Assessment of the *in vitro* efficacy of aspirin and aspirin analogues in combination with standard chemotherapeutics in glioma cell lines

by

Preet Chheda

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Abstract

Glioblastoma multiforme (GBM) is the most common and most malignant primary brain tumour. The prognosis for patients remains poor with a median survival time falling well short of a year in spite of surgical removal of tumour followed by radiotherapy. GBM is resistant to the current treatment regimen, often due to therapeutic resistance, however combination therapies have been proven to have a beneficial effect on the prognosis of patients. Aspirin, one of the most commonly used drugs reduces the viability and proliferation of glioblastoma both *in vitro* and *in vivo*. This study examined the combination of aspirin or its analogue PN517 with the standard chemotherapeutic (cisplatin) and determined effects on cell viability, apoptosis and caspase activity. Either aspirin or PN517 combined with cisplatin significantly reduced the viability of U87-MG glioblastoma cells. The apoptosis induced by cisplatin was augmented in combination with either PN517 or aspirin. These results for the first time suggest a potential for improved treatment of glioblastoma multiforme where aspirin or its analogue PN517 are combined with the standard chemotherapeutic drugs cisplatin.

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Abbreviations

3-MA	3-methyladenine
4HBZ	4-hydroxy benzoate zinc
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
CDDP	Cisplatin
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CFSE	Carboxyfluorescein succinimidyl ester
CNS	Central Nervous System
COX	Cyclooxygenase
CRC	Colorectal cancer
CT	Computerised tomography
CYP450	Cytochrome P450
DISC	Death Inducing Signal Complex
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagles Minimum Essential Medium
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum

FITC	Fluorescein isothiocyanate
FS	Forward Scatter
GBM	Glioblastoma Multiforme
GSH	Glutathione
GY	Gray Units
IC_{50}	Inhibitory concentration 50%
IDH1	Isocitrate dehydrogenase 1
LC-MS	Liquid chromatography-mass spectroscopy
LSM	Laser Scanning Microscope
MDC	Monodansylcadaverine
MGMT	O^6-alkylguanine DNA alkyltransferase
MMR	Mismatch Repair
MRI	Magnetic Resonance Imaging
MTIC	3-methyl-(triazen-1-yl)imidazole-4-carboxamide
MTOR	Mammalian target of rapamycin
NEAA	Non-essential amino acids
NER	Nucleotide Excision Repair
NICE	National Institute for Health and Clinical Excellence
NSAID	Non-steroidal anti-inflammatory drug
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
SEM	Standard error of mean
SS	Side Scatter

Stat3	Signal transducer and activator of transcription 3
TMZ	Temozolomide
TNF	Tumour Necrosis Factor
WHO	World Health Organisation

1 Introduction

1.1 Introduction – Central Nervous System Tumours

Brain tumours, while rare in comparison to cancers such as breast or prostate, represent a significant challenge both in terms of diagnosis and treatment. The UK annual incidence rate of brain tumours ranges from 14.8 per 100,000 for males and 14.6 per 100,000 for females (Cancer Research UK, 2014). Primary brain tumours are those arising within the central nervous system (CNS) and represent 1.6% of all the tumours diagnosed, whereas secondary brain tumours, resulting from metastasis of non CNS tumours, represent 6% of all tumours detected in the UK (Cancer Research UK, 2014). While low grade tumours that are slow growing with well-defined borders can usually be removed surgically, their location within the CNS presents challenges due to the risk of significant neurological sequelae (Veeravagu *et al*., 2013). This risk increases with high grade tumours that grow rapidly and are highly invasive, but most studies show a significant improvement in survival that correlates the extent of tumour resection (Hervey-Jumper & Berger, 2014).

The main cause of brain tumours, like several other cancers still remains unidentified. However, several risk factors have been established which include exposure to radiation, previous incidence of cancer, genetic conditions such as neurofibromatosis, and other medical conditions such as AIDS (Reilly, 2010). Age is also a major factor as the risk of acquiring the disease increases with age, with the age group of 50-70 being the most affected (Eheman *et al*., 2012). Improvements in diagnosis, treatment, and prevention of cancer has resulted in a significant improvement in the median survival time for all cancers following diagnosis, from 1 year in 1971-72 to 5.8 years in 2007, with the biggest increase being in colon cancer, improving from 0.6 to 10 years (Macmillan, 2012). However, brain tumour survival time in UK has

shown little improvement, increasing from 0.3 years in 1971 to 0.5 years in 2007. This report emphasises the significant challenges that remain in the diagnosis and treatment of brain tumours, and highlights the importance of the development of new treatment regimens for brain tumours.

1.2 Classification and Grading of Central Nervous System Tumours

Classification and grading is used to design individual patient treatment protocols and to determine their likely prognosis. Additionally, it creates a system that can be accepted worldwide allowing for global clinical trials and epidemiological studies (Louis *et al*., 2007a). In 2007, the World Health Organisation (WHO) ratified a new wide-ranging classification of neoplasms affecting the CNS (Louis *et al*., 2007b). The classification of brain tumours is based on the abnormal growth of a specific cell type, and grading helps in understanding the extent of malignancy or aggressiveness of the tumour, with higher grade corresponding to higher malignancy (Loukopoulos & Robinson, 2007). The new WHO system is particularly useful in this regard with only a few notable exceptions for example, either all or almost all gemistocytic astrocytomas are actually anaplastic and hence named grade III or even IV rather than grade II. The WHO classification also provides a corresponding grading system for each type of tumour where most named tumours are of a single defined grade. An outline of this classification is provided below (Table 1.1).

Table 1.1: WHO classification of CNS tumours (adapted from Louis *et al.*, 2007)

Tumours of Neuroepithelial Tissue	
Astrocytic Tumours	Choroid plexus Tumours
Oligodendroglial Tumours	Neuronal & mixed neuronal-glial tumours
Oligoastrocytic Tumours	Tumours of the pineal region
Ependymal Tumours	Embryonal tumours
Other neuroepithelial tumours	
Tumours of Cranial and Paraspinal Nerves	
Schwannoma	Perineuroma
Neurofibroma	Malignant peripheral nerve sheath tumour
Tumours of the Meninges	
Tumours of melingothelial cells	Primary melanocytic lesions
Mesenchymal tumours	Other neoplasms related to the meninges
Lymphomas and Haematopoietic Neoplasm	
Malignant lymphomas	Granulocytic sarcoma
Plasmacytoma	
Germ Cell Tumours	
Germinoma	Choriocarcinoma
Embryonal carcinoma	Teratoma
Yolk sac tumour	Mixed germ cell tumour
Tumours of the Sellar Region	
Craniopharyngoma	Pituicytoma
Granular cell tumour	Spindle cell oncocytoma

Tumour grading plays a very important role in the choice of therapy, especially while ascertaining the adjuvant radiotherapy and chemotherapy protocols (NICE, 2006; Araujo *et al*., 2011). The grading of CNS tumours by WHO involves a scheme of grading that serves as a malignancy scale (Bratasz *et al*., 2008). Grade I includes tumours that have very low potential to proliferate and higher possibility to cure by surgery alone. Grade II tumours are usually infiltrative with low level of proliferation, although some grade II tumours may progress to higher malignancy grades. Grade III neoplasms have morphological evidence of malignancy such as high mitotic activity and nuclear atypical. Lesions designated grade IV are cytologically malignant, necrosis prone, involving widespread infiltration of surrounding tissue and a tendency for craniospinal dissemination (Louis *et al*., 2007). WHO grading, in combination with several other factors, also aids in predicting the patients response to treatment and in an overall prediction of prognosis (Kleihues *et al*., 2007).

1.3 Glioma

Primary brain tumours that arise from neuroglial cells, including astrocytes and oligodendrocytes, are referred to as glioma (Laws *et al*., 2003; Nikkhah *et al*., 1992; Fleming *et al*., 2011). Gliomas are the most common primary tumours of the brain and they comprise about 2% of all newly diagnosed cancers every year in the UK (The National Institute for Health and Clinical Excellence Cancer Service Guidance, 2007), but despite this low incidence, glioma is the leading cause of cancer-related death in men aged 20-39 years (Habela *et al*., 2009). The incidence of primary brain tumours in the UK is similar to that found in the US and with an annual incidence rate of 7 per 100,000 of the population (McKinney, 2004; Sehmer *et al*., 2014). To put the incidence rate of malignant glioma in perspective, the rate roughly equals that of leukaemia

With respect to tumours of the CNS, gliomas account for 40-60% of primary brain tumours in adults, of which half are malignant gliomas (Brandes *et al*., 2008). Primary glioblastoma multiforme (GBM) represents approximately 95% of cases, with the remaining 5% of cases being secondary GBM typically arising over a period of years from either low grade astrocytoma (WHO grade II), or from anaplastic astrocytoma (WHO grade III) (Rivera *et al*., 2008; Jaeckle *et al*., 2011). Following diagnosis, the 5-year survival rate for low grade glioma is 97% following surgical resection of greater than 90% of the tumour, but in high grade glioma where the tumour is more aggressive and recurrence more common, the median survival is 1-3 years (Hervey-Jumper & Berger, 2014).

There are several classes of glioma including astrocytoma, which develop anywhere in the brain or spinal cord (Zhou & Mark, 2013). Brain stem gliomas arise in the lowest part of the brain, ependymomas develop inside the brain in the lining of the ventricles, while oligodendrogliomas usually develop in the cerebrum. This latter form is very rare, representing just 3% of all primary brain tumours (Dolecek *et al*., 2012) (Table 1.2). Overall, gliomas are the most common forms of primary brain tumours accounting for 80% of all cases (Hervey-Jumper & Berger, 2014).

1.4 Glioma Classification and Grading

Depending on their cell of origin, gliomas are classified into three main types, astrocytoma, oligodendroglioma, and ependymoma (Table 1.2).

Table 1.2: WHO classification of Gliomas (Louis *et al*., 2007).

Categories in italics are also not recognized by the new WHO classification system, but are in common use

WHO Designation	Grade	Incidence (% of all brain tumours)
Astrocytic Tumours		
Pilocytic Astrocytoma *Hemispheric, diencephalic, optic*	I	5-6%
Diffuse Astrocytoma Variants: protoplasmic, fibrillary, mixed	II	10-15%
Anaplastic Astrocytoma *Hemispheric, diencephalic, brain stem, cerebellar*	III	10-15%
Glioblastoma Multiforme Variants: giant cell glioblastoma, gliosarcoma	IV	12-15%
Oligodendroglial Tumours		
Oligodendroglioma	II	2.5%
Anaplastic oligodendroglioma	III	1.2%
Ependymal Tumours		
Subependymoma	I	0.7%
Ependymoma	II	4.7%
Anaplastic ependymoma	III	1%
Mixed and other common types of glioma		
Angiocentric glioma	I	
Oligoastrocytoma	II	1.8%
Anaplastic oligoastrocytoma	III	1.2%

Astrocytomas develop from star shaped glial cells called astrocytes found throughout the CNS which functions include maintaining extracellular ion levels and repairing of tissues (Goodden *et al*., 2014). They represent the most common type of glioma, forming 75% of all cases detected and their occurrence increases with age (Berkhard *et al*., 2003; Dolecek *et al*., 2012). Astrocytomas are majorly divided into two classes; encapsulated and diffuse tumours.

Oligodendrogliomas arise from oligodendrocytes, the cells which form the myelin sheath around the axons of nerve cells within the CNS. They can be well differentiated oligodendrogliomas which are grade II and have slow growth or anaplastic oligodendrogliomas which are of grade III and grow rapidly (Macmillan, 2014). Oligodendrogliomas form 20% of all tumours of the CNS. They've been found to be more prevalent in males as compared to females (Dolecek *et al*., 2012)

Ependymal cells produce and circulate the cerebrospinal fluid, are found lining the ventricles and central canal of the spinal cord, and give rise to ependymomas. They are considered to be a rare type of glioma, accounting for 5% of all tumours of the CNS (Macmillan, 2014). They include tumours of grade I (myxo-papillary ependydoma and sub-ependydoma), II (low grade ependymoma) and III (anaplastic ependymoma) (Louis *et al*., 2007).

The WHO system further grades the tumours on the basis of histological degrees of malignancy with Grade I being the least malignant to Grade IV being the most malignant (Louis *et al*., 2007). The degree of malignancy is based on either the presence or absence of increased cellularity, nuclear atypia, mitosis, endothelial

proliferation and necrosis. Grade I and Grade II tumours are considered to be low grade and are usually circumscribed and grow slowly over a period of time while Grade III and Grade IV are high-grade tumours and are diffuse and comparatively aggressive having poor prognosis (Chandana *et al*., 2008). 10% of the low-grade gliomas undergo malignant transformation to high-grade neoplasms with time and age (Ohgaki & Kleihues, 2007). High-grade gliomas are the most common type of brain tumours in the adult age group and represent a major cause of morbidity and mortality with a median survival time of 12-15 months (Dolecek *et al*., 2012; Fisher *et al*., 2010) Glioblastoma multiforme (GBM) is the most malignant and aggressive high-grade glioma (WHO Grade IV) (Preusser *et al*., 2011).

1.5 Molecular Markers of Glioma

Glioma progression is a combined effect of over activation of signalling pathways that oversee normal cell growth, abnormal expression by the cellular oncogenes and deletion or suppressed activity of tumour suppressor genes (Bernardi *et al*., 2006).

p53 is a tumour suppressor and transcription factor found to be mutated in 50% of tumours (England *et al*., 2013). Activation of p53 is induced by events occurring during carcinogenesis which include: genotoxicity, abnormal growth signals, DNA damage, hypoxia and loss of cell contacts (Harris, 2005). Various genes upregulated by p53 include the ones involved in apoptosis (PUMA, Bax, CD95/Fas, Apaf1), cell cycle arrest (p21, 14-3-3σ), autophagy (DRAM, Sestrin 1/2), senescence (PAI-1), angiogenesis (TSP1), DNA Repair (R2) and metabolism (PTEN, SCO2) (Harris, 2005).

Gliomas often display mutations in the ARF-MDM2-p53 and p16INK4a-CDK4-RB tumour suppressor pathways resulting in increased genomic instability, loss of G_1 cell cycle checkpoint control, and evasion of apoptosis (Hede *et al*., 2011).

Stat3 is a member of the Stat family of transcription factors which get activated via several cytokine and growth factor receptors, normally leading to transient phosphorylation of Stat 3 (Bromberg *et al*., 1999; Darnell *et al*., 1994; Zammarchi *et al*., 2011). However, in 70% of tumours persistent Stat3 phosphorylation has been observed (Zammarchi *et al*., 2011), initiating transcription of genes promoting anti-apoptosis, cell cycle-progression, cell survival, migration and invasion (Brantley & Benveniste, 2014). Activity of Stat3 is essential to tumour growth and has been noted to be of high frequency in gliomas (Luwor *et al*., 2013).

IDH1 is a member of isocitrate dehydrogenase family found in the cytoplasm and aids in catalytic oxidative decarboxylation of isocitrate. Somatic mutations of IDH1 are found in 70% of high grade gliomas (Lv *et al*., 2011), and while the wild type converts isocitrate to α-ketoglutarate (α-KG), mutated IDH1 results in formation of D-2-hydrohygluterate (2-HG). This aids in glioma pathogenesis either *via* downstream effects of 2-HG or by inhibiting isocitrate metabolism (Ohka *et al*., 2014).

1.6 Astrocytic Tumours

Astrocytic tumours belong to the neuroepithelial tissue WHO classification and are sub-divided into circumscribed (grade I) versus diffuse tumours (grades II-IV) (Louis *et al*., 2007) (Table 1.2). They may also be sub-classified as invasive or non-

invasive, although this is not formally part of the WHO system. They are the most common glioma and occur in most parts of the brain and occasionally the spinal cord; however, these tumours rarely metastasise outside the CNS (Habela *et al*., 2009). There are two broad classes of astrocytoma recognized in the literature, the first having narrow zones of infiltration that often are clearly outlined on diagnostic images (mostly invasive tumours; e.g., pilocytic astrocytoma, subependymal giant cell astrocytoma, pleomorphic xanthoastrocytoma), and the second having diffuse zones of infiltration, that share various features including the ability to arise at any location in the CNS, but with a preference for the cerebral hemispheres (e.g., low-grade astrocytoma, anaplastic astrocytoma, glioblastoma) (Srinivasan *et al*., 2011; Sonabend *et al*., 2014).

Astrocytomas can develop at any age, however, low-grade is more often found in children or young adults, while high-grade is more prevalent in adults (Grier & Batchelor, 2006). In children more than 80% of astrocytomas are low grade while 20% are high grade (St. Judes Research, 2014). As a proportion of all gliomas detected, low-grade astrocytomas in adults represent 10% of cases, whereas in children this is 25% (Ononiwu *et al*., 2012).

In adults, grade I astrocytoma are rare and comprise a distinct entity called pilocytic astrocytoma which occurs most commonly in the cerebellum in children. Grade II astrocytomas account for approximately 10-15% of all astrocytic brain tumours with an incidence rate of 1.4 new cases / million people a year (Bigner *et al*., 1998). They affect any region of CNS but predominately occur in the cerebrum followed by the brain stem and spinal cord (Wintersperger *et al*., 2007). Grade II astrocytomas are well

differentiated, slow growing and exhibit hyper-cellularity and cellular plemorpheins (Louis *et al*., 2007).

Anaplastic astrocytomas represent 10-15% of all intra-cerebral tumours and about 25-30% of all gliomas (Yarbro *et al*., 2005) (Table 1.2). They generally appear between the fourth and fifth decades of life and are more common in males than in females (Mohapatra *et al*., 1998). Grade III astrocytomas show strong mitotic activity along with hyper-cellularity and anaplasia, and may be defined as an infiltrating lesion with either focal or dispersed anaplasia or marked proliferative potential.

1.7 Grade IV (Glioblastoma Multiforme GBM)

Glioblastoma multiforme (GBM) is the most common and most malignant primary brain tumour accounting for 16% of all diagnosed primary tumours with an annual incidence of 2-3 cases per 100,000 (Dolecek *et al*., 2012; Fisher *et al*., 2010). When the tumour shows either vascular endothelial proliferation and/or the presence of necrosis, it is graded as grade IV glioblastoma (Louis *et al*., 2001; Reiser *et al.*, 2007; Chadduck *et al.*, 1991). Regional heterogeneity and highly invasive growth is commonly observed, whereas cellular polymorphisms, nuclear atypia, vascular thrombosis, may also be observed, albeit not as commonly (Brandes *et al.*, 2008). Primary GBM proliferates and spreads to other parts of the brain rapidly and can become very large before producing symptoms, which often begin abruptly with seizures. Less than 10% are secondary GBM which form more slowly following degeneration of low-grade astrocytoma or anaplastic astrocytoma, and are more common in younger patients (mean age 45 *versus* 62 years) (Ohgaki and Kleihues, 2009). It occurs more commonly in males, 1.6 times more when compared to females

(Dolecek *et al*., 2012). Prognosis remains poor for those with grade IV gliomas with few patients surviving beyond 3 years, a median survival time of 17 weeks without treatment, 30 weeks with radiation, and 37 weeks with surgical removal of most of the tumour followed by radiation therapy. Long term survival (at least five years) falls well under 5% in adults, while slightly better at 21% in children (Stupp *et al*., 2009).

1.8 Diagnosis of Glioma

Symptoms of glioma depend on the area of the brain affected. The most commonly observed symptoms are headaches, seizures, memory loss, physical weakness, cognitive decline, and loss of muscle control. Symptoms can exacerbate as the tumour progresses (Fox *et al*., 2007). In the first stage of diagnosis a history of symptoms is recorded followed by a basic neurological exam, including an eye exam and tests of vision, balance, coordination and mental status (Taylor, 2010). Diagnosis is confirmed using either a computerized tomography (CT) scan or magnetic resonance imaging (MRI) of the patient's brain.

1.9 Treatment of Glioma

The treatment of malignant glioma includes surgery, chemotherapy and radiotherapy (Lonardi *et al*., 2005). Surgical removal remains the mainstay of treatment, provided that unacceptable neurologic injury can be avoided, however, the extremely infiltrative nature and ill-defined margins of gliomas makes complete surgical removal impossible (Lonardi *et al*., 2005). Depending on the site of the lesion and the condition of the patient, surgery can include gross total excision of the tumour using image guidance or may be restricted to biopsy (Wheeler *et al*., 2008). Maximal resection is

performed whenever possible in patients with both low-grade and malignant gliomas as it has proven to increase the 1-year survival rate by 76.5% when compared to lesser extent of resection (Orringer *et al*., 2012).

Technical advances in neuro-imaging like MRI, MR spectroscopy and surgical technology like image guided surgery, intra operative ultrasound and, CT have improved and maximized tumour resection, while decreased procedure - related morbidity (Dhermain, 2014). A study from the Eastern Co-operative Oncology and Radiation Therapy Group (USA) showed a positive correlation between survival and extent of resection (Kuhnt *et al*., 2011).

1.9.1 Radiotherapy

Radiation therapy is one of the most important treatments for glioma, and although it rarely cures glioma, it increases the median survival time of patients when compared to supportive care alone. Standardised radiation treatment of glioma involves irradiating with 60 gray units (GY), delivered in 30 fractions of 2GY, with conventional external beam radiotherapy followed by surgery (Ricard *et al*., 2013). This dose is too low to sterilize the tumour and prevent it's reoccurrence, but high radiation doses using standard fractionation can pose a greater risk of radiation injury to normal brain without any survival benefits (Frappaz *et al*., 1999).

Radio-immunotherapy is a beneficial treatment technique as it aims at selectively destroying tumour cells by using radio-labelled monoclonal antibodies which target specific antigens expressed only by tumour cells while sparing normal

tissues (Westphal *et al*., 2013). Three step avidin-biotin has been targeted to the ^{90}Y-biotin to the tumour in glioma patients with encouraging results (Paganelli *et al*., 2001).

1.9.2 Chemotherapy & Alkylating Agents

Several randomized clinical trials have assessed the role of chemotherapy in the improvement of survival for glioma patients (Lonardi *et al*., 2005), with various chemotherapeutic agents having been administered before (neo-adjuvant), concomitantly, or post radiotherapy (Ohka *et al*., 2011). The two major classes of chemotherapeutic drugs that are being used currently in the treatment of gliomas include the alkylating agents and alkylating-like agents.

Alkylating agents were the first compounds identified to treat cancer (Espinosa *et al*., 2003). These compounds react directly with electron rich atoms in biological molecules to form covalent bonds. The chemotherapeutic and cytotoxic effects are directly related to the alkylation of DNA (Middleton & Margison, 2003). Nitrosourea alkylating agents are commonly used in the treatment of gliomas as they are highly lipid soluble and readily cross the blood brain barrier (Zhelev *et al.*, 2008). The main nitrosureas employed in glioma treatment are nimustine, carmustine and lomustine (Weller *et al*., 2013).

1.9.2.1 Temozolomide

Temozolomide (TMZ) (trade name: Temadol in Europe, Temador in the USA) is an orally administrated, non-classical alkylating agent derived from dacarbazine and was first synthesised in 1984 (O'Reilley *et al.*, 1993; Friedberg, 2001). It is an

interesting drug in part because its development was based on chemosensitivity assays using a wide variety of cultures of brain-tumour cells (Bower *et al*., 1997). Surprisingly, the other common chemotherapy agents used for brain cancer were developed, not on their effect in the laboratory on cultured brain tumour cells, but on cell cultures from other types of cancer (Prasad *et al*., 1980). It is recommended for the treatment of patients with malignant gliomas showing recurrence or progression after standard therapy *(Walker et al*., 2011). TMZ shows excellent penetration of the blood brain barrier due to its small size and lipophilic properties, with studies showing the CNS concentration of the drug to be 30-40% of its plasma concentration (Agarwala, 2000). Typically, it is administered orally, once a day for 5 days in a 28-day cycle, has high bioavailability and once it crosses the blood-brain barrier it is spontaneously hydrolysed to its active form (Wick *et al*., 2009). TMZ toxicity results from induction of the DNA adduct O6-methylguanine (O6M-G) which activates the mismatch repair (MMR) system, causing double strand breaks in the DNA, cell cycle arrest in G2/M phase, and ultimately apoptosis (Roos *et al*., 2007; Caporali *et al*., 2004; Hickman & Samson, 1999).

The most influential randomized study on TMZ, undertaken by the National Cancer Institute of Canada, led to the establishment of concurrent temozolomide and radiation therapy for glioma (Hegi *et al*., 2005). It reported that the two-year survival rate of patients increased from 10.4% with radiation alone, to 26.5% with the concomitant therapy, establishing TMZ as the current standard treatment for anaplastic astrocytomas. TMZ is also being tested in combination with a spectrum of other drugs extensively in low-grade gliomas (Dinca *et al*., 2007).

O6-alkylguanine-DNA alkyltransferase (AGT), the MMR pathway and Poly (ADP-ribose) polymerase (PARP) are the three different mechanisms of resistance to TMZ. With AGT, the alkyl group is removed from the O6 position of guanine bases which in turn reverses the cytotoxic lesion of TMZ (Tisdale, 1987). Guanine alkylation by TMZ can be counteracted by MGMT (O6-methylguanine DNA methyltransferase). Thus, tumour sensitivity to O6-alkylating agents inversely correlates with MGMT expression/activity levels, and as MGMT expression is controlled by hypermethylation of the promoter gene in tumour tissue, methylation can be used to predict sensitivity to TMZ (Hegi *et al*., 2005; Reifenberger *et al*., 2012; Bady *et al*., 2012; Qian *et al*., 1997). During DNA replication O6-MG mispairs with thymine, a pairing recognised by the MMR system which removes the thymine leading to repeated insertion of O6-MG and ultimately production of DNA strand breaks, p53 activation and apoptosis induction (Tentori *et al*., 2013). However, mutations in one or more protein complexes result in MMR pathway deficiency, meaning that cells can tolerate the methylation and cytotoxic effects of TMZ. This leads to the unrepaired O6-MG adducts produced by the TMZ causing DNA replication to pass the O6-MG adducts without apoptosis (Wedge *et al*., 1996). In addition to generating O6-MG, TMZ also methylates DNA resulting in adducts that are excised by N-methylpurine DNA glycosylase, producing apruinic endonuclease target sites, resulting in gaps that are repaired by the base excision repair system (Tentori *et al*., 2013). PARP-1 is involved in both strand break detection and repair processes and in patients treated with TMZ, can be upregulated, leading to resistance to treatment effects.

1.9.2.2 Cisplatin

Cis-diamminedichloroplatinum (CDDP) or cisplatin is a platinum containing chemotherapeutic drug that despite lacking an alkyl group in its structure is considered to be an alkylating-like agent due to its mechanism of action. Currently it is one of the most potent drugs used in the treatment of testicular, ovarian, head and neck tumours (Janjetovic *et al*., 2011) and is also used in the treatment of paediatric brain tumours (Singh *et al*., 2010). It is used in both adjuvant and neo-adjuvant treatment of glioma (Seifart *et al*., 2005; Diaz *et al*., 2005), as a monotherapy or in combination with other chemotherapeutic agents to treat recurrent glioblastoma and low grade glioma, and in combination with radiotherapy has proven to be effective (Agholme *et al*., 2012).

The main mechanism of cisplatin antitumor activity is *via* dative covalent interaction with purine bases in the DNA following aquation of the one of the chloride ions in cisplatin. The formation of DNA-protein and DNA-DNA crosslinks block the proliferation of the tumour cell causing a transient cell cycle arrest at S-phase leading to a final G2/M phase arrest, ultimately leading to apoptosis (Janjetovic *et al*., 2011). Cisplatin has other mechanisms of toxicity, including changing cellular transport mechanisms, damaging mitochondria, and activation of the p53 and MAPK pathways. Several genes activated by p53 are associated with cell cycle arrest, DNA repair, apoptosis (CDK inhibitor $p21^{Waf1/Cip1}$), growth arrest and pro-apoptosis (bax). When DNA damage goes beyond a critical threshold, and overwhelms the repair mechanisms, apoptosis is induced (Wu *et al*., 2000; Siddik 2003; Hershberger *et al*., 2002).

As with other chemotherapeutic drugs, several different mechanisms of resistance to cisplatin have been described, typically with multiple forms being found at

a same tumour site. The mechanisms include a reduction in the intracellular accumulation of cisplatin which is generally caused by an inhibition of the drug's uptake due to reduced expression of the high affinity copper transporter (Chen and Kuo, 2013), an increase in cellular efflux by MRP2, a member of the subfamily C of the human ABC (ATP-binding cassette) superfamily (Borst *et al*., 2000; Leslie *et al*., 2005), or both (Kelland, 2000; Yoshida *et al*., 1994). Resistance also occurs through inactivation by thiol-containing molecules such as glutathione (GSH) and metallothionein that increase in concentration when cisplatin is active in the chloride deficient cytoplasm, changes that appear to be mediated by the upregulation of the transcription factor c-jun (Pan *et al*., 2002). Similarly to TMZ, the nucleotide excision repair (NER) pathway becomes upregulated following treatment, resulting in an increase in DNA damage repair following formation of DNA adducts (Galluzzi *et al*., 2012). Finally, high expression of HER-2/neu and PI3-K/Akt pathways can lead to resistance. HER-2/neu is a proto-oncogene which encodes a receptor tyrosine kinase (p185) homologous to the epidermal growth factor receptor (EGFR) that on activation sends a downstream signal, which activates the PI3-K/Akt pathway. Akt subsequently promotes the phosphorylation of Mdm2 and its translocation to the nucleus where it downregulates p53, leading to drug resistance (Oren *et al*., 2002; Hung and Lau, 1999; Siddik, 2003). Along with side effects like ototoxicity, nephrotoxicity and neurotoxicity (Hartmann & Lipp, 2003), these factors can limit both the dose and use of cisplatin.

1.10 Non-steroidal Anti-inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are a drug class that can have anti-pyretic, analgesic and anti-inflammatory effects depending on their dosage (Ligett *et al.*, 2014). The class contains a variety of drugs like salicylates, propionic acid

derivatives and acetic acid derivatives (Hwang *et al*., 2004). They mainly function *via* inhibition of cyclooxegenase-1 and 2 (COX-1 and 2) enzymes, which in turn reduces the synthesis of pro-inflammatory prostaglandins and thromboxanes. The NSAIDs, aspirin, ibuprofen, and naproxen, are all widely used and available over the counter in most countries (Lichtenberger *et al*., 1995).

1.10.1 Aspirin

Acetylsalicylic acid (Fig 1.1), also known as aspirin, is one of the most widely used medicines in the world (Zhang *et al*., 2014). It was formulated as a stable compound by mixing acetic and salicyclic acids in 1897 by Felix Hoffman, in a lab owned by Friedrich Bayer (Hafizullah & Hafiz, 2013), and by 1899, had become Bayer's best-selling drug worldwide (Budge *et al*., 2014). In the same year it was registered under the name 'Aspirin' and by 1904 it was available in the tablet form, achieving widespread global use for the treatment of several conditions such as fever, pain, and rheumatic inflammation (Vane & Botting, 2003).

However, despite its extensive use, the mechanism of its action wasn't well understood until in 1971, when John Vane published his results in the journal *Nature*, explaining that the mechanism of action involved the inhibition of prostaglandin production, a discovery that led to him being awarded the Nobel Prize for his work on prostaglandins in 1982 (Oates, 1982).

Aspirin, an acetyl derivative of salicyclic acid synthesized *via* esterification of salicylic acid, is a white, crystalline substance with a very weak acidic nature and boiling point of 140^{o}C and melting point of 136^{o}C (Kim *et al*., 1984; Wheatley, 1964).

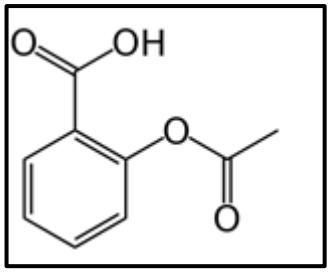

Figure 1.1: Chemical structure of aspirin

Obtained from:
http://dailymed.nlm.nih.gov/dailymed/archives/fdaDrugInfo.cfm?archiveid=56141

In addition to irreversibly inhibiting the COX-1 and COX-2 enzymes *(Pascale et al*., 2012), aspirin has also been shown to uncouple oxidative phosphorylation in mitochondria, act as a proton transporter, and modulate the NF-κB signalling pathway (Somasundaram *et al*., 2000; Shi *et al*., 1999; Stark *et al*., 2001). In addition, a study on the proliferation of coronary artery endothelial cells demonstrated that aspirin inhibits proliferation by upregulating p53, leading to cell cycle arrest (Ranganathan *et al*., 2003).

1.10.2 Aspirin and Cancer

The first evidence which supported the therapeutic use of aspirin in the treatment of cancer was reported by Kune and colleagues in 1988, who demonstrated a decreased risk of developing colorectal cancer (CRC) with the use of aspirin (Kune *et al*., 1988). The trial involved over 700 patients with CRC and a similar number of controls and found a reduction of 40% in the risk of developing CRC amongst participants who were administered aspirin on a regular basis. Following this seminal publication, several studies have proved the importance of aspirin in the prevention of cancer (Rosenberg *et al*., 1991; Muscat *et al*., 1994; Smalley *et al*., 1999), and meta-analysis of several trials examining the effects of aspirin on cardiovascular system have

also concluded that regular use of aspirin reduced the risk of cancer development *(Chan et al., 2011; Lee et al*., 2005).

Each meta-analysis has revealed different positive effects of aspirin use in the treatment and prevention of CRC. Two large randomized trials involving more than 7000 participants, the British Doctors Aspirin Trial and UK-TIA Aspirin Trial, where the long term effects of aspirin was studied, concluded that use of as much as 300 mg of aspirin daily for a term of five years was effective in reducing the risk of CRC (Flossman *et al*., 2007). Meta-analysis of four randomized trials involving more than 14,000 participants that had the primary aim of determining the efficacy of aspirin in the prevention of cardiovascular disease revealed that daily aspirin use reduced the twenty year CRC mortality by 34% (Chan *et al*., 2011). Additionally, the study showed that the risk of developing advanced adenomas was reduced by 28%. However, it was concluded that further investigation regarding the daily dose of aspirin required to reduce cancer risk, and to determine its underlying mechanism, was required.

Aspirin has also been suggested to have a protective role in several other cancers besides CRC (Spector *et al*., 2005; Baron, 2004; Jacobs *et al*., 2007). One large study that involved the meta-analysis of fifty one different trials involving more than 77,000 patients revealed that aspirin reduced the risk of cancer following only three years of use, and the risk of cancer related death following five years of use (Rothwell *et al*., 2012). Importantly, the results showed that the effect was independent of sex, age or smoking status. The study also suggested that a low dose of aspirin (75-300mg) reduced the overall risk of cancer by 25% within a period of 3 years, and the rate dropped even further to 37% after 5 years of regular consumption. However, an

increased risk of major bleeding was observed, although this risk disappeared following 3 years of use.

1.10.3 Aspirin and Metastasis

As well as reducing overall cancer incidence and related deaths, recent studies have established a protective effect of aspirin in relation to metastases, demonstrating benefits not just linked to primary tumour development (Algra & Rothwell, 2012; Rothwell *et al*., 2012). Meta-analysis of five separate trials examining the use of aspirin either by itself or combination with other NSAIDs, but where aspirin was the most commonly used drug, reported that regular use of aspirin reduced the risk of cancers with distant metastasis by 31% (Algra & Rothwell, 2012). Another pooled study which involved five randomized trials with more than 17,000 patients demonstrated a 36% reduced risk of death by distant metastases (Rothwell *et al*., 2012). It also showed that the reduction in risk of death by metastasis involving adenocarcinoma was 46%.

1.10.4 Aspirin and Glioma

While there is insufficient clinical data to currently support the use of aspirin in the treatment or prevention of brain tumours, several *in-vitro* studies on human glioblastoma cell lines and rat glioma cell lines have reported a cytotoxic effect of aspirin (Hwang *et al*., 2004; Kim *et al*., 2009a). A study combining an *in vitro* rat glioma cell line and *in vivo* rat models concluded that, in rat cell lines, aspirin administered at a dosage recommended for treatment of rheumatoid arthritis, inhibited the growth of the cells (Aas *et al*., 1995). *In vivo*, inoculated tumours showed reduced

growth following aspirin treatment, but other NSAIDS such as indomethacin and piroxicam didn't show similar effects, leading to the conclusion that the anti-proliferative effect was not due to the inhibition of prostaglandin production. Interestingly though, Hwang and colleagues demonstrated that both aspirin and indomethacin inhibited the synthesis of prostaglandin E2 in the C6 rat glioma cell line, that low dose aspirin was as effective as high dose, and suggested a link between prostaglandin production and cancer prevention (Hwang *et al*., 2004). More recently, an *in-vitro* study using the A172 human glioblastoma cell line demonstrated that aspirin induced apoptosis *via* the down regulation of IL-6 dependent Stat3 signalling (Kim *et al*., 2009). Given some of the conflicting reports in the literature, and the current lack of clinical data, further research is required to determine the effects of aspirin in human glioma patients and the therapeutic pathway/s involved.

1.11 Aspirin Analogues

In addition to its therapeutic effects, aspirin produces side-effects, especially at higher doses. These include increased risk of bleeding, and both gastrointestinal and renal disturbances, resulting in a drive to develop novel derivatives (analogues) of aspirin and other NSAIDs which retain the potent antineoplastic effects with reduced adverse effects (Deb *et al*., 2011; Hursthouse *et al*., 2010). Subsequently, several promising analogues have been developed and tested on human cancer cell lines (Deb *et al*., 2011; Basudhar *et al*., 2013). One such analogue, IPA/NO, which is a nitric oxide (NO) releasing aspirin-derivative, was shown to reduce cell viability of the non-small cell lung carcinoma A549 cell line with an IC_{50} of 90 μM, a concentration at which aspirin had no effect on viability (Basudhar *et al*., 2013). Another novel aspirin

analogue, 4-hydroxy benzoate zinc (4HBZ) reduced cell viability of primary chronic lymphatic leukemia cells with an IC_{50} of 330μM, significantly higher than that of aspirin on the same cell line (Pepper *et al*., 2011).

Deb and colleagues investigated the efficacy of several novel aspirin analogues *in vitro* using the SW480 colorectal cell line (Deb *et al*., 2011). In comparison to a 20% reduction in cell viability by aspirin at a concentration of 0.5mM, one analogue, PN517 (Fig 1.2) reduced cell viability by 65% at the same concentration (Deb *et al*., 2011). The efficacy of PN517 in viability assays was similar to that of irinotecan, a standard chemotherapeutic drug used in the treatment of CRC. They demonstrated that treatment with PN517 for forty eight hours induced apoptosis at a rate significantly greater than non-treated controls.

The preparation of PN517 involves modifying bis(2-carboxyphenyl) succinate (PN508), which is effectively a double aspirin molecule, meaning that PN517 resembles two aspirin molecules bound together by a double carbon bond (Fig 1.2). It is suggested that this double carbon bond provides the molecule with rigidity when compared to PN508 (Deb *et al*., 2011).

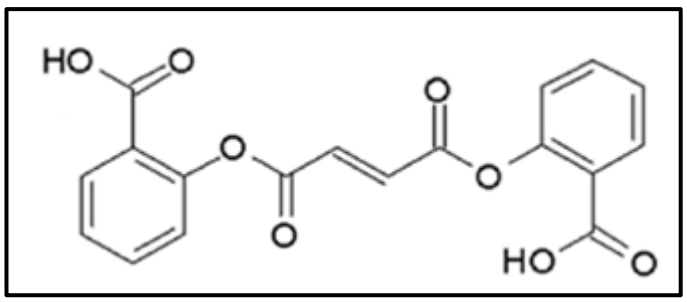

Figure 1.2: Chemical structure of PN517

1.12 Combination Therapies

Glioblastoma multiforme is highly resistant to the current treatment regimens, which is evident from its poor prognosis. This resistance is, to a large extent, due to the high invasiveness and proliferation rate of GBM, and as a result current treatment strategies are mainly palliative (Torres *et al.* ,2011). As has been described, diminishing responses to standard therapeutic drugs like temozolomide and cisplatin over the course of a chemotherapy can result from tumours acquiring defence mechanisms by overexpressing drug efflux pumps, increasing drug metabolism, enhancing self-repairing ability or expressing mutations at drug targets (Gottesman, 2002). To reduce drug resistance, combination chemotherapy has long been accepted in cancer treatment (Hu *et al*., 2010).

It has been suggested that applying multiple drugs with different molecular targets at doses reduced in comparison to monotherapies, can raise the genetic barriers that need to be overcome for glioblastoma mutations, thereby delaying its adaptation process. Conversely, it has been reported that multiple drugs targeting the same cellular pathways can function synergistically for higher efficacy and target-selectivity *(Lehàr et al.,* 2010).

Several studies and clinical trials have used drug combinations in treatment of glioblastoma with varying results (Kondo *et al*., 1995; VanderSpek *et al*., 2008; Mishima *et al*., 2000; Baumann *et al*., 2004; Friday & Adjei, 2008). Temozolomide has shown moderate therapeutic effect on its own, however, when combined with radiotherapy or other anticancer drugs, it has displayed increased efficacy (Zhu *et al*.,

2014). Combining aspirin with a highly resistance-forming drug like cisplatin could help improve the cytotoxicity of the chemotherapeutic drug by altering the cellular response (Cheng *et al.*, 2014). Aspirin can also be beneficial in reducing the extent of cisplatin-induced side effects, mainly related to hearing and balance (Pathak et al., 2014).

1.13 Apoptosis

Apoptosis was first described in 1972 (Kerr *et al*., 1972) with the term originating from the Greek meaning falling (*ptosis*) off (*apo*). It is an active form of cell death whose control and mediation is cell-specific and contextual, is highly conserved throughout evolution, and is important during development and homeostasis in the adult (Bai *et al*., 1999; Hetts, 1998). The process allows for the removal of damaged, injured, infected and incompetent cells from the body both quickly and efficiently. Apoptosis is different from necrosis, an uncontrolled form of cell death characterized by cell swelling and mitochondrial damage leading to rapid depletion of energy levels; a breakdown of homeostatic control; cell membrane lysis and release of the intracellular contents. This leads to an inflammatory response, with oedema and damage to the surrounding cells (Yeh, 1997).

The course of apoptosis is in stark contrast to that of necrosis. It is a controlled and contained process, activated by either external or internal stimuli (Kuan & Passaro, 1998). Initially, the cell shrinks, loosing contact with surrounding cells, and starts to display intracellular proteins on the cell surface. Chromatin condensation and DNA cleavage occurs which can be observed as DNA laddering during gel electrophoresis (Steller, 1995; Yeh, 1997). With the cell membrane still intact, blebbing occurs

releasing fragments of the cell known as apoptotic bodies (Hetts, 1998). Unlike necrosis, this process is relatively rapid, reaching completion in approximately two hours (Kuan and Passaro, 1998).

Triggers of apoptosis can be either extrinsic or intrinsic (Fig 1.3), activating separate pathways initially which ultimately converge on the caspase system of enzymes to execute their function. Extrinsic triggers include activation of death receptors, such as Tumour Necrosis Factor receptors, or removal of survival promoting signals such as the growth factor PDGF (Rich *et al*., 2000; Best *et al*., 1999). Intrinsic activators of apoptosis include increased oxidative stress (Desagher & Martinou, 2000).

Both extrinsic and intrinsic pathways converge on caspase enzymes, an evolutionary conserved family of cysteine proteases, the "central executioners" in apoptotic cell death. Caspases are synthesized as enzymatically inert zymogens, requiring proteolytic cleavage to induce their activation (Gibbons & Pollman, 2000; Hengartner, 2000), generally resulting in a serial sequence of caspase activation known as the caspase cascade. Activation of caspase 8 and caspase 9, known as initiator caspases, results in subsequent cleavage and activation of downstream effector caspases such as caspase 3, caspase 6 and caspase 7. The effector caspases are responsible for the induction of the biochemical and morphological changes associated with apoptosis, and are usually more abundant and active than the initiator caspases (Gibbons and Pollman, 2000; Hengartner, 2000).

Caspase 9 is activated as part of the intrinsic pathway through association with the apoptosome which consists of cytochrome c, an adapter molecule Apaf-1 (apoptosis

protease-activating factor), and pro-caspase 9 (Hengartner, 2000; Gupta, 2001). Activation of death receptors leads to the formation of the Death Inducing Signalling Complex (DISC), the formation of which leads to the cleavage and activation of pro-caspase 8. Ultimately, caspase 8 and 9 cleave and activate caspase 3, caspase 6 and a number of other substrates resulting in the biochemical and morphological characteristics of an apoptotic cell.

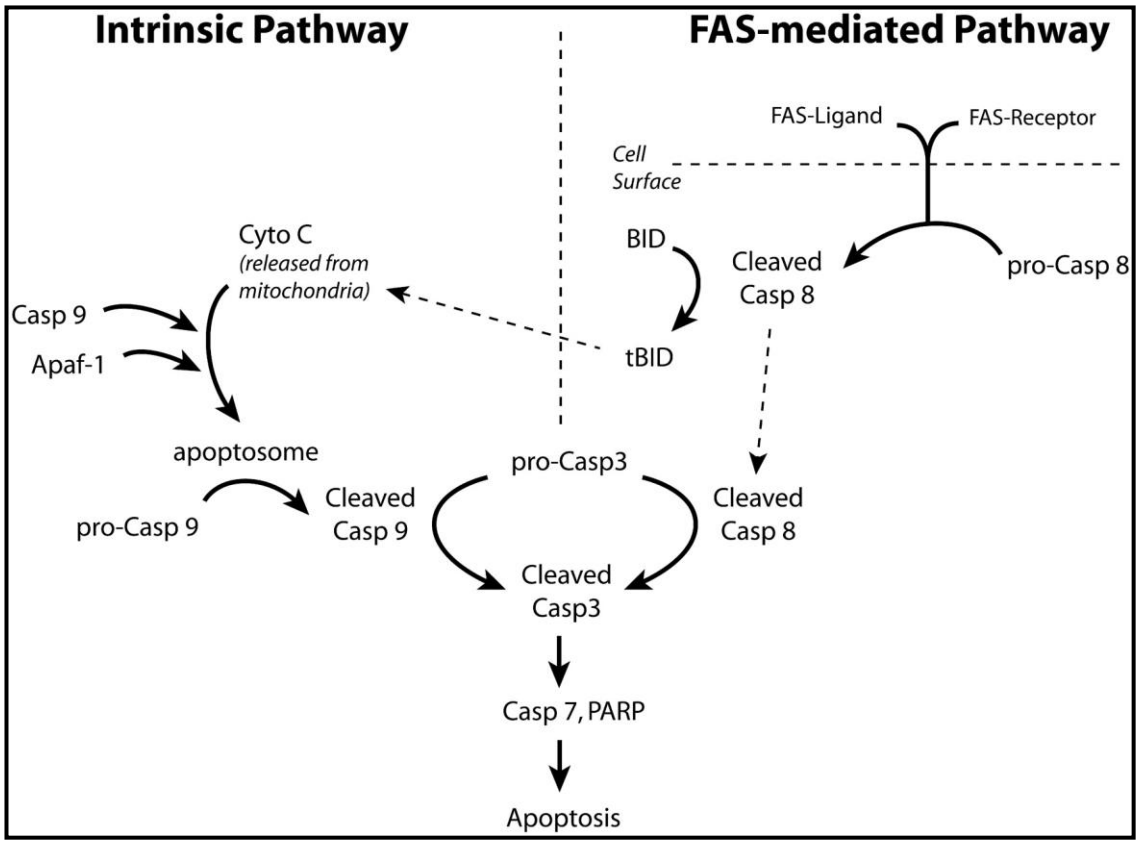

Figure 1.3: Schematic representation of the intrinsic and extrinsic apoptosis pathways

(Zacks *et al*., 2004).

1.14 Autophagy

As with apoptosis, autophagy is an evolutionary conserved process where double-membrane vesicles form containing sections of the cytosol and intracellular organelles, preparing them for lysosomal degradation (Yang & Klionsky, 2010; Sui *et al*., 2011). This process can eliminate damaged organelles and macromolecules, providing protection against carcinogenesis, but can also lead to tumour cell survival in response to stressors such as hypoxia or chemotherapeutic damage (Kondo *et al*., 2005; Maycotte & Thorburn, 2011; Janku *et al*., 2011). Conversely, persistent autophagy can stimulate cell death following chemotherapy by inducing apoptosis. Intracellular signalling pathways involved in autophagy are still being characterised, but phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) and AMP-activated protein kinase (AMPK) are critical regulators (Glick *et al*., 2010) (Fig 1.4).

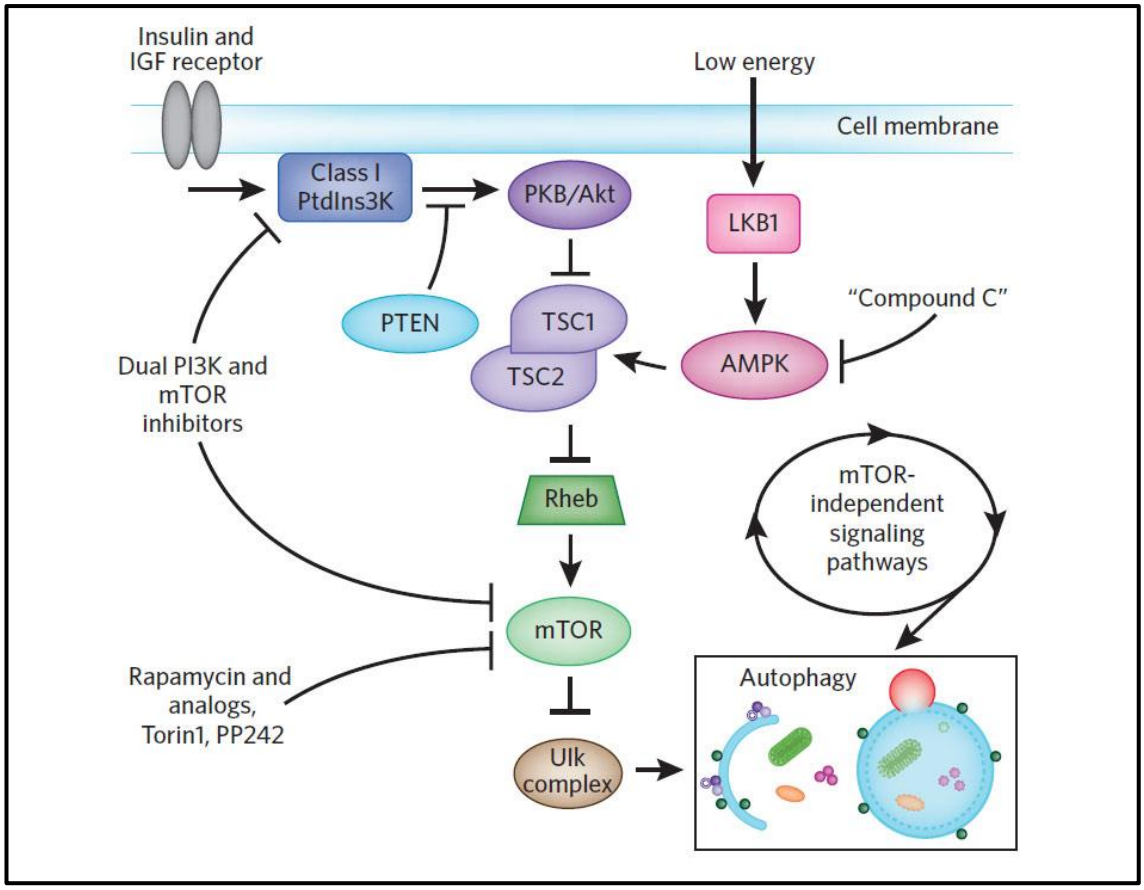

Figure 1.4: Schematic representation of the PI3K/mTOR signalling pathways in autophagy

(Fleming *et al*., 2011)

In GBM, autophagy is enhanced in response to hypoxia promoting tumour cell survival (Hu *et al*., 2012). In response to this, a recent clinical trial was launched to examine the effect of combining an autophagy inhibitor with temozolomide and radiation for the treatment of GBM. The results from the trial suggested a mechanistic interaction between temozolomide and the inhibitor (Rosenfeld *et al*., 2010). The theory is that autophagy inhibitors such as chloroquine will result in the accumulation of autophagic vacuoles, resulting in the induction of cell death in an non-apoptotic manner (Geng *et al*., 2010).

1.15 Hypothesis and Aims

Working Hypothesis: Can combined treatment of aspirin or aspirin analogues with other chemotherapeutics lead to enhanced efficacy in *in vitro* models of glioma?

Main Aim: The main aim of the study is to investigate the effect of the chemotherapeutics temozolomide and cisplatin and aspirin and aspirin analogues alone or in combination on glioma cell death and to determine the mechanism of action of these drugs in combination.

Specific Aims:

1. To determine both time course and dose dependent effects for chemotherapeutics and an aspirin-like drug by themselves or in combination, on glioma cell lines.
2. To determine the effects of drug treatment on cell proliferation
3. To investigate the mechanism of action of cell death for the combined treatments by examining apoptotic markers and pathways such as caspase-8 and 9 or the possible involvement of autophagy.
4. To confirm the cytotoxic effects of the drug treatment via confocal microscopy.

2 Materials and Methods

2.1 Materials

All reagents used were of the highest grade commercially available and, with the exception of PN517 which was supplied by Dr Iain Nicholl (Wolverhampton University, UK), obtained from the following suppliers:

Fisher Scientific (Loughborough, UK)

Ethanol, 25 & 75 cm^2 filter cap, angled neck tissue culture flasks, serological pipettes, 50 & 15 ml centrifuge tubes, multi well dishes, PBS tablets, Foetal Bovine Serum, 8-well LabTek Chamber slides

Sigma-Aldrich (Poole, UK)

Trypan blue, 3-methylanadine, temozolomide, cisplatin, aspirin, bovine serum albumin, DMSO

Life Technologies (Paisley, UK)

PrestoBlue™ Cell Viability Reagent, Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit for Flow Cytometry, CFDA-SE

Lonza (Slough, UK)

MEM Eagle with EBSS w/o L-Glut, MEM Eagle NEAA (100x), L-Glutamine 200 mM, Trypsin/EDTA 10x, Sodium Pyruvate Solution 100 mM

BD Biosciences (Plymouth, UK)

FACS Tubes, BD FACSTM Shutdown Solution, BD FACSTM Clean Solution, BD FACSFlowTM Sheath Fluid, BD FACSRinse Solution

Promega (Southampton, UK)

Caspase-Glo(R) 8 Assay, Caspase-Glo(R) 9 Assay

ECACC (Porton Down, UK)

U-87 MG Grade IV Human Glioblastoma Cell Line

ATCC (Manassas, VA, USA)

SVG-P12 human foetal astroglial cell line

2.2 Cell Culture Methods

2.2.1 Cell Maintenance

U-87 MG and SVG-p12 cells were maintained in Eagles Minimum Essential Media (EMEM), supplemented with 10 % Foetal Bovine Serum (FBS), L-glutamine (2 mM), 1 % Non-Essential Amino Acids (NEAA), sodium pyruvate (1 mM) in a 37^{o}C humidified atmosphere containing 5% CO_2. When a maximum confluence of 80% was reached, cell monolayers were washed with phosphate buffered saline (PBS) solution, 2 ml of 1 x trypsin-EDTA was added, the flasks returned to the incubator to allow cells to detach, and 8 ml of media was added and the cells pipetted gently to ensure a single cell suspension. Cells were either passaged 1:4 into flasks to maintain the cell line or seeded into dishes for experimental analysis.

2.2.2 Growth Curve

To determine the growth rate of the cell lines, growth curve analysis was performed over a period of nine days. Cells were seeded in 6 well plates at a density of 20,000 cells per well, and each day 3 wells were trypsinised and the cell number determined by counting using a haemocytometer.

2.2.3 PrestoBlue® Cell Viability Assay

PrestoBlue® is a resazurin-based cell permeable viability indicator that is reduced in living cells, changing in colour from blue to red (absorbance 570 nm), and becoming highly fluorescent (excitation 535 nm/emission 612 nm), with it being possible to quantitatively measure both changes.

As the greatest variable in viability assays is cell number, linearity of fluorescence versus cell number was determined. Cells were seeded at 0, 50, 100, 250, 500, 1000, 5000 and 10,000 cells/well in a 100 µl volume of media(EMEM) and cultured for 24 or 48 hours before incubating with PrestoBlue® (10 µl/well) for 1 hour and measuring fluorescence.

Concentration response assays were performed to determine the IC_{50} values for aspirin, cisplatin, temozolomide and PN517 in both U-87 MG and SVP-p12 cell lines. Briefly, cells were seeded at 1000 cells per well in 90 µl of media, cultured for 24 hours before drug treatment with a range of drug concentrations(10µM – 10mM) of volume 10 µl each, and viability determined by PrestoBlue® assay following either 24 or 48 hours of treatment. Appropriate media and vehicle controls were also added in a 10 µl volume.

Drug combination assays were performed by seeding both U-87 MG and SVP-p12 cell lines at 1000 cells per well in 90 µl of media and culturing for 24 hours before drug treatment (10 µl) with the IC_{50} value for each drug as determined by the concentration response assays. With cisplatin, two different concentrations were used: **Cisplatin high** – concentration corresponding to the IC_{50} at 24 hoursin the U87-MG cells; **Cisplatin low** – concentration corresponding to the IC_{50} at 24 hours in the SVG-P12 cells.

Three different combination protocols were performed. The first simply combined drug treatments and determined cell viability by PrestoBlue® assay following 2, 4, 8, 12, 24 and 48 hours of treatment. The second protocol staggered drug additions, treating cells

with aspirin or PN517 for 24 hours before adding cisplatin for a further 24 hours and determining cell viability by PrestoBlue® assay. The third protocol involved multiple treatments with aspirin or PN517, with fresh drugs being added at 12, 24 and 36 hours and the old media being removed. At the 36 hour time point, cisplatin was added in combination and cell viability subsequently determined at 48 and 72 hours by PrestoBlue® assay.

The contribution of autophagy to changes in cell viability was determined using the selective autophagy inhibitor, 3-methyladenine (3-MA) (Heckmann *et al.*, 2013). Cells were treated with 3-MA (0.5mM) for 1 hour prior to the addition of either cisplatin or PN517 and incubation for 24 hours, before determination of cell viability by PrestoBlue® assay.

2.2.4 CFDA-SE Cell Proliferation Assay

In order to examine cell proliferation, both the U-87 MG and SVG-p12 cell lines were seeded at a density of 150,000 cells per well in 6 well plates. Cells were treated with the proliferation marker CFDA-SE (0.5 μM) in PBS for 30 minutes, 22 hours after seeding (Shedlock *et al.*, 2010). The PBS-CFDA-SE solution was discarded and 3 ml of fresh media added to each well. 24 hours after seeding, non-CFDA-SE treated cells and non-drug treated control CFDA-SE treated cells were harvested for flow cytometric analysis. The drug treatments (0.1 mM) were added, and the plates incubated for a further 24, 48 and 72 hours prior to harvesting for flow cytometric analysis. Cells were harvested by trypsinisation and centrifugation, washed with PBS

before suspension in 100µl of PBS-0.1% BSA in FACS tubes, and maintained on ice prior to analysis.

2.2.5 Apoptosis Assay, Annexin-V/Propidium Iodide

Induction of apoptosis was examined in both the U-87 MG and SVG-p12 cell lines following drug treatment for 24 and 48 hours. Briefly, cells were seeded at a density of 150,000 cells per well in 6 well plates, incubated for 24 hours prior to drug treatment prior to harvesting by trypsinisation and centrifugation following a further 24 and 48 hours of incubation. As per the manufacturers protocol, cell pellets were resuspended in 1 x binding buffer containing propidium iodide (1:200 dilution of stock) and annexin-V (1:40 dilution of stock) and incubated at room temperature for 15 minutes prior to analysis. Unless otherwise stated, at all stages following harvesting, cells were maintained at 4^{o}C.

2.2.6 Flow Cytometry

All cells were analysed using a FACSAria (BD Bioscience, Franklin Lakes, New Jersey, USA) equipped with an air-cooled 15 mW argon laser emitting at a fixed wavelength of 488 nm. The fluorescence filters and detectors used were all standard with green fluorescence collected in the FL1 channel (530 ± 30 nm) and red in the FL2 channel (615 ± 25 nm) . Samples were gated on forward scatter (FS) versus side scatter (SS) to exclude debris and clumps. The cells were analysed using algorithmic amplifier to determine the percentage of stained cells and their mean fluorescence intensity. During data acquisition, a “live gate” was set on the appropriately stained cell population and a total of 10,000 gated events were acquired for each treatment.

2.2.7 Assay of Caspase 8 & 9 Activity

The Caspase-Glo® assays are luminescent assays that determined caspase activity based on the production of a luminogenic substrate following cell lysis (Fig 2.1). As per manufacturer's instructions, cells were seeded at a density of 10,000 cells per well in white walled opaque clear bottomed 96 well plates and incubated overnight. Cells were drug treated for 12, 24 and 48 hours prior to a one step lysis and detection protocol with either the caspase 8 or 9 reagent. Prior to addition of the mixture in a one to one ratio with the media under low light conditions, the cells were equilibrated at room temperature for 10 minutes. Following addition, the plate was protected from light using foil, shaken at 500 rpm for 30 seconds using an orbital shaker, and incubated at room temperature for 1 hour. The luciferase based luminescent signal, proportional to the amount of caspase activity, was detected using a TECAN plate reader.

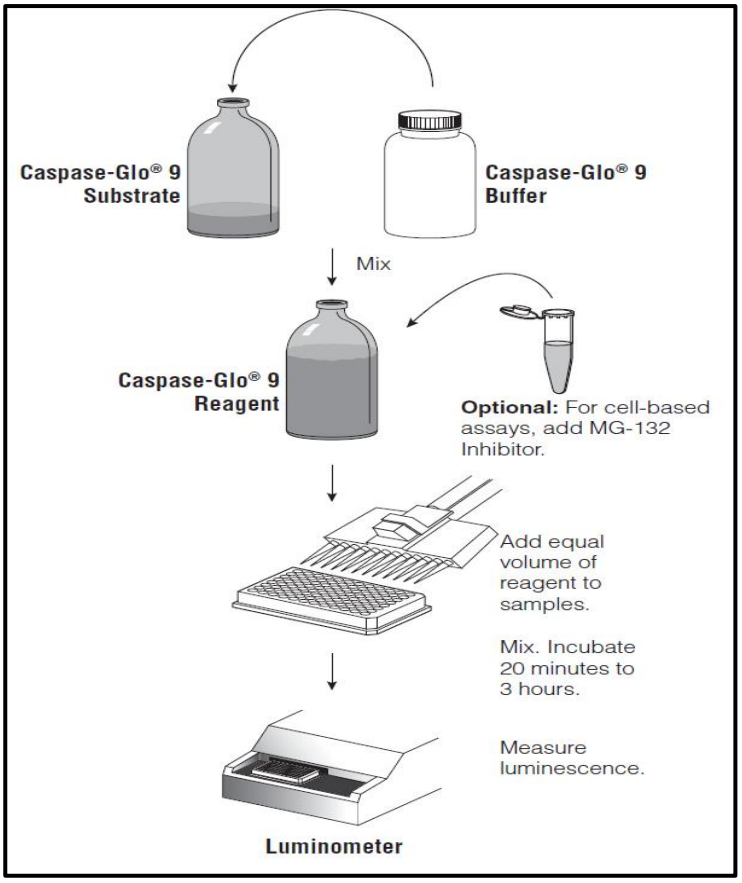

Figure 2.1: Schematic diagram depicting the Caspase-Glo assay protocol

(Adapted from www.promega.co.uk)

2.2.8 Confocal Laser Scanning Microscopy

The U-87 MG and SVG-p12 cell lines were seeded on 8 well chamber slides at a density of 5000 cells per well and cultured overnight prior to drug treatment as per the cell viability drug combination assay (Section 2.2.3). After 24 hours incubation, the media was removed and the cells washed twice with PBS at 37^{o}C, before being stained with annexin-V and propidium iodide as per the flow cytometry apoptosis protocol (Section 2.2.5). Following staining, the cells were washed with PBS before a final 100 µl of PBS was added prior to confocal analysis.

Cells were observed using a Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany) using a Zeiss Plan-Neofluar 40 x 1.3 oil immersion objective and pinhole of 100 μm. Annexin-V and propidium iodide were excited using a 488 nm argon / krypton laser, with annexin-V being detected with a 505-530 nm band pass filter and propidium iodide with a 560 nm long pass filter. The images were processed with Zeiss LSM Image Browser.

2.2.9 Statistical Analysis

Unless otherwise indicated, results of each assay were normalised and expressed as a percentage of untreated control cell populations. Data were expressed as mean ± SEM of at least three independent experiments with different cell passages. Comparison between experimental groups was performed using either two-way or one-way ANOVA with Bonferroni's Post-Hoc test. p - values ≤ 0.05 were considered as statistically significant.

3 Results

3.1 Growth Curves

As one factor influencing the results of viability assays is the proliferation of cells, growth curves were performed with both the U-87 MG and SVG-p12 cell lines to identify any lag phase in their initial growth period or plateau following prolonged incubation (Fig 3.1). The growth rate of both cell lines was approximately linear for the first six days of incubation, subsequently approaching a plateau at day eight. The assay indicates a doubling time of approximately two days for the U-87 MG cell lines, and three days for the SVG-p12 cell line.

3.2 Cell Viability

3.2.1 Effect of increasing cell number on cell viability

The relationship between cell number and fluorescence using the PrestoBlue cell viability assay was determined by performing an assay using increasing number of cells (Fig 3.2). Following 24 hours of culture, a linear relationship between cell number and fluorescence is observed for both the U-87 MG (R^2 = 0.93) and the SVG-p12 cell lines (R^2 = 0.93) across the initial seeding density range of 0 – 10,000 cells per well. Following 48 hours of culture, fluorescence was linear up to 2500 cells per well (fit not shown), but when all cell densities were included, fluorescence began to saturate above that cell number for both cell lines.

3.2.2 Concentration Response Assays

Initial concentration response assays aimed to establish the IC_{50} values for aspirin, PN517, and the standard chemotherapeutics temozolomide and cisplatin using the glioblastoma cell line U-87 MG and the control SVG-p12 cell line (Figs 3.3 & 3.4).

Following 24 hours of drug treatment, aspirin reduced the viability of both cell lines, with an IC_{50} value in U-87 MG cells of 3.2 mM and in the SVG-p12 cell line of 3.9 mM (Fig 3.3). Following 48 hours of drug treatment, these values were 3.5 mM and 2.7 mM respectively. Cisplatin also reduced cell viability in a concentration dependent manner following both 24 and 48 hours of incubation, with IC_{50} values at 24 and 48 hours in the U-87 MG cell line of 100 μM and 9 μM respectively. In the SVG-p12 cell line, respective IC_{50} values of 8 μM and 22 μM were observed. The aspirin analogue PN517 also reduced cell viability in a concentration dependent manner in both cell lines. In the U-87 MG cell line, following 24 and 48 hours of drug treatment, respective IC_{50} values of 1.4 mM and 1.9 mM were observed (Fig 3.4). In the SVG-p12 cell line, the IC_{50} value for PN517 was 1.7 mM following 24 hours of drug treatment, and 1.8 mM following 48 hours. The current gold standard chemotherapeutic drug for the treatment of glioblastoma, temozolomide, was also tested in both cell lines, but following both 24 and 48 hours of drug treatment in the two cells lines, the maximum effect of treatment resulted in a 33% reduction in viability (Fig 3.4, panel D), meaning that no reliable IC_{50} value for temozolomide could be determined.

A Two-Way ANOVA was performed on the combined data from aspirin, cisplatin, PN517, and temozolomide treatments, and it was found that the U-87 MG data displayed homogeneity of variance (Shapioro-Wilks and Levene's Test both $p < 0.05$). There was a significant main effect for drug ($F_{3,120} = 127.719$, $p < 0.001$), for concentration ($F_{7,120} = 164.264$, $p < 0.001$), and also a significant interaction effect ($F_{13,120} = 16.709$, $p < 0.001$). Subsequent Post-Hoc analysis, Bonferroni and a One-Way ANOVA comparison, was performed to determine where the differences lay. The effect of cisplatin was found to be significantly different from aspirin ($p < 0.03$) and

temozolomide ($p < 0.001$), and importantly, the effect of PN517 was significantly different from temozolomide ($p < 0.01$).

The SVG-p12 data also displayed homogeneity of variance (Shapioro-Wilks and Levene's Test both $p < 0.05$). There was a significant main effect for drug ($F_{3,120}$ =301.538, $p < 0.001$), for concentration ($F_{7,120}$ = 166.944, $p < 0.001$), and also a significant interaction effect ($F_{13,120}$ = 23.782, $p < 0.001$). Subsequent Post-Hoc analysis, Bonferroni and a One-Way ANOVA comparison, was performed to determine where the differences lay and found that the effect of cisplatin was significantly different from aspirin ($p < 0.001$), temozolomide ($p < 0.001$) and PN517 ($p < 0.001$). Again, the effect of PN517 was significantly different from temozolomide ($p < 0.001$).

3.2.3 Combination Treatment Assays – Simultaneous Drug Addition

Following the determination of IC_{50} values from the concentration response assays, these values were used for the subsequent combination treatment assays. The initial assays simply tested these IC_{50} concentrations on their own or in combination, with treatments being added simultaneously and incubated for 2, 4, 8, 12, 24 and 48 hours prior to determination of viability using the PrestoBlue® assay. Again, both the U-87 MG (Fig 3.5) and the SVG-p12 (Fig 3.6) cell lines were tested.

Using the U-87 MG cell line, following 2, 4 or 8 hours of drug treatment, no significant change in cell viability was observed (Fig 3.5, panels A - C). However, following 12, 24 and 48 hours of treatment, significant changes were observed (Fig 3.5, panels D – F). A One-Way ANOVA was performed on the results as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$). Analysis found that

there was a significant difference between groups at 12 hours ($F_{9,29}$ = 25.007, p < 0.001), 24 hours ($F_{9,29}$ = 31.951, p < 0.001), and 48 hours ($F_{9,29}$ = 68.768, p < 0.001), so Post-Hoc tests were performed to determine where the differences lay. All treatments at 12 hours were significantly different from control (p < 0.001) apart from cisplatin (low) (p = 0.093). The combination of PN517 with cisplatin (high) was significantly different from cisplatin (high) alone (p < 0.005). Additionally, the combination of aspirin with cisplatin (high) was significantly different from cisplatin (high) alone (p < 0.05). Following 24 hours of drug treatment it was found that all treatments were significantly different from control ($p<0.05$) apart from cisplatin (low) (p = 0.215). The combination of PN517 with cisplatin (low) was significantly different from cisplatin (low) alone (p < 0.05), as was the combination of cisplatin (high) with PN517 when compared to cisplatin (high) alone (p < 0.001). Additionally, the combination of aspirin with cisplatin (high) was significantly different from cisplatin (high) alone (p < 0.005). Lastly, it was found that all groups following 48 hours of drug treatment were significantly different from control (p < 0.001) apart from cisplatin (low) (p = 0.083) and aspirin in combination with cisplatin (low) (p = 0.112). The combination of PN517 with cisplatin (high or low) was significantly different from cisplatin (high or low respectively) alone (p < 0.001). Additionally, the combination of aspirin with cisplatin (high) was significantly different from cisplatin (high) alone (p < 0.005).

A similar pattern was observed using the SVG-p12 cell line (Fig 3.6). No significant effect on cell viability was found following 2 or 4 hours of drug treatment (Fig 3.6, panels A & B), but again, significant decreases in viability were recorded with the longer incubation times (Fig 3.6, panels C - F). A One-Way ANOVA was performed on results as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-

Smirnovs, $p > 0.05$), and a significant difference between groups at 8 hours ($F_{9,29} = 3.444$, $p = 0.01$), 12 hours ($F_{9,29} = 11.353$, $p = 0.001$), 24 hours ($F_{9,29} = 10.033$, $p = 0.001$), and 48 hours ($F_{9,29} = 14.354$, $p = 0.001$) was observed, so Post-Hoc tests were performed to determine where the differences lay. At 8 hours, no individual treatment produced a significant effect. All treatments at 12 hours were significantly different from control ($p < 0.001$) apart from aspirin, cisplatin (low) and aspirin-cisplatin (low) ($p > 0.05$). The combination of PN517 with cisplatin (low) was significantly different from cisplatin (low) alone ($p < 0.05$). Following 24 hours of drug treatment, all groups were significantly different from control ($p < 0.001$) apart from aspirin, cisplatin (low and high) and aspirin-cisplatin (low) ($p > 0.05$). The combination of PN517 with cisplatin (low) was significantly different from cisplatin (low) alone ($p < 0.05$). Finally, all treatments at 48 hours were significantly different from control ($p < 0.001$) apart from aspirin, cisplatin (low) and aspirin-cisplatin (low) ($p > 0.05$). The combination of PN517 with cisplatin (low) was significantly different from cisplatin (low) alone ($p < 0.005$).

3.2.4 Combination Treatment Assays - Staggered

The next series of experiments investigated the effect of staggering the combination treatments, where cells were treated with aspirin or PN517 for 24 hours before adding cisplatin for a further 24 hours (Fig 3.7). The combination of PN517 with cisplatin reduced U-87 MG cell viability to 25 % (cisplatin low) and 18 % (cisplatin high) of control. A One-Way ANOVA was performed on U87-MG results (Fig 3.7, panel A) as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$), and a significant difference between the groups was observed ($F_{9,29} = 30.387$, $p < 0.001$). Post-Hoc tests were subsequently performed to determine where the

differences lay and all treatments were significantly different from control ($p < 0.001$), however, the combination of PN517 with cisplatin (low or high) was not significantly different from the respective cisplatin mono-treatment.

Similar results were observed in the SVG-p12 cell line (Fig 3.7, panel B) with the combination of cisplatin (low and high) with PN517 reducing cell viability to 31 % and 12 % of control. A One-Way ANOVA was performed on the results as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$) and a significant difference between the groups was found ($F_{9,29} = 29.127$, $p < 0.001$). Post-Hoc analysis found that all treatments were significantly different from control ($p < 0.001$), but again, the combination of cisplatin (high or low) with PN517 was not significantly different from the cisplatin treatment alone.

3.2.5 Combination Treatment Assays - Multiple

As the major cytochrome P450 enzyme responsible for the metabolism of aspirin is expressed in the U-87 MG cell line (data not shown), a third protocol was investigated where aspirin or PN517 were added repeatedly at 12, 24 and 36 hours before subsequent treatment with cisplatin and determination of viability at 48 and 72 hours (Fig 3.8). It was assumed that repeated drug treatment might help in maintaining the drug concentration following it's metabolism via cytochrome P450 enzyme, thereby enhancing its cytotoxic effect.

In the U-87 MG cell line, PN517 or cisplatin alone reduced cell viability to 80% and 35% of control respectively after 48 hours of drug treatment (Fig 3.8, panel A). The combination of PN517 with cisplatin reduced viability to 34% of control at 48 hours. Following 72 hours of drug treatment (Fig 3.8, panel C), cisplatin alone or in

combination with PN517 reduced cell viability by more than 50%, but the PN517 treatment alone was found to have little effect on viability. Statistical analysis using a One-Way ANOVA was performed as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$), and found that there was a significant difference between groups at 48 hours ($F_{6,20} = 7.851$, $p = 0.001$), and 72 hours ($F_{6,20} = 90.573$, $p < 0.001$). Post-Hoc analysis found that all treatments at 48 hours were significantly different from control ($p < 0.05$) apart from aspirin and PN517 ($p = 1$), and at 72 hours, all treatments were significantly different from control ($p < 0.05$) apart from aspirin and PN517 ($p = 1$). Interestingly, while, the combination of PN517 with cisplatin was not significantly different from cisplatin alone, the combination of aspirin with cisplatin was significantly different from cisplatin alone ($p < 0.05$).

A similar pattern of results was observed in the SVG-p12 cell line (Fig 3.8, panels B & D), although with smaller reductions in cell viability when compared to U-87 MG cell line. After 48 hours of treatment, PN517 reduced cell viability to 80% of control while cisplatin on its own was the most potent treatment, reducing the viability to approximately 50% of control (Fig 3.8, panel B). The combination of PN517 with cisplatin reduced viability to 44% of control. Following 72 hours of treatment (Fig 3.8, panel D), similar results were observed with the PN517 combination treatment with cisplatin reducing cell viability to 62% of control, while PN517 and cisplatin on their own reduced viability to 64% and 50% of control. A One-Way ANOVA was performed as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$) and found a significant difference between groups at 48 hours ($F_{6,20} = 9.949$, $p < 0.001$), and 72 hours ($F_{6,20} = 61.514$, $p < 0.001$). Post-Hoc tests determined that all treatments at 48 and 72 hours were significantly different from control ($p < 0.05$) apart from aspirin

and PN517 (p = 1). Again, however, the combination of PN517 with cisplatin was not significantly different from cisplatin alone.

3.3 Cell Proliferation

One factor which can affect cell viability is cell proliferation, so CFDA-SE staining was used to examine any changes following drug treatment. CFDA-SE enters the cells via diffusion through the plasma membrane. Once it enters the cell, the cellular esterases convert the CFDA-SE to CFSE which is fluorescent in nature. CFSE forms a covalent bond with the amine groups in proteins, thereby resulting in retention of the dye inside the cell. Following cell division, each daughter cell receives approximately half of the dye from the parent cell. Analysing the fluorescence intensity via the flow cytometer helps in determining the number of generations the cell has progressed through after the addition of the stain (Wang *et al*., 2005).

In the U-87 MG glioblastoma cell line (Fig 3.9), the biggest effect on proliferation rate was observed following treatment with cisplatin, with a rightward shift in the proliferation curve when compared to control. The affect was biggest at 24 and 48 hours, with cells appearing to recover at 72 and 96 hours where fluorescence intensity approached that of control treatment. Neither treatment with aspirin, nor PN517 alone affected proliferation. Interestingly, the combination of either aspirin or PN517 with cisplatin reduced the effect of cisplatin on proliferation. When proliferation in the SVG-p12 cell line was examined (Fig 3.10), none of the treatments, either mono or combination, had any effect on cell proliferation when compared to control.

Sample results for the proliferation data are shown in figures 3.11 (U-87 MG) and 3.12 (SVG-p12), where a shift in peak to the left indicates cell proliferation, with each colour indicating a different timepoint of analysis. In the U-87 MG cell line (Fig

3.11), it can be clearly seen that compared to control (panel A), cisplatin treatment has reduced the leftward shift in the fluorescence peak (panel F). However, little difference is observed in the leftward shift of peaks compared to control for any of the drug treatments in the SVG-p12 cell line (Fig 3.12).

3.4 Apoptosis

3.4.1 Annexin-V and Propidium Iodide

Another factor which can affect cell viability is cell death, commonly as a result of apoptosis, so staining with annexin-V and propidium iodide was used to examine any changes following drug treatment for 24 and 48 hours.

In the U-87 MG glioblastoma cell line, drug treatment induced apoptosis at both time points, with staining indicating both early (annexin-V alone) and late (annexin-V and PI) apoptotic populations, and a low level of necrosis (PI alone) (Fig 3.13). After 24 hours of drug treatment, only 8 % of cells remained unstained following treatment with cisplatin (high), while 32 % were unstained after treatment with aspirin (Fig 3.14, panels A and B). The combination of PN517 with cisplatin (low or high) increased the proportion of late apoptotic cells when compared to cisplatin alone following 24 hours of treatment, while the levels of unstained (live) cells did not vary. Following 48 hours of treatment, a general trend towards a decrease in staining was observed (Fig 3.13, panels C and D). The combination of PN517 with cisplatin (high) resulted in the smallest proportion of living cells (19 % of control), while 56 % of cells remained unstained (live) following aspirin alone (panel D). The combination of PN517 with cisplatin (high) showed the greatest induction of apoptosis with 81 % of cells in either the early or late phases. Cisplatin (high) along produced the largest necrotic population (3 % of control).

Results for the SVG-p12 cell line showed lower levels of apoptosis (Fig 3.14), particularly at 48 hours, when compared to the U-87 MG cells. Following 24 hours of

incubation, treatment with aspirin alone resulted in the highest proportion of live cells, 44 % of control (Fig 3.14, panel B), while less that 20 % of cells were living following treatment with the combination of PN517 and cisplatin (high). The same combination treatment also induced early apoptosis in the highest percentage of cells, 90 % of control, while aspirin had the least at 56 % (Fig 3.14, panels A and B). The combination of PN517 and cisplatin (high) also had the highest proportion of late-apoptotic cells, 52 % of control, while cisplatin (low) had the lowest at 13 % of control. Little necrosis was observed with a maximum of 2 % being found in the mono-treatments (Fig 3.14, panel B). After 48 hours of drug treatment (Fig 3.14, panels C and D), an increase in the proportion of live cells was observed following most treatments, with 83 % of cells living following cisplatin (high) treatment. However, the combination of PN517 with cisplatin (high) resulted in the lowest proportion of living cells at only 5 % of control. In each case, the combination of PN517 with cisplatin (low or high) resulted in the largest populations of early and late apoptotic cells. The treatment with the highest proportion of necrotic cells was cisplatin (high) at 7 % of control.

Representative dot plots for annexin-V and propidium iodide staining in the U-87 MG and SVG-p12 cell lines following 24 and 48 hours of drug treatment are shown in figures 3.15 and 3.16 respectively. The increase in annexin-V and propidium iodide staining following drug treatment in U-87 MG cells (panels B-D) and SVG-p12 cells (panels F-H) in comparison to their respective controls (panels A and E) can clearly be observed.

3.4.2 Caspase 8 and 9 Activation

In order to examine the mechanism of apoptosis, the activation of caspase 8 (extrinsic pathway) and caspase 9 (intrinsic pathway) was examined using the luminescent Caspase-glo® assay, following 12, 24 and 48 hours of drug treatment (Fig 3.17 and 3.18).

After 12 hours of treatment, with the exception of cisplatin (low or high), all treatments increased caspase 8 activity when compared to control (Fig 3.17, panel A). The combination of PN517 and cisplatin (high) produced the largest increase in caspase 8 activity, 191 % of control. Following 24 hours of treatment aspirin and its combination with cisplatin showed high caspase activity. However, cisplatin had not managed to induce any significant activity as compared to control. After 48 hours of drug treatment, cisplatin (low and high) induced caspase 8 activity when compared to control, but the combination of cisplatin with PN517 reduced caspase 8 activity when compared to cisplatin alone (Fig 3.17, panel E). Unusually, PN517 treatment alone actually reduced caspase 8 activity when compared to control. A One-Way ANOVA was performed as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$), but no significant difference was found between groups at any time point ($p > 0.05$).

In the SVG-p12 cell line, following 12 hours of drug treatment, no increase in caspase 8 activity was observed when compared to control (Fig 3.17, panel B), with all treatments showing a decreased compared to control. PN517 treatment resulted in the biggest decrease in activity, reducing it to 37 % of control. Following 24 hours of drug treatment, most treatments increased caspase 8 activity when compared to control with

the combination of cisplatin (low) and aspirin showing activity of 198 % of control (Fig 3.17, panel D). Interestingly, aspirin treatment alone showed activity of 192 % of control, and the combination of PN517 with cisplatin (high) showed the lowest activity. However, following 48 hours of incubation, activity appeared to change significantly again, with cisplatin (low) showing the largest activation at only 126 % of control (Fig 3.17, panel F). PN517 in combination with cisplatin (low), was shown to decrease activity to only 7% of the media control. A One-Way ANOVA was performed as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$) and a significant difference between groups was found at 12 hours ($F_{8,27} = 4.790$, $p = 0.001$), and 48 hours ($F_{8,27} = 4.081$, $p < 0.001$). Post-Hoc analysis found that at 12 hours PN517 was significantly different from control ($p < 0.05$).

In a similar manner, caspase 9 activation was examined following drug treatment for 12, 24 and 48 hours (Fig 3.18). In the U-87 MG cell line, as with caspase 8, cisplatin alone did not increase caspase 9 activity following 12 hours of treatment (Fig 3.18, panel A). However, aspirin and PN517 alone and in combination with cisplatin did increase caspase 9 activity when compared to control. Following 24 hours of drug treatment, a very similar pattern was observed with little effect found following treatment with cisplatin (low or high), but both aspirin, PN517, and their combination with cisplatin increasing caspase 9 activity compared to control (Fig 3.18, panel C). Aspirin in combination with cisplatin (high) induced the highest caspase-9 activity at 219 % of control. After 48 hours of incubation, cisplatin (low) did increase caspase 9 activation to 356 % of control (Fig 3.18, panel E). A One-Way ANOVA was performed as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$), but no significant difference between groups was found at any time point ($p > 0.05$).

In the SVG-p12 cell line, following 12 hours of incubation none of the treatments had any effect on caspase 9 activity, with PN517 treatment resulting in activity of 56 % of control (Fig 3.18, panel B). A similar pattern was observed after 24 hours with the only exception being aspirin in combination with cisplatin (low), increasing activity to 116 % of control (Fig 3.18, panel B). Even after 48 hours of treatment, no significant change in caspase-9 activity was observed (Fig 3.18, panel F). However, cisplatin (low) did increase activity to 136 % of control. A One-Way ANOVA was performed as the data exhibited normality (Shapiro-Wilk's and Kolmogorov-Smirnov, $p > 0.05$), but no significant difference between groups was observed at any time point ($p > 0.05$).

Given some of the unexpected results of the Caspase-glo® assays, images of cells were taken to determine if any morphological changes consistent with apoptosis were observed. In comparison to control cells (Fig 3.19), it can clearly be seen that at 24 and 48 hours (Fig 3.20 and 3.21 respectively), PN517 treatment resulted in a rounding of cells and also a decrease in cell density, changes consistent with apoptosis. These effects were observed in both the U-87 MG and SVG-p12 cell lines.

3.4.3 Laser Scanning Confocal Microscopy

In a similar manner, confocal microscopy was also used to qualitatively confirm the induction of apoptosis following drug treatment, using staining with annexin-V and propidium iodide. Annexin-V binding (green) indicates binding to the cell surface early apoptotic marker, phosphatidylcholine, and propidium iodide staining (red) of the nucleus indicates late apoptosis and a compromised cell membrane.

In the U-87 MG cell line (Fig 3.22), control treatment showed sparse staining with annexin-V, indicating early apoptosis in a few cells, but no late apoptosis was observed. Following 24 hours of drug treatment, aspirin treatment induced early apoptosis with a small population of late apoptosis, while PN517 treatment resulted in the induction of early apoptosis in most the cells and a larger proportion of late apoptosis when compared to aspirin treatment. In a similar fashion to PN517 treatment, cisplatin treatment induced early apoptosis in the majority of cells. The combination of cisplatin with PN517 or aspirin resulted in the induction of early apoptosis in all observed cells, and late apoptosis in a large proportion also.

Treatment of the SVG-p12 cell line showed a similar pattern of staining with a small number of cells showing annexin-V staining in control, induction of early apoptosis in cells following aspirin, PN517 or cisplatin treatment alone, with a small proportion of late apoptotic cells. Again, combination treatments resulted in high intensity staining indicating induction of early apoptosis but little evidence of late stage apoptosis.

3.5 Autophagy

The potential involvement of autophagy in the reduction of cell viability following drug treatment was examined using the selective autophagy inhibitor, 3-methylanadine (3-MA). Initial experiments examined the effect of 3-MA alone on cell viability (Fig 3.24). A concentration response assay found that concentrations below 5mM did not affect cell viability, thus 0.5mM 3-MA was chosen as a co-treatment in further experiments.

A dose-dependent reduction in cell viability was observed following treatment with either PN517 or cisplatin following 24 and 48 hours of incubation in U-87 MG cell line (Fig 3.25), with IC_{50} values of 0.96mM & 0.58mM and 125µM & 10µM respectively. Addition of 3-MA (0.5mM) did not significantly change the concentration response observed for either PN517 or cisplatin at either time point ($p > 0.05$). Similar results were observed in the SVG-p12 cell line, with 3-MA treatment not significantly changing the concentration response to PN517 or cisplatin treatment following 24 or 48 hours of drug treatment.

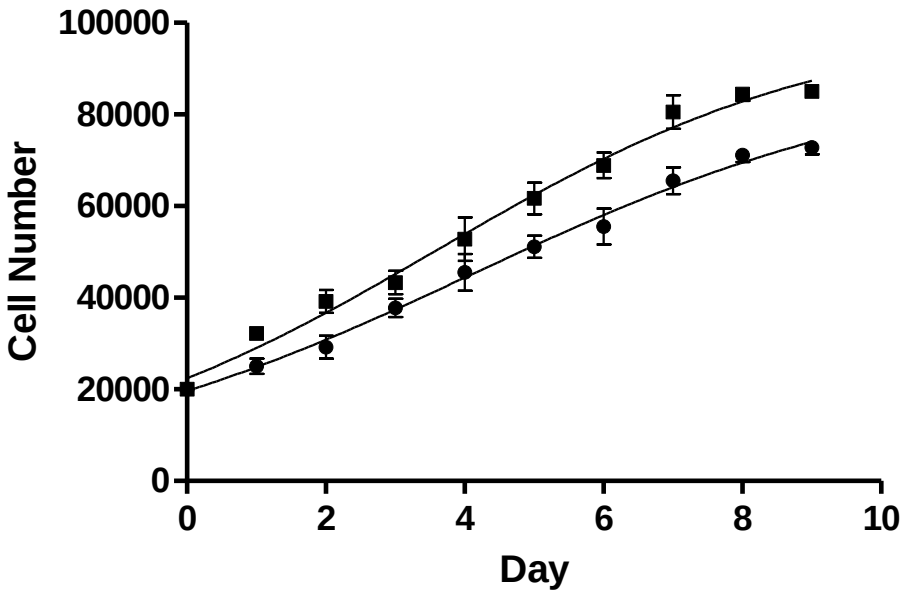

Figure 3.1: Growth curve for U-87 MG and SVG-p12 cell lines.

Following an initial seeding density of 20,000 cells per well, proliferation was determined by cell counting on nine subsequent days. U-87 MG and SVG-p12 cell lines demonstrated doubling times of approximately 2 and 3 days respectively. Data illustrate mean cell number ± SD generated from three independent experiments.

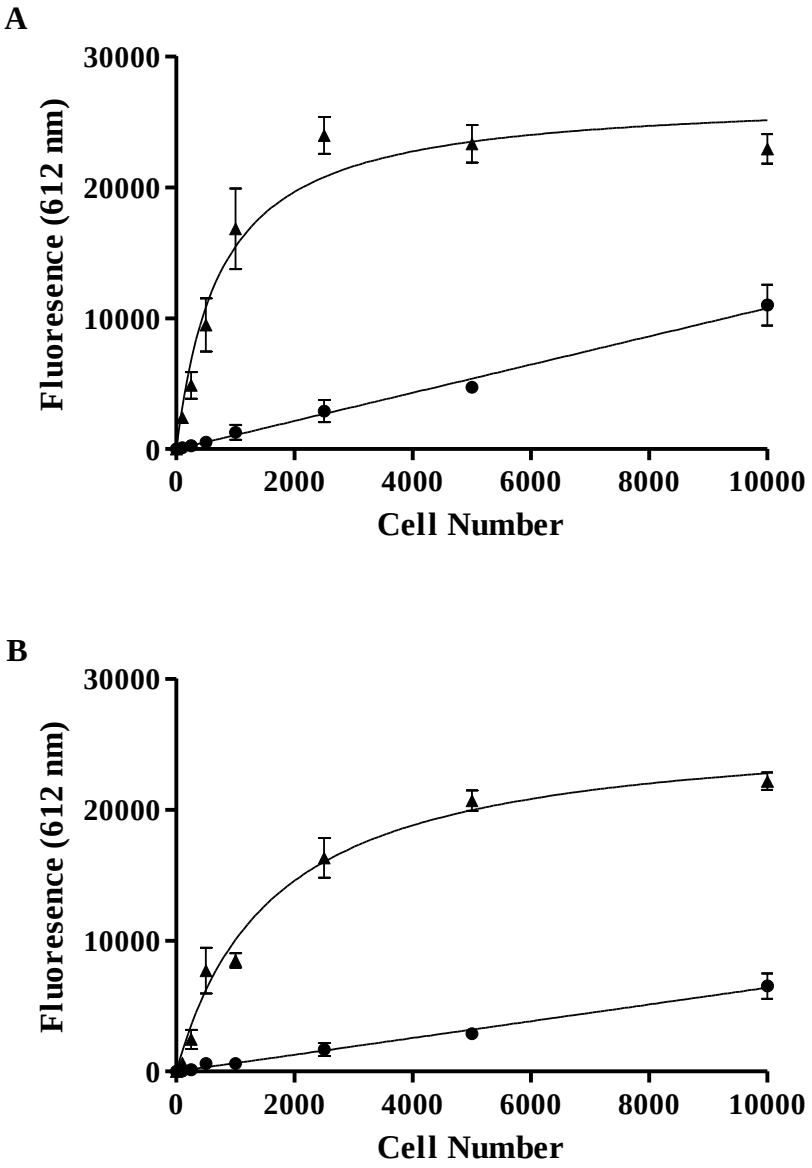

Figure 3.2: Effect of increasing cell number on fluorescence determined by PrestoBlue® assay in U-87 MG and SVG-p12 cell lines.

The relationship between fluorescence and U-87 MG (panel A) or SVG-p12 (panel B) cell number illustrated following 24 (closed circles) and 48 (closed triangles) hours of incubation. Data illustrate mean fluorescence ± SD generated from three independent experiments.

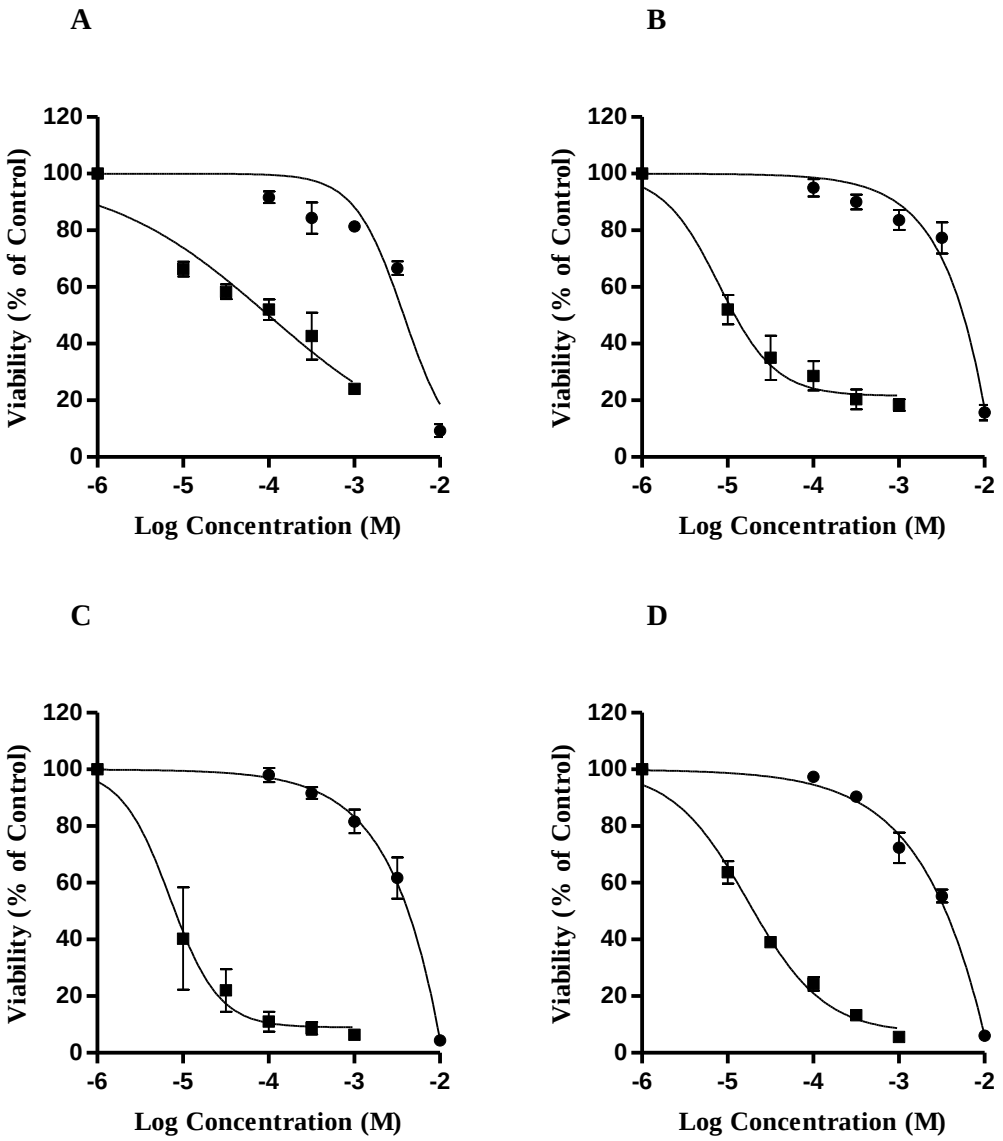

Figure 3.3: Data illustrating the effect of dose-dependent treatment of aspirin (closed circle) and cisplatin (closed square) on U87-MG and SVG-P12 cell lines.

Cell viability was determined using PrestoBlue® assay for cell viability. Panel A and C show the effect of the drugs on U87-MG cell line (1000cells/well) at 24 and 48 hours respectively. Panel B and D illustrate the effect of the drugs on SVG-P12 (1000cells/well) at 24 and 48 hours respectively. The data represents mean ± SEM of three experiments.

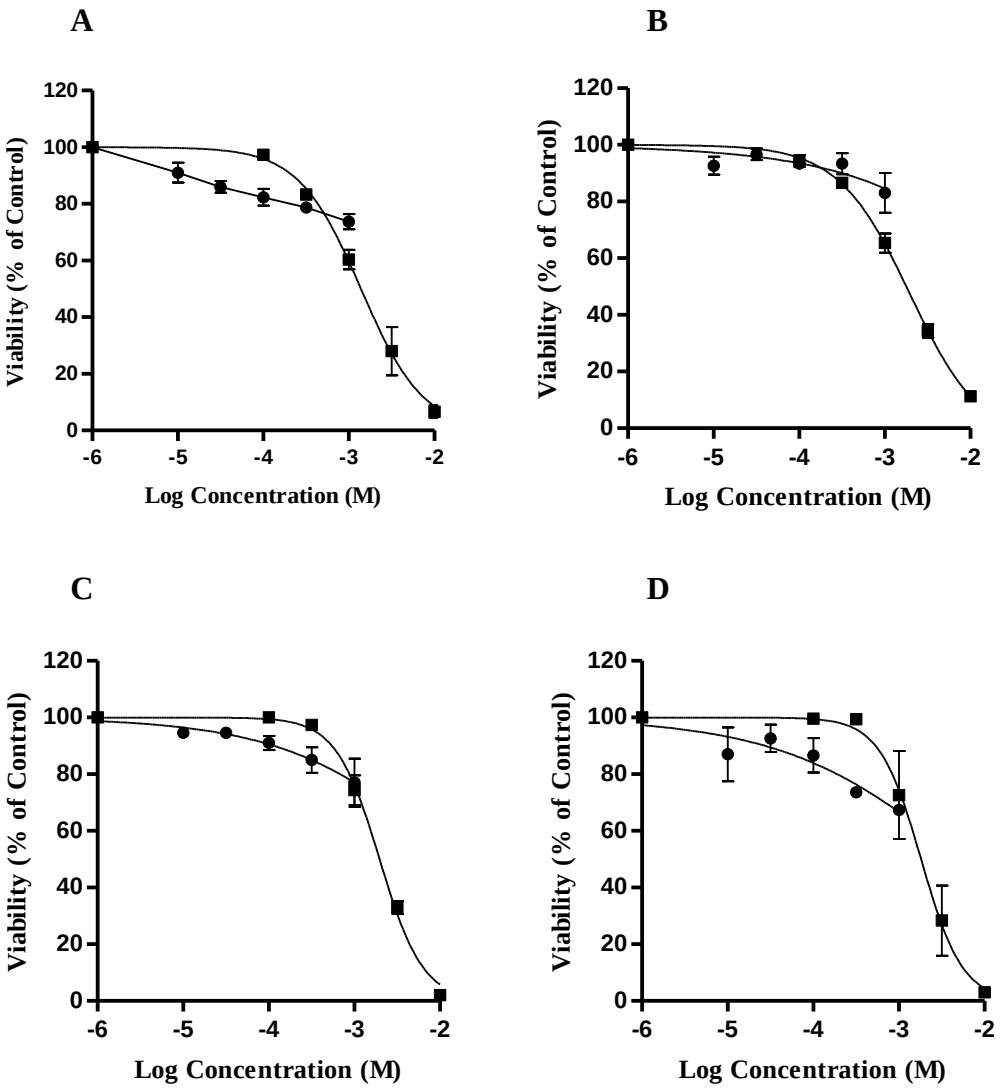

Figure 3.4: Dose dependent effects of temozolomide (closed circle) and PN517 (closed square) on cell viability in U87-MG and SVG-P12 over 24 and 48 hours.

Data illustrating the effect of dose-dependent treatment of temozolomide (closed circles) and PN517 (closed squares) on U87-MG and SVG-P12 cell lines (1000cells/well). Cell viability was determined using PrestoBlue® assay for cell viability. Panel A and C show the effect of the drugs on U87-MG cell lines at 24 and 48 hours respectively. Panel B and D illustrate the effect of the drugs on SVG-P12 at 24 and 48 hours respectively. The data represents mean ± SEM of three experiments.

	IC_{50} value	
	24 hours	48 hours
Aspirin	3.2 mM	3.5 mM
Cisplatin	100 µM	9 µM
PN517	1.4 mM	1.9mM

Table 3.1: IC_{50} values for U-87 MG cell line following 24 and 48 hours incubation with aspirin, cisplatin and PN517.

	IC_{50} value	
	24 hours	48 hours
Aspirin	3.9 mM	2.7 mM
Cisplatin	8 µM	22 µM
PN517	1.7 mM	1.8 mM

Table 3.2: IC_{50} values for SVG-P12 cell line following 24 and 48 hours incubation with aspirin, cisplatin and PN517.

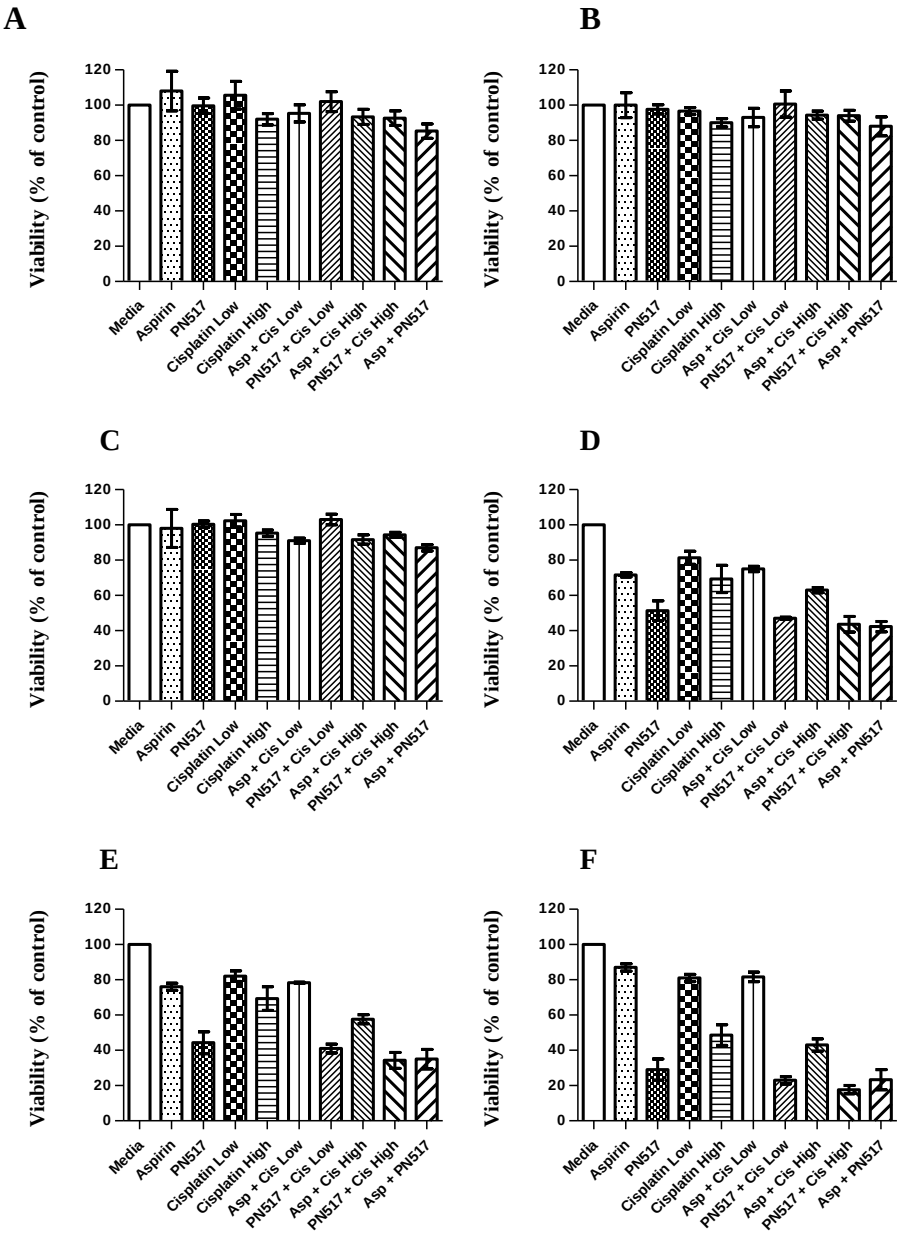

Figure 3.5: Cell viability following combination treatment of aspirin and PN517 with cisplatin and of aspirin with PN517 in a time dependent manner in U87-MG cell line.

Cell viability was determined using the PrestoBlue® viability assay following 2, 4, 8, 12, 24 and 48 hours of incubation. Panels A, B, C, D, E and F represent cell viability at 2, 4, 8, 12, 24 and 48 hours respectively. Data is a mean ± SEM of three experiments.

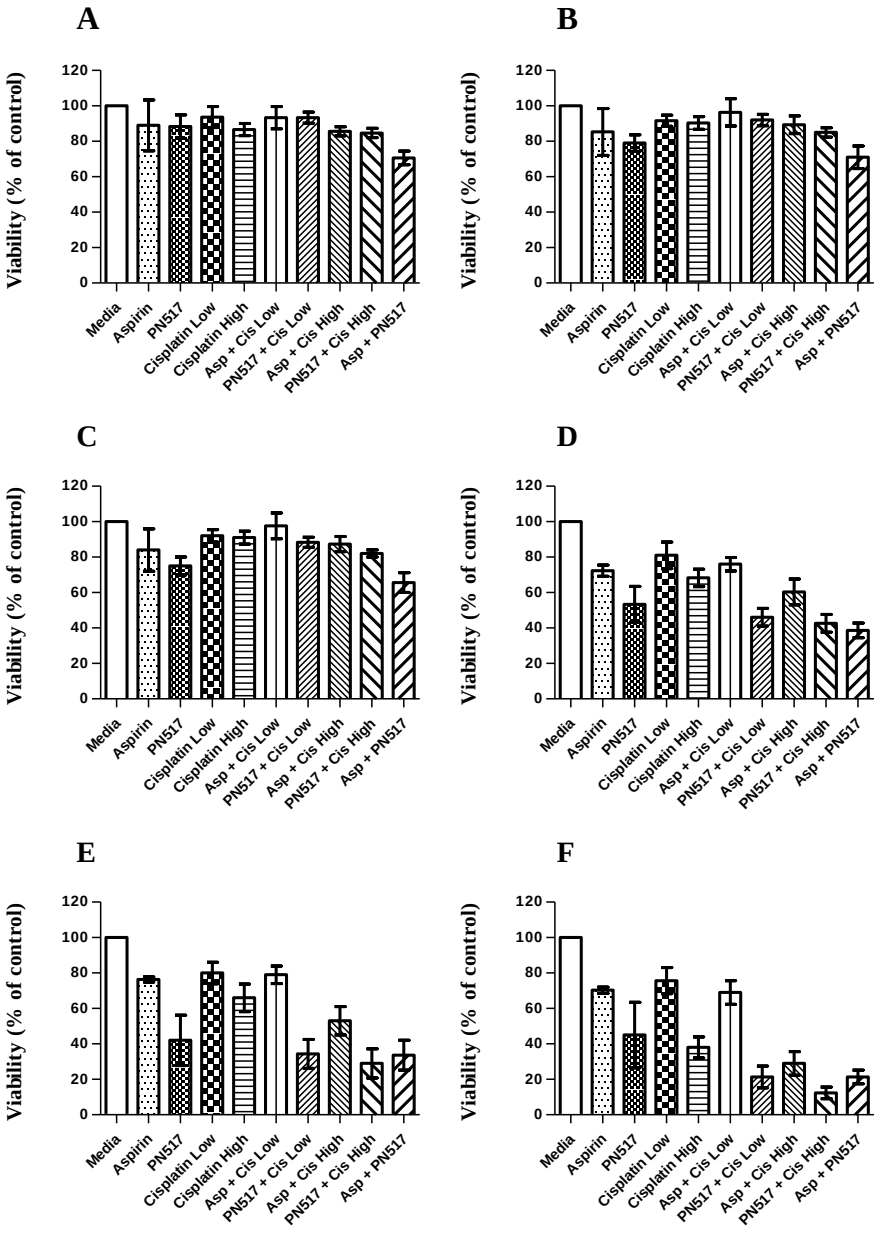

Figure 3.6: Cell viability following combination treatment of aspirin and PN517 with cisplatin and of aspirin with PN517 in a time dependent manner in SVG-P12 cell line.

Cell viability was determined using the PrestoBlue® viability assay following 2, 4, 8, 12, 24 and 48 hours of incubation. Panels A, B, C, D, E and F represent cell viability at 2, 4, 8, 12, 24 and 48 hours respectively. Data is a mean ± SEM of three experiments.

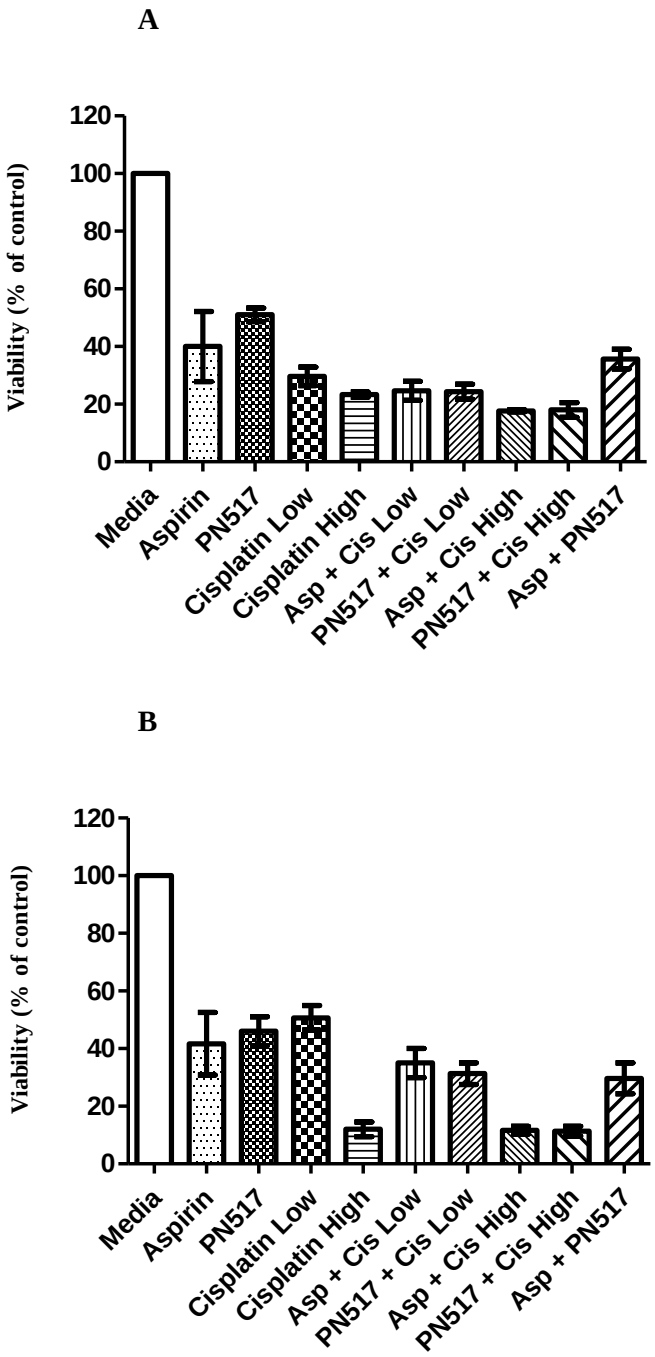

Figure 3.7: Cell viability after staggered treatment of aspirin/PN517 followed by cisplatin.

Cell viability was determined using PrestoBlue viability assay following 48 hours of incubation. Panel A illustrates data for U87-MG cell line while panel B indicates data for SVG-P12 cell line. Data is a mean ± SEM of three experiments.

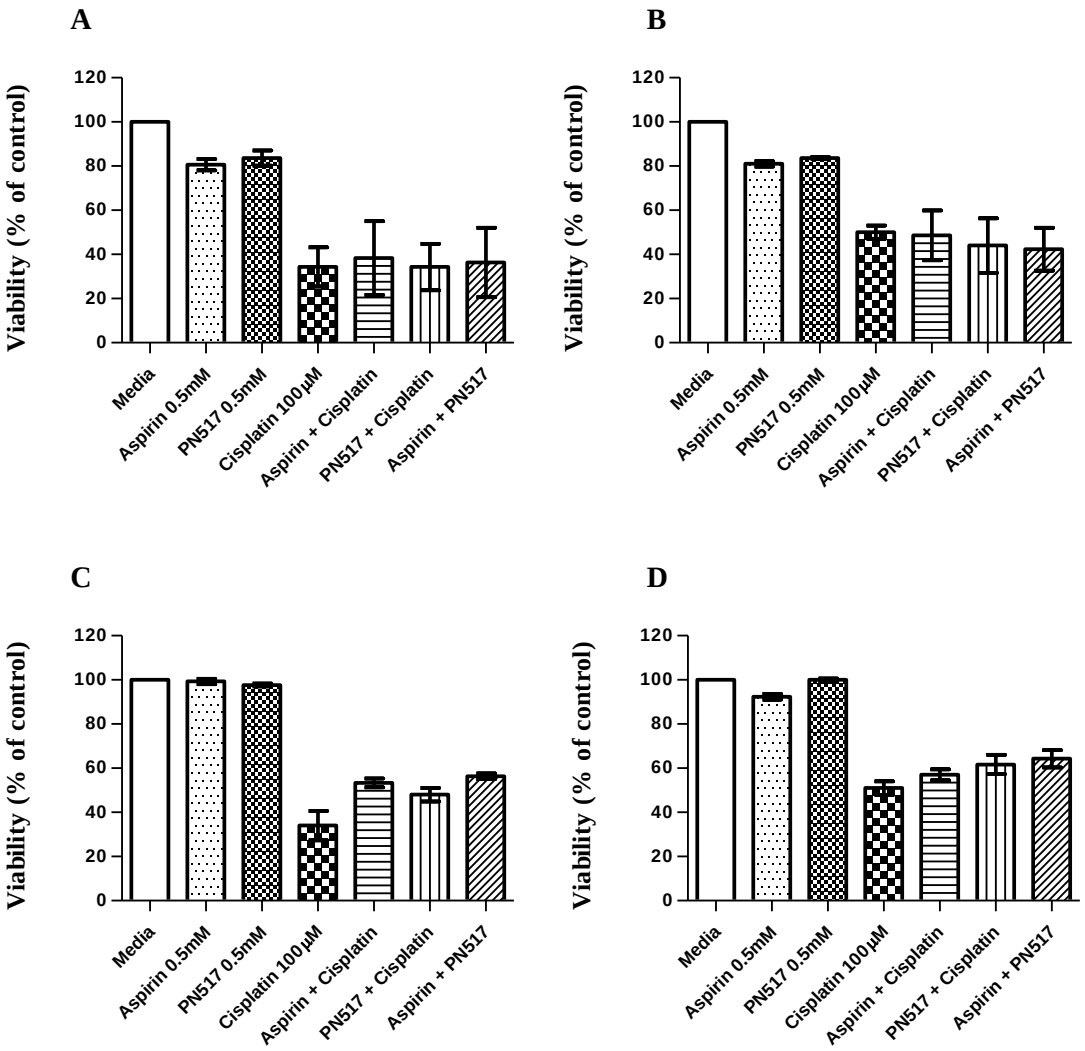

Figure 3.8: Cell viability after prolonged treatment with aspirin/ analogue followed by cisplatin.

Cell viability was determined using PrestoBlue viability assay. Panel A and C illustrate data for U87-MG cell line at 48 hours and 72 hours while panel B and D indicate data for SVG-P12 cell line at 48 and 72 hours respectively. Data is a mean ± SEM of three experiments

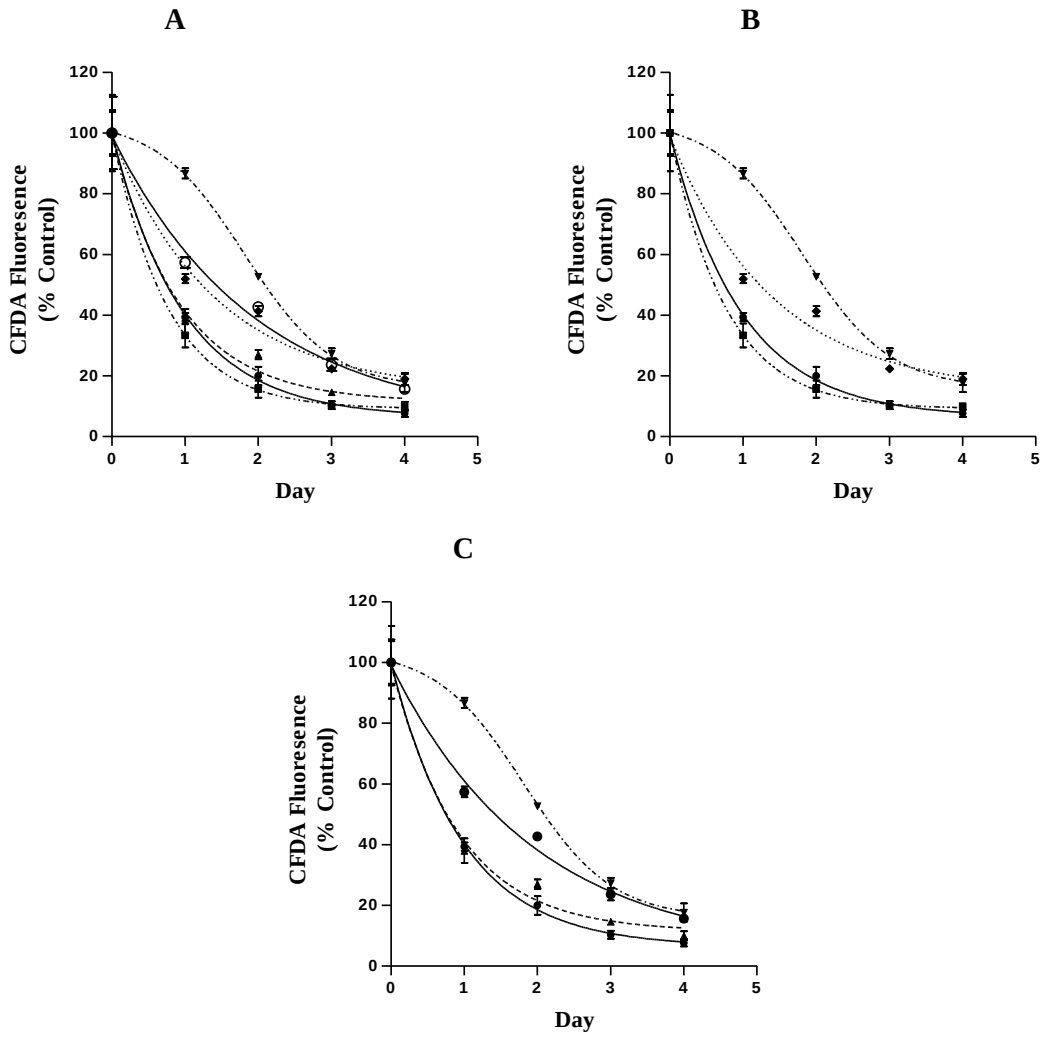

Figure 3.9: Cell proliferation following drug treatment in U87-MG cell line.

The data illustrates the cell proliferation observed in U87-MG cell line (150,000cells/well) using CFDA-SE assay following drug treatment (0.1mM) over a period of 5 days. Panel A displays the effect of aspirin (closed square), PN517 (closed triangle), cisplatin (closed inverted triangle) and the combinations of aspirin-cisplatin (closed diamond) and PN517-cisplatin (open circle) along with the control (closed circle) on cell proliferation where a shift towards the right depicts reduction in proliferation. Panel B shows the effect of aspirin, cisplatin and their combination, panel C shows the effect of PN517, cisplatin and their combination treatment on proliferation.

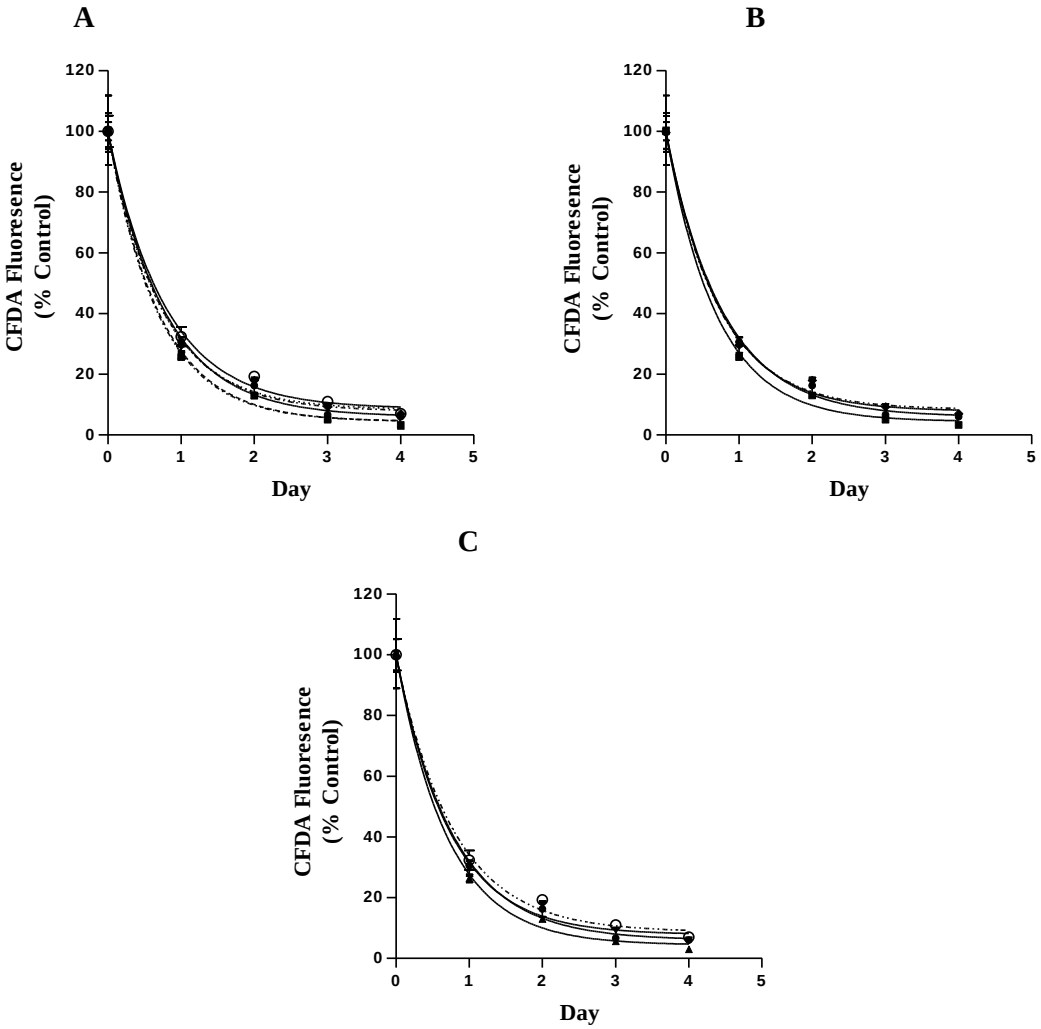

Figure 3.10: Cell proliferation following drug treatment in SVG-P12 cell line.

The data illustrates the cell proliferation observed in SVG-P12 cell line (150,000 cells/well) using CFDA-SE assay following drug treatment (0.1mM) over a period of 5 days. Panel A displays the effect of aspirin (closed square), PN517 (closed triangle), cisplatin (closed inverted triangle) and the combinations of aspirin-cisplatin (closed diamond) and PN517-cisplatin (open circle) along with the control (closed circle) on cell proliferation where a shift towards the right depicts reduction in proliferation. Panel B shows the effect of aspirin, cisplatin and their combination, panel C shows the effect of PN517, cisplatin and their combination treatment on proliferation.

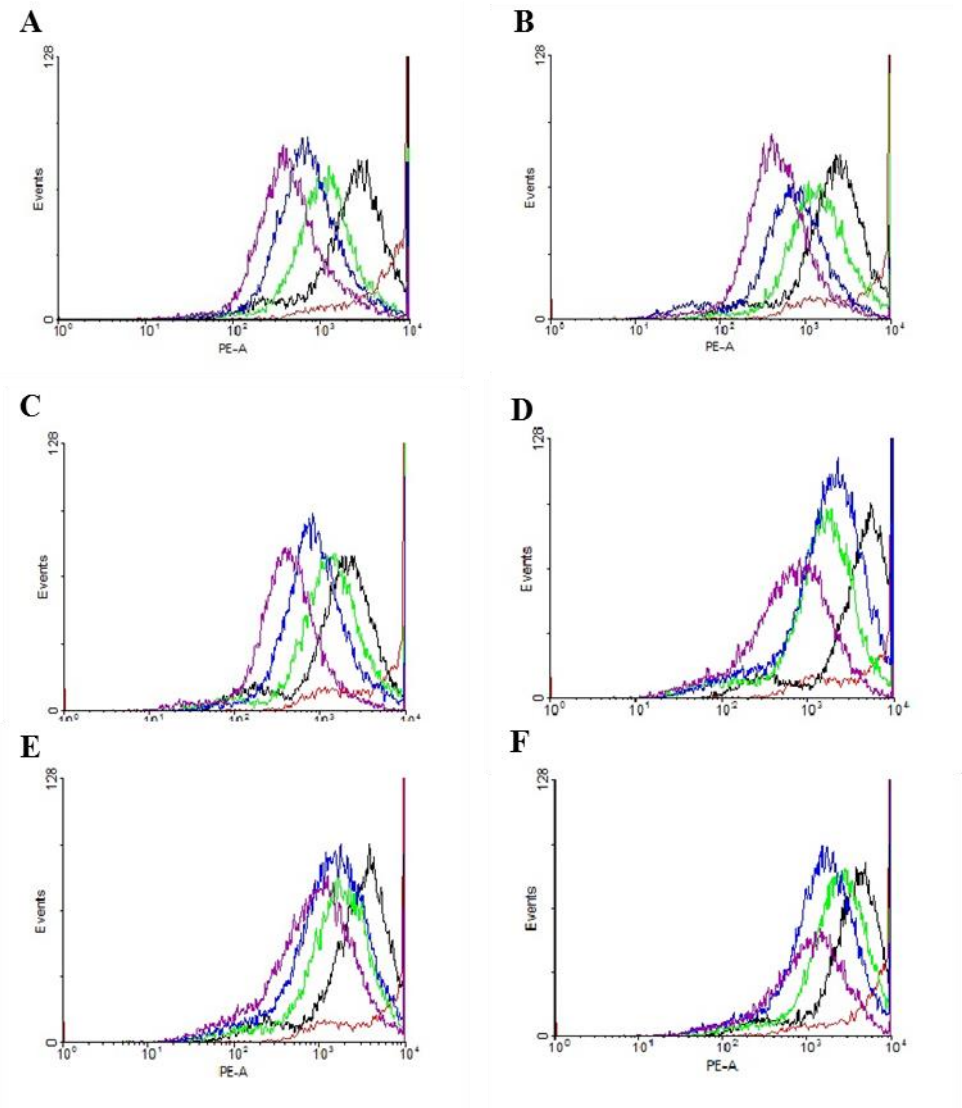

Figure 3.11: Cell proliferation following drug treatment in U87-MG cell line.

The data illustrates the effect ensuing drug treatment over a period of five days, a leftward shift indicating cell proliferation. Panel A depicts control treatment, where a shift towards the left can be observed. Panel B and C are for Aspirin and PN517 treatments respectively, panel D shows effect following cisplatin treatment, where over lapping peaks suggest inhibition of proliferation. Panel E indicates aspirin-cisplatin combined treatment effects while panel F indicates effects following PN517-cisplatin combined treatment.

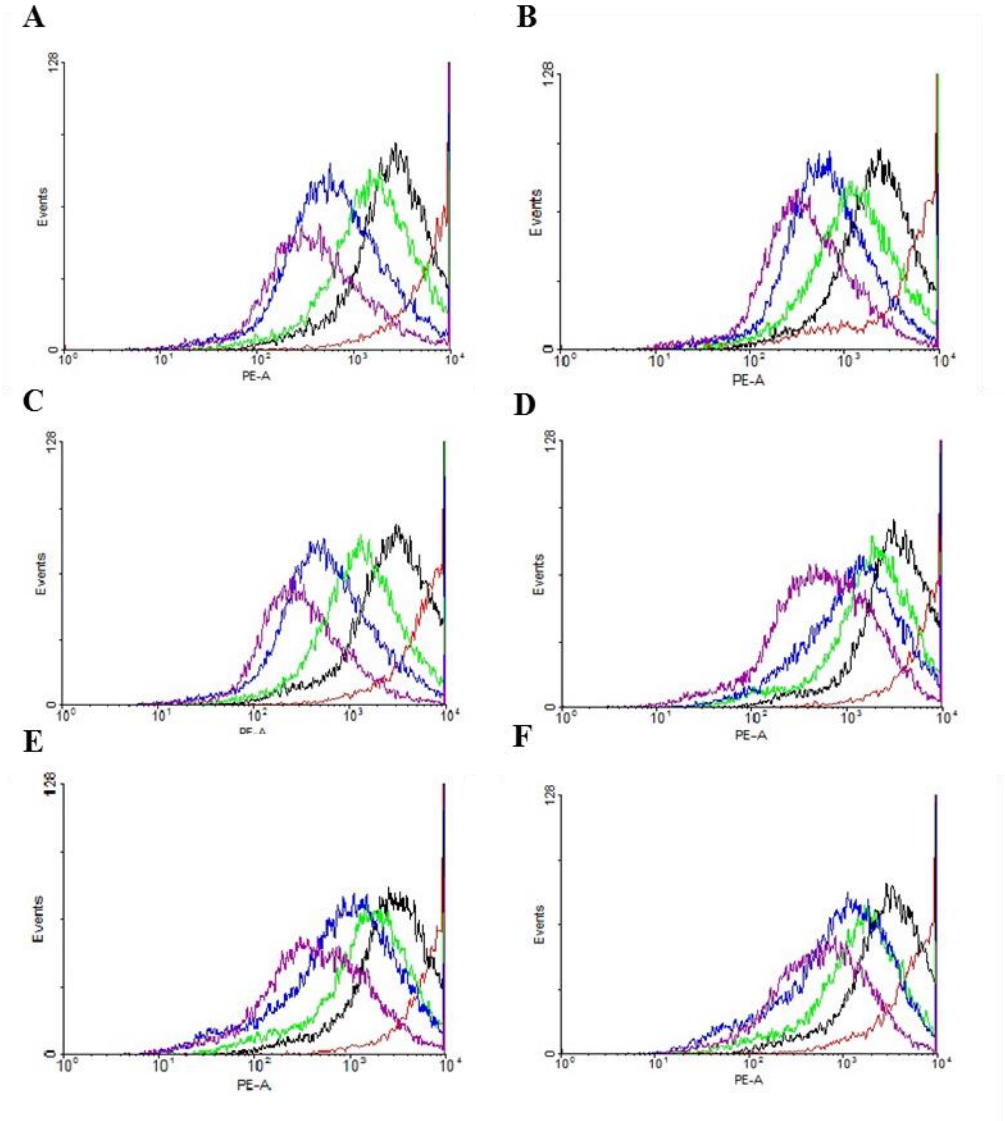

Figure 3.12: Cell proliferation following drug treatment in SVG-P12 cell line

The data illustrates the effect ensuing drug treatment over a period of five days, a leftward shift indicating cell proliferation. Panel A depicts control treatment, where a shift towards the left can be observed. Panel B and C are for Aspirin and PN517 treatments respectively, panel D shows effect following cisplatin treatment, where over lapping peaks suggest inhibition of proliferation. Panel E indicates aspirin-cisplatin combined treatment effects while panel F indicates effects following PN517-cisplatin combined treatment.

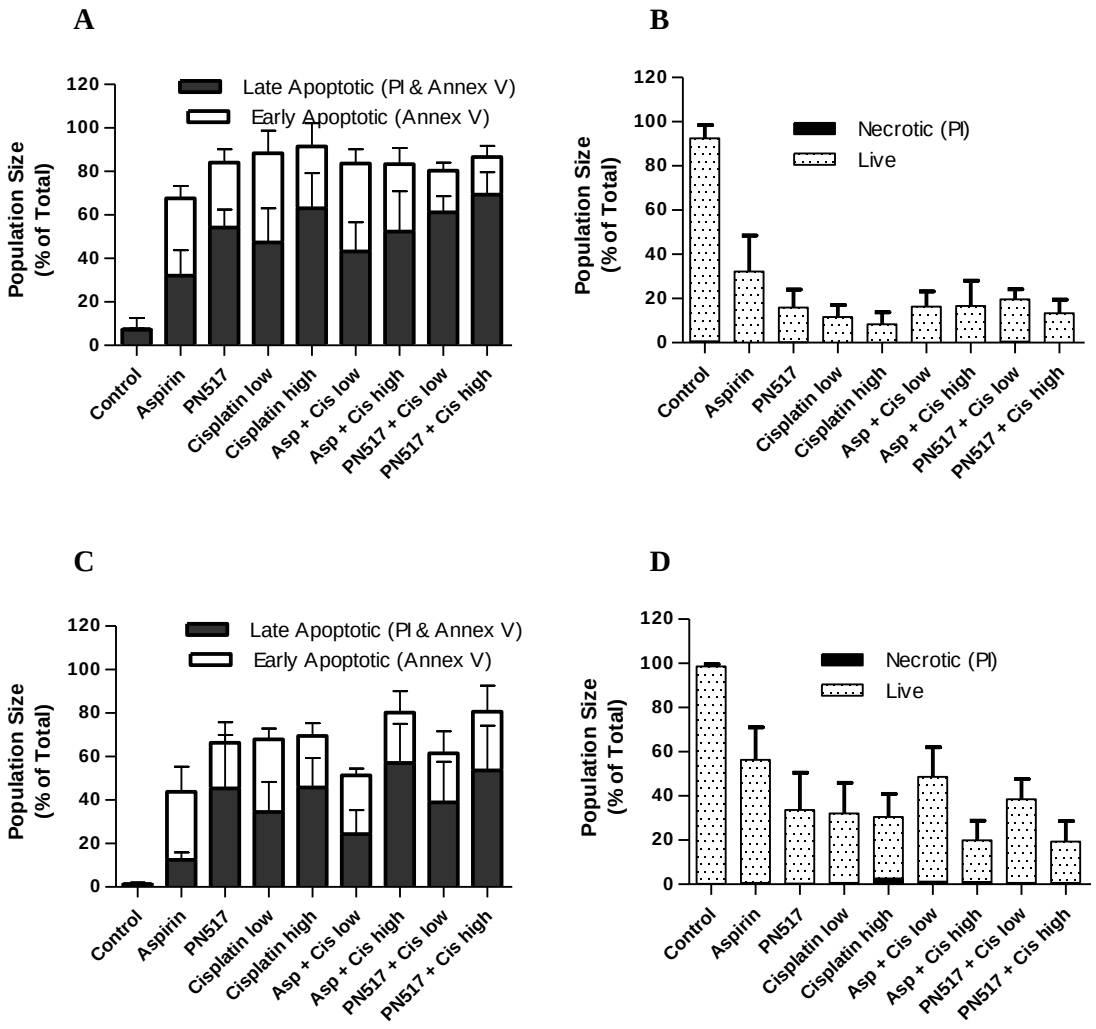

Figure 3.13: Apoptosis following drug treatment in U87-MG cell line.

Data illustrates the apoptosis observed in U87-MG cell line with the help of Annexin-V and Propidium iodide (PI) staining. Panel A and B show the relative proportions of cell in healthy, early and late apoptotic or necrotic state following 24 hours of drug treatment. Panel C and D illustrate the same data as seen at 48 hours post-drug treatment.

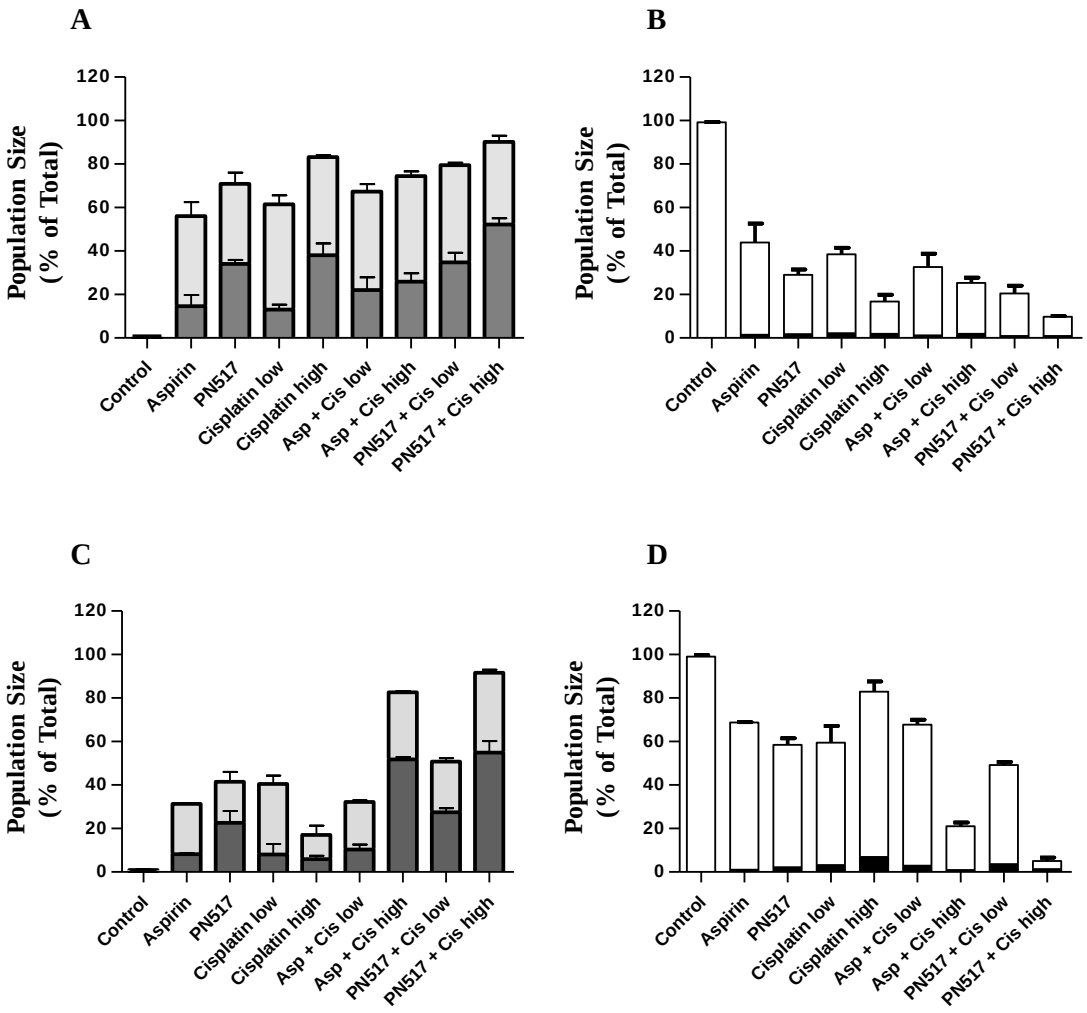

Figure 3.14: Apoptosis following drug treatment in SVG-P12 cell line.

Data illustrates the apoptosis observed in SVG-P12 cell line with the help of Annexin-V and Propidium iodide (PI) staining. Panel A and B show the relative proportions of cell in healthy (colourless), early (light grey) and late apoptotic (dark grey) or necrotic (black) state following 24 hours of drug treatment. Panel C and D illustrate the same data as seen at 48 hours post-drug treatment.

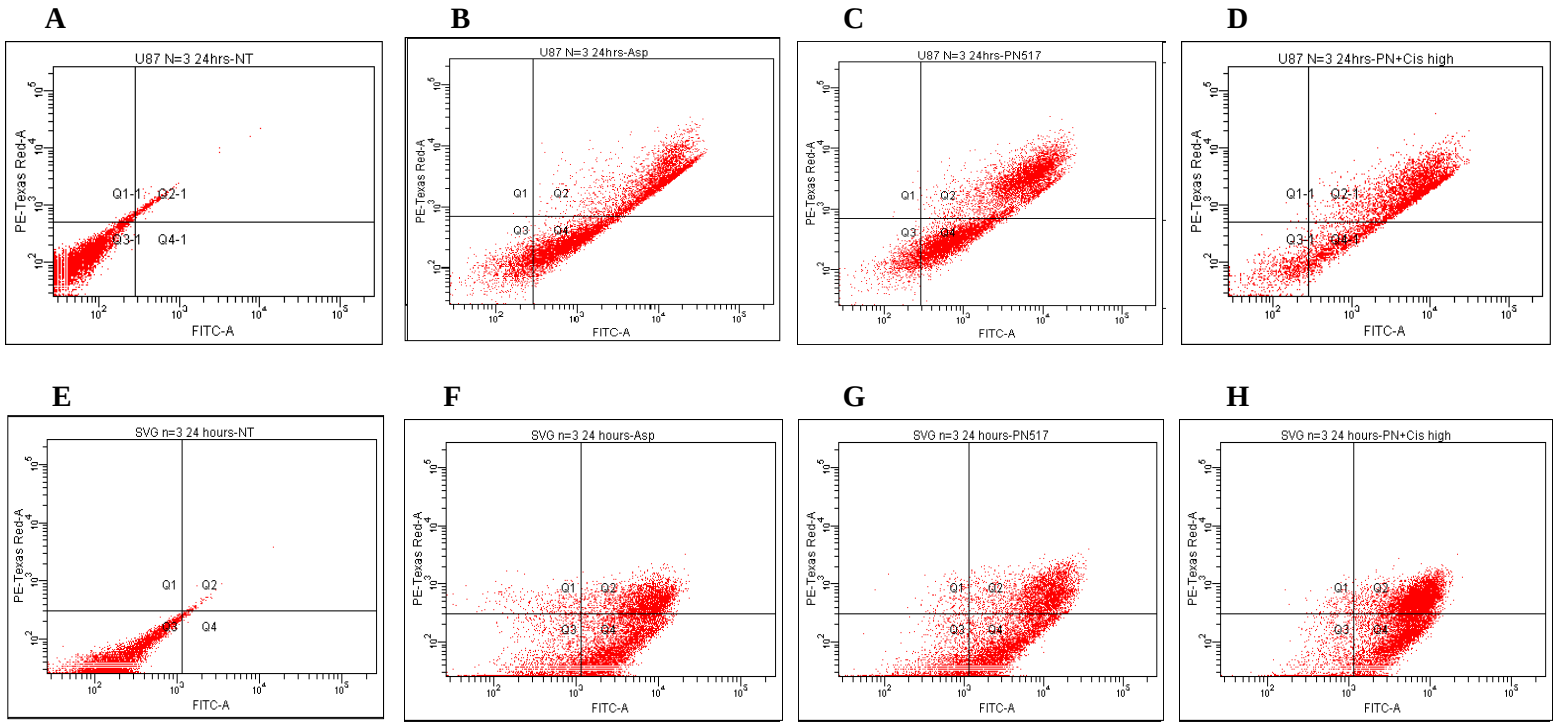

Figure 3.15: Dot-plots for Apoptosis assay following 24 hours of drug treatment in both the cell lines.

Images obtained from the flow-cytometer following analysis of the apoptosis assay at 24 hours. Panels A,B,C,D are treatments for U87-MG cell while panels E,F,G,H are for SVG-P12 cells The treatments are as follows: A & E) Control, B & F) Aspirin, C & G) PN517, D & H) PN517+Cisplatin-high. Quadrant 1 indicates necrotic cells; quadrant 2 indicates late apoptosis; quadrant 3 depicts live cells whereas quadrant 4 indicates early apoptosis.

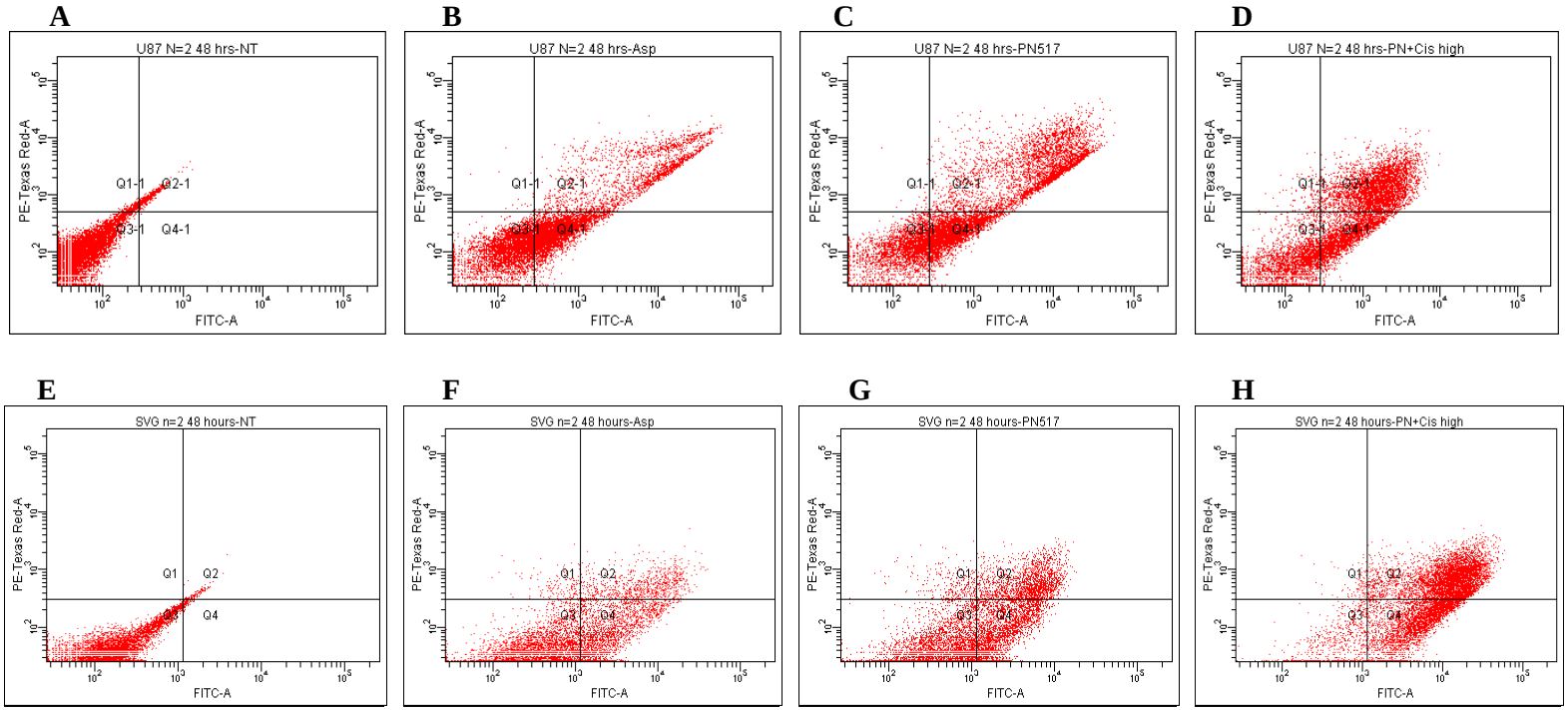

Figure 3.16: Dot-plots for Apoptosis assay following 48 hours of drug treatment in both the cell lines.

Images obtained from the flow-cytometer following analysis of the apoptosis assay at 48 hours. Panels A,B,C,D are treatments for U87-MG cell while panels E,F,G,H are for SVG-P12 cells The treatments are as follows: A & E) Control, B & F) Aspirin, C & G) PN517, D & H) PN517+Cisplatin-high. Quadrant 1 indicates necrotic cells; quadrant 2 indicates late apoptosis; quadrant 3 depicts live cells whereas quadrant 4 indicates early apoptosis.

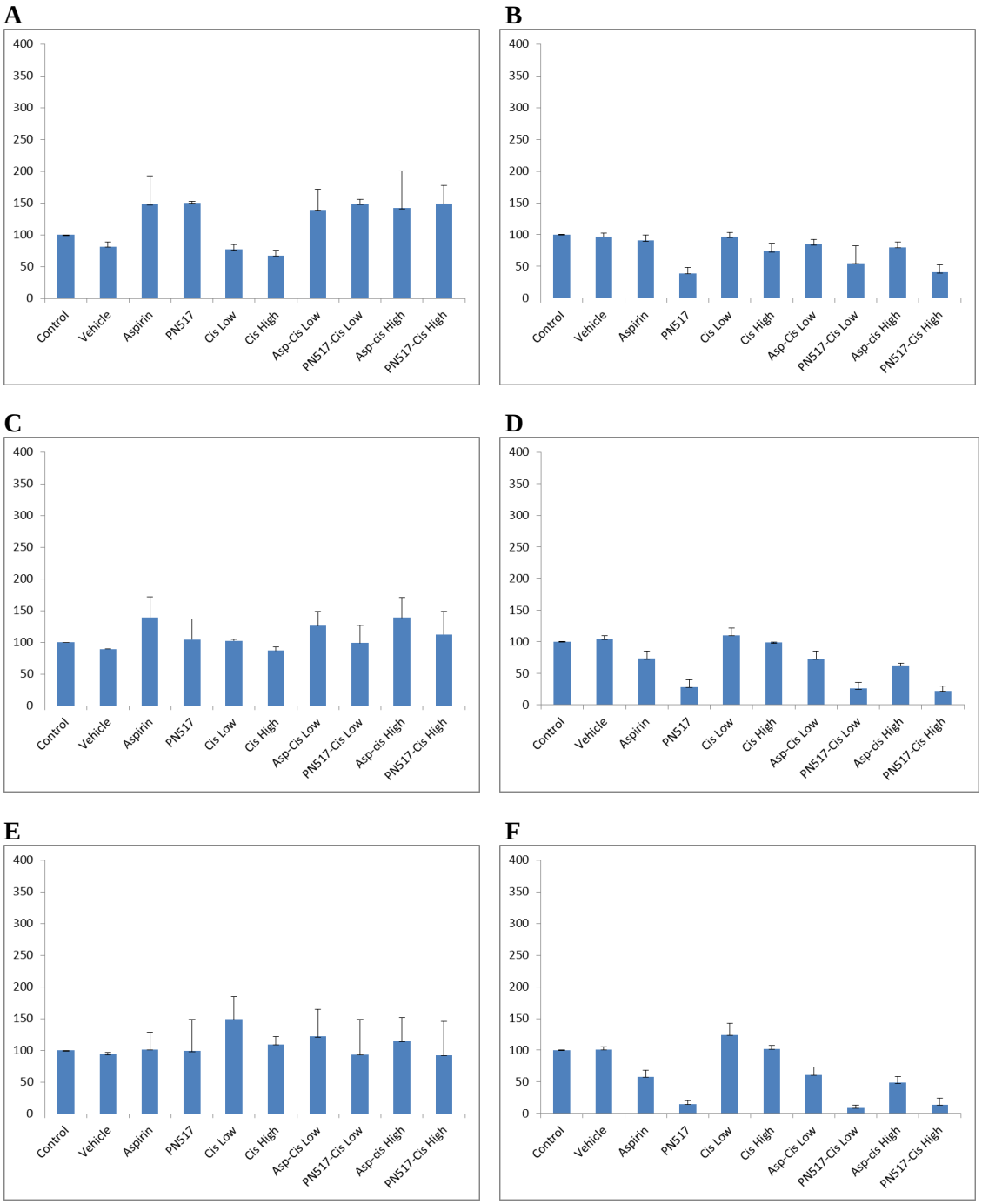

Figure 3.17: Caspase-8 activity seen U87-MG and SVG-P12 cell lines.

Data illustrates caspase-8 activity in both the cell lines following drug treatment. Panels A, C, and E show effects in U87-MG cell line following 12, 24 and 48 hours of incubation following drug treatment. Panels B, D and F show caspase-8 activity in SVG-P12 cells following same time points respectively. Data are mean ± SEM of four experiments.

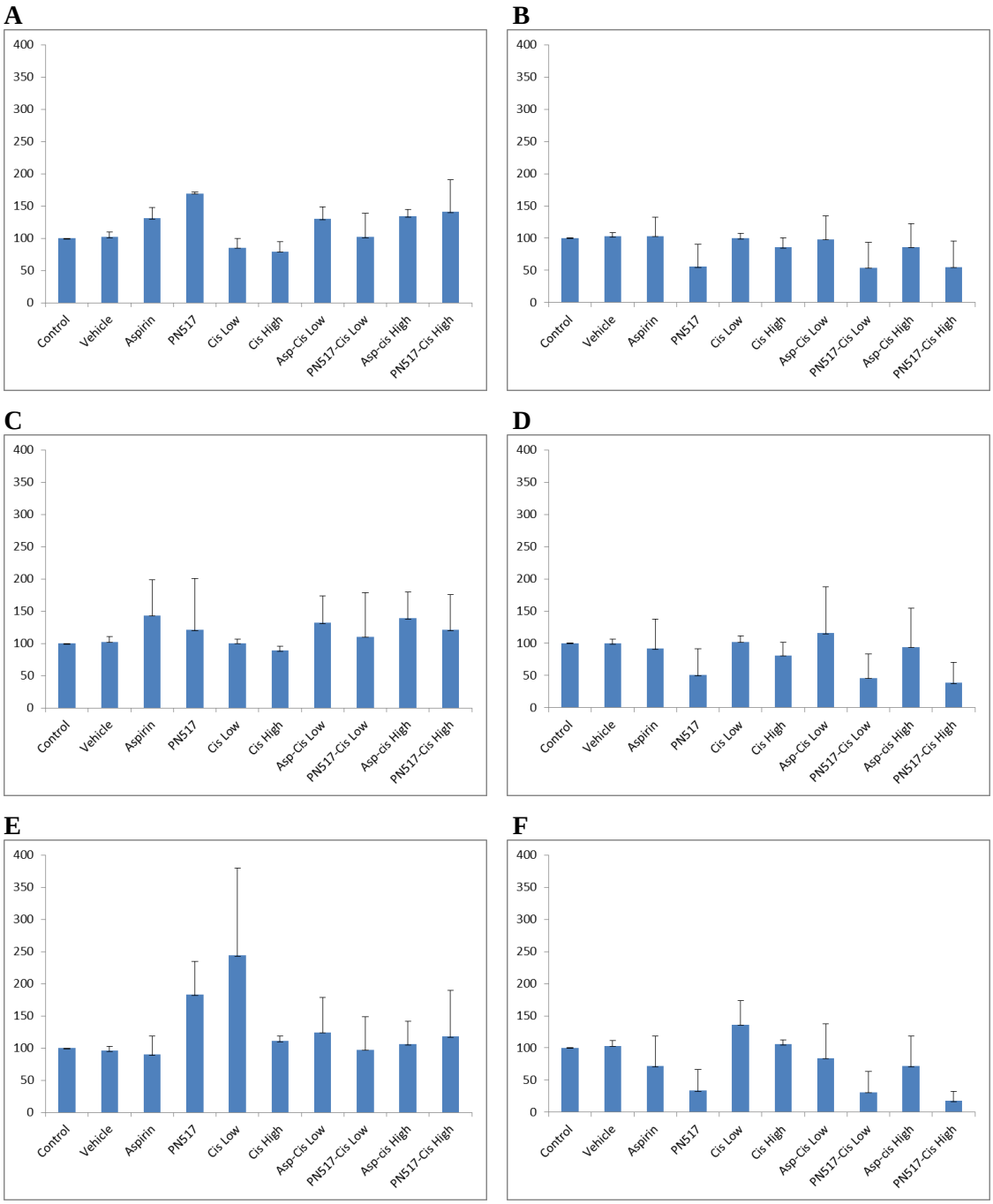

Figure 3.18: Caspase-9 activity seen U87-MG and SVG-P12 cell lines.

Data illustrates caspase-9 activity in both cell lines following drug treatment. Panels A, C, and E show effects in U87-MG cell line following 12, 24 and 48 hours of incubation following drug treatment. Panels B, D and F show caspase-9 activity in SVG-P12 cells following same timepoints respectively. Data are mean ± SEM of four experiments.

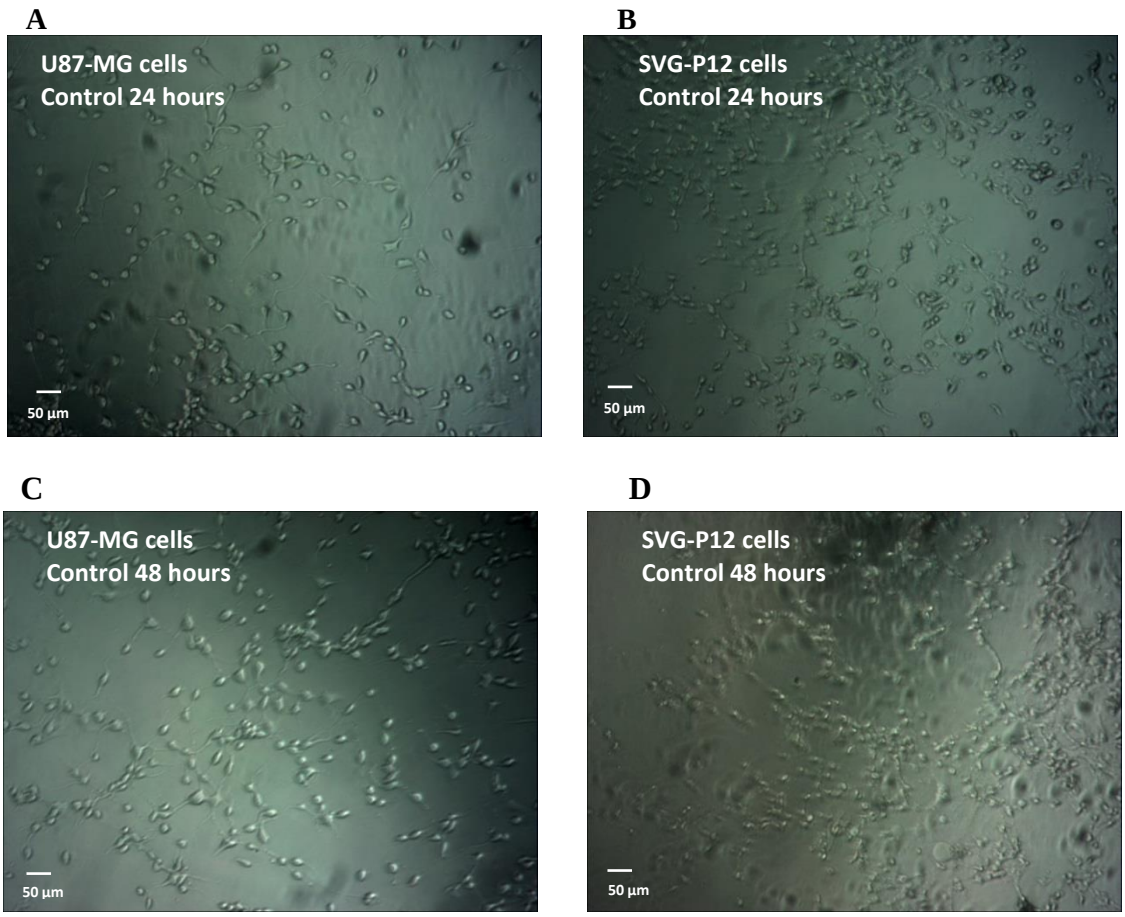

Figure 3.19: Cell images of control treatments in U87-MG and SVG-P12.

Images show the effects of control (media) treatments on the cells prior to caspase analysis detected via Brightfield microscopy. Panels A and C show the effects at 24 hours and 48 hours in U87-MG cells respectively. Panels B and D illustrate the effects seen at 24 and 48 hours for SVG-P12 cells.

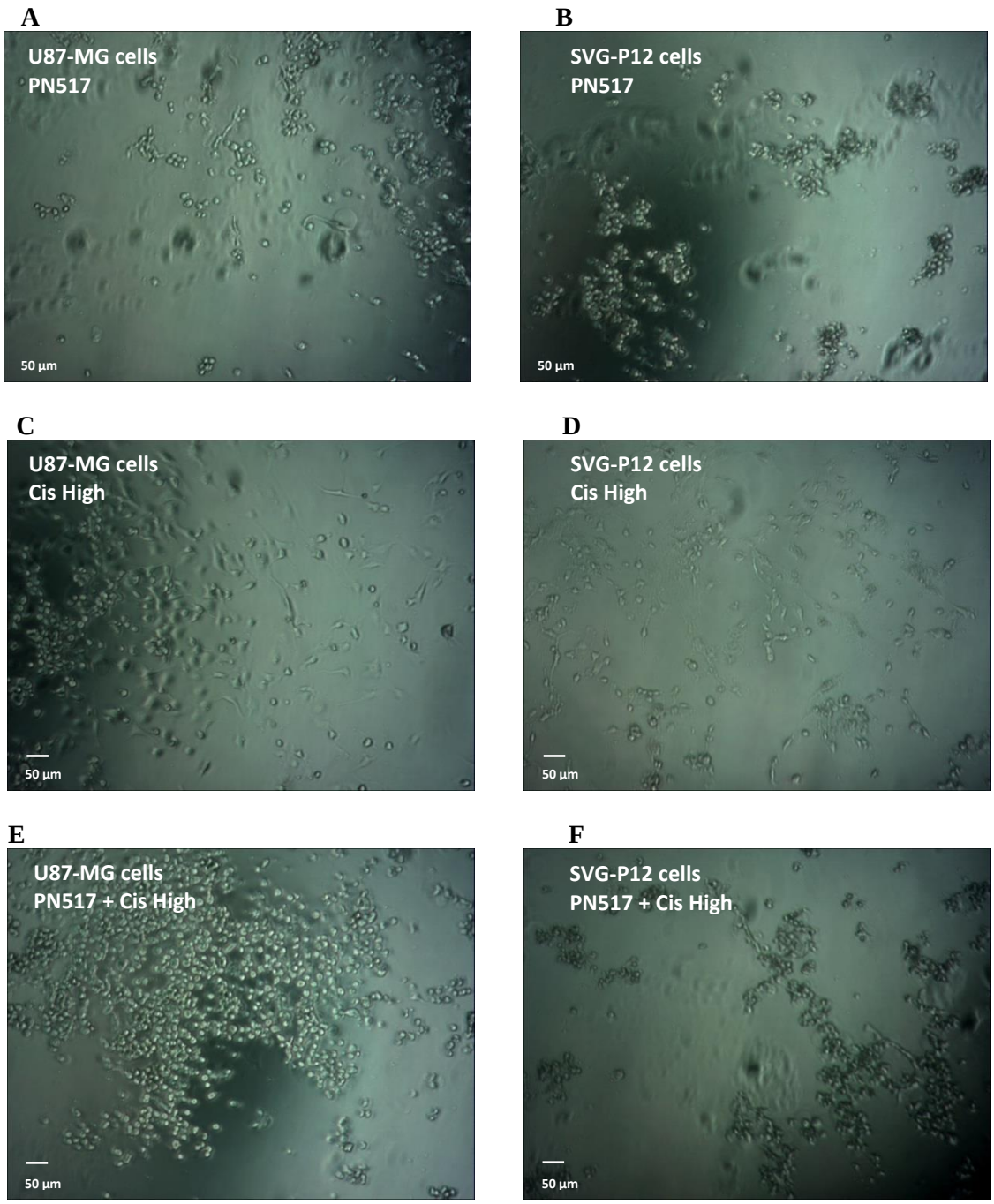

Figure 3.20: Brightfield microscopy images following drug treatment in U87 and SVG cells at 24 hours.

Images show the effect of drug treatment prior analysis of caspase activity at 24hours. Images A, C and E illustrate effect of PN517, cisplatin (high-dose) and PN517-cisplatin (high-dose) combination effects in U87-MC cells respectively. Images B, D and F show the effect of same treatments in SVG-P12 cells respectively.

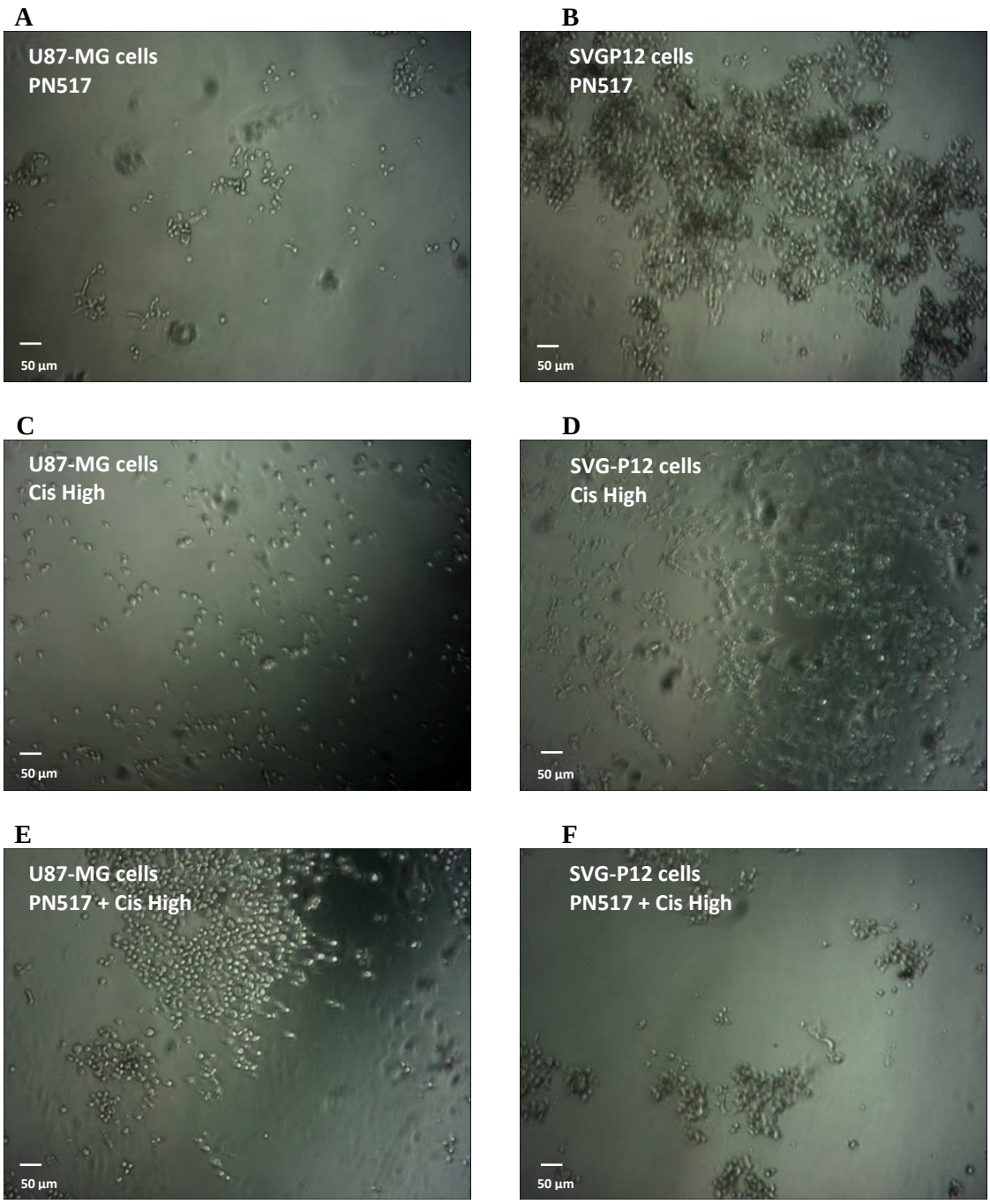

Figure 3.21: Brightfield microscopy images following drug treatment in U87 and SVG cells at 48 hours.

Images show the effect of drug treatment prior analysis of caspase activity at 48 hours. Images A, C and E illustrate effect of PN517, cisplatin (high-dose) and PN517-cisplatin (high-dose) combination effects in U87-MC cells respectively. Images B, D and F show the effect of same treatments in SVG-P12 cells respectively.

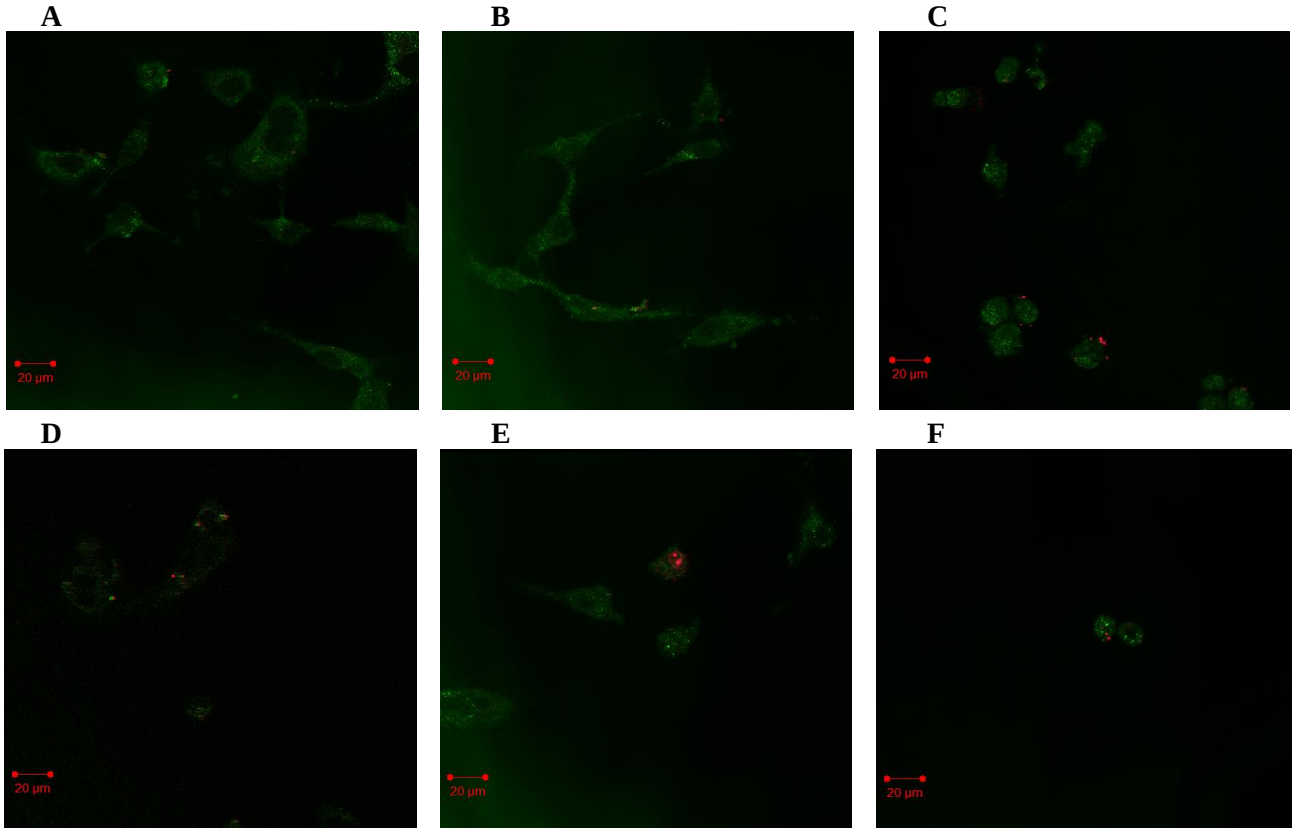

Figure 3.22: Confocal images showing induction of apoptosis following drug treatment in both the cell lines.

Panel A, B and C show the effects of drug treatment on apoptosis at 24 hours in U87-MG cells whereas D, E and F in SVG-P12 cells. The treatments are as follows: A & D) control; B & E) aspirin; C & F) PN517

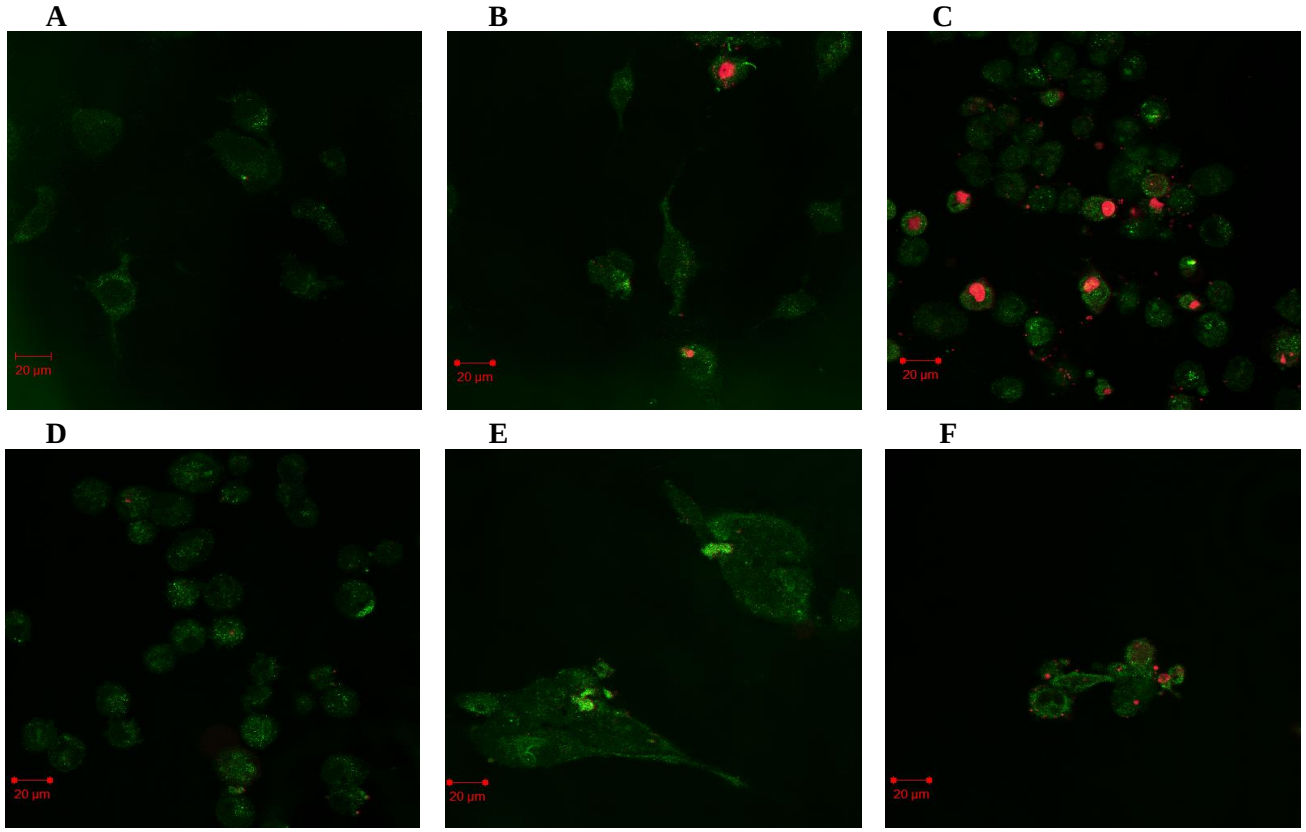

Figure 3.23: Confocal images showing induction of apoptosis following drug treatment in both the cell lines.

Panel A, B and C show the effects of drug treatment on apoptosis at 24 hours in U87-MG cells whereas D, E and F in SVG-P12 cells. The treatments are as follows: A & D) cisplatin; B & E) aspirin+cisplatin; C & F) PN517+cisplatin

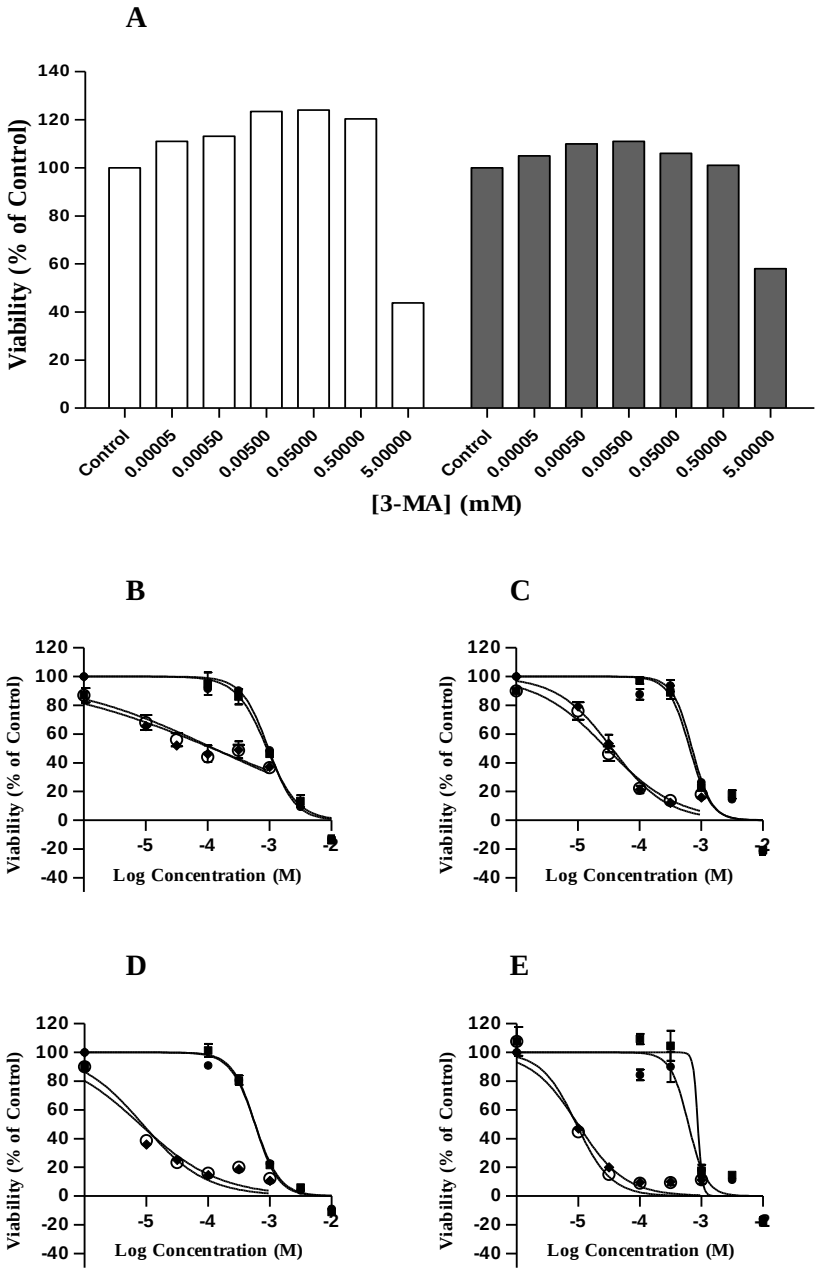

Figure 3.24: 3-MA dose response curve for U87-MG and SVG-P12 cell lines at 24 and 48 hours.

Data illustrates the effects of autophagy inhibitor 3-MA on the cell viability. Panel A shows the effect of 3-MA by itself on cell viability. Panels B and D show the effect of the dose–dependent drug treatments with or without the inhibitor in U87-MG cells following drug treatment with PN517 (closed square) and cisplatin (open circle) after 24 and 48 hours of incubation respectively. Panel C and E show the effects in SVG-P12

cells following 24 and 48 hours of incubation respectively. Individual drug treatments are shown as PN517 (closed circle) and cisplatin (closed diamond).

4 Discussion

The main aim of this study was to examine the effect of combined treatment involving standard chemotherapeutics and aspirin or its analogue PN517 on a glioblastoma cell line *in vitro*. It was hypothesised that the combined treatment would reduce cell viability and proliferation of U87-MG glioblastoma cell line to a greater extent than individual treatments. While variation was observed between assays, the cell viability results provide the first evidence that PN517 might have therapeutic potential for the treatment of glioma. The remaining aims of the project focused on identifying the mechanisms involved in cell death following drug treatment of the U87-MG cell line.

4.1 Growth Curve and PrestoBlue® Linearity

As would be expected of a cancer cell line, the U87-MG cells demonstrated a higher rate of proliferation as compared to non-cancerous foetal SVG-P12 control cell line (Fig. 3.1) (Hanahan & Weinberg, 2011). The U87-MG cells doubled in number over forty eight hours after initial seeding and the trend continued till the eighth day demonstrating a log phase of expansion, after which a decline in proliferation rate was observed indicating a lag phase. The PrestoBlue® assay uses the reducing nature of the cell environment as an indication of viability, but when measuring the response of a population of cells, the largest variable impacting response is the cell number (www.invitrogen.co.uk). The linearity assay established that over the period of incubation and with a two day doubling time, fluorescence was proportional to cell density (Fig. 3.2). Taken in combination, these results established that drug treatment after a twenty four hour incubation period following seeding of one thousand cells per well was appropriate for subsequent viability assays.

4.2 Cell Viability

Aspirin demonstrated its cytotoxic effects by reducing the cell viability of both the U87-MG and SVG-P12 cell lines in a concentration dependent manner (Fig. 3.3), displaying similar efficacy to that found in previous studies (Hwang *et al*., 2004; Kim *et al*., 2009b; Deb *et al*., 2011). The IC_{50} value however, did not significantly change with either twenty-four or forty eight hours of treatment (Tables 3.1 & 3.2). A possible explanation for this result would be the metabolism of aspirin *in vitro* by cytochrome P450 enzymes.

In vivo, low dose aspirin has a half-life of approximately four hours because following rapid de-acetylation to salicylic acid, aspirin is further metabolized by glucuronidation, hydroxylation, and glycine conjugation (Hutt *et al*., 1986; Takanashi *et al*., 2000). However, higher doses can extend the half-life to approximately thirty hours because the pathways concerned forming salicyluric acid and salicyl phenolic glucuronide products become saturated (Schror, 2009). The cytochrome P450 enzymes, essential for detoxification of foreign chemicals, are a large family of fifty enzymes, six of which are responsible for the metabolism of ninety percent of drugs (Guengerich, 2008). The enzymes are highly expressed in liver, but are also found in the small intestine, lungs, and at lower levels in most tissues throughout the body. The major cytochrome P450 involved in the metabolism of aspirin is CYP2C9 (Miners & Birkett, 1998; Takanashi *et al*., 2000), which is polymorphic, and could also affect the metabolism of aspirin analogues. Importantly for this study, CYP2C9 is expressed in glioma, and its expression increases with grade, thus high expression is found in glioblastoma (Knüpfer et al., 2006). This provides a potential expression as to why the

IC_{50} remains constant for aspirin and its analogues, and may suggest a limiting factor for the use of aspirin or its analogues in the future treatment of GBM.

PN517 is effectively two aspirin molecules joined by a double bond, a site which may represent a target for enzyme mediated hydrolysis. While it displayed greater efficacy than aspirin in both the U87-MG and SVG-P12 cell lines (Fig. 3.4), the metabolic mechanism already described could negatively impact its IC_{50} value, resulting in an underestimate of its potency.

There are a number of mechanisms that could be involved in the effects of aspirin or its analogues on cell viability, including the Wnt/β-catenin/TCF pathway. The signalling of the said pathway has been previously shown to be highly dysregulated in several cancers, with glioma being an example (Logan and Nusse, 2004; Cadigan and Peifer, 2009; Pu *et al.*, 2009). The pathway is activated by the β-catenin/TCF complex and serves as the main signalling node and activates several effectors like Stat3, AKT1 and AKT2 (Chen *et al.*, 2011; Huang *et al*., 2011; Yue *et al*., 2010). A study has shown that aspirin antagonizes the activity of the β-catenin/TCF complex in U87-MG and A172 cell lines, thereby exerting its anti-neoplastic activity (Lan *et al*., 2011).

Additionally, COX-2 has shown to be overexpressed in several glioblastoma cell lines, very notably in U87-MG which leads to excessive production of prostaglandins (Kardosh *et al*., 2011). COX-2 prostaglandins promote cell division, angiogenesis and metastasis while bringing about inhibition of apoptosis, which in turn leads to growth of the tumour (Grösch *et al*., 2006). This could be a possible explanation of the stronger inhibitory effect of aspirin and the PN517 analogue on the U87-MG cell line viability as compared to the SVG-P12 cell line (Tables 3.1 & 3.2).

The efficacy of aspirin and PN517 in vitro was compared to both cisplatin and temozolomide (Figs. 3.3 & 3.4), the leading drug in the treatment of glioblastoma multiforme (Zhang *et al*., 2012; Ding *et al*., 2012). TMZ is a prodrug which is converted into the active alkylating agent MTIC under physiological conditions (Friedman *et al*., 2000). However, temozolomide does not display similar effects *in vitro* due to its prodrug nature, instead displaying schedule-dependent activity. This could explain its weak effect against cell viability of both cell lines and failure in achieving an accurate IC_{50} value.

Cisplatin is a potent anticancer chemotherapeutic due to targeting of multiple intracellular pathways to induce death in tumour cells (Rosenberg *et al*., 1975). *In vitro*, it displayed a time dependent efficacy, with a lower IC_{50} value following forty eight hours of treatment, an effect easily explained by its mechanism of action where it causes DNA damage by affecting its replication, ultimately inducing apoptosis (Florea & Büsselberg, 2011). Although cisplatin is cell cycle non-specific, during S-phase, toxicity is increased resulting in G2-phase arrest. Hence, as the cells replicate over time, a higher cytotoxic effect is seen, a mechanism that normally provides some selectivity over healthy cells when tumours are rapidly growing in comparison.

Aspirin and PN517 in combination with cisplatin (high) significantly reduced the viability of the glioblastoma cells following twelve, twenty four and forty eight hours of incubation as compared to cisplatin on its own (Figs. 3.5 & 3.6). One possible explanation for this combined effect could be due to aspirin re-sensitizing the cisplatin-induced resistance in tumour cells *via* inhibition of β-catenin transactivation. It has been

noted that Akt phosphorylates Mdm2 subsequently down regulating p53 which causes drug resistance of cisplatin (Hung and Lau, 1999; Siddik, 2003). β-catenin/TCF4 transactivational complex serves as a central activating node for effectors such as Akt and Stat3 (Shi *et al*., 2013). As mentioned above, aspirin has shown to inhibit the effect of the β-catenin/TCF4 complex in U87-MG cells and could thereby desensitize the cells to the cisplatin resistance induced by high dose cisplatin. It was also noted that following twenty four and forty eight hours of incubation of the glioblastoma cells with PN517 and cisplatin (low) caused a significant reduction in cell viability when compared to cisplatin (low) alone(Fig 3.5, panels E and F, Fig 3.7). This indicates that PN517 is augmenting the effect of cisplatin and a lower dose of cisplatin when combined with aspirin analogue can cause higher level of cytotoxic effect.

These combination effects are significant if they can be reproduced *in vivo*, as lowering the dose of a highly toxic chemotherapeutic drug such as cisplatin, should significantly improve its side effect profile, while still producing similar treatment efficacy due to the addition of the second chemotherapeutic agent. Importantly, the side effect profile of aspirin is typically very good, and as previously mentioned the positive effects of aspirin on cancer outweigh the more serious side effects such as increased bleeding (Rothwell *et al*., 2012a). Given the high structural similarity to aspirin, it could be predicted that PN517 might have a similar side effect profile, while producing increased therapeutic efficacy.

The study also found that prolonged treatment with aspirin followed by cisplatin significantly decreased cell viability in the U87-MG glioblastoma cell line when compared to cisplatin alone (Fig. 3.8). This could be due to aspirin mediated inhibition

of molecules central to regulation of cell cycle, cell survival and apoptosis. Kumar and Singh (2012) have shown that pre-treatment with aspirin in a T-cell lymphoma murine model, decreases the expression of several regulatory molecules such as Bcl-2, cyclin D and cyclin B1, changes which would leave the cells susceptible to damage by an added dose of cisplatin. The study also found the p53 levels to be decreased in cells pre-treated with aspirin and subsequently treated with cisplatin, providing a simple explanation for the results obtained in the multiple-treatment assay. Unfortunately, no significant results were obtained in the staggered treatment of cisplatin followed by aspirin and PN517 (Fig. 3.7). This could possibly be due to the cells recovering following the individual treatment, and again be linked to drug metabolism, emphasizing that a concomitant treatment is essential for combined efficacy.

4.3 Cell Proliferation

No significant effect was produced by either aspirin or PN517 alone on the cell proliferation of both the cell lines (Fig. 3.9-3.12), a result that might be due to the low concentration (100 μM) of each drug in the assay. While it was previously reported that treatment using relatively low concentrations of aspirin, less than 500 μM, doesn't affect the cell growth of glioblastoma cells in a significant way (Amin *et al*., 2003), this concentration was chosen as one which was not going to significantly affect glioblastoma cell viability and prevent any independent measurement of proliferation. Low concentration cisplatin (10 μM) however, had a significant inhibitory effect on the cell proliferation over twenty four and forty eight hours, an effect that appeared to be overcome with further incubation at seventy two and ninety six hours. This effect was quite expected as already explained, the mechanism of action of cisplatin involves the

impedance of cell cycle and DNA replication (Siddik, 2003). The combination of either aspirin or PN517 with cisplatin did not significantly affect proliferation when compared to cisplatin treatment alone, a result that was not unexpected given the lack of efficacy of the aspirin and analogue treatments on their own. However, although there wasn't any combined effect, no antagonism was observed which implicates that low dose of aspirin with cisplatin could be of beneficial use.

4.4 Apoptosis and Autophagy

The high percentage of early as well as late apoptotic cells following drug treatment show that PN517 and aspirin reduce cell viability in glioblastoma cells *via* induction of apoptosis (Fig. 3.13-3.16). Cisplatin (high) combined with either aspirin or PN517 had a significant effect on apoptosis when compared to cisplatin alone, an effect maintained over twenty four and forty eight hours. Interestingly though, in all the other treatments, the apoptotic cell population was reduced at forty eight hours, suggesting that the cells were recovering. This could suggest that while a monotherapy would require daily dosing to maintain efficacy, a combination treatment might have a greater treatment interval. Combined with the lower doses of each individual drug in combination, this decreased treatment frequency might also improve the side effect profile of the chemotherapy. A further important result, was that apoptosis in the SVG-P12 control cell line was lower than that in the GBM cell line, indicating that the combined therapy produced less damage to normal cells in terms of apoptosis, which could be of clinical importance.

Results from the confocal microscopy provided morphological and immunofluorescent evidence of apoptosis induction following drug treatment. The

rounding of cells observed following treatment with PN517 on its own or in combination with cisplatin proved that it had caused permanent damage to the cells (Fig. 3.22 & 3.23) (Purow *et al*., 2005). The red staining, as a result of propidium iodide nuclear binding, confirmed late apoptosis with compromised cell membrane, and scattered nuclear fragments. In comparison to individual treatments, the combination of PN517 and cisplatin resulted in almost all observable cells demonstrating binding of annexin-V, thus indicating early apoptosis, and a significant proportion with propidium iodide staining, suggesting late apoptosis. The patterns from SVG-P12 cell treatments showed a similar pattern however, with lower proportion of late apoptotic cells.

Apoptosis is associated with elevated levels of intra-cellular mediators such caspase-8 and caspase-9 (Chandra *et al*., 2002). The two major pathways of apoptosis, extrinsic and intrinsic pathways involve activation of caspase-8 and caspase-9 respectively. Cisplatin treatment induces apoptosis independent of caspase-8, 9 which can be seen from the low and constant activity of both following high and low concentration treatments of the drug (Fig. 3.17-3.21). Aspirin and PN517 showed similar effects on caspase activity in the glioblastoma cell line which seemed to decrease over time which suggested their involvement in induction of apoptosis. However, PN517 showed extremely low caspase activity in SVG-P12 cells suggesting a different pathway of apoptosis. In fact, the activity was 4 times lower than the activity seen in control treatments, suggesting inhibition of caspase activation by the drug. The combined drug treatments showed similar caspase 8 and 9 activity as seen in either aspirin alone or PN517 which was as expected due to low activity displayed by cisplatin, which didn't change over time.

Following the caspase activation results, it was decided to examine the potential role of autophagy following drug treatment. The selective autophagy inhibitor, 3-methyladenine, which inhibits activation of phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway, was used at a concentration which did not itself reduce cell viability (Glick *et al*., 2010). While these provisional results might suggest that neither PN517 nor cisplatin involve autophagy in their mechanism of reducing cell viability, no positive control was utilised to confirm either autophagy induction, or its inhibition by 3-MA (Fig. 3.24). Hence, the results have to be treated with caution.

4.5 Conclusion

The results of this study have demonstrated that PN517 or aspirin potentiate the effect of cisplatin in reducing viability and inducing apoptosis in the U87-MG glioblastoma cell line. This study provides the first evidence that the combination of a platinum based chemotherapeutic with aspirin or an aspirin analogue could be of therapeutic potential for the future treatment of glioblastoma multiforme.

4.6 Future Work

Further tests need to be performed to confirm the mechanism of induction of apoptosis and verify the involvement of different proteins. Substrates such as PARP or phosphorylated histones can be detected via immunohistochemistry. Western blots can be used to detect caspase activation.

High pressure liquid chromatography could be used to confirm the metabolism of aspirin. The direct quantitation of aspirin or its metabolites salicylic acid, salicyluric

acid, gentsic acid can also be achieved via LC-MS/MS. The levels of CYP 450 enzymes could be determined in both U87-MG and SVG-P12 cell lines by western blotting in addition to activity assays.

The involvement of β-catenin pathway needs to be examined. This could be achieved by evaluating the activity of the b-catenin/TCF4 transcriptional complex using luciferase reporter constructs based assays.

Although the results indicate the absence of autophagy, the lack of positive control could have led to generation of false-negative results. To overcome this, the experiments should be repeated using a positive control such as rapamycin. Other compounds testing involvement of autophagy can be utilised such as monodansylcadaverine (MDC), which accumulates in the autophagic vacuoles. It is an auto-fluorescent compound which can be visualised using confocal-laser scanning and autophagy is measured as the proportion of stained vacuoles.

Better drug delivery methods could be used for dual-drug delivery which might include carriers such as microspheres or nano-particles. This approach could help to achieve higher concentrations of the drug in a tumour environment with reduced toxicity and side effects. Recent findings suggest that combining both the drugs to develop a prodrug can be highly beneficial. It can help in overcoming the cisplatin resistance and improve the cytotoxicity of the prodrug by altering the cellular uptake pathways (Cheng *et al.*, 2014; Pathak *et al.*, 2014)

Ultimately, the efficacy of combination treatments would need to be confirmed using both primacy glioblastoma cultures and also testing *in vivo*. The best available *in vivo* model would involve the induction of intracranial tumours in nude mice using both established cell lines and primary cells. This would allow for the

peripheral delivery of the combination therapy, establishing both efficacy and blood brain barrier penetration in a single model.

5 References

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