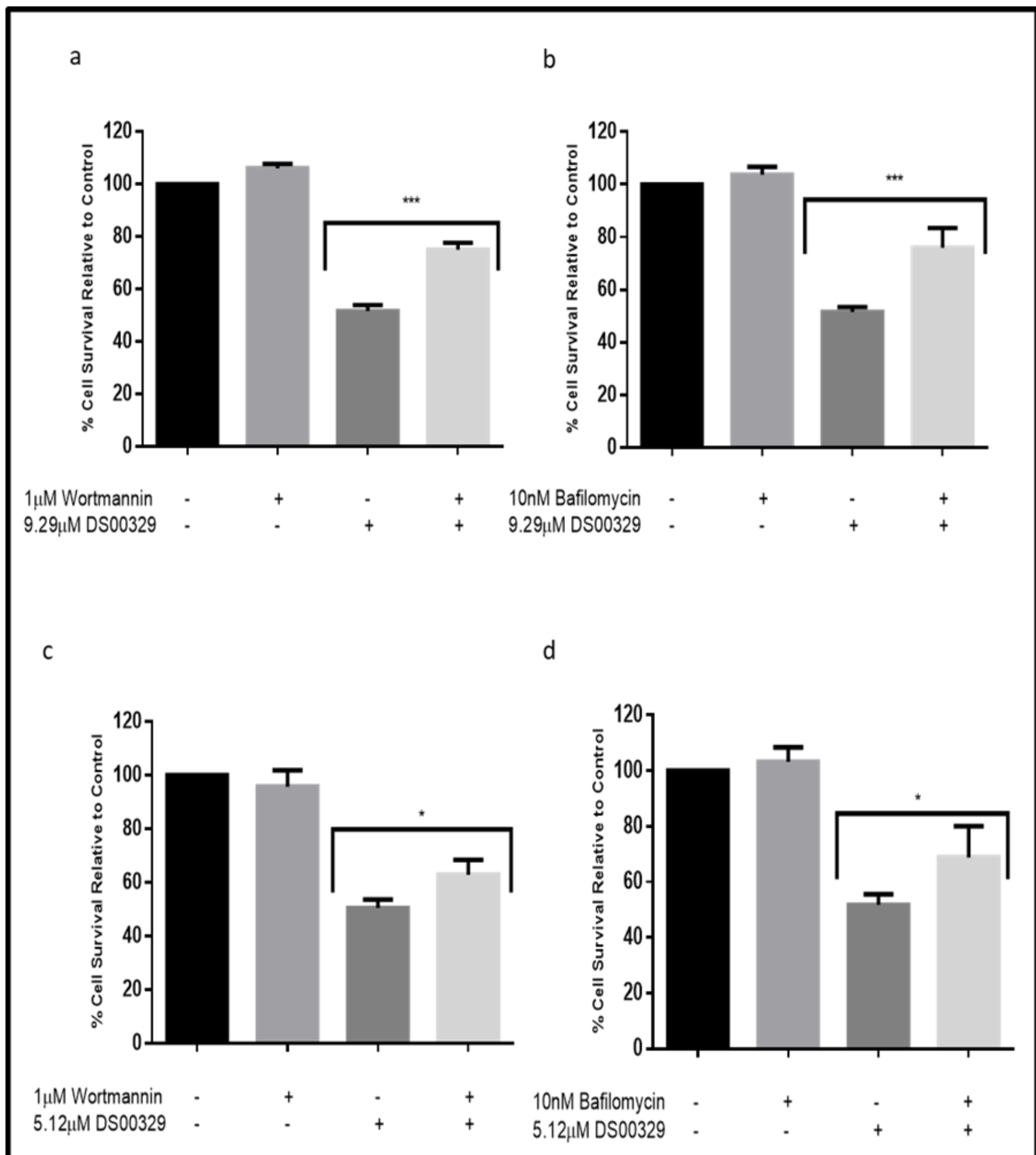


Figure 4.14. Inhibition of autophagy inhibited DS00329 induced cell death in glioblastoma cells. U87 and U251 were plated on 96 well plates and treated with either vehicle, inhibitors of autophagy (wortmannin and bafilomycin) or DS00329 for 48 hours and MTT assay was performed after treatment. Percentage cell survival of U87 cells treated with DS00329 following inhibition with wortmannin **(a)** and bafilomycin (**b**). Percentage cell survival of U251 cells treated with DS00329 following inhibition with wortmannin (**c**) and bafilomycin (**d**). Results show bars with the mean percentage ± SEM of data of at least three experiments performed in quadruplicate and significance ($P \leq 0.05$) indicated by * was tested between cells treated with DS00329 and the corresponding inhibitor.

4.9 DS00326 and DS00329 trigger ERK and p38 MAPK pathways in glioblastoma cells

The MAPK comprises of three distinct signalling molecules, JNK, p38 and extracellular signal-regulated kinase (ERK1/2). When activated due to phosphorylation, they could transmit extracellular signals to regulate cell proliferation, differentiation, migration and cell death (McCubrey et al., 2006, Torii et al., 2006, Dhillon et al., 2007, Sui et al., 2014). In response to chemotherapeutic agents and other stimuli, MAPKs have been implicated in the induction of both apoptotic and autophagic cell death in various cancers (Cobb, 1999, Bode and Dong, 2005, Hundley and Rigas, 2006). Activation of p38 MAPK was also reported to induce cell death and a G1 arrest of the cycle in glioblastomas (Yao et al., 2008). Thus, to determine whether DS00326 and DS00329 modulate the MAPKs to elicit their activity in U87 and U251 malignant glioblastoma cells, protein extracts from treated and untreated cells were analysed by western blotting with antibodies to pp38 (phosphorylated p38), and pERK1/2 (phosphorylated ERK1/2). Results show that DS00326 treatment led to the activation of p38 (pp38) as well as the ERK (pERK1/2) MAPK pathway for both cell lines tested and pp38 was seen as an early response in the U87 cells (**Figure 4.15a**). Furthermore, U87 and U251 cells treated with DS00329 showed sustained activation of ERK and a slight increase in pp38 levels (**Figure 4.15b**). Taken together, these results may suggest that DS00326 and DS00329 modulate the ERK and the p38 MAPK pathways to possibly exert their anti-cancer activity in malignant glioblastomas. A previous study also reported that phenothiazines induced the activation of the MAPKs in oral squamous cell carcinoma (OSCC) to promote cell death (Wu et al., 2016).

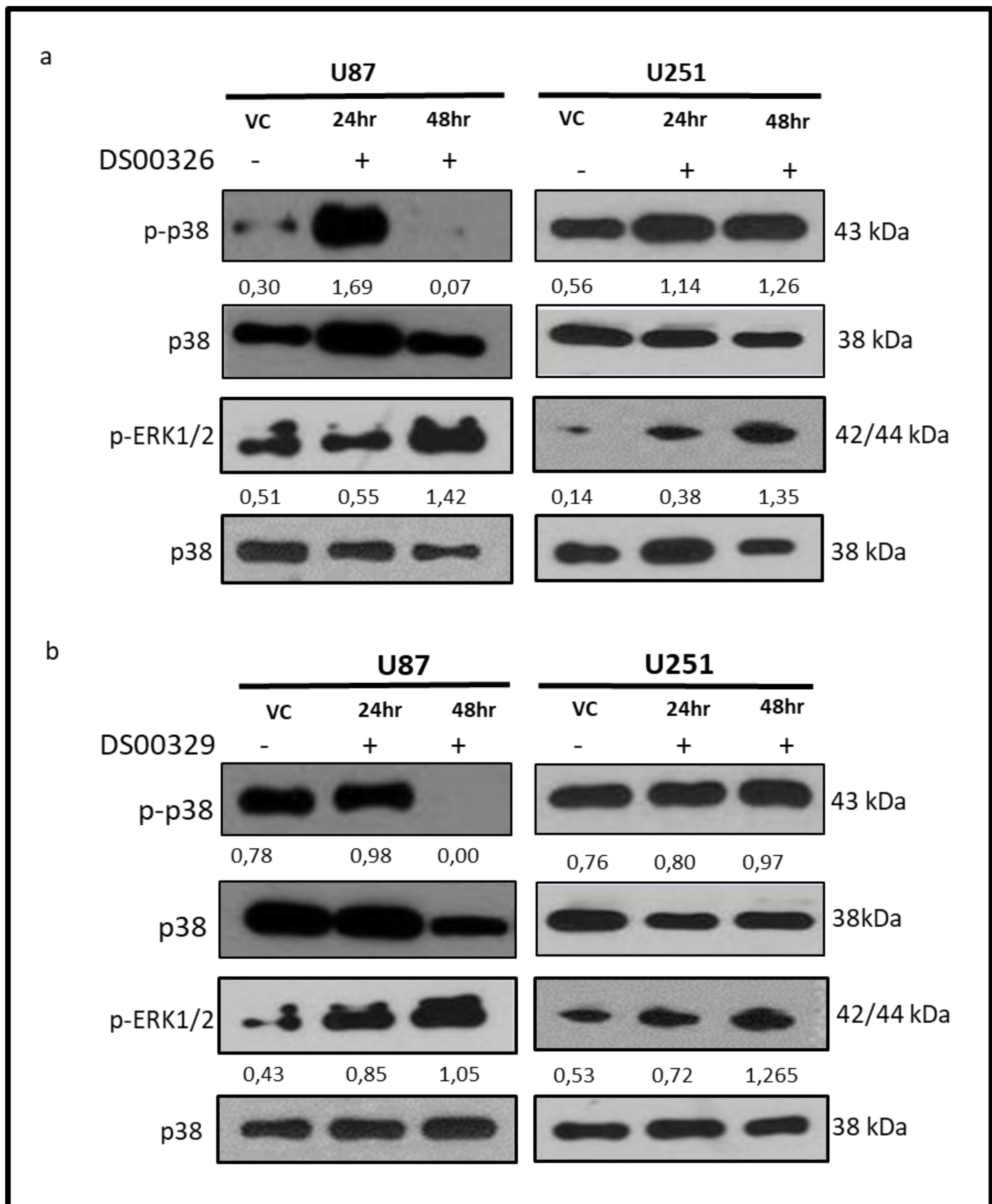


Figure 4.15. Impact of DS00326 and DS00329 on the p38 and ERK MAPK pathway. U87 and U251 cells were treated with either vehicle (VC) or DS00326 **(a)** or DS00329 **(b)** for 24 and 48 hours. Proteins were extracted and analysed by western blotting with antibodies to pp38, pERK1/2 and p38 (loading control). Densitometric values (numbers under each representative blots) show the ratio of pp38 and pERK1/2 to that of p38.

CHAPTER FIVE

ANTI-CANCER ACTIVITY OF DS00326 AND DS00329 IN GLIOBLASTOMA CELLS FROM PATIENT-DERIVED TUMOUR XENOGRAFT (PDX) MODELS

This section of the thesis investigates the anti-cancer activity of the phenothiazines DS00326 and DS0329 on glioblastoma cells from patient-derived tumour xenografts (PDX). The rationale for this was that PDXs resembles more closely tumours *in vivo* because they consist of all the different cell types that contribute to tumours and they are thus more predictive of the likely efficacy of a chemotherapeutic agent in tumours *in vivo* (Laks et al., 2009). To this end, T10 and T91 primary glioblastoma cell cultures derived from brain tumour biopsies of patients which were xenografted in mice were used for this study.

5.1 DS00326 and DS00329 inhibit T10 and T91 PDX cell viability

To investigate the effects of DS00326 and DS00329 on PDX cell viability, T10 and T91 cells were seeded at a density of 3000 cells per well in a 96 well plate. Following this, cells were exposed to increasing concentrations of compounds from 0 µM to 15 µM for 48 hours and cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega). Results show that DS00326 and DS00329 effectively reduced cell viability in a dose-dependent manner for both cell lines tested (**Figure 5.1**). Furthermore, DS00326 is more efficacious than DS00329 as it showed lower IC_{50} values in both PDX cell lines and the T91 cells were more sensitive to both compounds. Indeed, the IC_{50} values for DS00326 and DS00329 were 1.60 µM and 5.4 µM respectively in the T91 cells compared to 5.06 µM and 12.53 µM in the T10 cells. Taken together, these results show that DS00326 and DS00329 inhibit cell viability differentially in primary PDX glioblastoma cells.

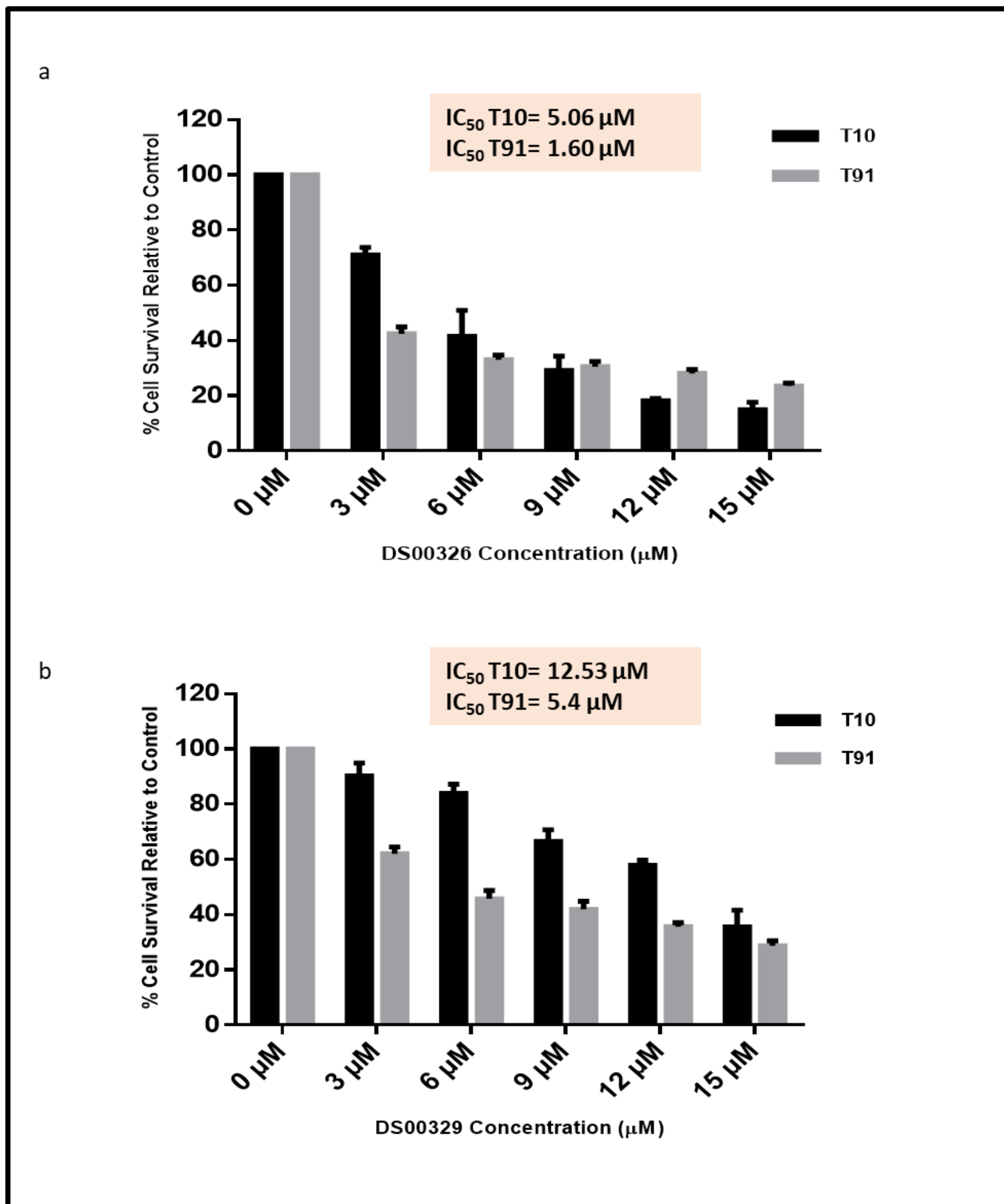


Figure 5.1. DS00326 and DS00329 induce cytotoxicity in T10 and T91 PDX cells. Bar graphs showing **p**ercentage cell viability (as measured by CellTitre Glo assays) in T10 and T91 cells exposed for 48 hours to vehicle or increasing concentrations (0-15µM) of **(a)** DS00326 and **(b)** DS00329. Results show bars with the mean percentage ± SEM of data of at least three experiments performed in quadruplicate. The concentration of DS00326 and DS00329 that killed 50% of the cells (IC_{50}) was calculated using GraphPad Prism Version 6.

5.2 Impact of DS00326 and DS00329 on neurosphere formation and cancer stem cell activity in PDX glioblastoma cells

PDX glioblastoma cells are heterogeneous in nature with defined cellular hierarchies and genomic hallmarks similar to their donor tumour (Xu et al., 2017). Notable amongst these cell types are the cancer stem cells with self-renewing properties that contribute to tumour initiation, resistance to chemotherapeutic agents as well as tumour recurrence (Lathia et al., 2015). Thus, the ability of an anti-cancer agent to target these tumour stem cells or tumour initiating cell populations will, in turn, inhibit tumour cell proliferation, drug resistance and recurrence (Azari et al., 2011). The ability of PDX glioblastoma cells to grow as neurospheres have been used over time to characterize and identify stem cells (Reynolds et al., 1992). Neurosphere forming cells are often termed ‘brain tumour stem cells’ due to their ability to form gliomas in mouse models (Singh et al., 2003). To evaluate the impact of DS00326 and DS00329 on stem cell activities in PDX cells, a neurosphere formation assay was conducted. Briefly, T10 and T91 PDX cells were plated at a density of 100 cells/well in a 12 well plate and exposed to the IC_{50} of DS00326 or DS00329 and then left in culture for 14 days to allow for neurosphere formation. The results show that, compared to the vehicle treated cells, sphere formation was significantly inhibited after incubation of T10 cells with DS00326 (**Figure 5.2a**) and DS00329 (**Figure 5.2b**). It is important to note that T91 cells did not form neurospheres after 14 days, perhaps they lack neurosphere forming capabilities or limited stem cell activities. These results indicate that the phenothiazines inhibited the self-renewing ability of the T10 PDX cells and consequently their survival.

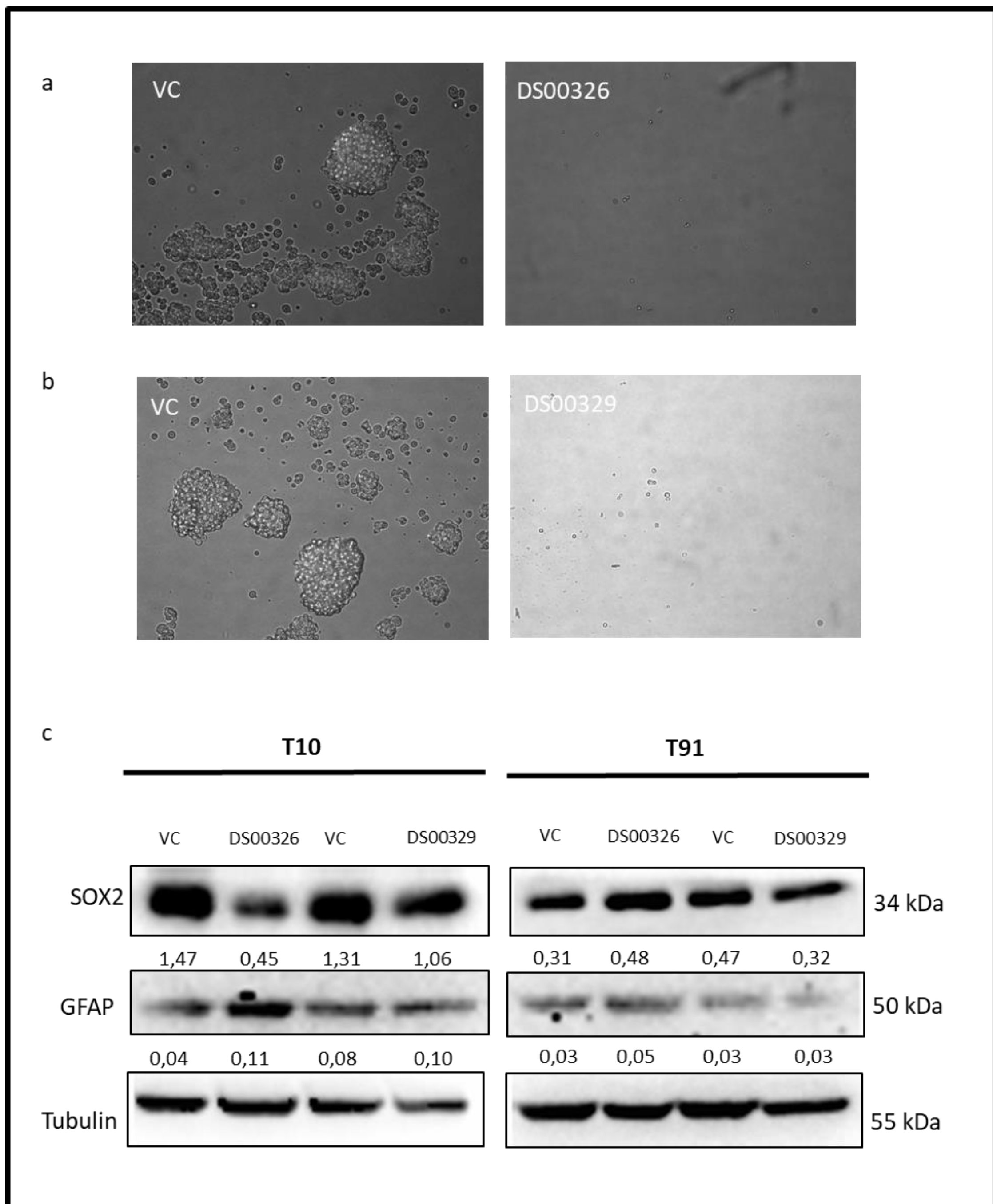


Figure 5.2. Anti-cancer stem cell activities of DS00326 and DS00329. Results from the neurosphere formation assay conducted for T10 cells plated at 100 cells per well in a 12 well plate and exposed to vehicle (VC), DS00326 **(a)** or DS00329 **(b)** for 14 days. **(c)** Western blot analyses of protein from T10 and T91 cells treated with either vehicle (VC) or DS00326 or DS00329 for 48 hours and probed with antibodies to SOX2, GFAP and Tubulin (loading control). Densitometric values (numbers under each representative blot) show the ratio of SOX2 and GFAP to Tubulin.

The anti-cancer stem cell activities of DS00326 and DS00329 were next investigated by western-blotting with antibodies to the stem cell markers Sex-determining region Y-box 2 (SOX 2) and Glial fibrillary acidic protein (GFAP). Briefly, T10 and T91 PDX cells were treated with the IC_{50} of DS00326 or DS00329 for 48 hours and total protein extracted from the cells. While SOX2 promotes the maintenance of neural stem cells and the regulation of stemness of glioma-initiating cells, GFAP is a differentiation marker in stem cells (Ikushima et al., 2009, Annovazzi et al., 2011). **Figure 5.2c** shows, as expected, that T10 cells treated with both DS00326 and DS00329 had a decrease in SOX2 levels which corresponded with an increase in GFAP levels. This indicates that DS00326 and DS00329 reduced stemness and induced differentiation in T10 cells. These results are encouraging because they suggest that treatment with these phenothiazines will prevent glioblastoma drug resistance and recurrence. Consistent with the data showing that T91 cells are unable to form neurospheres, they exhibited no remarkable changes in SOX2 and GFAP levels. Taken together, these results suggest that DS00326 and DS00329 displayed anti-cancer stem cell activities in T10 PDX glioblastoma cells.

5.3 Impact of DS00326 and DS00329 on cell cycle profile of T10 and T91 PDX glioblastoma cells

To ascertain the mechanism by which the phenothiazines DS00326 and DS00329 induce anti-cancer activity in T10 and T91 PDX cells, FACs analyses were firstly performed to determine their impact on the cell cycle. **Figure 5.3** shows that there was a slight, albeit not significant, increase in sub-G1 T10 cells treated with DS00326 and DS00329 for 48 hours and this corresponded with a slight decrease in G2/M cells. The cell cycle profile obtained for the T91 cells treated with DS00326 and DS00329 showed a significant increase in the number of G1 cells with a reduction in cells in S phase (**Figure 5.4**). It is worth stating that the cell cycle profiles of T10 cells were manually gated into the various

cell populations as the flowjo software did not automatically recognise the sub-G1 cell population, hence the difference in appearance. These results indicate that DS00326 and DS00329 treatment led to only small changes in the cell cycle profile of PDX glioblastoma cells, and that they appear to induce a sub-G1 apoptotic peak and exert a G1 arrest in the T10 and T91 cells respectively.

5.4 DS00326 and DS00329 induce double-strand DNA breaks and the DNA damage response in T10 and T91 PDX cells

Many anti-cancer drugs, as well as phenothiazines, are known to induce double-strand DNA breaks in cancerous cells and this was also shown for DS00326 and DS00329 in the U87 and U251 glioblastoma cells (**Figure 4.6**). To determine if this is also the case for PDX glioblastoma cells, T10 and T91 cells were plated on coverslips and treated with either vehicle, or with IC_{50} of DS00326 or DS00329, and then processed for immunofluorescence microscopy with antibodies to γH2AX. As shown in representative micrographs, there was a marked increase in the intensity of γH2AX, indicated by the fluorescent puncta in the nuclei of the T10 and T91 cells following treatment with the compounds (**Figures 5.5 and 5.6**). Though some artefacts from the geltrex used to enhance adhesion of the cells to coverslips picked up the secondary antibody, this was distinguished from real cells by the DAPI nuclei stain.

Changes in levels of key proteins involved in the canonical DNA damage response pathway were next investigated in T10 and T91 PDX cells following treatment with IC_{50} of DS00326 and DS00329 for 48 hours. Proteins were harvested and subjected to western blotting with antibodies to γH2AX, pATM, ATM, p53 and pcdc2 (phosphorylated cell division control 2). The cdc2 also known as cyclin-dependent kinase1 lies downstream of the DDR signalling and when bound to its cyclin partners, it phosphorylates thus leading to the progression of the cell cycle (Enserink and Kolodner, 2010).

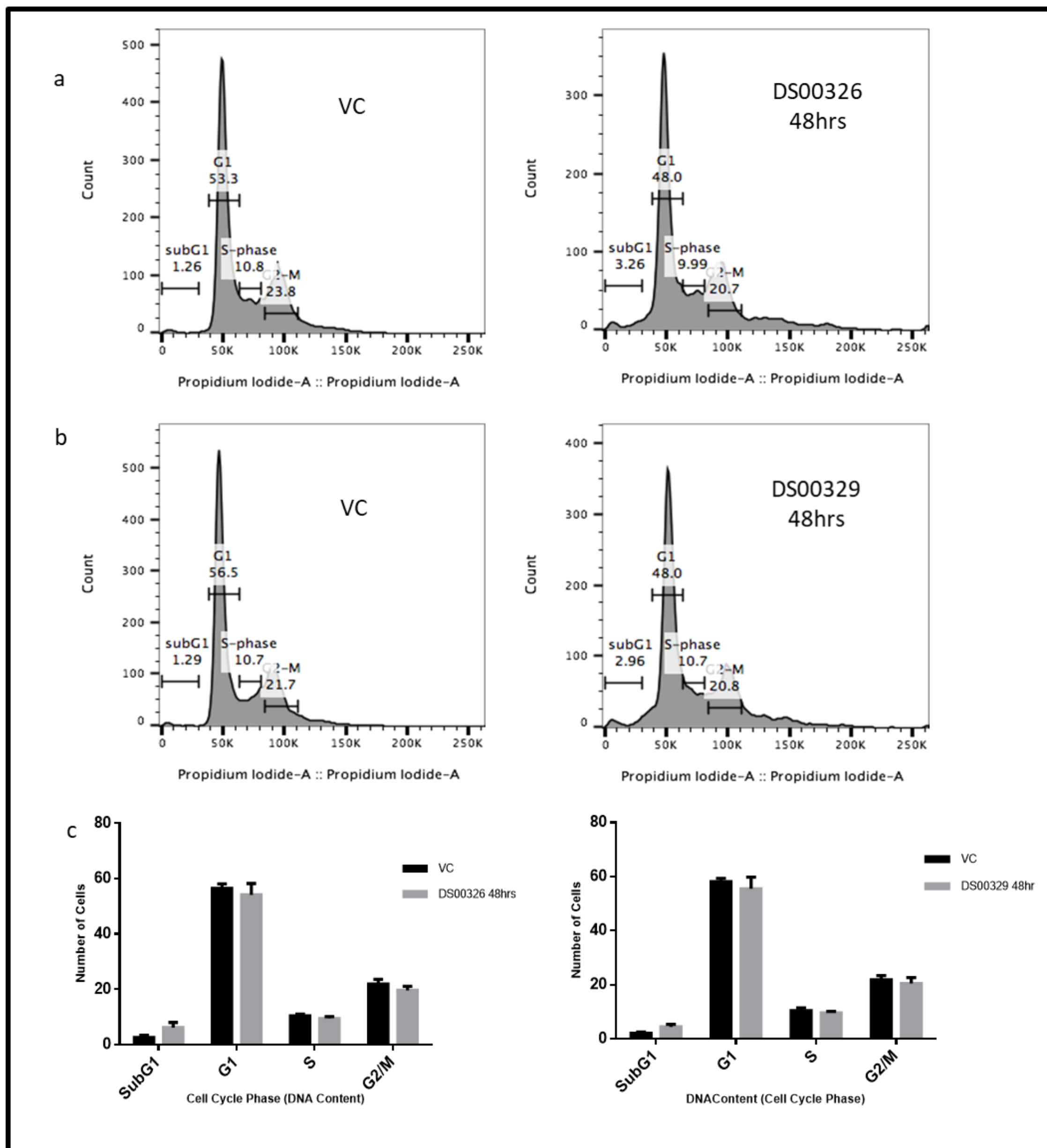


Figure 5.3. Cell cycle profile of T10 cells treated with DS00326 and DS00329. (a, b) Flow cytometry of T10 cells exposed to either vehicle (VC) or DS00326 or DS00329 for 48 hours and stained with propidium iodide. The number of cells in each phase of the cell cycle was expressed as a percentage of the total number of cells analysed. **(c)** Bar graph showing the percentage of cells in the Sub-G1, G1, S and G2/M phases of the cell cycle. Bars show the average of three independent experiments with the mean percentage ± SEM as calculated using GraphPad Prism Version 6 and significance ($P \leq 0.05$) indicated by * was tested between treated cells and vehicle control.

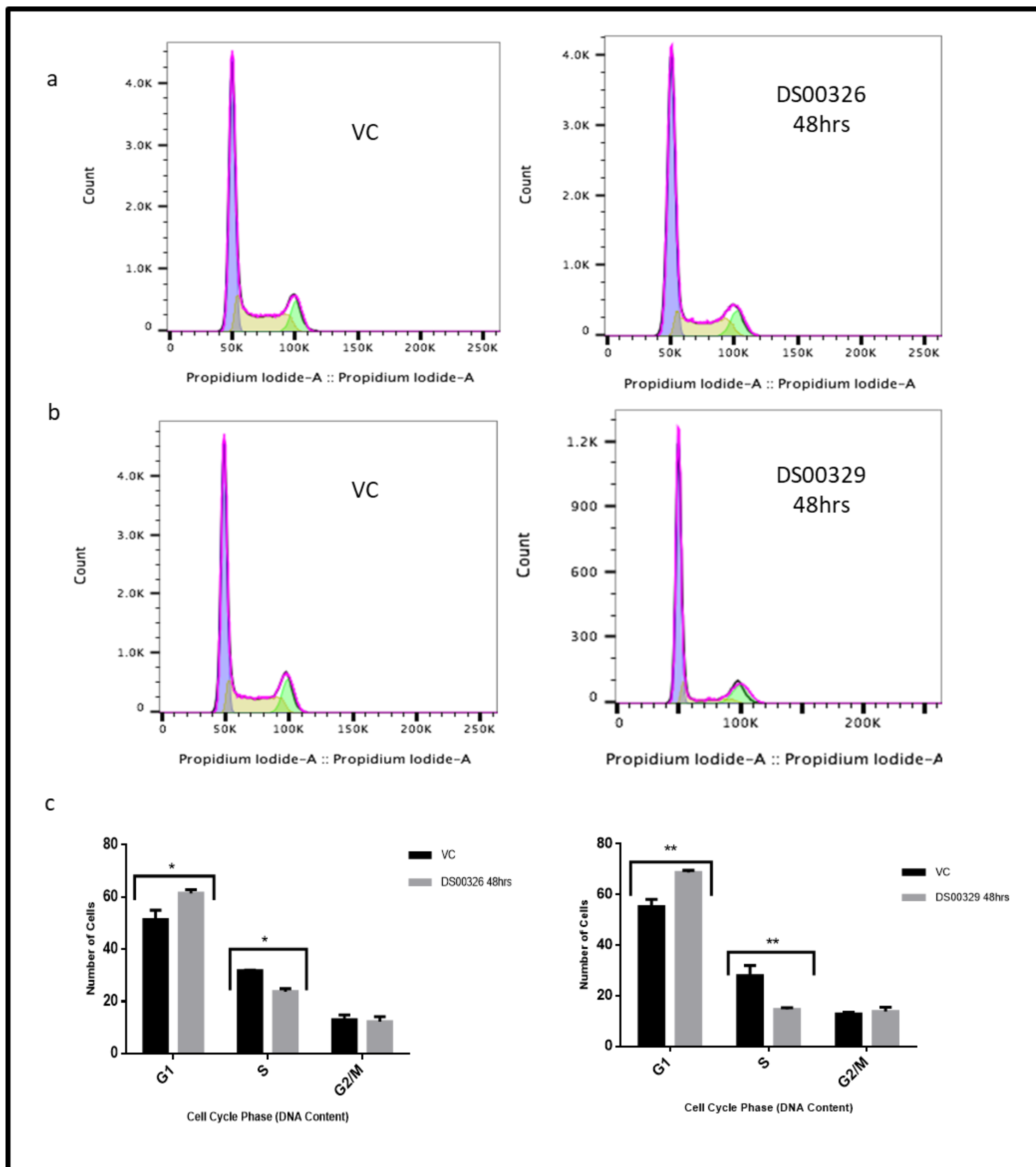


Figure 5.4. Cell cycle profile of T91 cells treated with DS00326 and DS00329. (a, b) Flow cytometry of T10 cells exposed to either vehicle (VC) or DS00326 or DS00329 for 48 hours and stained with propidium iodide. The number of cells in each phase of the cell cycle was expressed as a percentage of the total number of cells analysed. **(c)** Bar graph showing the percentage of cells in the Sub-G1, G1, S and G2/M phases of the cell cycle. Bars show the average of three independent experiments with the mean percentage ± SEM as calculated using GraphPad Prism Version 6 and significance ($P \leq 0.05$) indicated by * was tested between treated cells and vehicle control.

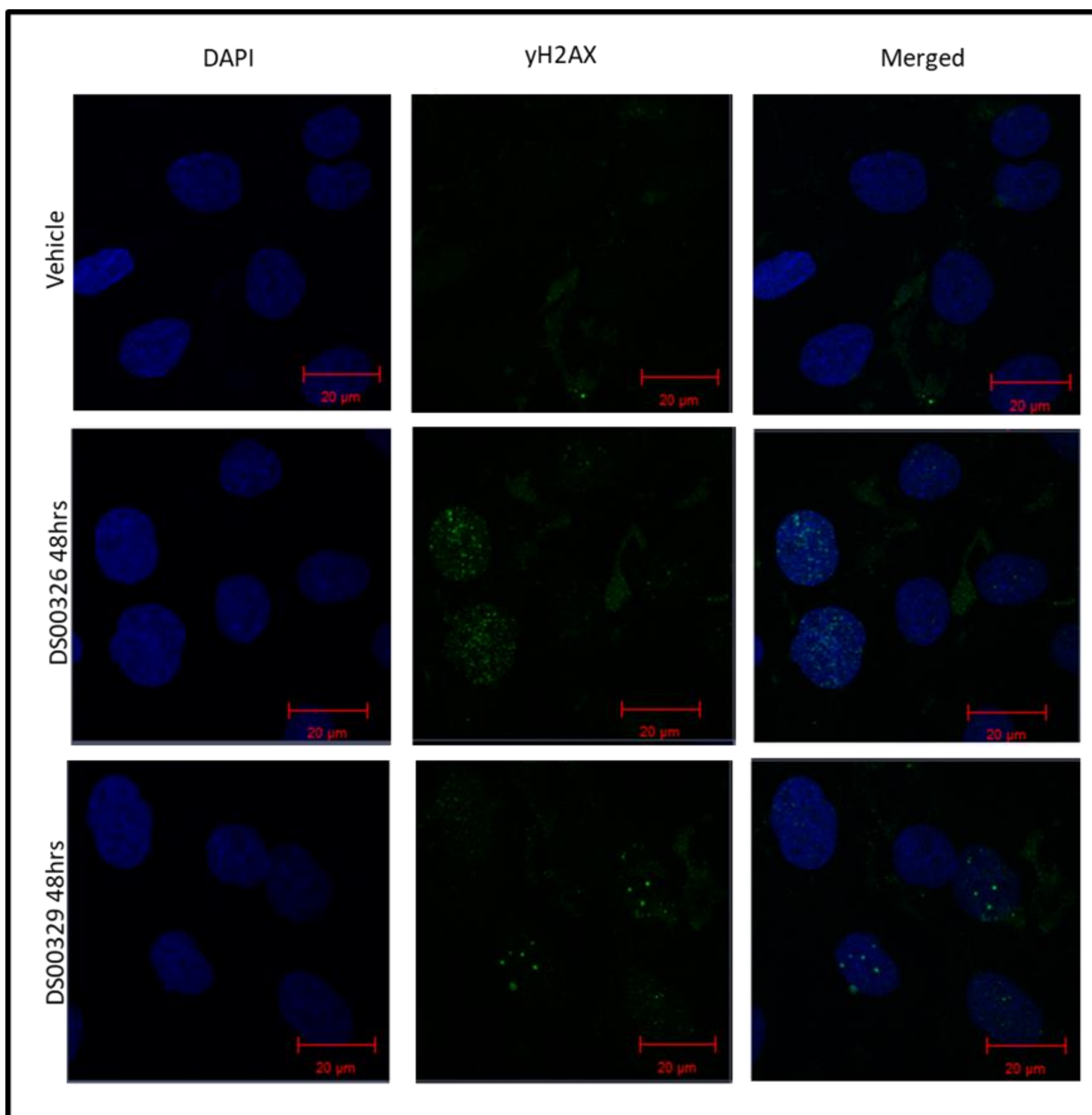


Figure 5.5. DS00326 and DS00329 induce γH2AX foci in T10 cells. Immunofluorescence microscopy showing γH2AX foci formation (green puncta) in nuclei of cells treated with either vehicle, DS00326 or DS00329 for 48 hours. Cells were counterstained with DAPI to visualise nuclei (left panels) and the right panel shows merged images.

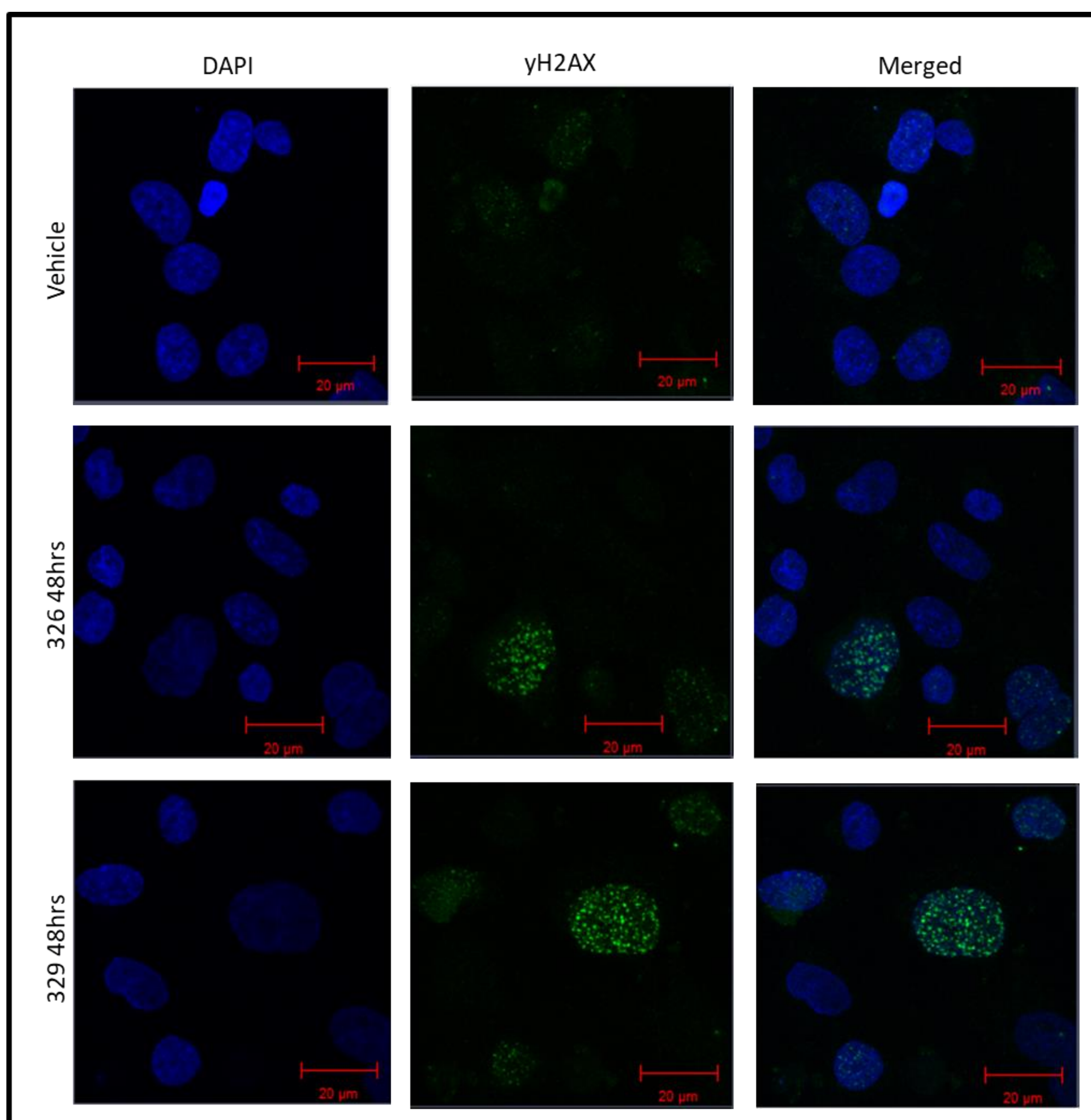


Figure 5.6. DS00326 and DS00329 induce γH2AX foci in T91 cells. Immunofluorescence microscopy showing γH2AX foci formation (green puncta) in nuclei of cells treated with either vehicle, DS00326 or DS00329 for 48 hours. Cells were counterstained with DAPI to visualise nuclei (left panels) and the right panel shows merged images.

Figure 5.7 shows that DS00326 and DS00329 induced the DDR pathway in both T10 and T91 cells as there was an upregulation of the DDR sensor γH2AX as well as an increase in levels of phosphorylated (active) ATM with no change in levels of total ATM. This corresponded with an increase in levels of the tumour suppressor p53 and a decrease in levels of pcdc2, which is a key driver of the cell cycle and has been reported to promote cell proliferation and tumourigenesis in glioblastoma as well as in other cancers (Chen et al., 2008, Zhang et al., 2011, Wei et al., 2013). Taken together, these results suggest that the anti-cancer activity induced by DS00326 and DS00329 in T10 and T91 PDX cells involves double-strand DNA damage.

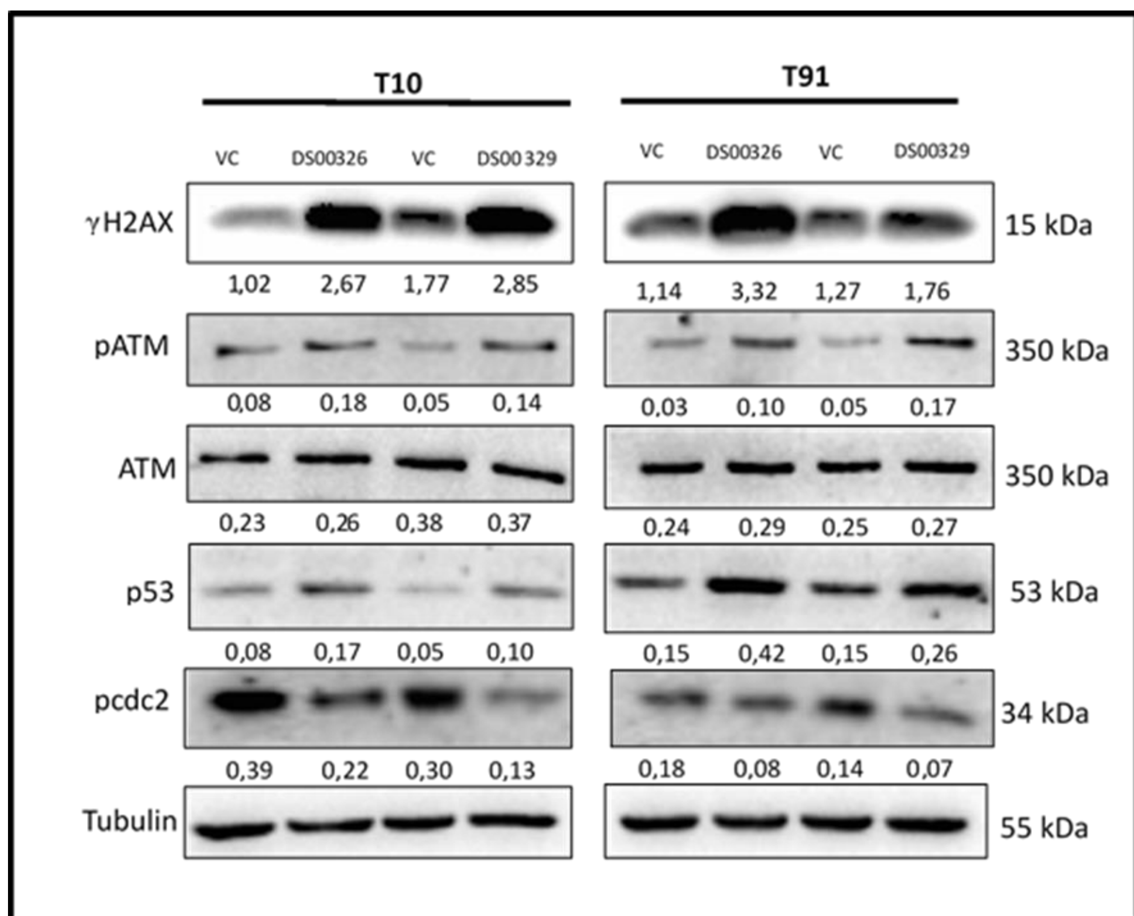


Figure 5.7. DS00326 and DS00329 trigger the DNA damage response. Western blot analyses of protein from T10 and T91 cells treated with either vehicle (VC), DS00326 or DS00329 for 48 hours and probed with antibodies to γH2AX, pATM, ATM, p53, pcdc2 and Tubulin (loading control). Densitometric values (numbers under each representative blot) show the ratio of γH2AX, pATM, ATM, p53, pcdc2 to Tubulin.

5.5 Impact of DS00326 and DS00329 on apoptosis in T10 and T91 PDX cells

To determine whether DS00326 and DS00329 induce apoptosis in T10 and T91 PDX cells, the cells were treated with the compounds for 24 hours and 48 hours and western blotting was performed to examine PARP cleavage. **Figure 5.8** shows that DS00326 induced apoptosis in T10 cells as shown by the increase in cleaved PARP levels following treatment. However, DS00329 treated T10 cells showed no remarkable change in cleaved PARP levels. Furthermore, for all treatment conditions, DS00326 and DS00329 lead to increased PARP cleavage in the T91 PDX cells. Together, these results suggest DS00326 and DS00329 activate the apoptotic pathway in PDX glioblastoma cells except for the T91 cells treated with DS00329.

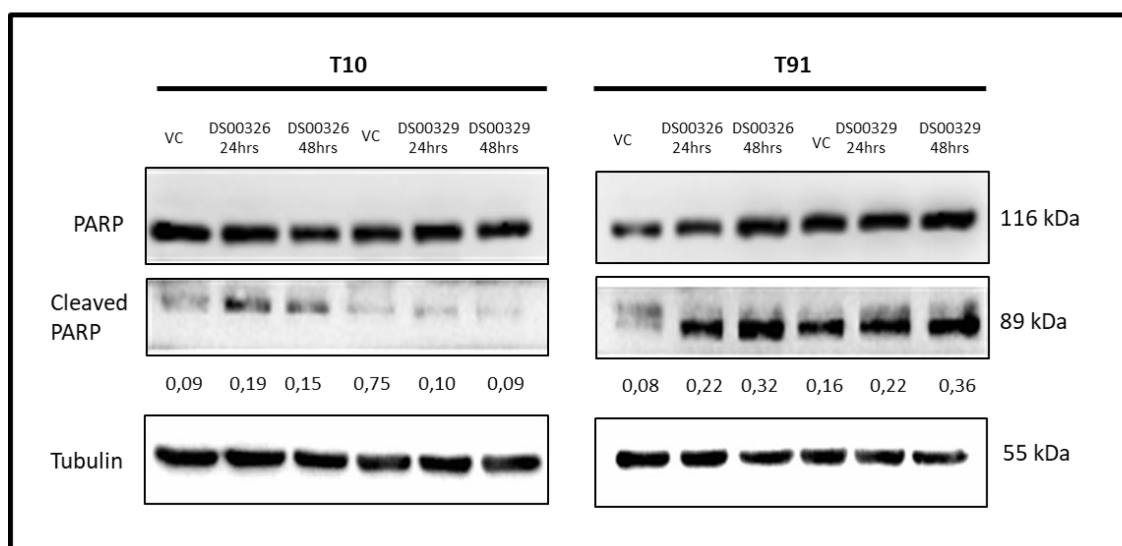


Figure 5.8. Impact of DS00326 and DS00329 on apoptosis in T10 and T91. Western blot analyses of protein from T10 and T91 cells treated with either vehicle (VC), DS00326 or DS00329 for 24 and 48 hours and probed with antibodies to PARP and Tubulin (loading control). Densitometric values (numbers under each representative blot) show the ratio of cleaved PARP to Tubulin.

5.6 Impact of DS00326 and DS00329 on autophagy and autophagic flux in PDX in PDX glioblastoma cells

As previously stated, the conversion of LC3-I to LC3-II serves as a hallmark for autophagic activity and thus the levels of LC3-II correlate with the extent of autophagic vesicle formation. To ascertain whether DS00326 and DS00329 lead to the accumulation of LC3-II in T10 and T91 PDX cells, western blotting was performed with an antibody against LC3. Results show that DS00326 and DS00329 lead to an increase in LC3-II in all treatment conditions, though LC3-II signal for the DS00329 treated T10 cells was not as significant compared to others (**Figure 5.9a**).

Increased LC3-II levels can be associated with either enhanced autophagosome synthesis or reduced autophagosome turnover, perhaps due to delayed trafficking to the lysosomes (Barth et al., 2010). Thus, to differentiate between these two processes, it is important to perform western blotting on protein extracts harvested from cells treated with inhibitors to autophagy, such as bafilomycin A1 (BAF). BAF is a lysosomal inhibitor, which blocks autophagy at the late stage by preventing the fusion of autophagosomes and lysosomes (Mauvezin and Neufeld, 2015). In the presence of BAF, accumulation of LC3-II-positive autophagosomes would be evident by efficient autophagic flux, while the failure of LC3-II protein to increase in the presence of such inhibitors, would indicate a defect or delay in the process, prior to degradation at the autolysosome**. Figure 5.9b** shows that treatment with BAF alone led to an increase in the amount of LC3-II for both cell lines. For all treatment conditions except the T10 cells treated with DS00329, the addition of BAF at the last two hours of the experiment to the cells treated DS00326 or DS00329 further increased the levels of LC3-II suggesting that treatment with these compounds enhanced autophagic flux. Together, these results suggest that DS00326 and DS00329 induce

autophagy as well as autophagic flux differentially in T10 and T91 PDX glioblastoma cells.

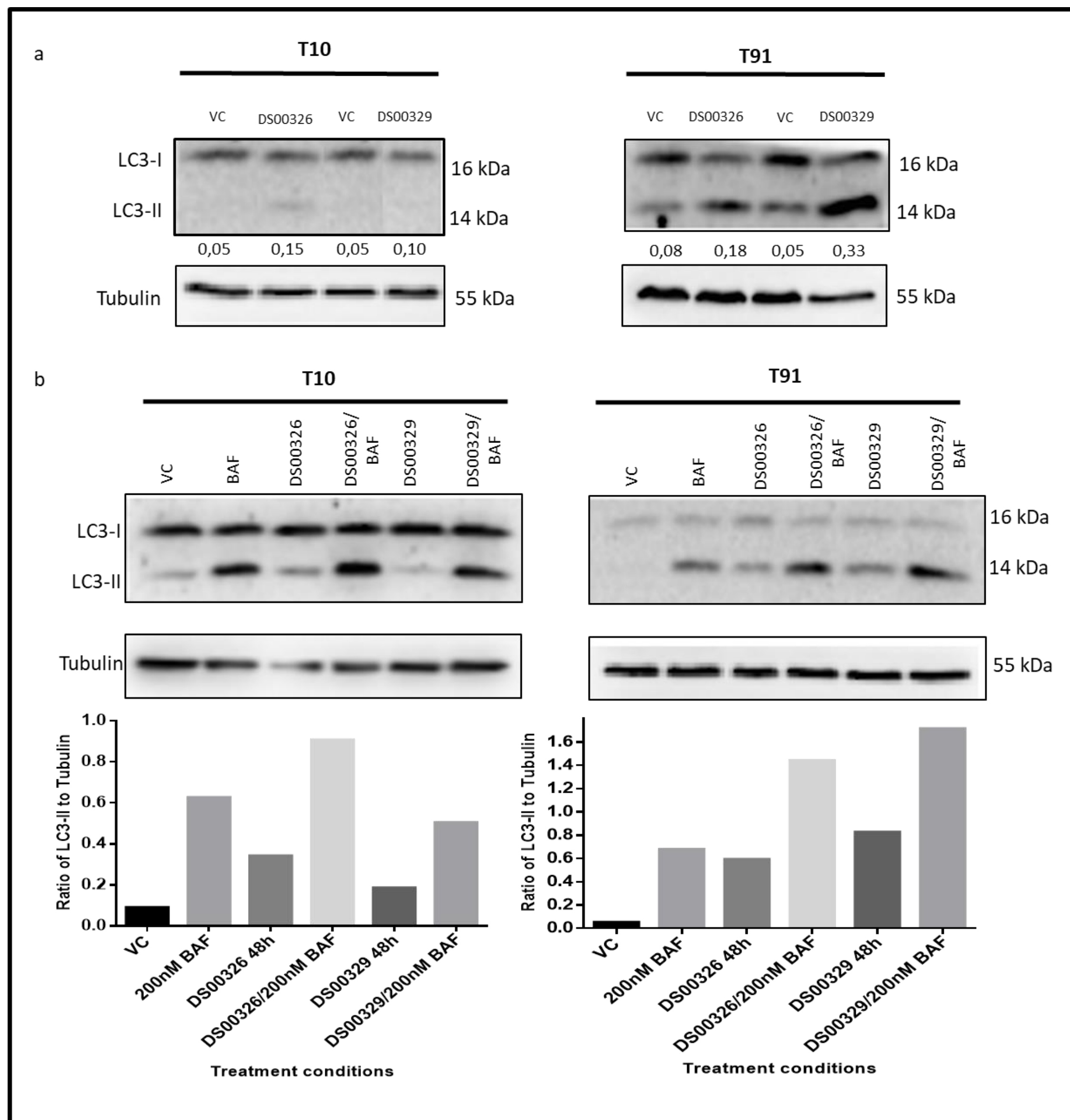


Figure 5.9. Impact of DS00326 and DS00329 on autophagy and autophagy flux in PDX cells. T10 and T91 cells were treated with either vehicle (VC), DS00326 or DS00329 for 48 hours as well as 200nM BAF and proteins were extracted and analysed by western blotting using antibodies to LC3 and Tubulin (loading control). **(a)** LC3 levels in cells treated without BAF showing densitometric values (numbers under each representative blot) indicating the ratio of LC3-II to tubulin. **(b)** LC3 levels in cells treated with compounds and BAF added at the last 2 hours of the experiment with bar graphs showing ratio of LC3-II to Tubulin.

5.7 Effect of DS00326 and DS00329 on MAPK signalling in T10 and T91 PDX glioblastoma cells

As previously reported, DS00326 and DS00329 triggered the activation of the p38 and ERK MAPKs in U87 and U251 glioblastoma cells. It was therefore next investigated if this is also the case for the T10 and T91 PDX glioblastoma cells treated with DS00326 and DS00329. Briefly, protein extracts from cells treated with or without compounds were subjected to western blot analyses with antibodies to total ERK, phosphorylated ERK1/2 (pERK1/2), JNK and phosphorylated JNK (pJNK). It would have been interesting to investigate the p38 MAPK in these cells, but as earlier stated this work was done with our collaborating laboratory in Denmark and experiments were conducted with available resources. Results however, show that under all conditions tested T10 did not express pERK1/2 but there was an increase in the levels of pJNK when the cells were treated with either DS00326 or DS00329 (**Figure 5.10**). Interestingly, while the levels of total JNK were not affected by treatment of T10 with either compound, total ERK levels slightly decreased following treatment and this has previously been attributed in part to a serine/threonine protein phosphatase (PP2A) which is known to impact on total ERK (Alessi et al., 1995). Moreover, it has been reported that phenothiazines have been shown to induce PP2A-mediated apoptosis in T-cell acute lymphoblastic leukaemia (Gutierrez et al., 2014b). T91 cells treated with either DS00326 or DS00329 showed an increase in the levels of both pERK1/2 and pJNK with no changes in total ERK and JNK expression. Together these results suggest that the ERK and JNK MAPKs may impact on the activity of DS00326 and DS00329 in T10 and T91 PDX glioblastoma cells possibly to induce cell death.

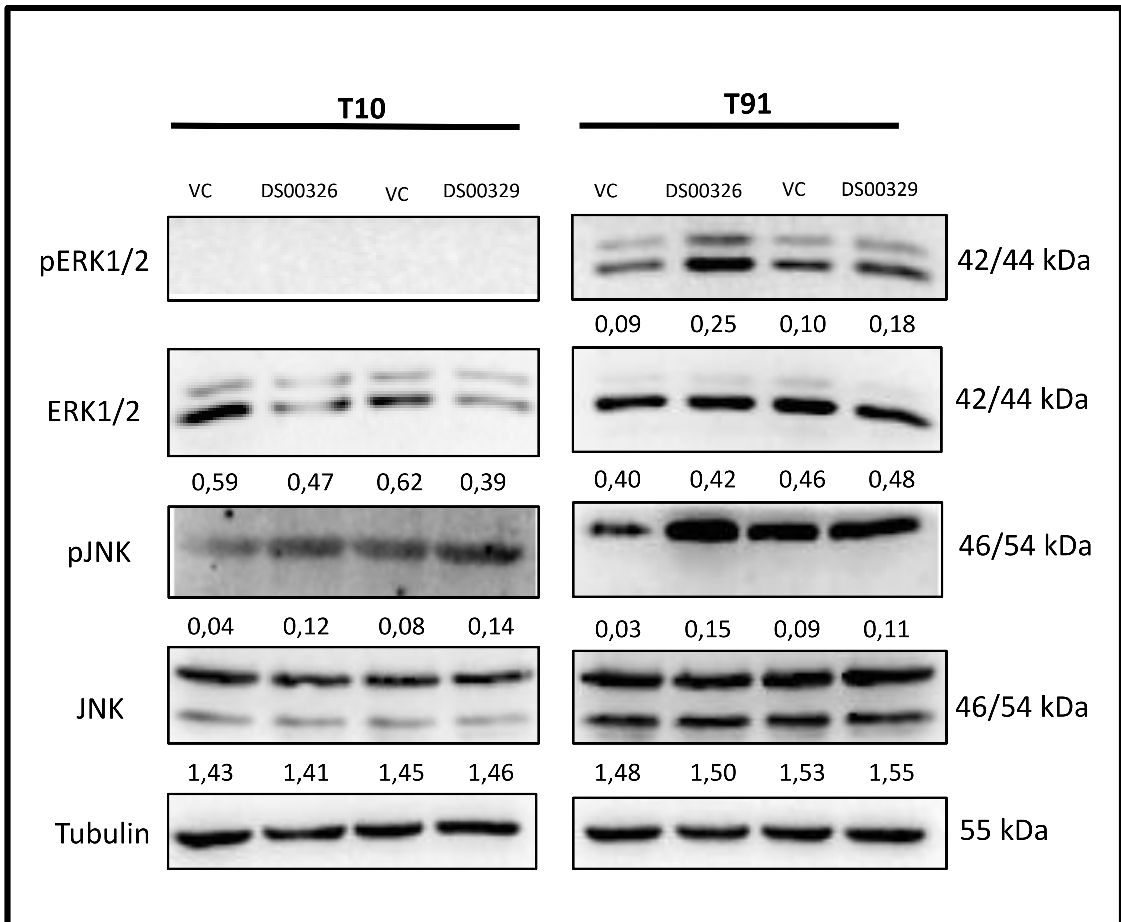


Figure 5.10. DS00326 and DS00329 modulate the ERK1/2 and JNK MAPKs. Western blot analyses of protein from T10 and T91 cells treated with either vehicle (VC), DS00326 or DS00329 for 48 hours and probed with antibodies to pERK1/2, pATM, ERK1/2, pJNK, JNK and Tubulin (loading control). Densitometric values (numbers under each representative blot) show the ratio of pERK1/2, pATM, ERK1/2, pJNK, JNK to Tubulin.

5.8 Profiling p53 in a panel of PDX glioblastoma cells

Mutations in p53, which occurs in most cancers including glioblastoma, have been found to predict a poor prognosis and outcome of chemotherapy in many cancer patients (Oren and Rotter, 2010). These mutations could be in the form of complete deletions demonstrating a loss of function or truncated mutations which could give rise to p53-null alleles (Levine, 1989, Liu et al., 2016). To determine the status of p53 in PDX glioblastoma cells used in this study, protein extracts from a panel of 6 (T10, T91, T131, T115, T133 and 4121) PDX cells were analysed by immunoblotting with the p53 D01 (antibody to detect both wild-type and mutant p53) and antibody to phosphorylated p53 serine 15 (antibody to detect activated p53). Results with both antibodies to p53 show that p53 was present in all the PDX cells except for the T131 cells which might be as a result of complete deletion of the p53 gene (**Figure 5.11a**). However, all other cells including T91 showed truncated p53 protein products which could be visible by the degraded protein bands under the upper p53 band (see arrows). No truncated protein product was detected in the T10 cells. This may be due to the low levels of p53 in these cells and a longer exposure time may be required to visualise their presence. Furthermore, phosphorylated p53 was picked up in all cells except for the T131 cells.

To confirm p53 status (whether it is mutant or wild-type) in T10 and T91 PDX glioblastoma cells, cells were plated on cover slips and processed for immunofluorescence microscopy with p53 D01 antibody. **Figure 5.11b** shows that all nuclei in the T10 and T91 cells stained positive for D01, thus indicating p53 mutations in these cells as cells with wild-type p53 will have more of cytosolic signals and few nuclei staining. Together, these results may suggest a mutation of p53 in the T91 and T10 PDX glioblastoma cells as well as in other PDX cells tested.

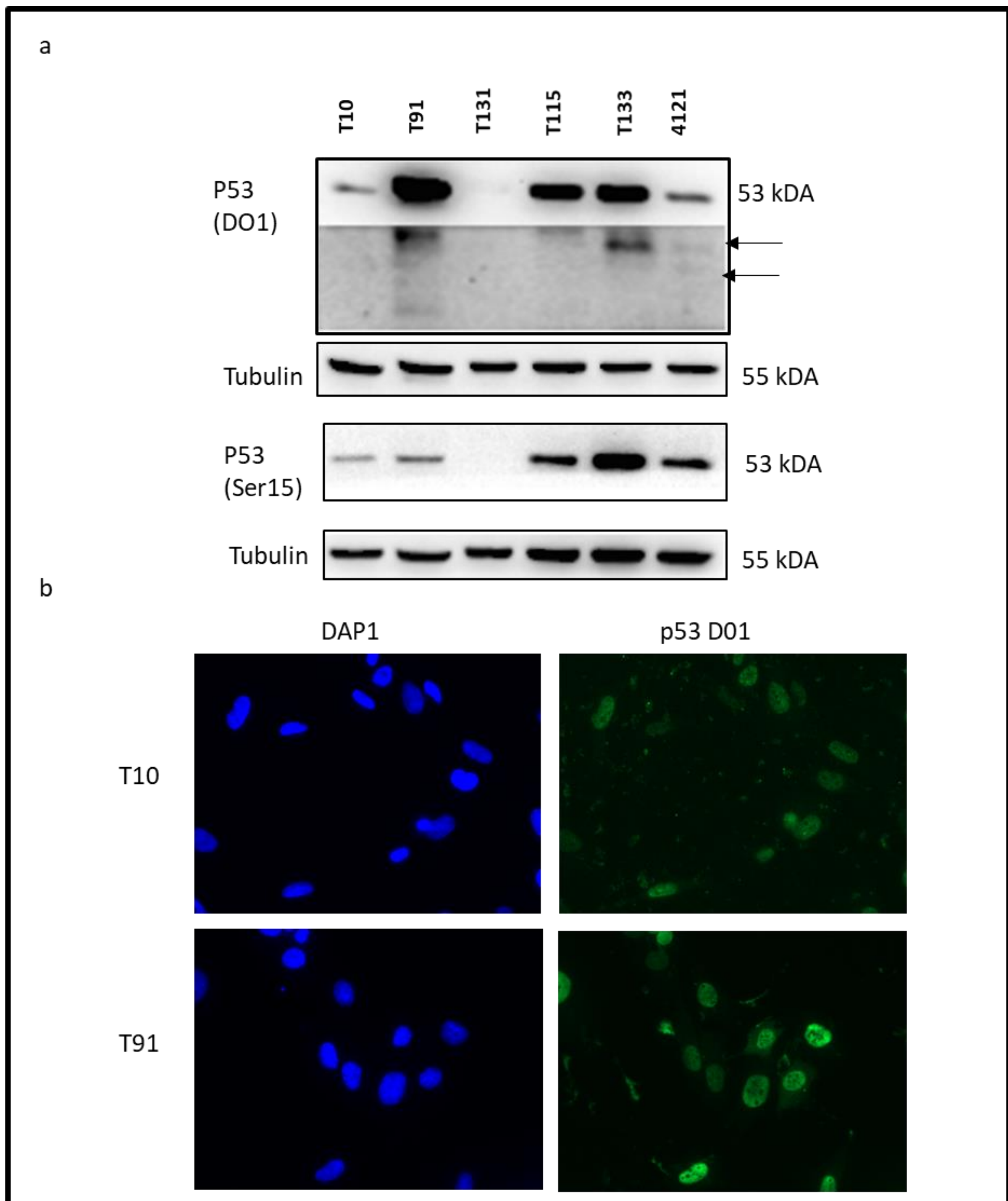


Figure 5.11. p53 status in a panel of PDX glioblastoma cells. (a) Protein extracts from T10, T91, T131, T115, T133 analysed by western blotting using two antibodies to p53 D01, p53 serine 15 and tubulin (loading control). **(b)** Micrographs of T10 and T91 cells labelled with DAPI and p53 D01 antibody to show expression of p53 in the cells.

CHAPTER SIX

DISCUSSION

Cancer is the second leading cause of death worldwide following cardiovascular diseases and poses both emotional and economic challenges to nations and patients. In 2015, it was reported that cancer-caused over 8.7 million deaths globally and despite improvements in diagnosis and treatments, cancer related deaths have been projected to reach 13.5 million cases in 2030 (Ferlay et al., 2015, Wang et al., 2016).

The most common and earliest chemotherapeutic agents for cancer treatment include 1-(2-chloroethyl) -1- nitrosoureas (e.g., BCNU and CCNU) and metallic compounds such as cisplatin and carboplatin amongst others. However, some of these agents are associated with side effects due to systemic toxicity as well as multidrug resistance. Despite technological advancements, curative treatment for cancer is still daunting and there is a dire need for effective chemotherapeutic agents which significantly reduce tumour burdens and slow tumour progression with minimal toxicity. It is also imperative for these novel therapies to speedily go through clinical trials and consequently approval for use in the clinic. An approach to this is the repurposing of already existing drugs or drugs that were abandoned in phase III clinical trials for new indications. The rationale for this is that these drugs are already approved and their safety profile and pharmacological properties would have been determined. This would dramatically shorten the drug discovery pipeline and expedite their progression to the clinic.

In recent times, a number of previously known drugs have been repurposed for glioblastoma treatment. Notable of such drugs which are already in clinical trials are metformin, mebendazole, disulfiram, chloroquine and chlorpromazine belonging to the metabolic, antihelminthic, nervous system, antiparasitic and antipsychotic class of drugs

respectively (Abbruzzese et al., 2017, Basso et al., 2018, Tan et al., 2018). Owing to the recorded successes of drug repurposing, the approach has continued to gain recognition in the drug discovery world as more FDA approved drugs are currently being explored for new indications including cancer and other disease conditions (Patwardhan and Chaguturu, 2016). Importantly, repurposing of antipsychotic drugs or drugs associated with the nervous system presents an advantage of readily crossing the blood-brain barrier (BBB) which is a well-known challenge to the treatment of glioblastoma.

Thus, the main focus of the current study was to repurpose derivatives of the antipsychotic drug phenothiazine for their anti-cancer activities in glioblastoma. This study screened novel phenothiazine-derivatives for their selective cytotoxicity in malignant glioblastoma cells and identified two lead compounds DS00326 and DS00329. It was further established that DS00326 and DS00329 elicit their cytotoxic activities by inducing double-strand DNA damage, cell cycle arrest as well as apoptosis and autophagy.

6.1 DS00326 and DS00329 show potency against malignant glioblastoma cells

Glioblastoma remains the most common and deadliest primary cancer of the brain, causing about 10 000 to 12 000 deaths annually in the U.S. alone (Purow, 2016). Though there has been an improvement in diagnosis, treatment of glioblastoma is still a challenge. This is largely due to tumour drug resistance, the inability of some chemotherapeutic drugs to pass through the blood-brain barrier (BBB) and the infiltrative nature of tumour cells which complicates the use of radiotherapy and surgery.

One reason why anti-cancer drugs display severe side-effects is the high concentrations at which they are administered. In the present study, DS00326 and DS00329, showed potent cytotoxicity against U87 and U251 malignant glioblastoma cells at relatively low

concentrations. Specifically, the IC_{50} values for DS00326 and DS00329 were 4.51 μM and 5.12 μM in the U251 cells and 6.11 μM and 9.29 μM in the U87 cells respectively. Further, temozolomide a DNA-damaging agent and the standard chemotherapeutic agent for glioblastoma was reported to have an IC_{50} of approximately 200 μM in both U87 and U251 glioblastoma cells, while bevacizumab showed an IC_{50} of approximately 671 μM (Baer et al., 1993, Kanzawa et al., 2004b, Mesti et al., 2014). Thus the IC_{50} values of DS00326 and DS00329 obtained from this study were about 20 to 40 times much lower than what has been reported for temozolomide and 71 to 130 times lower than that of bevacizumab. Importantly, DS00326 and DS00329 had minimal effects in normal fibroblasts indicating their selectivity to glioblastoma cells. Selectivity index ranged from 2.47 to 5.33 and it was previously reported that a selectivity index greater than 2 is used as a cut-off to determine selectivity. Although the safety of these compounds still needs to be confirmed by further *in vivo* toxicity studies, previous studies reported that cancer patients most times tolerate phenothiazines (Hait et al., 1989, Sridhar et al., 1993). These results suggest that DS00326 and DS00329 may be promising drugs for the treatment of malignant glioblastoma.

6.2 DS00326 and DS00329 are effective against primary PDX glioblastoma cells

A major reason for the low success rate of novel anti-cancer agents in clinical trials has been attributed to the unavailability of suitable preclinical models that epitomize the heterogeneous nature of malignancies in patients (Kamb, 2005). Although successes in drug development have been achieved with the use of conventional cell lines, they are also limited due to their inability to completely recapitulate tumour properties in patients which in turn, hampers the translation of findings to clinical practice (Johnson et al., 2001, Gillet et al., 2013).

In an attempt to bypass the challenges associated with the use of conventional cell lines, patient-derived xenograft (PDX) tumours which are sometimes not accessible to many laboratories due to high cost, are now being used for cancer drug discovery (Tentler et al., 2012). The use of PDX models is predicated on the premise that they will be more predictive of patient's tumour biology since they are likely to retain key genetic and histopathological features of donor tumours, thus predicting patient's response to treatments (Zanoni et al., 2016). Several studies have shown that PDX glioblastoma models yielded better platform in the screening of potential drug candidates for glioblastoma and serve as a potential for the development of personalized treatments for cancer (Willey et al., 2016, William et al., 2017, Patrizii et al., 2018).

In the current study, we provide the first evidence that phenothiazines as antipsychotics via DS00326 and DS00329 at low concentrations show potent cytotoxicity to primary PDX glioblastoma cultures T10 and T91. Indeed, the IC_{50} values for DS00326 and DS00329 were 5.06 μM and 12.53 μM in the T10 and 1.60 μM and 5.4 μM in the T91 PDX cells respectively. The findings from this study show that DS00326 and DS00329 may be effective in the treatment of glioblastoma and suggests the need for *in vivo* testing.

6.3 DS00326 and DS00329 inhibit long-term survival in glioblastoma cells

Following inhibition of cell viability by DS00326 and DS0039, we next determined their ability to inhibit long-term survival of glioblastoma cells as this will give an indication of how useful they will be *in vivo* (Fiebig et al., 2004). For an anti-cancer agent to be considered effective, it should be able to induce cytotoxicity (short-term) as well as inhibit the long-term survival of cancer cells long after drug withdrawal (Pessina et al., 2005). The current study determined the ability of DS00326 and DS00329 to inhibit long-term survival via a clonogenic assay and the findings show that DS00326 and DS00329 inhibited colony formation in malignant glioblastoma cells U87 and U251 dose-

dependently. In line with these findings, other phenothiazines in particular trifluoperazine was reported to inhibit colony formation in lung cancers (Zong et al., 2011b, Yeh et al., 2012). Together, these results suggest that DS00326 and DS00329 inhibit long-term survival of glioblastoma cells and could serve as a template for further studies to determine their anti-tumour activity *in vivo* and their subsequent enrolment in to clinical trials for glioblastoma treatment.

6.4 DS00326 and DS00329 trigger DNA damage response pathway

The mode of action of the majority of anti-cancer agents involves the induction of double-strand DNA breaks (DSBs) which is the most severe of all mammalian DNA lesions (Annovazzi et al., 2017).

In response to this damage, the DDR pathway is triggered and cancerous cells may try to repair the damaged DNA, activate checkpoints to halt cell cycle progression and in cases of lethal damage, cells may be programmed for death (Kastan and Bartek, 2004, Maréchal and Zou, 2013). This pathway involves a cascade of events leading to the activation of γH2AX response, ATM-Chk2 complexes, the p53 cell cycle regulator and its downstream target p21 cyclin-dependent kinase inhibitor to induce a G1 arrest of the cycle (Celeste et al., 2002, Lukas et al., 2003, Buscemi et al., 2004).

This study provides the first evidence that phenothiazines as a class of antipsychotics induce DSBs and trigger the canonical DDR pathway in glioblastoma cells. Indeed, DS00326 and DS00329 treatment led to the induction of γH2AX as well as the activation of ATM in the PDX glioblastoma cells. In addition, Chk2 activation was observed in the U87 and U251 glioblastoma cells following treatment with these compounds. Accumulating evidence shows that phenothiazines mitigate DNA repair by potentiating the DDR signalling pathway. Indeed, phenothiazines have previously been shown to hinder repair of DSBs by inhibiting tousled-like kinases (TLKs) responsible for the

maintenance of genomic stability (Ronald et al., 2013). Further, it was reported that phenothiazines inhibited the DNA-PK, a serine/threonine nuclear kinase critical for the repair of DSBs in lung cancer cells, melanomas and larynx carcinoma (Eriksson et al., 2001) (Gangopadhyay et al., 2007). It has been previously reported that cells defective in DNA-PK were more sensitive to DNA damaging agents as well as ionising radiation (Varga et al., 2017). More recently, phenothiazines were shown to induce DNA damage via generation of reactive oxygen species in oral squamous cell carcinoma (Wu et al., 2016). However, whether DS00326 and DS00329 also induce DNA damage by reactive oxygen species was not analysed but would be interesting to investigate.

Furthermore, as typical of the canonical DNA damage response pathway, Chk2 phosphorylates its substrate p53 to increase its stability and function. p53, which is a tumour suppressor has been linked to programmed cell death pathways and cell cycle regulation by transcriptionally activating p21. Induction of p21 in turn, leads to the inactivation of cyclin/cyclin-dependent kinases leading to cell cycle arrests including a G1 arrest (Cazzalini et al., 2010). The findings from the current study showed that DS00326 and DS00329 induce a G1 arrest in the U87 and U251 (p53 mutant) glioblastoma cells as well as the T91 primary PDX glioblastoma cells which is likely to be p21-dependent. Since U251 has a mutant inactive p53, this suggests that other mechanisms must be involved in p21 activation in these cells.

Also activated, following DNA damage induced by DS00326 and DS00329 were the p38, ERK and JNK MAPK pathways which are involved in various cellular processes Activation of ERK, JNK and p38 MAPK by stress stimuli is associated with cell cycle arrest, apoptotic and autophagic cell death (Munshi and Ramesh, 2013). Importantly, both ERK and p38 MAPK activation have been reported to mediate p21-dependent cell

cycle arrests independently of p53 (Sanchez-Prieto et al., 2000, Tang et al., 2002, Kumari et al., 2013). In particular, activation of p38 has been associated with G1 cell cycle arrest in glioblastoma cells (Yao et al., 2008). Findings from this study thus suggest that that the induction of p21 by DS00326 and DS00329 may also involve the activation of the MAPKs. However, to conclusively elucidate the direct impact of MAPKs on cell cycle arrest, apoptosis and autophagy induction, further experiments involving the inhibition of these pathways either with chemical inhibitors or siRNAs will have to be conducted. Taken together, the current findings, suggest that DS00326 and DS00329 induce DNA damage response leading to a G1 cell cycle arrest and that activation of the MAPKs are in part responsible for the cell cycle arrest.

6.5 Apoptosis is involved in DS00326 and DS00329 induced cell death

Loss of apoptotic signals which leads to continuous growth and proliferation is typical of tumour cells and it is expected that a good chemotherapeutic agent should induce apoptosis in cancer cells. The findings from this study showed that DS00326 and DS00329 induced apoptosis. Indeed, morphological analysis of the cells showed membrane blebbing and cell shrinkage. Also noticeable is the externalization of phosphotidylserine which is typical of cells undergoing apoptosis as determined by FACS analyses following annexin V/PI staining of cells.

At a molecular level, apoptosis is characterized by the cleavage of cellular substrates by the cysteine proteases (caspases) and a notable substrate is the zinc finger protein PARP that responds to DNA damage in a bid to repair damaged DNA (Soldani and Scovassi, 2002, Crawford et al., 2013, Julien et al., 2016). In this study, DS00326 and DS00329 were shown to induce PARP cleavage in both malignant glioblastoma cells and the primary PDX glioblastoma cells. However, whether these compounds induce the intrinsic or extrinsic apoptotic pathway was not investigated in this study and it has been

reported that resistance of tumour cells to chemotherapeutic agents usually arises as a result of alterations in the intrinsic pathway machinery (Schimmer, 2004, Fulda, 2009). Thus, an effective anti-cancer drug should trigger both intrinsic and extrinsic pathways as this will ensure that tumour cells that have bypassed the intrinsic pathway will be targeted. Though phenothiazines have been shown to induce both intrinsic and extrinsic apoptotic pathways, it will be important in future to know if this is also the case for DS00326 and DS00329. For example, thioridazine has been reported to induce apoptosis via the extrinsic apoptotic ligand TRAIL and it also triggered caspase 8 (extrinsic marker) and 9 (intrinsic marker) activities in renal carcinoma Caki cells (Min et al., 2014a).

The mechanism of apoptosis induction observed in the glioblastoma cells can be attributed in part to the activation of the p53 tumour suppressor. In support of this, it is widely known that activation of the p53 pathway following DNA damage mediates apoptosis in several cancers including glioblastoma (Sahra et al., 2010, Zhang et al., 2010, Munagala et al., 2011, Villalonga-Planells et al., 2011, Aliwaini et al., 2013). p53 may induce apoptosis via its targets Puma and Noxa which encodes pro-apoptotic BH3 proteins (Oda et al., 2000, Yu et al., 2001). Upregulation of these genes by p53 has been known to rapidly trigger apoptosis in cancer cells and when silenced or inactivated, they confer resistance to cytotoxic agents or stimuli (Han et al., 2001, Nakano and Vousden, 2001, Jeffers et al., 2003, Akhter et al., 2014). However, there might be other mechanisms triggering apoptosis observed in this study as the U251 cells with mutant p53 also showed induction of apoptosis. Indeed, phenothiazines have been previously reported to induce apoptosis in both a p53 and non-p53 dependent manner (Karmakar et al., 2001, Gil-Ad et al., 2004, Gil-Ad et al., 2006, Min et al., 2014a, Spengler et al., 2016, Zhang et al., 2017b). Trifluoperazine was reported to induce apoptosis even in the presence of mutant p53 in L1210 leukemic lymphocyte cells and this was attributed to the ability of

trifluoperazine to inhibit the Ca^{2+}/calmodulin complex, an activity associated with phenothiazines in general (Sullivan et al., 2002). Taken together, the current findings indicate that DS00326 and DS00329 induced apoptotic cell death in both malignant glioblastoma cells and primary PDX glioblastoma cells.

6.6 DS00326 and DS00329 induce autophagic cell death

Autophagy initiation is characterized by the formation of double-membrane vesicles referred to as autophagosomes which engulf damaged cytoplasmic constituents. These autophagosomes, in turn, fuse with lysosomes to promote recycling of cytoplasmic cargoes (Degenhardt et al., 2006). The conjugation of phosphatidyl-ethanolamine, to LC3 thus giving rise to LC3-II protein, is commonly used to detect autophagosome formation in cells and consequently autophagy induction (Mizushima et al., 2001, Rubinsztein et al., 2009). Results from the current study show autophagy induction in the cells treated with DS00326 and DS00329 as observed by increased LC3-II levels.

However, an increment in LC3-II does not necessarily translate to autophagy induction as the autophagy pathway which is a complex process can be blocked at the point of fusion of autophagosomes and lysosomes (Barth et al., 2010). A blockage at this point will lead to increase accumulation of autophagosome which corresponds with increase LC3-II levels. Thus, for autophagy to be complete, there must be a fusion of autophagosome membranes to form an intact vesicle which will be degraded by lysosomal enzymes upon fusion with lysosomes (Rubinsztein et al., 2009). It is important to state that acidification of the lysosomal compartments is critical for the completion of the autophagy process. A notable pharmacological inhibitor of autophagy at the late stage is bafilomycin A1, an H+-ATPase inhibitor which blocks autophagy by inhibiting the acidification of autophagic vesicle (Mauvezin and Neufeld, 2015). The findings from this study show that DS00326 and DS00329 led to the increase in acidification of lysosomal

compartments as shown by increased formation of acidic vesicular organelles (AVOs) as well as induce autophagic flux as seen from the increased levels of LC3-II following the addition of BAF.

Furthermore, it is now known that some anti-cancer agents, including temozolomide, 5-fluorouracil (5-FU), resveratrol and doxorubicin, trigger both apoptosis and autophagy in several cancer cells (Kanzawa et al., 2004a, Lambert et al., 2008, Pan et al., 2013, Selvaraj et al., 2016). However, there has been opposing views on the role of autophagy in cancer cells either as pro-survival or pro-death. Though, cancer cells have been reported to rely on autophagy for survival or as a defence mechanism majorly as a result of increased metabolic demands initiated by altered proliferation (White, 2015). Basal autophagy has also been thought to be upregulated in the hypoxic core of tumours, where it is necessary for survival (Degenhardt et al., 2006).

Emerging evidence suggests that autophagy induction can trigger cell death in cancer referred to as autophagic or type II PCD. Studies have shown that palladium complexes, resveratrol, cannabinoid and arsenic trioxide (As2O3) induced autophagic cell death in several cancer cells including (glioma, leukaemia, hepatocellular carcinoma, melanoma and prostate cancer) and blocking autophagy reversed their cytotoxicity (Kanzawa et al., 2004a, Kanzawa et al., 2005, Qian et al., 2007, Vara et al., 2011, Aliwaini et al., 2013, Selvaraj et al., 2016). The results from the current study show that, inhibition of autophagy induced by DS00326 and DS00329 using either wortmannin or bafilomycin in the U87 and U251 cells treated with compounds increased cell survival thus suggesting a reversal of cytotoxic effects of these compounds. This is in line with the theory that the role of autophagy as a cell death or cell survival mechanism depends on the anti-cancer agent used or the nature of the cell (Mathew et al., 2007b). Additionally, it is consistent

with other reports that phenothiazines induced autophagy as a form of cell death in both *in vitro* and *in vivo* systems (Choi et al., 2008, Shin et al., 2013, Yin et al., 2015, Wu et al., 2016). Taken together, the current study suggests that autophagy induced by DS00326 and DS00329 is pro-death.

6.7 DS00326 and DS00329 inhibit the PI3/Akt pathway

The PI3/Akt signalling pathway is known to play a critical role in the regulation of several cellular processes and it can be deregulated by various genetic and epigenetic mechanisms, in a wide range of cancer cells (Ouyang et al., 2012). In this pathway, the phosphatidylinositol 3-kinase (PI3K) enzyme is activated by diverse signalling agents, including platelet-derived growth factor, epidermal growth factor and insulin-like growth factor-1, thus leading to the functional activation of Akt (protein kinase B) (Shin et al., 2013). Activated Akt in turn, phosphorylates a series of protein substrates that function as regulators of cell proliferation, growth and cell survival. The PI3K/Akt signalling is constitutively active in several cancers including glioblastoma and it is usually associated with poor prognosis (Sekulić et al., 2000, Altomare and Testa, 2005, Grecu, 2018). The lipid phosphatase PTEN or MMAC1 (mutated in multiple advanced cancer) negatively regulates the PI3K/Akt pathway (Koul et al., 2006). Loss of PTEN activity usually as a result of chromosomal gene mutation or epigenetic silencing due to DNA methylation in the promoter region leads to deregulation and activation of PI3K/Akt signalling (Steck et al., 1997, Mehrian-Shai et al., 2007). It is well documented that the U87 and U251 glioblastoma cells show a mutation in PTEN which is accompanied by hyperactivation of the PI3/Akt pathway (Haas-Kogan et al., 1998, Ren et al., 2010, Grecu, 2018).

The present study shows that DS00326 and DS00329 treatment inhibit the PI3/Akt pathway in malignant glioblastoma cells. These findings are consistent with what has been previously reported for some members of the phenothiazine group. Specifically,

thioridazine was shown to inhibit angiogenesis and tumour progression in ovarian cancer cells as well as in xenografts by negatively targeting PI3K/Akt/mTOR pathway (Rho et al., 2011, Park et al., 2014). Thioridazine has also been reported to sensitize renal carcinoma Caki cells to TRAIL-induced apoptosis through reactive oxygen species-mediated inhibition of Akt signalling (Min et al., 2014a). Additionally, both clozapine and chlorpromazine were reported to inhibit the PI3/Akt pathway in PTEN mutant U87 glioblastoma cells via impairment of the Ca2+/CaM complex (Shin et al., 2006, Shin et al., 2013).

There is also accumulating evidence suggesting that the PI3K/Akt pathway plays a crucial role in the regulation of autophagy (Sun et al., 2013, Roy et al., 2014, Lin et al., 2018). This is made possible via the ability of the PI3K/Akt pathway to regulate the downstream target mTORc1 (Pattingre et al., 2008). mTOR belongs to the class of the PI3K-related kinase family and contributes to cell metabolism, growth regulation and proliferation (Wullschleger et al., 2006). Inhibition of mTOR as a result of downregulation of Akt is known to induce autophagy as well as autophagic cell death (Yun et al., 2014, Wong et al., 2017). Chlorpromazine as well as curcumin were reported to inhibit the PI3/Akt pathway in PTEN-mutant U87 glioblastoma cell lines to induce a non-apoptotic autophagic cell death (Aoki et al., 2007, Shin et al., 2013). Together, these findings suggest that the inhibition of the Akt signalling may be critical for DS00326 and DS00329 induced cell death.

6.8 DS00326 and DS00329 show anti-cancer stem cell activity

Glioblastomas are among the first solid cancers in which tumour cells with stem cell-like activities were identified. Accumulating evidence shows that the majority of cancer cells are heterogeneous and are hierarchical in nature with the stem-like cells (cancer stem cells) being at the apex of this hierarchy (Chen et al., 2016). It is believed that these

cancer stem cells (CSCs) make up a subpopulation of the entire tumour and are responsible for the propagation of tumour cells and contribute greatly to metastasis, chemoresistance and tumour recurrence (Zhang et al., 2008b, Visvader, 2009, Chen et al., 2010, Cojoc et al., 2015, Zhao, 2016). In glioblastoma, the stem cell populations are called the GSCs or glioma-initiating tumour cells and are known to drive tumourigenesis. Thus a potent and effective anti-cancer agent should be able to target cancer stem cells as doing so will lead to the reduction of overall tumour population (Maitland and Collins, 2008, Cojoc et al., 2015, Iqbal et al., 2016). The results from the current study show that DS00326 and DS00326 confer anti-cancer stem cell activities in primary PDX glioblastoma cells as they inhibited neurosphere formation.

Stem cell transcription factors, including SOX2, Oct4, Nanog, c-Myc, Olig2, and Bmi1 have been reported to be crucial for the maintenance and survival of stem cell population in glioblastoma (Heddleston et al., 2009, Dahlrot et al., 2013). In particular, SOX2 an essential requirement for embryonic development and a well-known marker of embryonic stem cells is critical for the self-renewing properties of stem cells (Gangemi et al., 2009, DeCarvalho et al., 2010). SOX2 has also been reported to be expressed at higher levels in brain tumour tissue than in normal brain tissue and this has been linked to the maintenance of CSC properties in glioma (Schmitz et al., 2007, Sutter et al., 2010, Alonso et al., 2011). Reports show that knocking down SOX2 in GSCs impair tumourigenicity and stemness as well as loss of neurospheres (Gangemi et al., 2009, DeCarvalho et al., 2010, Lee et al., 2012). Therefore, targeting SOX2 provides a good therapeutic approach to glioblastoma. The findings from the current study show that DS00326 and DS00329 lead to a reduction of SOX2 and an increase in the differentiation marker GFAP expression in the T10 PDX glioblastoma cells, thus inhibiting the self-renewing ability of GSCs. Previous studies have also shown that phenothiazines induced

anti-cancer stem cell activities in neoplastic human pluripotent stem cell (hPSC), lung, gastric and colorectal cancers (Sachlos et al., 2012, Yeh et al., 2012, Mu et al., 2014, Zhang et al., 2017a).

6.9 Limitation to study and future work

Since this study was limited to only *in vitro* models of glioblastoma, preclinical studies to further elucidate the anti-tumour efficacy of DS00326 and DS00329 in animal models will be imperative. Furthermore, the safety and toxicity profile of the compounds used in this study need to be ascertained and studies to establish the absorption, distribution, metabolism and excretion (ADME) profiles of these compounds in *in vivo* models, will help to further elucidate their mechanisms of action.

6.10 Concluding remarks

This study describes the repurposing of novel phenothiazine-derivatives DS00326 and DS00329 as anti-cancer agents in glioblastoma and thus provides the first evidence of the anti-cancer activity of phenothiazines in primary PDX glioblastoma cultures. In addition, the study shows a detailed mechanism underpinning the anti-cancer activities of these compounds in glioblastoma. The data obtained from this study shows that DS00326 and DS00329 induce DSBs which activates the DDR and other pathways including the MAPKs to trigger p53 and p21 response leading to cell cycle arrest and apoptosis. This study also shows that DS00326 and DS00329 induce autophagy to promote cell death which may in part be due to the inhibition of Akt signalling and the modulation of MAPKs. Furthermore, this study shows that DS00326 and DS00329 target GSCs via the inhibition of neurosphere formation and SOX2 expression in a primary PDX glioblastoma model. Together, the findings from this study suggest that DS00326 and DS00329 may be effective in the treatment of glioblastoma and provides a strong

rationale for further clinical studies that explore phenothiazines and their derivatives as potential chemotherapeutic treatment for glioblastoma.

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APPENDIX

7.1 Mycoplasma mounting fluid

20mM citric acid
50% Glycerol
55mM Na2HP04.2H20
PH to 5.5, store in 4°C

7.2 10x phosphate buffered saline (PBS):1L

80g NaCl
12.6g Na2HPO anhydrous
2g KCl
2.4g KH2PO4
Dissolve in 800ml dH20 (using stirrer bar)
PH to 7.4 with concentrated HCl
Fill up to 1L and autoclave
For use dilute to 1x (100ml10xPBS+900ml dH2O)
Store at room temperature

7.3 2x Boiling blue

1M Tris HCL, PH 6.8
10% SDS
B-Mercaptoethanol
Glycerol
dH2O
Bromophenol Blue
Store in -20°C

7.4 Lysate preparation

Whole lysis buffer

Stock concentration	Volume (mL)	Final Concentration
0.5M Tris-HCl pH 6.8	100	50 mM
99.5% Glycerol	10	10%
10% SDS	20	2%
H_2O	62	

- Lysis buffer was aliquoted in eppendorf tubes and heated for 5 minutes at 95°C
- Suitable amount was added to tubes to pellets and vortexed while heating for 5 minutes at 95°C at 1400 rpm (on the machine programme S2: at 95°C, 1500 RPM) (If lysate is sticky add more buffer)

7.5 BCA Assay

Reagents

Thermo Scientific: PierceTM BCA Protein Assay Kit
Reagent A
Reagent B
BSA 2 mg/ml
4x loading dye + 10% β-Mercaptoethanol
Water
Solution C: Mix solution B and A 1:50
BSA stock 2 mg/ml: dilute with water 1:1 to 1 mg/mL = 1µg/µL

Steps

- Samples were plated in triplicates : 9µl H_2O + 1µl sample + 200µl solution C (x3 wells per sample)
- BSA was plated with rising concentration (µg/µl) in triplicates:
    - 0µl BSA + 10µl H_2O + 200µl solution C (x3 wells per concentration)
    - 2µl BSA + 8µl H_2O + 200µl solution C (x3 per wells per concentration)
    - 4µl BSA + 6µl H_2O + 200µl solution C (x3 per wells per concentration)
    - 6µl BSA + 10µl H_2O + 200µl solution C (x3 per wells per concentration)
    - 8µl BSA + 2µl H_2O + 200µl solution C (x3 per wells per concentration)
    - 10µl BSA + 0µl H_2O + 200µl solution C (x3 per wells per concentration)
- Plate was incubated for 30 minutes at 37°C (Incubator in freezer room) and after incubtion, plate was read on Victor Machine Victor – Q – PerkinElmer – Photometry – Absorbance@490 (0.1s).
- Following this, a trend line equation was determined and was used to calculate protein concentration (20µG) per sample.
- Loading samples consisted of protein, water and 4X loading dye

7.6 4X loading dye (10mL)

2.0 ml 1M Tris-HCl pH 6.8.
0.8 g SDS.
4.0 ml 100% glycerol.
0.4 ml 14.7 M β-mercaptoethanol.
1.0 ml 0.5 M EDTA.
8 mg bromophenol Blue

7.7 Sodium Dodecyl Sulphate (SDS)-polyacrylamide gels

Resolving gel:

Acrylamide/bis-acrylamide, 30% solution (percentage depending on size of protein)
0.375 M Tris (pH 8.8)
0.1% SDS
0.1% TEMED
0.1% Ammonium persulphate

Stacking gel:

Acrylamide/bis-acrylamide, 30% solution
0.192 M Tris (pH6.8)
0.1% SDS
0.1% TEMED
0.1% Ammonium persulphate

7.8 Running buffer

1 g SDS
3.03 g Tris
14.41 g Glycine
Make up to 1 litre

7.9 Transfer buffer

2.9 g Glycine
5.8 g Tris
0.37 g SDS
200 ml isopropanol
Make up to 1 litre and store at 4°C.