

**Anti-cancer effects and mechanism of actions of
aspirin-like drugs in the treatment of gliomas**

by

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STUDENT DECLARATION FORM

I declare that no material contained in the thesis has been used in any other submission for an academic award and is solely my own work

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Abstract

In the past two decades only modest advancements in glioma treatment have been made, with patient prognosis and median survival time following diagnosis only increasing from 3 to 7 months. A substantial body of clinical and preclinical evidence has suggested a role for aspirin in the treatment of cancer with multiple mechanisms of action. Aspirin is one of the most widely used drugs, successfully taken as an analgesic, antipyretic, anti-inflammatory agent and for prevention of strokes and ischemic diseases. The effects on cell viability, proliferation, apoptosis and migration of aspirin and aspirin derivatives were tested on primary glioblastoma cell cultures, BTNW911 and BTNW 914, and the well-established cell lines, SVG-p12, 1321N1, GOS-3, U87 MG, using the PrestoBlue assay, CFDA-SE, PI/annexin V, and live imaging receptively. The effects on cell viability following 24 and 48 hour incubation of four aspirin derivatives (PN508, PN517, PN526 and PN529) were compared to cisplatin, aspirin and di-aspirin, establishing IC_{50} values, showing PN517 to be the most potent analogue, and in some cases greater efficacy than cisplatin. Aspirin analogues showed greatest efficacy in the first 24 hours, while cisplatin increased in efficacy with time showing a lower IC_{50} value in all cell lines at 48 hours. Cell proliferation was assessed over 3 to 10 days, with each treatment decreasing proliferation and the largest effect of PN517 found in BTNW914 cells. PN517 treatment decreased the population of G0/G1 phase cells in cell cycle analysis, decreased cyclin D1 and EGFR activation, and total EGFR expression. Apoptosis was induced by PN517 in a concentration and time dependent manner in both the cell lines and short term cultures, with activation of both intrinsic and extrinsic pathways. Finally, PN517 reduced migration in both the Boyden chamber and scratch assays, but did not inhibit invasion. In conclusion, these data support the further development of PN517 as a novel therapeutic drug for the treatment of glioma.

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Abbreviations

ACDP	Advisory Committee on Dangerous Pathogens
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor 1
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid <i>assay</i>
Bcl-2	B-cell lymphoma 2
Bcl-X _L	B-cell lymphoma-extra large
BCNU	Carmustine
CCNU	Lomustine
CD34	Cluster of Differentiation 34
CD90	Cluster of Differentiation 90
CDDP	Cisplatin
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CNS	Central Nervous System
COX	Cyclooxygenase
CSCs	Cancer Stem Cells
CT	Computerised tomography
CuSO ₄	Copper(II) sulfate
CYP2C9	Cytochrome P450 2C9
CYP450	Cytochrome P450

DAPI	4',6-diamidino-2-phenylindole
DISC	Death Inducing Signal Complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMEM	Eagles Minimum Essential Medium
EtOH	Ethanol
FACS	Fluorescence Activated Cell Sorting
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
FS	Forward Scatter
G0	Resting phase
G1	Growth phase
GBM	Glioblastoma Multiforme
GFAP	Glial fibrillary acidic protein
HSCs	Hematopoietic stem cells
IC ₅₀	Inhibitory concentration 50%
LSM	Laser Scanning Microscope
M	Mitotic Phase
MGMT	O ⁶ -alkylguanine DNA alkyltransferase
MMR	Mismatch Repair
MRI	Magnetic Resonance Imaging

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NEAA	Non-essential amino acids
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Non-steroidal anti-inflammatory drug
p53	Phosphoprotein p53
PB	Presto Blue
PBS	Phosphate Buffered Saline
PET	Positron emission tomography
PGG ₂	Prostaglandin-endoperoxide synthase 2
PGH ₂	Prostaglandin H2
PGs	Prostaglandin
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PUMA	p53 upregulated modulator of apoptosis
RCS	Rabbit Aorta Substance
RIPA	Radio-Immunoprecipitation Assay
S	Synthesis phase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SS	Side Scatter
STAT3	Signal transducer and activator of transcription 3
TEMED	Tetramethylethylenediamine

TMZ	Temozolomide
TNF	Tumour Necrosis Factor
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Brain Tumours

There are over 130 types of tumour that can occur in the brain and other parts of the CNS or intracranial region with around 9400 people diagnosed with a brain, other CNS or intracranial tumour in 2011 in the UK (26 people every day), half of which were males, and half females. 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). Of those diagnosed in 2011 approximately 4500 died of brain cancer, (13 people a day) 100 of which are children. The greatest incidence was reported in over 65s (57%), however, 400 children were diagnosed, and this accounts for the second most common childhood cancer. The most common types of brain, other CNS and intracranial tumours in England in 2006-2010 were astrocytomas (34%) and meningiomas (21%) (Cancer Research UK, 2014). Primary brain tumours, those arising within the central nervous system (CNS) 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).

Primary brain tumours that originate in the brain only rarely metastasize; and secondary, or metastatic tumours arise from other cancer sites in the body, such as lung cancer, breast cancer, kidney cancer, melanoma and other types (Cancer Research UK, 2013). In addition, primary brain tumours can be benign or malignant. Benign tumours are typically slow-growing, usually grade I and II, and rarely spread to other areas of the body. They often have well-defined borders, so they can be surgically removed effectively. Benign brain tumours can be considered malignant if they are located in areas of the brain that control vital functions like breathing (NHS Choices, 2013). Malignant tumours tend to grow faster, are typically grade III and IV, and can be more invasive than benign tumours (Cancer Research UK, 2013).

Metastatic or secondary brain tumours are carried to the brain by the blood, or spread from adjacent tissue and are the most common type of brain tumour, particularly in the elderly (NHS Choices, 2013). The most commonly metastasizing primary cancers to the brain, are lung cancer (50%), breast cancer (20%), melanoma (10%), and colon cancer (5%). Around 80% of brain metastases occur in the cerebral hemispheres, 15% occur in the cerebellum, and 5% occur in the brain stem (Patchell *et al.*, 2003). Primary brain tumours rarely spread to other areas of the body, but they can spread to other parts of the brain and to the spinal axis. Low grade tumours that are slow growing with well-defined borders can usually be removed surgically; however, 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 and Berger, 2014). Improvement has been seen in the last four decades in patient survival with approximately 40% of patients diagnosed with malignant brain tumours surviving more than a year, 15% surviving five years, and 10% up to ten years from diagnosis (Cancer Research UK, 2014). However the chances of surviving longer relates to the age of the patient as more than 70% children survive for at least five years, 50% of young adults will survive five years, but only 2% of over 70s. The main cause of brain tumours still remains unidentified. However, several risk factors have been established which include exposure to radiation, previous incidence of cancer or genetic conditions such as neurofibromatosis (Reilly, 2010).

1.2 Tumour Grading

Classifying, grading and staging of tumours are helpful to determine the appropriate treatment and to better understand the patient's current health and prognosis. Grading helps the understanding of the aggressiveness, or malignancy of a tumour with grading classified as Grade I through Grade IV and the more aggressive and dangerous the tumour is, the higher the grade (Scheithauer, 2008). The description of tumour grade is based on the World Health Organization (WHO) grading system (Louis *et al.*, 2007).

Grade I: These are the least malignant tumours and are usually associated with long-term survival and the potential for cure following surgical resection alone. They grow slowly and have an almost normal appearance when viewed through a microscope.

Grade II: These tumours are slow-growing and look slightly abnormal under a microscope. Typically these neoplasms are infiltrative in nature, often recur following treatment, and can develop into higher grade tumours.

Grade III: Although there is not a big difference between grade II and grade III tumours, these tumours display a malignant phenotype. The cells of a grade III tumour are actively reproducing abnormal cells which invade normal brain tissue and following treatment tend to recur, often as a grade IV.

Grade IV: These are the most malignant tumours that reproduce rapidly, have an abnormal appearance when viewed under the microscope, and invade normal brain tissue. The

neoplasm is associated with a necrotic core and the formation of new blood vessels to maintain their rapid growth.

In spite of the relative clarity of this grading system it must be recognised that tumours can contain more than one grade of cell where the highest or most malignant grade of cell determines the overall grade of the tumour (Scheithauer, 2008). A summary of CNS tumour type and their current WHO grading can be found in the review by David Louis and colleagues, the authors of the official WHO publication (Louis *et al.*, 2007).

1.3 Glioma

There are more than 130 different types of primary brain tumours, named by the type of normal cell they most closely resemble, cell location, and grading from the least aggressive to the most according to World Health Organization (WHO) classification system (Louis *et al.*, 2007). While there is no single definitive cause of brain tumours, there are trends among people who get them such as age, environment influences, geography and genetics (Cancer research UK, 2013). Gliomas, neoplastic glial cells, are responsible for the majority of malignant brain tumours, and are the most common type of primary brain tumour. They are classified by cell type as astrocytoma, oligodendroglioma, mixed oligoastrocytoma, and ependymoma (Figure 1.1) (Louis *et al.*, 2007).

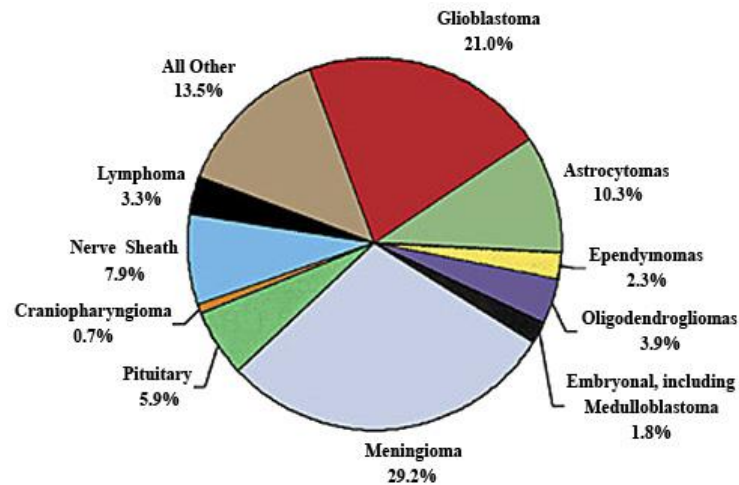


Figure 1.1 Classification of primary brain tumours by histology (Shonka and Gilbert, 2010).

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% after 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 and Berger, 2014).

1.3.1 Astrocytoma

This form of tumour originates from astrocytes, which are star-shaped glial cells found throughout the CNS that have a variety of functions including maintenance of extracellular ion levels and tissue repair. Astrocytic tumours are the most common type of glioma and are divided into encapsulated (e.g. non-invasive pilocytic astrocytoma) and diffuse tumours (e.g. glioblastoma multiforme (GBM)). While this form of glioma can occur at any stage, the risk

increases with age (Macmillan Cancer Support, 2013). Examples of glioma at each WHO grade are:

- Pilocytic astrocytoma (WHO grade I): typically found in the cerebellum, hypothalamus and optic nerve, and while uncommon, have favourable life expectancy
- Diffuse astrocytoma (WHO grade II): strongly associated with the TP53 mutation (Peraud *et al.*, 2002), survival is linked to progression to higher grade astrocytoma.
- Anaplastic (malignant) astrocytoma (WHO grade III): represents a progression of low to high grade lesion and characterised by progression to GBM, on average within 2 years (Ohgaki *et al.*, 2004).
- Glioblastoma multiforme (WHO grade IV): the most prevalent form of brain tumour with a median survival time of 1 year (Ohgaki *et al.*, 2009), strongly associated with mutations in NADP⁺-dependent isocitrate dehydrogenase 1 (IDH1) (Parsons *et al.*, 2008; Dang *et al.*, 2010). In addition to IDH1 mutations, overexpression and mutation of the epidermal growth factor receptor (EGFR) is a hallmark of GBM (Ohgaki *et al.*, 2004)

1.3.2 Oligodendroglioma

This type of glioma originates from oligodendrocytes, the cells which form the myelin sheath covering the axons of nerve cells within the CNS. It is the second most common form of primary brain tumour (Ohgaki *et al.*, 2009), and can be found as grades II (oligodendroglioma) and III (anaplastic oligodendroglioma) (Louis *et al.*, 2007). In addition

to being found more often in males than in females, the most common marker of this type of glioma is the frequent loss of heterozygosity on the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) (Jeuken *et al.*, 2004). This loss is associated with up to 90% of oligodendrogliomas and approximately 60% of anaplastic oligodendroglioma (Cairncross and Jenkins, 2008), and is thought to be as a result of translocation between chromosomes 1 and 19 (Griffin *et al.*, 2006; Jenkins *et al.*, 2006). In the UK population, this form of glioma occurs in approximately 5% of glial tumours (Macmillan Cancer support, 2013).

1.3.3 Ependymoma

Ependymomas are a rare type of glioma, constituting approximately 5% of all neuroepithelial tumours, including tumours of grade I, II and III (Louis *et al.*, 2007).

- Myxopapillaryependymoma and subependymoma (WHO grade I)
- Ependymoma (low-grade) (WHO grade II)
- Anaplastic (malignant) ependymoma (WHO grade III)

These tumours arise from the ependymal cells, which line the ventricles (fluid-filled spaces in the brain), and from the central canal of the spinal cord. Spinal ependymomas are more common in adults and have a more favourable prognosis than intracranial tumours, the majority of which are paediatric cases, which have a five year survival rate of less than 70% (Korshunov *et al.*, 2004). Unlike astrocytoma, ependymoma is not associated with any unique molecular changes, but overexpression of EGFR in intracranial tumours and TP53 in anaplastic ependymoma is associated with a poorer prognosis (Mendrzyk *et al.*, 2006; Korshunov *et al.*, 2002).

1.4 Symptoms

Intracranial pressure is usually the first symptom, which may be caused by a blockage in the ventricles and build-up of the cerebrospinal fluid (CSF). Raised intracranial pressure can cause headaches and seizures, sickness (vomiting) and vision problems. There are also symptoms involved according to the area of the brain affected, for instance, the frontal lobe may cause changes in mood and personality, weakness or numbness of one side of the body. The temporal lobe can cause problems with coordination and speech, and it may affect memory, and the parietal lobe may cause problems with writing and weakness or numbness of one side of the body (Macmillan UK, 2014; National Cancer Institute, 2014). A study analysing 103 patients from glioma grade II- IV, showed that patients often experienced more than one symptom, while 40% had presented only one symptom; ophthalmological symptoms in 28.8%), impaired coordination (28.8%), paresis (21.2%), pain (21.2%), gait ataxia (18.3%), dysarthria and dysphagia (13.5%), signs of raised intracranial pressure (12.5%), organic psycho-syndrome (7.7%), nausea and vomiting (6.7%), myoclonus (2.9%), tinnitus or auditory disturbances (1.9%), incontinence (1.9%) (Reithmeier *et al.*, 2014). By the time a glioma becomes symptomatic, it has progressed to the end of its biological course with stem cells having migrated far beyond the imaging-defined tumour mass (Kelly, 2010).

1.5 Diagnosis

In the first stage, diagnosis typically is assessing the history of symptoms followed by a basic neurological exam, including an eye and tests of vision, balance, coordination and reflexes, and the ability to think and remember (Taylor, 2010). Diagnosis is confirmed using either a computerized tomography (CT) scan or magnetic resonance imaging (MRI) of the patient's brain. CT helps direct differential diagnosis. MRI has superior soft-tissue resolution and can

better detect isodense lesions or tumour enhancement. In post therapy imaging, single-photon emission computed tomography (SPECT) and positron emission tomography (PET) may be useful in differentiating tumour recurrence from radiation necrosis (Hutter *et al.*, 2003; Schaefer *et al.*, 1996).

Once a brain tumour is detected on CT or MRI scans, a biopsy for examination under a microscope can be done either before surgery by needle biopsy or at the time of surgery, to identify the tumour type and grade. This would determine treatment options, and also give important information about prognosis (Brain Tumour Centre USA, 2005).

1.6 Treatment

The best treatment for each individual patient should take into account the tumour location, symptoms, and age to assess the different treatment options. Surgery is usually the initial treatment for glioma (Johns Hopkins Medicine, 2014). It has been suggested that patients with smaller solid tumours, have better prognosis when undergoing other treatments such as chemotherapy and radiation therapy. Radiation therapy is an important part of the treatment of high-grade gliomas, and would begin radiation treatments within 2 to 4 weeks after tumour resection, and will be done for around 4 to 6 weeks. After radiotherapy, patients usually obtain an MRI scan to assess the effect of the treatment (Brain Tumour Centre USA, 2005). Studies showed a significant survival benefit with adjuvant chemotherapy with an increase in survival of 10% at 1 year (Lonardi *et al.*, 2005). The most commonly used drugs today are: temozolomide (TMZ), procarbazine, carmustine (BCNU), lomustine (CCNU), vincristine and cisplatin (Cancer Research UK, 2014).

1.6.1 Temozolomide

Temozolomide is derived from dacarbazine, was first synthesised in 1984 by Aston University as one of a series of novel imidazotetrazinones (Stevens *et al.*, 1984), and is orally administered (O'Reilly *et al.*, 1993; Friedman, 2000). These agents were structurally unique because of their three adjacent nitrogen atoms which conferred unique physicochemical properties and much greater antitumor activity than the previously synthesized bicyclic triazenes, which contained only two adjacent nitrogen atoms (Stevens *et al.*, 1984). Temozolomide represents a new class of second-generation imidazotetrazine prodrug that undergoes spontaneous conversion under physiological conditions to the active alkylating agent MTIC (Stevens *et al.*, 1987) and does not require hepatic metabolism for activation (Clark *et al.*, 1995). TMZ crosses the blood brain barrier due to its small size and lipophilic properties (Agarwala, 2000). It works by the induction of the DNA adduct O6-methylguanine (O6M-G) which activates the mismatch repair system (MMR), causing double strand breaks in the DNA, cell cycle arrest in G2/M phase, and apoptosis (Roos *et al.*, 2007; Caporali *et al.*, 2004; Hickman and Samson, 1999). Preclinical studies showed that TMZ demonstrated distribution to all tissues, including penetration into the CNS and low toxicity compared to mitozolomide (parent compound), and antitumor activity against a broad range of tumour types, including glioma, (Stevens *et al.*, 1984). TMZ was absorbed rapidly, exhibited 100% bioavailability within 1–2 h of administration, and also showed antineoplastic activity in recurrent high-grade glioma (Newlands *et al.*, 1992). Results suggested TMZ is preferably administered once daily for 5 days in a 4-week cycle. Though it is the first choice of therapy for gliomas, due to its pro-drug nature, it is not preferred *in vitro* use for control experiments (Friedman *et al.*, 2006).

1.6.2 Cisplatin

Cisplatin is also used in some cases of glioma (Cancer Research UK, 2014). Cisplatin, cisplatinum, or otherwise cis-diamminedichloridoplatinum (II) (CDDP) is a chemotherapy drug, used in various cancer treatments, such as sarcoma, small cell lung cancer, germ cell tumours, lymphoma testicular cancer, breast cancer, bladder cancer, and ovarian cancer. It is often given as a combination chemotherapy regimen with other drugs, but can be used on its own. It is an alkylating-like drug, even though it has no alkyl groups, is platinum based and the first designed in that class of drugs. Cisplatin, often called the “penicillin of cancer” because it is used so widely, was the first blockbuster chemotherapy drug (Tanida *et al.*, 2012). It has a simple molecule of only 11 atoms, a chemical formula of $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$, molecular weight of 300.045, and solubility in water allowing delivery in aqueous form (Chemical and Engineering News, 2005). Cisplatin was first described by Michel Peyrone in 1845, and was known for a long time as Peyrone's salt or chloride, and by 1893 the structure was deduced by Alfred Werner.

In the 1960s scientists started testing the drugs biological effects, and it was first tested by Barnett Rosenberg, a professor of biophysics and chemistry at Michigan State University, on bacteria, where it was found that it prevented the cell division, but not the other growth processes. The researchers were able to deduce that this effect was due not to the electrical fields, but to a compound that was formed in a reaction between the "inert" platinum electrodes and components of the solution containing the bacteria (Rosenberg *et al.*, 1965). Rosenberg then tested cisplatin on mice with cancerous tumours in 1968, and there were significant effects on tumour growth (Rosenberg *et al.*, 1969). Consequently, cisplatin went

into clinical trials for cancer therapy in 1971, and has been widely used under the brand name Platinol® following its approval in 1978 by the FDA for cancer treatment (Chemical and Engineering News, 2005). It is now known that cisplatin forms a platinum complex inside of a cell which binds to and cross-links DNA. When DNA is cross-linked this way, it causes cells to undergo apoptosis, following damage to the DNA so that the repair mechanisms are activated and the cells are found to not be salvageable.

The shape of the cisplatin molecule is square and flat. Its covalent bonds readily exchange with other ligands, for example water, so in the aqueous body environment, the chlorine atom in the cisplatin molecule is replaced by a hydroxyl group, and later by part of the DNA (Tanida *et al.*, 2012). The name “alkylating” comes from cisplatin mechanism of action; an alkyl group signifying a single, covalently bonded carbon atom. Alkylating agents are capable of forming from strong electrophiles usually *via* a carbonium ion or carbon radical. These electrophilic compounds naturally seek out nucleophilic atoms and functional groups to form their covalent bonds, and one atom that is particularly susceptible to alkylation is the nitrogen 7 in the guanine nucleotide of DNA. The cisplatin molecule binds with a protein on one side and the DNA molecule on the other with the nitrogen atom at position 7 of guanine being highly susceptible to alkylation. This protein is critical as it protects the cisplatin molecule from being removed by DNA repair mechanisms in the cell (Tanida *et al.*, 2012).

1.7 Aspirin

1.7.1 History of Aspirin

Aspirin was first synthesized by Felix Hoffman in 1897 when he acetylated the hydroxyl group on the benzene ring of salicylic acid to form acetylsalicylic acid (Vane and Botting, 2003). By 1899 aspirin powder had been used to treat patients, rapidly becoming the number one selling drug worldwide. Only a year later was available in a water-soluble tablet form and in 1915 it was made available without prescription (Mahdi, 2006). This revolutionary medication was used widely in the flu pandemic in Europe (1918), despite nothing being known about its mechanism of action (Schorr, 2009). By 1924, its use as a medication included the treatment of flu (3 tablets a day- 75mg each), rheumatism, lumbago and neuralgia, and aspirin was well known by the general public (Bayer HealthCare LLC, 2009; Mahdi, 2006).

Despite its widespread use, it wasn't until 1971 that Sir John Vane published an article in Nature suggesting a mechanism of action for aspirin involving the inhibition of prostaglandin synthesis, explaining the pain-relief and anti-inflammatory effects (Vane, 1971). For the first time, a mechanism of action of aspirin explained the multiple biological activities of the compound by one pharmacological effect, and this discovery resulted in him being awarded the Nobel Prize in Physiology or Medicine in 1982 (Bayer HealthCare LLC, 2009; Schror, 2009).

1.7.2 Aspirin and Colorectal Cancer

The first epidemiological evidence to support the use of aspirin in cancer treatment was published by Kune and colleagues in 1988, who reported a decreased risk of developing colorectal cancer with aspirin use (Kune *et al.*, 1998). Their study examined a total of 715 colorectal cancer patients and 727 age and sex-matched controls and found a statistically significant decrease in both colon and colorectal cancer development in control patients taking aspirin, an effect found in both males and females. The decrease was dramatic, with a 40% reduction in colorectal cancer development in participants regularly taking aspirin (Kune *et al.*, 1988).

Subsequently, a link between aspirin and cancer prevention was demonstrated. Case-control studies established that the risk of colorectal cancer may be reduced in regular aspirin users (Kune *et al.*, 1988; Rosenberg *et al.*, 1991; Suhet *et al.*, 1993; Muscat *et al.*, 1994; Peleg *et al.*, 1994). Supporting studies showed a protection of between 20% - 40% in regular aspirin users (Thun *et al.*, 1991, 1993; Schreinemachers and Everson, 1994; Giovannucci *et al.*, 1994, 1995). Other trials such as the American Nurses' Health Study, suggest the protection was only evident for frequent use (four times per week) and after 20 years of use (Giovannucci *et al.*, 1995). These individual trial findings have been confirmed by more recent studies, where meta-analysis of clinical trials and epidemiological studies originally performed to examine the cardiovascular effects of aspirin, have demonstrated conclusively that regular aspirin (daily), reduces the risk of cancer development in the general population and genetically susceptible individuals (Smith *et al.*, 2000; Menter *et al.*, 2010; Dibra *et al.*, 2011; Rothwell *et al.*, 2012).

A hospital based case control study of 1326 colorectal cancer and 4891 control patients (both cancer and non-cancer) examined the effect of aspirin use on the risk of developing large-bowel cancer (Rosenberg *et al.*, 1991). In addition to finding a reduced risk of cancer with continued aspirin use that was proportional to the period of time that aspirin was taken for, the study established that if aspirin was discontinued for a period of a year, there was no associated decrease in risk, suggesting that sustained use is required. The relationship between the frequency of aspirin use and its protective effect has also been examined (Suh *et al.*, 1993; Muscat *et al.*, 1994; Peleg *et al.*, 1994). The studies found that the risk of colorectal cancers declined progressively as the frequency of aspirin use increased compared with control groups, with patients taking aspirin two to three times daily, having a greater decrease in risk than those taking aspirin once a day, compared to those taking no aspirin. The studies also found a dose related decrease in the incidence of polyps, a predictive marker for colorectal cancer development.

1.7.3 Aspirin and Other Cancer

In addition to the accepted protective effect of aspirin in colorectal cancer, there is emerging evidence that NSAID use is protective in a wide range of cancers, an effect that is closely linked with inflammation (Ulrich *et al.*, 2006). Epidemiological evidence suggests that aspirin or other NSAID use produces risk reductions of 39% for breast and prostate, 36% for lung, 73% oesophageal, 62% stomach, 63% in colon and 47% in ovarian cancer, after five or more years of daily intake (Harris *et al.*, 2005; Corley *et al.*, 2003; Terry *et al.*, 2004). However, this reduction does not apply to cancer in general, with results published on pancreatic, urinary bladder and renal cancers showing that incidence did not decrease significantly (Ulrich *et al.*, 2006). Other work has investigated the effect of aspirin on cancer

in general through retrospective analysis of trials designed to test vascular protective effects of aspirin (Elwood *et al.*, 2009). The results showed that taking 500mg of aspirin daily for a period of 6 years reduced overall cancer deaths by 18%.

Meta-analysis of published clinical trial data from 51 studies involving over 77,000 participants has shown that aspirin reduces cancer risk following just 3-5 years of daily use, much sooner than the previously suggested 10 years (Rothwell *et al.*, 2012). The study also showed that the protective effect was produced with a low daily dose (75-300mg) reducing cancer incidence by one quarter in just three years (9 cancer patients in 1000 compared to 12 in 1000 patient in the placebo group). This reduction in cancer rates increased with time, falling to 37% after five years. It was also noted in the results that while the risk of heart attacks and strokes were reduced, there was an increase in major bleeding incidences in the high dose patients leading to the conclusion that aspirin treatment should be favoured in individuals with family history of cancers.

There is an on-going debate amongst clinicians over whether or not aspirin should be prescribed as a chemopreventative agent, in particular, to individuals at high risk of cancer development. The exact mechanism of cancer prevention or treatment remains today unknown although there are some proposed involving apoptosis, angiogenesis, growth factors etc. (Langley *et al.*, 2011). A recent study published in the Lancet argued that there is still not enough proof to support aspirin use in cancer prevention, but that its side effects are well known, and severe (Rothwell *et al.*, 2012), supporting the development of aspirin analogues (Dibra *et al.*, 2011).

1.7.4 Aspirin and Glioma

The association between use of aspirin and other NSAIDs and risk of adult glioblastoma multiforme (GBM) was evaluated by the San Francisco Bay Area Adult Glioma Study which included 236 incident GBM cases and 401 population-based controls frequency-matched on age and gender. Cases with self-reported GBM reported less use of all types of NSAIDs combined during the 10-year prediagnostic period than did controls (aspirin (OR = 0.51, 95% CI: 0.3, 0.8), ibuprofen (OR = 0.41, 95% CI: 0.2, 0.8), and naproxen/other NSAIDs (OR = 0.34, 95% CI: 0.1, 0.8)). Their findings showed an inverse association between NSAID use and GBM (Sivak-Sears *et al.*, 2004).

The Columbia University and the University of California San Francisco study with 517 glioma cases and 400 controls compared the intake of NSAIDs for more than six months establishing two comparator groups of at least twice weekly use, *versus* a lower or no use. NSAIDs showed significant inverse trends between duration of drug use and glioma risks (OR = 0.68, 95% CI 0.49, 0.96) (Ferris *et al.*, 2012). However, some studies have found no association between NSAIDs and brain cancer (Daugherty *et al.*, 2011).

1.7.5 Aspirin and Metastases

The beneficial effect of aspirin is not just restricted to primary tumours; it also decreases metastases (Chan, 2012; Rothwell *et al.*, 2012). Meta-analysis of five randomized placebo controlled trials involving over 17,000 participants taking 75 mg of aspirin daily over a period of 6.5 years, results showed a reduction in metastasis (Chan, 2012). Aspirin reduced the risk for cancer with distant metastasis by 36% and the risk for adenocarcinoma with metastasis by 46%. A reduction in death due to cancer by 50% was found in patients who developed

adenocarcinoma without metastasis at diagnosis, and an overall 35% decrease in risk for fatal adenocarcinoma among participants taking aspirin. Rothwell and colleagues also found a decrease in metastasis, concluding that with aspirin treatment, one in five cancers would be prevented (Rothwell *et al.*, 2012)

1.8 Aspirin Mechanism of Action

Before 1971, little was known about the mechanism of action of aspirin-like drugs (Vane and Botting, 2003). Guzman *et al.*, 1964 and Lim *et al.*, 1964 provided the first definitive evidence of the peripheral analgesic activity of aspirin-like drugs, and Piper and Vane in 1969 used isolated lungs perfused with Krebs' solution from sensitised guinea pigs in order to detect substances released during the anaphylactic reaction, including histamine and SRS-A (anaphylaxis mediators). They also found the previously unreported substances PGE₂, PGF_{2α}, and the "rabbit aorta contracting substance" (RCS) which was later identified by Hamberg *et al.*, 1975 as thromboxane A₂. Vane published in 1971 that there was a dose-dependent inhibition of PG formation by aspirin, and in addition Smith and Willis (1971) investigated the effects of aspirin on platelet behaviour. They obtained blood samples one hour after taking 600 mg of aspirin orally, isolated the platelets, washed and incubated them with thrombin and tested the supernatant for the presence of various substances including PGs and found that there was only a change in the release of PGs. Collier and Flower (1971), also reported that administration of aspirin inhibited human seminal PG production, detecting PGs in inflammatory exudates (Di Rosa *et al.*, 1971).

Aspirin selectively acetylates the hydroxyl group of serine residue (Ser 530) located 70 amino acids from the C terminus of the COX enzyme (Roth *et al.*, 1975), leading to irreversible

COX inhibition. Acetylation of the enzyme by aspirin places a bulky substituent on the Ser 530 oxygen that inhibits binding of arachidonic acid (De Witt *et al.*, 1990). Cyclooxygenases have two main isoforms COX-1 and COX-2. COX-1 is responsible for the synthesis of prostaglandin and thromboxane while COX-2 plays a major role in prostaglandin biosynthesis in inflammatory cells and in the central nervous system, thus COX-2 has analgesic and anti-inflammatory activity by blocking the transformation of arachidonic acid into prostaglandin H₂ selectively (Figure 2) (Vane and Botting, 2003). Aspirin binds to Ser 516 in the active site of COX-2 in the same way as it binds to Ser 530 in the active site of COX-1, but it has been shown that the active site of COX-2 is slightly larger than that of COX-1 (Vane *et al.*, 1998).

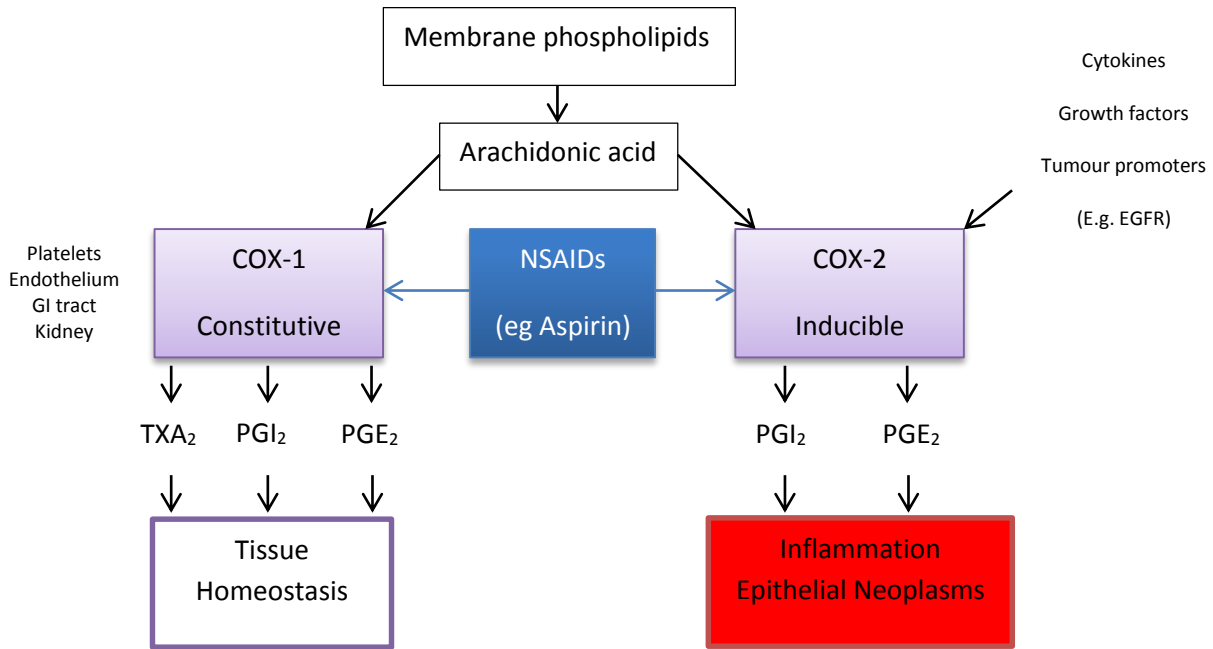


Figure 1.2 Aspirin inhibition of COX-1 and COX-2 activity. COX-1 is expressed in many tissues and PGs produced by COX-1 mediate the normal functions such as platelet aggregation, unlike COX-2 which is not detected in most normal tissues, but its expression is rapidly induced by both inflammatory and mitogenic stimuli resulting in increased synthesis of PGs in inflamed and neoplastic tissue (Adapted from Konturek *et al.*, 2005).

1.9 COX Expression in Brain Tumours

As stated previously, COX enzymes catalyze arachidonate metabolism, resulting in PG production (Figure 1.2). Two isoforms of the enzyme have been identified, COX-1 and COX-2; COX-1 is expressed in several cell types of normal mammalian tissues and is involved in the maintenance of tissue homeostasis, while COX-2 is responsible for PG production at sites of inflammation (Eberhar *et al.*, 1995). The expression of COX-1 and COX-2 has been linked with a variety of diseases of the brain, and colorectal tumourigenesis (Sano *et al.*, 1995; Sairanen *et al.*, 1998; Joki *et al.*, 2000). Joki *et al.*, 2000 demonstrated the expression of

COX-2 protein in the tumour cells of gliomas and normal brain tissue including, neurons. The same study also showed that high-grade glioma tissues expressed higher levels of COX-2 protein when compared with low-grade glioma. Since overexpression of COX-2 results in PG production in colon and human brain tumour tissue (Kokoglu *et al.*, 1998; Maxwell *et al.*, 1990), it has been suggested that PGs play a role in tumour development. Interestingly, growth factors, tumour promoters, cytokines, and other inflammatory mediators have been found to induce COX-2 expression (Smith *et al.*, 1995, Eberhar *et al.*, 1995).

In a study on childhood brain tumours, COX-2 has been shown to induce resistance in neoplastically transformed cells to chemotherapeutic agents or radiation (Bodey *et al.*, 2006). *In vitro* experimental systems showed that selective COX-2 inhibitors caused a decrease in cell proliferation, an increase in apoptosis and modulated cell cycle regulation at gene levels. Elevated COX-2 expression has been linked to a broad range of human cancers, including 80% of cancers of the breast, colon, oesophagus, liver, lung, pancreas, prostate, cervix, and head and neck (Choy and Milas, 2003). Although there is a variation in COX-2 expression among tumours, it is generally associated with a more malignant phenotype, aggressive tumour behaviour, worse prognosis and the development of metastatic disease (Choy and Milas, 2003). COX-2 derived prostaglandins have also shown to stimulate production of angiogenic growth factors (Liu *et al.*, 1999; Tsujii *et al.*, 1998).

1.10 EGFR and Brain Tumours

As discussed previously, increased COX-2 expression directly correlates with glioma grade and is associated with shorter survival rate in glioblastoma patients. COX-2 is also regulated by epidermal growth factor receptor (EGFR) signalling which is important in the pathogenesis of GBMs (Figure 2) (Joki *et al.*, 2000). EGFR is expressed at high levels in various types of cancer, suggesting a role in the pathogenesis of multiple cancer types (Gullick *et al.*, 1991), and there is substantial experimental evidence supporting a role for aberrant EGFR signalling in cancer pathogenesis and resistance to treatment (Huang *et al.*, 2009). EGFR amplification is found in 40–50% of primary GBM, with approximately half of these amplification events involving a variant of the receptor termed EGFRvIII. The most common EGFR mutant is EGFRvIII (EGFR type III, EGFRvIII, de2-7, Δ EGFR) (Ekstrand *et al.*, 1991; Wond *et al.*, 1992), which results from a deletion of exons 2 to 7 of the EGFR gene, which results in an in-frame deletion of 267 amino acids from the extracellular domain of the receptor (Mishima *et al.*, 1998). Reports suggest that EGFRvIII expression in cells lead to activation of PI3K/Akt pathways, which results in downregulation of p27 and inhibition of proapoptotic factors such as procaspase 9 (Choe *et al.*, 2003; Narita *et al.*, 2002). A more specific study showed the expression of EGFR mutant in U87MG cells leads to increased Bcl-XL, and result in resistance in apoptosis (Moscatello *et al.*, 1998). EGFR activation triggers a diverse array of signals EGFR stimulation is found to activate STAT-3 and triggers signalling cascades like MAPK which trigger NF- κ B activation (Indranil *et al.*, 2013), as well cyclin D1 expression stimulation, resulting in increased proliferation and invasion, and a reduction in apoptosis (Wang, 2011). There is substantial evidence suggesting that EGFRvIII signalling plays a key role in gliomagenesis (Nicholas *et al.*, 2006), and increased EGFRvIII expression has suggested to influence multiple aspects of tumour

biology, including survival, proliferation of cells, motility and invasiveness, and resistance to treatment (Nagane *et al.*, 1996). EGFR gene amplification and overexpression are a striking feature of glioblastoma but are rare in low-grade gliomas, suggesting a causal role for aberrant EGFR signalling in the pathogenesis of GBM (Hatanpaa *et al.*, 2010).

VEGF expression is upregulated in tumor cells by NF- κ B and STAT3 signaling. VEGF signalling also plays an important role in GBM biology and its expression is upregulated in GBM. Activation of its receptor, VEGFR-2, which is found on brain endothelial cells, promotes tumor growth by increasing blood supply to the highly metabolic tumour (Atkinson *et al.*, 2010).

1.11 NF- κ B and Brain Tumours

The transcription factor NF- κ B has been shown to be important in inflammation, suppression of apoptosis, and in cell proliferation (Poligone and Baldwin, 2001). Reports indicate that NF- κ B can function upstream of COX-2 and that the cyclopentenone prostaglandins can inhibit NF- κ B activation via the inhibition of the I κ B kinase. Upon stimulation of the cell, such as with TNF- α , I κ B is phosphorylated, ubiquitinated, and degraded. This allows the free NF- κ B to accumulate in the nucleus where it can activate transcription. In the nucleus NF- κ B can regulate the expression of many genes involved in inflammation such as ICAM-1, IL-2, IL-6, IL-8, and complement factors (Figure 1.3) (Baldwin, 1996). Strong evidence indicates that chronic dysregulation of NF- κ B may underlie most inflammatory diseases and contribute to oncogenesis (Tak and Firestein, 2001). Levels of NF- κ B activity, assessed by serine phosphorylation, are much higher in GBM tissue compared with non-GBM tissue (Wang *et al.*, 2004; Nozell *et al.*, 2008), and correspond with increasing grade in astrocytic

tumors (Korkolopoulou *et al.*, 2008; Angileri *et al.*, 2008). A number of proteins and pathways may be dysregulated in GBMs that may cause NF- κ B activation. TNF- α for instance is produced in the CNS by microglia, astrocytes, endothelial cells and some neurons (McCoy and Tansey, 2008). TNF- α and other proinflammatory soluble factors, such as IL-6 and IL-1 β , are important regulators of paracrine signaling in brain tumor cells. TNF- α signaling through TNFR1 promotes NF- κ B activation and subsequent anti-apoptotic responses (Otsuka *et al.*, 1999; Koul *et al.*, 2006). Moreover, the levels of TNFR1 expression are elevated in GBM as compared with low-grade gliomas (Hayashi *et al.*, 2001). NF- κ B is activated by EGF and EGFR and PDGF via a PI3K–Akt–IKK-dependent mechanism (Downward, 1998; Romashkova and Makarov, 1999). Literature has shown that inhibition of NF- κ B activity or NF- κ B-regulated genes, reduce brain tumor growth, invasion and angiogenesis (Xie *et al.*, 2008). Thus, there is a strong correlation between constitutive NF- κ B activation and gliomagenesis.

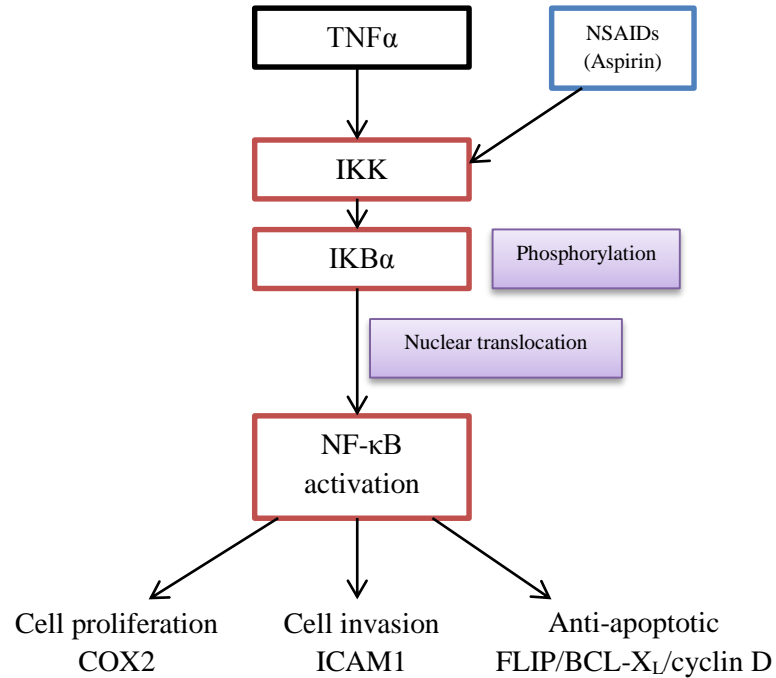


Figure 1.3 Role of NF- κ B in cancer development. Upon stimulation TNF α leads to the activation of IKK and subsequently, IKB phosphorylation occurs. NF- κ B translocates to the nucleus and activates gene transcription. NF- κ B activation can result in upregulation of genes that are involved in cell proliferation, cell invasion and cell death (anti-apoptotic genes) (Adapted from Dey *et al.*, 2008).

1.12 Wnt/ β -catenin and Brain Tumours

Canonical Wnt signalling (β -catenin dependent) regulates a wide set of genes that in turn orchestrate diverse cellular functions such as morphogenesis, differentiation, and proliferation. Consequently, aberrant activation of the canonical Wnt pathway has been found to be the driver of several human cancers including glioma. Early evidences for the involvement of Wnt signalling in brain tumours came when similar germline mutations of APC were found in gliomas and medulloblastomas (Lowenstein *et al.*, 1992). EGFR and

Wnt/ β -catenin pathways are known to interact under malignant conditions. Silencing of EGFRvIII reduced the expression of factor β -catenin, with a decrease in cell cycle progression which was shown on U87MG cells (Yamoutpour *et al.*, 2008). Small-molecule inhibitors of PI3K/Akt signalling pathway result in degradation of β -catenin and reduced expression of its target genes Cyclin D1 and c-Myc (Baryawno *et al.*, 2010). β -catenin is overexpressed in human glioblastoma and knockdown of β -catenin inhibits glioblastoma cell proliferation and invasive ability, and induces apoptotic cell death. Furthermore, intratumoral introduction of siRNA targeting β -catenin into established subcutaneous gliomas also delayed the tumour growth. Both *in vitro* and *in vivo* studies have confirmed that downregulation of β -catenin leads to reduced expression of EGFR, STAT3, Cyclin D1, MMP2, MMP9, and Akt1 mRNA as well as protein with a concomitant decrease of their active forms (Indranil *et al.*, 2013).

1.13 Aspirin Analogues

With any medicine, there is a balance between the risks and benefits of treatment, and the benefits need to outweigh the potential harms (NHS Choices, 2011). As a result, it has been widely suggested that significant effort should be directed into producing novel NSAID derivatives that do not produce the adverse gastrointestinal and cardiovascular effects associated with long term aspirin use, but retain the multiple and potent protective actions that are involved in suppressing cancer formation (Baron *et al.*, 2003; Chan *et al.*, 2009; Thun *et al.*, 2002; Dibra *et al.*, 2011). Various aspirin derivatives have been made and tested on cancer to this day. Nitric oxide-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs) have shown promising effects in colorectal and ovarian cancer cells (McIlhatton *et*

al., 2007; Selvendiran *et al.*, 2008, Bed *et al.*, 2011). *In vitro* studies also used the primary aspirin compound, salicylic acid, which inhibits the growth of rat glioma cells (Aas *et al.*, 1995). Sulindac, another NSAID, which inhibits both COX-1 and COX-2, has been found to inhibit glioblastoma cell growth, proliferation and invasion in a number of studies, a result previously observed in gastric carcinoma cell lines and colon cancer cells (Fujiwara *et al.*, 1993; Yamamoto *et al.*, 1999; Kambe *et al.*, 2009; White *et al.*, 2013; Bernardi *et al.*, 2006).

However, most emphasis has been given to COX-2 selective inhibitors (e.g. NS-398, celecoxib, meloxicam, rofecoxib or etoricoxib) which have all shown inhibition of proliferation in glioma cell lines (Kuipers *et al.*, 2007). However findings also suggest that celecoxib was able to exert pronounced pro-apoptotic effects *in vitro* and *in vivo* in the absence of any apparent involvement of COX-2 (Schönthal, 2010). Joki *et al.*, 2000 has described the effect of COX-2 inhibitor NS-398 on monolayer cell cultures and three-dimensional glioma spheroids was investigated using U-87MG and U-251MG human glioblastoma cell lines. NS-398 was found to reduce the proliferation and induce apoptosis of monolayer cell cultures, as well as the growth of spheroids and tumour cell migration in a dose-dependent manner. Matsuo *et al.*, 2004 also used the same COX-2 inhibitor *in vivo* using KMG4 tumour xenografts on SCID mice, which slowed tumour growth.

To this end, aspirin analogues have been synthesized and tested successfully on colorectal cancer cells (Deb *et al.*, 2011, Claudius *et al.*, 2014). It was shown that in comparison to aspirin, which reduced SW480 cell viability by 20%, an analogue called di-aspirin (PN508) had a significantly larger effect, reducing viability by approximately 50%. Di-aspirin as its name suggests has a structure that compromises two aspirin molecules. The work also

described other aspirin analogues, such as PN517, that reduced SW480 viability by a greater degree (Deb *et al.*, 2011). The analogues described in this study are PN508 (di-aspirin biscarbxylyphenol succinate), PN517 (fymaroyl di-aspirin), PN526 (2-carboxyphenyl carbonate) and PN529 (isopropyl m-bromobenzoylsalicylate), with a focus on PN508 and PN517 with molecular weights of 358 and 356 respectively (Fig 1.4). These two analogues are structurally two aspirin molecules bound together with a single (PN508) or a double bond (PN517). The presence of the double bond restricts rotation around the C=C bond, and cannot occur at room temperature, because pi bonds should be broken and thus more energy is required. Double bonds, also known as alkenes, are more stable, stronger and shorter than single bonds, and are also electron-rich making them reactive. However the double bond means that PN517 is in the trans isoform, while PN508 which can rotate, can be both cis and trans forms.

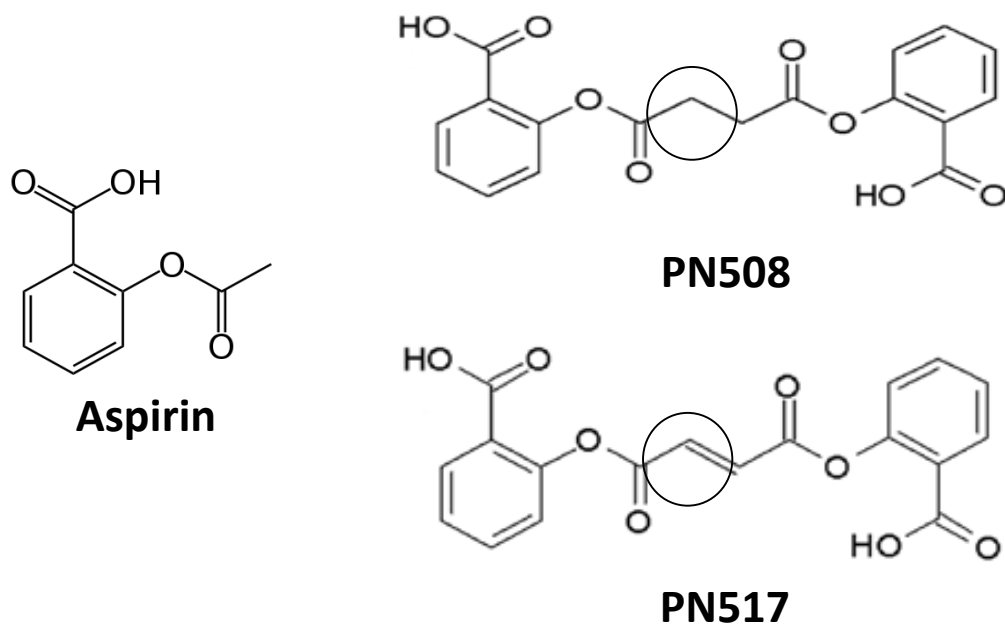


Fig 1.4 Chemical structure of aspirin, PN517 and PN 508

1.14 Cell Culture Model Systems

1.14.1 Established Cell Lines

Established cancer cell lines are the main stay of much research and the development of new therapies, with each passage the population becomes more homogenous allowing for the replication of experiments between researchers and laboratories. Three glioma cell lines were used in this study; a grade I astrocytoma (1321N1), grade II/III astrocytoma/oligodendroglioma (GOS-3), and grade IV glioblastoma (U87 MG). The U87 MG cell line was harvested from 44 year old male patient and is an adherent cell with an epithelial like morphology and the cell line is classified as a grade IV glioblastoma using the 2007 WHO grading. The GOS-3 cell line was harvested from a 55 year old male patient, is an adherent monolayer, and has fibroblast morphology, whereas the 1321N1 cell line is also an adherent cell but with a glial morphology classed as grade I astrocytoma according to 2007 WHO grading. An embryonic cell line served as control (SVG-p12), which are adherent brain cells from foetus, in the first trimester, with a fibroblast morphology.

1.14.2 Primary Cell Lines

In addition to the established cell lines, primary short term cell cultures were obtained from the Royal Preston Hospital (BTNW911 and BTNW914). Primary cultures are established directly from patient tissue and contain a heterogeneous population of cells including both cancer cells and those of surrounding tissue. Both primary cell lines utilised in this project were prepared from high grade glioma; the BTNW911 cells came from a male patient in his 60s and the BTNW914 cells came from a male patient in his 30s.

1.15 Summary

Glioma is a type of aggressive insocranial tumours with extremely narrow window for operative approaches, which is mostly palliative and rarely curative, and has a poor long term survival rate from diagnosis. Currently, the most common drug used in treatment is the prodrug TMZ, however a range of other chemotherapeutics are used including cisplatin. Increasing evidence from the meta-analysis of randomised controlled clinical trials supports aspirin for the treatment or prevention of cancer, with other trials involving aspirin currently still in progress. However, due to aspirins side effects, a need for derivatives with efficacy in the treatment of cancer that reduce the side effect profile are required.

1.16 Hypothesis

Aspirin analogues will show increased efficacy over aspirin and represent a potential therapeutic for the prevention or treatment of glioma.

1.17 Specific Aims

The overall aim of this project is to characterise the effects of aspirin and its analogues on both established and primary cell cultures *in vitro*. The specific aims are:

1. To use cell viability as a screen to assess aspirin analogue efficacy in both established and primary cells
2. To establish any cell type or tumour stage specific efficacy of aspirin analogues.
3. To subsequently select an efficacious analogue and further characterise its effects on proliferation, cell cycle regulation, apoptosis and migration/invasion
4. To identify mechanism(s) involved in the action of aspirin analogues

Chapter 2: Materials and Methods

2.1 Materials

The aspirin analogues used in this study PN508, PN517, PN526 and PN529 were supplied by Dr Iain Nicholl (Wolverhampton University, UK).

All other reagents used were of the highest quality commercially available and were obtained from the following suppliers:

ATCC (Manassas, VA, USA)

SVG P12 human foetal astroglial cell line

BD Biosciences (Plymouth, UK)

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

BTNW (Brain Tumour North West)

BTNW911, BTNW914

Cayman Chemical (Michigan, USA)

COX Fluorescent inhibitor screening assay kit

Cell Signalling, New England Biolabs (Hitchin, UK)

Caspase-3 control cell Extracts, Caspase-8 (1C12) Mouse mAb, Cleaved Caspase-3 (Asp175) (5A1E) Rabbit mAb, Caspase-9 Antibody (Human Specific), Cyclin D1 Rabbit

mAb, Phospho-Cyclin D1 XP Rabbit mAb, EGF Receptor XP Rabbit mAb, Phospho- EGF Receptor XP Rabbit mAb, Anti-rabbit IgG HRP-linked Antibody

ECACC (Porton Down, UK)

U-87 MG Grade IV Human Glioblastoma Cell Line, 1321N1 Cell Line human Glial cells from brain astrocytoma, GOS-3 Cell Line human Glioblastoma (derivative of U-343-MG)

Fisher Scientific (Loughborough, UK)

Phosphate buffered saline tablets, Foetal Bovine Serum, 8-well LabTek Chamber slide, Amersham ECL Prime, Tris Hydrochloride (HCl), Glycine, Sodium dodecyl sulphate (SDS), Glycerol, Bromophenol Blue, Dithiothreitol (DTT), Triton X-100, Sodium Phosphate, Sodium Chloride, HEPES, Protran nitrocellulose transfer membrane

Life Technologies (Paisley, UK)

PrestoBlue™ Cell Viability Reagent, Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Vybrant® CFDA-SE Cell Tracer Kit, MagicMark XP Western Protein Standard, SimplyBlue™ SafeStain, GFAP Mouse Monoclonal Antibody (clone 131-17719) Alexa Fluor® 594 Conjugate, CD34 Mouse Anti-Human mAb (clone 581) Fluorescein (FITC) Conjugate, β -Actin Mouse Monoclonal Antibody AC-15, Goat Anti-Mouse IgG (H+L) Horseradish Peroxidase Conjugate, Foetal Bovine Serum Qualified Australia Origin

Lonza (Slough, UK)

EMEM (2.2 g/l sodium bicarbonate 1 g/l glucose Earle's salt 0.0053 g/l phenol red), DMEM (25 mM Hepes 1.0 g/l glucose 1.0 mM sodium bicarbonate 0.011 g/l phenol red), NEAA (100x), L-Glutamine 200mM, Trypsin/EDTA 10x, Sodium Pyruvate Solution 100mM

Millipore

QCM 96-Well Cell Invasion Assay, Guava Mitochondrial Depolarization Kit for Flow Cytometry, Milli-Mark™ Anti-CD90-FITC Antibody clone 5E10, Millicell 12 mm 8.0 µm polycarbonate Cell Culture Insert

Promega (Southampton, UK)

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

Sigma-Aldrich (Poole, UK)

Cisplatin, Aspirin, Dimethyl Sulfoxide (DMSO), Trypan blue, Bovine Serum Albumin (BSA), sucrose, ColorBurst Electrophoresis Marker, RNase, Paraformaldehyde (PFA), Mounting media, Phosphatase Inhibitor Cocktail 2, Protease Inhibitor Cocktail, Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), Copper Sulphate, Sodium pyrophosphate decahydrate, Sodium Deoxycholate, Sodium Fluoride, Ponceau-S, Nutrient Mixture F-10 Ham, Trizma Base, Salicylic Acid, Acrylamide/Bis-acrylamide, 30% solution, ColorBurst™ Electrophoresis Marker, Phenylmethanesulfonyl fluoride, Thiazolyl Blue Tetrazolium Bromide, N,N,N',N'-Tetramethylethylenediamine

Thermo Scientific (Paisley, Scotland)

BCA Protein Assay Reagent A, Western Blotting Filter Paper, Restore Western Blot Stripping Buffer

Vector Laboratories (Peterborough, UK)

Vectashield Mounting Medium with DAPI, ImmEdge Hydrophobic Barrier Pen

The glioma cell lines purchased from the European Collection of Cell Cultures (ECACC) and the American Type Culture Collection (ATCC) were of human origin with no evidence of the presence of infectious viruses or toxic products. ECACC stated that they should be handled as recommend by the Advisory Committee on Dangerous Pathogens (ACDP) for Category 2 containment.

Drugs were prepared as 100mM stock solutions in a maximum of 10% DMSO. Untreated and appropriate vehicle controls were added in all experiments in addition to aspirin and cisplatin treated samples.

2.2 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). 1321N1 and GOS-3 cells were maintained in Dulbecco's Modified Eagle's Medium with 10 % Foetal Bovine Serum (FBS) and L-glutamine (2 mM). The cell lines were cultured in a 37°C humidified atmosphere containing 5% CO₂. The BTNW911 and BTNW914 primary cultures were maintained in HEPES buffered Ham F-10 nutrient mixture supplemented with 10% FBS (Australian Origin) and L-glutamine (2 mM) and were cultured in a 37°C humidified atmosphere. When a maximum confluence of 80% was reached, cell monolayers were washed with phosphate buffered saline (PBS) solution, 3 ml of 1 x trypsin was added, the flasks were returned to the incubator to allow cells to detach, and 7 ml of media was added to deactivate trypsin and resuspend the cells to achieve a single cell suspension. Cells were passaged 1:4 into flasks to maintain the cell line or seeded into dishes for experimental analysis.

2.2.2 Growth Curve

To determine cell growth rates, growth curve analysis was performed over a period of seven days for cell lines. One million cells were seeded in 25 cm² flasks, harvested by trypsinisation daily and cell number determined using a haemocytometer.

2.2.3 Cell Characterization:

Immunofluorescence

The U87 MG, 1321N1, GOS-3 and SVGp12 cell lines were seeded at a density of 20,000 cells per well and cultured overnight on glass slides where 8 chambers had been drawn with a PAP Pen HT™ Slide Marker. After 24 hours incubation, the media was removed and the cells washed three times with PBS before being fixed with 4% paraformaldehyde in 4% sucrose for 15 minutes. The cells were washed three times and permeabilised with 0.4% triton X-100 in PBS for 5 minutes. The cells were again washed three times with PBS, and the appropriate antibody was added, (GFAP, CD34, CD90) in 1:100 dilution for 1 hour. Following a further three washes in PBS, mounting media containing DAPI (2.5 µl per well) was added and the coverslip was secured in place with clear nail varnish. DAPI is a blue-fluorescent nucleic acid stain preferentially staining double stranded DNA but also binds RNA (Tanious *et al.*, 1992). It is a popular nuclear specific counterstain for use in multicolour fluorescent techniques as its blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes of other structures (Life Technologies UK, 2014).

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 1. The images were processed with Zeiss LSM Image Browser.

2.2.4 COX Inhibition Assay

Inhibition of isolated COX-1 and COX-2 enzymes was determined using a COX Fluorescent Inhibitor screening Assay Kit (Cayman Chemical (USA) as per manufacturer's instructions. The COX Fluorescent Inhibitor Screening Assay, screens both COX-1 (ovine) and COX-2

(human recombinant) for isozyme-specific inhibitors, by the peroxidase component of COX, PGG₂, which when exposed to ADHP produces resorufin, a highly fluorescent compound (Cayman Chemical, USA, 2014). Briefly, using black 96-well plates 75 µl of assay buffer, 5 µl of HEME, 5 µl of the enzyme being assayed (COX-1 or COX-2) and 5 µl of each drug (cisplatin, aspirin, PN508, PN517, PN526 & PN529) was added at three final concentrations (10, 1 and 0.1 mM). Following incubation at room temperature for 5 minutes, ADHP (5 µl) was added to all wells followed by 5 µl Arachidonic Acid. After a further 2 minute incubation at room temperature fluorescence was read using an excitation wavelength of 530 nm and an emission wavelength of 585 nm.

2.2.5 Cell Viability:

Thiazolyl Blue Tetrazolium Blue (MTT) Assay

Cells were seeded in 96-well plates at a density of 1×10^5 /ml in a final volume of 90 µl/well. After 24 h incubation, where required drug treatments were added in a 10 µl volume per well and after 24 h treatment, 10 µl of MTT (5mg/ml stock solution in media) was added to each well and the plate returned for 1 h to the incubator. The media was removed, 100 µl of 10% SDS solution was added to each well and the plate was incubated at room temperature overnight, wrapped in foil. Absorbance at 570 nm was determined using a spectrophotometer.

PrestoBlue™ Assay

Cells were seeded in 96-well plates at a density of 1×10^4 /ml in a final volume of 90 µl/well. After 24 h incubation, where required drug treatments were added in a 10 µl volume per well and after 24 and 48 hour treatment, 10 µl PrestoBlue™ was added to each well and incubated

for 2 h. Fluorescence was measured with excitation at 535nm and emission at 610nm using a spectrophotometer.

Linearity Assay

The greatest variable in viability assays is cell number, thus linearity of fluorescence versus cell number was determined. Cells were seeded at 0, 50, 100, 250, 500, 750, 1000, 1500, 2500, 5000, 10,000 and 20, 000 cells/well in a 100µl volume of media and cultured for 24 and 48 hours before incubating with PrestoBlue[®] or MTT as above and the fluorescence or absorbance of each sample being determined.

Drug Concentration Response Assay

Concentration response assays were performed to determine the IC₅₀ values for each of the aspirin analogues in comparison to the commercially available drugs salicylic acid, aspirin and cisplatin on U-87 MG, 1321N1, GOS-3 and SVP-p12 cell lines. Cells were treated with a range of drug concentrations for 24 or 48 hours unless otherwise stated, and viability determined by PrestoBlue[®] assay. Appropriate media and vehicle controls were also added in a 10 µl volume.

Spheroid Viability Assay

Spheroid cultures were established for the U87 M cell line using the method described by Vinci M.et al., 2012. Briefly, cells were seeded at 4x10⁴/ml density per well in round bottomed low adherence plates and typically cultured for x time until the formation of spheroids was observed. Following spheroid formation, drug treatment and cell viability assays were performed as described above using both MTT and PrestoBlue[®] methods.

2.2.6 CFDA-SE Cell Proliferation Assay

Proliferation was determined by 5[6] Carboxyfluoresceindiacetate, succinimidyl ester (CFDA-SE) labelling of cells. Cells were seeded at different densities to take into account individual doubling times (Table 2.1) determined by the growth curve assay.

Table 2.1 Seeding density and assay period for cell proliferation assay

Cell line/culture	Cell Density	Assay Length (days)
SVG P12	1.5x10 ⁵ cells/ml	4
GOS-3	1x10 ⁵ cells/ml	4
1321N1	1x10 ⁵ cells/ml	4
U87 MG	1.5x10 ⁵ cells/ml	4
BTNW911	1.5x10 ⁵ cells/ml	10
BTNW914	1.5x10 ⁵ cells/ml	10

Cells were harvested when 70-80% confluent, and seeded in 6-well plates at the density stated in table 2.1 in a final volume of 1 ml/well. After 24 h incubation, cell monolayers were washed with PBS and 1 ml of PBS-CFDA-SE (5µM) was added to each well and returned to the incubator for 30 min. The PBS-CFDA-SE solution was discarded and the appropriate media was added to each well and incubated for 4 h prior to drug treatment with aspirin, double dose aspirin, cisplatin, PN508 and PN517 at a final concentration of 0.1 mM in 1.5ml of media. Prior to flow cytometric analysis, cells were harvested by trypsinisation and centrifugation and washed with PBS before resuspension in 100 µl of PBS-0.1% BSA in

FACS tubes. If required cells were fixed using a 70% ethanol solution and stored at -20°C before washing with PBS and resuspension in 100 µl of PBS-0.1% BSA in FACS tubes.

Cells were analysed using a FACS-Aria flow cytometer (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). 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 Cell Cycle Analysis

Cell cycle distribution was determined by propidium iodide staining. Cells were harvested when 70-80% confluent, and seeded in 6-well plates at the density stated in table 2.1 in a final volume of 1 ml/well. After 24 h incubation the cells were drug treated with aspirin, double dose aspirin, cisplatin, PN508 and PN517 at a final concentration of 0.1 mM in 1.5ml of media. After a further 24 and 48 h incubation cells were harvested by trypsinisation, washed with 0.5ml of 1% BSA in PBS and centrifuged at 224 G for 5 minutes. The wash solution was discarded and the cells were fixed in 500µl of ice cold 70% EtOH-PBS while vortexing before being stored at -20°C. Prior to labelling with PI, the cells were centrifuged at 1000rpm for 5 minutes and resuspended in 300µl total volume of RNase (10mg/ml) and PI (50µg/ml), and incubated in room temperature for 30 minutes.

Cells were analysed using a FACS-Aria flow cytometer as previously described (2.2.6). The DNA profile was optimized to adjust the G0/G1 peak to appear around channel50. Cell-cycle software used algorithms that correctly modelled the four cell-cycle compartments to determine the percentage of cells in each of the cell cycle phases G0/G1, S, and G2/M phases. A total cell population of 10,000 gated events were acquired for each treatment.

2.2.8 SDS-PAGE and Western Blotting

Sample Preparation

Following drug treatment cells were harvested by scraping in ice cold PBS and pelleted by centrifugation. Lysates were prepared using a standard RIPA buffer in the presence of protease and phosphatase inhibitors by shaking at 4°C for 1 h. The resulting lysate was centrifuged at 224 G for 5 minutes and the resulting supernatant retained.

Bicinchoninic Acid (BCA) Protein Assay

BCA working reagent is prepared fresh by mixing CuSO_4 and the BCA reagent A (1:50). Standards (0 to 2.0 mg/ml BSA) and unknown lysate samples (diluted 1:5 and 1:10) (20 μl) were incubated with 0.2ml of the working reagent for 1 h at 37°C and absorbance measured at 562 nm. Calculation of unknown lysate protein concentration was by comparison to the BSA standard curve fitted using linear regression. Lysates were mixed with 4x loading buffer (2% SDS, 2mM beta-mercapto-ethanol, 4% glycerol, 0.04 M Tris-HCL and 0.01% Bromophenol blue) sample buffer lysis buffer) to give a final volume of 40 μl containing 100 μg of protein and heated at 95°C for 5 min prior to analysis.

SDS-PAGE

Samples were separated using either 10% (EGFR, Cyclin D1) or 15% (caspases 3, 8 & 9) mini gels using a standard protocol. Briefly, the separating gel mix was prepared and poured upon the addition of TEMED, overlaid with 0.5ml of 0.1% SDS solution, and left to polymerise for 45 min. The SDS solution was discarded and the stacking gel was added and again allowed 45 min for polymerization. The comb and excess un-polymerized stacker were removed, and 40 µl samples were wet-loaded, and samples separated using a 100V constant current through the stacker raised to 200V constant current in the resolving gel. Prior to semi-dry transfer, the gel, nitrocellulose membrane and filter paper were placed in transfer buffer for 20 minutes. Transfer was performed using the Bio-Rad Trans-Blot Semi-Dry kit assembled as described in kit guide with a constant 30 volts applied for 25 minutes.

Table 2.2 SDS-PAGE gel constituents

	10% Separating Gel	15% Separating Gel
H ₂ O	2.2 ml	3.8 ml
Acrylamide	5 ml	3.4 ml
1.5 Tris	2.6 ml	2.6 ml
10% SDS	0.1 ml	0.1 ml
10% ammonium Persulfate	0.1 ml	0.1 ml
TEMED	0.01 ml	0.01 ml

Stacking Gel	
H ₂ O	2.975 ml
Acrylamide	0.67 ml
0.5 Tris-HCL	1.25 ml
10% SDS	0.05 ml
10% ammonium Persulfate	0.05 ml
TEMED	0.005 ml

Western Blotting

The membrane was blocked with TBS-T solution containing 5% skimmed milk powder for 2 hours before being exposed to diluted primary antibody (1:1000 unless otherwise stated) in TBS-T containing 5% BSA and 0.1% tween-20 at 4°C with gentle shaking overnight. Following three 10 minute washes with TBS-T solution containing 5% skimmed milk powder the membrane was incubated for 2 hours with the appropriate HRP conjugated secondary antibody (1:5000 unless otherwise stated) in 5% milk TBS-T. Finally, the membrane was washed three times for 5 minutes with TBS-T and exposed to ECL solutions for 5min prior to visualisation using a BioRad ChemiDoc system. Membranes were typically re-probed after stripping with Restore Western Blot Stripping Buffer as per manufacturer's instructions and confirmation of the strip using ECL solution.

2.2.9 Apoptosis Assay, Annexin-V/Propidium Iodide

Induction of apoptosis was examined in the U-87 MG,1321N1, GOS-3 and SVG-p12 cell lines following drug treatment for 4, 8, 12 24 and 48 hours. Cells were seeded at a density described in table 2.1, cultured for 24 hours and drug treated with cisplatin, aspirin, PN517 and PN508 at 0.1 and 1 mM. Subsequently, the cells were washed with PBS and harvested by trypsinisation and centrifugation. A further 100µl of binding buffer was added and the samples transferred to FACs tubes.

Cells were analysed using a FACSaria as described previously (section 2.2.7) with green fluorescence collected in the FL1 channel (530 ± 30 nm) and red in the FL2 channel (615 ± 25 nm).

2.2.10 Caspase- Glo 8 & 9 Assay (Promega, UK)

Caspase-Glo[®] are luminescent assays that determined caspase activity based on the production of a luminogenic substrate following cell lysis. As per manufacturer's instructions, cells were seeded at a density of 10,000 cells per well in 90 µl of media in white walled opaque clear bottomed 96 well plates and incubated overnight. Following drug treatment with cisplatin, aspirin or PN517 (10µl addition) at 1mM for 12, 24 and 48 hours 100 µl of Caspase-Glo 8 or 9 was added to each well, the plate 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.

2.2.11 Migration

2.2.11.1 Migration

Complete media (300 µl) was added to each well of a 24-well plate, where appropriate containing either drug treatments (cisplatin, aspirin, PN517) at 0.1 mM or vehicle control (0.1% DMSO). Millicell 8.0 µm polycarbonate cell culture inserts were placed in each well and following a brief equilibration period cells were seeded in serum free media in the inserts at a density of 1.5×10^5 /ml in a final volume of 100 µl/chamber also containing drug treatment or vehicle control where appropriate. After 6 hours incubation, the inserts were transferred to a fresh 24-well plate containing 200 µl PBS and following a brief wash placed into another fresh plate, this time containing 200 µl trypsin to allow cells to be detach. Following 5 minutes incubation at 37°C, the optimum time determined for trypsinisation, 200 µl media was added, and cell density calculated using Trypan blue staining and counting on a haemocytometer.

2.2.11.2 Migration Scratch Assay

Cells were plated in a 24-well plate for 24 hours at a density of 250,000 cells per well. Prior to drug treatment, a sterile cocktail stick was used to make a scratch in the middle of the well, and media was replaced and drugs treatments added. Each well was imaged every two hours over an 18 hour period and the scratch size measured using Zeiss ZEN imaging software.

2.2.12 QCM 96-Well Cell Invasion Assay (Millipore, UK)

The cell invasion assay was performed as per manufacturer's instructions. Briefly, prior to seeding for the invasion assay, the cells were placed in serum free media for 24 hours. Before addition of cells, the plate was rehydrated/equilibrated with 100 µl of serum free media for 2

hours at room temperature. The equilibration media was removed, and 150 μ l of complete media (chemo attractant) added to the lower chamber. Following standard trypsinisation, the cells were seeded at 2×10^4 cells per well in the 96 well plate in a 100 μ l volume of serum free media containing where appropriate either drug treatment, aspirin, cisplatin or PN517 at 0.1mM, or vehicle control, 0.1% DMSO. The plate was then incubated for 12 hours at 37°C. Following incubation, the media was discarded from the upper chamber and the inserts were placed in a fresh 96-well plate containing 150 μ l PBS and incubated for 1 minute. The PBS was replaced with 150 μ l of pre-warmed cell detachment solution and incubated for 30 minutes at 37°C. Following gentle tilting of the plate to ensure complete detachment, lysis buffer and dye solution (50 μ l) were added to each well of the 96-well plate and incubated for 15 minutes at room temperature. 150 μ l of the resulting mixture was transferred to a fresh 96 well plate and fluorescence was read using 480 nm excitation and 520 nm emission filters.

2.2.13 Statistical Analysis

Results of cell viability and proliferation assays were expressed as a percentage of control untreated cell populations and are expressed as means \pm SEM of at least three independent experiments with consecutive cell passages. Comparison between experimental groups was performed using two-way ANOVA with Tukey's post-hoc test. P-values ≤ 0.05 were considered as statistically significant and indicated in the figures as follows: *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

Chapter 3: Cell and Drug Characterization and Cell Viability

3.1 Introduction

The project aims to characterise the effects of a number of aspirin analogues initially in glioma cell lines. To confirm the validity of these results a standard approach is to first characterise the cell lines using immunohistochemistry. This method combines anatomical, immunological and biochemical techniques to identify discrete tissue components by target antigens with antibody tagged labels to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context. IHC is used in diagnosis, prognostication, therapeutic decisions to individual of patients, and investigations into the pathogenesis of a disease (Ramos-Vara and Miller, 2014).

In the characterisation of glioma cells, a range of markers are commonly used including Glial Fibrillary Acidic Protein (GFAP), CD34 and CD90. GFAP is a protein that is expressed in the central nervous system in astrocyte cells (Halliday *et al.*, 1996). GFAP is important in astrocyte motility and shape by providing structural stability to astrocytic processes. If brain or spinal cord cells are injured through trauma or disease, astroglial cells react by rapidly producing more glial fibrillary acidic protein (Eng *et al.*, 2000). As such, the filament protein GFAP is used as a specific astrocytic cell marker (Jacque *et al.*, 1978). CD34 a cell surface glycoprotein and functions as a cell-cell adhesion factor and has been widely used as a marker to assist in the identification of hematopoietic stem cells (HSCs) and progenitors in preparation for bone-marrow transplantation (Nielsen and McNagny 2008). It functions as an angiogenesis marker, as prognostic marker of recurrence for prostate cancer (Bettencourt *et al.*, 1998), and to identify endothelium in glioblastoma tissue (Charalambous *et al.*, 2006). CD90, also known as the Thy-1 antigen, is a glycosyl phosphatidylinositol-linked cell surface protein (Buccisano *et al.*, 2004) that is part of the immunoglobulin supergene family

(Mansour Haeryfar *et al.*, 2005). CD90 is expressed on primitive hematopoietic stem cells in normal bone marrow, cord blood, and foetal liver cells and has been identified as a stem cell marker in glioma (He *et al.*, 2012).

While it is known that the aspirin analogues act against colorectal cancer cells, the mechanisms involved have not been fully elucidated, and nothing is known about their effects in glioma. The cyclooxygenase (COX) enzymes, COX-1 and COX-2, convert arachidonic acid to prostaglandins resulting in pain and inflammation *in vivo*. Cyclooxygenase converts arachidonic acid to a hydroperoxyendoperoxide (PGG₂), and the peroxidase component reduces the endoperoxide to the corresponding alcohol (PGH₂), the precursor of PGs, thromboxanes, and prostacyclins (Vane and Botting, 2003). Traditional NSAIDs are considered nonselective because they inhibit both COX-1 and COX-2, but the inhibition of COX-2 accounts for the anti-inflammatory effect of the drugs while the inhibition of COX-1 can lead to toxicity and associated side effects including ulcers, prolonged bleeding time, kidney problems (Schorr, 2009). Aspirin produces greater inhibition of COX-1 than COX-2 and thus it has been suggested that there is a need to synthesise analogues that show greater selectivity for COX-2 to undesirable side effects (Deb *et al.*, 2011).

Cell viability testing gives an overview of the effects of drug treatment on the general health of a population of cells and is the primary method of drug screening for characterisation of new compounds. A wide variety of assays are available including the MTT, or (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and PrestoBlue assays, each with their advantages and disadvantages (Mosmann, 1983; Lu *et al.*, 2012).

The first *in vitro* chemo-sensitivity test was the colony forming (clonogenic) assay, established in the 70s-80s which examines the ability of a treatment to alter colony formation (Rosenblum *et al.*, 1975). However, as the clonogenic assay is time consuming, a wide range of colorimetric assays were developed, one of which that is still widely used in research being the MTT assay. MTT, or (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole first described by Tim Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase enzyme to reduce the yellow coloured substrate to purple formazan product in the mitochondria of living cells (Mosmann, 1983). A major disadvantage of this assay is that the MTT formazan product is a crystalline precipitate that requires solubilisation before readings can be taken. In addition to requiring the use of an appropriate solvent, the solubilisation causes cell lysis and death preventing the use of samples for further assay. An additional limitation of the MTT assay is the amount of time needed to accurately measure a treatment effect. Cultured cells that are undergoing apoptosis *in vitro* undergo secondary necrosis as well. For instance, extended incubation would cause apoptotic cells shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium. Therefore if apoptosis is the primary mechanism of cell death, understanding the kinetics of the cell death process is critical (Cory *et al.*, 1991). Bearing this in mind, an assay that requires only a few minutes to produce a measurable signal would give information representing a snapshot in the specific time, rather than after several hour incubation generated signal (Dawson *et al.*, 2011).

In response to these disadvantages, the PrestoBlue™ cell viability reagent was developed to offer highly reproducible cell viability results and instant time savings (Fejerskov and Zelikin, 2012). The PrestoBlue™ reagent is a resazurin-based solution that functions as a cell

viability indicator by using the reducing power of living cells to quantitatively measure the proliferation of cells (Lu *et al.*, 2012).

Hypothesis

GFAP staining, as an astrocytic cell marker, should stain the higher grade glioma cell line more intensely than the control cell line, (SVG-p12 < 1321N1 < GOS-3 < U87 MG). Also, the cell lines should not contain stem cell like cells, and therefore staining with CD34 and CD90 should not be present at all, working as negative controls.

Aspirin is known to inhibit both COX-1 and COX-2 enzymes, affecting COX-1 to a greater degree, and was used as a positive control in comparison to the aspirin analogues. The aspirin analogues will inhibit COX enzyme activity, but with differing efficacies. Cisplatin, not a known inhibitor of COX, will serve as a negative control.

The MTT and PrestoBlue assays differ in mechanism, but largely reflect changes in proliferation and apoptosis, so the results of the two assays will be very similar, and justify the use of the latter assay for subsequent screening. Aspirin reduces glioma cell viability in a concentration and time dependent manner, thus it is expected that the analogues will display similar activity, but with differing efficacies.

3.2 Results

3.2.1 Immunohistochemistry

Each of the established cell lines were probed with directly labelled fluorescent antibodies to glial fibrillary protein and CD34 with a DAPI nuclear counterstain (Fig 3.1; Table 3.1). The four cell lines labelled positively for GFAP, although the degree of staining varied, with the SVG-p12 cell line displaying the lowest level of GFAP staining. Unexpectedly, the 1321N1 cell line also showed specific labelling of the endothelial cell marker CD34.

As each of the short term cultures were probed with the CD90 antibody to identify cancer stem cells, although these would not be present in the cell lines it was decided to test them as a negative control (Fig 3.2; Table 3.1). Interestingly, both the 1321N1 and GOS-3 cell lines displayed a high level of CD90 antibody staining in a pattern consistent with other published results (He *et al.*, 2012). Neither the SVG-p12 nor U87 MG cell lines showed any specific CD90 binding.

GFAP, as expected, stained more intensively in the higher grade glioma cell line, (SVG P12, 1321N1, GOS-3 and then U87 MG). Unexpectedly, there was CD34 binding in the 1321N1 cell line, and CD90 binding in 1321N1 and GOS-3. As a series of antibody dilutions were performed and a lack of binding was observed in some cell types, the staining is not due to non-specific binding, but could be as a result of binding to an unknown protein that contains a similar antigenic sequence.

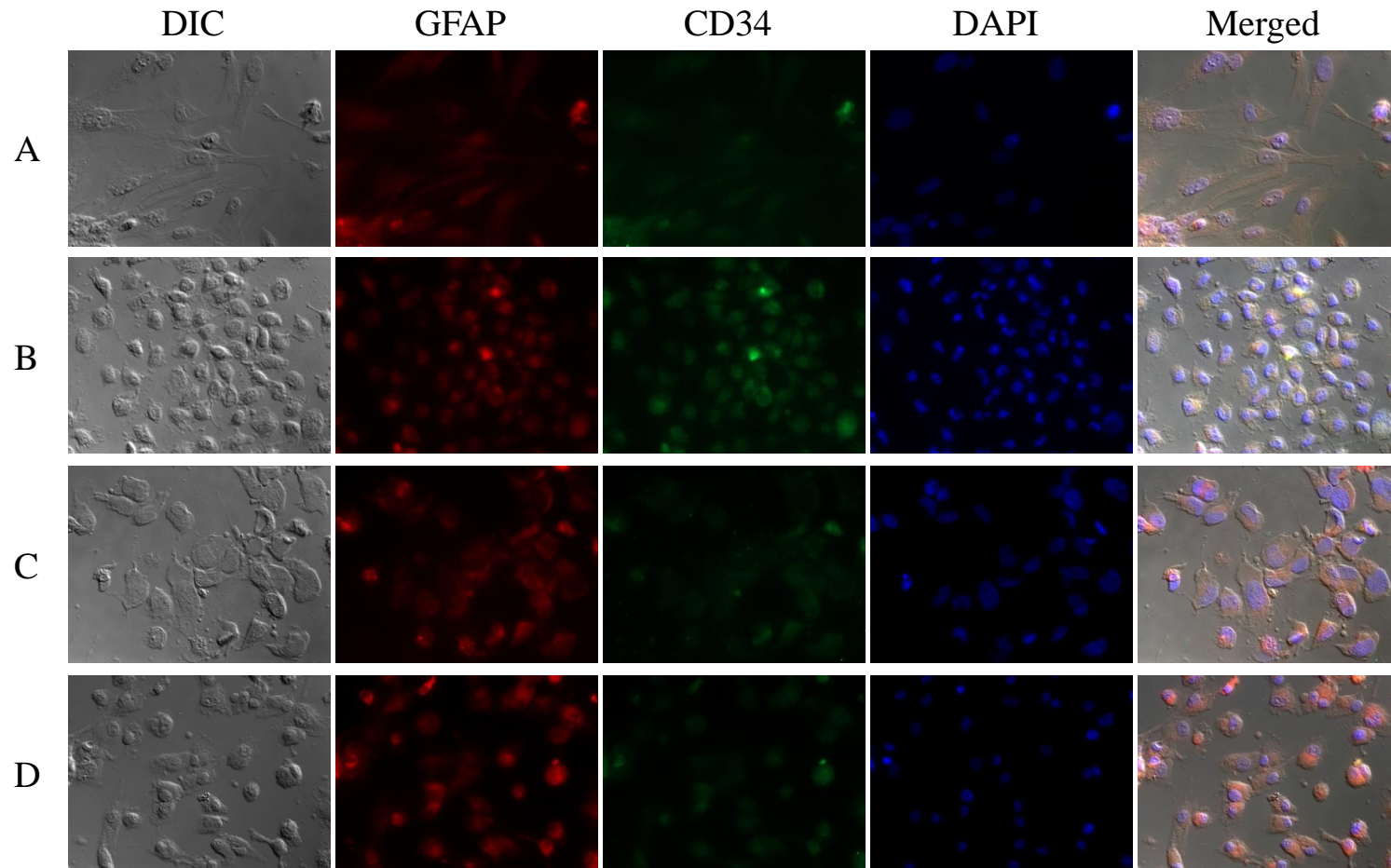


Figure 3.1 Immunofluorescent staining of the SVG-p12, 1321N1, GOS-3 and U87 MG cell lines for glial fibrillary protein (GFAP) and CD34 with a DAPI nuclear counterstain. GFAP was labelled with an alexa flour 594 conjugated mouse monoclonal antibody (clone 131-17719) and CD34 with FITC conjugated mouse anti-human monoclonal antibody (clone 581). Row A represents SVG-p12 cells, row B 1321N1, row C GOS-3, and row D U87 MG.

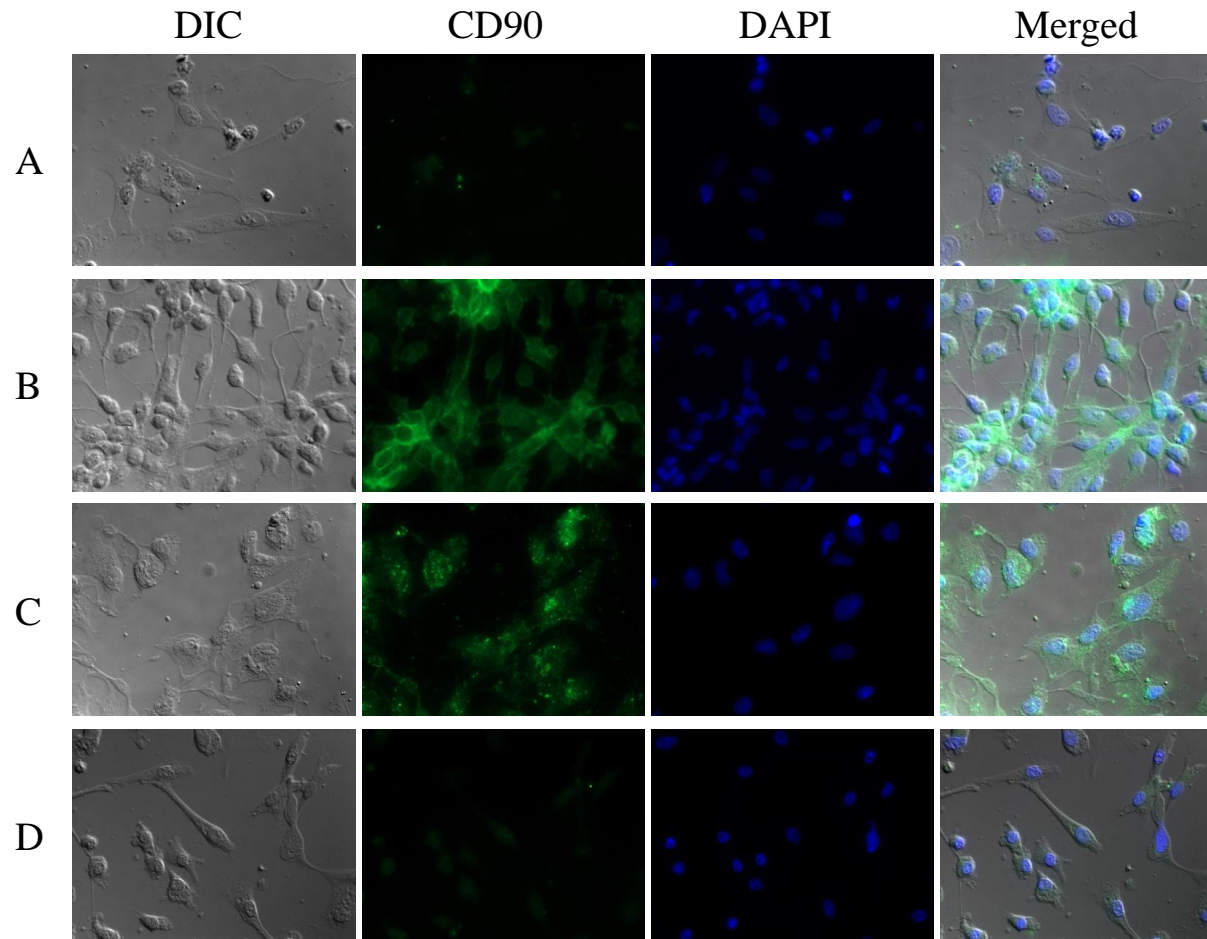


Figure 3.2 Immunofluorescent staining of the SVG-p12, 1321N1, GOS-3 and U87 MG cell lines for CD90 with a DAPI nuclear counterstain. CD90 was labelled with a FITC conjugated mouse monoclonal antibody (clone 5E10). Row A represents SVG-p12 cells, row B 1321N1, row C GOS-3, and row D U87 MG.

Table 3.1 Fluorescence intensity quantification of CD34, CD90 and GFAP staining in cell lines. CD34, CD90 and GFAP expression were detected using directly labelled immunofluorescent antibodies in sequential cell passages, 1321N1, GOS-3, U87-MG glioma and SVG-p12 astrocyte cell lines. Fluorescence was quantified using Zeiss Zen software and median values determined from three independent experiments.

	<i>1321N1</i>			<i>GOS-3</i>			<i>U87 MG</i>			<i>SVG p12</i>		
	Median	Min	Max	Median	Min	Max	Median	Min	Max	Median	Min	Max
<i>CD34</i>	322	266	1510	284	226	1334	282	225	1311	306	247	1138
	334	270	1117	317	242	1490	370	293	1440	320	248	1129
	406	316	1208	332	264	1618	353	284	1407	320	251	1128
<i>Mean</i>	354	284	1278	311	244	1481	335	267	1386	315	249	1132
<i>CD90</i>	767	352	3425	434	294	1511	406	332	1487	319	256	1542
	643	322	3256	538	334	2575	344	271	1443	340	271	1264
	453	280	2670	383	259	1400	299	239	1354	339	271	1310
<i>Mean</i>	621	318	3117	452	296	1829	350	281	1428	333	266	1372
<i>GFAP</i>	357	284	2042	358	278	1102	329	261	1847	925	784	2134
	366	294	1272	375	281	1090	394	304	1910	822	706	1987
	357	274	1133	347	283	3261	414	328	2063	730	632	1928
<i>Mean</i>	360	284	1482	360	281	1818	379	298	1940	826	707	2016

3.2.2 COX Inhibition

There are two distinct isoforms of COX, COX-1 and COX-2, and the inhibition of both by the aspirin analogues used in this study (PN508, PN517, PN526 and PN529) in addition to the control drugs were tested. As expected, in comparison to a non-treated control, the selective inhibitors SC-560 and DuP-697 significantly reduced COX-1 and COX-2 activity. The control drug SC-560 reduced COX-1 activity to 8% of control, while DuP-697 reduced COX-2 activity to 38% of control ($p < 0.01$).

COX inhibition by aspirin and the analogues was found to be concentration dependent over the range used 0.1, 1 and 10mM ($p < 0.05$). COX-1 (28.5% of control) was inhibited more than COX-2 (85% of control) by 10 mM aspirin ($p < 0.001$) (Fig 3.3, panel A and B). Cisplatin showed a greater inhibition of COX-2. The analogues showed a range of effect with PN529 producing the greatest effect in COX-1, reducing activity to 25.6% of control ($p < 0.0001$), and PN526 producing the smallest effect for COX-1, reducing activity to 80% of control (panel C) ($p < 0.05$). In all cases, the lowest concentration did not significantly reduce COX-1 activity compared to control. At 10mM, PN508 reduced COX-1 activity to 76% of control, and PN517 to 61.5% ($p < 0.001$). With respect to COX-2 activity, aspirin was found to inhibit activity to 85% of control at 10mM (panel B), and each analogue showed a concentration dependent inhibition of activity (panel D). Again, PN529 showed the greatest inhibition of enzyme activity, reducing it to 51% of control at 10mM, followed by PN526 (57.6%), PN517 (73%) and PN508 (76.6%) ($p < 0.05$).

In summary, COX-1 activity was reduced most by aspirin and PN529 followed by PN517, PN508, PN526 and then cisplatin. The aspirin analogues produced the largest inhibition of COX-2 activity (PN529 > PN526 > PN517 > PN508), greater than that of aspirin.

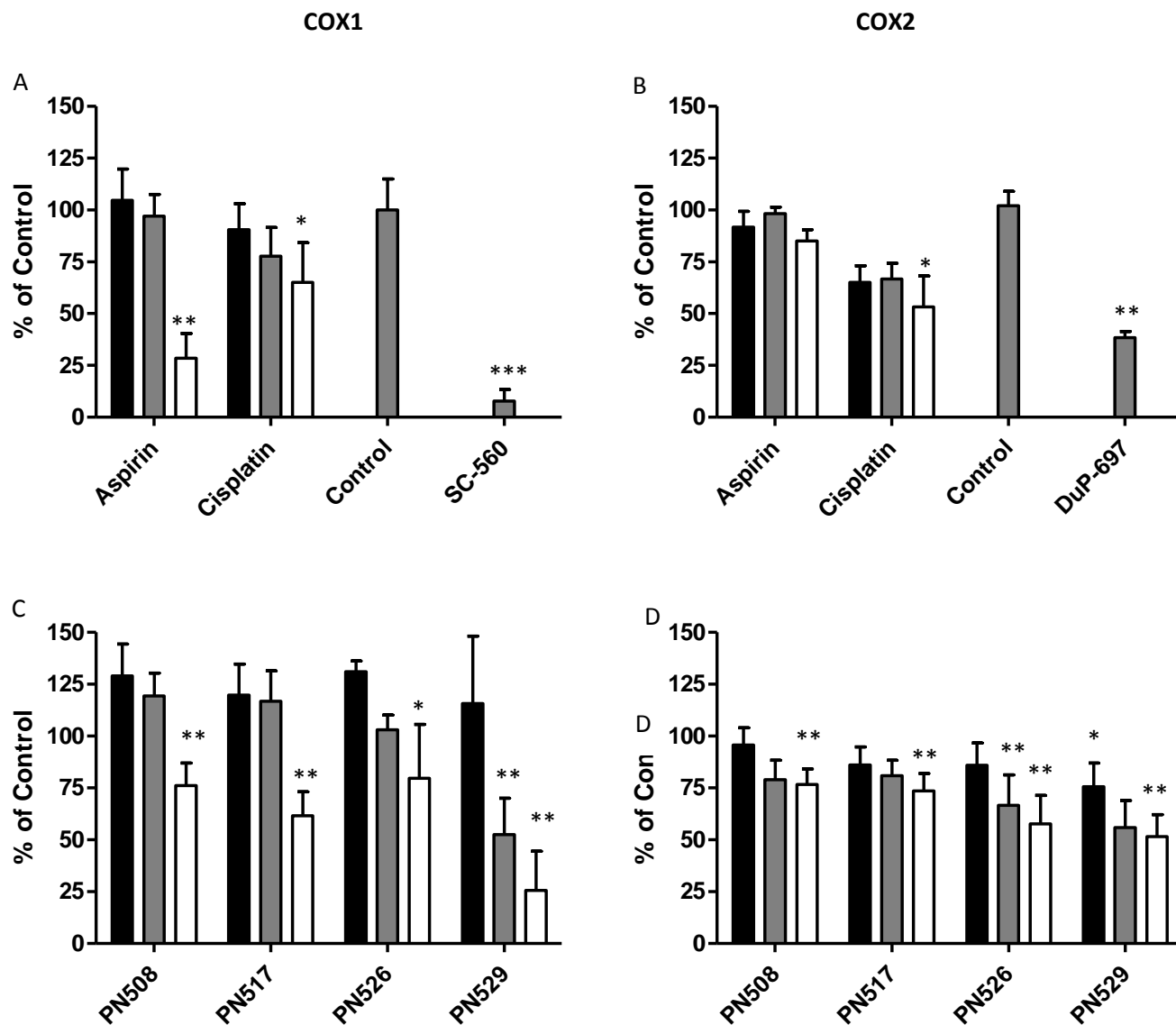


Figure 3.3 Inhibition of COX-1 and 2 enzymes by the aspirin analogues using the COX Fluorescent Inhibitor Screening Assay Kit by Cayman Chemical. The data illustrate COX inhibition by the aspirin analogues PN508, PN517, PN526 and PN529 and the control drugs aspirin and cisplatin at three concentrations (0.1 mM black bar, 1 mM grey bar and 10 mM white bar) for 7 minutes. Panels A and C illustrate COX-1 inhibition (SC-560 standard inhibitor) and panels B and D COX-2 inhibition (DuP-697 standard inhibitor). Enzyme inhibition is expressed as a percent of control with significance ($p < 0.05$) calculated by ANOVA. Data are representative of mean \pm SEM of three independent experiments.

3.2.3 Growth Curves

Growth curves can provide vital information, doubling time, lag time, and saturation density. This is normally the time needed for recovery after subculturing and the time needed to attach and spread, the phase where the number of cells increase, and plateau phase where the growth rate slows because the cells are confluent (Mather *et al.*, 1998). As a number of cell lines were used, growth curves were performed to identify the doubling times of each cell line and any significant difference between them (Fig 3.4). Cells were plated at 1×10^4 /ml, and were counted using a hemocytometer. SVG-p12 cell line showed a doubling time of 48 hours, U87 MG doubled every 36 hours, and both GOS-3 and 1321N1 cell lines doubled every 24 hours. There was no significant difference in proliferation between the 1321N1 and GOS-3 cell lines over the period of the assay, with both proliferating significantly more rapidly than the U87MG and SVG-p12 cell lines ($p < 0.001$). A difference in doubling time between U87 MG and SVG-p12 cells was only significant after day 5 ($p < 0.001$).

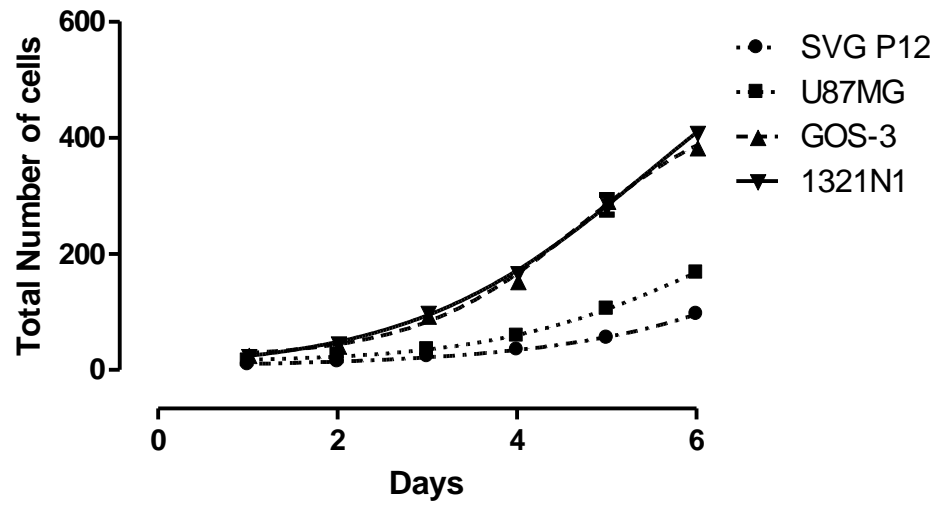


Figure 3.4 Growth curves over six days for the SVG-p12, 1321N1, GOS-3 and U87 MG cell lines. Growth curves for SVG-p12 (circle), U87 MG (square), GOS-3 (triangle) and 1321N1 (inverted triangle) are plotted over 6 days, showing the growth and plateau phase. Data are representative of mean \pm SEM of three independent experiments.

3.2.4 Cell Viability

MTT *versus* PrestoBlue

As the PrestoBlue assay has only recently been developed, it was decided to perform a comparison between it and the established MTT assay. MTT is reduced to a formazan product when mitochondrial reductase enzymes are active; consequently the conversion is related to the number of viable cells. The amount of purple formazan product produced by untreated control cells is quantified by absorbance spectroscopy and compared to the levels in treated cells. Using this simple comparison the effect of drug treatment on cell viability can be deduced by constructing a concentration-response curve. The MTT method has been used to measure cell viability in response to mitogens, antigenic stimuli, growth factors and other cell growth promoting reagents, and forms the basis of cytotoxicity studies, and in the derivation of cell growth curves. In the PrestoBlue assay the blue cell-permeant substrate, risazurin, is non-fluorescent when added to the cells, but when modified by the reducing environment of viable cells is converted into resorufin, a red and highly fluorescent product. As a result, cell viability can be rapidly detected with either fluorescence or absorbance measurements. Additionally, as neither the risazurin substrate nor the resorufin product are cytotoxic, the PrestoBlue™ reagent allows the development of live-cell assays for real-time monitoring of cell metabolism and viability and the recovery of cells following assay for further culturing or use in a subsequent experiments.

This comparison examined both the relative sensitivity of the assay, in terms of its ability to detect changes in cell number (Fig 3.5 and 3.6, panels C and D), and also in terms of IC₅₀ value determined for the control drug cisplatin (Fig 3.5 and 3.6, panels C and D). Using the primary cells, a direct comparison of the PrestoBlue and MTT assays showed an equal ability

to detect large cell numbers ($p < 0.0001$) (Fig 3.5 and 3.6, panel C) but that the sensitivity of the PrestoBlue assay was greater at low cell numbers (Panel D) and was in fact able to detect as few as 10 cells (data not shown). In terms of drug induced reductions in cell viability, in the BTNW911 cells, no difference was found in the IC_{50} values for aspirin or cisplatin (Fig 3.5), however, in the BTNW914 cells, the PrestoBlue assay appeared more sensitive to drug induced changes in viability for both drug treatments, with a significant difference in aspirin treatment between the two assays ($p < 0.0001$) (Fig 3.6). This demonstrates that the results of standard MTT were confirmed with PrestoBlue, and therefore is appropriate to use the latter assay for subsequent testing.

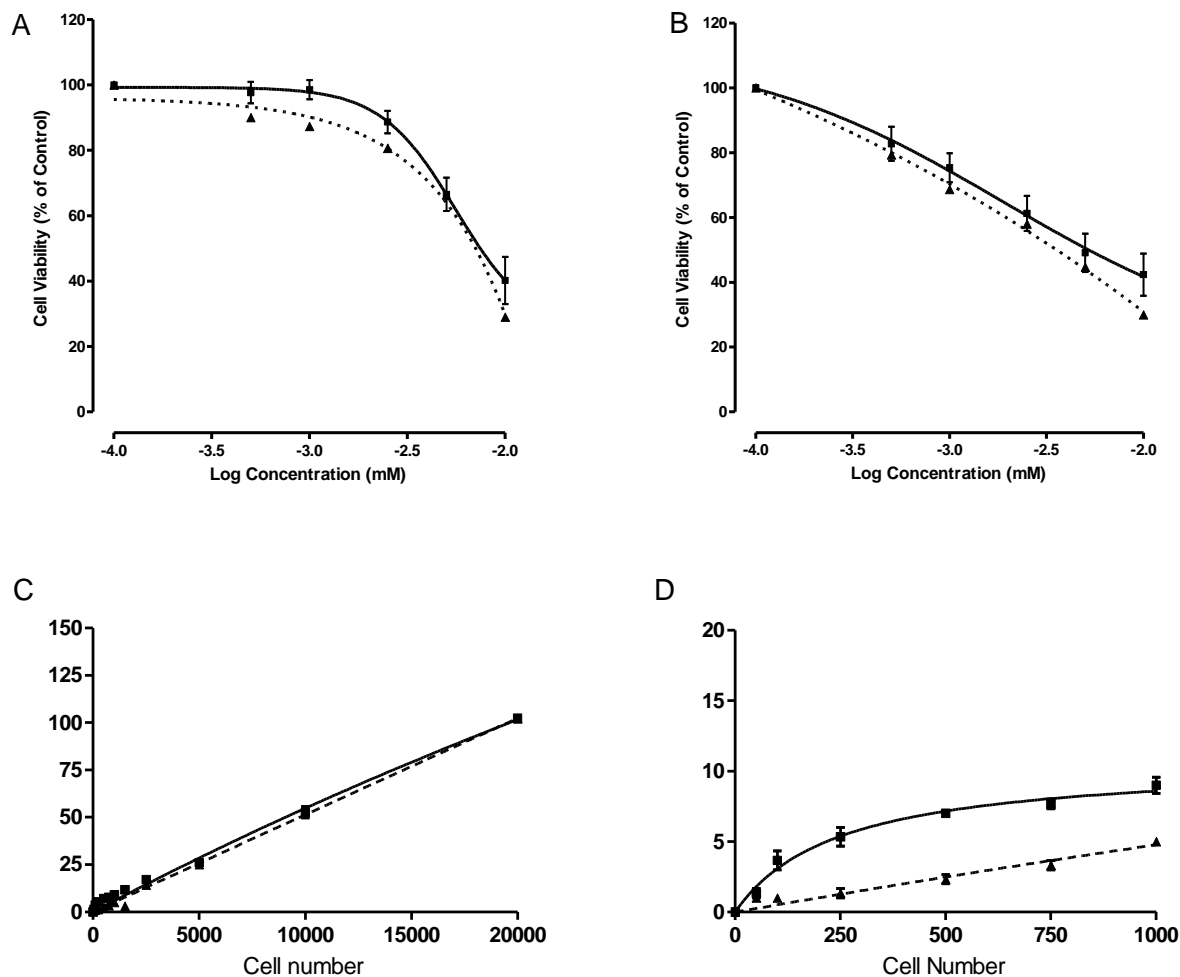


Figure 3.5 Comparison of MTT and PrestoBlue cell viability assays following 24 hours drug treatment in the BTNW 911 primary culture. The data illustrate cell viability expressed as a percent of control determined using the PrestoBlue™ (squares) and MTT (triangles) assays in the BTNW 911 primary culture following drug treatment for 24 hours (aspirin in panel A & cisplatin in B) using 1000 cells per well, and with increasing cell numbers (50 – 20,000 cells per well - panel C; 50 – 1000 cells per well – panel D). ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$), and no significant difference between MTT and PrestoBlue IC_{50} values, with data representing results from 3 independent experiments.

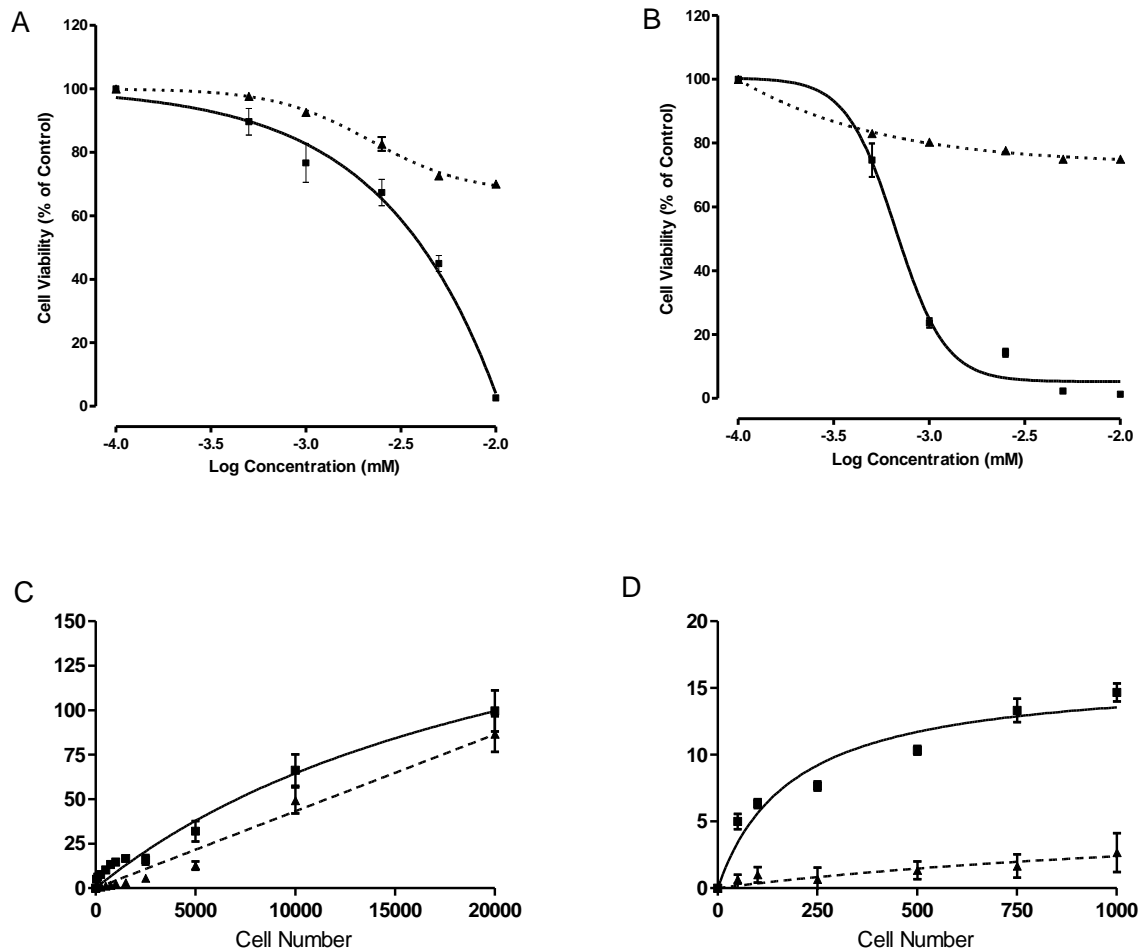


Figure 3.6 Comparison of MTT versus PrestoBlue assay for the detection of cell viability following 24 hours drug treatment in the BTNW 914 primary cell culture. The data illustrate cell viability determined using the PrestoBlue™ and MTT assay on BTNW 914 cell culture following drug treatment for 24 hours. Viability of MTT (triangles and dashed line) and PB (squares and solid line) assay to detect effects on cell viability following aspirin (panel A) and cisplatin (panel B) drug treatment for 24 hours. The lower panels illustrate the relationship between cell number and viability for both the MTT and PB assays, up to 1000 cells per well (panel D) and 20 000 cells per well (panel C). ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$), and significant differences in IC_{50} values for cisplatin (panel A) and aspirin (panel B), data representing results from 3 independent experiments.

Aspirin Analogue Characterisation

Initial investigations aimed to characterise the effect of aspirin and its analogues on cell viability using established cell lines for grade II – IV glioma, specifically 1321N1 - a grade II astrocytoma, GOS-3 - a grade II/III mixed astro-oligodendroglioma, and U-87 MG - a grade IV glioblastoma. Experimental controls included the SVG P12 foetal glial cell line and the standard chemotherapeutic drug cisplatin.

SVG P12 Viability

All compounds tested decreased the viability of the SVG P12 cell line in a concentration dependent manner following both 24 (Fig 3.7) and 48 hours of drug treatment (Fig 3.8). The aspirin analogue PN517 showed the greatest inhibition of cell viability with an IC_{50} value of 1.2 mM at 24 hours ($p < 0.001$), approximately half that of cisplatin (Fig 3.7, panel A) ($p < 0.05$). The IC_{50} values for aspirin and aspirin analogues remain similar at 48 hours, but as expected given its mechanism of action, the IC_{50} for cisplatin decreased to 0.6 mM (Fig 3.8 and 3.9) ($p < 0.0001$).

1321N1 Viability

In a similar manner to the control cell line, each of the compounds tested decreased the viability of 1321N1 cell line in a time and dose response manner (Fig 3.10). Following 24 hours of treatment, with the exception of PN508 which only reduced cell viability to approximately 60% at its highest concentration, the aspirin analogues PN517, 526 and 529 displayed similar potency. Following 48 hours of incubation, the IC_{50} value for PN517 of 2 mM ($p < 0.001$) was similar to the value observed with the control cell line (Fig 3.11, panel A). The control drug cisplatin produced a consistent large decrease in cell viability, with an

IC₅₀ value of 0.5 mM at 24 hours, dropping to 0.2 mM at 48 hours ($p < 0.001$) (Fig 3.11, C and 3.12, C).

GOS-3 Viability

The GOS-3 cell line represents a grade II/III mixed astro-oligodendroglioma. As with the previous cell lines, each of the compounds reduced cell viability in a concentration dependent manner following 24 hours of treatment (Fig 3.13), however, the analogue PN529 only reduced viability by approximately 50%. Interestingly, at 48 hours the same analogue had only a marginal effect on cell viability (Fig 3.14). As with the previous cell lines, PN517 displayed the greatest effect on cell viability, with an IC₅₀ value at 24 hours of 0.6 mM ($p < 0.001$), very similar to cisplatin with an IC₅₀ value of 0.5 mM ($p < 0.001$) (Fig 3.13 panel C). In addition to using aspirin and salicylic acid as controls, a treatment that represented a double dose of aspirin was used and its effect on cell viability assessed following 24 and 48 hours (Fig 3.14 and 3.15, panel B). Following 24 hours the three treatments produced very similar results with IC₅₀ values of approximately 3 mM ($p < 0.001$) (Fig 3.13, panel B). However, after 48 hours, while salicylic acid had only a marginal effect on cell viability, aspirin had an IC₅₀ of 3.1 mM ($p < 0.001$) and the double dose 1.7 mM ($p < 0.001$) (Fig 3.14, panel B).

U87 MG Viability

The most common primary brain tumour in adults is glioblastoma multiforme (GBM), a tumour represented by the U87 MG cell line. Following both 24 (Fig 3.16) and 48 (Fig 3.17) hours of treatment, all the compounds tested decreased cell viability. In line with results from the previous cell lines, the most potent analogue at both 24 and 48 hours was PN517 with an

IC₅₀ value of 1.5 mM and 1.8 mM respectively ($p < 0.001$) (Fig 3.16 and 3.17). Interestingly, PN517 appeared to be more potent in this cell line at reducing viability than the control drug cisplatin which had IC₅₀ values of 3.1 mM and 2.5 mM in 24 and 48 hours respectively. However, this apparent effect was not statistically significant. As with the GOS-3 cell line the PN529 analogue had the least effect on viability producing a maximum reduction at 24 hours of only 40% ($p < 0.001$) (Fig 3.16).

To summarise, based on glioma grade, the treatment with the greatest efficacy in comparison to control was cisplatin in 1321N1, PN517 in GOS-3, PN517 in U87 MG and also PN517 in the SVG-p12 embryonic cell line. This shows that PN517 is the most potent aspirin analogue in terms of reduction of cell viability, with comparable results to cisplatin even after 48 hours, although the analogues displayed a greater effect in some cases at 24 hours. These results have identified the most potent aspirin analogue and identified treatment concentrations for subsequent experiments.

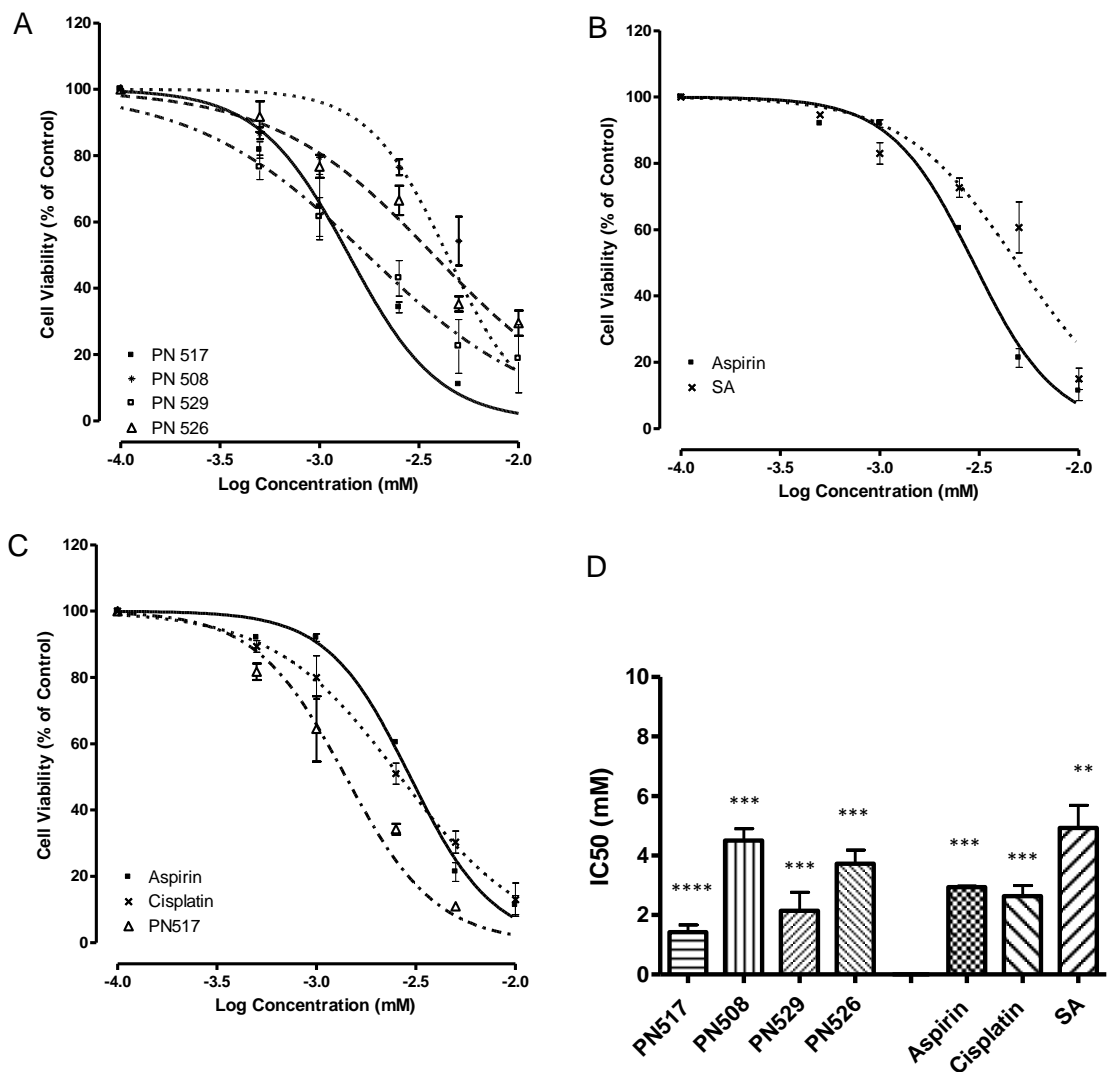


Figure 3.7 Cell viability following 24 hours drug treatment in SVG-p12 cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on the SVG-p12 cell line following drug treatment for 24 hours. Panel A illustrates cell viability following treatment with aspirin analogues, PN517, PN508, PN526 and PN529. In panel B, control drugs aspirin and salicylic acid decrease cell viability. Panel C illustrates a comparison between PN517, cisplatin and aspirin showing that at 24 hours the most potent drug is PN517. Panel D illustrates the IC₅₀ values following 24 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

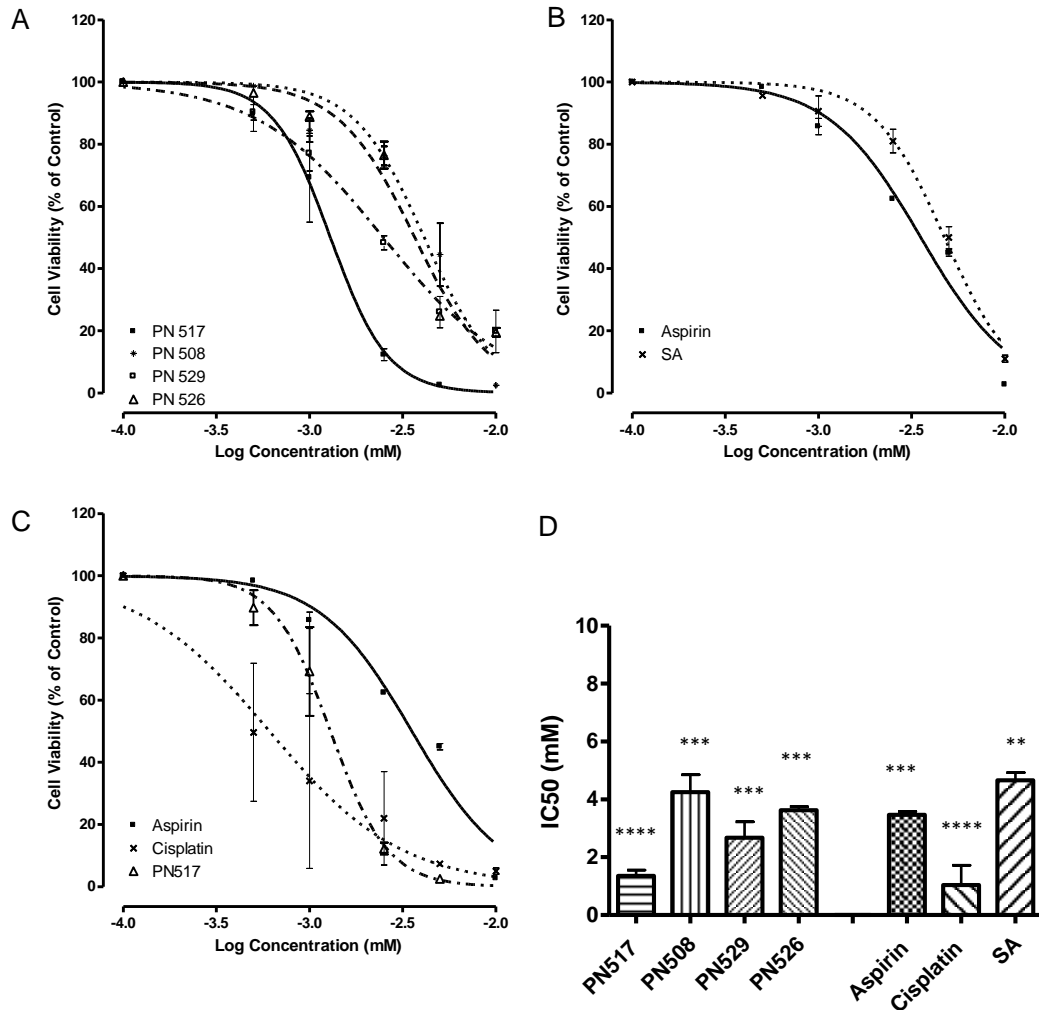


Figure 3.8 Cell viability following 48 hours drug treatment in SVG-p12 cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on SVG-p12 cell line following drug treatment for 48 hours. Panel A illustrates cell viability following treatment with aspirin analogues, PN517, PN508, PN526 and PN529, where it is shown that PN517 is the most potent. In panel B, control drugs aspirin and salicylic acid show a decrease in a dose dependent manner. Panel C compares PN517 with cisplatin and aspirin showing that the most potent drug is cisplatin following PN517 and then aspirin. Panel D represents the IC₅₀ values following 48 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.001$) with data representing results from 3 independent experiments.

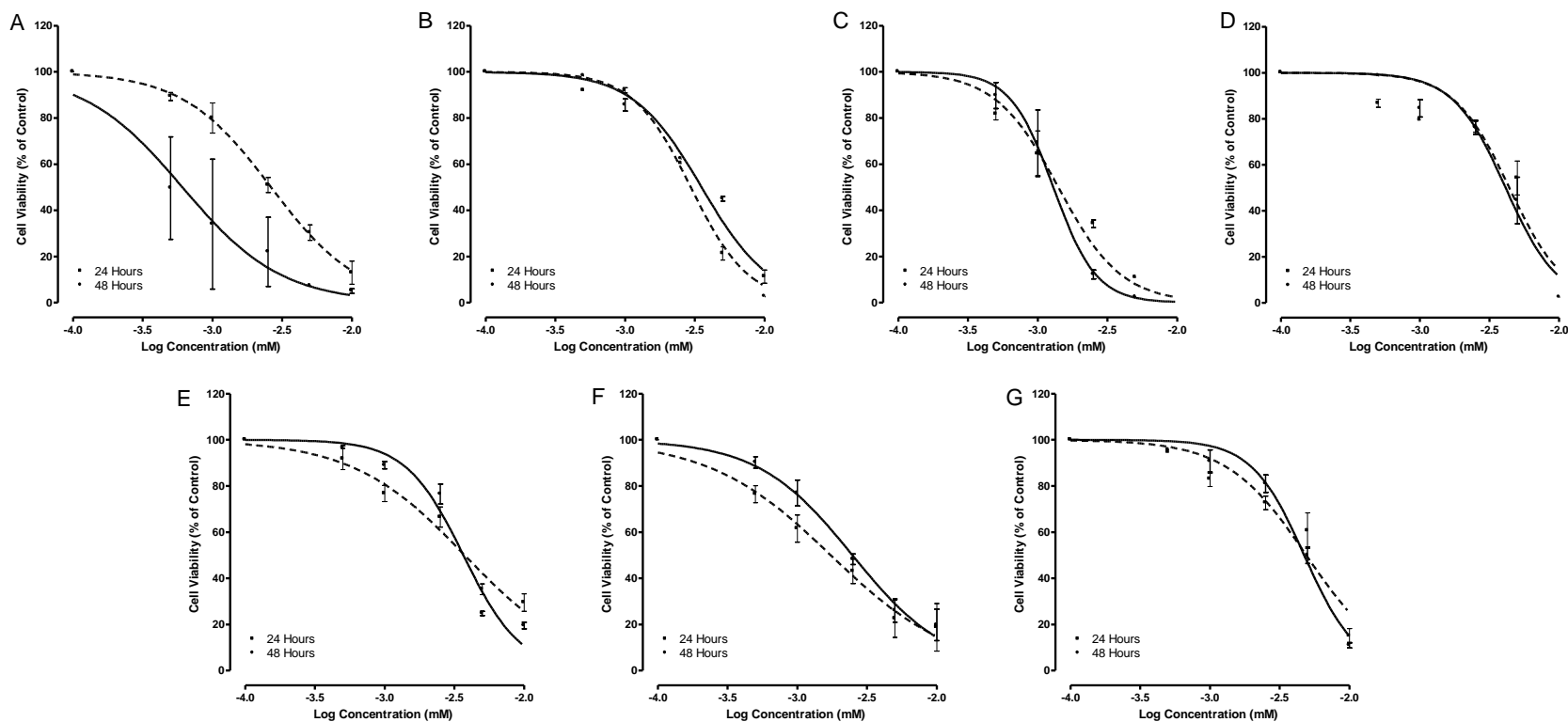


Figure 3.9 Cell viability following 24 and 48 hours drug treatment in SVG-p12 cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on SVG-p12 cell line following drug treatment for 24 and 48 hours. Panel A illustrates cell viability following treatment with cisplatin at 24 and 48 hours. Panel B shows the decrease in cell viability over 24 and 48 hour treatment with aspirin. Panel C demonstrates cell viability decrease following drug treatment with PN517, panel D shows cell viability decrease with PN508, panel E, F and G show the effect on cell viability following drug treatment with PN526, PN529 and SA. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

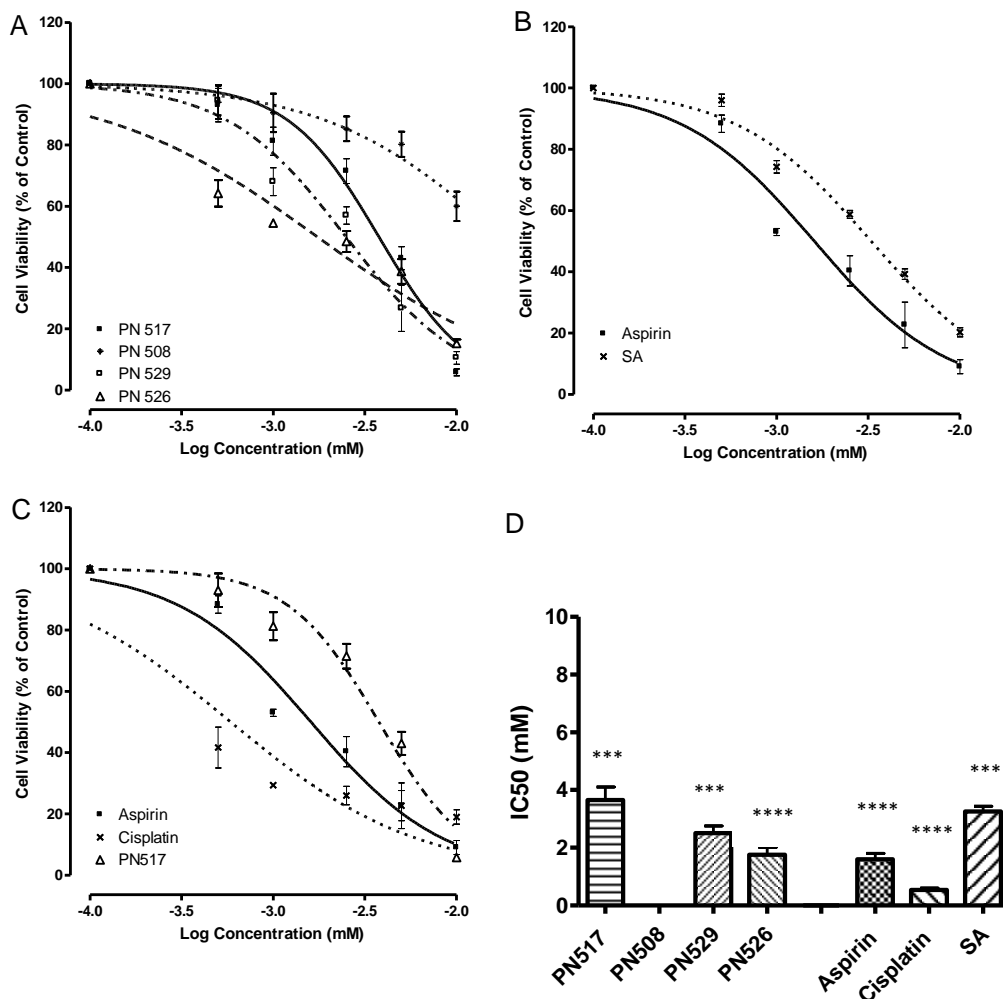


Figure 3.10 Cell viability following 24 hours drug treatment in 1321N1 cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on 1321N1 cell line following drug treatment for 24 hours. Panel A illustrates cell viability following treatment with aspirin analogues, PN517, PN508, PN526 and PN529, where it is shown that PN526 is the most potent. In panel B, control drugs aspirin and salicylic acid show a decrease in a dose dependent manner. Panel C compares PN517 with cisplatin and aspirin. Panel D represents the IC₅₀ values following 24 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

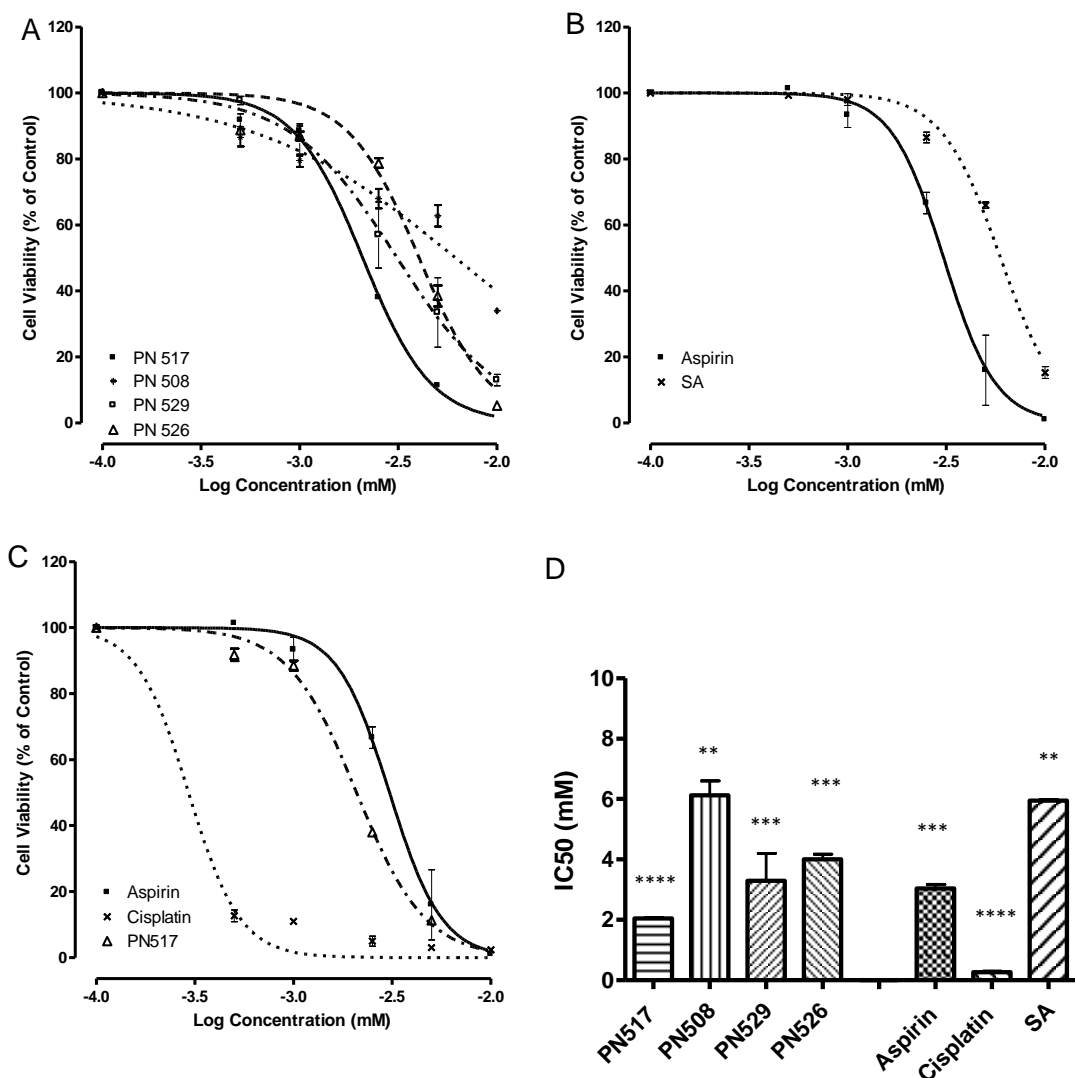


Figure 3.11 Cell viability following 48 hours drug treatment in 1321N1 cell line. The data illustrates cell viability determined using the PrestoBlue™ assay on 1321N1 cell line following drug treatment for 48 hours. Panel A illustrates cell viability following treatment with aspirin analogues, PN517, PN508, PN526 and PN529, where PN517 is the most potent. In panel B, control drugs aspirin and salicylic acid show a decrease in a dose dependent manner. Panel C compares PN517 with cisplatin and aspirin. Panel D represents the IC₅₀ values following 48 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

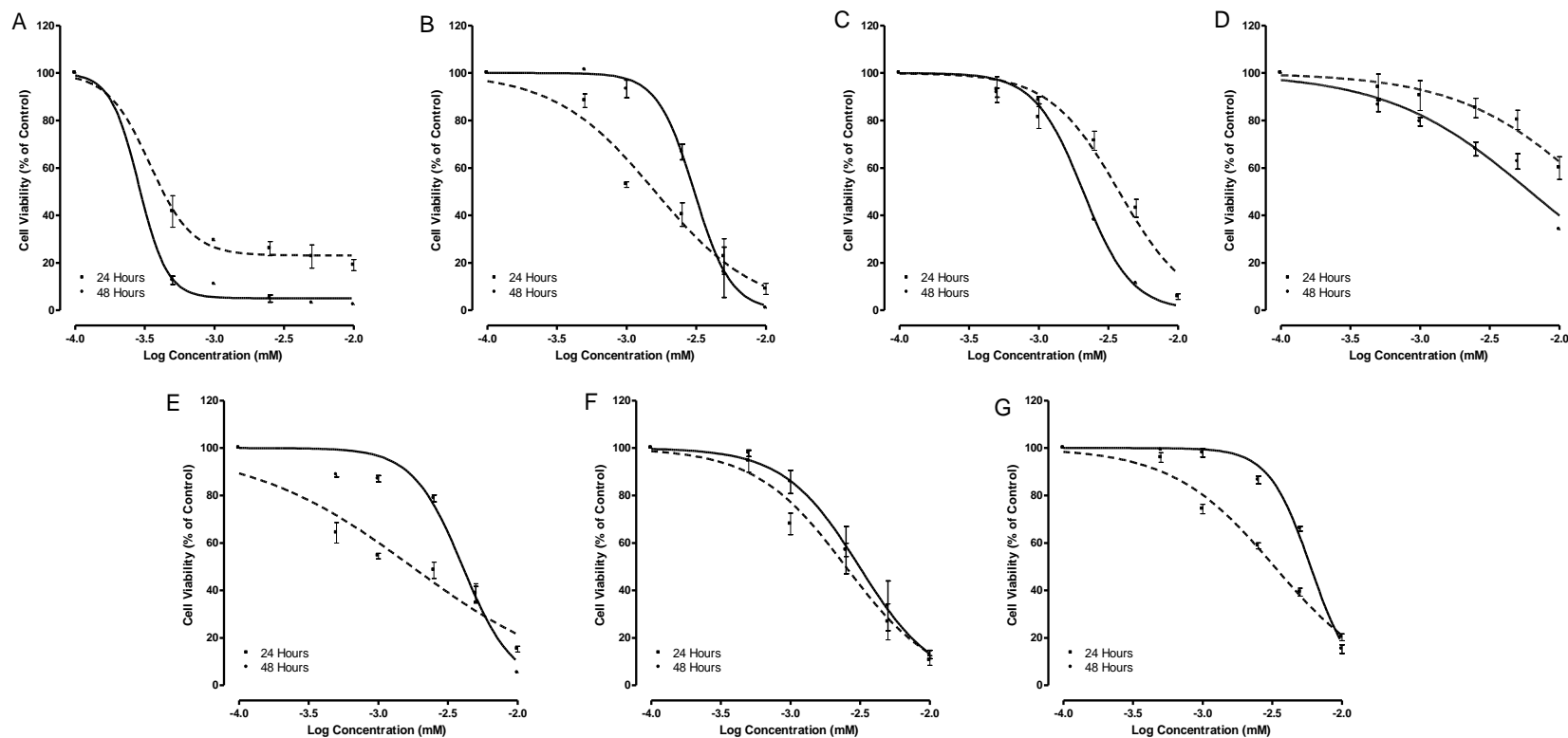


Figure 3.12 Cell viability following 24 and 48 hours drug treatment in 1321N1 cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on 1321N1 cell line following drug treatment for 24 and 48 hours. Panel A-G illustrate the effect on cell viability following drug treatment of cisplatin, aspirin, PN517, PN508, PN526, PN529 and SA respectively over 24 and 48 hours. Cisplatin has shown a large decrease in cell viability compared to the rest of the drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

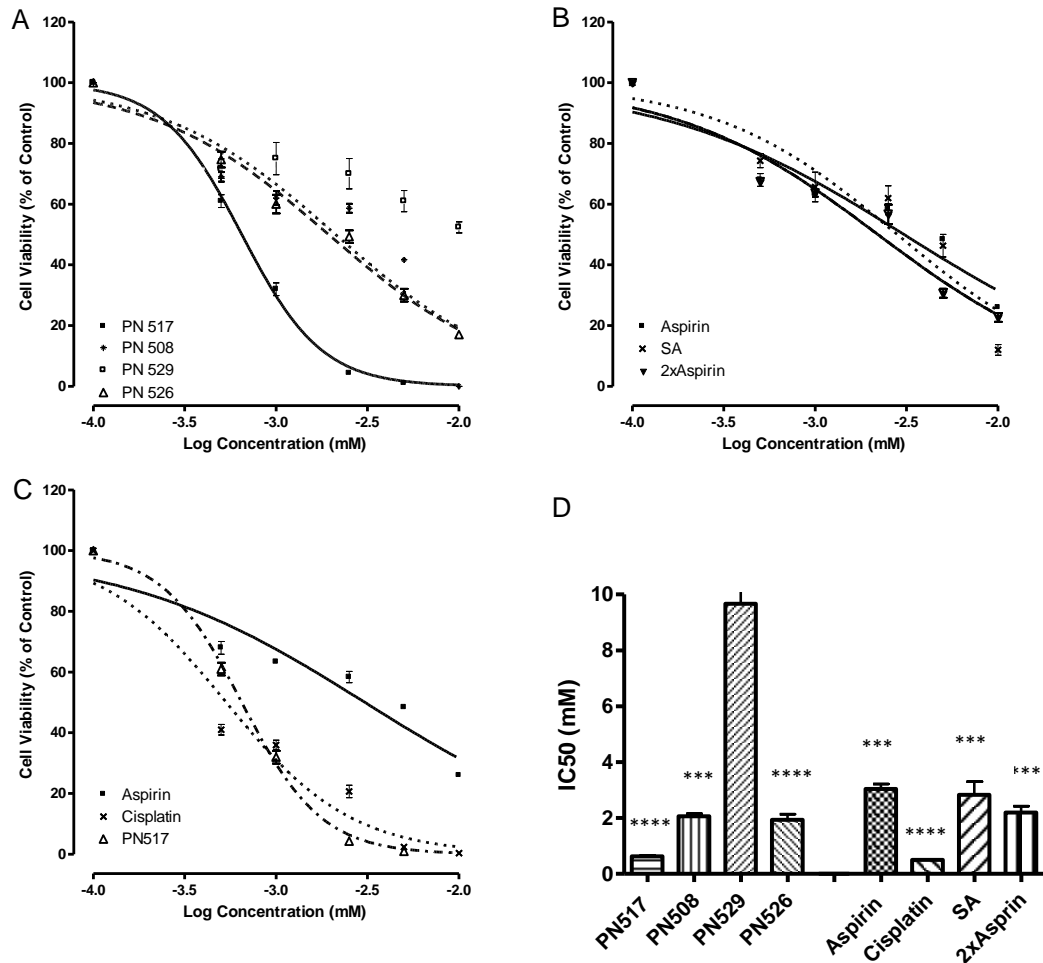


Figure 3.13 Cell viability following 24 hours drug treatment in GOS-3 cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on GOS-3 cell line following drug treatment for 24 hours. Panel A illustrates cell viability following treatment with aspirin analogues, PN517, PN508, PN526 and PN529, where PN517 shows a large effect in cell viability. Panel B, compares control drugs aspirin, double aspirin and salicylic acid. Panel C compares PN517 with cisplatin and aspirin. Panel D represents the IC₅₀ values following 24 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

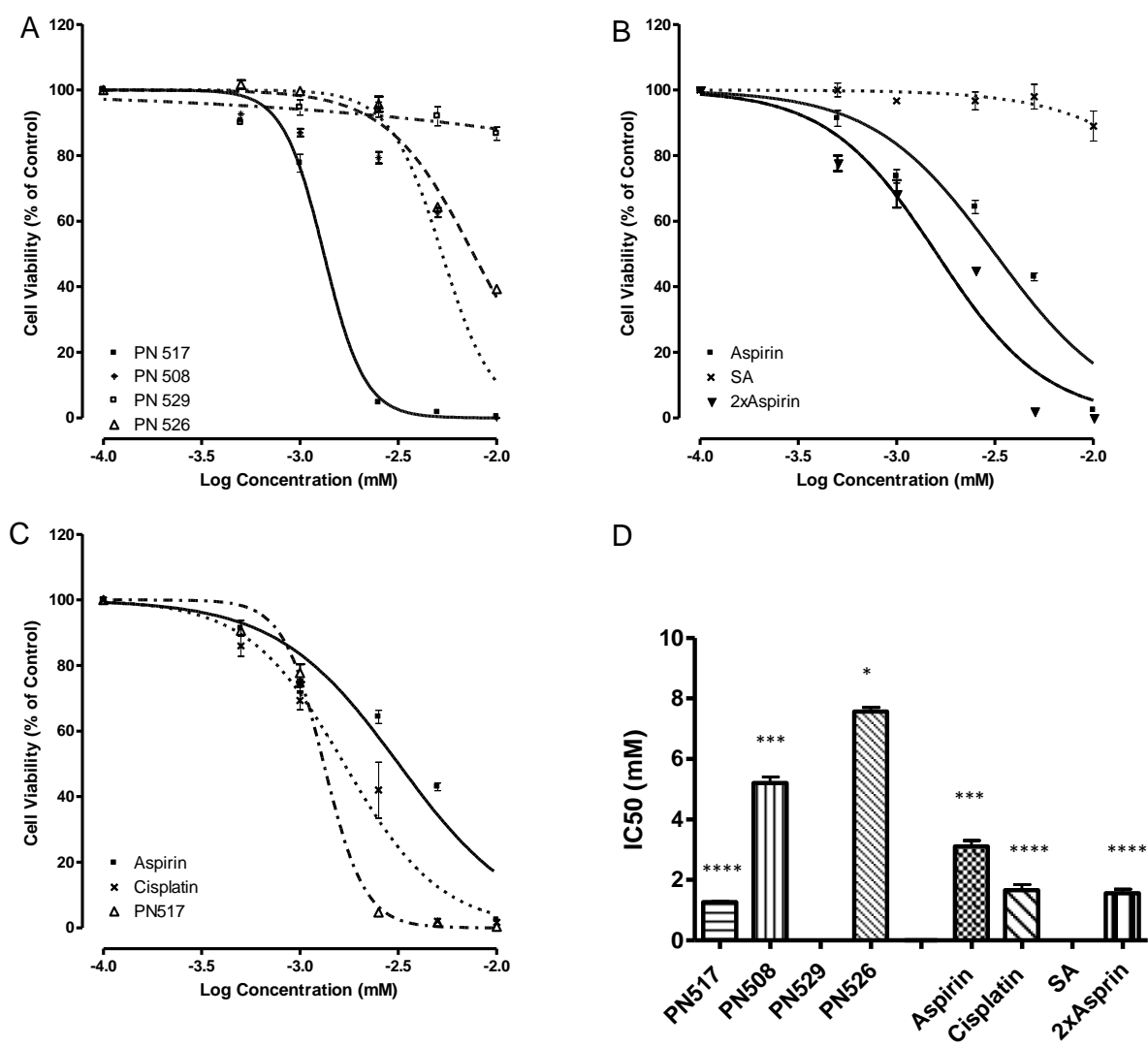


Figure 3.14 Cell viability following 48 hours drug treatment in GOS-3 cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on GOS-3 cell line following drug treatment for 48 hours. Panel A illustrates cell viability following treatment with aspirin analogues, PN517, PN508, PN526 and PN529. In panel B, there is a comparison of aspirin, double aspirin and SA. Panel C compares PN517 with cisplatin and aspirin. Panel D represents the IC₅₀ values following 24 hour treatment of the aspirin analogues and control drugs. Salicylic acid and PN529 had no recorded IC₅₀ value in the range of drug concentrations used, so were left blank. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

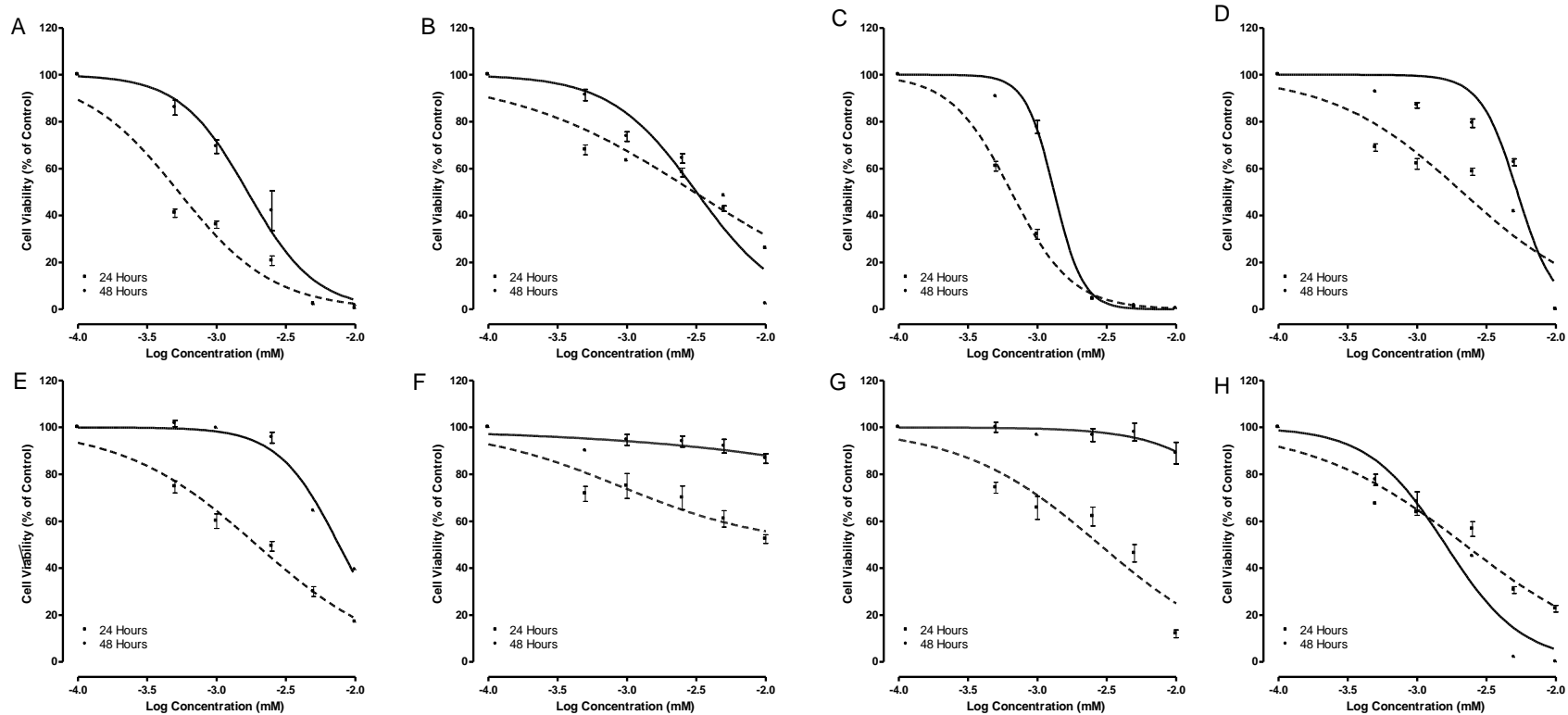


Figure 3.15 Cell viability following 24 and 48 hours drug treatment in GOS-3 cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on GOS-3 cell line following drug treatment for 24 and 48 hours. Panel A-H illustrate the effect on cell viability following drug treatment of cisplatin, aspirin, PN517, PN508, PN526, PN529, SA and double aspirin respectively over 24 and 48 hours. It is shown that the drugs decrease cell viability in both dose and time dependent manner. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

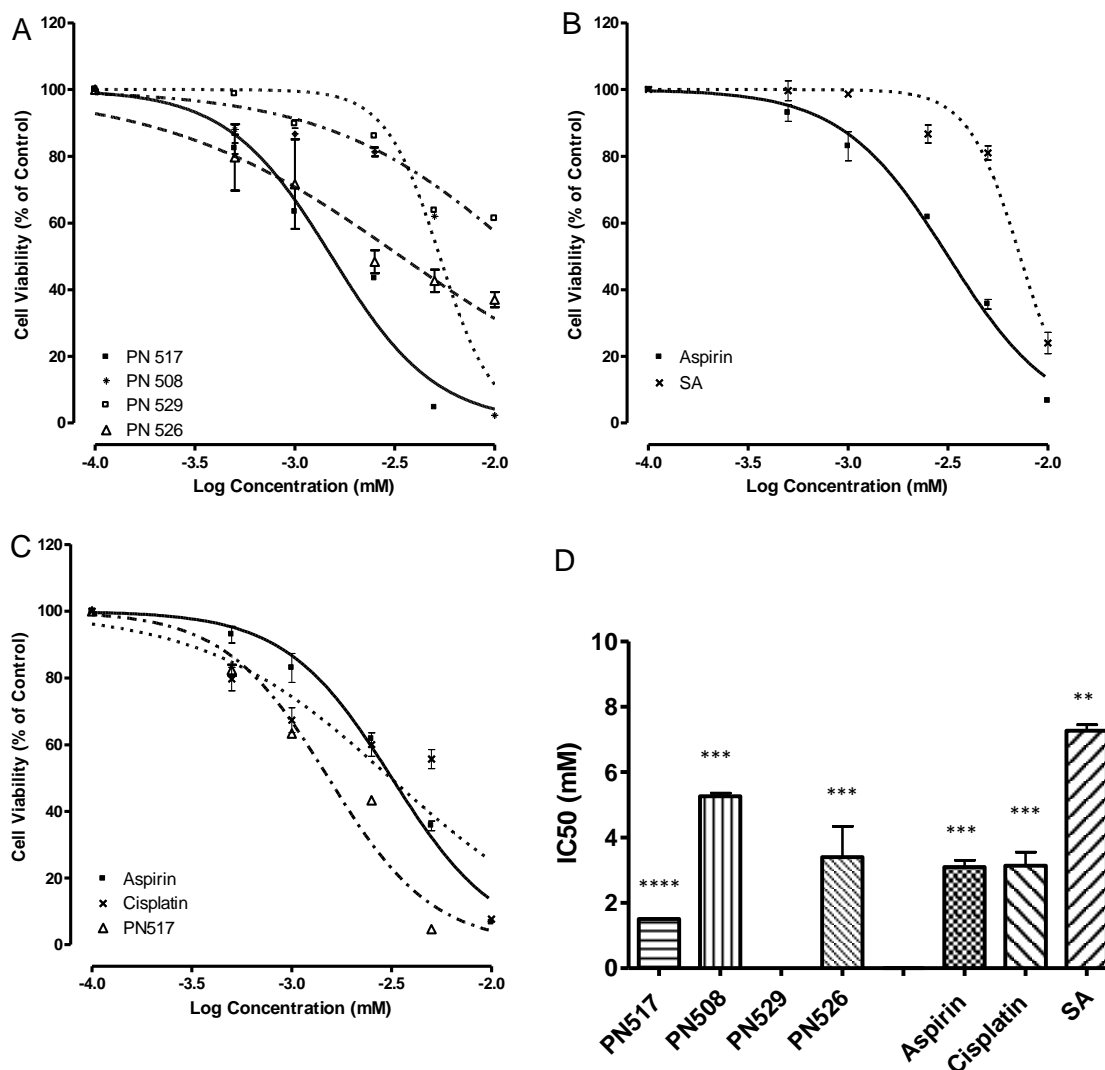


Figure 3.16 Cell viability following 24 hours drug treatment in U87 MG cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG cell line following drug treatment for 24 h. The results for the aspirin analogues are illustrated in panel A and aspirin with salicylic acid in panel B. The data for the most potent analogue, PN517 is illustrated with the control drugs cisplatin and aspirin (panel C). The final panel (D) illustrates the individual IC₅₀ values for each treatment with the exception of PN529 where no IC₅₀ value could be determined. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

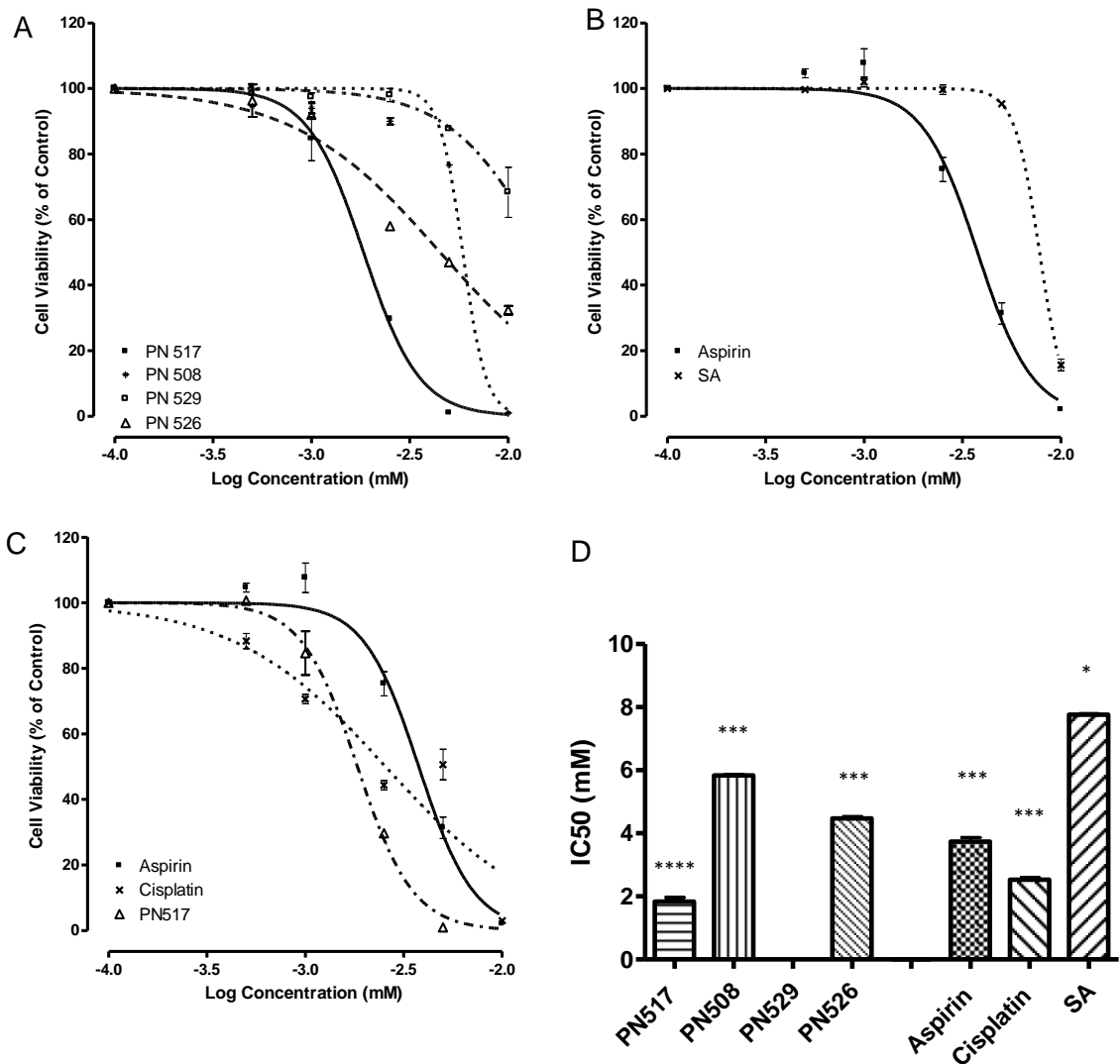


Figure 3.17 Cell viability following 48 hours drug treatment in U87 MG cell line. The data illustrate cell viability determined using PrestoBlue™ assay on U87 MG cell line following drug treatment of 48 hours. Panel A illustrates the effect of the aspirin analogues, and the control graph (panel B) shows the effect of aspirin and salicylic acid. Panel C shows that PN517 has similar potency to cisplatin, with a significant difference to aspirin. IC₅₀ values are shown individually showing that PN517 is the most potent and PN529 non-reactive. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

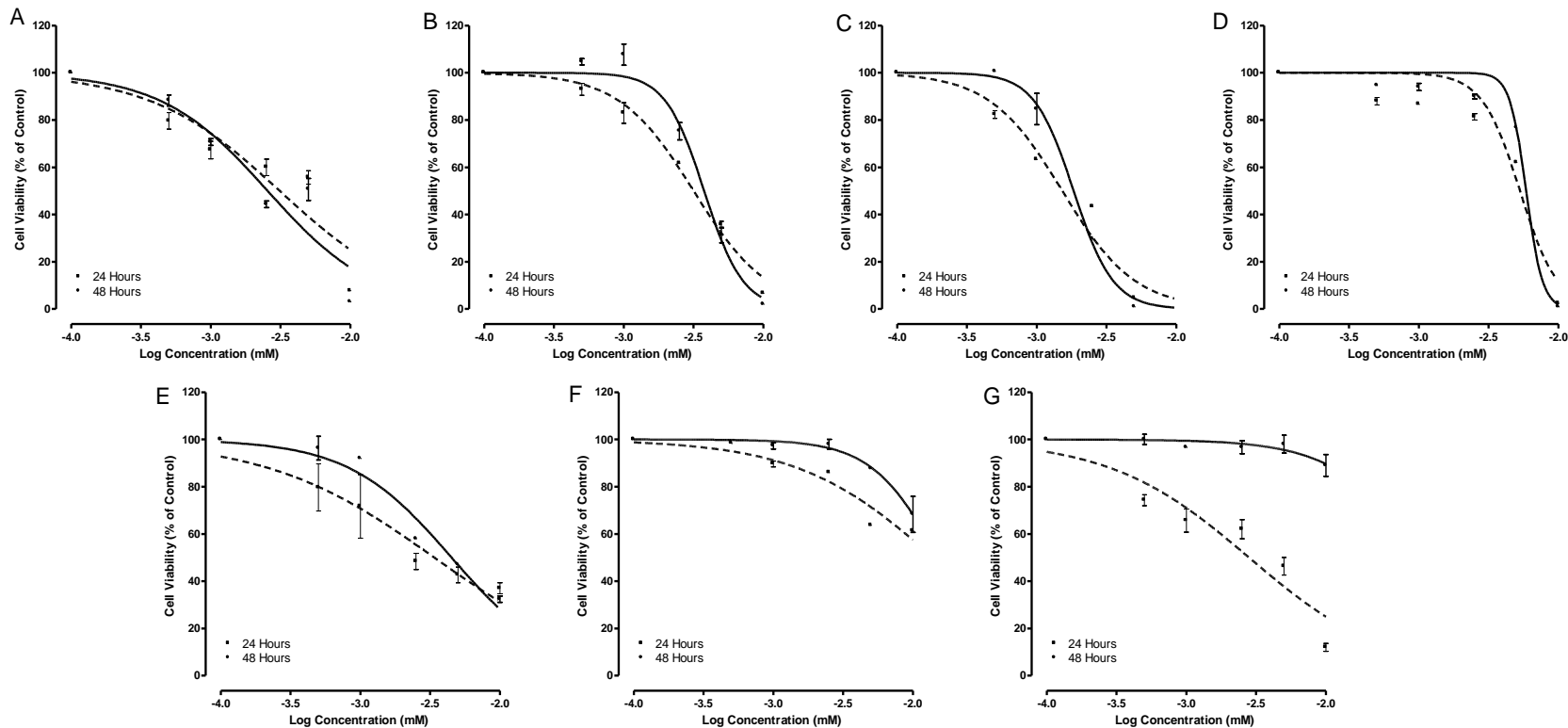


Figure 3.18 Cell viability following 24 and 48 hours drug treatment in U87 MG cell line. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG cell line following drug treatment for 24 and 48 hours. Panel A-G illustrate the effect on cell viability following drug treatment of cisplatin, aspirin, PN517, PN508, PN526, PN529 and SA respectively over 24 and 48 hours. The decrease in cell viability is similar in both 24 and 48 h. ANOVA analysis of results show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

3.2.5 Comparison of Drug Effects on Viability across Cell Lines

The purpose of the data illustrated in figures 3.19 to 3.25 is to allow the assessment of individual drug effects across the different cell lines utilised in the study, meaning that any cell specific effects can be identified.

Control Drugs

The effect of cisplatin on cell viability over 24 and 48 hours was compared in the three glioma, and the control embryonic cell line (Fig 3.19). At both time points, the IC_{50} value for the 1321N1 and GOS-3 cell lines was significantly lower than in the U87 MG and SVG-p12 cell lines. Although aspirin did not show any significant difference in IC_{50} value between the cell lines at either the 24 or 48 hour time points, the values following 24 hours of incubation were lower than those following 48 hours (Fig 3.20). Interestingly, the primary metabolite of aspirin, salicylic acid may show some cell selective differences in viability following 24 hours of incubation with an IC_{50} value of approximately 5 mM for both the 1321N1 and GOS-3, and an IC_{50} closer to 10 mM for the U87 MG and SVG-p12 cell lines (Fig 3.21).

Aspirin Analogues

Unlike aspirin which did not show any cell type selectivity, the analogue PN517 was significantly more potent in its ability to reduce cell viability in GOS-3 cells than in either the U87 MG or the 1321N1 cells (Fig 3.22). Significantly, it was least potent in the control cell line, SVG-p12, suggesting selectivity for cancer cells. Neither PN508, nor PN526 displayed such selectivity, with no significant difference in IC_{50} value between cell types (Fig 3.23 and 3.24 respectively). The aspirin analogue PN529 was consistently the least

efficacious treatment and additionally reduced viability in SVP-p12 cells at least as well as the cancer cell types and in fact better following 48 hours of treatment (Fig 3.25).

In conclusion, the data identify PN517 as the most potent aspirin analogue in the cell viability assays, displaying greater efficacy than aspirin. PN517 demonstrates glioma cell line selectivity, with the highest efficacy in the GOS-3 cell line, followed by the U87 MG and then 1321N1. However, as viability is a combination of many factors including proliferation and apoptosis, the mechanism by which PN517 is producing this effect has not been established by these results.

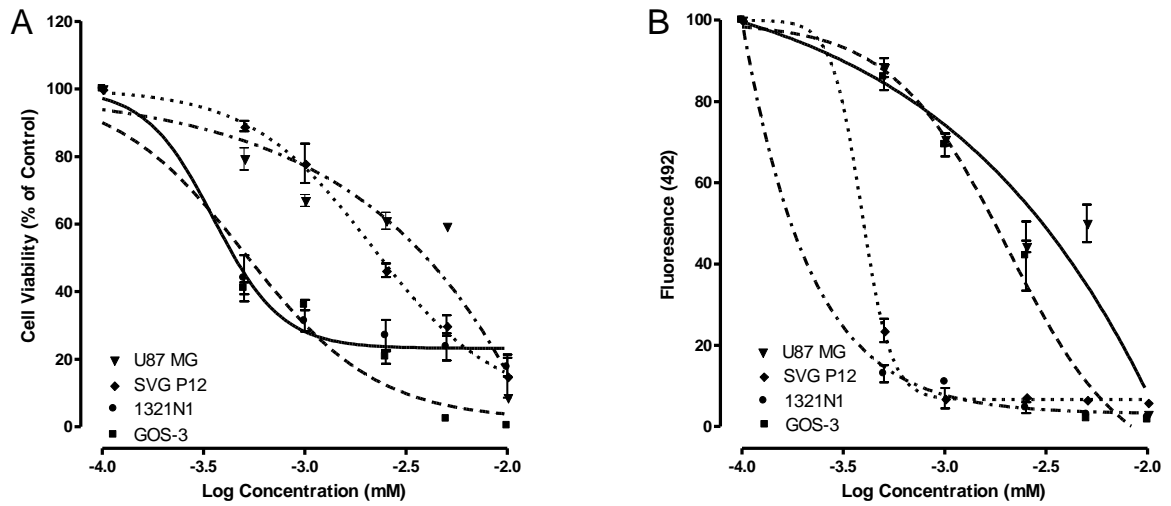


Figure 3.19 Cell viability following drug treatment with Cisplatin at 24 and 48 hours in cell lines SVG-p12, GOS-3, 1321N1 and U87 MG. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG, SVG-p12, 1321N1 and GOS-3 following drug treatment for 24 and 48 hours. In panel A the data for 24 hours show the cell line effect is significantly different between them. Panel B illustrates the effect following cisplatin drug treatment at 48 hours. The cell lines affected the most are 1321N1 and GOS-3 however only the 1321N1 remain consistent at 48 hours. The drug seems to have little effect on U87 MG cell line. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

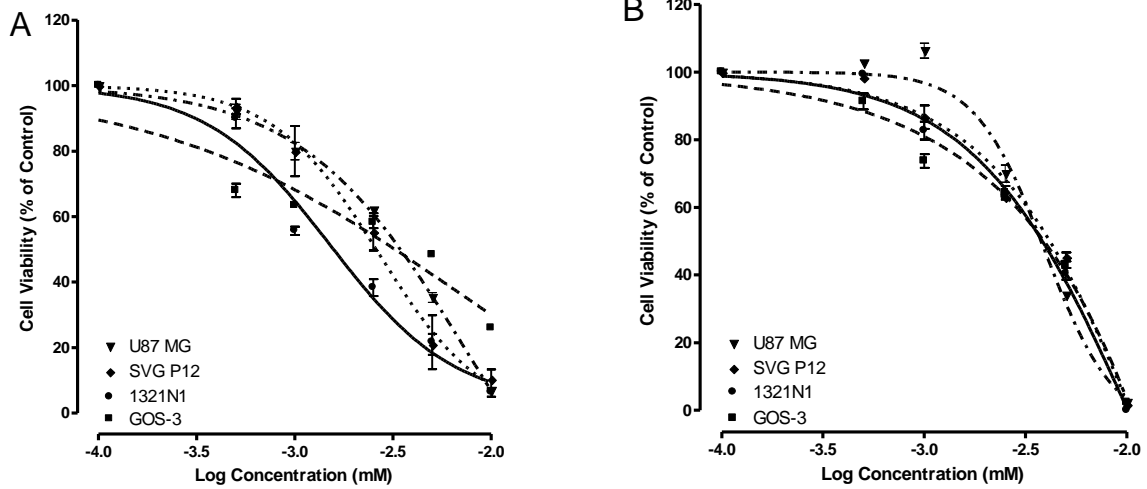


Figure 3.20 Cell viability following drug treatment with Aspirin at 24 and 48 hours in cell lines SVG-p12, GOS-3, 1321N1 and U87 MG. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG, SVG-p12, 1321N1 and GOS-3 following drug treatment for 24 and 48 hours. In panel A the data for 24 hours are shown. Panel B illustrates the effect following aspirin drug treatment at 48 hours. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

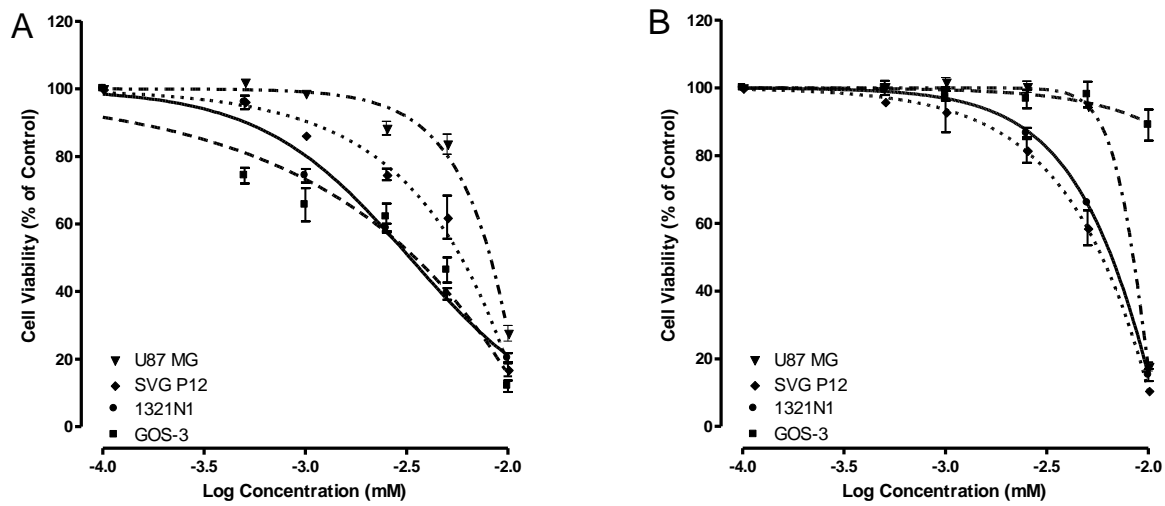


Figure 3.21 Cell viability following drug treatment with Salicylic acid at 24 and 48 hours in cell lines SVG-p12, GOS-3, 1321N1 and U87 MG. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG, SVG-p12, 1321N1 and GOS-3 cell lines following drug treatment for 24 and 48 hours, panel A and B respectively. In 24 hours SA shows a large effect on GOS-3 cell line which recovers at 48 hours. SA shows no effect after 48 hours of treatment in any of the cell lines. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

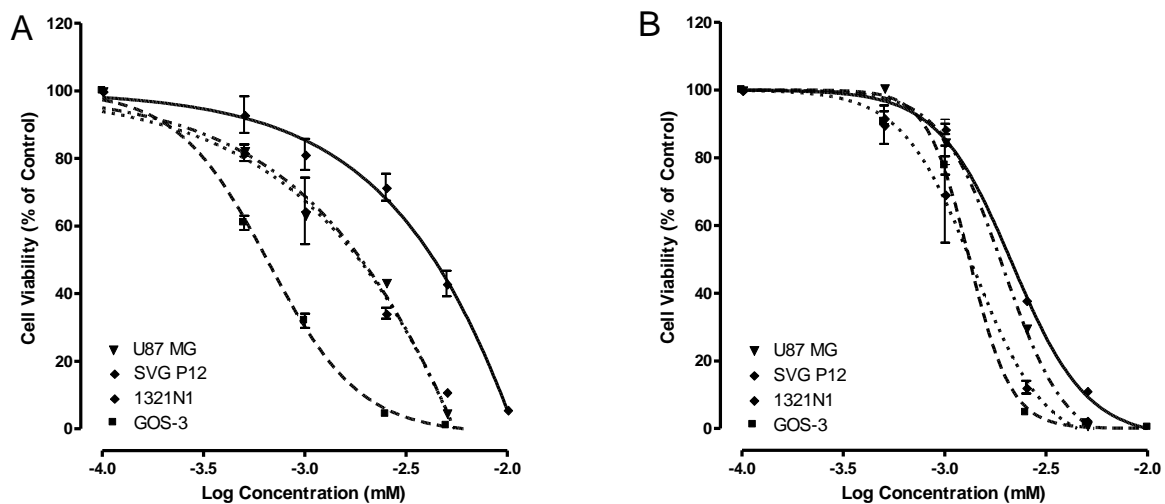


Figure 3.22 Cell viability following drug treatment with PN517 at 24 and 48 hours in cell lines SVG-p12, GOS-3, 1321N1 and U87 MG. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG, SVG-p12, 1321N1 and GOS-3 following drug treatment with aspirin for 24 and 48 hours. GOS-3 shows the biggest effect in cell viability in both 24 (panel A) and 48 hours (panel B) while the less effected cell line is 1321N1. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

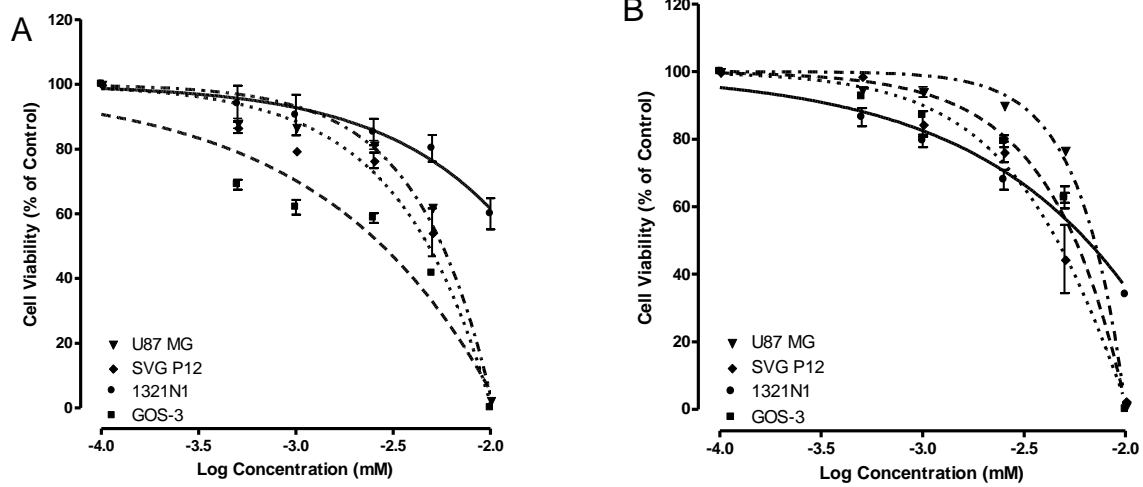


Figure 3.23 Cell viability following drug treatment with PN508 at 24 and 48 hours in cell lines SVG-p12, GOS-3, 1321N1 and U87 MG. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG, SVG-p12, 1321N1 and GOS-3 cell lines following drug treatment for 24 hours (panel A) and 48 hours (panel B). The results show that GOS-3 shows the largest effect at 24 hours and 1321N1 non effective; however at 48 hours 1321N1 show an increase in cell death. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

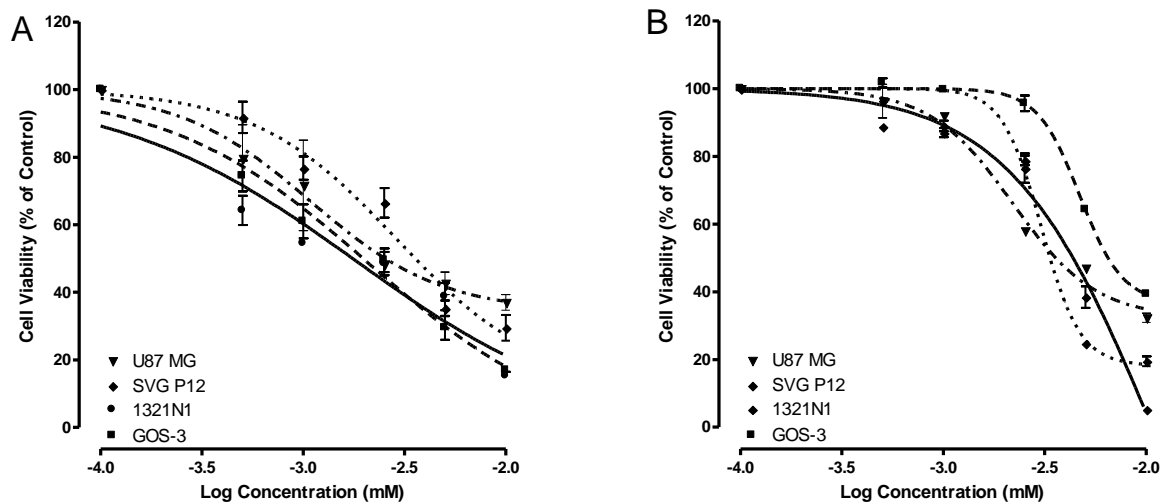


Figure 3.24 Cell viability following drug treatment with PN526 at 24 and 48 hours in cell lines SVG-p12, GOS-3, 1321N1 and U87 MG. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG, SVG-p12, 1321N1 and GOS-3 cell lines following drug treatment with PN 526 for 24 hours (panel A) and 48 hours (panel B). Data show that at 24 hours the drug is more effective on 1321N1 following GOS-3 then U87 MG and SVG-p12, in 48 hours the drug does not affect GOS-3 cell line, but is more effective on U87 MG.. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

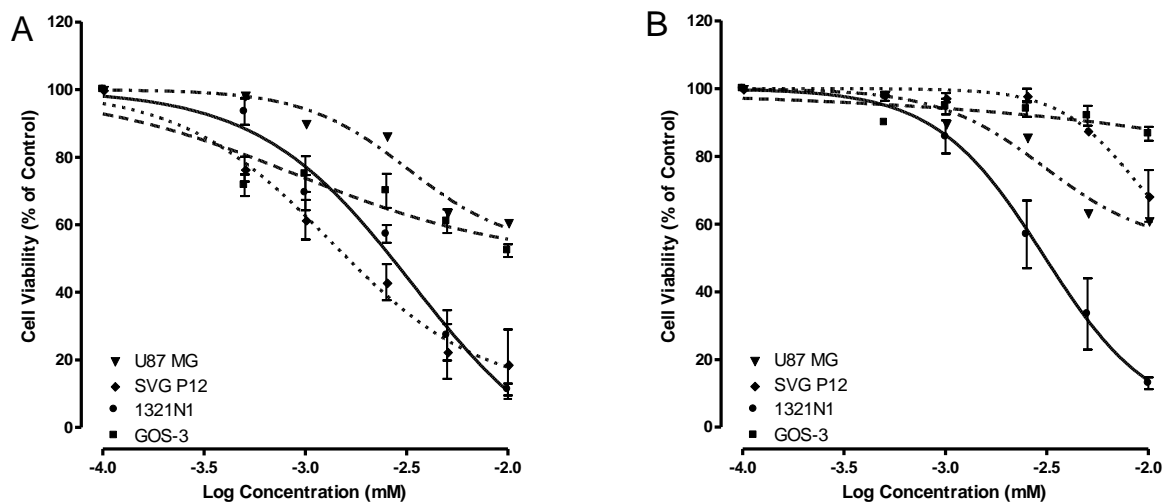


Figure 3.25 Cell viability following drug treatment with PN529 at 24 and 48 hours in cell lines SVG-p12, GOS-3, 1321N1 and U87 MG. The data illustrate cell viability determined using the PrestoBlue™ assay on U87 MG, SVG-p12, 1321N1 and GOS-3 cell lines following drug treatment for 24 hours (panel A) and 48 hours (panel B). In this case PN529 at 24 hours is more toxic to SVG-p12 cell line and least potent on U87 MG. In 48 hours PN529 affects 1321N1 cell line more. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

3.2.6 Spheroid cell viability

Spheroids are more representative of *in vivo* conditions than cell monolayers, and tumour cells in these microenvironments typically exhibit several traits including relevant morphology, increased cell survival, and a hypoxic core in comparison to traditional monolayer cultures (Smith *et al.*, 2012; Godugu *et al.*, 2013; Lee *et al.*, 2013).

Using the U-87 MG cell line it was decided to perform a comparison between PrestoBlue and the MTT assay to determine the sensitivity to detect changes in cell number (Fig. 3.26 panel A), and response to standard drug treatments (Fig. 3.27 panels B to F). A direct comparison of the PrestoBlue and MTT assays showed no significance ($p > 0.05$) (Fig 3.28, panel A). In terms of drug induced reductions in cell viability, no difference was found comparing the two different methods (panel B and panel C) in the treatments for aspirin and PN517 ($p > 0.05$), but a significant difference was seen in cisplatin treatment ($p < 0.05$). Spheroids seem to be more sensitive in cisplatin treated cells ($p < 0.05$) (panel A). IC_{50} values recorded for monolayer treatment for aspirin was 3 mM, cisplatin 3.1 mM and PN517 1.5 mM, in comparison to spheroids with aspirin 1.2 mM, cisplatin 5.1 mM and PN517 1 mM ($p < 0.001$).

In conclusion, there was no significant differences in results from the two different cell viability methods used (MTT vs. PB), and apart from cisplatin, no difference in efficacy was observed between spheroid and monolayer cultures. This suggest that the results from the monolayer investigations are representative of a 3D culture environment.

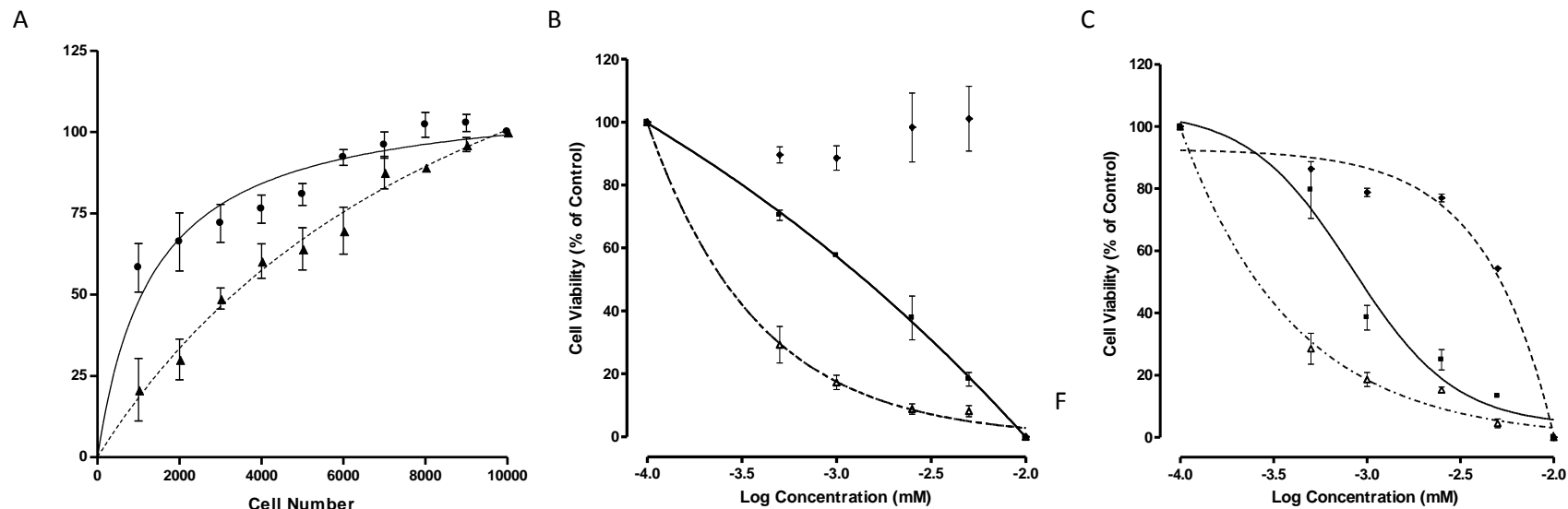


Figure 3.26 PrestoBlue assay for the detection of cell viability following 24 hours drug treatment in the spheroid U-87 MG cell line. The data illustrate growth curve determined using the PrestoBlue™ (circle solid line) and MTT assay (triangles and dashed line) on U-87 MG cell line on increase in cell number after 24 hours (panel A) ($p > 0.05$). Viability of MTT (panel B) and PB assay (Panel C) to detect effects on cell viability following aspirin (squares) cisplatin (circles) and PN517 (triangles) drug treatment for 24 hours. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

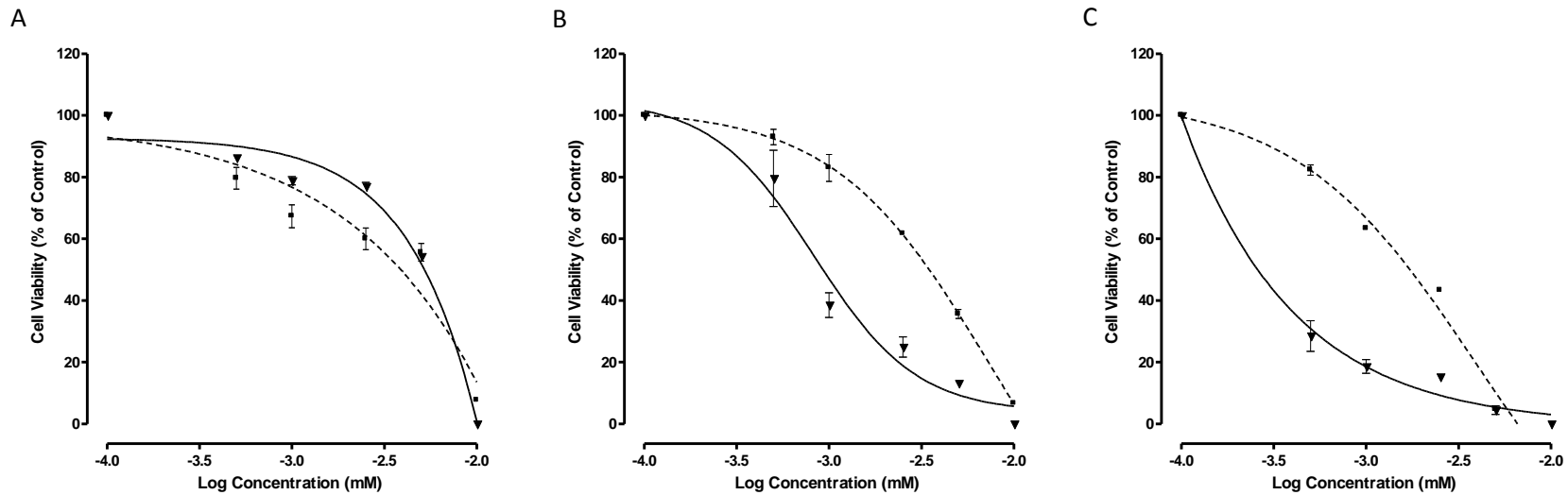


Figure 3.27 Comparison of PrestoBlue assay in Spheroid and Monolayer for the detection of cell viability following 24 hours drug treatment in the U-87 MG cell line. The data illustrate cell viability determined using the PrestoBlue™ on U-87 MG cell line following 24 hours treatment with cisplatin (panel A), aspirin (panel B) and PN517 (panel C) in spheroids (triangles and solid line) and monolayers (squares and dashed line). ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

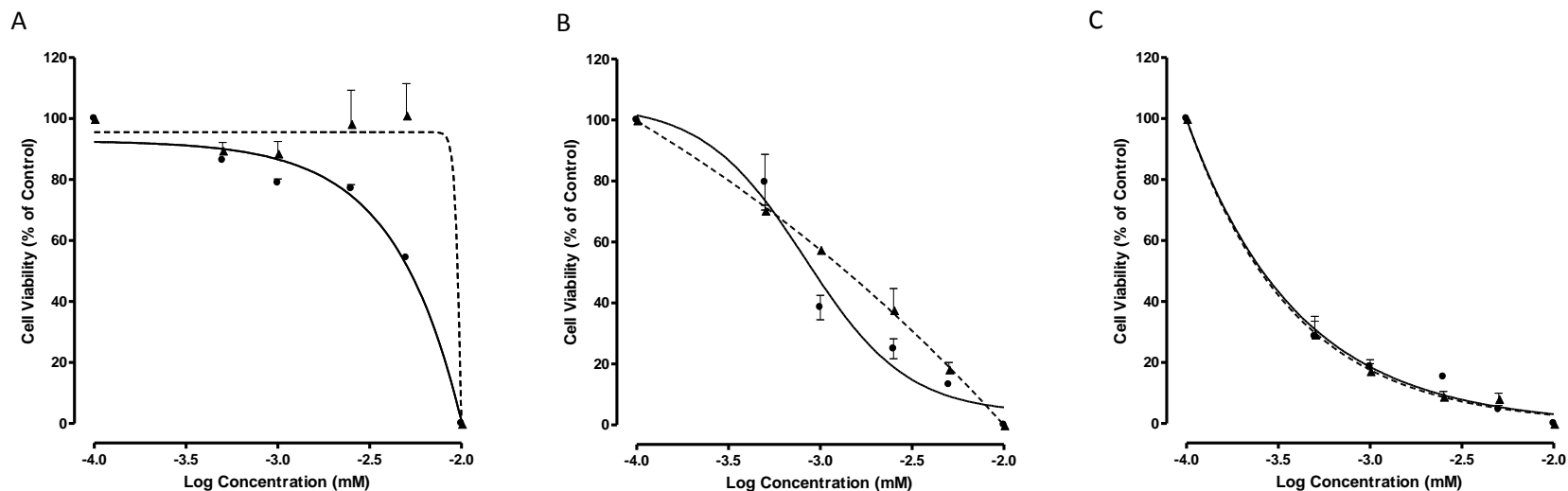


Figure 3.28 Comparison of PrestoBlue and MTT assay in Spheroids for the detection of cell viability following 24 hours drug treatment in the U-87 MG cell line. The data illustrate cell viability determined using the PrestoBlue™ (triangles dotted line) and MTT (circles solid line) on U-87 MG cell line following 24 hours treatment with cisplatin (panel A), aspirin (panel B) and PN517 (panel C) in spheroids. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

3.3 Discussion

The first aim of this project was to characterize the effect of newly synthesized aspirin analogues on glioma cell viability to allow identification of the analogue with highest efficacy. The initial step was to confirm the cell lines used were glioma cell lines and then to identify the inhibition of COX enzyme isoforms on the drugs provided. Results from GFAP staining confirmed that the higher the grade of glioma, the more GFAP binding was shown. Results of the COX enzyme inhibition assay have suggested the analogues have indeed inhibited the enzymes in a concentration dependant manner, while indicating that PN529 was the most efficacious, followed by PN517 and PN508, and lastly by aspirin.

Cell viability results provide the first evidence that PN517 might have therapeutic potential for the treatment of glioma. Identifying PN517 as the compound with highest efficacy, these initial aims have been met while generating many intriguing results.

To date, the majority of literature examining a role for aspirin, selective COX-2 inhibitors or prostaglandin signalling has made use of established cell lines (Xu *et al.*, 2013; Suzuki *et al.*, 2013; White *et al.*, 2013; Gomes and Colquhoun, 2012; Lan *et al.*, 2011; Lo *et al.*, 2010; Kang *et al.*, 2009; Kambe *et al.*, 2009; Annabi *et al.*, 2009; Kim *et al.*, 2009; Lee *et al.*, 2005; Amin *et al.*, 2003; Arrieta *et al.*, 2001; Aas *et al.*, 1995). Anti-proliferative effects of aspirin or selective COX-2 inhibitors have been discussed in the literature in a range of different cell lines. NS398 was demonstrated an effect in glioma (Joki *et al.*, 2000), as well as aspirin, acetaminophen and ibuprofen (Casper *et al.*, 2000). NO-ASA, a nitric oxide has shown to reduce proliferation in a range of cancer cell lines, including colon, ovarian,

pancreas, skin, cervical, oesophageal and breast (Fiorucci *et al.*, 2003; Troyano *et al.*, 2001; Chang *et al.*, 2003; Zhi *et al.*, 2006; Gao and Williams, 2012). Both aspirin and indomethacin inhibit *in vitro* glioma cell proliferation in the COX-1 and COX-2 positive T98G cell line in a time and concentration dependent manner (Amin *et al.*, 2003).

With respect to the concentrations of aspirin used in studies, these vary in the literature and are dependent on assay and cell type. In aspirin experiment with prolonged incubations, low concentrations such as 0.001mM have been used (Casper *et al.*, 2000), but studies have also utilised treatments with concentrations as high as 10 mM (Pathi *et al.*, 2012).

In this study, as well as using cisplatin as a control treatment, aspirin was also used as any developed analogue would at least have to improve on the effects of its parent compound. In addition, a treatment equivalent to a double dose of aspirin was also used as a control as the structures of two of the analogues, PN508 and PN517, closely resemble two aspirin bound together with either a single or double carbon bond (Fig 3.28). The central double or single carbon bond either gives the molecule rigidity, or allows for rotational flexibility between to the two aspirin groups.

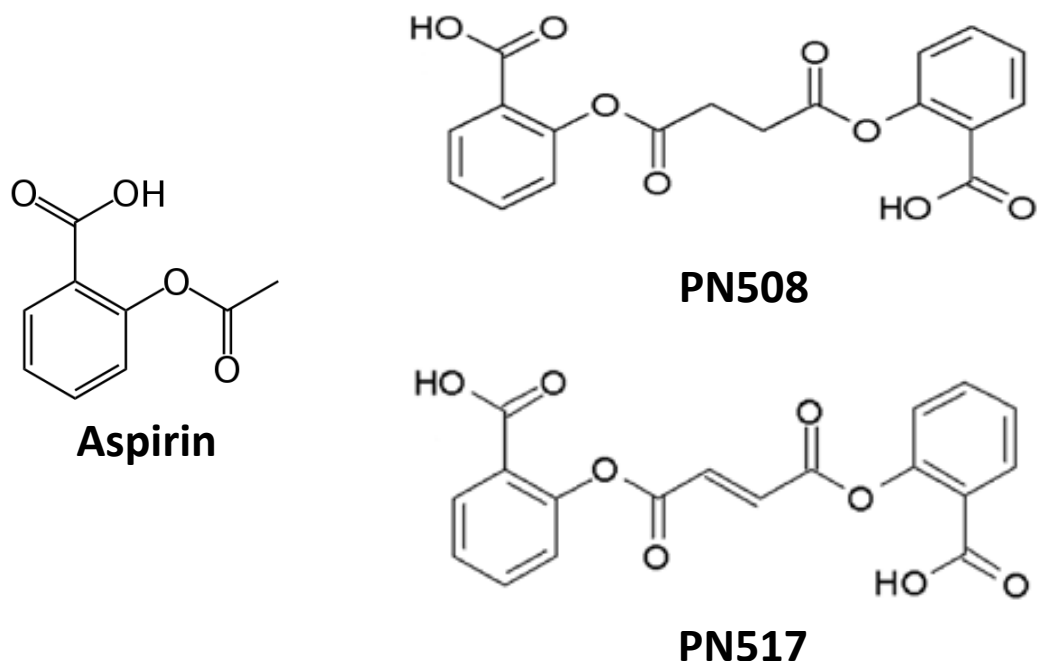


Figure 3.28 Chemical structures of aspirin, PN508 and PN517

This site may also represent a target for enzyme mediated hydrolysis, yielding two aspirin molecules and if this was, then case and any increased efficacy of these compounds would be replicated by adding a double dose of aspirin. However, this control treatment seldom replicated the potency of PN517 in the cell viability assay (e.g. Fig 3.22), suggesting that its efficacy is due to some other structural characteristic and not simply because it resembles two aspirin molecules.

Cells are most commonly cultured as monolayers *in vitro*, but spheroid cell cultures were first described in the 1980, focusing on the extracellular matrix and the ability of cultures in artificial 3D matrices to produce physiologically relevant multicellular structures (Sutherland *et al.*, 1981). The extracellular matrix regulates the cell dynamic behaviour,

and helps the cells move within their spheroid similar to the way cells would move in living tissue (Pampaloni *et al.*, 2007). Spheroids are true representative models for cell migration, survival, and growth (Lee *et al.*, 2008), thus are used to evaluate cellular responses to pharmaceutical compounds in drug discovery applications. Unlike classical monolayer-based models, spheroids mirror the 3D cellular context and therapeutically relevant pathophysiological gradients of *in vivo* tumours and because of this have the potential to either eliminate poor drug candidates before *in vivo* or clinical testing, or to identify promising drugs that would fail in classical 2D cell assays (Hirschhaeuser *et al.*, 2010).

It is known that 3D structures have a transient G2 delay, apoptosis induction and late onset of DNA strand breaks, and thus therapeutic approaches are found to be less effective in 3D than in 2D cultures. A major reason for this is that tumour spheroids have been known to develop chemical gradients (e.g. of oxygen, nutrients, and catabolites) at diameters between 200 and 500 μm with a central secondary necrosis typically established at sizes $>500 \mu\text{m}$ (Hirschhaeuser *et al.*, 2010). This, however, cannot be generalized as Frankel *et al.*, (2000) tested the activity of PS-341 in a spheroid/solid tumour context using four different human ovarian carcinoma and three prostate carcinoma cell lines. PS-341 showed equal or greater activity in spheroids than in the respective monolayer cell cultures, including the slow growing prostate cancer spheroid model, inducing apoptotic cell death in both culture systems. Similarly, the COX-2 selective inhibitor NS-398 was found to reduce proliferation rate equally in both monolayer and spheroid cultures (Joki *et al.*, 2000).

Here, spheroid cultures were produced using the U87 MG cell line to allow comparison with monolayer cell suspension following drug treatments and it was observed that PN517 showed no significant difference in the effect of the aspirin analogue on cell viability between monolayer and spheroid cultures.

These results with COX inhibitors are in contrast to much of the literature, such as to results from Li *et al.*, (2008) where they compared the effects of cisplatin and other drugs on cell viability using 2D and 3D cell cultures of HepG2 cells. They concluded that the multicellular morphology resulted in a differentiated phenotype resulting in increased cell-cell adhesion and G1 phase cell cycle arrest, enhanced cellular resistance to apoptosis, and upregulated angiogenic potential. This altered phenotype resulted in decreased efficacy of cisplatin and an increased viability of 3D cultures. A study that developed a multicellular spheroid model of malignant mesothelioma to investigate molecular mechanisms of acquired apoptotic resistance found that the spheroids developed resistance to a variety of apoptotic stimuli, including combinations of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), ribotoxic stressors, histone deacetylase, and proteasome inhibitors that were highly effective against mesothelioma cells when grown as monolayers (Barbone *et al.*, 2008). Inhibitors of the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway, particularly rapamycin, blocked much of the acquired resistance of the spheroids, suggesting a key role for mTOR.

The aim of drug discovery is to develop a new compound that improves on the current “best in class”. The introduction of temozolomide (TMZ), a prodrug of the alkylating agent 5-(3 methyltriazen-1-yl) imidazole-4-carboximide (MTIC), has improved glioblastoma

patient median survival time by 2.5 months (Stupp *et al.*, 2005). Due to its prodrug nature, it does not have reproducible effects *in vitro* so is commonly replaced in research with other standard chemotherapeutic drugs, and as such, throughout this project cisplatin was used as a control. Cisplatin has proven to be one of the more effective anticancer chemotherapeutic agents due to targeting of multiple intracellular sites to induce death in tumour cells (Rosenberg *et al.*, 1965).

The cell viability results for cisplatin typically show a decrease in IC₅₀ value from 24 to 48 hours of incubation, an effect that is related to its mechanism of action. Platinum based compounds damage tumours *via* induction of apoptosis, which is mediated by the activation of various signal transduction pathways (Rosenberg *et al.*, 1965). Cisplatin becomes activated intracellularly by aquating one of two chloride-leaving groups and covalently binding to DNA, forming DNA adducts. The drug binds with DNA to form intrastrand crosslinks and adducts that cause changes in the conformation of the DNA and affect DNA replication. Other mechanisms of cisplatin cytotoxicity include mitochondrial damage, decreased ATPase activity, and altered cellular transport mechanisms. Although cisplatin is cell cycle non-specific, cytotoxicity is increased with exposure during S-phase. Cisplatin causes cell cycle arrest in the prolonged G2-phase and deregulation of signal transduction pathways involved in growth, differentiation, and stress responses which then induces programmed cell death or apoptosis (Florea and Busselberg, 2011). Cisplatin affects cells which turn over rapidly (e.g. tumour cells, gastrointestinal cells, bone marrow cells), meaning that cell death will occur at a faster rate than in other cells with a slower proliferation rate. The mode of cell death has been related to drug concentration, with

necrosis occurring with high concentrations and apoptosis with lower concentrations. While cisplatin is not selective for cancer cells over normal cells, it will affect rapidly growing cells more quickly (Sancho-Martinez *et al.*, 2011). Thus, its mechanism of action explains why fast growing cell lines like 1321N1 and GOS-3 showed reduced viability in the first 24 hours following treatment with cisplatin giving IC_{50} values of 0.5 mM for both cell lines (Fig 3.19). The slower proliferating cell lines SVG P12 and U87 MG responded more slowly to cisplatin treatment with cell viability IC_{50} values of 0.6 and 2.5 mM at 48 hours (Fig 3.19) respectively. The fivefold higher IC_{50} value for the U87 MG cell line could indicate that a longer incubation with cisplatin (e.g. 72 hours) would decrease the IC_{50} further.

The data have also revealed a number of other cell line selective responses. For example, the analogue PN517 displayed greater efficacy to cisplatin in the U87 MG cell line (Fig 3.16 to 3.18), in contrast to the other lines where cisplatin was more potent. The anti-cancer effects of aspirin have largely been linked to its ability to inhibit the cyclooxygenase enzymes and a decrease in the production of prostaglandins. Expression of the COX-1 enzyme plays an important role in platelet aggregation and is largely associated with the gastro-intestinal tract and the side effects of chronic aspirin treatment. However, the expression of COX-2 protein is more varied, and is found in both normal brain and glioma specimens, and is significantly higher in high-grade glioma than low-grade glioma and normal brain tissue. Additionally, its expression is higher in more in slower growing cells than faster growing (Joki, 2000). The U87 MG cell line is both high grade and relatively slow growing with a doubling time of approximately 30 hours, both characteristics

associated with high COX-2 expression. COX-1 inhibition is particularly higher compared to COX-2 inhibition of all the drugs tested (Fig 3.3) PN529 being the most effective in both COX isoform inhibition, however no data has been recorded in decreasing cell viability in glioma cell lines U87 MG and GOS-3, although it has been observed that it was toxic in SVG P12 cell line in 24 hours (Fig 3.9). Although there is a two-fold difference in COX-1 inhibition of PN517 compared to that of aspirin, it is the closest to aspirin compared to the other drugs, even though it affects COX-2 more too. This provides a potential explanation for the high efficacy of PN517 in U87 MG cell line, and warrants further investigation as it could contribute to future patient specific treatment based on COX expression. Even though COX-2 is associated with growth factors, oncogenes and carcinogens, it is suggested that the role of COX-1 in the induction of COX-2 explains in epidemiologic studies, why aspirin reduces risk of colorectal cancer at very low doses that could not sustain COX-2 inhibition in nucleated cells (Thun *et al.*, 1991, Giovannucci *et al.*, 1995). COX-2 expression between cell lines (Elder *et al.*, 2000; Sheng *et al.*, 1998) and is correlated with the cell characteristics, but not directly with malignancy. Since reports have shown that even non-cancerous cell lines express COX-2, this suggests that prostaglandins and lipid metabolites formed by astrocytes may be involved in central nervous system pathology and physiology (Pistritto *et al.*, 1998; Koyama *et al.*, 1999).

While the effect of cisplatin on cell viability increased from 24 to 48 hours, little increase was observed between time points with either aspirin or its analogues. In fact the IC₅₀ values for the aspirin analogues typically increased between time points in the 1321N1 and GOS-3 cell lines, indicating a recovering in cell viability following 48 hours (Fig 3.14 and

3.17 respectively). The only exception to this observation is the most potent analogue, PN517 in 1321N1 cells. The simplest explanation for this result is that the drugs are rapidly undergoing metabolism to form inactive metabolites.

The chemical stability of a drug is of great importance since it becomes less effective as it undergoes degradation. In humans, aspirin is rapidly deacetylated to salicylic acid, which is further metabolized by glucuronidation, hydroxylation, and glycine conjugation (Hutt *et al.*, 1986; Takanashi *et al.*, 2000). In low concentrations (250 mg) salicylates half-life is about 2-5 hours, but higher doses can increase the half-life up to 30 hours, because the biotransformation pathways concerned with the formation of salicyluric acid and salicyl phenolic glucuronide become saturated (Schorr, 2009).

Cytochrome P450 enzymes are essential for the detoxification of foreign chemicals and the metabolism of drugs and consist of a family of 50 enzymes, six of which metabolize 90 per cent of drugs (Guengerich, 2008). Cytochrome P450 enzymes are called so because they are bound to membranes within a cell (cyto) and contain a heme pigment (chrome and P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide. These enzymes are largely expressed in the liver, but they also occur in the small intestine, lungs, placenta, and kidneys, and in fact throughout all cells of the body at lower levels of expression.

Metabolism of NSAIDs involves oxidation by CYP enzymes and conjugation, particularly glucuronidation by phase II enzymes (Bigler *et al.*, 2001). Aspirin is rapidly deacetylated

to salicylic acid which is further metabolized by glucuronidation, hydroxylation, and glycine conjugation with the major enzymes involved being *CYP2C9* (Takanashi *et al.*, 2000; Miners and Birkett, 1998) and *UGT1A6* (Ciotti *et al.*, 1997). Both of these enzymes are polymorphic and produce slow-metabolizing enzymes, which does affect the quick metabolism of aspirin and could affect the metabolism of the aspirin analogues in the different cell lines. Significantly, the *CYP2C9* enzyme is expressed in glioma and its expression increased in higher grade glioma compared to low grade (Knupfer *et al.*, 2006). This expression of *CYP2C9* provides a potential explanation as to why the cell viability IC_{50} values for aspirin and its analogues did not decrease at 48 hours, and in addition also explains why they were least effective against the U87 MG cell line which was derived from high grade GBM (Fig 3.16 to 3.18).

Conclusion

In conclusion, while each of the analogues decreased cell viability and proliferation, PN517 consistently displayed the greatest efficacy. The effect of PN517 was greater than that of aspirin, and in high grade glioma (U87 MG) was greater than that of cisplatin. The results from both the cell viability and COX assays suggest that the aspirin analogues will produce tumour grade selective effects. Further investigation is needed to determine the mechanism of action of the most efficacious drug (PN517) and compare it to that of aspirin because cell viability is the measure of the overall health of a population and is affected by a range of parameters including cell proliferation, cell death and enzyme activity.

Chapter 4: Cell proliferation and Cell cycle analysis

4.1 Introduction

While cell viability is an important screening tool in the characterisation of novel compounds, it is a reflection of the overall health of a population and is affected by a range of parameters including cell proliferation, cell death, and metabolic activity, and thus, accurate characterisation of drug effects requires more specific assays.

Examining the cell cycle status of individual cells of a proliferating population can be used to further characterise novel compounds and can be quickly and accurately determined using flow cytometry and propidium iodide staining (Cecchini *et al.*, 2012). The cell cycle is a highly controlled series of events which has two major phases, interphase and the mitotic phase (Nojima, 1997). The first of these, interphase, can be further subdivided into three stages, G1 where cells initiate RNA and protein synthesis to induce growth, S phase where the cells replicate their DNA, and G2 phase where cells continue to prepare for division. The mitotic phase, as the name suggests, is where a mother cell divides and produces two identical daughter cells. Propidium iodide (PI) is a red-fluorescent dye agent that intercalates between DNA bases with a stoichiometry of one dye molecule per 4-5 base pairs, and following a maximum excitation at 535 nm, PI emits at a maximum wavelength of 617 nm (Life Technologies UK, 2014). This property of PI can be exploited to determine the cell cycle phase distribution of a population, because as the cell progresses through S-phase, the DNA content ultimately doubles, and consequently PI intercalation increases proportionally, as does the resulting fluorescence meaning the fluorescence of cells in the G2/M phase will be twice as high as that of cells in the G0/G1 phase.

As already mentioned, the cell cycle is a highly regulated process where, following DNA replication, identical chromosomal copies are distributed to two daughter cells. An important regulator of this process is the cyclins, with progression between phases initiated after cyclins are phosphorylated by an activating kinase (Israels and Israels, 2000). Cyclin dependent kinases form complexes with partner cyclins and regulate specific protein substrates through phosphorylation to control progression through the cell cycle. The expression of cyclins D 1, 2 and 3 is associated with G1 phase of the cell cycle where DNA replication initiates (Sherr and Roberts, 2004). Their accumulation reaches a maximum before S-phase and the formation of complexes with CDK4 and CDK6 regulates the phosphorylation of retinoblastoma (RB) protein which controls G1 progression (Baldin *et al.*, 1993; Israels and Israels, 2000).

While cyclin D1 expression is observed in normal brain tissue, it is overexpressed in glioma (Zhang, 2005), and this overexpression increases proliferation and invasion, while reducing apoptosis (Wang, 2011). Increasing evidence has shown that cyclin D1 overexpression in the tumour cells is partly dependent on the mitogenic effects of EGF signalling through the EGFR in a number of cancers (Rieber and Rieber, 2006; Poch *et al.*, 2001; Perry *et al.*, 1998). Thus, downregulation of cyclin D1 expression may provide a new gene therapy approach for patients with malignant glioma.

Epidermal growth factor receptor (EGFR) and its ligands are frequently upregulated in cancer including GBM and typically is a hallmark for high grade tumours resistant to therapy (Lo *et al.*, 2006; Yarden, 2001), however, is not always a marker for survival

(Faulkner *et al.*, 2014). EGFR is involved in DNA synthesis, enhanced cell growth, invasion, and metastasis and specific abrogation of EGFR results in cell cycle arrest, apoptosis, or differentiation of cancer cells (Lui and Grandis, 2002). It has also been demonstrated that PGE₂ enhances cell proliferation through EGFR transactivation (Fernández-Martínez and Cazaña, 2013; McCarty, 2014). Establishing EGFR expression and the impact of drug treatment on its activation is important in the development of novel therapies in glioma.

Thus, having established that the aspirin analogues decreased cell viability, it was subsequently decided to determine if cell proliferation was affected using the fluorescent proliferation marker CFDA-SE and flow cytometry. Additionally, cell cycle analysis was performed to assess the status of the cells following drug treatment, and finally, due to the association of EGFR with cyclin D1, the effect of drug treatment on Cyclin D phosphorylation in EGFR treated cells was determined.

Hypothesis

It known that NSAID treatment effects cell cycle in many cell lines, with reports of G0/G1 phase arrest and changes cell cycle distribution, including in M/G1 phase, as well as G2/M and S/G2 phases. As a result, it is expected that the analogues PN517 and PN508 will have an effect on both proliferation and cell cycle and regulatory proteins including cyclin D1.

4.2 Results

Cell proliferation is regularly assayed using CFDA-SE (carboxyfluoresceindiacetate, succinimidyl ester), a colourless and non-fluorescent dye that passively diffuses into cells where its acetate groups are cleaved by intracellular esterases producing an amine-reactive carboxyfluoresceinsuccinimidyl ester with a green fluorescence (Weston, 1990). Importantly, the succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates, and these dye–protein adducts are retained by cells throughout development, mitosis, and *in vivo* tracing and is not transferred to adjacent cells in a population. Importantly for proliferation, the label is inherited by daughter cells after cell division, resulting in a 50% reduction in fluorescence following each cell division (Weston and Parish, 1990). Initial studies examined the effect of drug treatment on the established glioma cell lines where a rightward shift in the data indicates an increase in the average fluorescence of the population and thus a decrease in proliferation compared to control (Fig 4.1 to 4.9).

The results from the 1321N1 cell line indicate that while both aspirin analogues significantly decrease proliferation compared to control (PN517, $p < 0.001$; PN508, $p < 0.001$), there wasn't a significant difference between the analogues. While both aspirin and the double dose of aspirin decreased the rate of proliferation (aspirin, $p < 0.0001$; 2x aspirin, $p < 0.001$), there was no significant difference between them. Interestingly, cisplatin had no effect on proliferation at 24 hours, but significantly reduced proliferation at 48 hours ($p < 0.001$). The treatment that reduced proliferation to the greatest extent compared to control in the GOS-3 cell line was cisplatin ($p < 0.001$). The aspirin analogues

PN517 also significantly reduced cell proliferation ($p < 0.0001$), with PN508 not having any effect ($p > 0.05$). The effect of PN517 was significantly greater than that of aspirin ($p < 0.001$). Results from the U87 MG cell line found that the treatment with the greatest effect on proliferation compared to control was cisplatin ($p < 0.001$), with aspirin having no effect. PN517 and PN508 both significantly reduced proliferation ($p < 0.001$ and $p < 0.05$ respectively), but were not significantly different from each other. Interestingly, while aspirin did not affect proliferation, 2x aspirin significantly reduced proliferation, producing a similar effect to the aspirin analogues. Finally, in the 1321N1 cell line there was the greatest effect on proliferation ($p < 0.001$), in cisplatin, aspirin and PN517. There was no significant effect between U87 MG and 1321N1 cell lines effect in aspirin and PN517. GOS-3 showed a similar effect to that of SVG-p12 in cisplatin, aspirin and PN517 ($p < 0.05$).

The effect of each treatment was also compared across the cell lines (Fig 4.5). In each case, treatments were most effective against the GOS-3 cell line with the exception of the aspirin analogues (panels D and E) which were most effective against the SVG-p12 cell line. A general trend of all treatments was displayed towards a greater effect on proliferation of the SVG-p12 and GOS-3 cell lines than U87 MG and 1321N1.

Figures 4.6 to 4.9 illustrate sample raw data as generated using the flow cytometer. In each case a leftward shift in the peaks indicates cell proliferation as the intensity of fluorescent signal decreases.

To summarise, proliferation was significantly reduced by treatment with PN517 when compared to control in the SVG-p12 cell line. This effect was not significantly different to that of treatment with double the concentration of aspirin. In the glioma cell lines, all treatments significantly reduced proliferation in the low grade 1321N1 cells, but were not significantly different from each other. In GOS-3 cells, PN517 also significantly reduced proliferation, however, in the high grade glioma U87 MG cells, the only significant reduction in proliferation was observed following cisplatin treatment.

Consistent with the cell viability results, the most effective analogue in the SVG-p12 and GOS-3 cell lines was PN517. However, in the 1321N1 and U87 MG cell lines the pattern was not repeated, potentially suggesting that other signalling pathways are involved.

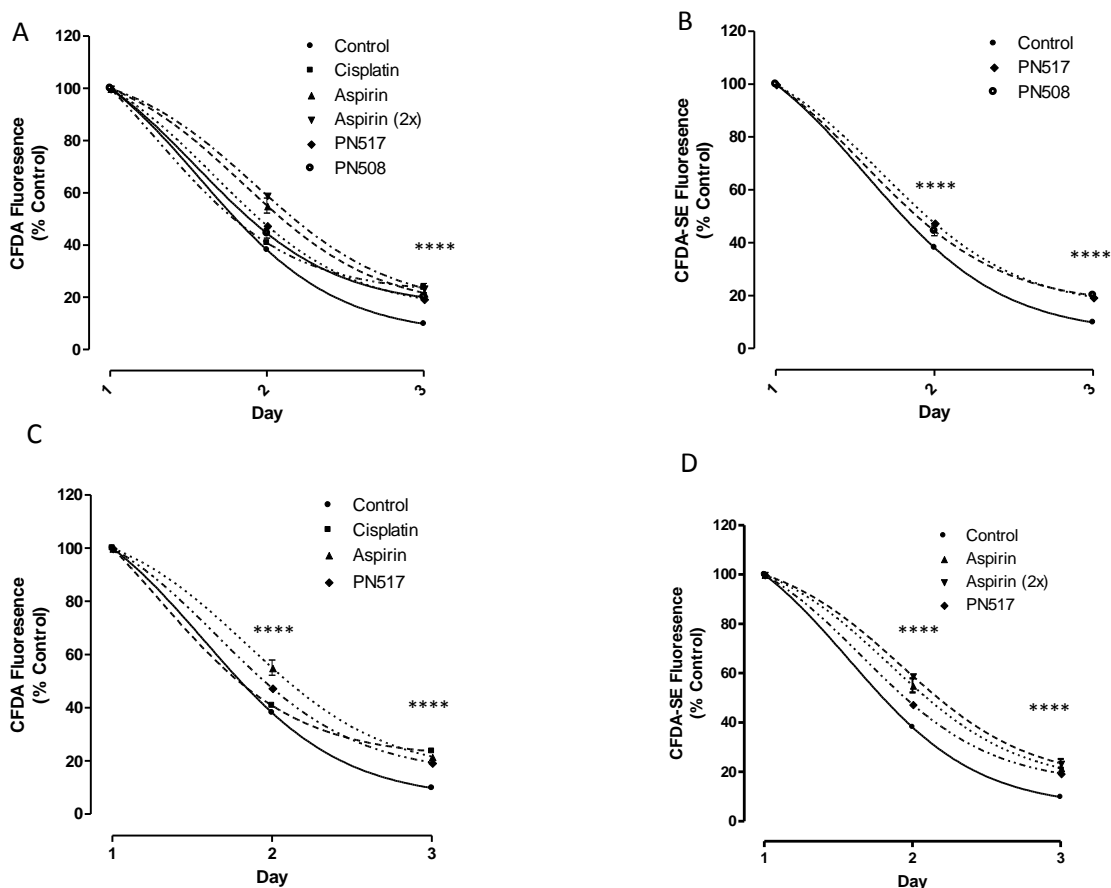


Figure 4.1 Cell proliferation measured by flow cytometry following drug treatment in 1321N1 cell line. The data illustrate cell proliferation determined using CFDA-SE staining of the 1321N1 cell line, following drug treatment (0.1mM) over a period of 3 days. Panel A shows the effect on all treatments on cell proliferation, where a rightward shift indicates a reduction in proliferation; panel B compares PN508 to PN517; panel C compares the most potent analogue PN517 with control, aspirin and cisplatin treatments; panel D illustrates the effect of aspirin and double aspirin in comparison to control and PN517 treatments. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

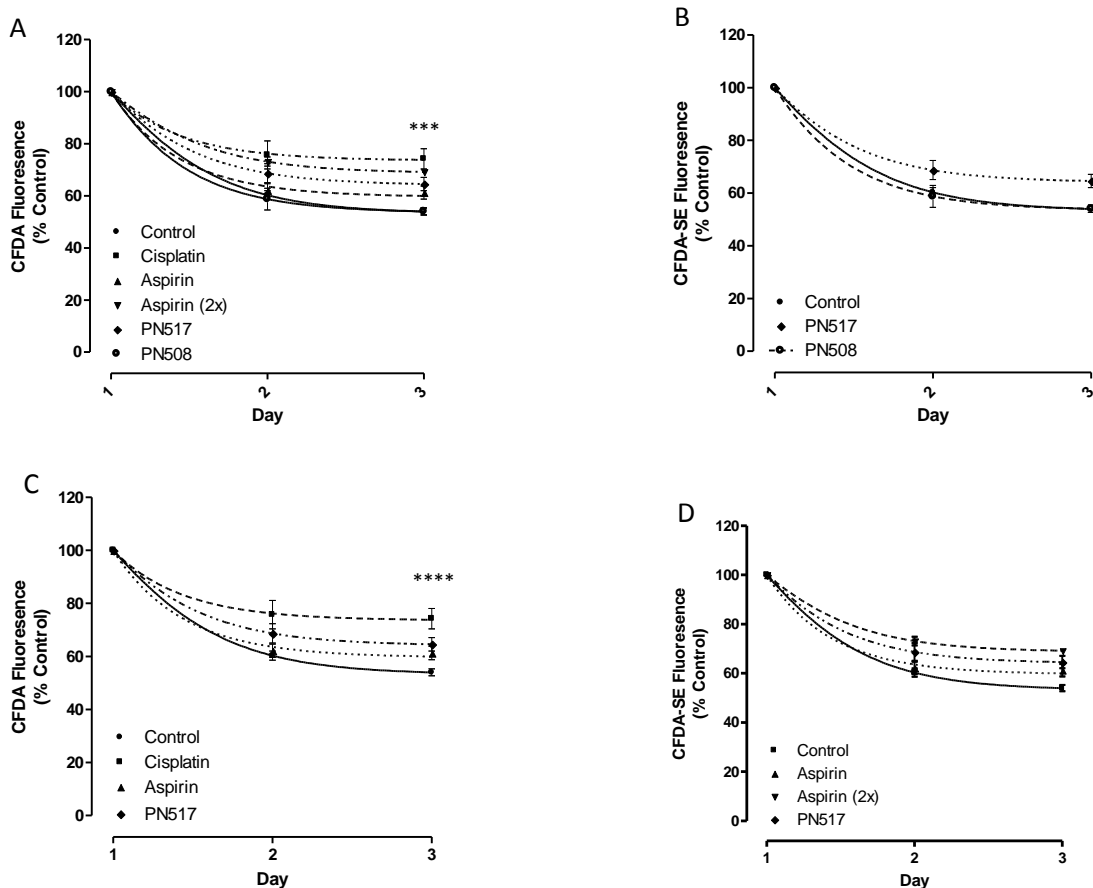


Figure 4.2 Cell proliferation measured by flow cytometry following drug treatment in GOS-3 cell line. The data illustrate cell proliferation determined using CFDA-SE staining of the GOS-3 cell line, following drug treatment (0.1mM) over a period of 3 days. Panel A shows the effect on all treatments on cell proliferation, where a rightward shift indicates a reduction in proliferation; panel B compares PN508 to PN517; panel C compares the most potent analogue PN517 with control, aspirin and cisplatin treatments; panel D illustrates the effect of aspirin and double aspirin in comparison to control and PN517 treatments. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

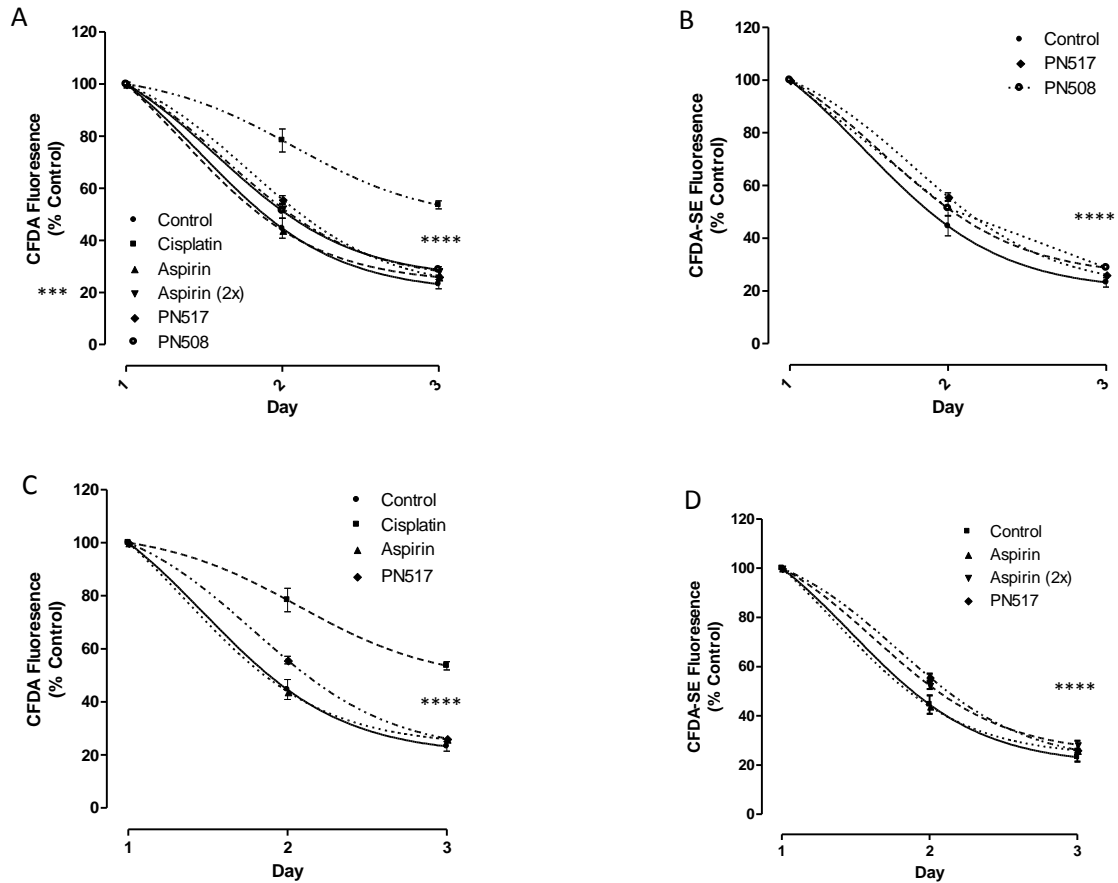


Figure 4.3 Cell proliferation measured by flow cytometry following drug treatment in U87 MG cell line. The data illustrate cell proliferation determined using CFDA-SE staining of the U87 MG cell line, following drug treatment (0.1mM) over a period of 3 days. Panel A shows the effect on all treatments on cell proliferation, where a rightward shift indicates a reduction in proliferation; panel B compares PN508 to PN517; panel C compares the most potent analogue PN517 with control, aspirin and cisplatin treatments; panel D illustrates the effect of aspirin and double aspirin in comparison to control and PN517 treatments. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

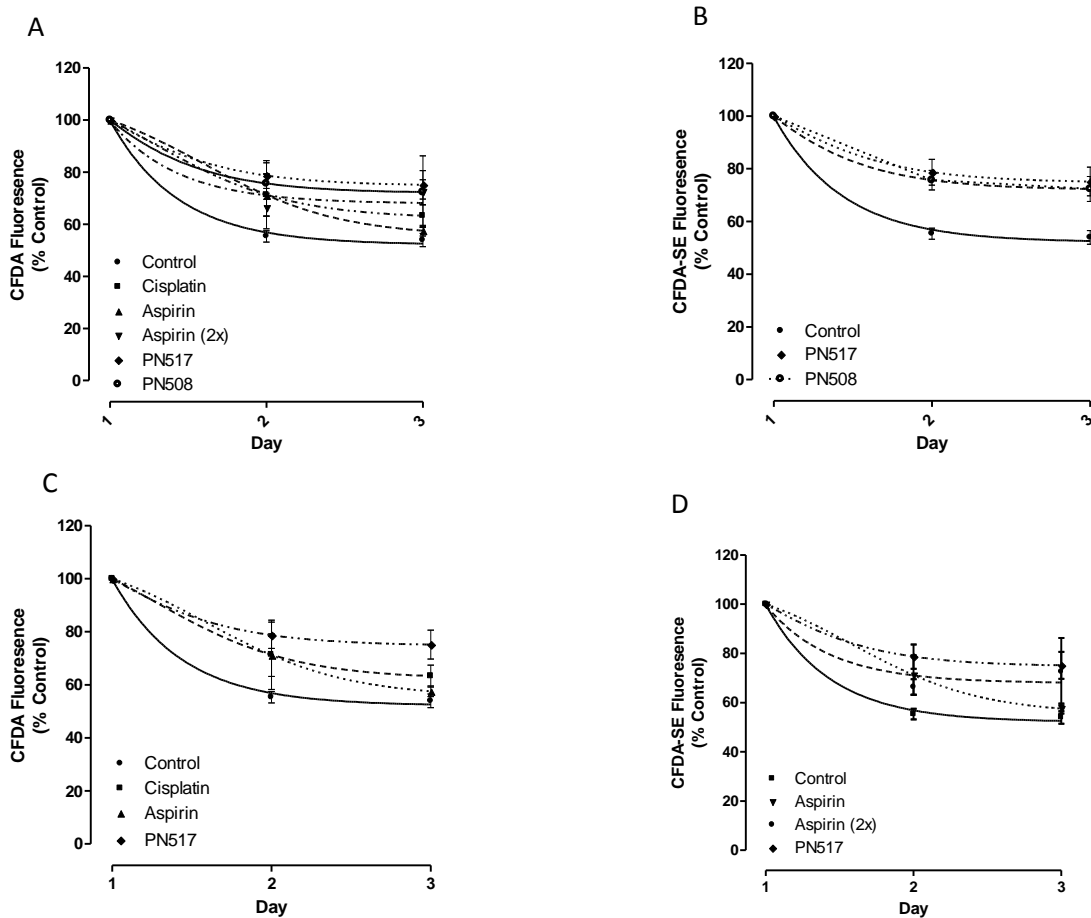


Figure 4.4 Cell proliferation measured by flow cytometry following drug treatment in SVG-p12 cell line. The data illustrate cell proliferation determined using CFDA-SE staining of the SVG-p12 cell line, following drug treatment (0.1mM) over a period of 3 days. Panel A shows the effect on all treatments on cell proliferation, where a rightward shift indicates a reduction in proliferation; panel B compares PN508 to PN517; panel C compares the most potent analogue PN517 with control, aspirin and cisplatin treatments; panel D illustrates the effect of aspirin and double aspirin in comparison to control and PN517 treatments. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

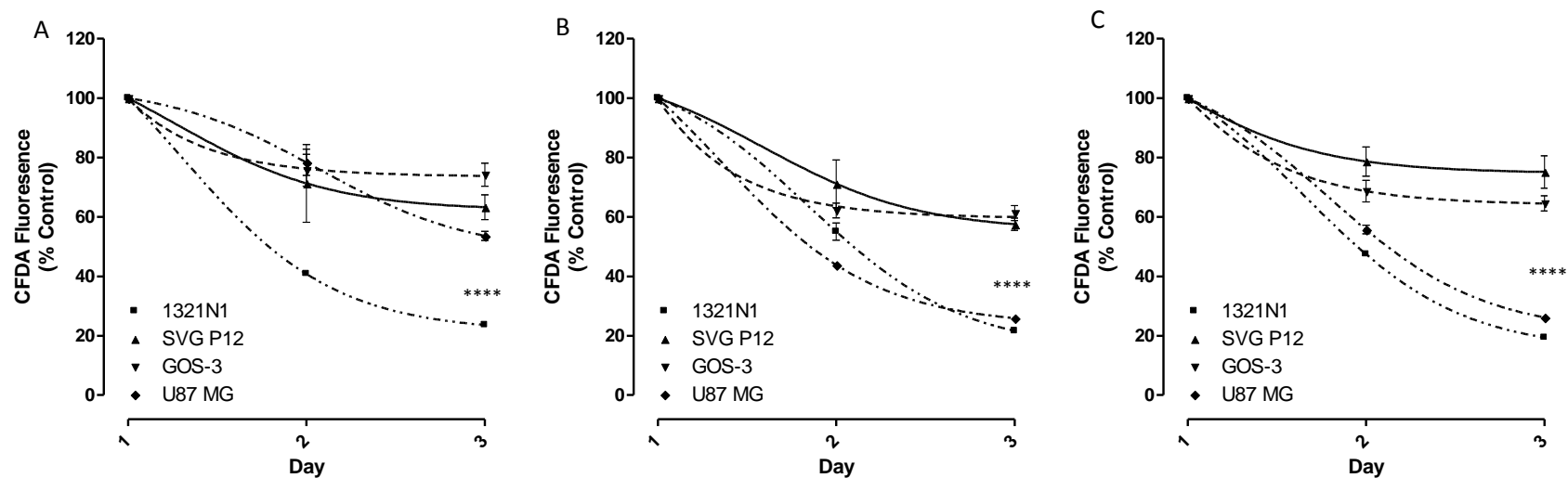


Figure 4.5 Comparison of proliferation measured by flow cytometry following drug treatment of the SVG-p12, GOS-3, 1321N1 and U87 MG cell lines. The figure illustrates the effect of each drug treatment (0.1mM) on proliferation of U87 MG (parallelogram), SVG-p12 (triangle), 1321N1 (square), and GOS-3 (inverted triangle) cell lines as measured by CFDA-SE staining. Panel A shows proliferation following treatment with cisplatin; panel B with aspirin; and panel C with PN517. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

Cell Cycle Analysis

Cell cycle analysis by flow cytometry was performed following PI staining and drug treatment of each of the cell lines over a 48 hour period (Figures 4.6 to 4.13).

In the SVG-p12 control cell line, treatment with cisplatin, aspirin or PN517 at 0.1mM did not significantly alter cell cycle populations following 4, 8 and 12 hours of treatment (Fig 4.6, panels A-C). Also, there was no significant change in the control population distribution over 48 hours. However, following 24 and 48 hours of treatment (Fig 4.6, panels D & E respectively), all three drug treatments produced significant changes in cell cycle distribution. Cisplatin produced overall the largest effect by 48 hours with the sub G1 population indicating apoptosis increasing from 1.3% to 24% ($p < 0.0001$), G1 decreasing from 88.3% to 35%, ($p < 0.0001$) with a large increase in the S phase population, increasing from 3.3% to 34.4%, ($p < 0.0001$). A small but significant increase in the M phase population was also observed (6% to 8%), ($p < 0.0001$). No significant difference was observed between aspirin and PN517 treatments, both producing small increases in the apoptotic population (0.5 and 3% respectively), with a significant decrease in the G1 phase (88.3% and 64% respectively) ($p < 0.0001$), and increases in both the S (1 and 13% respectively) and M phases (9.6 and 20% respectively) ($p < 0.0001$). A similar trend was observed following treatment at 1mM (Fig 4.7), however a greater increase in the S phase population was observed for each of the drug treatments (cisplatin 30%, aspirin 14%, PN517 12%) ($p < 0.0001$).

The 1321N1 cells produced some unexpected results. The control population in both the 0.1mM (Fig 4.8) and 1mM (Fig 4.9) treatment groups displayed an unusually high M phase population, in addition to a larger than expected S phase population at some time points. However, it can be seen that cisplatin treatment (0.1mM, Fig 4.10) significantly increased the apoptotic and S phase populations compared to control at each time point ($p < 0.0001$ and $p < 0.0001$, respectively). Aspirin treatment (0.1mM) also increased the apoptotic population significantly ($p < 0.0001$) apart from at 8 hours, with an increase in the S phase population found at each time point ($p < 0.0001$). PN517 treatment resulted in a gradual but significant increase in the S phase population (8% $p < 0.0001$ at 8 hrs; 26% $p < 0.0001$ at 12 hrs; 42% $p < 0.0001$ at 24 hrs; 65% $p < 0.0001$ at 48 hours). Treatment at 1mM produced similar results, with cisplatin significantly increasing the apoptotic and S phase populations at all time points compared to control ($p < 0.0001$), aspirin significantly increasing the S phase population at all time points ($p < 0.0001$) and apoptotic population at 12 (23 %, $p < 0.0001$), 24 (17 %, $p < 0.0001$) and 48 (6 %, $p < 0.0001$) hours. PN517 treatment significantly increased the S phase population at all time points compared to control ($p < 0.0001$).

A different pattern of drug effects was observed for the GOS-3 cell line. While the control population displayed a normal distribution at 4, 24 and 48 hour time points, a large increase in the M phase population can be seen at both 8 and 12 hours (Fig 4.10). Additionally, cisplatin (0.1mM) produced a significant increase in the apoptotic population at each time point compared to control ($p < 0.0001$) with particularly large increases in the S phase population ($p < 0.0001$). While treatment with aspirin initially produced a significant

increase in the apoptotic population (17% at 12 hours, $p < 0.0001$), this effect was lost by 24 and 48 hours when a significant increase in the M phase population can be seen (53.4%, $p < 0.0001$, and 54% $p < 0.0001$, respectively). PN517 treatment resulted in a significant increase in the apoptotic population at 12 (23 %, $p < 0.0001$) and 48 (28%, $p < 0.0001$) hours with significant increase in the S and M phase populations compared to control ($p < 0.0001$) at all time points apart from 48 hours. While the control populations display a normal distribution over each time point in the 1mM treatment group (Fig 4.11), cisplatin again produced significant increases in the apoptotic population ($p < 0.0001$) at all time points, and a similar increase in the S phase population ($p < 0.0001$). At all time points, PN517 produced a significant decrease in the G1 phase population compared to control ($p < 0.0001$), with an increase in the M phase population at 4, 8 and 48 hours treatment ($p < 0.0001$), with aspirin producing a similar pattern of results.

A similar pattern of cell cycle distribution was observed between the SVGP12 and U87 MG cell line, where treatment at 0.1mM had little effect at up to 12 hours (Fig 4.12, panels A – C), with a decrease in the G1 phase population and corresponding increases in the apoptotic and S and M phase populations following 24 hours of treatment with all three drugs (Fig 4.12, panel D). A further increase in the S and M phase populations can be observed following 48 hours of cisplatin treatment (Fig 4.12, panel E), however, while no greater effect of aspirin was observed, the apoptotic population further increased with PN517 treatment. At 1mM, cisplatin significantly increased the apoptotic population following 24 (17%) and 48 hours (37%) of treatment compared to control ($p < 0.0001$). A significant increase in the S phase population was also observed at both time points (47

and 27% respectively, $p < 0.0001$). Again, while an increase in the apoptotic population (8%, $p < 0.0001$) and decrease in G1 phase population (79%, $p < 0.0001$) following aspirin treatment for at 24 hours, no enhancement of this effect was found at 48 hours. Treatment with the aspirin analogue PN517 decreased the G1 phase population significantly at both 24 and 48 hour time points (52%, $p < 0.0001$, and 36%, $p < 0.0001$, respectively), with corresponding increases in both S (24%, $p < 0.0001$, and 29%, $p < 0.0001$, respectively) and M (16.5%, $p < 0.0001$, and 30%, $p < 0.0001$, respectively) phase populations. One unexpected result was an increase in the M phase population following both 0.1 and 1mM treatments at 8 hours.

To summarise, a similar effect of treatment was observed in the SVG-p12 and U87 MG cell lines with the greatest effects observed following cisplatin treatment for 48 hours. In the lower grade glioma cell lines, 1321N1 and GOS-3, again the most potent treatment was cisplatin, but all drugs altered cell cycle distribution even at early timepoints, with phase distribution changing over time.

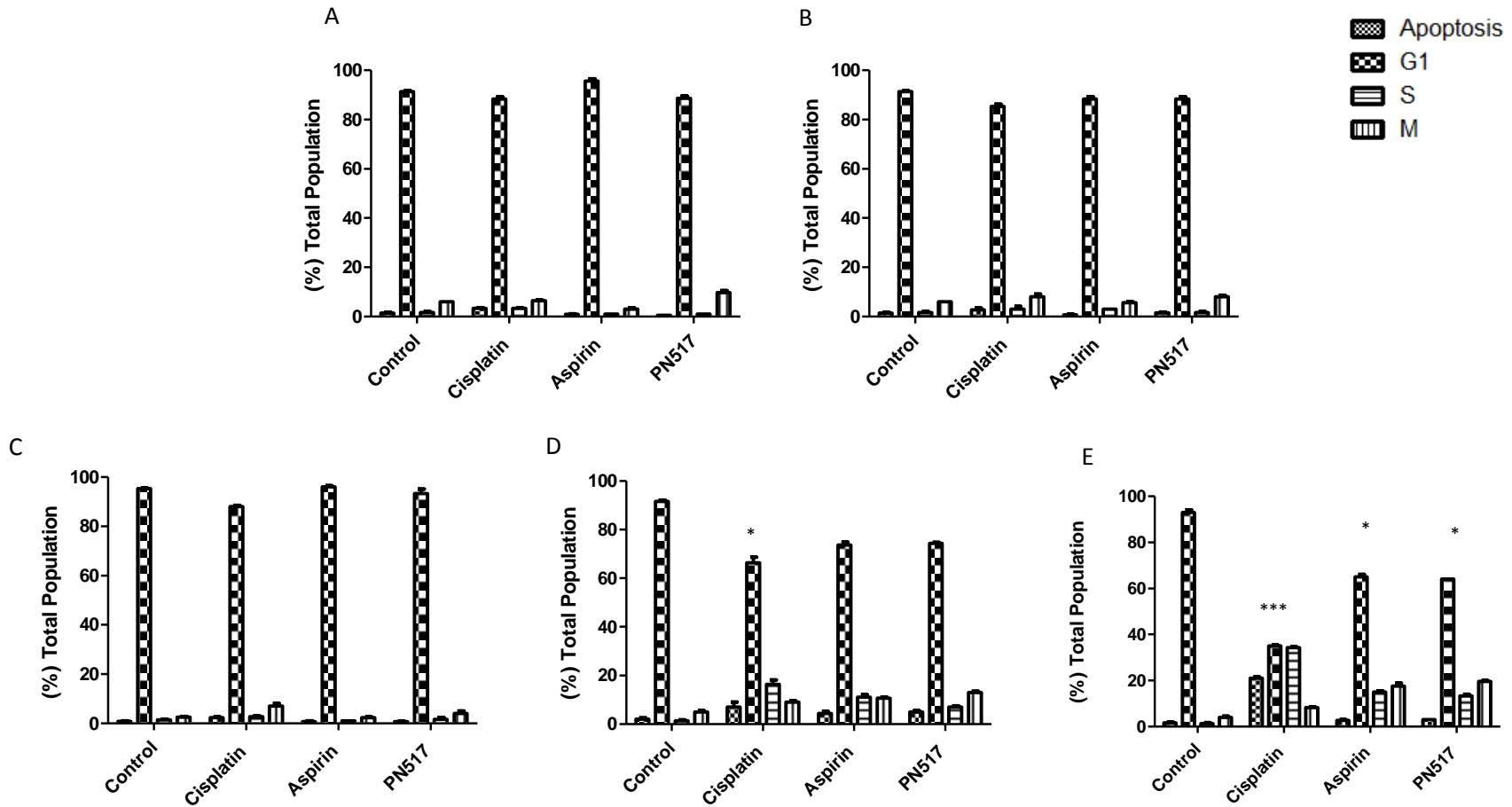


Figure 4.6 Cell cycle distribution of the SVG P12 cell line following drug treatment 0.1 mM. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 4 (panel A), 8 (panel B), 12 (panel C), 24 (panel D) and 48 (panel E) hours respectively. The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

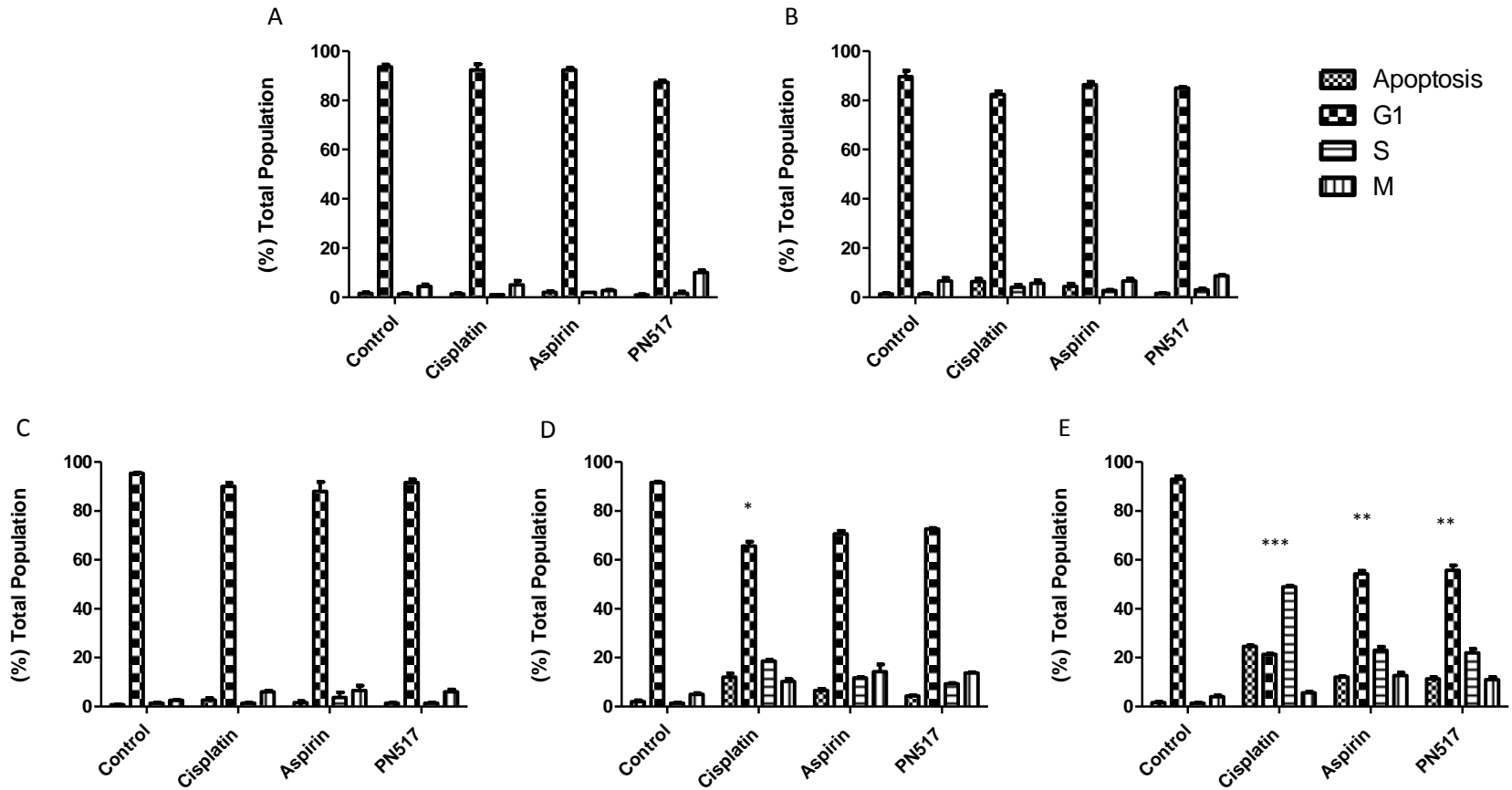


Figure 4.7 Cell cycle distribution of the SVG P12 cell line following drug treatment 1 mM. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 4 (panel A), 8 (panel B), 12 (panel C), 24 (panel D) and 48 (panel E) hours respectively. The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

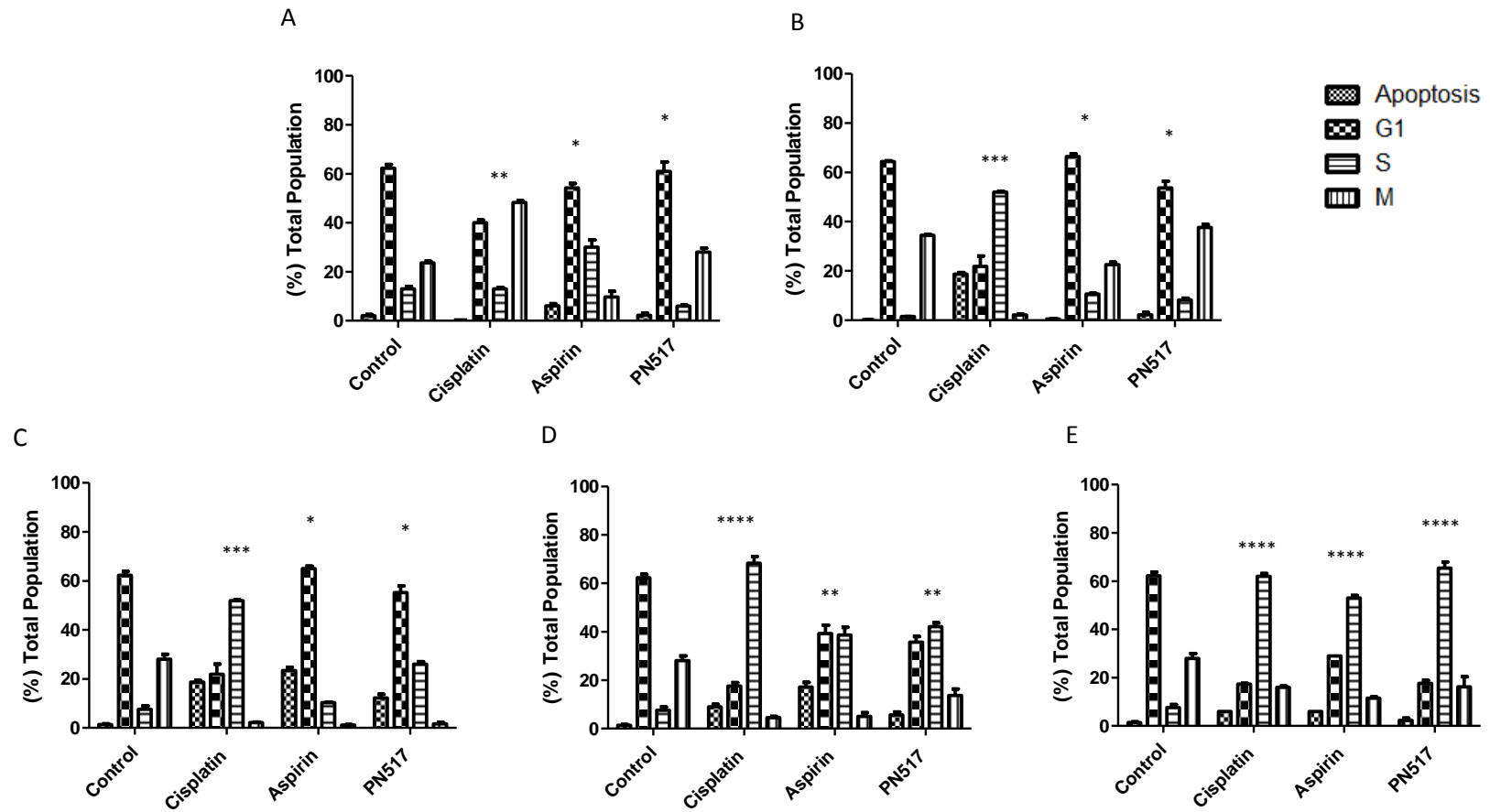


Figure 4.8 Cell cycle distribution of the 1321N1 cell line following drug treatment 0.1 mM. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 4 (panel A), 8 (panel B), 12 (panel C), 24 (panel D) and 48 (panel E) hours respectively. The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

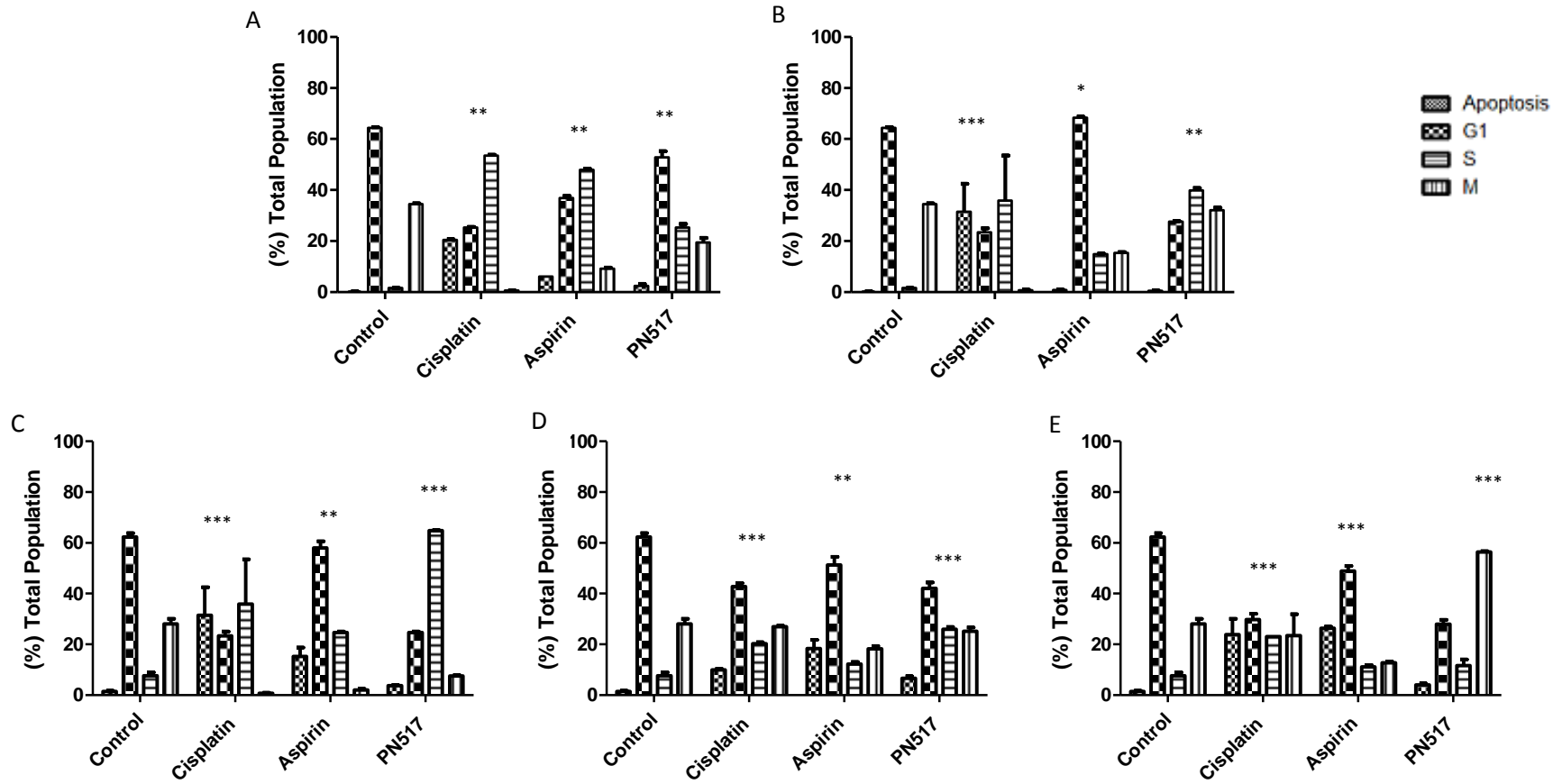


Figure 4.9 Cell cycle distribution of the 1321N1 cell line following drug treatment 1 mM. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 4 (panel A), 8 (panel B), 12 (panel C), 24 (panel D) and 48 (panel E) hours respectively. The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

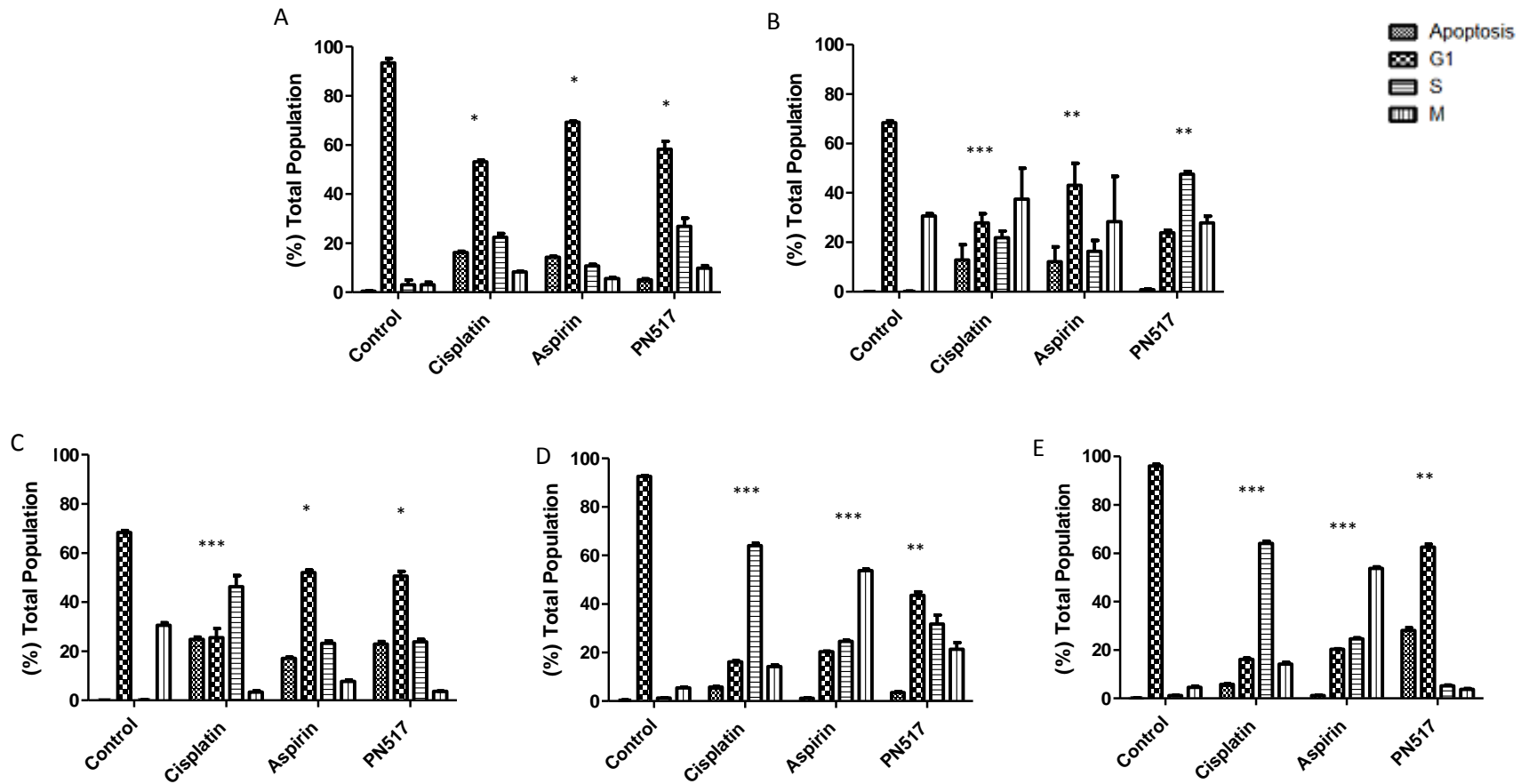


Figure 4.10 Cell cycle distribution of the GOS-3 cell line following drug treatment 0.1 mM. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 4 (panel A), 8 (panel B), 12 (panel C), 24 (panel D) and 48 (panel E) hours respectively. The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

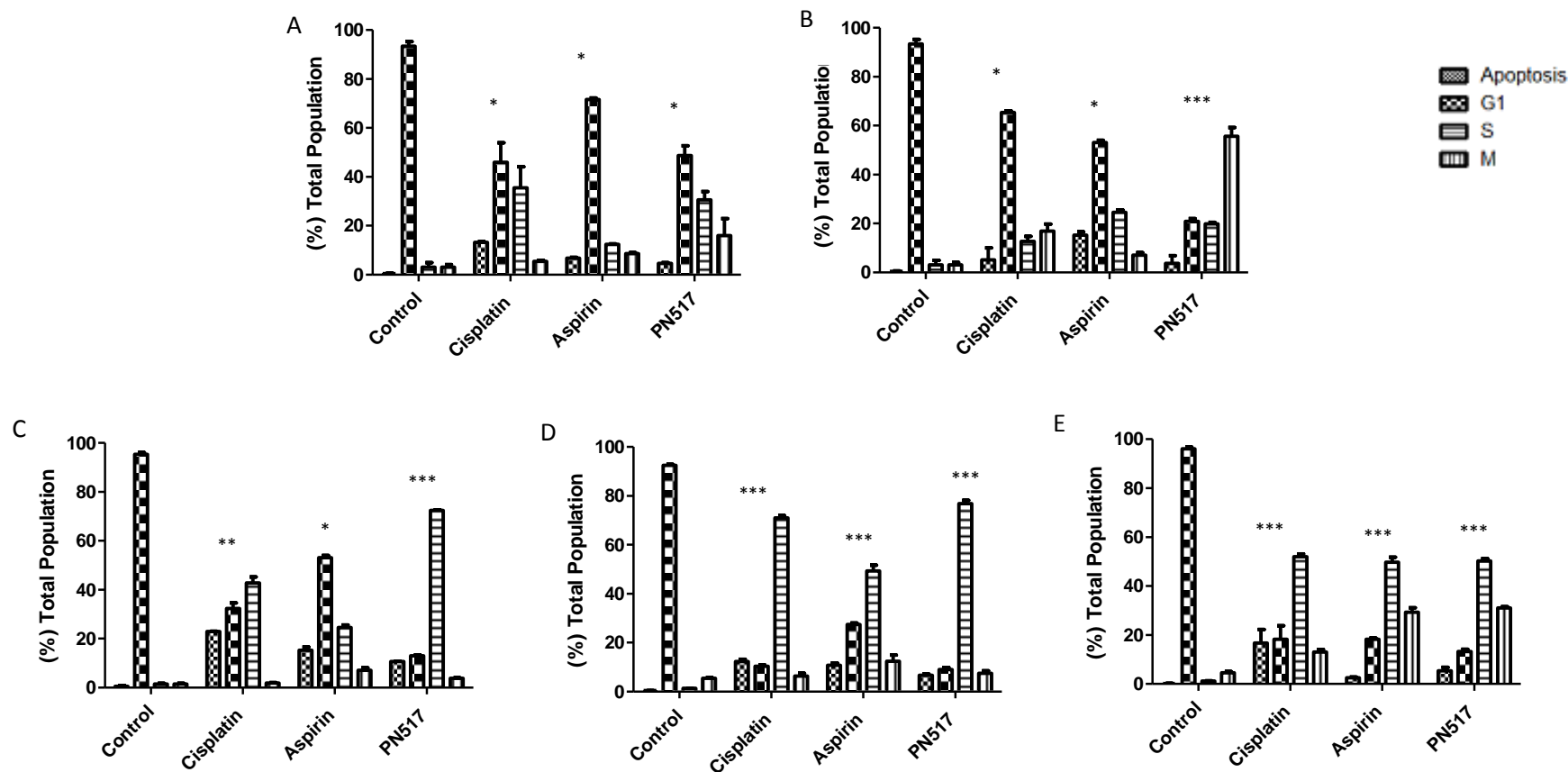


Figure 4.11 Cell cycle distribution of the GOS-3 cell line following drug treatment 1 mM. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 4 (panel A), 8 (panel B), 12 (panel C), 24 (panel D) and 48 (panel E) hours respectively. The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

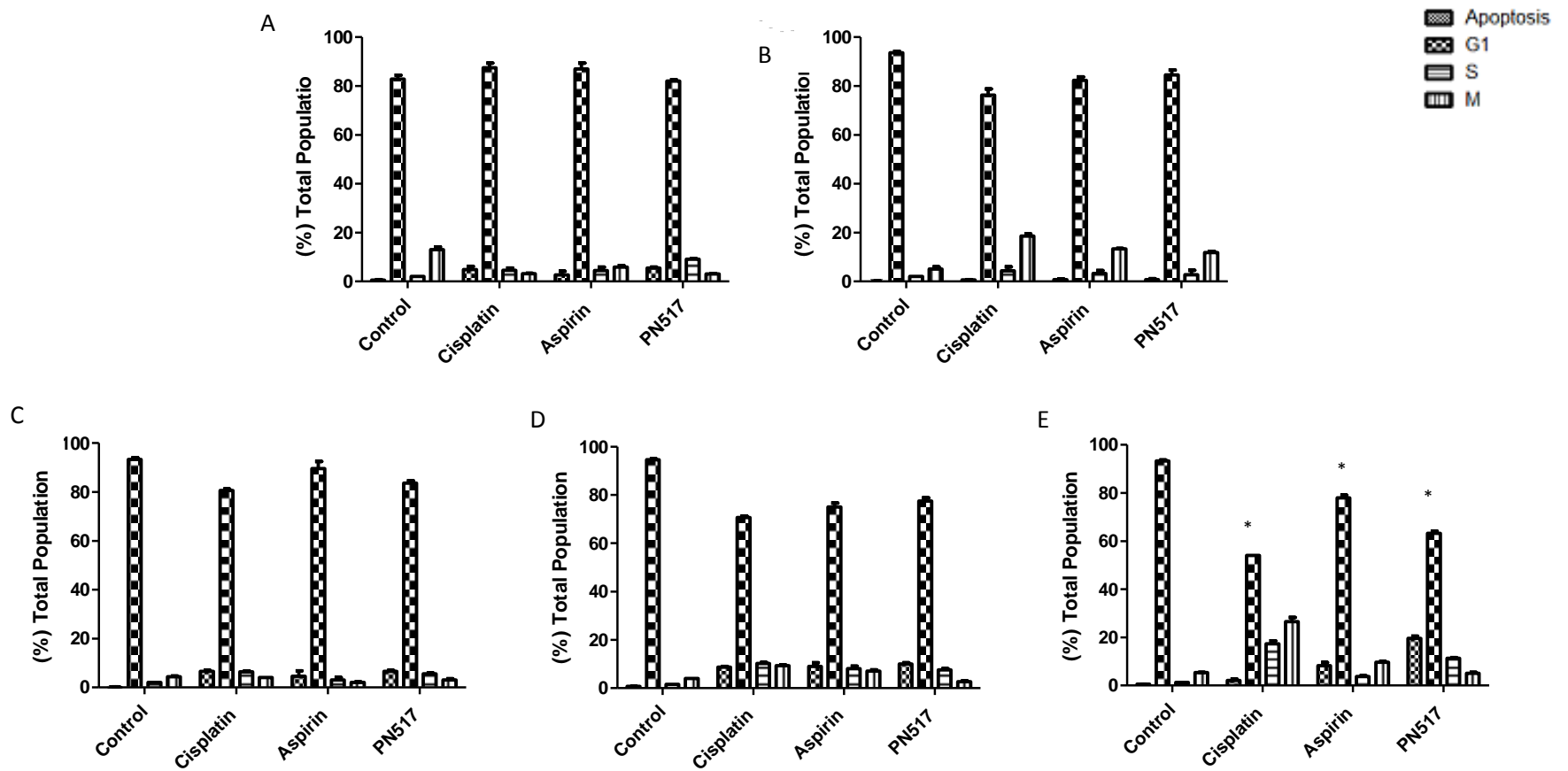


Figure 4.12 Cell cycle distribution of the U87 MG cell line following drug treatment 0.1 mM. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 4 (panel A), 8 (panel B), 12 (panel C), 24 (panel D) and 48 (panel E) hours respectively. The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

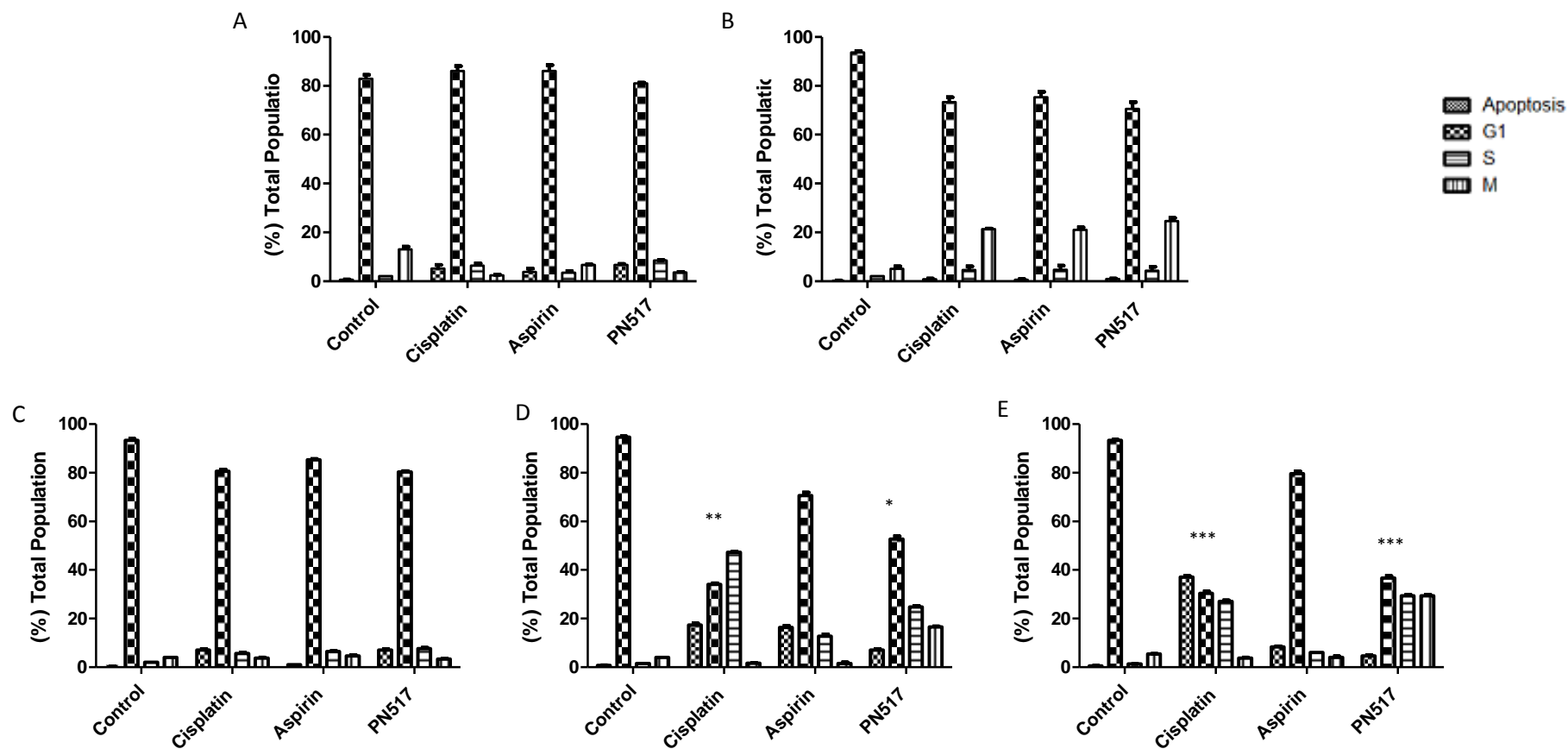


Figure 4.13 Cell cycle distribution of the U87 MG cell line following drug treatment 1 mM. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 4 (panel A), 8 (panel B), 12 (panel C), 24 (panel D) and 48 (panel E) hours respectively. The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

Cyclin D

The effect drug treatment on cyclin D1 activation in the U87 MG cell line was examined after 24 hour treatment at 1 mM, following EGF activation for 0, 5, 15 and 60 minutes (Fig 4.14). Using densitometry, cyclin D1 activation, indicated by phosphorylation (pCyclin D1), was expressed as a percentage of the phosphorylation found in cells that were not drug treated, but activated with EGF. The total expression of cyclin D1 was also determined for each sample and used to correct for any loading differences between lanes. Cisplatin and PN517 treatments produced the greatest reduction in phosphorylation following 5 and 15 minutes of activation, with no significant difference between respective time points. Aspirin treatment did not significantly reduce phosphorylation at any of the time points tested.

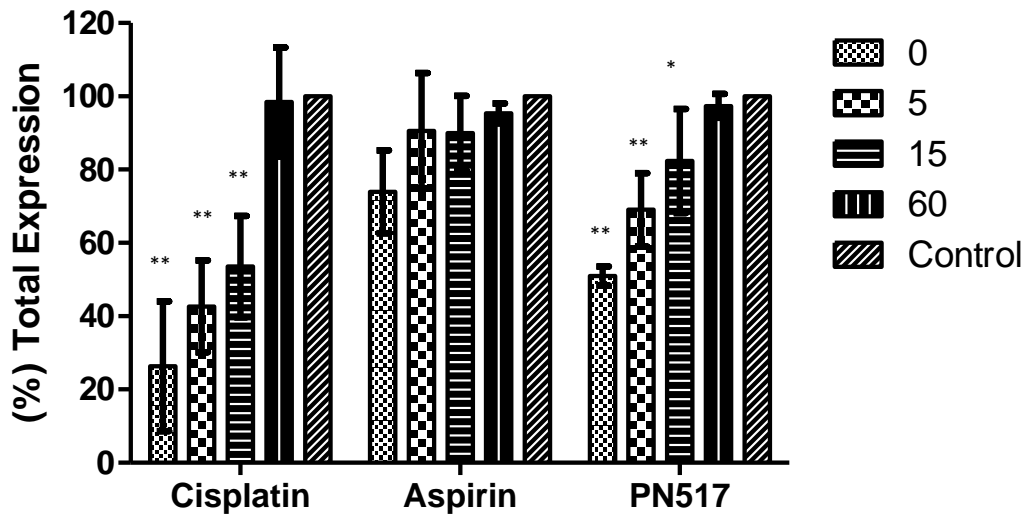
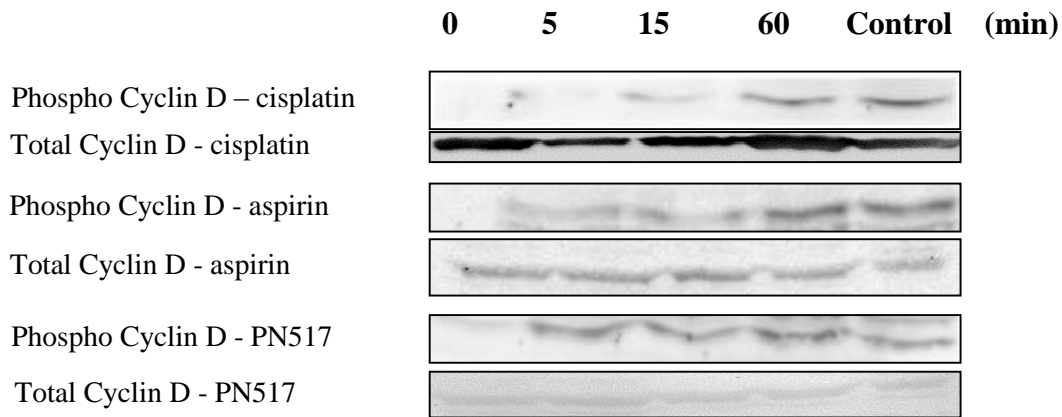


Figure 4.14 Phosphorylation of Cyclin D following drug treatment for 24 hr at 1 mM and EFG activation in the U87 MG cell line. The data illustrate cyclin D phosphorylation in U87 MG cell line after 24 hour drug treatment and EGF stimulation for 0, 5, 15 and 60 minutes as determined by densitometry. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.05$) with data representing results from 3 independent experiments.

EGFR Activation

Initial western blotting examined EGFR expression in the cell lines (Fig 4.15). It was observed that EGFR expression was lowest in the SVG-p12 cell line with a similar level of expression in GOS-3, and highest in the 1321N1 cells with a similar level of expression in U87 MG.

EGFR activation following EGF stimulation was examined in the U87 MG cell line (Fig 4.16) and phosphorylation compared to non-treated control following EGF stimulation. Total EGFR expression and EGFR phosphorylation were determined by densitometry, with any differences in sample loading corrected using the total EGFR results. EGFR phosphorylation was subsequently expressed as a percentage of non-treated control phosphorylation following 60 minutes of EGF activation. Both aspirin and PN517 significantly reduced the levels of phosphorylation following 60 minutes of EGF stimulation in comparison to control, but no significant difference was observed in phosphorylation between aspirin and cisplatin treatments.

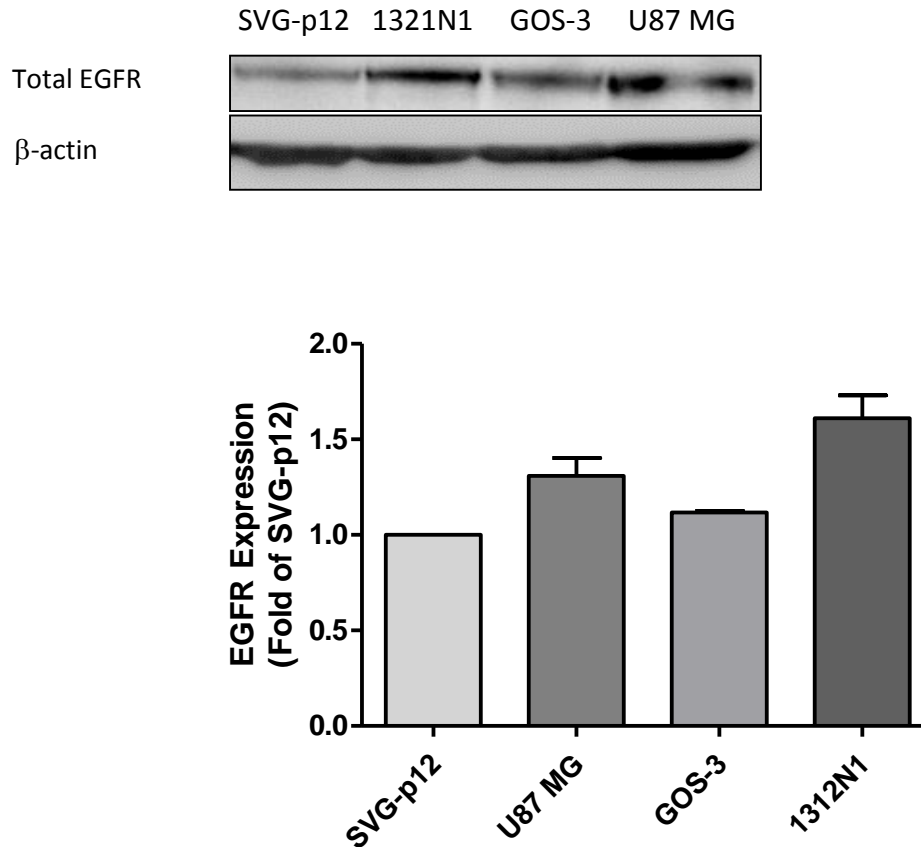


Figure. 4.15 Expression of the EGF receptor in the SVG-p12, 1321N1, GOS-3 and U87 MG cell lines. The data represents the total EGFR expression determined by western blotting in each cell line and expressed as a fold difference from the lowest expressing cell line, the SVG-p12. SVG-p12 showed the lowest level of expression followed by GOS-3, 1321N1, with U87 MG showing the highest. Data are representative of results from 3 independent experiments.

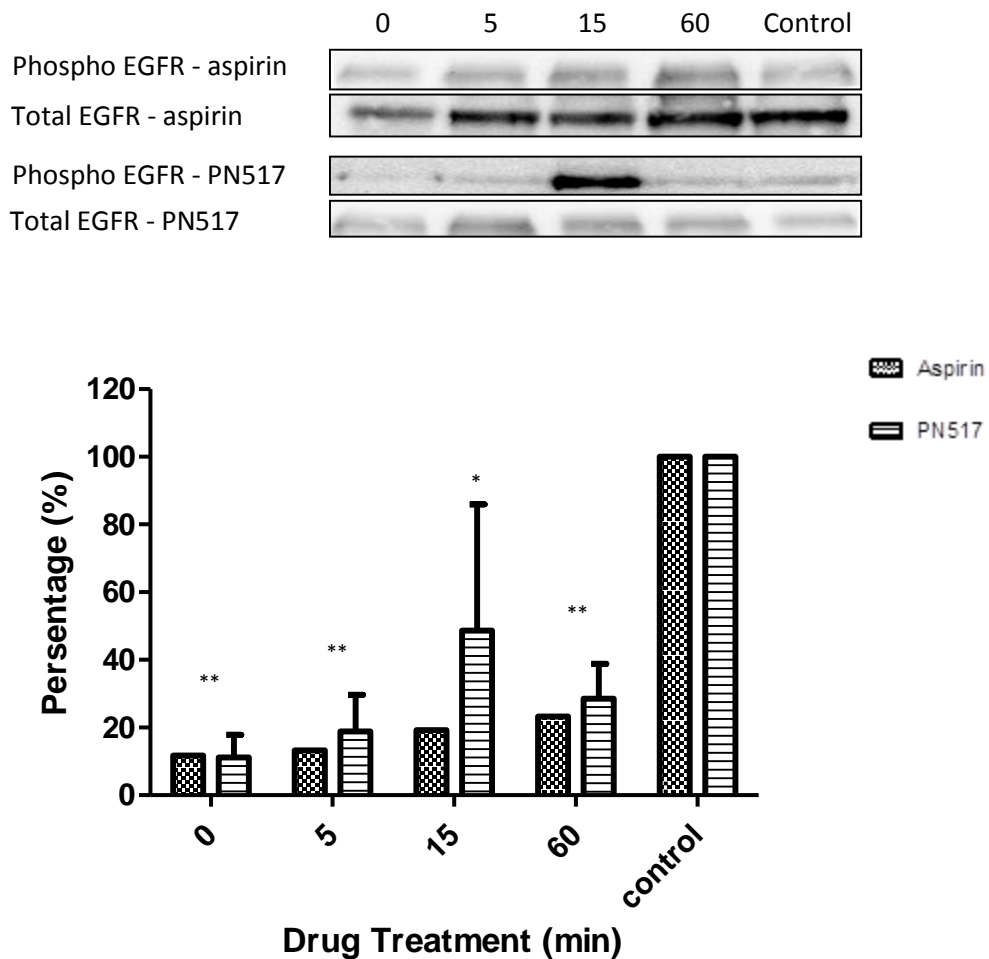


Figure 4.16 Phosphorylation of the EGF receptor following drug treatment for 24 hr at 1 mM and subsequent EGF activation in the U87 MG cell line. The data illustrate EGF activated phosphorylation of the EGFR at 0, 5, 15 and 60 minutes in the U87 MG cell line after 24 hour drug treatment **as determined by densitometry**. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.05$) with data representing results from 3 independent experiments.

4.3 Discussion

As already stated, cell viability is dependent on a range of factors including cell death, proliferation and metabolic activity and with this in mind, the aim of this series of experiments was to determine the effect of aspirin and the analogues on cell proliferation.

There is a wealth of literature on the anti-proliferative effects of aspirin or selective COX-2 inhibitors in a range of different cancers and cell lines. Since the analogues short-listed have shown an effect of both COX-1 and COX-2 it is fair to relate the enzymes with the supporting literature on proliferation. Selective COX-2 inhibition has been shown to inhibit proliferation in colon, prostate and pancreatic cell lines (Tucker *et al.*, 1999; Elder *et al.*, 2000; Tsujii *et al.*, 1998; Liu *et al.*, 2000). Importantly, the COX-2 selective inhibitor NS398 was shown to inhibit proliferation in glioma (Joki *et al.*, 2000). Casper and colleagues found similar effects with millimolar concentrations of aspirin, acetaminophen and ibuprofen using human and rodent glioma cell lines (Casper *et al.*, 2000). The COX-2 selective inhibitor SC-236 inhibits proliferation in the HT-29 colorectal cancer cell line, significantly, an effect that is reversed by co-treatment with PGE₂ (Doherty *et al.*, 2009).

In addition, other novel aspirin analogues have demonstrated anti-proliferative effects, including NO-ASA, a nitric oxide releasing form of acetylsalicylic acid. This compound has been shown to reduce proliferation in a range of cancer cell lines, including colon, ovarian, pancreas, skin, cervical, oesophageal and breast (Fiorucci *et al.*, 2003; Troyano *et al.*, 2001; Chang *et al.*, 2003; Zhi *et al.*, 2006; Gao and Williams, 2012).

Further support for a role of COX-2 in cancer cell proliferation comes from the finding that increased prostaglandin levels increase cell proliferation amongst other effects (Maxwell *et al.*, 1990; Zimmermann *et al.*, 1999). In human oesophageal squamous cell carcinoma cultures, COX-2 is highly overexpressed compared to normal squamous epithelium, and expression was positively correlated with tumour progression (Zhi *et al.*, 2006). The importance of COX-2 was confirmed using siRNA knockdown which dramatically inhibited PGE₂ production and cell growth. Li and colleagues also demonstrated a positive correlation between COX-2 inhibition, a reduction in PGE₂ levels, and the time and concentration dependent inhibition of oesophageal squamous carcinoma cells (Li *et al.*, 2009).

With respect to brain tumours, elevated PGE₂ and arachidonic acid levels have been found in glioma and meningioma (Kokoglu *et al.*, 1998), a finding that correlates well with the long established role for lipoxygenase products of arachidonic acid metabolism as positive modulators of glioma cell line division in vitro (Wilson *et al.*, 1989). Additionally, both aspirin and indomethacin inhibit in vitro glioma cell proliferation in the T98G cell line in a time and concentration dependent manner (Amin *et al.*, 2003).

However, these are not universal findings, because a lack of correlation between COX-2 expression and proliferation and apoptosis has been observed in human colorectal carcinoma cell lines, where cell lines lacking COX-2 expression responded similarly to NS398 treatment to cell lines expressing COX-2 (Elder *et al.*, 1997). In pancreatic adenocarcinomas, cell lines negative for COX-2 expression were found to respond

similarly to selective COX-2 inhibitor treatment as those expressing the enzyme (Molina *et al.*, 1999). Zhi and colleagues also found that the concentration of NS398 required to inhibit cell proliferation was ten times higher than the concentration required to inhibit PGE₂ production, suggesting non-COX-2 dependent pathways (Zhi *et al.*, 2006). Similarly, one of the original publications examining the effect of NSAIDs on glioma cell proliferation found that while aspirin decreased cell growth *in vitro*, neither indomethacin, nor piroxicam had any effect, suggesting a prostaglandin independent pathway (Aas *et al.*, 1995).

The effect of NSAID treatment on cell cycle distribution in a range of cancer cell lines has also produced conflicting results. Early publications suggested that treatment of the colorectal carcinoma COX-2 positive cell line HT29, and the COX-2 negative cell line S/KS with NS398 was reported to have no effect on cell cycle distribution in either cell line (Elder *et al.*, 1997), a finding that was in contrast to treatment with non COX-2 selective NSAIDs where accumulation in the G₀/G₁ phase was found following sulindac or salicylate treatment in a range of colorectal cancer adenoma or carcinoma cell lines (Shiff *et al.*, 1995; Elder *et al.*, 1996).

These early findings are in contrast to more recent publications however, where using the same colorectal carcinoma cell line, HT29, Doherty and colleagues reported cell cycle arrest in the G₀/G₁ phase following treatment with the COX-2 selective inhibitor SC-236, and this effect was reversed by co-treatment with PGE₂, confirming the dependence on COX activity (Doherty *et al.*, 2009). G₀/G₁ arrest following aspirin treatment has also been

observed in human hepatoma HepG2 cells (Raza *et al.*, 2011) and oesophageal squamous carcinoma cells (Li *et al.*, 2009). However, these results are in contrast to results following treatment with NO-ASA, where using a wide range of human carcinoma cell lines including colon, pancreatic, skin, cervix and breast, consistent cell cycle arrest in the G2/M phase was observed (Gao and Williams, 2012). Also, treatment with the COX-2 selective inhibitor NS-398 induced G1/S phase arrest in oesophageal carcinoma cell lines (Zhi *et al.*, 2006), pancreatic tumour cell lines and ovarian carcinoma cells (Yip-Schneider *et al.*, 2001; Denkert *et al.*, 2003).

The impact of COX expression and subsequent prostaglandin production on regulators of the cell cycle has been examined in a range of cell lines. Over 24 and 48 hours, aspirin treatment of human pancreatic endocrine BON1 and human bronchopulmonary NCI-H727 cells reduced expression of the cyclin dependent kinase 4 (CDK4) and cyclin D3 (Spampatti *et al.*, 2014). A more common finding is that NSAID treatment decreases the expression of cyclin D1. Treatment with the aspirin analogue NO-ASA results in a decrease in the expression of cyclin D1, and an increase in the expression of cyclin B1 in the SW480 colon cancer cell line (Gao and Williams, 2012), the former change decreasing progression from M to G1 phase, and the latter combining with changes in Cdk1 activity to result in G2/M arrest. Yip-Schneider and colleagues determined the effect of a number of COX inhibitors on cyclins and reported that sulindac, indomethacin and NS-398 all decreased cyclin D1 expression in PaCa-2 and BxPC-3 pancreatic carcinoma cells (G1/S phase) (Yip-Schneider *et al.*, 2001). In the same study, NS-398 was also found to decrease the expression of cyclin A in both cell lines (S/G2 phase). Pathi and colleagues examined the

effect of aspirin treatment for 24 or 48 hours on cyclin D1 expression in a range of colon cancer cell lines, including RKO, SW480, HT29 and HCT116, finding a significant decrease in all cases at 10mM (Pathi *et al.*, 2012). In contrast to observing decreased cyclin expression following COX inhibition, hepatocyte treatment with PGE₂ amplifies subsequent epidermal growth factor stimulation of cyclin D1 expression (Dajani *et al.*, 2007).

Taken together, these reports suggest that there is a great deal of variation in cell specific responses to NSAID treatment, that each NSAID may possess a unique efficacy profile, and that COX expression is not always a requirement for NSAID activity might support the range of effects on proliferation and the cell cycle observed in the different glioma cell lines.

For example, while PN517 displayed greater efficacy than cisplatin in reducing cell viability in the U87 MG cell line, this was not replicated in the proliferation assay, where cisplatin decreased proliferation to a much greater degree (Fig 4.12), suggesting a difference in the signalling pathways altered by analogue treatment in cell viability and proliferation. As mentioned already, one pathway known to be targeted by aspirin in it's the regulation of cell proliferation is the epidermal growth factor receptor (EGFR) signalling. EGFR stimulation results in activation of signalling cascades like MAPK which result in changes in NF- κ B and c-Myc, and regulation of proliferation, migration and differentiation (Indranil. *et al.*, 2013). EGFR gene amplification and over-expression has been reported in glioblastoma (e.g. U87 MG) but is rare in low-grade glioma (e.g. 1321N1

and GOS-3) (Hatanpaa *et al.*, 2010; Mestre *et al.*, 1995). This cell type specific expression of EGFR could provide an explanation for the effects of aspirin and its analogues, where EGFR expression would correlate with analogue reduction of cell proliferation. It is important to note that there are no data in the literature describing EGFR expression in the control cell line, SVG-p12, and thus it is not currently possible to relate growth factor receptor expression to drug efficacy in this cell line.

In the U87 MG cell line it was observed that treatment with aspirin or PN517 significantly decreased EGFR expression over 24 hours (Fig 4.5), and additionally, the same drug treatment decreased subsequent EGF activation of cyclin D1. Coupled with the known overexpression of COX-2 and subsequent increase in PGE₂ levels, these results correlate well with the finding that PGE₂ amplifies subsequent epidermal growth factor stimulation of cyclin D1 expression (Dajani *et al.*, 2007), and that COX-2 inhibition and reduced PGE₂ levels result in an inhibition of proliferation (Li *et al.*, 2009).

Goel *et al.*, (2003) tested the effect of aspirin on COX-independent mechanisms in HCT116 and SW480 human colon cancer cell lines at 1, 2.5 and 5 mM concentrations, and showed a significant decrease in cell growth, induction of apoptosis, and arrest in G₀/G₁ phase at 48 and 72 hours. In another study HT29, HCT116 and HCT116^{p53^{-/-}} were cultured in the presence of 2.5, 5, and 10 mM aspirin for 48 h. HCT116 cells arrested in G₁ almost completely HCT116^{p53^{-/-}} and HT29 cells showed a less complete arrest (REF). The results suggested that p53 had only a partial role in the G₁ arrest, although cell cycle arrest was seen in all cell lines at G₁/G₂ phase, and to a lower extent, in apoptosis. There was no

significant effect on the cell cycle parameters when SW480 and Caco-2 cells were treated with NS-398, although aspirin treatment resulted in an increase in the proportion of cells in the S phase (Lin *et al.*, 1998).

Another important factor that has recently emerged to be a significant determinant of glioma initiation and progression is the canonical Wnt/ β -catenin signalling pathway. Additionally, studies have linked prostaglandin E₂ (PGE₂) to tumour growth by activating EGFR (Pai *et al.*, 2002) and β -catenin (Castellone *et al.*, 2005). In breast cancer it has been shown that Wnt overexpression activates signalling via EGFR (Faivre and Lange 2007; Musgrove.2004), and that EGFR activation induces nuclear accumulation of β -catenin via PI3K/Akt pathway in prostate cells (Figure 4.3.1) (Sharma *et al.*, 2002).

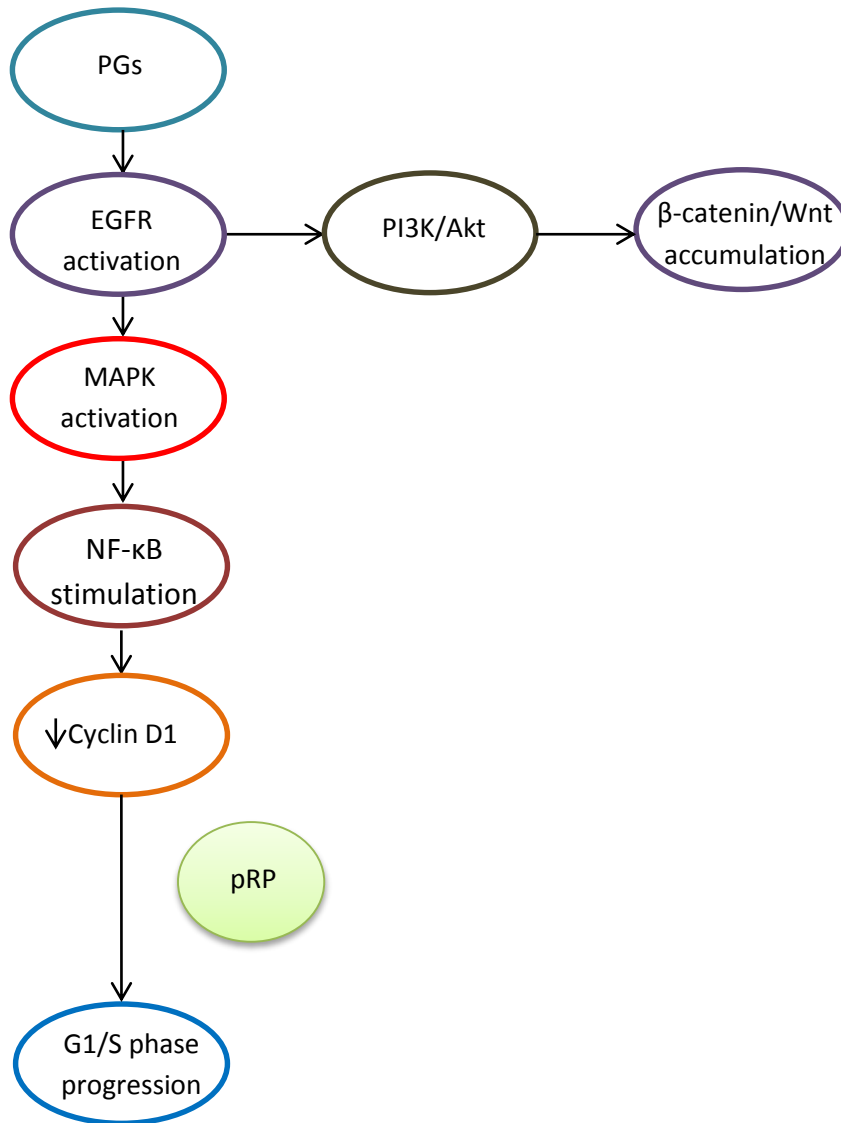


Figure 4.3.1. PGE activation of EGFR to stimulate tumour growth. EGFR stimulation results in activation of signalling cascade including MAPK which results in changes in NF- κ B levels and regulation of proliferation (cyclin D1), migration and differentiation. Wnt overexpression activates signalling via EGFR activation, which induces accumulation of β -catenin via the PI3K/Akt pathway.

Aspirin has been shown to exert its antineoplastic action by inhibition of the β -catenin/TCF signalling pathway in glioma cells (Lan *et al.*, 2011), as well as in colorectal cancer which has been linked to decreased transcription and translation of cyclin D1 (Hawcrift *et al.*, 2002; Dihlmann *et al.*, 2003). Conversely, increased β -catenin levels in colon cancer results in the activation of the cyclin D1 gene promoter by the heterodimeric complex formed between β -catenin and LEF-1, which, in turn, results in the elevation of cyclin D1 gene expression and protein level, and thus increased proliferation (Michael *et al.*, 1999).

Recent findings by Claudius *et al.*, (2014) showed that treatment of the SW480 colorectal cancer cell line with aspirin results in degradation of I κ B, nuclear translocation of NF- κ B/RelA complexes and suppression of NF- κ B activity. However, they reported that the analogues PN517 and PN508 failed to induce nucleolar translocation of RelA or cell cycle arrest. They suggested that these differences could be explained by the aspirin analogues retaining the salicylate part of the aspirin molecule, but losing the acetyl component. This could mean that NF- κ B stimulation is directly linked with the salicylate component, and the nucleolar translocation of RelA and cell cycle arrest with the acetylating potential. These findings are in contrast with the results reported here using glioma cell lines treated with the aspirin analogue PN517, as an effect on cell cycle was observed with a decrease in G1 and increase in the S cell populations, an effect linked to changes in cyclin D1, and a prolonged M phase which could potentially be linked to cyclin B, although this has not been investigated. A change in cyclin B1 has been previously reported following treatment of a colon cancer cell line with the aspirin analogue NO-ASA, which showed an increase in the expression of cyclin B1 (Gao and Williams, 2012).

It has been reported that selective COX-2 inhibitors cause G1/S cell cycle arrest in the U87 MG cell line *in vitro* (Kang *et al.*, 2009), an effect that was p53 dependent and accompanied by p21 activation. In addition to p21 activation, it has also been reported that COX-2 inhibition results in activation of the tumour suppressor p27 (Han *et al.*, 2004; Maier *et al.*, 2004; Narayanan *et al.*, 2003). COX dependent activation of p53 is not unique to glioma, with similar finding in colon and oral cancer (Grosch *et al.*, 2005; Ho *et al.*, 2003; Swamy *et al.*, 2003). The report of Kang and colleagues is in contrast to our results with PN517, aspirin and the U87 MG cell line, where a decrease in G0/1 population was observed with a corresponding increase in S/M phase populations. However, PN517 and aspirin were used at concentrations 10-fold higher with celecoxib where G1/S phase arrest was observed, providing two possible explanations for this difference. Additionally, mutational activation of p53 is only found in approximately 60% of high grade glioma (Newcomb *et al.*, 1993), thus this COX-2 effect is dependent on expression of functional p53 and thus is not a universal mechanism.

It has also been observed, that while COX-2 expression correlates with increasing histological grade in glioma, COX-2 expression does not correlate with p53 expression or loss of p16 or retinoblastoma protein (Shono *et al.*, 2001), although COX-2 tended to correlate with p16 expression ($p < 0.05$), providing another potential mechanism for G1 phase cell cycle arrest as p16 is a tumour suppressor protein that inhibits progression from G1 to S phase. p16 is an inhibitor of cyclin dependent kinases such as CDK4 and CDK6 which mediate the progression from G1 phase to S phase through

their interaction with cyclin D1 (Hara. *et al*, 1996). Immunohistochemistry has found a correlation between high grade glioma and the expression of p53 and p21, with p14 and p16 more frequently present in low grade tumours (Zolota *et al*, 2008). These differences in expression could provide some explanation for why PN517 alters the cell cycle distribution and proliferation of the 1321N1 and GOS-3 (grade 2-3) cell lines at an earlier time point and at the lower drug concentration than was found in the U87 MG (grade 4) cell line.

There is also a link between p53 and COX-2 expression, as wild type p53 has been shown to inhibit COX-2 expression in vitro (Subbaramaiah *et al*, 1999). So in addition to a loss of p53 causing a disruption in cell cycle regulation, it may also result in an increase in the expression of COX-2 in glioma. Increased COX expression and the resulting production of PGE₂ provide a further potential mechanism for changes in proliferation. It is known that PGE₁ and PGE₂ increase cell number and incorporation in the T98G human glioma cell line in vitro with PGE₂ producing consistently larger effects than PGE₁ (Gomes and Colquhoun 2012). Activation of the EP4 receptor by PGE₂ has been shown to correlate with proliferation, with COX inhibitors suppressing expression of the receptor (Kambe *et al.*, 2008; Kambe *et al.*, 2009).

Conclusion

We have observed both a decrease in proliferation and inhibition of cell cycle progression following treatment with the novel aspirin analogue PN517. Additionally, PN517 both decreased EGFR expression and inhibited EGF stimulation of cyclin D1 activation. While

it is clear from the literature that COX enzymes play a significant role in the regulation of proliferation and cell cycle in glioma, the pathways are complex with evidence suggesting that decreases in cell proliferation following treatment with COX inhibitors might be mediated by both COX-2 and by COX independent pathways; cell cycle arrest may occur in each stage of the cell cycle, an effect that may be concentration dependent; and expression of a range of cyclins and tumour suppressor proteins may be involved. In summary, PN517 has shown an effect in proliferation, mostly in GOS-3 cell line, a results replicated by aspirin, and a G1 arrest was observed, followed by an increase in S/M phase. An effect on phosphorylated Cyclin D1 has supported cell cycle data, as well as EGFR. The results support the therapeutic potential of PN517 for the treatment of glioma.

Chapter 5: Apoptosis

5.1 Introduction

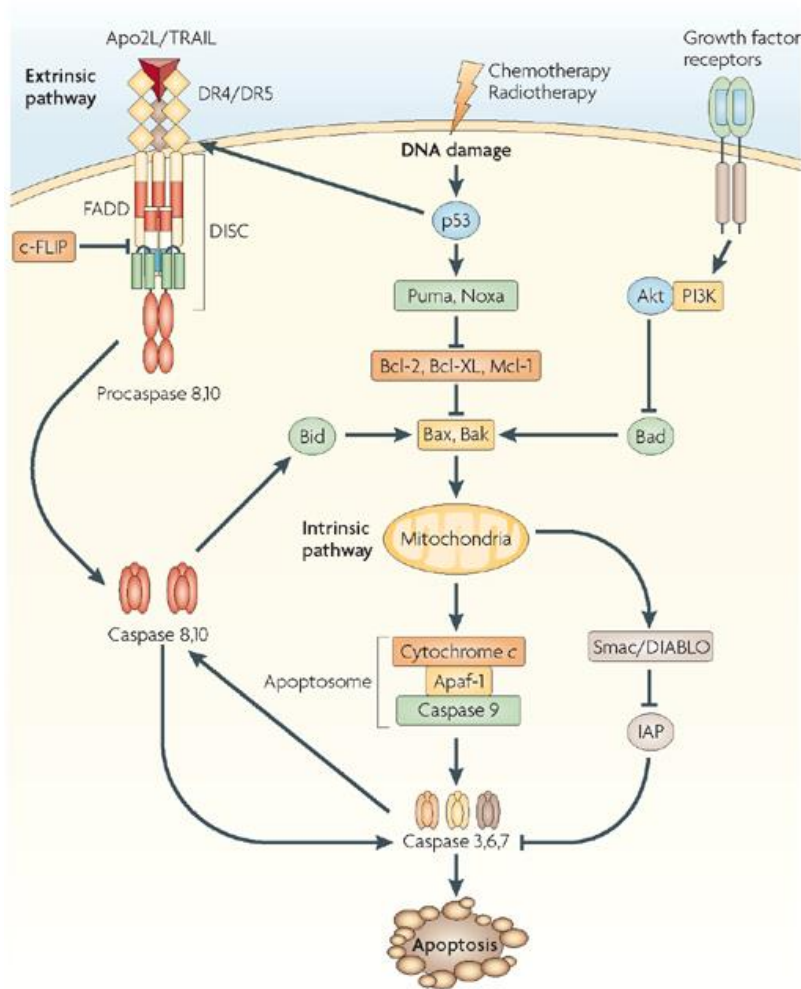
The cells in a multicellular organism are members of a highly organized community which are tightly regulated from the rate of cell division and cell death where in general cells die through either of two distinct processes, cell apoptosis and cell necrosis. Apoptosis is the process of programmed cell death that avoids eliciting inflammation (Fink *et al*, 2005). It involves a series of biochemical events that lead to a variety of morphological changes, like membrane blebbing, loss of membrane asymmetry, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Elmore, 2007). Necrosis on the other hand is the premature death of cells and living tissue and is a passive process of accidental cell death resulting from environmental perturbations with uncontrolled release of inflammatory cellular contents, a process that prevents phagocytosis, leading to a build-up of dead tissue and cell debris (Edinger and Thompson 2004). Thus, the disposal process of cellular debris that does not damage the organism differentiates apoptosis from necrosis. Apoptosis often provides beneficial effects to an organism, whereas necrosis is almost always detrimental.

The first indication that apoptosis might influence the malignant phenotype was by Kerr and colleagues in 1972, where they described how apoptosis contributed to the high rate of cell loss in malignant tumours and could promote tumour progression (Kerr *et al.*, 1972). Apoptosis has since been separated in three stages, early, intermediate and late-stage apoptotic events with methods and reagents developed to identify the various apoptotic stages, and to clearly distinguish them from necrotic processes (Wlodkowic *et al*, 2011).

Early to mid-stage apoptosis is characterized by the translocation of phosphatidylserine (PS), a lipid located in the cellular membrane. PS in live, intact cells is cytosolic but translocates to the extracellular portion of the membrane in early/mid-stage apoptosis (Kawasaki Y., et al 2000). Annexin V, a cellular protein of unclear function, can be used for PS detection in addition to cell impermeable dyes, such as Propidium Iodide (PI), that are used as a counterstain to differentiate early apoptosis from late apoptosis or necrosis (Waehrens 2009). Late stage apoptosis is characterised by DNA fragmentation and can be detected using PI.

In addition to differentiating apoptosis from necrosis, caspase enzymes can be used to determine the apoptotic pathway activated following a drug treatment, either the intrinsic/mitochondrial pathway, or the extrinsic pathway (Fig 5.1). The intrinsic pathway is often activated in response to signals resulting from DNA damage, loss of cell-survival factors, or other types of severe cell stress (Fulda *et al*, 2010). The intrinsic pathway is complex and involves a wide variety of signalling molecules, but can involve p53 induction of the expression of PUMA, p53 upregulated modulator of apoptosis (Bai and Wei-Guo 2006). Upon its activation PUMA interacts with anti-apoptotic members of the Bcl-2 family of proteins, resulting in their release from apoptotic family members such as Bax, which subsequently insert into the mitochondrial membrane causing permeabilisation, break down of the membrane potential, and release of cytochrome *c* (Krakstad and Chekenya 2010). Cytochrome C, Apaf-1 and caspase-9, the initiator caspase, form the apoptosome and initiate a further cascade of signalling resulting in the activation of the caspase-3, the terminal caspase (Baliga and Kumar, 2003).

The extrinsic signalling pathway involves the activation of transmembrane death receptors that are members of the tumour necrosis factor (TNF) receptor gene superfamily (Wang and El-Deiry 2003). These receptors bind extrinsic ligands and transduce intracellular signals that induce apoptosis with the most well characterized ligands being TNF-alpha, Apo3L, and Apo2L, while known receptors include FasR, TNFR1, DR3, and DR4/DR5 (Elmore 2007). Ligand binding leads to the formation of a death-inducing signalling complex (DISC), activating procaspase-8 which in turn can either directly activate caspase-3 or can regulate the bcl-2 family leading to activation of the intrinsic pathway (Elmore 2007). Having established that the aspirin analogues decreased cell viability, one potential factor contributing to this result is the induction of apoptosis, and thus it was decided to examine its contribution using a variety of assays including flow cytometric analysis of cells stained with annexin V and PI following drug treatment.



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Fig 5.1 Extrinsic and intrinsic pathways of apoptosis. Diagrammatic representation of signalling pathways involved in the induction of apoptosis (Ashkenazi, 2012).

Hypothesis

Aspirin and its analogue PN517, reduced both cell viability and proliferation in each of the cell lines tested, but the pattern of results from the assays did not correlate directly, suggesting a role for another mechanism. In addition to cell proliferation, cell viability also includes apoptotic cell death, thus it is hypothesised that treatment with aspirin and PN517

will induce apoptosis, and this effect will be greatest in the GOS-3 cell line, the glioma cells most sensitive to treatment in previous assays. There are conflicting results in the literature as to which pathway is most important for the induction of apoptosis by NSAIDs in glioma, extrinsic or intrinsic, thus it is expected to find effects on both pathways.

5.2 Results

The first assay performed to examine the induction of apoptosis used differential staining of cells by annexin V and PI to distinguish between early and late apoptotic cells. As already stated, annexin V conjugated to green-fluorescent FITC dye detects the externalization of phosphatidylserine in apoptotic cells, and red fluorescent PI staining to distinguish early from late apoptotic cells. Additionally, PI can be used to indicate necrotic cells, where the cell membrane is no longer intact, but phosphatidylserine staining is not observed as translocation has not occurred. In control living populations little or no fluorescence was observed, thus in all cases, no apoptosis was recorded at the zero timepoint thus these results were not shown on the graphs. The effect of drug treatment with aspirin and PN517 at 0.1 and 1mM was examined using the established cell lines for grade II – IV glioma, specifically 1321N1 - a grade II astrocytoma, Gos-3 - a grade II/III mixed astro-oligodendroglioma, and U-87 MG - a grade IV glioblastoma. Experimental controls included the SVG-p12 foetal glial cell line and the standard chemotherapeutic drug cisplatin.

Following drug treatment of the SVG-p12 cell line, induction of apoptosis was observed in both a time and concentration dependent manner for cisplatin, aspirin and PN517 (Fig 5.2). A significant increase in both early and late apoptotic cells was observed following 24 hours of drug treatment with cisplatin at both 0.1 mM ($p < 0.0001$) and 1mM ($p < 0.0001$), an effect that increased further following 48 hours of treatment ($p < 0.0001$ and $p < 0.0001$ respectively). A similar trend was observed with aspirin at 24 ($p < 0.0001$ for 0.1mM and $p < 0.0001$ for 1mM) and 48 hour timepoints ($p < 0.0001$ for 0.1mM and $p <$

0.0001 for 1mM), and also with PN517 following 24 ($p < 0.0001$ for 0.1mM and $p < 0.0001$ for 1mM) and 48 hours of treatment ($p < 0.0001$ for 0.1mM and $p < 0.0001$ for 1mM). Of the three treatments, cisplatin produced the largest induction of apoptosis with early and late phase apoptosis at 55% and 83% following treatment at 0.1 mM and 1 mM respectively at 48 hours. Aspirin and PN517 produced similar results, inducing 35% and 33% apoptosis respectively after 48 hour treatment at 1 mM concentration.

In a similar manner to the control cell line, each of the drug treatments induced apoptosis in the 1321N1 cell line (Fig 5.3). However, in contrast to the SVG-p12 cell line, an increase in apoptosis is observed at an earlier time point following treatment with all three drugs, with significant increases in apoptosis observed at either the 4 or 8 hour timepoints. At 0.1mM, a similar level of apoptosis is observed following treatment with each drug at 48 hours with cisplatin resulting in 70%, aspirin 60% and PN517 62.5% of apoptotic cells. At 1mM, cisplatin induces apoptosis at the earliest timepoint and to a greater extent than was observed at 0.1mM. However, little difference was observed between 0.1 and 1mM treatments for aspirin apart from a greater proportion of apoptotic cells at the 4 hour timepoint (13% versus 36% respectively). An increase in the proportion of apoptotic cells at the 4 hour timepoint was also observed following PN517 treatment at 1mM (27%), a trend that was reproduced at each subsequent timepoint apart from at 48 hours, where the proportion of apoptotic (22%) cells dropped unexpectedly.

A similar pattern of apoptosis induction was observed with the GOS-3 cell line (Fig 5.4) as was observed in the 1321N1 cell line, with each drug inducing apoptosis at both

concentrations and from the earliest timepoints. The largest apoptotic population in the GOS-3 cell line was observed following treatment with cisplatin at both 0.1 and 1mM (Fig 5.4, panels A and B). Interestingly, the proportion of apoptotic cells was greater at 1mM following aspirin treatment than PN517 treatment at 24 (60% for aspirin and 83% for PN517) and 48 hours (53% for aspirin and 86% for PN517), but at the earlier timepoints, PN517 produced a larger effect.

The effect of drug treatment on apoptosis was also examined in the U87 MG grade IV GBM cell line with both a concentration and time dependent effect observed for each drug (Fig 5.5). The largest apoptotic population was observed following 1mM cisplatin treatment for 24 and 48 hours, 64% and 66% respectively. The size of this population is almost three times that observed at the equivalent 0.1mM timepoints (18% and 19% respectively). While aspirin generally increased the apoptotic population in a time dependent manner, following 48 hours of treatment the population decreased significantly in comparison to the 24 hour timepoint at both concentrations ($p < 0.0001$ at 0.1mM and $p < 0.0001$ at 1mM). PN517 also induced apoptosis in a time dependent manner at both drug concentrations, but while little difference was observed in the total apoptotic population at 48 hours following either 0.1mM (31%) or 1mM (35%) treatments, the proportion of late apoptotic cells was greater at the higher drug concentration (10% versus 29%).

Representative sample dot plots for the annexin V/PI apoptotic flow cytometry analysis of each of the cell lines can be found in the appendix. A shift from quadrant three (Q3), live cells with little or no staining, into quadrant four (Q4 – early apoptosis annexin V positive)

and quadrant two (Q2 – late apoptosis, both annexin V and PI positive) is observed where treatments induce apoptosis. The cells in quadrant one (Q1 – PI positive) may indicate necrotic cells.

To summarise, as expected the effect on all cell lines was greater at 1 mM drug treatment as compared to 0.1 mM. The lowest level of apoptosis induction by all treatments was found in the control SVG-p12 cell line, although both time and concentration dependent effects were observed, with cisplatin producing the largest effect. In the glioma cell lines, little difference was seen between the levels of apoptosis induced by PN517 or cisplatin across all grades, with aspirin being less efficacious. An inverse correlation was observed between apoptosis and glioma grade, with typically the highest levels of apoptosis found in the 1321N1 cell line, and the lowest levels of apoptosis in the U87 MG cell line.

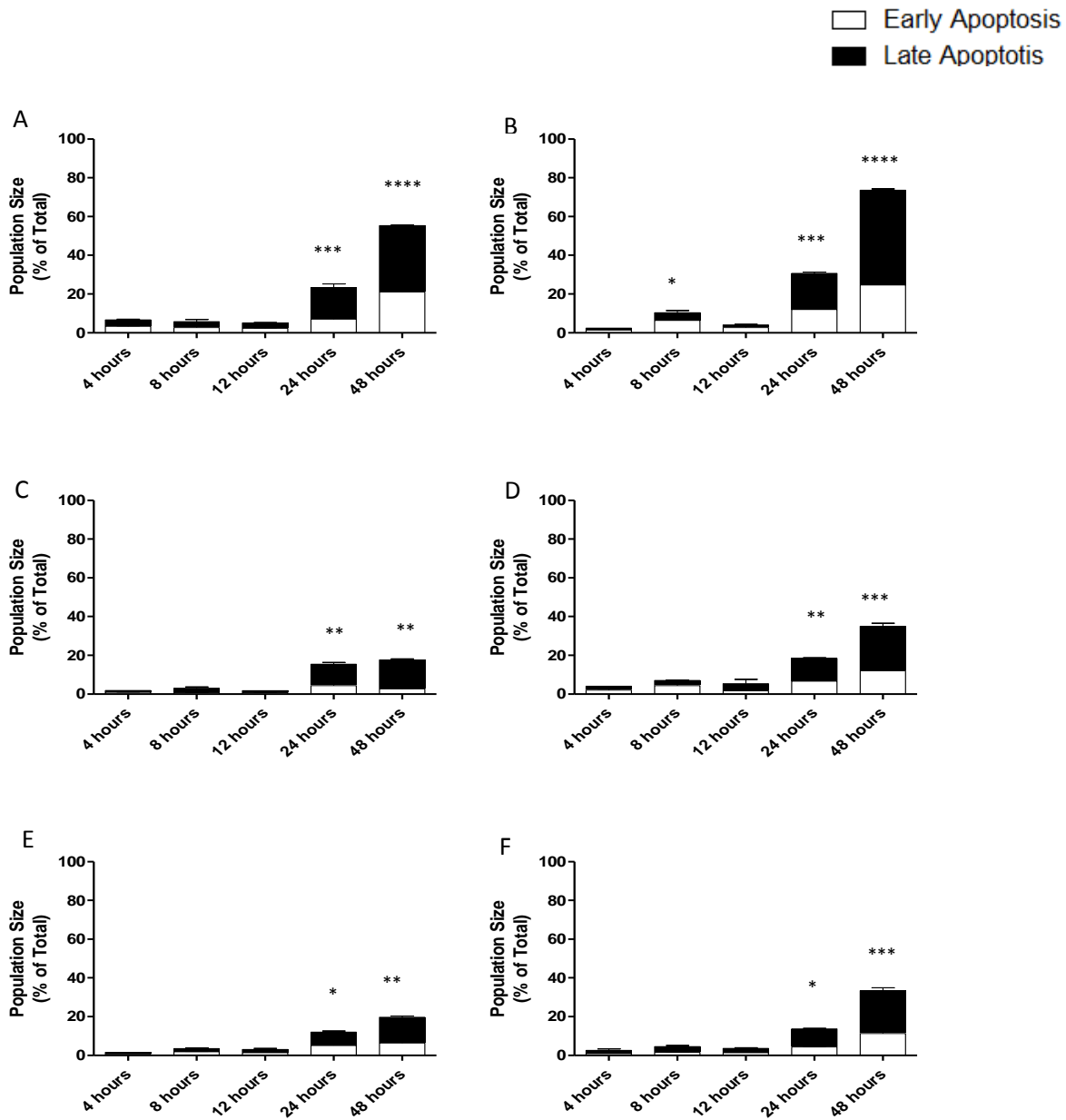


Figure 5.2 Induction of apoptosis determined by Annexin V/PI staining following drug treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in the SVG-p12 cell line. The data represent the proportion of apoptotic cells (white - early apoptotic, black - late apoptotic) in SVG-p12 cell line as determined by flow cytometry following drug treatment over time. Panel A and B illustrate cisplatin treated cells at 0.1 and 1 mM respectively, and similarly C and D aspirin treated cells and E and F panel show PN517 treated cells. The data represent results of 3 independent experiments with an overall significant effect of treatment in comparison with control ($p < 0.0001$) determined by ANOVA.

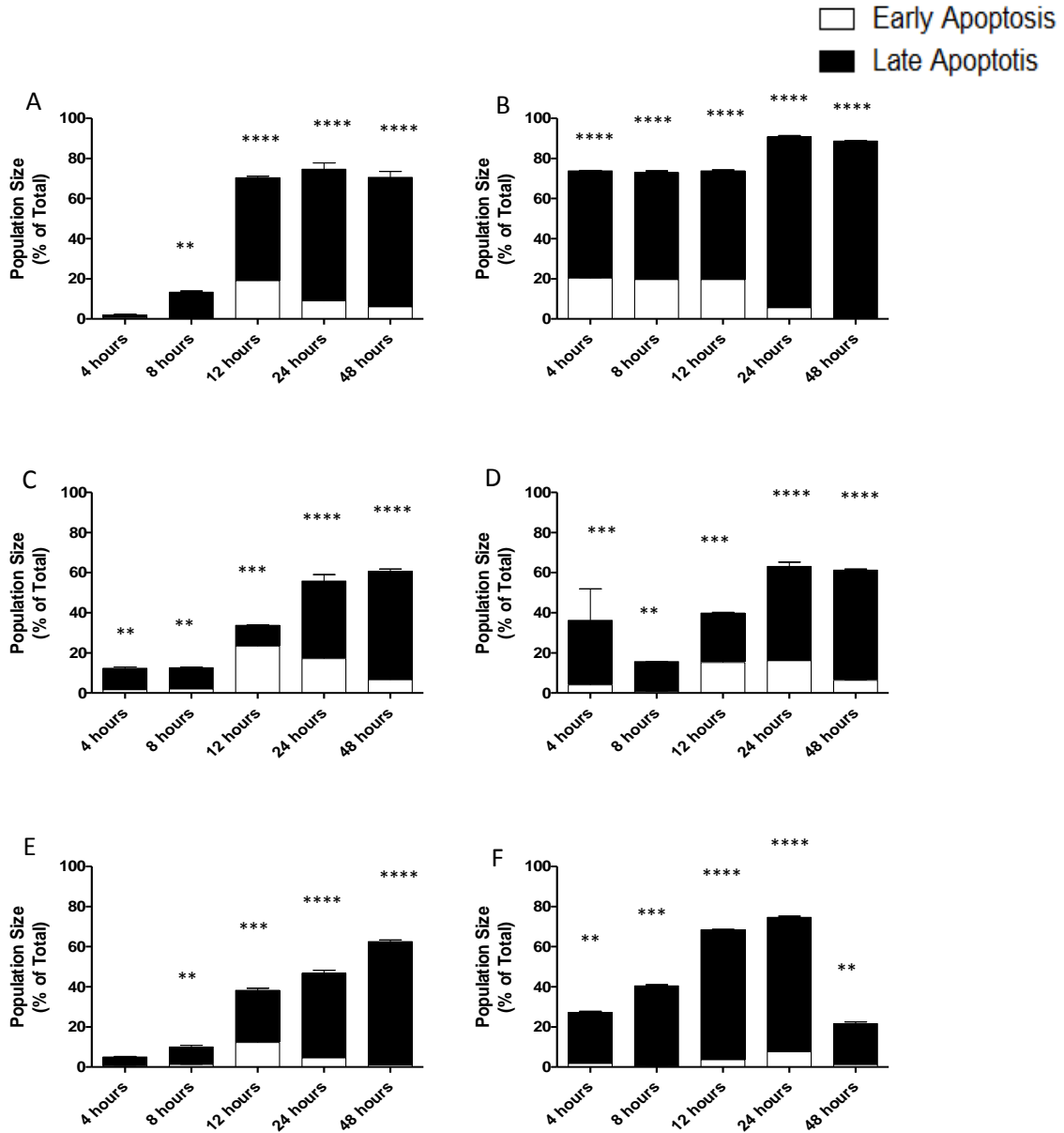


Figure 5.3 Induction of apoptosis determined by Annexin V/PI staining following drug treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in the 1321N1 cell line. The data represent the proportion of apoptotic cells (white - early apoptotic, black - late apoptotic) in 1321N1 cell line as determined by flow cytometry following drug treatment over time. Panel A and B illustrate cisplatin treated cells at 0.1 and 1 mM respectively, and similarly C and D aspirin treated cells and E and F panel show PN517 treated cells. The data represent results of 3 independent experiments with an overall significant effect of treatment in comparison with control ($p < 0.0001$) determined by ANOVA.

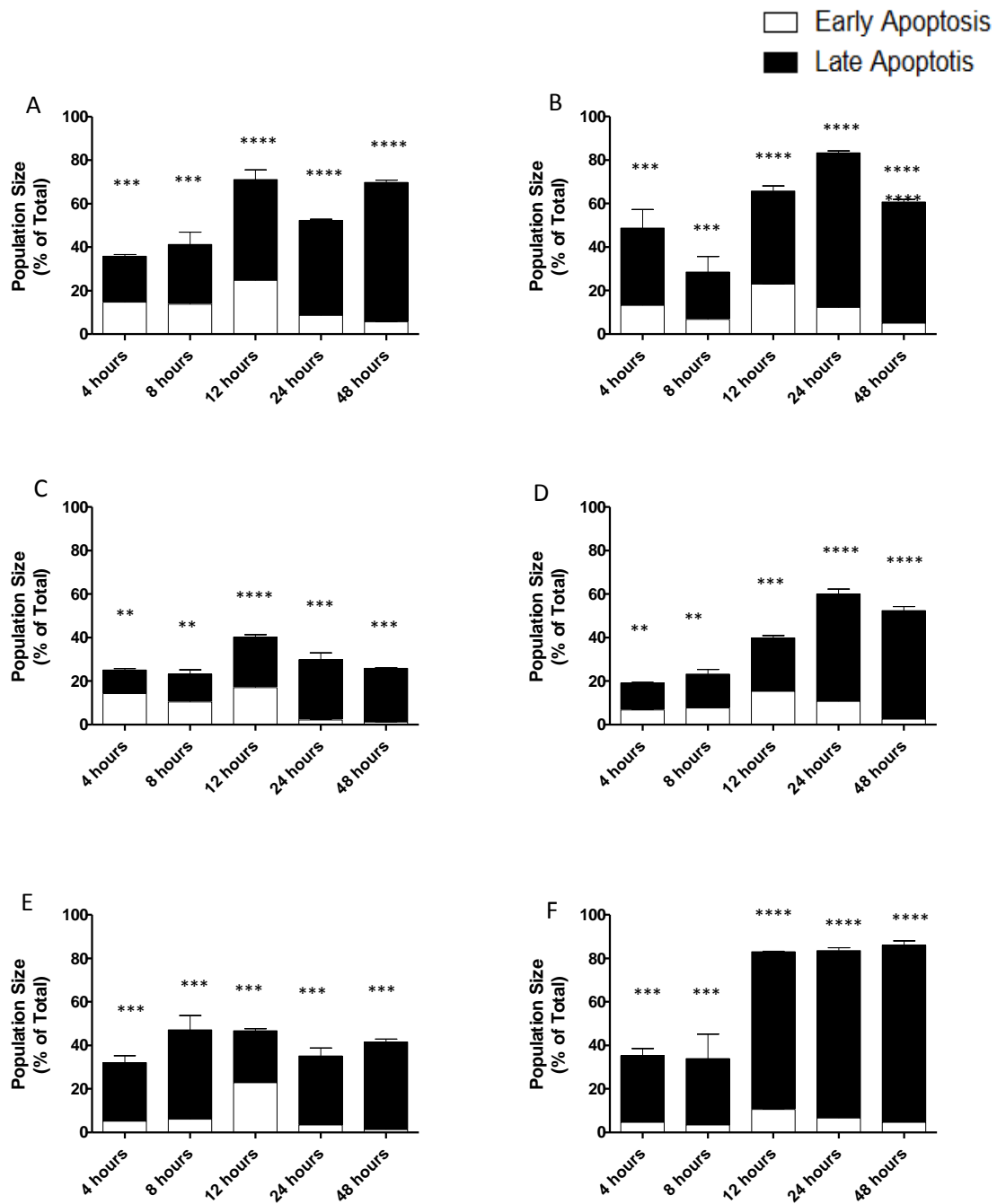


Figure 5.4 Induction of apoptosis determined by Annexin V/PI staining following drug treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in the Gos-3 cell line. The data represent the proportion of apoptotic cells (white - early apoptotic, black - late apoptotic) in Gos-3 cell line as determined by flow cytometry following drug treatment over time. Panel A and B illustrate cisplatin treated cells at 0.1 and 1 mM respectively, and similarly C and D aspirin treated cells and E and F panel show PN517 treated cells. The data represent results of 3 independent experiments with an overall significant effect of treatment in comparison with control ($p < 0.0001$) determined by ANOVA.

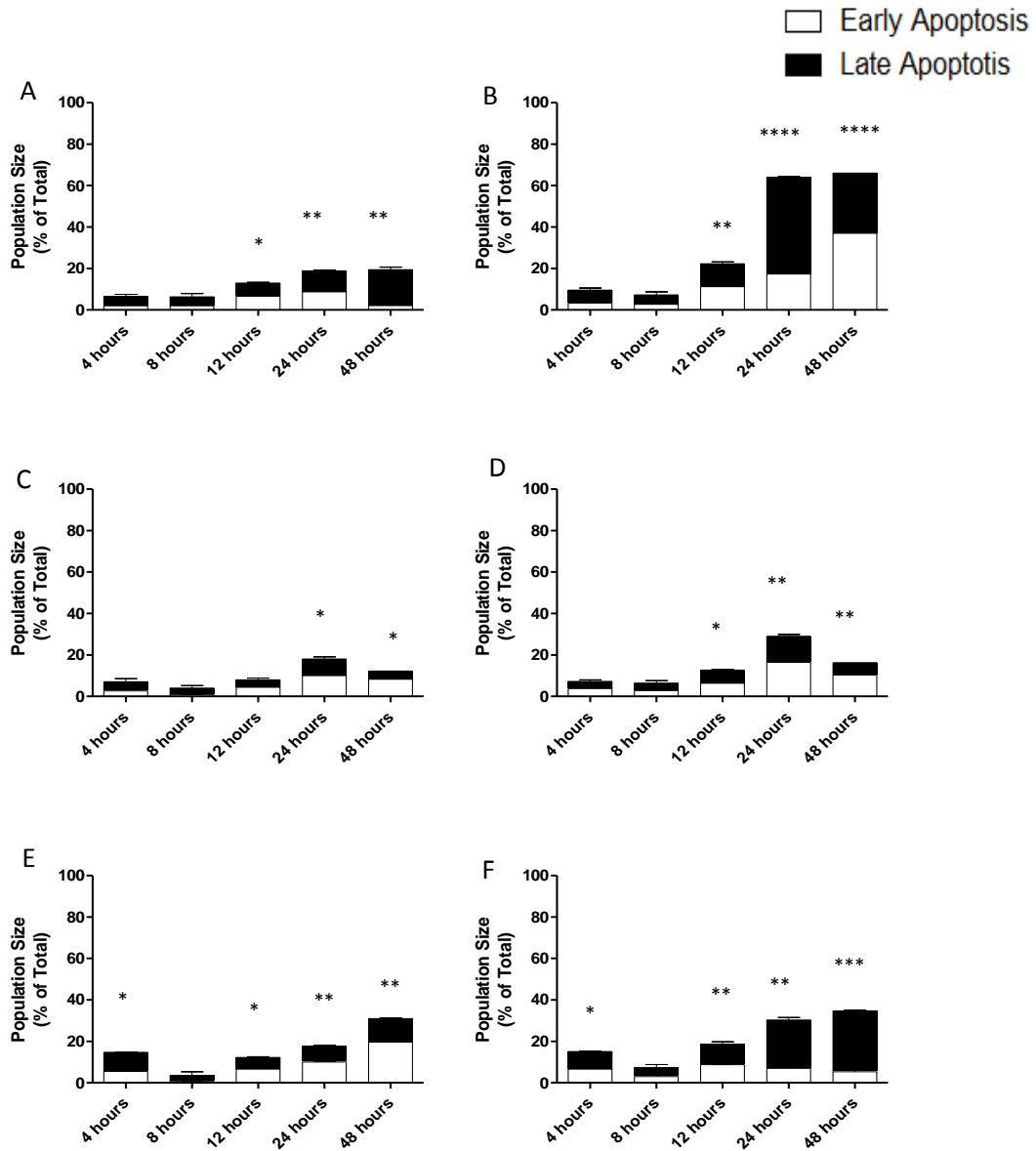


Figure 5.5 Induction of apoptosis determined by Annexin V/PI staining following drug treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in the U87 MG cell line. The data represent the proportion of apoptotic cells (white - early apoptotic, black - late apoptotic) in U87 MG cell line as determined by flow cytometry following drug treatment over time. Panel A and B illustrate cisplatin treated cells at 0.1 and 1 mM respectively, and similarly C and D aspirin treated cells and E and F panel show PN517 treated cells. The data represent results of 3 independent experiments with an overall significant effect of treatment in comparison with control ($p < 0.0001$) determined by ANOVA.

Caspase 8 & 9

In parallel to the flow cytometric determination of apoptosis, the luminescent Caspase Glo assay was performed to quantify the activation of both caspase 8 and 9 in the U87 MG and SVG-p12 cell lines (Fig 5.6 and 5.7).

In the SVG-p12 cell line in the case of both caspase 8 and 9, each drug treatment showed a time dependent activation of the enzymes with the exception of PN517 where activity dropped between the 24 and 48 hour timepoints. The greatest activation of both caspase 8 and 9 was observed following aspirin treatment, with PN517 showing a similar efficacy for the activation of caspase 9 but peaking at 24 hours, and cisplatin showing the lowest degree of activation in both assays.

In the U87 MG cell line, again a time dependent activation of both enzymes was observed by each drug treatment with the exception of aspirin activation of caspase 9 where a high degree of activation was observed at the 12 hour timepoint. Both PN517 and aspirin showed a similar efficacy in the activation of caspase 9, but aspirin produced a greater activation of caspase 8. As with the SVG-p12 cell line, the lowest activation in both enzymes was observed following cisplatin treatment.

When compared to the results of the annexin V and PI staining, the activation of caspase 8 and 9 as measured using the caspase glo assay do not always directly correlate. An example of this can be observed in the U87 MG cells, where aspirin treatment results in the highest level of caspase 8 activity at 48 hours, but the lowest level of apoptosis by flow cytometry.

This is not entirely unexpected though, as caspase activation precedes events like membrane degradation and PI staining. Overall, both assays demonstrate a time and concentration dependent activation of apoptosis by each treatment.

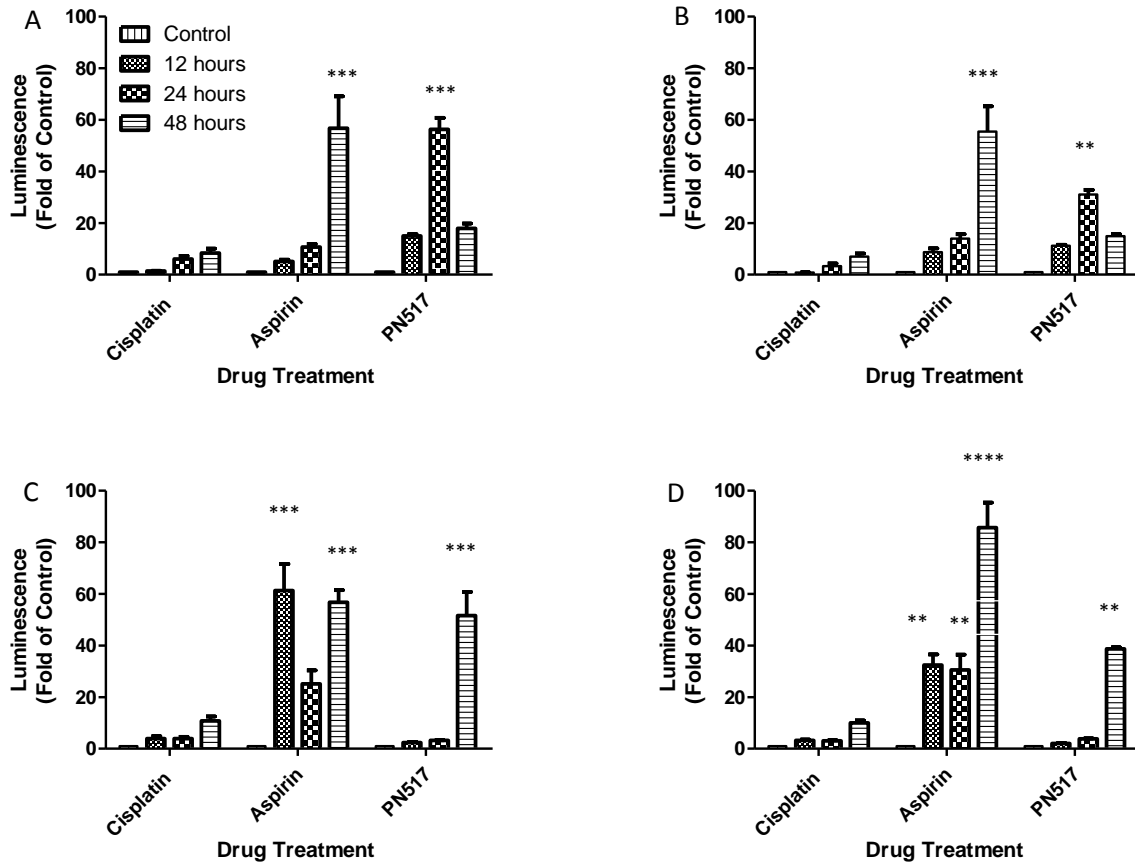


Figure 5.6 Activation of caspase 9 and 8 following drug treatment for 12, 24 and 48 hr at 1 mM in SVG-p12 and U87 MG cell lines. The data illustrate fold change in caspase activity determined by the Caspase Glo luminescent assay with results grouped by drug. Activation of caspase 9 (panel A and C) and caspase 8 (panel B and D) in SVG-p12 cell line (top panels of graph) and U87 MG cell line (bottom panels of graph). The data shows a time dependant activation of both caspases by each drug treatment. An overall significant effect of treatment in comparison with control ($p < 0.005$) was found by ANOVA with data representing results from 3 independent experiments.

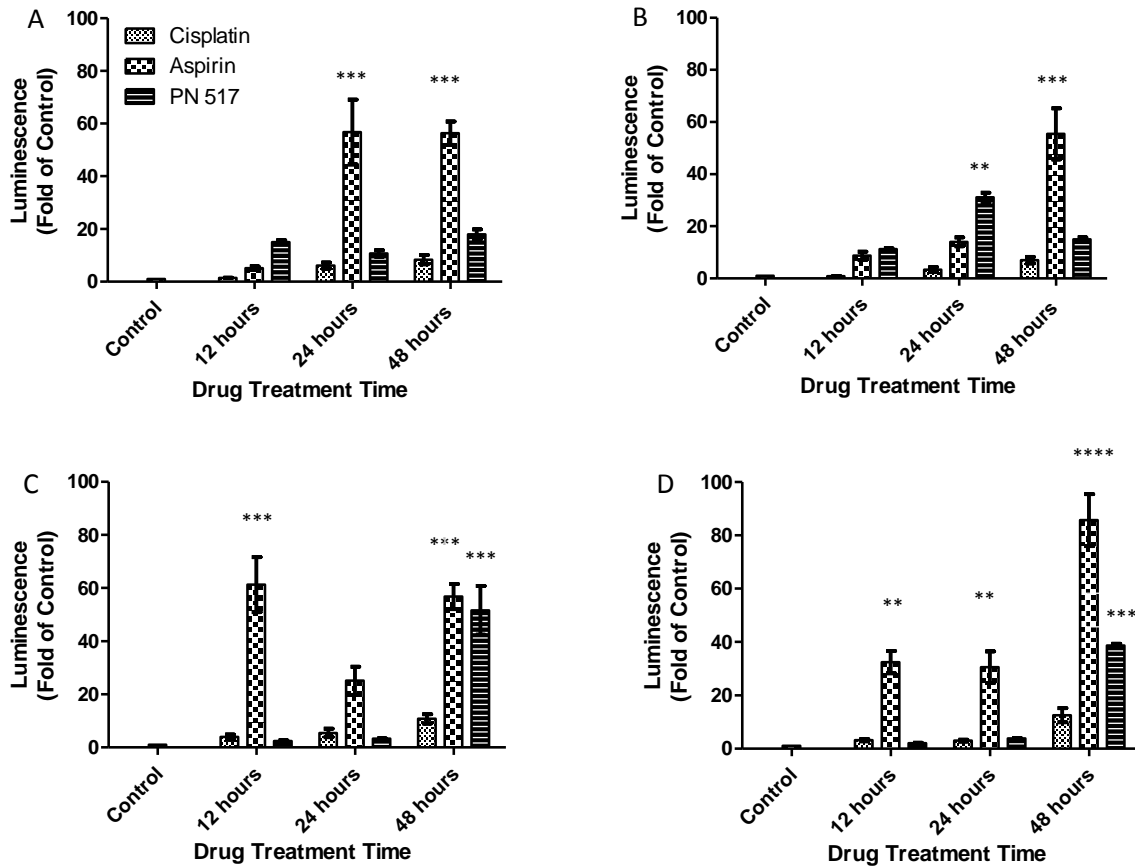
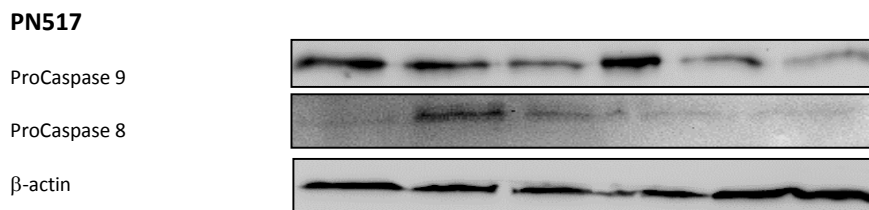
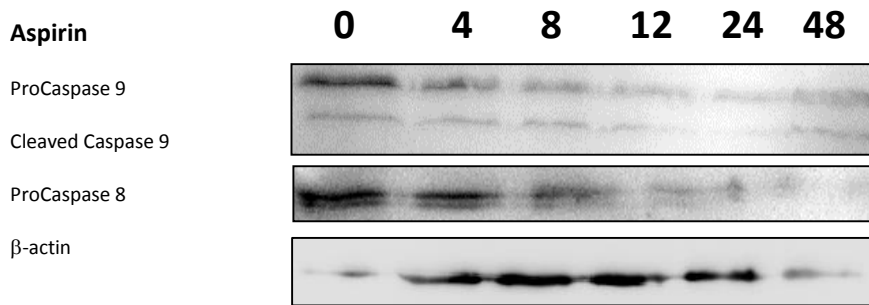


Figure 5.7 Activation of caspase 9 and 8 following drug treatment for 12, 24 and 48 hr at 1 mM in SVG-p12 and U87 MG cell lines. The data illustrate fold change in caspase activity determined by the Caspase Glo luminescent assay with results grouped by timepoint. Activation of caspase 9 (panel A and C) and caspase 8 (panel B and D) in SVG-p12 cell line (top panels of graph) and U87 MG cell line (bottom panels of graph). The data shows aspirin producing the greatest activation, followed by PN517 and lastly cisplatin. An overall significant effect of treatment in comparison with control ($p < 0.005$) was found by ANOVA with data representing results from 3 independent experiments.

In parallel with the annexin V/PI flow cytometry assay, western blotting was used in an attempt to examine the activation of caspases 8, 9 and 3 following drug treatment in the various cell lines. However, following difficulty with primary antibodies only provisional data were generated for the 1321N1 and U87 MG cell lines (Fig 5.8). Treatment with aspirin and PN517 seemed to indicate cleavage of procaspase 9 and an appearance of cleaved caspase 9 over the time course examined, with loss occurring at an earlier timepoint in the 1321N1 cell line than in U87-MG cell line, a finding that correlates with the flow cytometric apoptosis data. Similarly, a loss of procaspase 8 was apparent in the 1321N1 cell line following aspirin treatment, and may be observed following PN517 treatment. The blots for the U87 MG cell line do not show as big a change in procaspase 8 expression, with an obvious decrease following 24 hours of aspirin treatment, but a less obvious effect with PN517 treatment.

1321N1



U87 MG

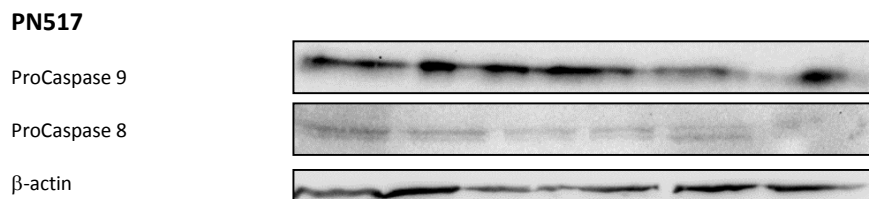
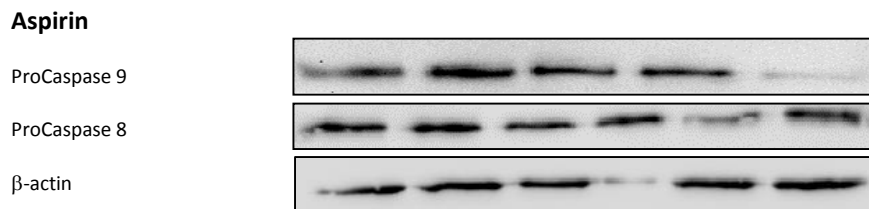


Fig 5.8 Activation of caspase 8 & 9 in the 1321N1 and U87 MG cell lines following drug treatment with aspirin and PN517. The data show representative western blots of caspase 8 and 9 in the 1321N1 and U87 MG cell lines following drug treatment at 1mM with aspirin and PN517 for 4, 8, 12, 24 and 48 hours. The data are preliminary and are representative of two independent experiments.

5.3 Discussion

An important mechanism for the anti-tumour activity of aspirin and other non-steroidal anti-inflammatory drugs is the induction of apoptosis. Aspirin has been shown to enhance apoptosis in several cancer cell lines including colorectal adenoma and carcinoma cells (Elder *et al.*, 1996), mouse Neuro 2a cells (Dikshit *et al.*, 2006) human hepatoma cells (Raza *et al.*, 2011), oesophageal squamous carcinoma cells (Li *et al.*, 2009), and myeloma cell lines (Ding *et al.*, 2014). COX-2 selective inhibitors have also been demonstrated to have similar activity, including nimesulide (Li *et al.*, 2009), NS-398 (Liu *et al.*, 1998; Elder *et al.*, 2000; Elder *et al.*, 2002), and SC326 (Doherty *et al.*, 2009). Importantly, an enhancement of apoptosis has been demonstrated following aspirin, indomethacin and ibuprofen treatment in T98G human glioblastoma cells (Amin *et al.*, 2003; Gomes and Colquhoun, 2012) and through COX-2 selective inhibition in U87 MG cells (Kang *et al.*, 2009).

Our results examining the induction of apoptosis confirm that short term aspirin treatment can enhance apoptosis in different glioma cell lines and also demonstrate for the first time that the aspirin analogue PN517 increases apoptosis with at least the same, if not greater efficacy. In the four cell lines tested, induction of apoptosis correlated with the known proliferation rate of the cells, with the greatest induction in the GOS-3 and 1321N1 cell lines which have a doubling time of 24 hours, and a lower degree of induction in the SVG-p12 and U87 MG cell lines which have a doubling time of 36 and 48 hours respectively. Using the *in vitro* luciferase assay we found that both aspirin and PN517 activated caspase

8 and caspase 9 in a time and concentration dependent manner, a finding supported by western blotting.

The control drug cisplatin enhanced apoptosis to a greater degree than aspirin or PN517 treatment in all cell lines, again correlating with proliferation rate. This finding is unsurprising given that the known mechanism of action for cisplatin induction of apoptosis is through the formation of inter and intra crosslinked DNA adducts (Tanida *et al.*, 2012). This DNA damage is detected during transcription or replication and activates downstream pathways including p53 which ultimately result in the induction of apoptosis. While the novel aspirin analogue PN517 enhanced apoptosis to a lesser extent than cisplatin, the induction of apoptosis was significant, and importantly *in vivo* PN517 does not display any obvious toxicity related side effects (Caludius *et al.*, 2014). This is in contrast to chemotherapeutic drugs like cisplatin whose toxicity limits their use.

Several mechanisms have been reported for aspirin induction of apoptosis including the downregulation of anti-apoptotic protein Bcl-2 (Li *et al.*, 2009; Raza *et al.*, 2011; Ding *et al.*, 2014), the upregulation of the pro-apoptotic protein Bax (Zhou *et al.* 2001; Gu *et al.* 2005; Diskshit *et al.*, 2006; Ding *et al.*, 2014), release of cytochrome *c* (Pique *et al.* 2000; Zimmermann *et al.* 2000; Diskshit *et al.*, 2006; Raza *et al.*, 2011), activation of caspase 8 (Ding *et al.*, 2014), caspase 9 (Diskshit *et al.*, 2006; Gomes and Colquhoun, 2012; Ding *et al.*, 2014), and caspase 3 (Diskshit *et al.*, 2006; Raza *et al.*, 2011; Gomes and Colquhoun, 2012; Ding *et al.*, 2014), inhibition of ATP synthesis (Raza *et al.*, 2011), and downregulation of VEGF (Ding *et al.*, 2014). The COX-2 selective inhibitor NS398 causes

a similar downregulation of Bcl-2 protein expression in the LNCaP prostate cancer cell line (Liu *et al.*, 1998), with an activation of poly-(ADP ribose) polymerase in HT29 colorectal cancer cells (Elder *et al.*, 2002). Interestingly, NSAID upregulation of Bax expression was not a universal finding, with Li and colleagues reporting that neither aspirin nor nimesulide altered its expression in oesophageal squamous carcinoma cells (Li *et al.*, 2009).

NF- κ B is found as a heterodimer of the RelA/P50 subunits in the cytoplasm bound to I κ B, but degradation of the latter allows NF- κ B nuclear translocation where it can regulate target gene expression. In addition to aspirin activation of this pathway resulting in the degradation of cyclin D1 and apoptosis induction in SW480 colorectal cancer cells (Thoms *et al.*, 2010), Claudius *et al.*, (2014) found that the aspirin analogue PN517 decreases I κ B levels leading to activation of NF- κ B, a reduction in cyclin D1 and induction of apoptosis. In contrast to the findings presented here using glioma cells, they did not observe cell cycle arrest in SW480 cells following PN517 treatment, and also reported effects using a higher concentration of analogue (3mM) than used in these studies. This higher concentration may suggest that the SW480 cell line is less sensitive to treatment with aspirin or aspirin analogues, and explain some of the differences observed in the timescale of responses. However, it is clear that NF- κ B activation is likely to play a role in glioma cell lines, and is therefore associated with the

Our results with PN517 and aspirin confirm the previously reported NSAID activation of caspase 8 and 9, suggesting the involvement of both the extrinsic and intrinsic pathways of

apoptosis induction. The extrinsic pathway involves the activation of death receptors such as Fas and TNFR1, leading to clustering and formation of a death-inducing signalling complex which includes the adapter protein FADD (Fas-associated death domain) and initiator procaspase-8, the latter of which is cleaved and activates downstream effector caspases (caspase-3) (Gu *et al* 2004). The intrinsic pathway is controlled by the Bcl-2 family. Anti-apoptotic proteins (e.g. Bcl-XL and bcl-2) antagonize the pro-apoptotic proteins Bax and Bak by binding to their BH3 domains. This antagonism is relieved by BH3-only, pro-apoptotic proteins (BIM, BID, BAD, NOXA, PUMA), which alternately bind to anti-apoptotic proteins. This leads to the release of cytochrome *c*, and apoptosome formation with Apaf-1 and caspase-9 which subsequently activates the effector enzyme, caspase 3.

While numerous studies have suggested the involvement of COX enzymes in the mechanism of apoptosis induction, it has been reported that different doses of aspirin effect different pathways (Arrieta *et al.*, 2001), with low aspirin concentrations inhibiting COX enzymes and the production of prostaglandins, but high concentrations producing effects through COX independent mechanisms such as β -catenin and wnt signalling (Lan *et al.*, 2011; Bos *et al.*, 2006). COX-1 and COX-2 are prostaglandin H synthetases and catalyses the conversion of arachidonic acid into prostaglandins and thromboxanes. COX-2 expression is inducible and increases response to various stimuli, including inflammatory signals, mitogens, cytokines, tumour promoters and growth factors, while COX-1 is expressed in nearly all normal tissues and mediates the synthesis of PGs required for physiological tissue homeostasis (Pairet and Engelhardt, 1996). COX-2 has been linked to

tumorigenesis in certain cancers, and more recently was shown to be upregulated in high-grade gliomas resulting in elevated prostaglandin levels (Joki *et al.*, 2000; Shono *et al.*, 2001; Kokoglu *et al.*, 1998). These increased PG levels have been linked to increased proliferation, decreased apoptosis, promotion of angiogenesis, and inhibition of immune surveillance (Maxwell *et al.*, 1990; Zimmermann *et al.*, 1999; Kokoglu *et al.*, 1998). This is supported by the concentration of aspirin and nimesulide required to inhibit PGE₂ production correlating with the inhibition of proliferation and induction of apoptosis (Li *et al.*, 2009). However, there are other reports which failed to find a correlation between COX-2 expression and NSAID induced apoptosis (Elder *et al.*, 2000; Molina *et al.*, 1999). Elder and colleagues also found no effect of PGE₂ addition on the induction of apoptosis (Elder *et al.*, 2000).

A further pathway implicated in the induction of apoptosis by aspirin was described in the A172 human glioblastoma cell line A172 where a reduction in the level of phosphorylated STAT3 (Tyr705), a transcription factor required for survival of glioblastoma, was found (Kim *et al.*, 2009). Aspirin reduced the expression of STAT3 target genes such as Cyclin D1, XIAP, and Bcl-2, all contribution to decreased proliferation and apoptosis induction. The same study showed that the expression and secretion of interleukin-6 (IL-6), led to STAT3 phosphorylation and activation, and this was inhibited by aspirin. When exogenous IL-6 was administered to aspirin-treated A172 cells, the phosphorylation of STAT3 and cellular apoptosis was reduced compared to aspirin only-treated cells. The description of this pathway is highly significant because interleukins such as IL-6 are often found expressed in human glioma cells (Sasaki *et al.* 2001), and phosphorylation and activation

of STAT3 has recently been described as an independent prognostic factor in GBM linked to poor clinical outcome (Lo *et al.*, 2008; Lin *et al.*, 2014a, b).

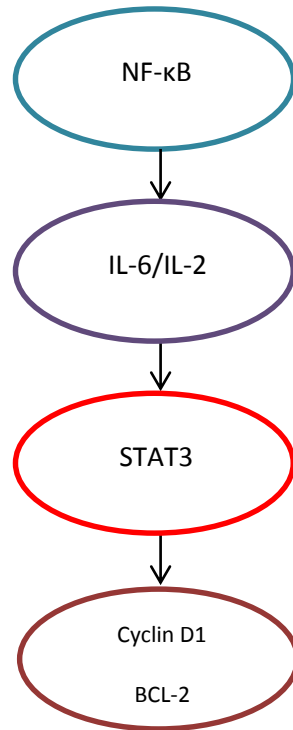


Figure 5.3.1. Activation of NF-κB represents an important step in the regulation of IL-6 gene expression. IL-6 expression and secretion leads to STAT3 phosphorylation and activation. Aspirin reduces the expression of STAT3 target genes such as cyclin D1, XIAP, and Bcl-2.

As well as the JAK/STAT pathway, another pathway involved in apoptosis in many cancers including brain tumours, is the upregulation of NF-κB (Noqueira *et al.*, 2011). NF-κB activity is high in GBM tissue and corresponds with increasing grade in astrocytic tumours (Korkolopoulou *et al.*, 2008; Angileri *et al.*, 2008). The exact mechanism of NF-κB activation in GBMs is not known, however, a variety of proteins and pathways affecting

GBM could be related to NF- κ B activation. TNF- α , IL-6 and IL-1 β , are important regulators of paracrine signalling in brain tumour cells which cause changes in gene expression in neighbouring cells through direct ligand–receptor interactions, and cause inflammation. TNFR1, expression of which correlates positively with glioma grade, activates TNF- α signalling which promotes NF- κ B activation and subsequent anti-apoptotic responses (Otsuka *et al.*, 1999; Koul *et al.*, 2006). Inhibition of NF- κ B activation has been demonstrated to reduce brain tumour growth, invasion and angiogenesis (Xie *et al.*, 2008), and importantly, aspirin is known to inhibit the NF- κ B pathway by inhibiting I(kappa)B kinase-beta activity (IKK β) (Yin *et al.*, 1998).

Crosstalk between the STAT3 and NF- κ B pathways has been observed at various points, including upregulation of VEGF expression in tumour cells (Wu *et al.*, 2004; Loeffler *et al.*, 2005). Additionally, EGF has been shown to activate NF- κ B in GBM cells (Hayashi *et al.*, 2001), and EGFR amplification at the protein level has been described in 60-90% of GBMs, with the expression level correlating with poor patient outcome (Shinojima *et al.*, 2003; Umesh *et al.*, 2009). A downstream effect of EGFR over-activation is stimulation of STAT3 activity in GBM cells (Rao *et al.*, 2005). This pathway has been reported to activate COX-2 gene expression in GBM (Lo *et al.*, 2010), thus NSAID inhibition of COX and subsequently NF- κ B activation may actually negatively regulate COX-2 expression, a finding that emphasises the complexity of the signalling pathways involved in tumourigenesis.

In conclusion, we have shown that aspirin and PN517 induce early and late apoptosis at 0.1 and 1 mM concentrations in a time dependent manner, and with an efficacy that correlates with proliferation rates. Both drugs activated caspase 8 and 9 *in vitro*, suggesting the involvement of both intrinsic and extrinsic pathways of apoptosis. In conjunction with our finding that PN517 and aspirin inhibit EGFR activation in U87 MG cells, the literature would suggest that STAT3 and NF- κ B signalling are involved, however further work is required to confirm this hypothesis.

Chapter 6: Invasion and Migration

6.1 Introduction

Glioma is the most common intracranial tumour with patients undergoing invasive surgery, radiotherapy and chemotherapy but despite this having a median survival time of approximately 12 months following diagnosis (Cancer Research UK, 2013). It has been observed that after surgical removal, tumours recur mainly due to the fact that cells from the tumour have already invaded normal brain tissue, making the invasive behaviour of glioma cells one of the most important hallmarks, with a median recurrence time of 7 months (Stupp *et al.*, 2005). GBM invasion occurs along nerve fibre tracts and regularly penetrates the glial limiting membrane resulting in cancer spread throughout the spinal fluid (Lee *et al.*, 2005). Invasion requires degradation of the local extracellular matrix and in GBM, the proteolytic activity of two matrix metalloproteinases (MMP-2 and MMP-9) has been identified to play a role, with expression being upregulated in line with tumour grade (Rao *et al.*, 1993; Forsyth *et al.*, 1998; Yang *et al.*, 2014).

PGE₂, the levels of which are elevated in brain tumours (Kokoglu *et al.*, 1998), plays an important role in proliferation, invasion, and metastasis in GBM (Sivak-Sears *et al.*, 2004). In primary cultures derived from head and neck tumours, it has been demonstrated that invasion, and MMP-2 and MMP-9 activity is reduced by the non-selective COX inhibitor sulindac, aspirin, and the COX-2 selective inhibitor NS398 (Koontongkaew *et al.*, 2010). The invasiveness of U87 MG cells was shown to be inhibited by sulindac and the COX-2 selective inhibitor celecoxib, a process unaffected by aspirin, ketoprofen, ketorolac and naproxen (Lee *et al.*, 2005). Inhibition of invasiveness was linked to MMP-2 activity.

Prior to metastasis, cells must acquire several properties in addition to increased invasiveness. These properties include the loss of cell adhesion, intravastation and increased survival and proliferation (Chaffer and Weinberg, 2011; Fidler, 2003; Joyce and Pollard, 2009). Survival of the cancer cells while circulating is thought to involve platelets (Gasic *et al.*, 1968; Gasic *et al.*, 1973 Bambace *et al.*, 2011; Gay and Felding-Habermann, 2011), a process which is reduced by aspirin (Bambace *et al.*, 2011; Gay and Felding-Habermann, 2011; Gasic *et al.*, 1972; Henschke *et al.*, 1977; Futakuchi *et al.*, 2002). The importance of this has been emphasised by the results of many clinical trials and described in a meta analysis of five major randomised controlled trials by Rothwell and colleagues who found that aspirin reduced the risk of cancer with distant metastasis in all cancers but mainly due to a reduction in metastatic adenocarcinomas (Rothwell *et al.*, 2012). Overall, aspirin reduced the risk of fatal adenocarcinoma significantly, an effect independent of age and gender. This finding has been demonstrated elsewhere, confirming that regular use of aspirin lowers the risk of distant metastasis for several cancers such as breast and biliary cancer (Zhang X, Smith-Warner *et a.,l* 2012). Based on these findings, we decided to determine the effect of PN517 on migration and invasion.

Hypothesis

Invasion is known to be reduced by NSAID treatment *in vitro*, and meta-analysis of clinical data has shown that aspirin reduces metastasis. Thus it is predicted that aspirin and the aspirin analogue PN517 will reduce invasion and migration in glioma cell lines *in vitro*.

6.2 Results

The effect of drug treatment (0.1mM) on cell migration was examined in each of the cell lines over a six hour period with the migrated population expressed as a percentage of control migration (Fig 6.1). Briefly, cells were seeded on polycarbonate inserts (8.0 µm) and following equilibration the media within the insert was replaced with serum free media, with complete media within each well acting as a chemoattractant. Drug treatments were placed in both the serum free and complete media. Following 6 hours incubation, cells were removed from the underside of the insert by trypsinisation and counted (Fig 6.1A).

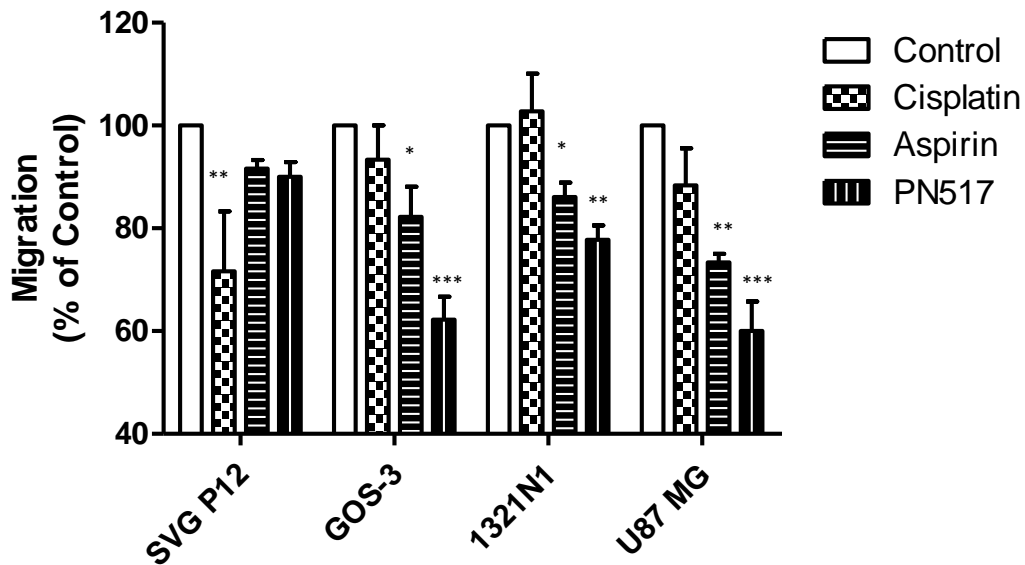
An important finding of the migration assay was that in the SVG-p12 foetal astrocyte cell line, neither aspirin nor PN517 significantly altered migration, demonstrating a selectivity for glioma cancer cell lines (Fig 6.1B).

With the exception of cisplatin treatment in the SVG-p12 cell line, PN517 treatment produced the greatest reduction in cell migration with aspirin producing the next largest reduction in migration. Cisplatin treatment reduced cell migration in the SVG-p12 and U87 MG cell lines but did not significantly alter migration in the GOS-3 or 1321N1 cell lines.

The greatest effect of PN517 was in the U87 MG cell line where migration was reduced to 60% of control, with reductions to 62%, 77% and 90% of control for GOS-3, 1321N1 and SVG-p12 cell lines respectively. Aspirin also produced its greatest reduction in migration in the U87 MG cell line (73% of control), followed by GOS-3 (82%), 1321N1 (86%), and

SVG-p12 (91%). The control drug cisplatin reduced migration in the control cell line, SVG-p12, to 71% of control and in the U87 MG cell line to 88% of control.

A



B

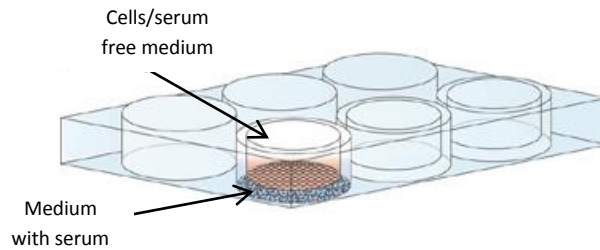


Fig 6.1 Effect of drug treatment on cell migration in SVG-p12, GOS-3, 1321N1 and U87 MG cell lines over 6 hours (A) and a diagrammatic representation of the assay (B). The data illustrate the effect of drug treatment at 0.1 mM on migration expressed as a percentage of control in the SVG-p12, GOS-3, 1321N1 and U87 MG cell lines. With the exception of the SVG-p12 cell line, PN517 treatment produced the largest reduction in migration. An overall significant effect of treatment ($p < 0.005$) was found by ANOVA analysis with data representing results from 3 independent experiments.

Migration was also examined in the cell lines using the wound healing/scratch assay (Fig 6.2). Once cells were confluent, a scratch was created using a sterile cocktail stick so as not to scratch the plastic surface and alter migration, and the media replaced (including drug treatment where appropriate) to remove any floating cells whose re-attachment might influence the results (Fig 6.2).

In control the slowest migration was observed with the SVG-p12 cell line with the scratch not completely closing over the 18 hour period (Fig 6.3). This is in contrast to the GOS-3 cell line where the scratch completely closed.

In each cell line, treatment with PN517 reduced migration with the scratch remaining open after 18 hours, with migration being reduced to approximately 60% of control. Aspirin also reduced migration, but to a lesser extent, to an average of approximately 80% of control in all cell lines.

The control drug cisplatin had the largest effect on migration in the scratch assay, reducing overall migration in the 18 hour period to between 40% and 80 % of control, with the greatest effect in the U87 MG cell line.

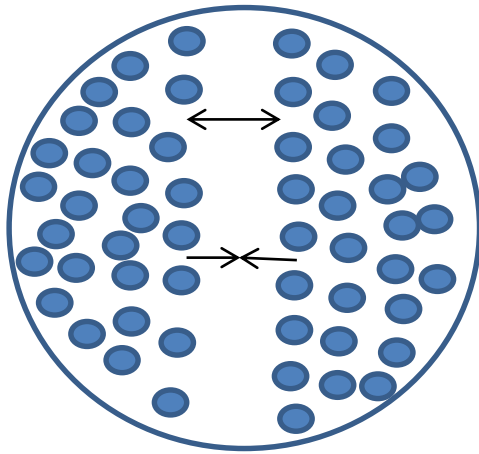


Fig 6.2 Diagrammatic representation showing a single well of the scratch assay. Following scratching with a cocktail stick, cells (indicated in blue) migrate over 18 hours to fill the resulting space.

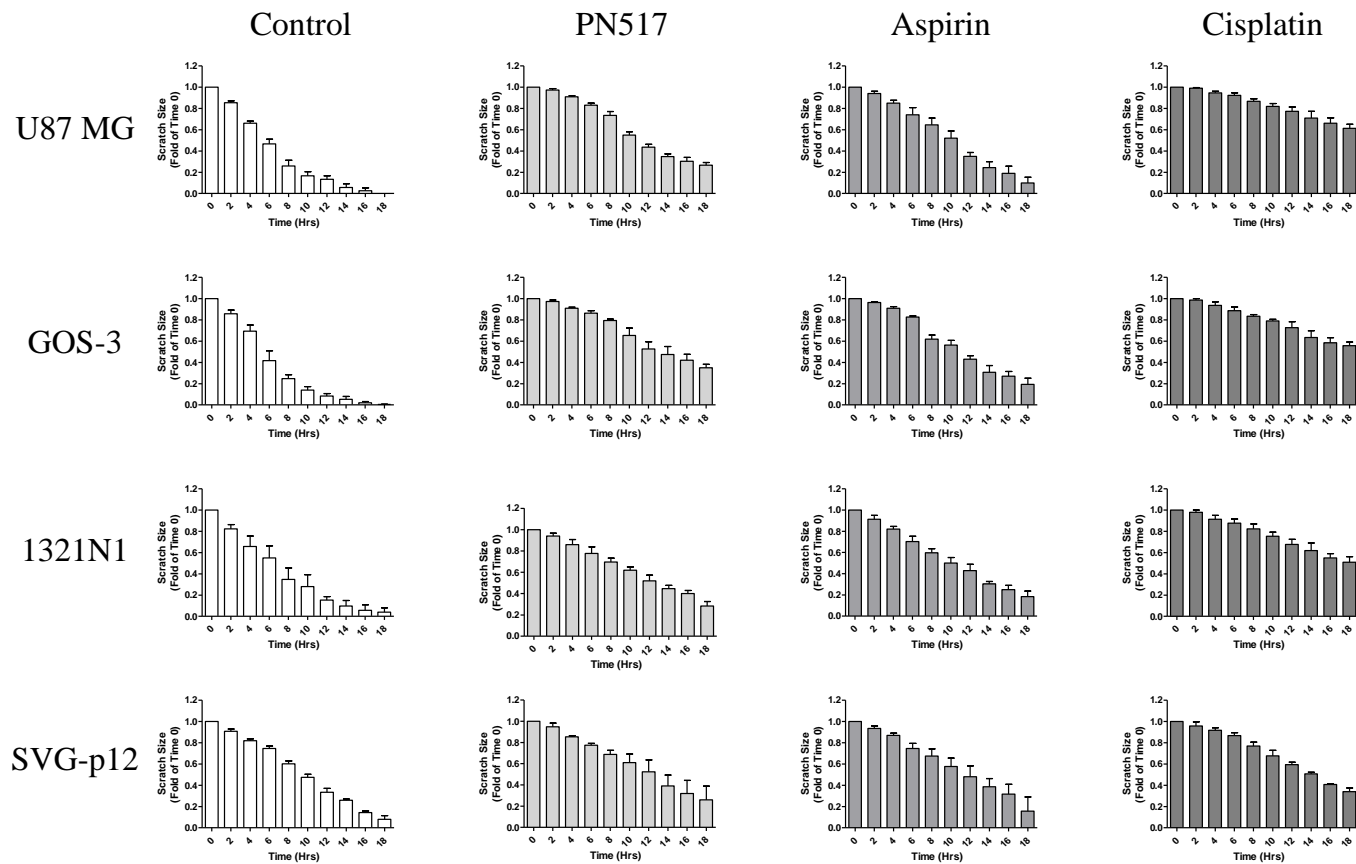


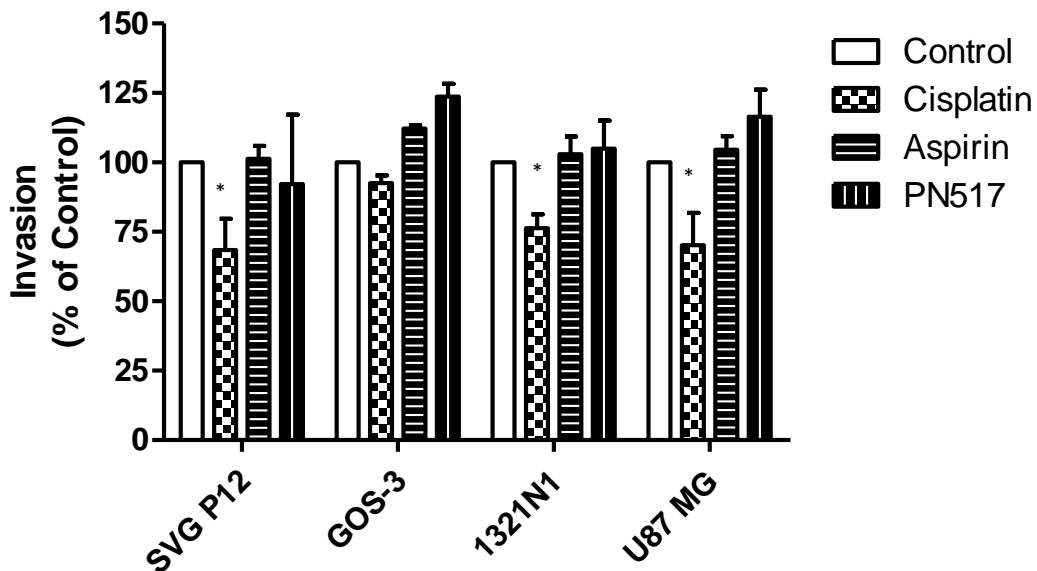
Fig 6.3 Effect of drug treatment on U87 MG, GOS-3, 1321N1 and SVG-p12 cell migration using the scratch assay. Migration was expressed as fold decrease over 18 hours in scratch width as measured by minimum distance following treatment with PN517, aspirin and cisplatin at 1mM. An overall significant effect of treatment ($p < 0.01$) was found by ANOVA analysis with data representing results from 3 independent experiments.

In contrast to migration, invasion of the established cell lines was not consistently reduced by drug treatment (Fig 6.4A), where invasion was measured over an 18 hour period with cells exposed to drug treatment at 0.1mM. Briefly, invasion was measured as the proportion of cells migrating from a serum free environment into complete media over 12 hours with quantification by enzyme activated fluorescence (Fig 6.4B)

Cisplatin was the only treatment found to significantly reduce invasion across each of the cell lines, reducing invasion to 68% of control in the SVG-p12 cell lines followed by U87 MG (70%), 1321N1 (76%), and GOS-3 (92%). While aspirin had no effect (SVG-p12 101%; 1321N1 102%; U87 MG 104%; GOS-3 112%), PN517 appeared to increase invasion in some cell lines (SVG-p12 92%; 1321N1 104%; U87 MG 116%; GOS-3 123%).

In control cells, the greatest proportion of invasion was observed in the SVG-p12 cell line, followed by U87 MG, 1321N1 and GOS-3 cell lines respectively (data not shown).

A



B

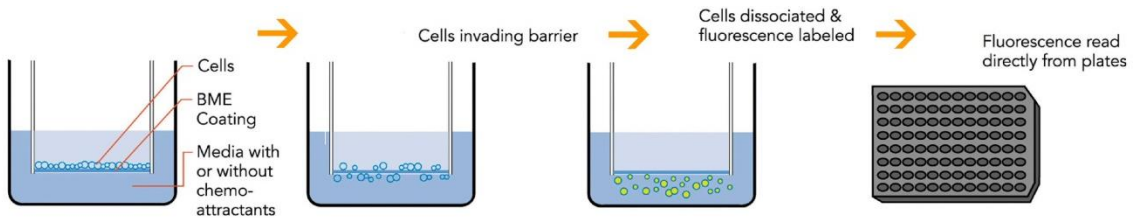


Fig 6.4 Effect of drug treatment on invasion in the SVG-p12, GOS-3, 1321N1 and U87 MG cell lines (A) and a diagrammatic representation of the assay (B). The data illustrate the effect of drug treatment (0.1 mM) over 18 hours on cell invasion in the SVG-p12, GOS-3, 1321N1 and U87 MG cell lines expressed as a percentage of control. Cisplatin was the only treatment found to reduce invasion, with aspirin and PN517 producing no effect. Statistical significance determined by ANOVA with data representing results from 3 independent experiments.

6.3 Discussion

Migration and invasion are two key properties of tumour development and represent an important drug target in the development of new therapeutics for cancer treatment. We have demonstrated that the novel aspirin analogue PN517 significantly reduces migration in glioma cell lines, but did not reduce invasion.

A wide range of literature supports a role for aspirin and COX selective inhibitors in the inhibition of both migration and invasion. In addition to the previously mentioned clinical trial data indicating that aspirin reduces metastasis (Rothwell *et al.*, 2012; Algra and Rothwell, 2012), *in vivo* results using a mouse model examining lung cancer metastasis demonstrated that aspirin significantly reduced LLC-GFP cell metastasis following injection into regional lymph nodes, an effect accompanied by a significant reduction in mortality (Ogawa *et al.*, 2014). A vital step in metastasis is invasion, an ability acquired after a cell goes through epithelial mesenchymal transition (EMT) (Yu *et al.*, 2012), a process inhibited by aspirin (Lou *et al.*, 2014). In addition to *in vivo* and clinical evidence, many *in vitro* studies have characterised the molecular mechanisms involved, establishing that COX-2 expression increases the metastatic potential of Caco-2 colon cancer cells in a matrigel invasion assay (Tsuji *et al.*, 1997), an effect replicated in LN229 glioma cells and found to be dependent on Id1 expression (Xu *et al.*, 2013). It was also reported that PGE₁ and PGE₂ treatment of T98G glioma cells increased migration measured by scratch assay and transwell Boyden chambers, with PGE₂ producing the greater stimulation (Gomes and Colquhoun, 2012). Also using transwell Boyden chambers and scratch assay, Lee and colleagues showed an inhibition of invasion in U87 MG cells by sulindac associated with

a downregulation of MMP-2 (Lee *et al.*, 2005), a result replicated in HN4, HN12, HT29 and HCT116 cell lines in addition to demonstrating a downregulation of MMP-9 (Koontongkaew *et al.*, 2010).

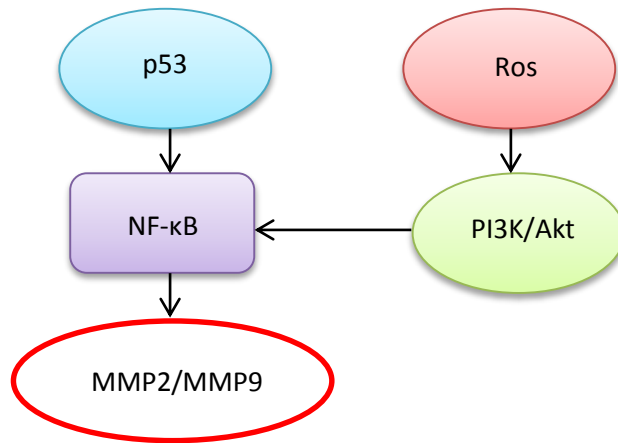


Figure 6.5 Growth factors and cytokines affect MMP-9 expression. MMP expression is regulated by the transcription factors NF-κB and AP-1 through the Ras/MAPK and PI3K/AKT signalling pathways. NF-κB reduces the levels and activities of MMP-2, MMP-9. NF-κB binding to the MMP-2 and MMP-9 promoter is involved in the induction of *MMP-2* and *MMP-9* gene expression associated with tumour cell invasion.

Our results with aspirin and PN517 using the transwell migration assay and scratch assay support these findings and further confirm the therapeutic potential of PN517 for the treatment of glioma. However, the failure of PN517 or aspirin to inhibit invasion is a confounding result given the literature, but there are a number of potential explanations. Firstly, one difficulty associated with the use of matrigel coated transwell inserts for the measurement of invasion is that it is very difficult to establish uniformity of coating, meaning that an increase in matrigel volume in a treatment well compared to a control well

may provide a false positive result. However, in our case, the invasion assay was provided by an independent company where quality control should eliminate such issues. The second, and more likely explanation for contradictory results is incubation time. We examined invasion over eighteen hours, an incubation time significantly greater than many published results where five hours (Koontongkaew *et al.*, 2010), and eight hours (Lee *et al.*, 2005) have been previously used. It could simply be that our incubation was too long and that any decrease in the rate of invasion by either PN517 or aspirin would have only been observed at an earlier timepoint.

One other potentially interesting finding of our results was that the GOS-3 and 1321N1 cell lines showed the greatest level of migration measured using the transwell chambers. This result correlates well with our report of CD90, also known as Thy-1, expression in these two cell lines (Fig 3.2), as it has been reported that up-regulation of CD90 is associated with increased invasion and metastasis in cancer (Cheng *et al.*, 2012).

In conclusion, the data presented in this chapter demonstrate that PN517 and aspirin reduce cell migration, an effect that supports its therapeutic potential for the treatment of glioma. This effect was found at micromolar concentrations, significantly lower than the millimolar concentrations of aspirin that are found *in vivo* after administration of a dose for the treatment of cardiovascular disease (Stark *et al.*, 2001; Paulus *et al.*, 1971; Tin *et al.*, 1998; Borthwick *et al.*, 2006).

Chapter 7. Short Term Cultures

7.1 Introduction

In vitro experiments are most often performed with immortalized cell lines because they are widely available and are able to expand to a high number of passages without any alterations, but they are often criticized because these properties further removes them from *in vivo* data. However, one clinically relevant tumour model system that has gained wide acceptance is the primary cell, or short term culture model, a time consuming process with a relative high success rate of 60% (Mullin *et al.*, 2013). Primary cultures are isolated from tissue and have a heterogeneous population of cell types but they have a limited life span and can lose their phenotype. They are more difficult to work with because of their slower proliferation rate, but should be passaged as little as possible to prevent epigenetic or genetic alterations.

The main purpose for generating *in vitro* models of brain tumours is for the identification of the mechanisms contributing to oncogenesis or tumour maintenance through analysis of distinct molecular patterns, and evaluation of potential therapeutic strategies. This model of testing can also provide a patient-individual sample for the use of individualized therapy. In this chapter, two very different high grade gliomas were supplied from the Preston Royal hospital, BTNW911 and BTNW 914. These two primary cultures have a different doubling time (data not shown), a small and simple difference, but one which allows comparison of drug efficacy in two cultures with contrasting proliferation.

As the short term cultures have a limited availability, quantification of cell viability was performed using PrestoBlue, a reagent with sufficient sensitivity to detect as few as ten

cells. Proliferation was also examined using CFDA-SE staining, but over a longer timecourse than with the cell lines due to the naturally longer doubling time of short term cultures. Finally, cell cycle distribution and apoptosis were examined using flow cytometry, a technique that is sensitive to changes in small cell populations.

Hypothesis

It was anticipated that the effects reported in the previous chapters using standard cell lines would be replicated to some degree with primary cultures. However, as it is known that differences in protein expression, such as EGFR and COX, are found in primary cultures, and that proliferation is much lower, differences in the results would be expected.

7.2 Results

Immunofluorescent staining of the short term cultures was performed to examine GFAP, CD34 and CD90 labelling (Fig 7.1, 7.2 and Table 7.1). Both BTNW911 and BTNW914 showed similar levels of GFAP staining in different passages, but the latter culture had a higher degree of labelling with the endothelial cell marker CD34, however this staining appeared to co-localise with GFAP. With respect to the stem cell marker CD90, both short term cultures displayed a high level of staining across a number of different passages.

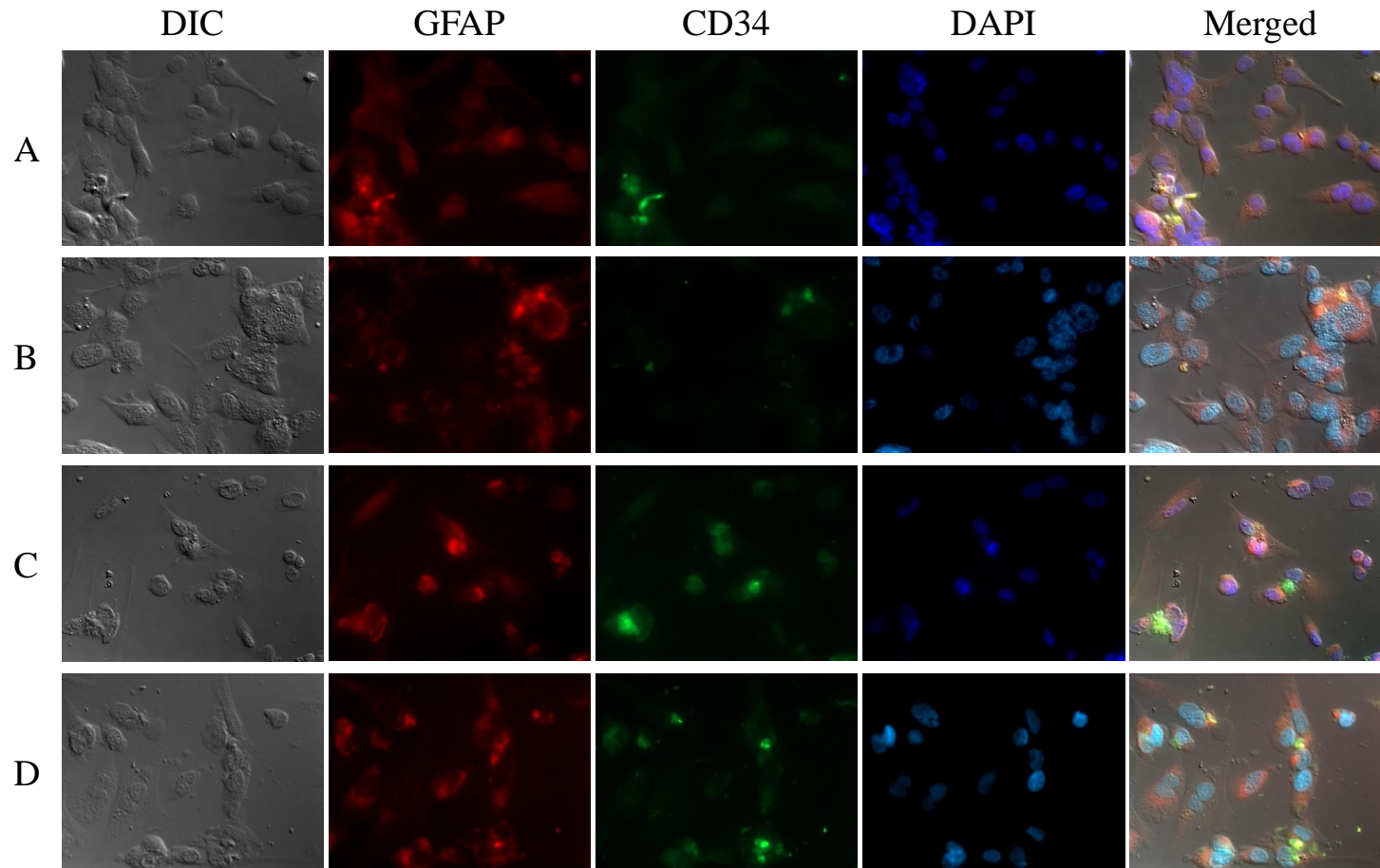


Figure 7.1 Immunofluorescent staining of the BTNW911 and BTNW914 short term cultures for glial fibrillary protein (GFAP) and CD34 with a DAPI nuclear counterstain. GFAP was labelled with an alexa flour 594 conjugated mouse monoclonal antibody (clone 131-17719) and CD34 with FITC conjugated mouse anti-human monoclonal antibody (clone 581). Row A represents BTNW911 passage 7, row B BTNW911 passage 10, row C BTNW914 passage 6, and row D BTNW914 passage 7.

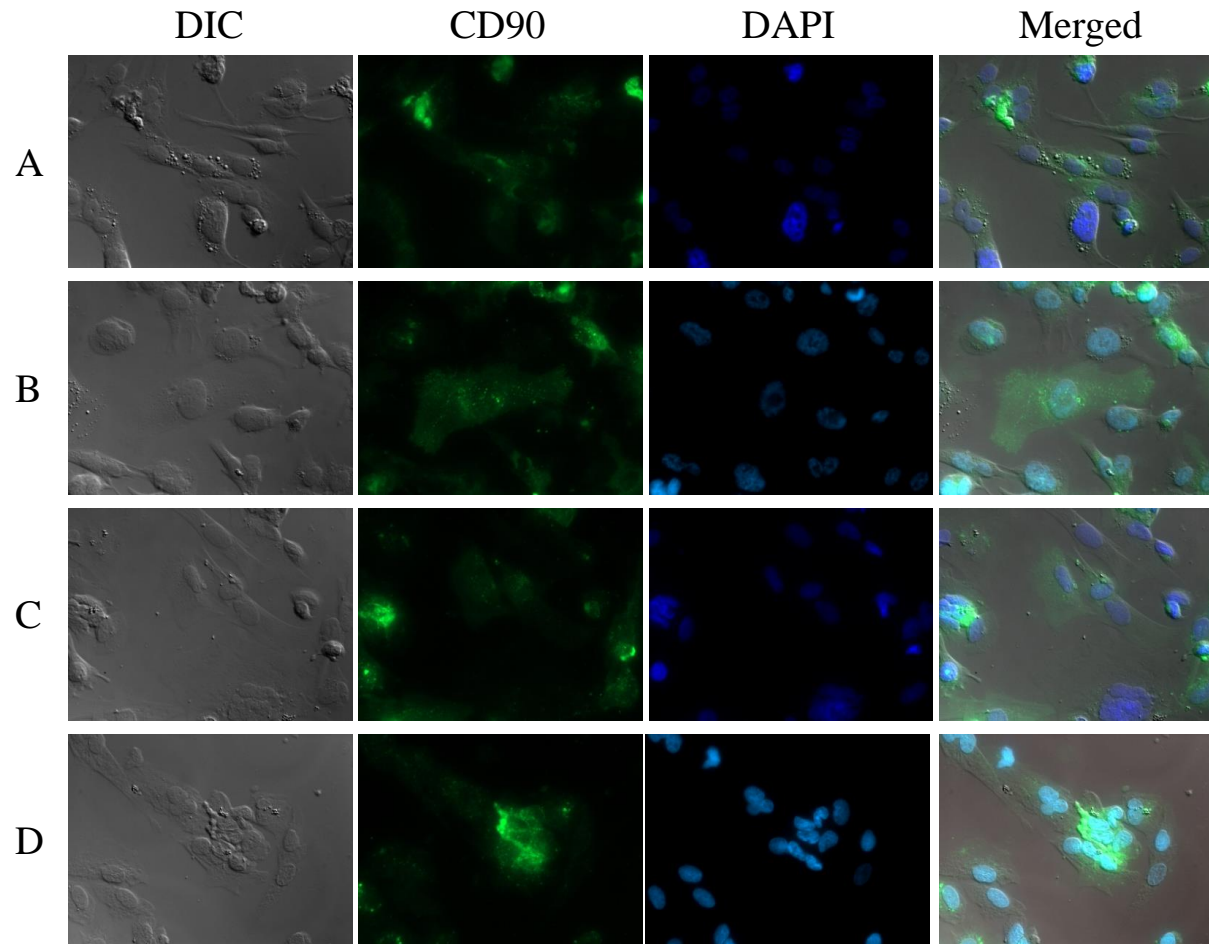


Figure 7.2 Immunofluorescent staining of the BTNW911 and BTNW914 short term cultures for CD90 with a DAPI nuclear counterstain. CD90 was labelled with a FITC conjugated mouse monoclonal antibody (clone 5E10) Row A represents BTNW911 passage 7, row B BTNW911 passage 10, row C BTNW914 passage 6, and row D BTNW914 passage 7.

Table 7.1 Fluorescence intensity quantification of CD34, CD90 and GFAP staining in short term cultures. CD34, CD90 and GFAP expression were detected using directly labelled immunofluorescent antibodies in sequential cell passages of BTNW911 and BTNW914 short term cultures. Fluorescence was quantified using Zeiss Zen software and median values determined from three independent experiments.

	<i>BTNW911</i>			<i>BTNW914</i>		
	Mediun	Min	Max	Mediun	Min	Max
<i>CD34</i>	467	336	2233	416	335	3471
	398	323	4095	428	338	3471
	423	325	3329	405	332	2259
<i>Mean</i>	429	328	3219	416	335	3067
<i>CD90</i>	333	263	1752	418	318	4013
	457	287	4095	455	341	3419
	505	277	4095	397	275	3797
<i>Mean</i>	432	276	3314	423	311	3743
<i>GFAP</i>	1104	914	2696	961	813	2382
	1095	935	2742	1891	1649	4070
	1107	954	2495	1817	1577	4095
<i>Mean</i>	1102	934	2644	1556	1346	3516

A direct comparison of the PrestoBlue and MTT assays showed an equal ability to detect large cell numbers (Fig 7.3 and 7.4) but that the sensitivity of the PrestoBlue assay was greater at low cell numbers (Panel D) and the ability of the assay to detect ten cells was confirmed but required approximately 12 hours of incubation with the reagent (data not shown). In terms of drug induced reductions in cell viability, in the BTNW911 cells, no difference was found in the IC_{50} values for aspirin and cisplatin (Fig 7.3), however, in BTNW914 cells the PrestoBlue assay appeared more sensitive to drug induced changes in viability for both drug treatments (Fig 7.4).

The effect on cell viability by the aspirin analogues was subsequently examined over 24 and 48 hours (Fig 7.5 – 7.10). In BTNW911 cells, PN517 was the most potent drug tested at both timepoints, with an IC_{50} value of 2.6 mM and 1.2 mM respectively (Fig 7.5 and 7.6), and as in the cell lines, PN529 failed to reduce cell viability by 50%. Cisplatin was the next most potent drug, with an IC_{50} at of 2.7mM in the PrestoBlue assay and of 3.0mM in the MTT assay. Interestingly, salicylic acid produced a greater reduction in cell viability at both timepoints than aspirin. In BTNW914 cells cisplatin was the most potent treatment at both timepoints with an IC_{50} value of 0.7mM and 1.9mM at 24 and 48 hours respectively (Fig 7.8 and 7.9). The most potent analogue at 24 hours was PN517 with an IC_{50} value of 1.7 mM, but PN508 showed greater efficacy at 48 hours with an IC_{50} value of 4 mM. In contrast to the BTNW911 short term culture, aspirin had greater efficacy than salicylic acid.

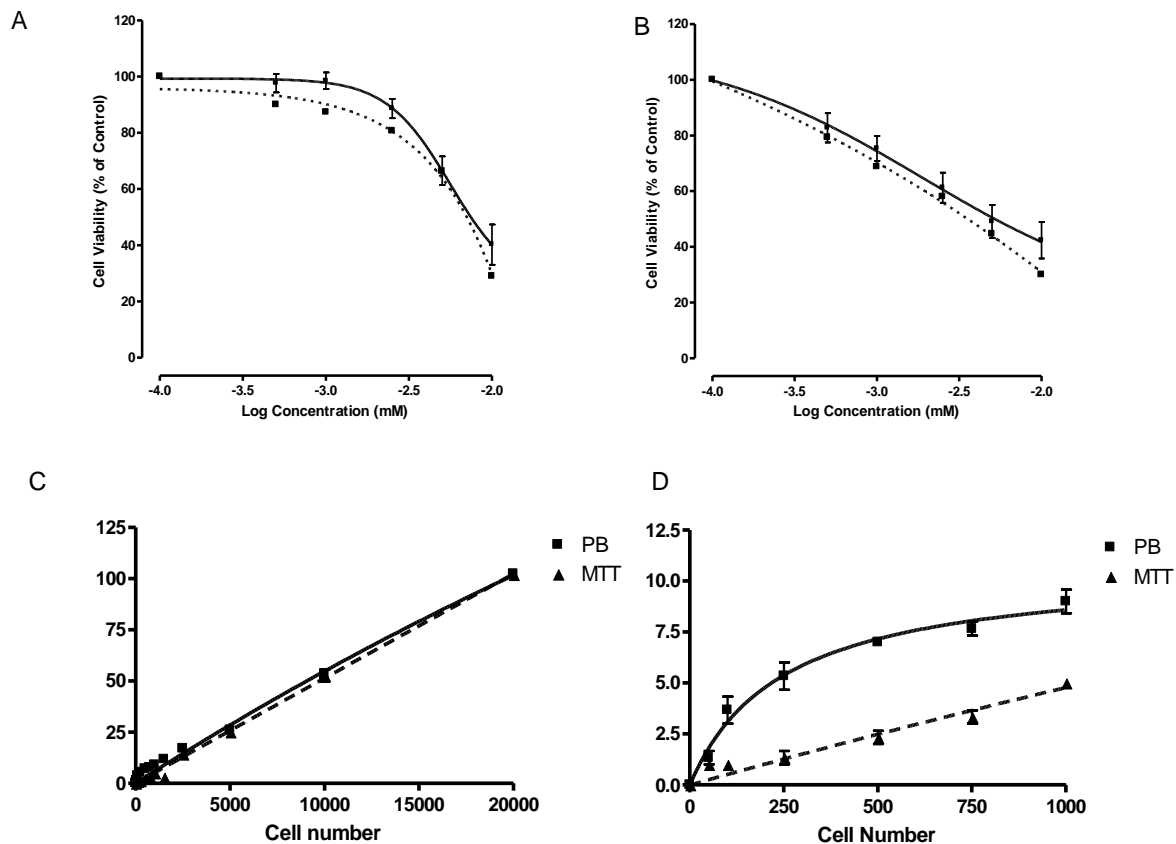


Figure 7.3 Comparison of MTT and PrestoBlue cell viability assays following 24 hours drug treatment in the BTNW 911 primary culture. The data illustrate cell viability expressed as a percent of control determined using the PrestoBlue™ (squares) and MTT (triangles) assays in the BTNW 911 primary culture following drug treatment for 24 hours (aspirin in panel A & cisplatin in B) using 1000 cells per well, and with increasing cell numbers (50 – 20,000 cells per well - panel C; 50 – 1000 cells per well – panel D). ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$), and no significant difference between MTT and PrestoBlue IC_{50} values, with data representing results from 3 independent experiments.

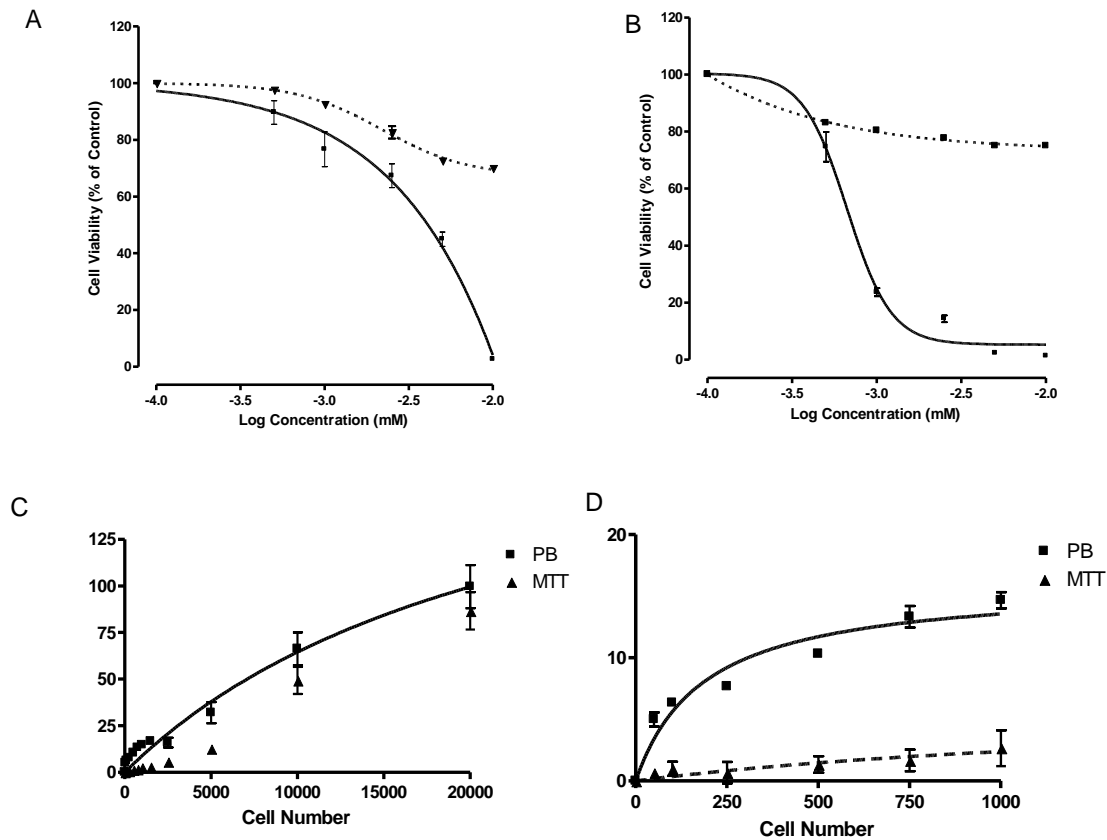


Figure 7.4 Comparison of MTT versus PrestoBlue assay for the detection of cell viability following 24 hours drug treatment in the BTNW 914 primary cell culture. The data illustrate cell viability determined using the PrestoBlue™ and MTT assay on BTNW 914 cell culture following drug treatment for 24 hours. Viability of MTT (triangles and dashed line) and PB (squares and solid line) assay to detect effects on cell viability following aspirin (panel A) and cisplatin (panel B) drug treatment for 24 hours. The lower panels illustrate the relationship between cell number and viability for both the MTT and PB assays, up to 1000 cells per well (panel D) and 20,000 cells per well (panel C). ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$), data represent results from 3 independent experiments.

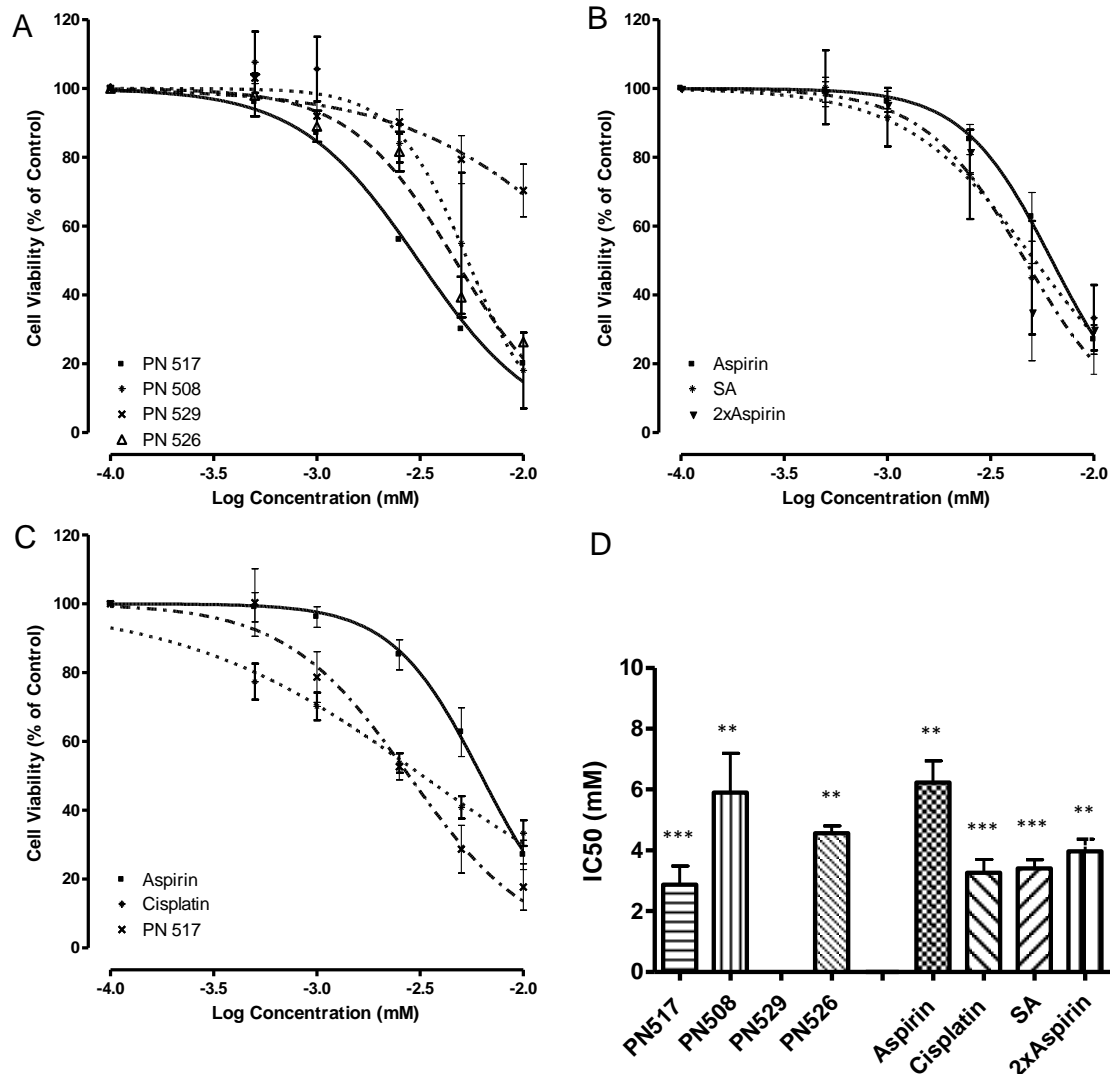


Figure 7.5 Cell viability following 24 hours drug treatment in BTNW911 short term culture. The data illustrate cell viability determined using the PrestoBlue™ assay on BTNW911 cells following drug treatment for 24 hours. Panel A illustrates the results for the aspirin analogues PN517, PN508, PN526 and PN529. In panel B, the control treatments aspirin, salicylic acid and double aspirin decrease cell viability. Panel C illustrates a comparison between PN517, cisplatin and aspirin showing that at 24 hours PN517 and cisplatin have similar efficacy. Panel D illustrates the IC₅₀ values following 24 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

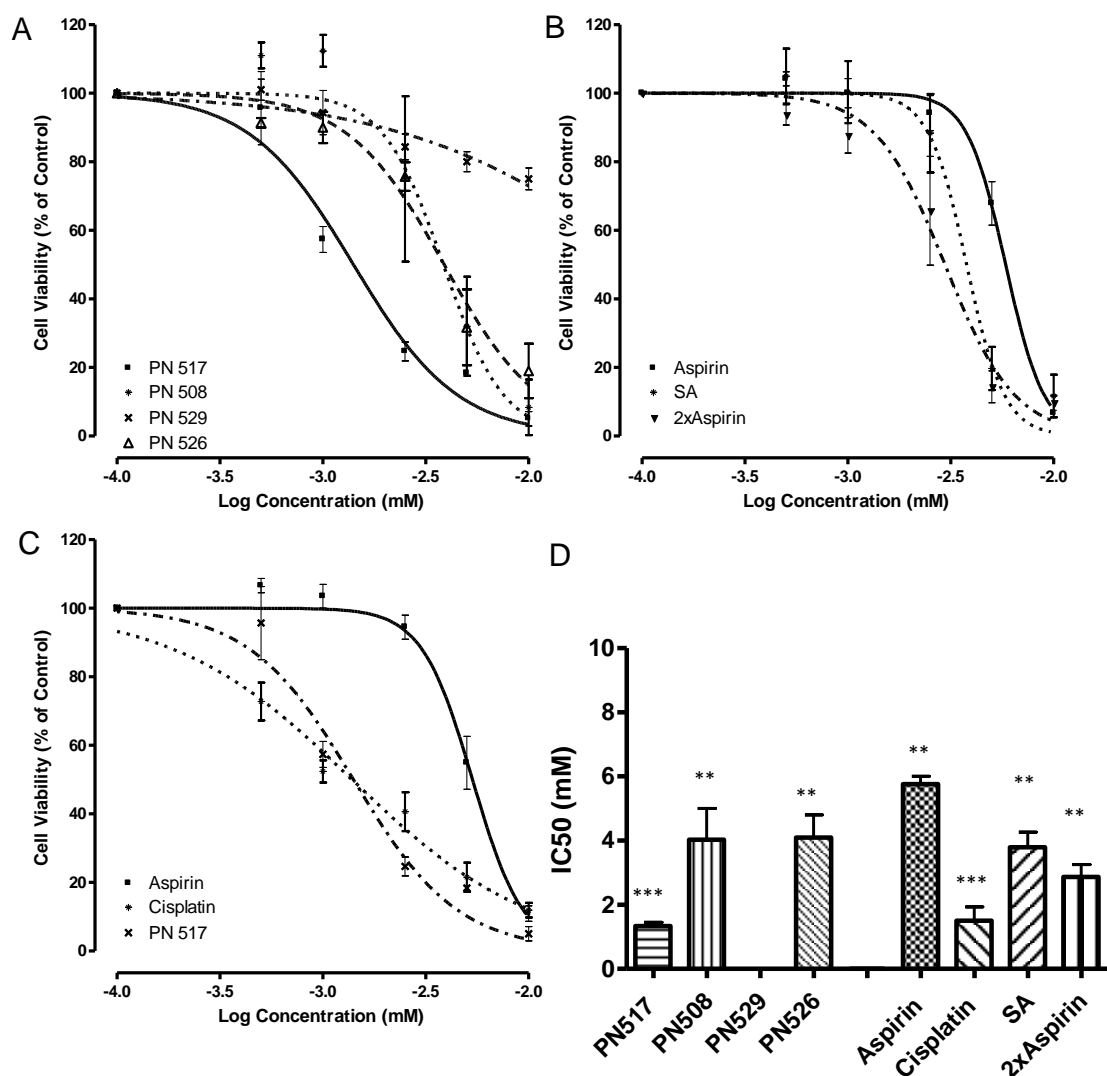


Figure 7.6 Cell viability following 48 hours drug treatment in BTNW911 short term culture. The data illustrate cell viability determined using the PrestoBlue™ assay on BTNW911 cells following drug treatment for 48 hours. Panel A illustrates the results for the aspirin analogues PN517, PN508, PN526 and PN529. In panel B, the control treatments aspirin, salicylic acid and double aspirin decrease cell viability. Panel C illustrates a comparison between PN517, cisplatin and aspirin showing that at 48 hours PN517 and cisplatin have similar efficacy. Panel D illustrates the IC₅₀ values following 48 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

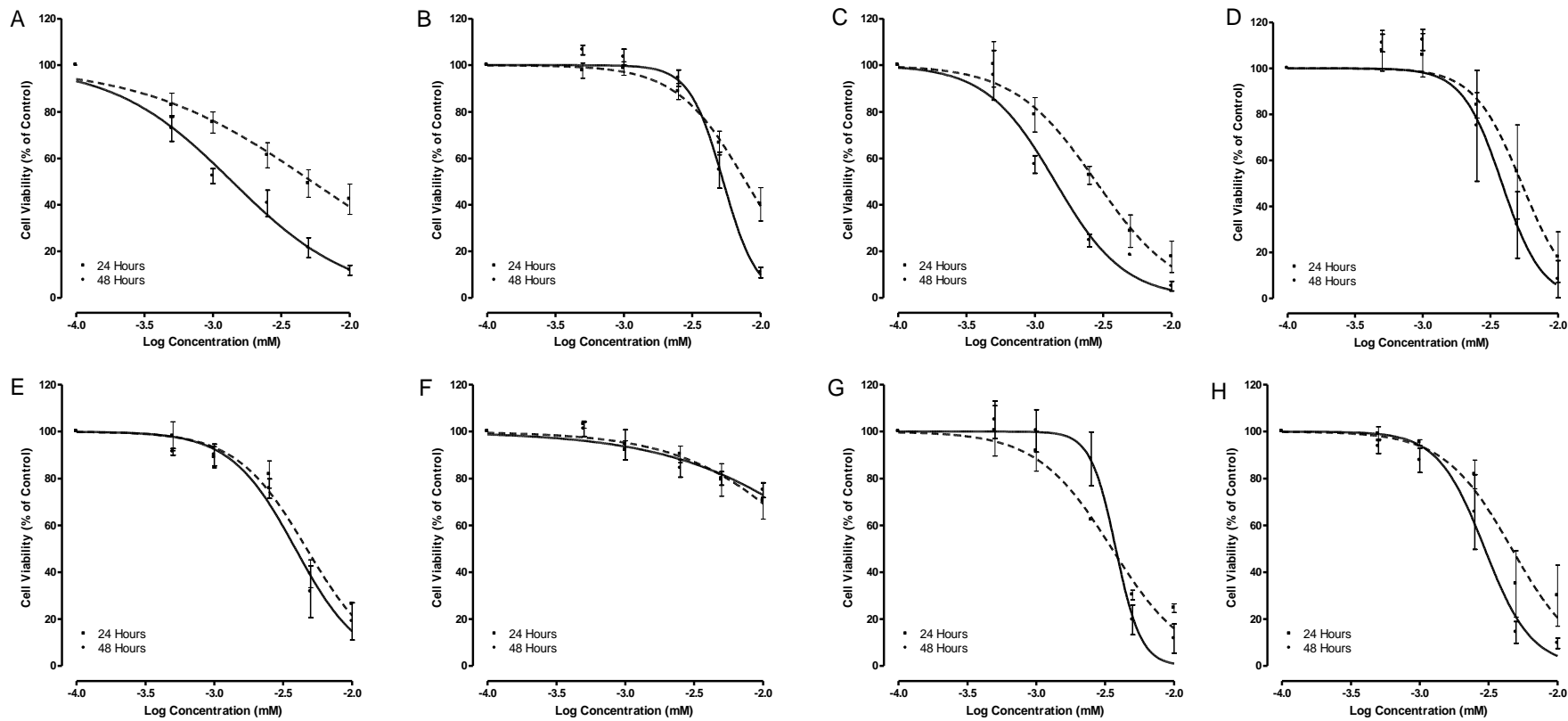


Figure 7.7 Cell viability following 24 and 48 hours drug treatment in BTNW911 short term culture. The data illustrate cell viability determined using the PrestoBlue™ assay in the BTNW911 cells following drug treatment for 24 and 48 hours. Panel A-G illustrate the effect on cell viability following drug treatment of cisplatin, aspirin, PN517, PN508, PN526, PN529 and SA respectively over 24 and 48 hours. The decrease in cell viability is similar in both 24 and 48 h. ANOVA analysis of results show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

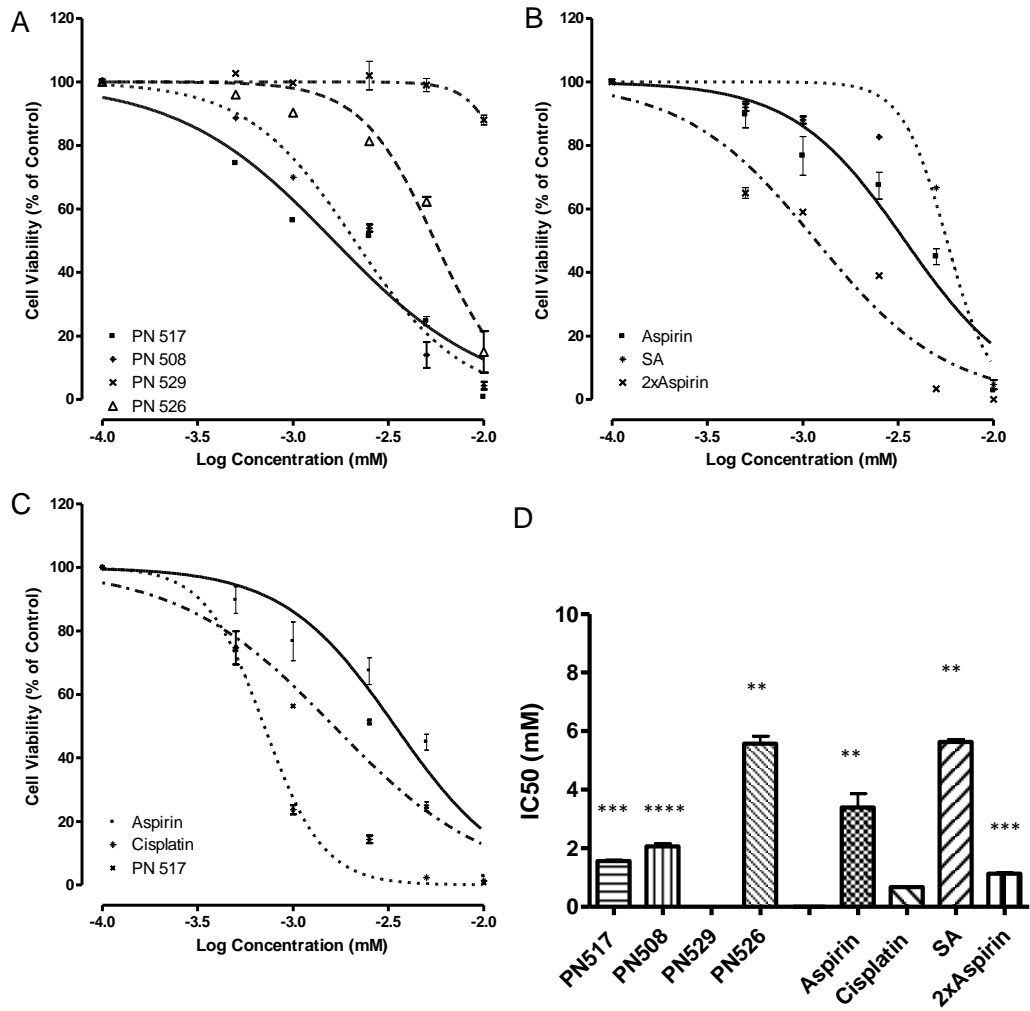


Figure 7.8 Cell viability following 24 hours drug treatment in BTNW914 short term culture. The data illustrate cell viability determined using the PrestoBlue™ assay on BTNW914 cells following drug treatment for 24 hours. Panel A illustrates the results for the aspirin analogues PN517, PN508, PN526 and PN529. In panel B, the control treatments aspirin, salicylic acid and double aspirin decrease cell viability. Panel C illustrates a comparison between PN517, cisplatin and aspirin. Panel D illustrates the IC₅₀ values following 24 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

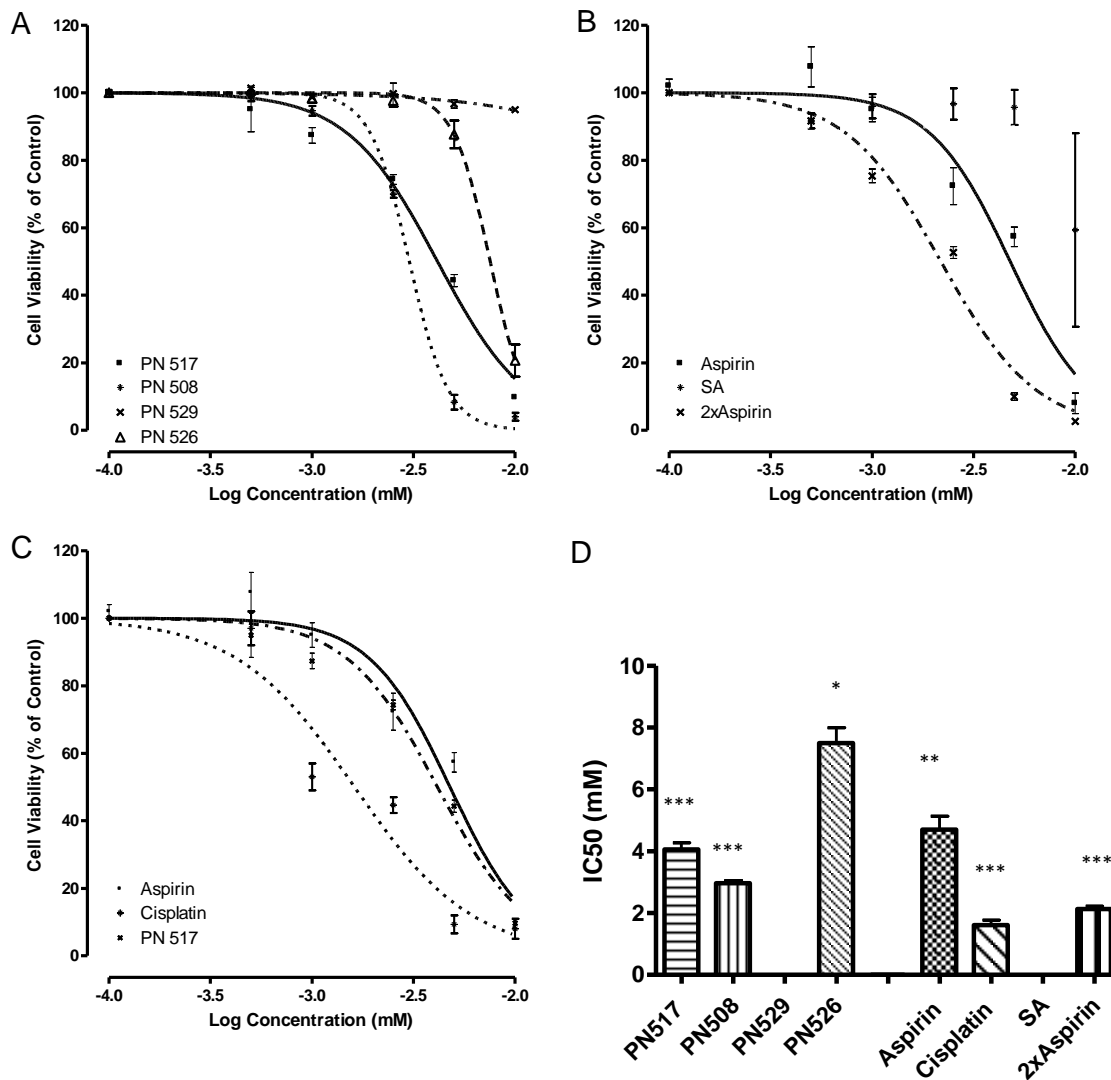


Figure 7.9 Cell viability following 48 hours drug treatment in BTNW914 short term culture.

The data illustrate cell viability determined using the PrestoBlue™ assay on BTNW914 cells following drug treatment for 48 hours. Panel A illustrates the results for the aspirin analogues PN517, PN508, PN526 and PN529. In panel B, the control treatments aspirin, salicylic acid and double aspirin decrease cell viability. Panel C illustrates a comparison between PN517, cisplatin and aspirin. Panel D illustrates the IC₅₀ values following 48 hour treatment of the aspirin analogues and control drugs. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

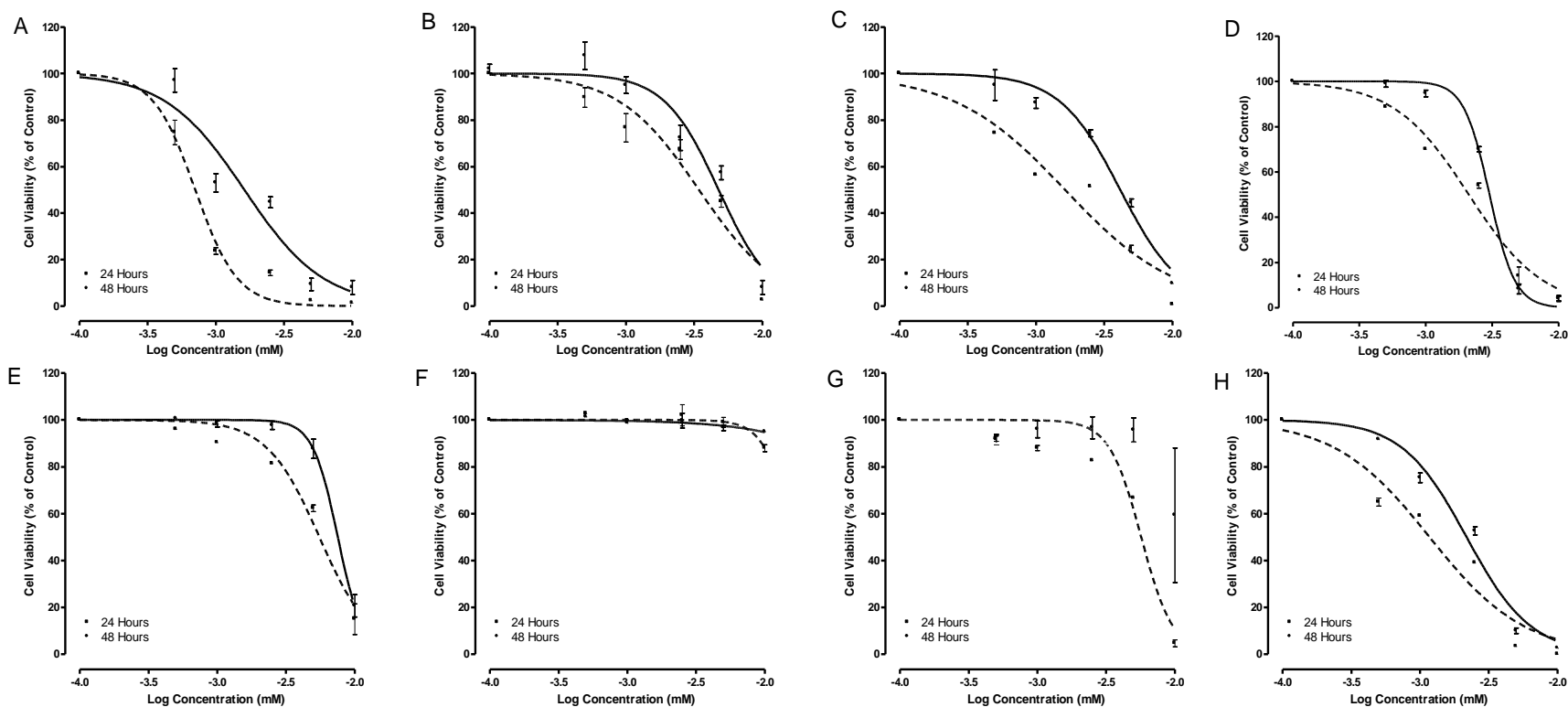


Figure 7.10 Cell viability following 24 and 48 hours drug treatment in BTNW911 short term culture. The data illustrate cell viability determined using the PrestoBlue™ assay in the BTNW911 cells following drug treatment for 24 and 48 hours. Panel A-G illustrate the effect on cell viability following drug treatment of cisplatin, aspirin, PN517, PN508, PN526, PN529 and SA respectively over 24 and 48 hours. ANOVA analysis of results show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

The effect of drug treatment on proliferation in the BTNW 911 and BTNW914 short term cultures was subsequently determined (Fig 7.11 and 7.12). It is important to note that as these cells divide at a much slower rate than the established cell lines, the experiment was run over 10 days and not 3 days as previously used in chapter four. Each drug treatment reduced the proliferation rate of the short term cultures, but most significantly, the largest effect was observed with the aspirin analogue PN 517 (Fig 7.11 and 7.12). In BTNW914 cells, the effect of PN517 on proliferation returned to levels similar to other drug treatments by day 6 and to the same level as control by day 10 (Fig 7.12).

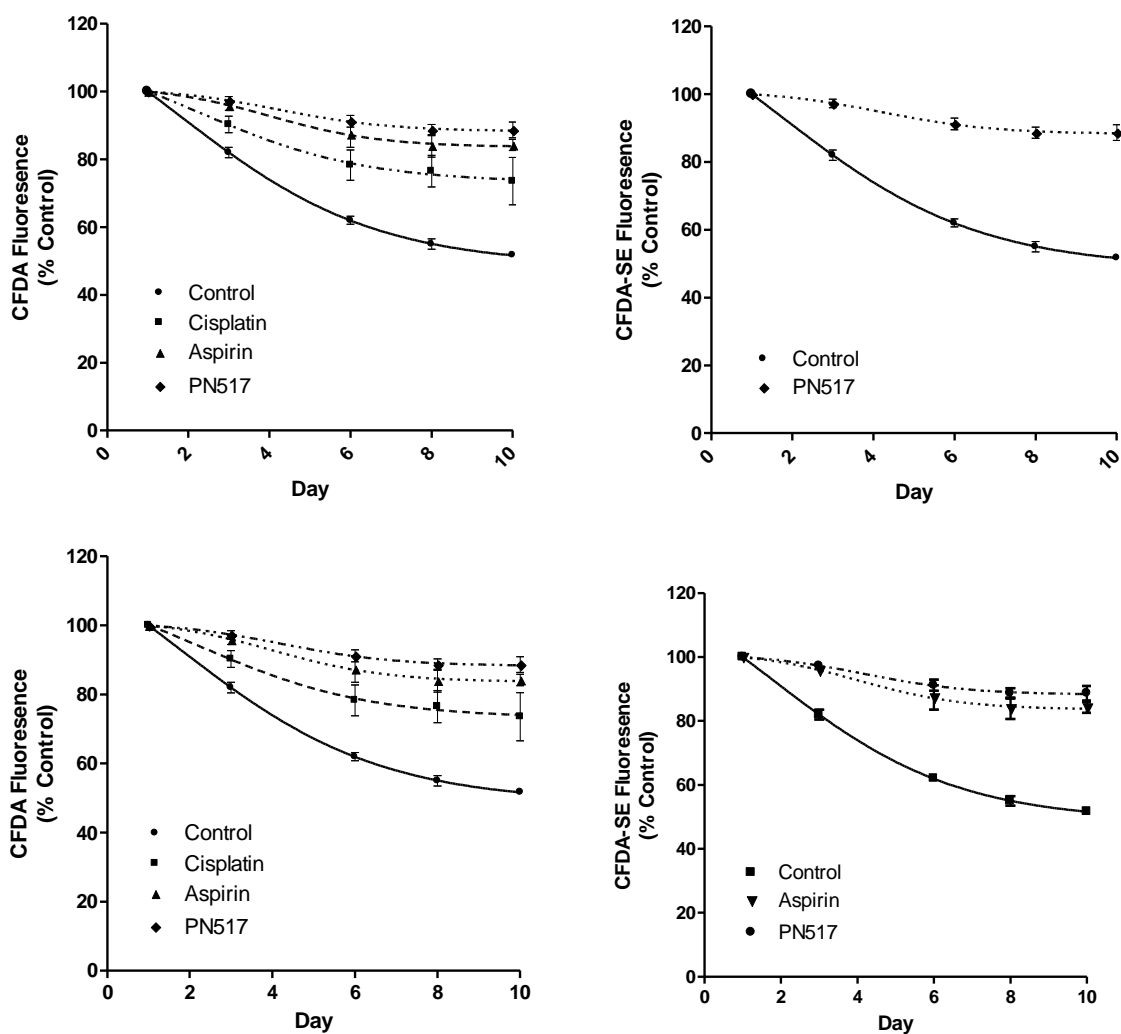


Figure 7.11 Cell proliferation measured by flow cytometry following drug treatment in the BTNW911 short term culture. The data illustrate cell proliferation determined using CFDA-SE staining of BTNW911 cells, following drug treatment (0.1mM) over two weeks (days 2, 4, 6, 8 and 10). Panel A shows the effect on all treatments on cell proliferation, where a rightward shift indicates a reduction in proliferation. Panel B compares control to PN517; panel C compares the most potent analogue PN517 with control, aspirin and cisplatin treatments; panel D shows a comparison of aspirin and PN517. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

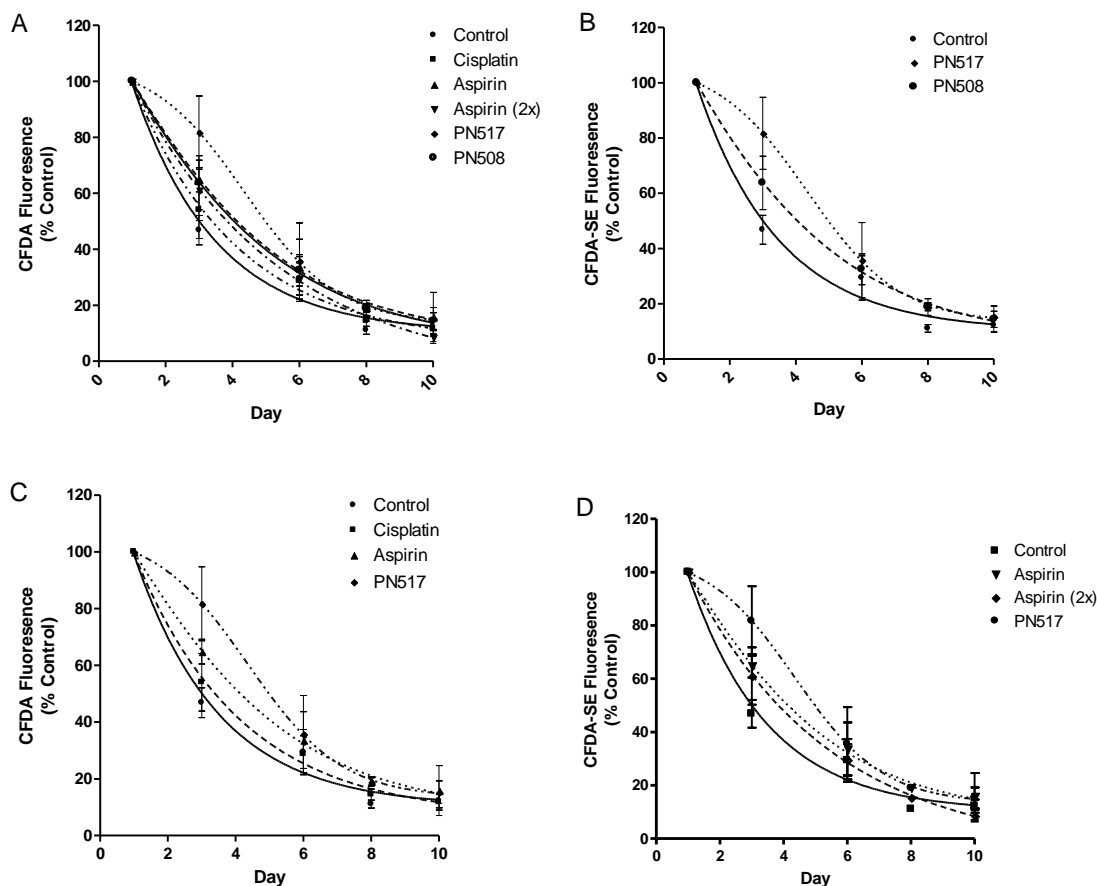


Figure 7.12 Cell proliferation measured by flow cytometry following drug treatment in the BTNW914 short term culture. The data illustrate cell proliferation determined using CFDA-SE staining of BTNW914 cells, following drug treatment (0.1mM) over two weeks (days 2, 4, 6, 8 and 10). Panel A shows the effect on all treatments on cell proliferation, where a rightward shift indicates a reduction in proliferation. Panel B compares control to PN517 and PN508; panel C compares the most potent analogue PN517 with control, aspirin and cisplatin treatments; panel D shows a comparison of aspirin, double aspirin and PN517. ANOVA analysis of results, show an overall significant effect of treatment in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

Cell cycle analysis was performed at 24 and 48 hours with a low (0.1mM) and high (1mM) concentration of cisplatin, aspirin and PN517 in the BTNW911 short term culture (Fig 7.13). PN517 increased the proportion of cells in the sub-G0 apoptotic population at both timepoints following treatment at both 0.1 and 1mM, decreased the G0/G1 population, and increased the S phase population of cells. While aspirin increased the sub G0 population at 24 hours with 0.1mM treatment it had little effect on either the S or M phases. At 48 hours it produced a similar effect to PN517, an affect replicated at 24 hours with 1mM treatment, but not at 48 hours where a larger G0/G1 population was observed. Treatment with cisplatin increased the sub-G0 apoptotic population at both concentrations and timepoints, with an increase in the S phase population apart from at 48 hours.

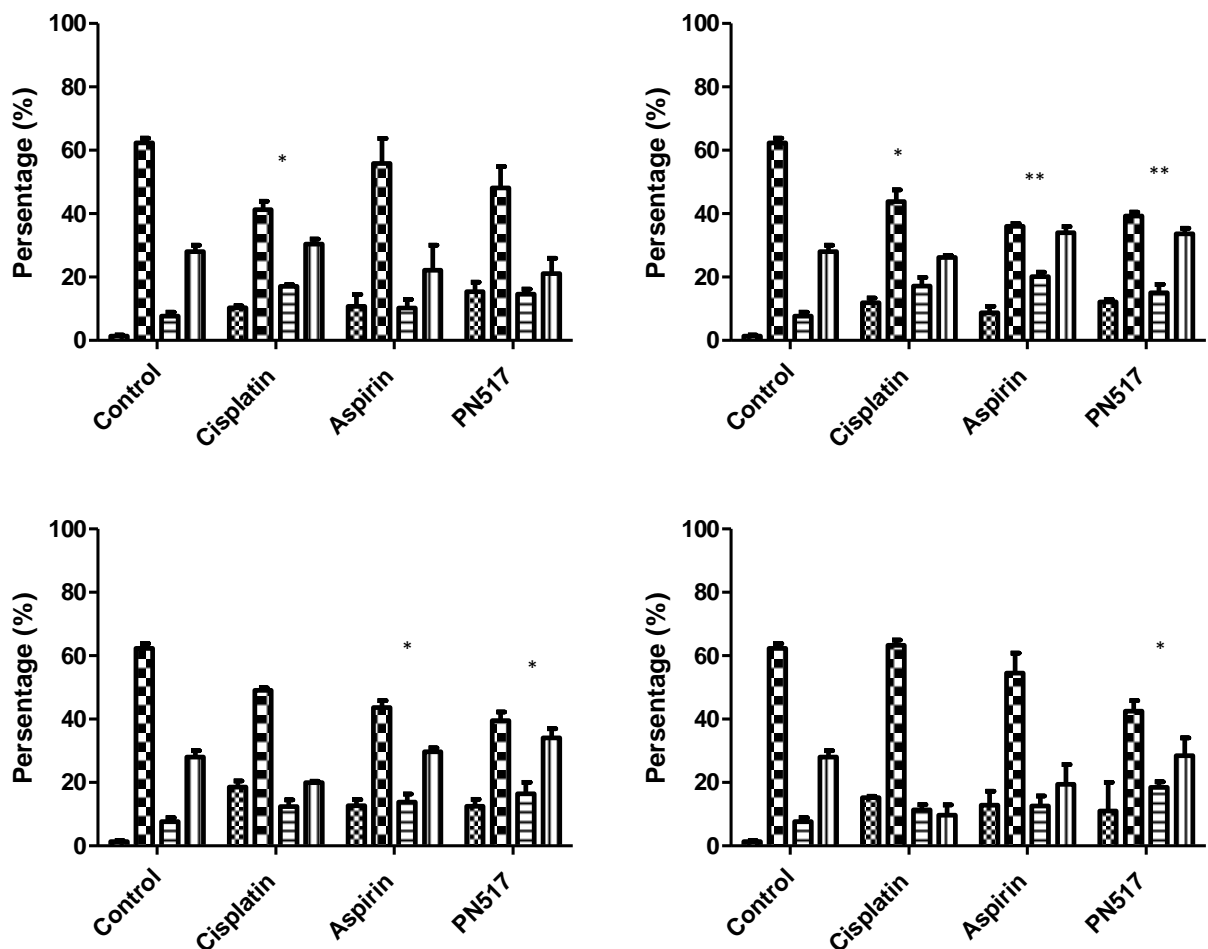


Figure 7.13 Cell cycle distribution of the BTNW911 short term culture following drug treatment. Cell cycle distribution was determined by PI staining and flow cytometry following drug treatment for 24 (panel A and C) and 48 hours (panel B and D) at 0.1mM (panel A and B) and 1mM (panel C and D). The proportion of the sub G1 apoptotic, G1, S and M phase populations is displayed as a percentage of the total population. ANOVA analysis of results, show an overall significant effect of treatment, in comparison with control ($p < 0.0001$) with data representing results from 3 independent experiments.

Apoptosis was examined using flow cytometry (Fig 7.14 and 7.15). PN517 induced apoptosis in the BTNW911 short term culture at both timepoints and at both drug concentrations, showing a time dependent effect. Interestingly, in contrast to cell viability and proliferation assays, aspirin induced apoptosis to a greater degree than PN517. Apoptosis in the BTNW914 short term culture was induced in both a concentration and time dependent manner by the aspirin analogue PN517 (Fig 7.15). In contrast to BTNW911 cells, a larger proportion of late apoptotic cells was observed. Aspirin again induced apoptosis to a greater degree than PN517, also with a larger proportion of late apoptotic cells in the 1mM treatment.

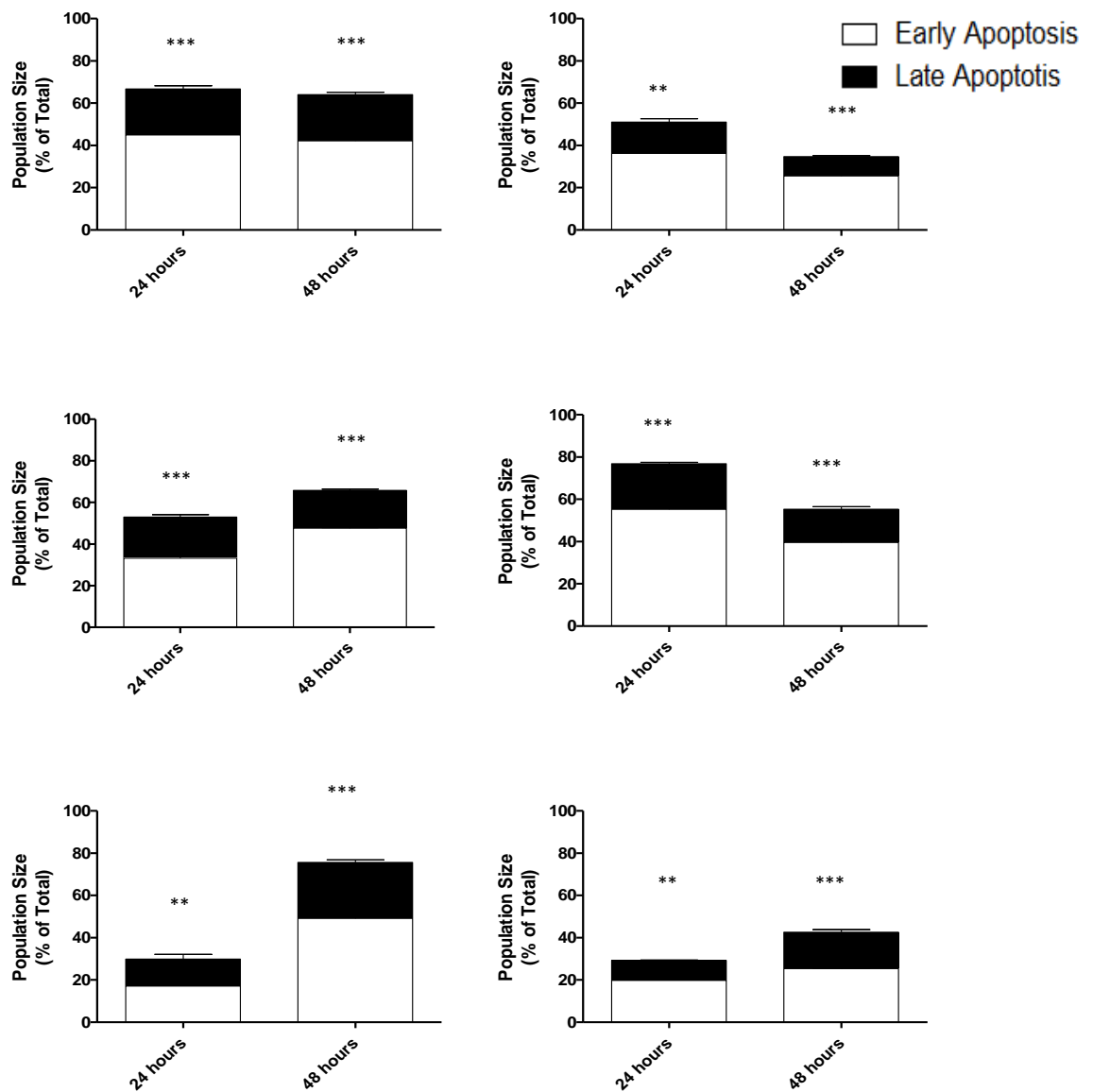


Figure 7.14 Induction of apoptosis determined by Annexin V/PI staining following drug treatment for 24 and 48 hrs at 0.1 mM and 1 mM in the BTNW911 short term culture. The data represent the proportion of apoptotic cells (white - early apoptotic, black - late apoptotic) in BTNW911 cells as determined by flow cytometry following drug treatment over time. Panel A and B illustrate cisplatin treated cells at 0.1 and 1 mM respectively, and similarly C and D aspirin treated cells and E and F panel show PN517 treated cells. The data represent results of 3 independent experiments with an overall significant effect of treatment in comparison with control ($p < 0.0001$) determined by ANOVA.

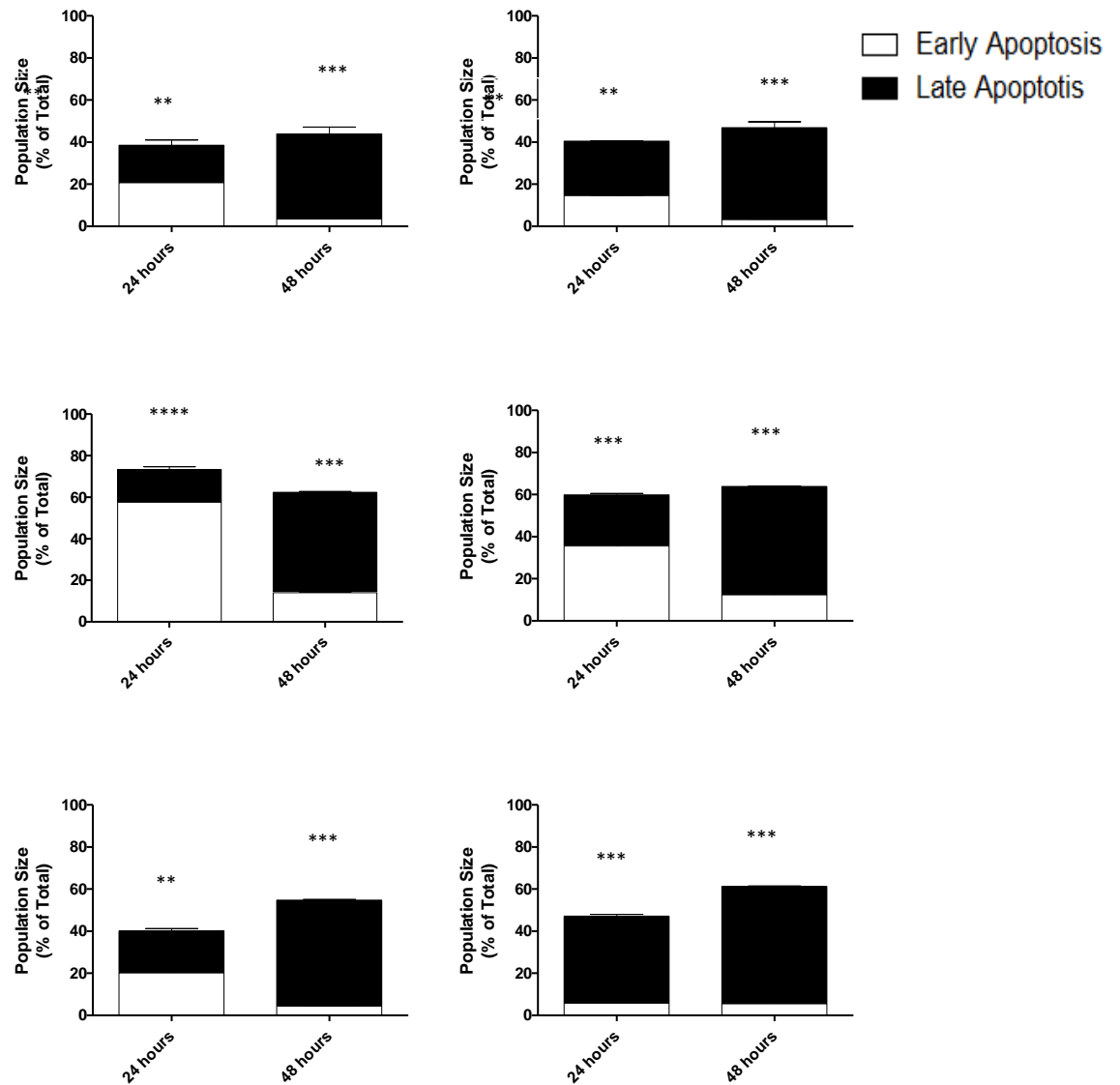


Figure 7.15 Induction of apoptosis determined by Annexin V/PI staining following drug treatment for 24 and 48 hrs at 0.1 mM and 1 mM in the BTNW914 short term culture. The data represent the proportion of apoptotic cells (white - early apoptotic, black - late apoptotic) in BTNW914 cells as determined by flow cytometry following drug treatment over time. Panel A and B illustrate cisplatin treated cells at 0.1 and 1 mM respectively, and similarly C and D aspirin treated cells and E and F panel show PN517 treated cells. The data represent results of 3 independent experiments with an overall significant effect of treatment in comparison with control ($p < 0.0001$) determined by ANOVA.

Migration was examined in the BTNW911 short term culture using the wound healing/scratch assay (Fig 7.16). As with the cell lines, once cells were confluent, a scratch was created using a sterile cocktail stick so as not to scratch the plastic surface and alter migration, and the media replaced (including drug treatment where appropriate) to remove any floating cells whose re-attachment might influence the results. In non-treated cells, migration did not completely close the scratch over the 18 hour period of the assay. Treatment with PN517 reduced migration to approximately 60% of control with the scratch remaining unclosed after 18 hours. In contrast to the cell lines, cisplatin did not reduce migration any further, showing similar efficacy to PN517. Aspirin also reduced migration, but only reduced it to approximately 80% of control.

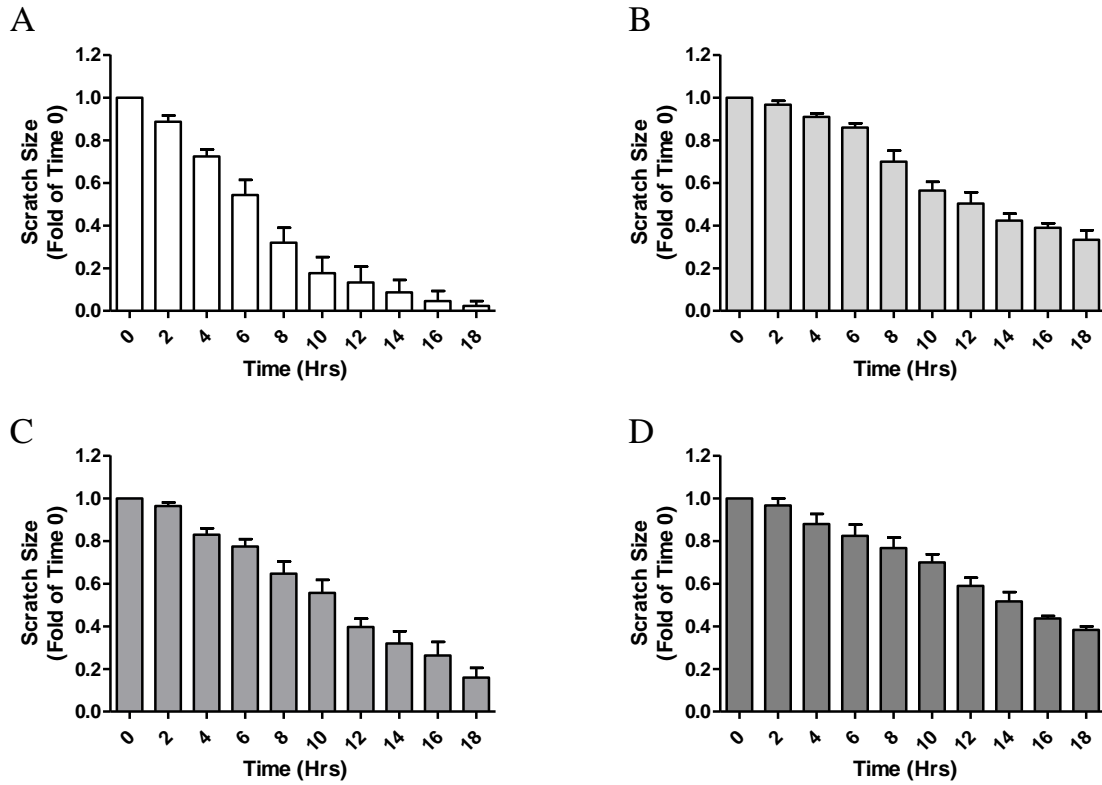


Fig 7.16 Effect of drug treatment on the BTNW911 short term culture migration using the scratch assay. Migration was expressed as fold decrease over 18 hours (panel A control) in scratch width as measured by minimum distance following treatment with PN517 (panel B), aspirin (panel C) and cisplatin (panel D) at 1mM. An overall significant effect of treatment ($p < 0.01$) was found by ANOVA analysis with data representing results from 3 independent experiments.

7.3 Discussion

In the present chapter the aspirin analogue PN517 was evaluated in comparison to the control drugs aspirin and cisplatin in the short term cultures BTNW911 and BTNW914. Although the same methodology was used as described in previous chapters, assays were adapted to allow for the slow proliferation rate when compared to the commercially available cell lines. Both BTNW911 and BTNW914 are high grade glioma, originating from male patients of 33 and 66 years of age respectively. Both cultures were passaged typically in a 1:2 ratio, in contrast to the normal 1:4 for the cell lines. BTNW911 had a doubling time of approximately 7 days, and was used up to passage 10 in the lab without any slowing of proliferation or change in IC_{50} for the control drugs. On the other hand BTNW914 initially had a doubling time of approximately 7 days, which increased by about 2 days at every passage, finally taking up to 3 weeks to double at passage 10.

Immunohistochemical results indicated that both short term cultures display a high level of GFAP staining, indicating that they are of an astrocytic cell origin (Jacque *et al.*, 1978; Halliday *et al.*, 1996), with CD34 staining found to a lesser extent suggesting only a relatively small population of endothelial cells in the culture (Charalambous *et al.*, 2006). In both the BTNW911 and 914 cultures, a high degree of CD90 positive cells were observed, indicating a large population of cancer stem cells (He *et al.*, 2012). However, following our previous observation of CD90 binding in the GOS-3 and 1321N1 cells, this conclusion would have to be treated with caution.

As previously mentioned, the sensitivity of the PrestoBlue assay made it ideal for assessing changes in cell viability in short term cultures where cells are in limited supply. However,

while we found no difference in IC₅₀ values for either aspirin or cisplatin in the BTNW911 short term culture, the PrestoBlue assay appeared to be significantly more sensitive to drug treatment in BTNW914 cells. It is possible that given the slow proliferation rate that the mitochondrial content of these cells is low, and thus the MTT assay, which is based on the ability of a mitochondrial dehydrogenase enzyme to reduce the yellow coloured substrate to purple formazan product (Mossman, 1983), is not sensitive to the effects of drug treatment. Whereas the PrestoBlue assay, that functions as a cell viability indicator by using the reducing power of cells (Lu *et al.*, 2012), might be more sensitive to treatment.

With respect to drug treatment, PN517 showed at least similar if not greater efficacy than cisplatin in cell viability, proliferation, apoptosis and migration assays. This is again a strong indicator of the therapeutic potential of this novel compound. One unexpected result in the apoptosis assay was that aspirin actually produced a greater reduction in cell viability when compared to either PN517 or cisplatin. This effect is unlikely to be due to differences in inhibition of COX enzymes, as we found both compounds to have similar efficacy in the isolated enzyme assay, suggesting a difference in COX independent pathways. These pathways may include inhibition of the transcription factor nuclear factor kappa B (NF- κ B) however, as earlier stated, this does contribute to activation of other signalling pathways which are COX dependant, thus, is not a truly independent pathway.

What is clear from the literature is that COX-2 plays an important role in aspirin mediated effects and as a result has been highly studied (Xu *et al.*, 2013; Suzuki *et al.*, 2013; White *et al.*, 2013; Gomes and Colquhoun, 2012; Lan *et al.*, 2011; Lo *et al.*, 2010; Kang *et al.*,

2009; Kambe *et al.*, 2009; Annabi *et al.*, 2009; Kim *et al.*, 2009; Lee *et al.*, 2005; Amin *et al.*, 2003; Arrieta *et al.*, 2001; Aas *et al.*, 1995). However, very few publications have examined aspirin effects or looked at COX expression and activity either *in vivo* or *in vitro* using primary cultures. Kokoglu and colleagues reported that prostaglandin and arachidonic acid levels are elevated in brain tumours (Kokoglu *et al.*, 1998). In 2000, it was reported that COX-2 expression which is greater in high-grade glioma (Shono *et al.*, 2001), is most elevated in more slowly growing cultures (Joki *et al.*, 2000). A decrease in proliferation and migration in a concentration dependant manner by the COX-2 selective inhibitor NS398 was also reported. However, only a small induction of apoptosis was found, in contrast to our findings with either aspirin which is relatively COX-1 selective, or the novel analogue PN517. This result might suggest that the induction of apoptosis in glioma is dependent on the constitutively expressed COX-1 enzyme and not the induced COX-2.

As previously mentioned, it is also possible that metabolism of aspirin or the PN517 analogue is occurring *in vitro* as the *CYP2C9* enzyme is expressed in glioma and correlates positively with tumour grade (Knupfer *et al.*, 2006). This expression of *CYP2C9* provides a potential explanation as to why the cell viability IC₅₀ values for aspirin and its analogues did not decrease further at 48 hours. Additionally, in the BTNW 914 short term culture, the cells appeared to recover at 48 hours following treatment with PN517, reflected in an increase in the IC₅₀ value. As the cells were derived from a high grade glioma, this might be a reflection of *CYP2C9* expression, but would require confirmation.

The epidermal growth factor receptor (EGFR) pathway is probably the most significant signalling pathway clinically implicated in glioma, with the EGF polypeptide playing an important role in the regulation of growth, proliferation (Lo *et al.*, 2010; Zhang *et al.*, 2013; Auf *et al.*, 2013). EGFR expression positively correlates with the glioma grade of cell lines, however, it was been reported that primary cells express the receptor at an even higher level. The aspirin analogue, NCX-4016, induces cell cycle arrest and apoptosis in cisplatin-resistant human ovarian cancer cells *via* down-regulation of EGFR/PI3K/STAT3 signalling and modulation of Bcl-2 family proteins (Selvendiran *et al.*, 2008). Aspirin is also known to inhibit EGFR activation, with inhibition having been demonstrated in COX-1 expressing OVCAR-3 cells, further demonstrating an interaction between EGFR-activated signalling pathways and COX (Cho *et al.*, 2013). Since aspirin can affect both COX-1 and COX-2, a correlation between both could be the key to activation of different pathways. The Wnt/ β -catenin signalling pathway which regulates proliferation in glioma also cross links with EGFR (Chen *et al.*, 2011). Tcf-4 protein expression is significantly increased in high-grade glioma when compared to low-grade glioma and is correlated with Akt-1 expression. β -catenin/Tcf-4 directly regulates Akt-1 in glioma, and these two proteins may cooperate with each other in exerting their oncogenic effects (Chen L. et al 2011).

Both BTNW911 and BTNW914 short term cultures show expression of the stem cell marker CD90, an important property for the development of new therapies. It has been shown that NSAID inhibition of COX-2 reduced the population of cancer stem cells in colon cancer (Moon *et al.*, 2014). This may also be related to NF- κ B signalling, as elevated NF- κ B signalling activates the Wnt pathway and induces de-differentiation of non-stem

cells that have acquired a tumour-initiating capacity (Schwitalla *et al.*, 2013). This aspirin and its analogues may reduce cancer stem cell populations through an anti-inflammatory action on the tumour microenvironment. In addition, the PI3K/Akt/mTOR pathway is activated for maintenance and proliferation of cancer stem cells, by AMPK (Kim *et al.*, 2014). Activation of AMPK induces cell cycle arrest by inhibiting the expression of cyclin D1 and activating p21/p27, resulting in apoptosis. As aspirin activates AMPK it could provide one possible explanation for the protective effects of NDAIS against development of glioma.

In conclusion, the aspirin analogue PN517 has demonstrated efficacy in primary cultures derived from high grade glioma, reducing viability, inhibiting proliferation, inducing apoptosis, causing cell cycle arrest and inhibiting cell migration. These results may provide a better assessment of therapeutic potential because they contain various tumour derived cell types and not just a single cell population which established cell lines contain.

Chapter 8 Final Discussion

This project characterised the effect of novel aspirin analogues using a range of assays in glioma cell lines and primary cultures, to allow identification of the analogue with best therapeutic potential. The primary aim examined cell viability and identified the compound displaying the greatest efficacy, with the remaining aims of the project focusing on characterising the mechanism of action of this lead compound, including the investigation of its effect on cell proliferation, apoptosis, and migration/invasion. In identifying PN517 as the compound with highest efficacy, these initial aims have been met while generating many intriguing results.

Currently, the biggest challenges in GBM treatment are the extremely narrow window for operative approaches, which is mostly palliative and rarely curative even when in combination with radiotherapy. A further issue is the blood brain barrier, which poses a major challenge in successful drug delivery, one which many NSAIDs successfully overcome (Goncalves *et al.*, 2010). Therefore, most investigators and clinicians believe that development of better chemotherapeutic agents targeting multiple pathways in glioma stand a better chance of improving patient survival. Many potential targets have been identified, including activation of the EGFR and PI3K/Akt pathways, NF- κ B and Wnt/ β -catenin signalling pathways, as well as cyclin D, STAT3, and interleukins, in addition to the common pathogenic pathway of p53/Rb. These pathways contribute to cell survival, proliferation, apoptosis, migration, invasion and angiogenesis, and thus are crucial for tumourigenesis. Vially, the COX enzymes have been shown to be involved in most of these pathways in various cancer models, and crucially are overexpressed in glioma and correlate with tumour grade and poor clinical outcome (Shono *et al.*, 2001). Therefore,

while the regulation of COX-2 in glioma is an attractive target, the complex signalling pathways and their crosstalk represent a significant challenge in the characterisation of novel drugs.

To date, the majority of literature examining a role for aspirin, selective COX-2 inhibitors or prostaglandin signalling has made use of established cell lines (Xu *et al.*, 2013; Suzuki *et al.*, 2013; White *et al.*, 2013; Gomes and Colquhoun, 2012; Lan *et al.*, 2011; Lo *et al.*, 2010; Kang *et al.*, 2009; Kambe *et al.*, 2009; Annabi *et al.*, 2009; Kim *et al.*, 2009; Lee *et al.*, 2005; Amin *et al.*, 2003; Arrieta *et al.*, 2001; Aas *et al.*, 1995), with few publications using either clinical tissue or primary cultures (Kokoglu *et al.*, 1998; Joki *et al.*, 2000; Shono *et al.*, 2001). The results of Joki and colleagues provides the best *in vitro* insight into the role of COX-2 inhibition in glioma. They characterised the specific COX-2 inhibitor NS-398 using both monolayer cell cultures and three-dimensional glioma spheroids using U-87MG and U-251MG human glioblastoma cell lines in addition to immunohistochemical analysis of glioma and normal brain tissue. They reported that proliferation rate was reduced in both monolayer and spheroid cultures. The effects of NS-398 were concentration dependant, suggesting the regulation of specific pathways as opposed to non-specific effects. Interestingly though, they only reported a moderate increase in the number of apoptotic cells in the treated spheroids, and NS-398 did not have an inhibitory effect on tumour invasion in the co-culture spheroid system.

The aspirin analogue PN517 has been investigated previously in colorectal cancer cell models (Deb *et al.*, 2011; Claudius *et al.*, 2014). It was reported to decrease cell viability,

activate NF- κ B signalling, decrease cyclin D1 expression, but unlike aspirin did not affect cell cycle *in vitro*. Importantly, in an *in vivo* tumour model, PN517 eliminated tumours but had no side effects in control animals (Claudius *et al.*, 2014). In characterising PN517, our results have supported the findings of Joki and colleagues but at times contradicted Claudius, emphasising the therapeutic potential for aspirin analogues in the treatment of glioma, but indicating that tumour specific differences may impact efficacy.

In many assays throughout the project, the effect of aspirin or its analogues reached a maximum at the twenty four hour timepoint, and in fact the IC₅₀ values for the aspirin analogues typically decreased between twenty four and forty eight hour time points in the 1321N1 and GOS-3 cell lines, suggesting a recovery in cell viability. The only exception to this observation was the most potent analogue, PN517 in 1321N1 cells. The simplest explanation for this result is that the drugs are rapidly undergoing metabolism to form inactive metabolites.

The chemical stability of a drug is of great importance since it typically becomes less effective as it undergoes degradation. In humans, aspirin is rapidly deacetylated to salicylic acid, which is further metabolized by glucuronidation, hydroxylation, and glycine conjugation (Hutt *et al.*, 1986; Takanashi *et al.*, 2000). The cytochrome p450 enzymes that are largely responsible for drug metabolism and detoxification of foreign chemicals are found at their highest concentrations in the liver, but are also distributed throughout the body (Guengerich, 2008). In the case of aspirin metabolism, the major enzyme involved is CYP2C9 (Takanashi *et al.*, 2000; Miners and Birkett, 1998), an enzyme that is

polymorphic, with fast and slow metabolising forms. While it is known that CYP2C9 is expressed in glioma, expression that correlates positively with grade (Knupfer *et al.*, 2006), it is not known whether there is any heterogeneity in the expression of the polymorphic forms. This provides not only an explanation for why IC₅₀ values do not decrease further with longer incubations, for example in the U87 MG cell line where high expression would be expected, but also a potential explanation for why aspirin or its analogues have greater efficacy in one cell culture versus another.

In this study, aspirin was included as a control because any developed analogue would at least have to improve on the effects of its parent compound. Throughout the results, with few exceptions this was true of PN517 in comparison to aspirin, identifying it as the lead analogue of interest. In addition, a treatment equivalent to a double dose of aspirin was also used as a control as the structures of two of the analogues, PN508 and PN517, closely resemble two aspirin bound together with either a single or double carbon bond (Fig 8.1). It has been suggested that the central double or single carbon bond either gives the molecule rigidity, or allows for rotational flexibility between the two aspirin groups and that the site might also be a target for hydrolysis, the result of which would essentially be two aspirin molecules.

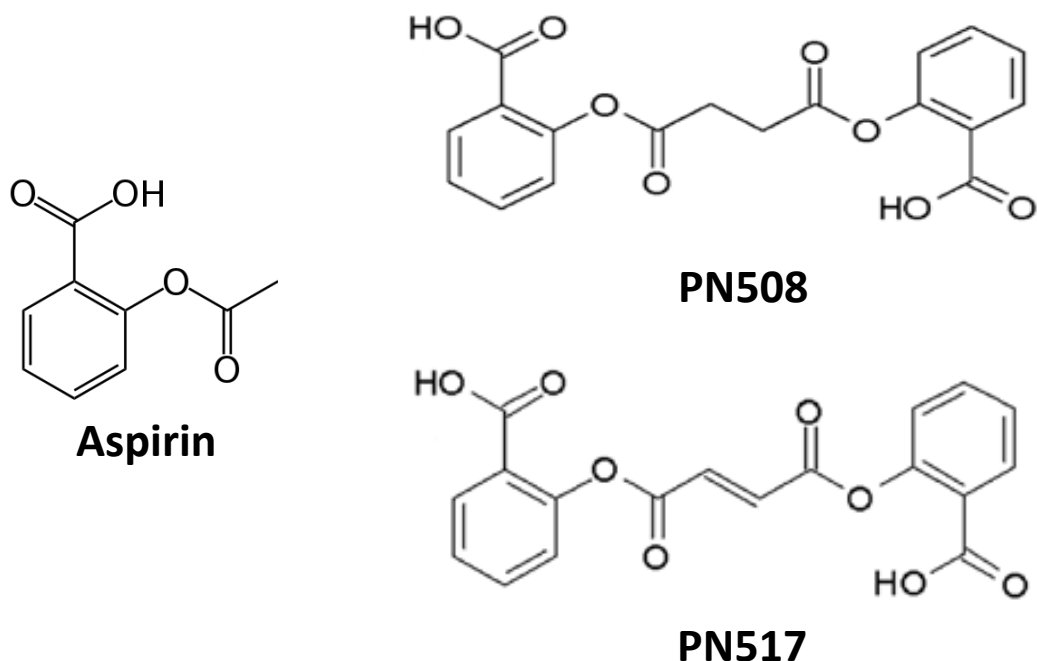


Figure 8.1 Chemical structures of aspirin, PN508 and PN517

If this was the truly the mechanism of action of PN517, then any increased efficacy of these compounds observed in comparison to aspirin would simply be replicated by adding a double dose of aspirin. However, this control treatment seldom replicated the potency of PN517 in any assay, suggesting that its efficacy is due to some other structural characteristic and not simply because it resembles two aspirin molecules. This fact supports its development as a unique chemical entity and not simply as an increased dose of aspirin.

In addition to CYP2C9 expression, a further explanation for variations in PN517 responses between the cell lines or short term cultures might simply be linked to COX expression and prostaglandin production. COX-2 protein expression is inducible and found in both normal brain and glioma specimens, and is significantly higher in high-grade glioma than low-

grade glioma and normal brain tissue. Additionally, its expression is higher in slow growing cells than fast growing (Joki, 2000). The U87 MG cell line is both high grade and relatively slow growing with a doubling time of approximately 36 hours, both characteristics associated with high COX-2 expression. This provides a potential explanation for the high efficacy of PN517 in this cell type and warrants further investigation as it could contribute to future patient specific treatment based on COX expression.

Interestingly, the cell viability efficacy of PN517 in the U87 MG cell line was not replicated in the proliferation assay, where cisplatin decreased proliferation to a much greater degree, suggesting a difference in the signalling pathways altered by analogue treatment in cell viability and proliferation. This may be related to EGFR, the expression of which is elevated in glioblastoma (e.g. U87 MG) but the literature would suggest is rare in low-grade glioma such as 1321N1 and GOS-3 (Hatanpaa *et al.*, 2010). However, while we did find that U87 MG expressed EGFR at the highest level, the expression in the GOS-3 and 1312N1 cell lines was still significantly elevated in comparison to the control SVG-p12 cell line. While the variation in expression may provide some explanation for cell specific proliferation responses it is unlikely to be the whole story. Also, it has been reported that primary culture show significantly elevated levels of EGFR compared to established cell lines (Tong, 1998), but as both the BTNW911 and BTNW914 cultures were slow proliferating, this would suggest that the receptor is unlikely to be linked to proliferation.

While many therapeutic approaches to cancer treatment promise patient individualised therapies, the cost of such developments are typically prohibitive and mean that the treatments which are developed are not widely available. With the development of a low cost and widely applicable therapy in mind, this *in vitro* project characterised the aspirin analogues in a range of glioma cell lines derived from tumours of differing grades, in addition to the short term cultures established from surgical tissue from Royal Preston Hospital. The primary cultures *more* closely mimic the true tumour environment, and combined with the cell lines allow comparison of results between labs. The results with PN517 in the cell lines demonstrate that both it and the control treatments aspirin and cisplatin generally show their greatest efficacy in the GOS-3 cell line (Table 8.1).

Table 8.1 Summary of assay results for the glioma and control cell lines. The table illustrates protein expression and efficacy of drug treatment in the cell lines, with * indicating the lowest expression or efficacy and **** the highest.

	Treatment	1321N1	GOS-3	U87 MG	SVG-p12
Doubling Time (Hrs)	n/a	24	24	36	48
EGFR Expression	n/a	***	***	****	*
CD90 Expression	n/a	****	****	-	-
COX-2 Expression (predicted)	n/a	**	***	****	*
Viability	PN517	*	****	**	*
	Aspirin	****	*	*	*
	Cisplatin	***	****	*	**
Proliferation	PN517	*	***	**	****
	Aspirin	*	****	**	***
	Cisplatin	*	****	**	***
Apoptosis	PN517	***	****	**	*
	Aspirin	****	***	*	**
	Cisplatin	****	***	**	*
Migration	PN517	***	****	**	*
	Aspirin	****	****	*	**
	Cisplatin	***	***	****	*

This finding has many potential explanations, one of which could simply be the doubling time of the cells, as cells that are more rapidly proliferating are more sensitive to drug treatment in a cell viability assay. In addition, the GOS-3 cell lines showed high levels of EGFR expression, a known target for aspirin related compounds *in vitro*, and would be predicted to have lower levels of CYP2C9 expression which would result in prolonged compound stability compared to higher grade cells.

Another model utilised in the project was spheroid cultures. 3D cell structures offer a high degree of clinical and biological relevance to *in vivo* models, stimulating the growth and micro-environmental conditions not replicated by monolayer cultures. First described over thirty years ago, they can be used to evaluate cellular responses novel compounds in the hope that *in vitro* results will more closely translate to the *in vivo* environment (Sutherland *et al.*, 1981; Lee *et al.*, 2008). Several studies have compared monolayer cell cultures to 3D cultures to assess the efficiency of drug toxicity in glioma (Thore, 1984; Glimelius *et al.*, 1988; Kolchinsky and Roninson, 1997; Joki *et al.*, 2000). U87 MG cells have been shown to form and proliferate *in vitro* as spheroids, whereas U251 MG cells formed spheroids, but did not continue to proliferate (Kolchinsky and Roninson, 1997). Interestingly, the monolayer and 3D structures of U87 MG cells did not show any difference in vinblastine resistance, but the U251 MG cells were 4.5 times more resistant in the spheroid form. The multicellular spheroids in most cases are more resistant than the corresponding monolayers to drug treatment, especially when limited drug penetration would be expected, suggesting that the spheroid system is more representative of the *in vivo* situation than monolayer cultures (Nederman *et al.*, 1981).

As already discussed, comparison of the U87 MG and U251 MG cell lines found that NS-398 (a COX-2 inhibitor) reduced the proliferation and migration in both models (Joki *et al.*, 2000). However, there was no effect of drug treatment on invasion in the spheroid model, a finding that we have confirmed with PN517. However, while the time period of both assays varied largely (18 hours for the current study versus 4 days), the lack of efficacy for drug treatment may be related to this prolonged treatment. Over 4 days, it is very likely that aspirin related compounds would be rapidly metabolised and thus have no effect on invasion. This may also be the case in our experiment, or it may be related to concentration where any inhibition of invasion was masked by giving the cells sufficient time to invade, while it might be at a slower rate.

Various pathways have been linked with aspirin and glioma, regulating processes such as proliferation, apoptosis, and migration. However, many of the pathways are interlinked, and either directly or indirectly regulated by COX enzymes or prostaglandins, making it difficult to isolate any one particular protein as being critical for efficacy (Fig 8.2).

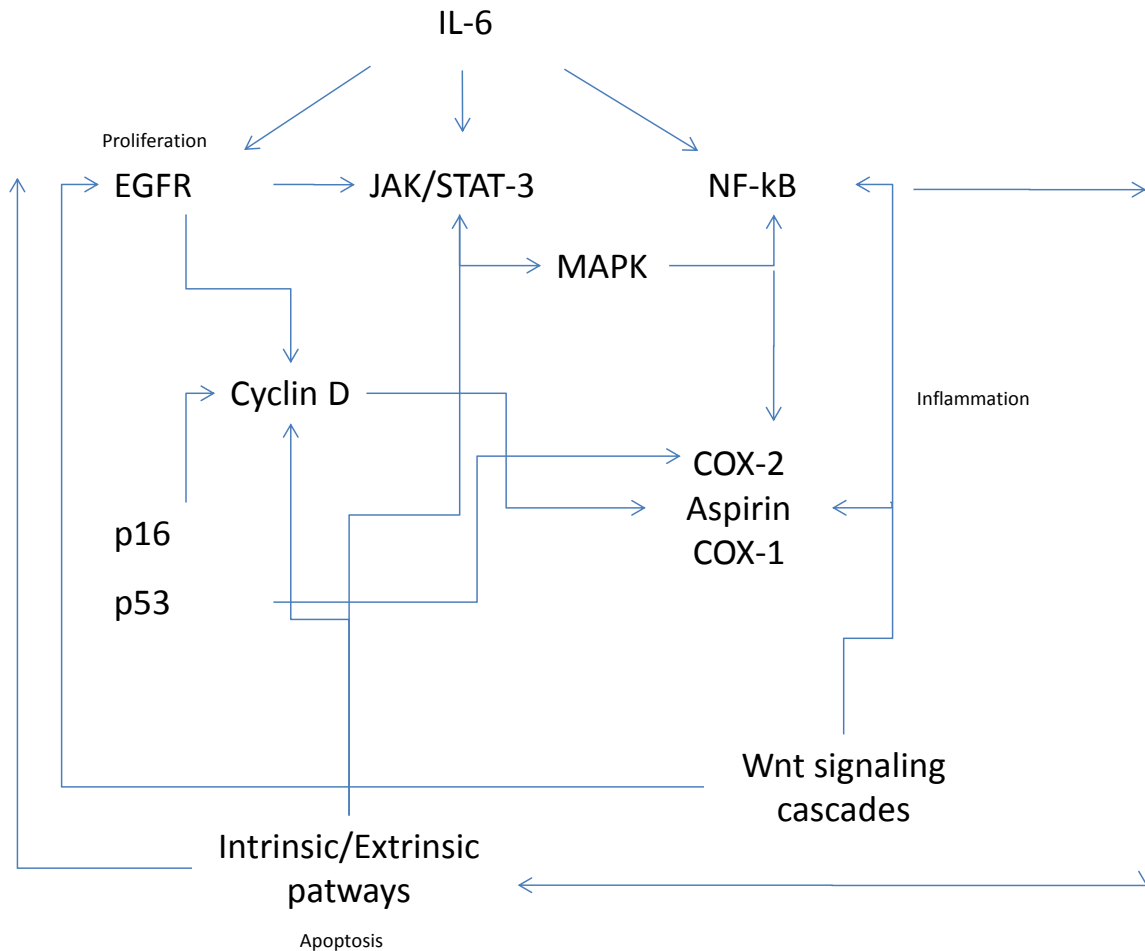


Figure 8.2 Pathways implicated in aspirin regulation

Aspirin was first linked to cancer prevention following the demonstration of low colorectal cancer incidence in long time aspirin users (Kune *et al.*, 1988). This effect is mainly believed to involve the suppression of inflammation, since colorectal cancer is caused by chronic inflammation (Itzkowitz and Yio, 2004). However, proteins such as NF-κB, which up-regulates major inflammatory factors, such as TNFα and IL-6, are critical for the process of tumourigenesis (Prasad *et al.*, 2010). IL-6 activates STAT3, a transcription factor known to be involved in cancer progression and a further link between inflammation

and tumourigenesis (Yihui *et al.*, 2012). STAT-3 has been also found to activate anti-apoptotic proteins such as Bcl-X_L and Bcl-2 in multiple myeloma cells (Bhardwaj *et al.*, 2007). A further protein linking aspirin and cancer is the EGFR, the stimulation of which activates STAT-3 and triggers signalling cascades like MAPK which result in turn can result in NF-κB activation (Indranil *et al.*, 2013). The phosphorylation, and thus activation of both EGFR and STAT-3 is inhibited by aspirin treatment (Karuppaiyah *et al.*, 2007), resulting in the down regulation of Bcl-2 and Bcl-X_L, and the up-regulation of Bax and Bak. EGFR activation has also been shown to stimulate cyclin D1 expression, resulting in increased proliferation and invasion, and a reduction in apoptosis (Wang, 2011). The Wnt/β-catenin pathway, involved in proliferation and tumourigenesis, can be activated by EGFR via PI3K/Akt (Sharma *et al.*, 2002), NF-κB (Agarwal *et al.*, 2005), and in glioma by STAT3 (Fig 8.3) (Paul *et al.*, 2013).

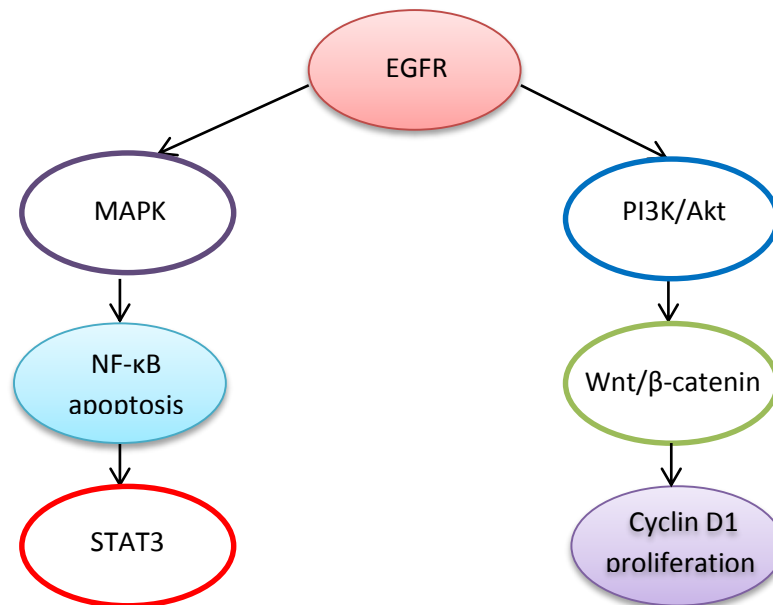


Figure 8.3 Pathways affected by EGFR

It is widely known that COX-2 and inflammatory cancers are linked, however the exact role of COX in glioma biology remains unclear. Deininger *et al.* 1999 detected COX-1 in 20-50% of all cells in both low- and high-grade gliomas, the COX-2 protein has been found in one study to be expressed in all human glioma specimens tested (Joki *et al.*, 2000) but in another was found more commonly in high-grade gliomas (70%) than in low-grade gliomas (30%) or normal brain cells (Shono *et al.*, 2001). COX-2 is involved in proliferation, apoptosis, angiogenesis and metastasis (Steele *et al.*, 2003; Farrow *et al.*, 1998; Prescott *et al.*, 2000; Hwang *et al.*, 1998), and the products of the COX-2 enzyme are prostaglandins, are key mediators of inflammation. The expression of COX-2 is regulated by NF- κ B (Lee *et al.*, 2004; Ulivi *et al.*, 2008) and the EGFR-STAT-3 pathway in GBM cells. Finally, wild-type p53 inhibits COX-2 expression (Subbaramaiah *et al.*, 1999), a pathway demonstrated in glioma (Seijiro *et al.*, 2008). The crosstalk of these pathways, each linked to aspirin, demonstrates the complexity of signalling and the challenges to characterising the mechanism of action of novel drugs.

Conclusion

Important molecular events have been described such as overactivation of the EGFR and PI3K/Akt pathways, and inactivation of the p53/Rb pathways to be important contributors for glioma development. NF- κ B and Wnt/ β -catenin signalling pathways have also been linked, as well as cyclin D, STAT3, IL-6 and many more. All of these pathways contribute to cell survival, proliferation, apoptosis, migration, invasion and angiogenesis, and thus are crucial for tumourigenesis. COX inhibition has been shown to be involved nearly in every pathway discussed, and also expressed, according to literature, in glioma cell lines and

primary tissue. Therefore, the regulation of COX-2 activity in glioma is likely to involve many factors, so further studies are required to fully elucidate the mechanism. In summary, the results of this project have shown that the aspirin analogue PN517 reduces viability, inhibits proliferation, induces apoptosis through both extrinsic and intrinsic pathways, induces cell cycle arrest, regulates the activation of cyclin D1 and the EGFR, and inhibits migration. It has demonstrated greater efficacy than aspirin, and in some assays and cell lines cisplatin, and has demonstrated efficacy in both established cell lines and short term cultures derived from high grade gliomas. In conclusion, PN517 represents a novel aspirin analogue with significant therapeutic potential for the treatment of glioma and warrants further investigation.

Future Work

Although high efficacy of the aspirin analogue PN517 was observed in various experiments, it is still essential to further characterise the mechanisms involved in its effects. These would include characterisation of intracellular signalling pathways and also the use of an *in vivo* model.

Throughout the thesis, a number of different signalling pathways have been described that may be involved in the mechanism of action of PN517, however, definitive assays would need to be performed to establish these. For example, we have demonstrated that PN517 inhibits both COX-1 and COX-2 in an isolated enzyme assay, but ideally the expression and activity of both enzymes in the cell lines and primary cultures should be established. This could be done using western blotting, a technique that could also be used to determine any inhibition of activation by PN517. A different approach could be to use a similar kit to the isolated enzyme assay, where instead of a purified enzyme, cell lysates are used and a direct assay of COX activity is obtained. It would also be interesting to compare PN517 with other NSAIDs such as the COX-2 selective compounds like NS398 or celecoxib, a comparison that may help to delineate drug specific pathways in each assay.

It has also been suggested that PN517 could be being metabolised *in vitro* by CYP2C9. An unrelated project in the school has actually demonstrated both expression and activity of this enzyme in U87 MG cells, however, this result would require confirmation and either HPLC or ICPMS could be used to detect both the loss of PN517 and appearance of

metabolites in the culture media over time. Similar assays should also be performed using the short term cultures to establish a stronger link to the *in vivo* pathological state.

With regards signalling pathways, the involvement of the β -catenin pathway needs to be examined. This could be achieved by evaluating the activity of the β -catenin/TCF4 transcriptional complex using luciferase reporter construct based assays. It is also essential to evaluate the NF- κ B pathway, as it is involved directly with COX-2 inhibition. The link between COX enzymes and the different signalling pathways could be confirmed using an siRNA approach, where either COX-1, COX-2, or a combination of both enzymes could be knocked down and the effects of PN517 again examined.

Glioblastoma cancer stem cells (GSCs) are a subgroup of tumour cells that are radiation and chemotherapy resistant and likely contribute to tumour recurrence. We've tentatively identified GSCs in our short term cultures using CD90 staining, but this result could be confirmed using other stem cell markers such as CD133, a marker linked to spheroid growth and tumour development in animal models. Using cell sorting, CD90 and CD133 positive cells could be isolated and their sensitivity to drug treatment compared to the remaining marker negative populations. This may help in the development of new therapeutic approaches to GBM treatment.

In addition to PN517 monotherapy, the efficacy of the novel aspirin analogue in combination with other chemotherapeutics should be examined. A recently completed project in the lab demonstrated significantly increased efficacy of the combination of

PN517 and cisplatin using the cell lines. This project, and the combination with other chemotherapeutics should be continued to confirm the results and also to establish the mechanism(s) of action involved.

Finally, while we have used monolayer and spheroid cultures in addition to short term cultures, the gold standard model would be to determine the efficacy of PN517 in a nude mouse model. This system is currently being established at UCLan, where intracranial tumours will be established using both cell lines like U87 MG and short term cultures. The efficacy of peripheral delivery of PN517 in either the prevention of tumour development or in the treatment of established tumours will be examined.

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Appendix

Cell Line Supplementary Data

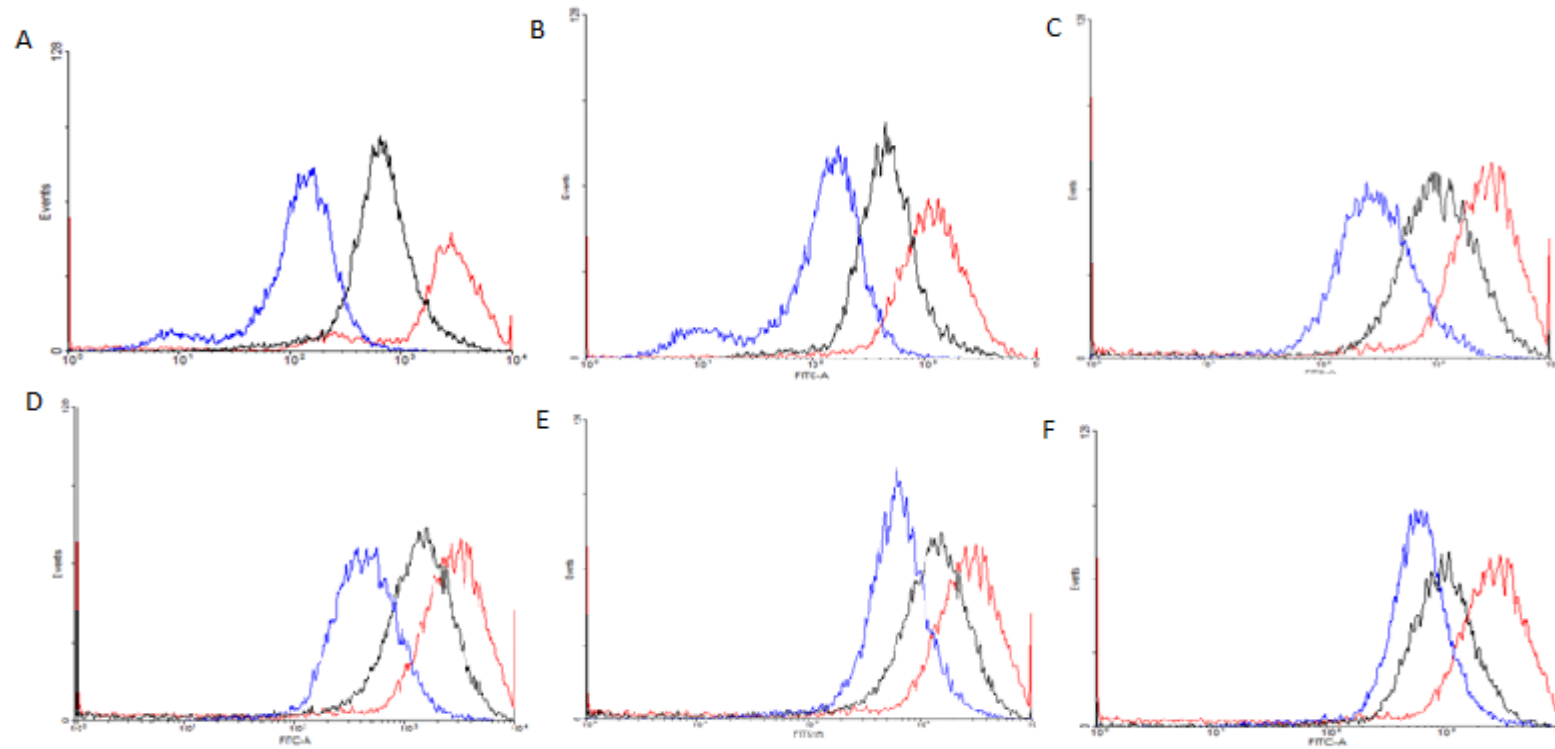


Figure 10.1 Cell proliferation measured by flow cytometry following drug treatment in SVG-p12 cell line. The figure illustrates the effect of drug treatment on proliferation over three days where the red peak corresponds to day 1, black to day 2, and blue to day 3. A leftward shift indicates a decrease in fluorescence indicating proliferation. Panel A illustrates representative results for control treatment; panel B cisplatin; panel C PN517; panel D PN508; panel E aspirin; and panel F double aspirin.

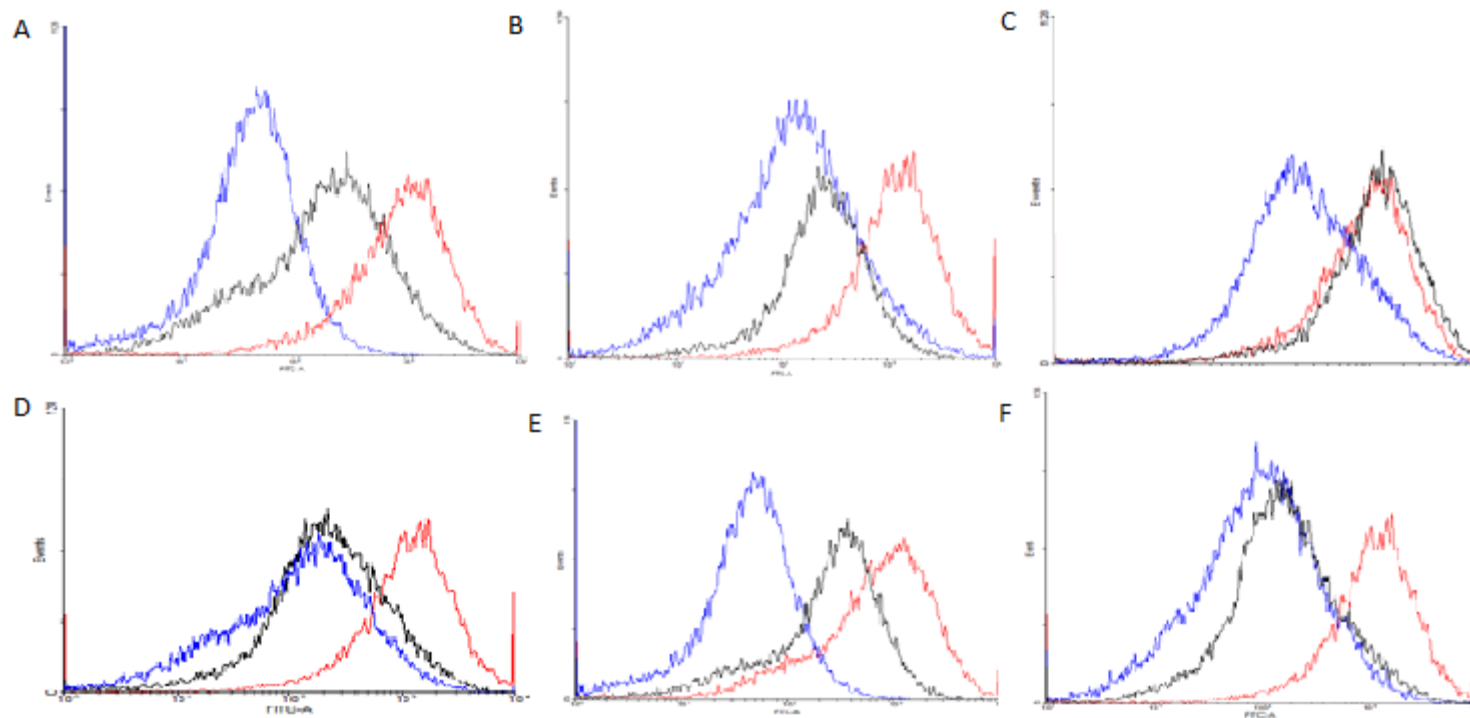


Figure 10.2 Cell proliferation measured by flow cytometry following drug treatment in 1321N1 cell line. The figure illustrates the effect of drug treatment on proliferation over three days where the red peak corresponds to day 1, black to day 2, and blue to day 3. A leftward shift indicates a decrease in fluorescence indicating proliferation. Panel A illustrates representative results for control treatment; panel B cisplatin; panel C PN517; panel D PN508; panel E aspirin; and panel F double aspirin.

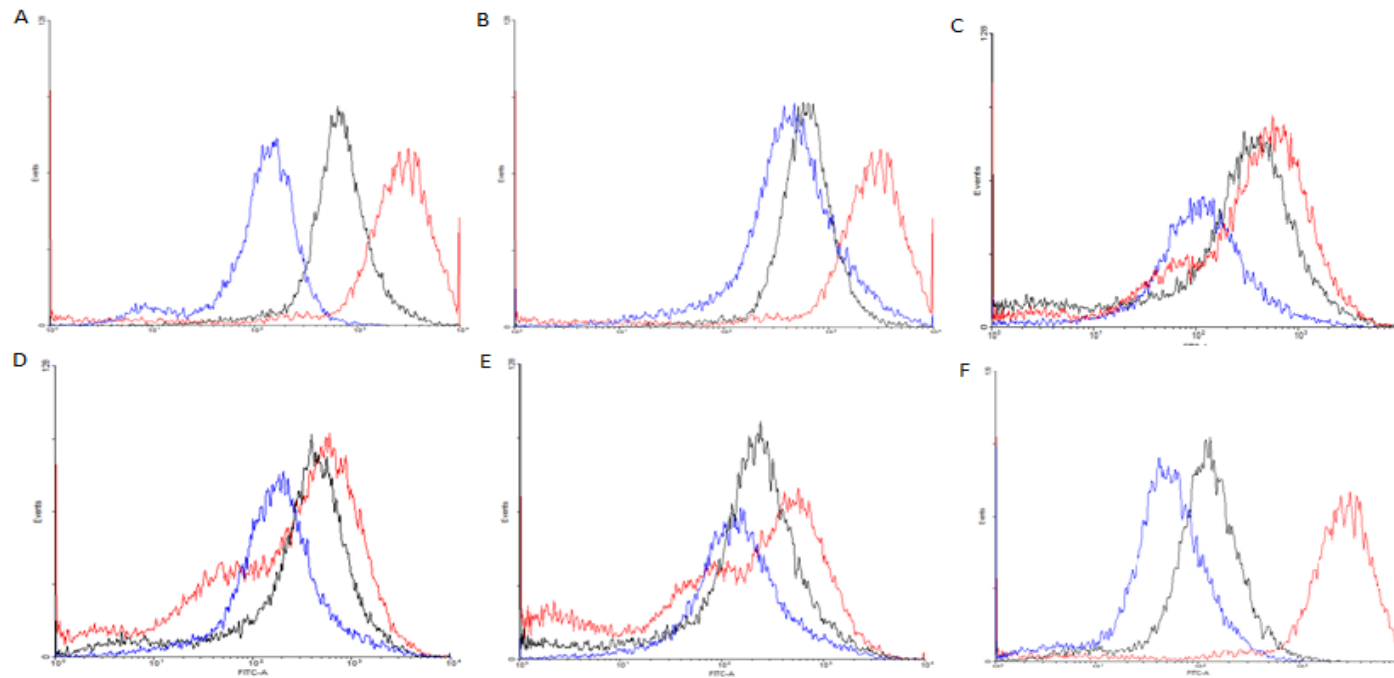


Figure 10.3 Cell proliferation measured by flow cytometry following drug treatment in U87 MG cell line. The figure illustrates the effect of drug treatment on proliferation over three days where the red peak corresponds to day 1, black to day 2, and blue to day 3. A leftward shift indicates a decrease in fluorescence indicating proliferation. Panel A illustrates representative results for control treatment; panel B cisplatin; panel C PN517; panel D PN508; panel E aspirin; and panel F double aspirin.

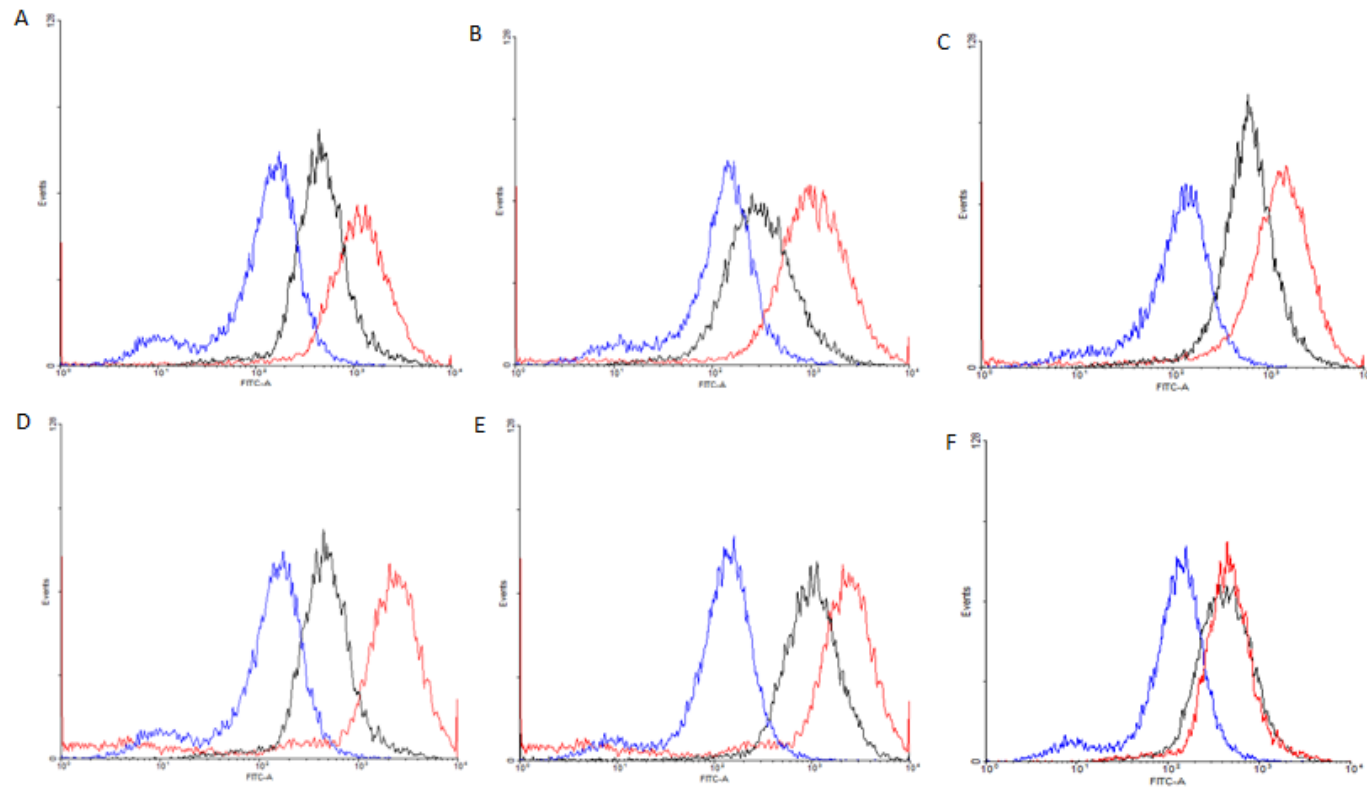


Figure 10.4 Cell proliferation measured by flow cytometry following drug treatment in GOS-3 cell line. The figure illustrates the effect of drug treatment on proliferation over three days where the red peak corresponds to day 1, black to day 2, and blue to day 3. A leftward shift indicates a decrease in fluorescence indicating proliferation. Panel A illustrates representative results for control treatment; panel B cisplatin; panel C PN517; panel D PN508; panel E aspirin; and panel F double aspirin.

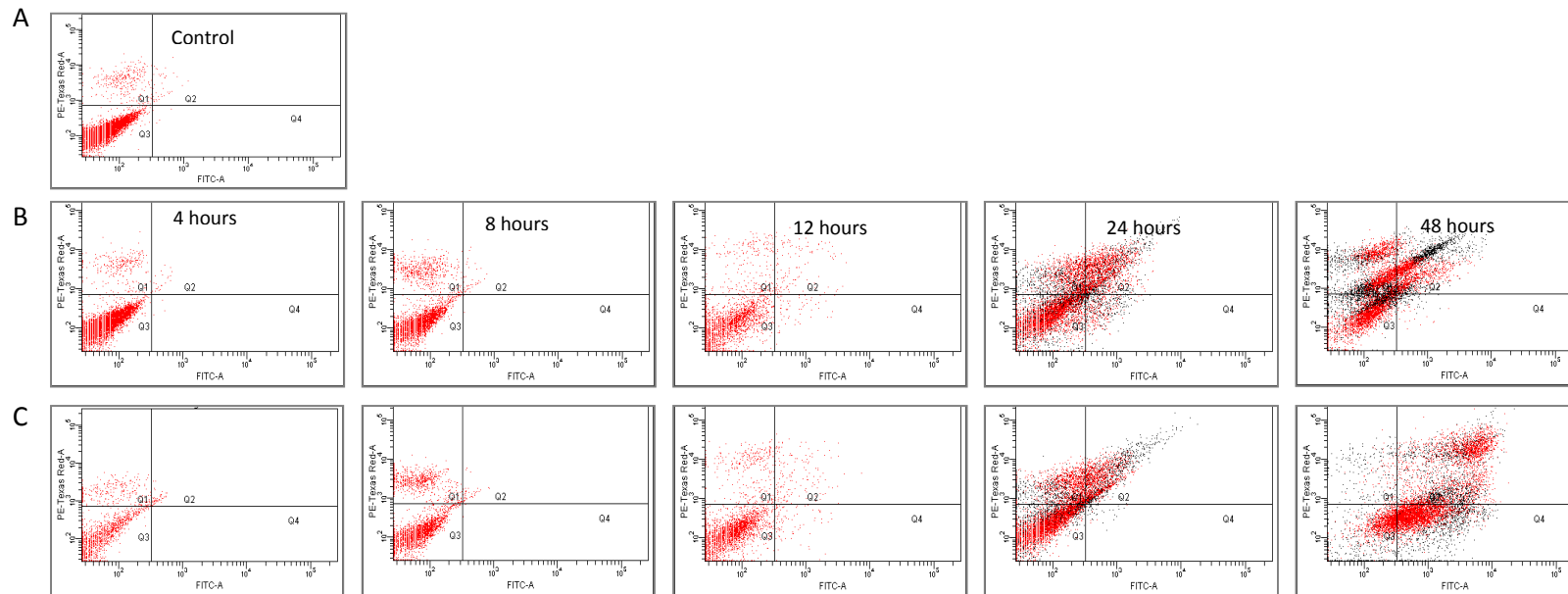


Figure 10.5 Representative dot plots illustrating the induction of apoptosis following cisplatin treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in SVG-p12 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with cisplatin at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

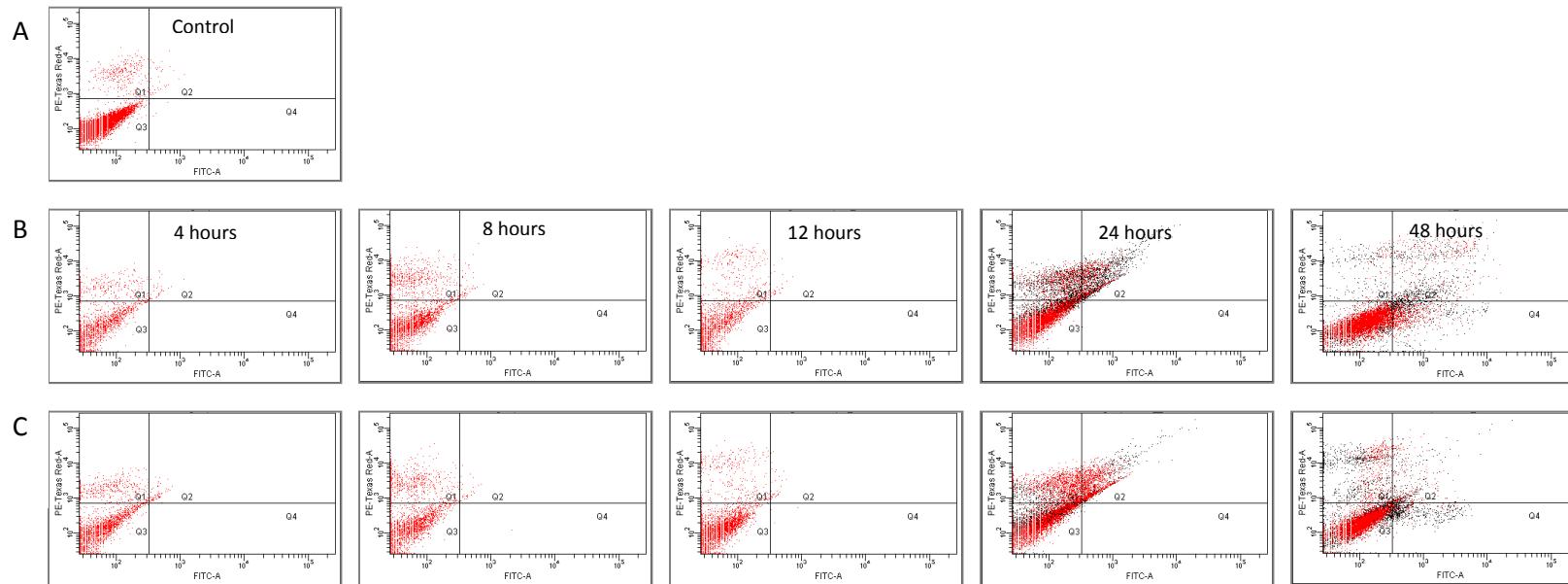


Figure 10.6 Representative dot plots illustrating the induction of apoptosis following aspirin treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in SVG-p12 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with aspirin at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

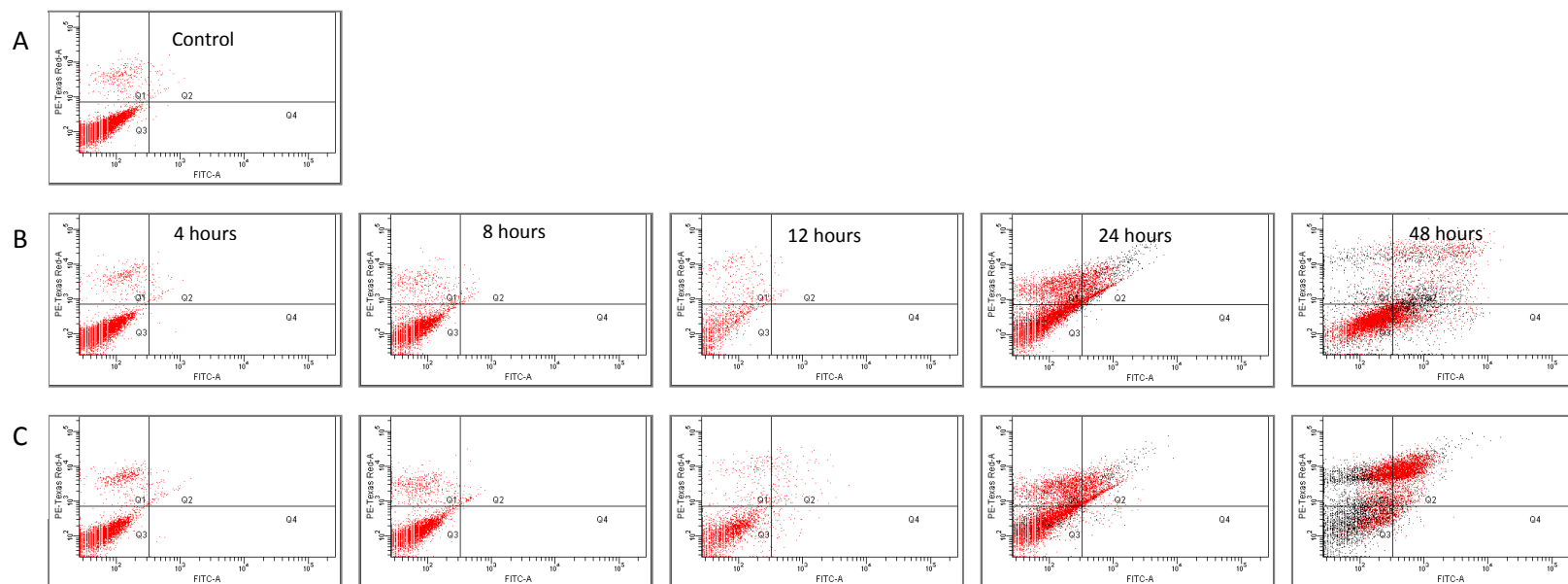


Figure 10.7 Representative dot plots illustrating the induction of apoptosis following PN517 treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in SVG-p12 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with PN517 at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

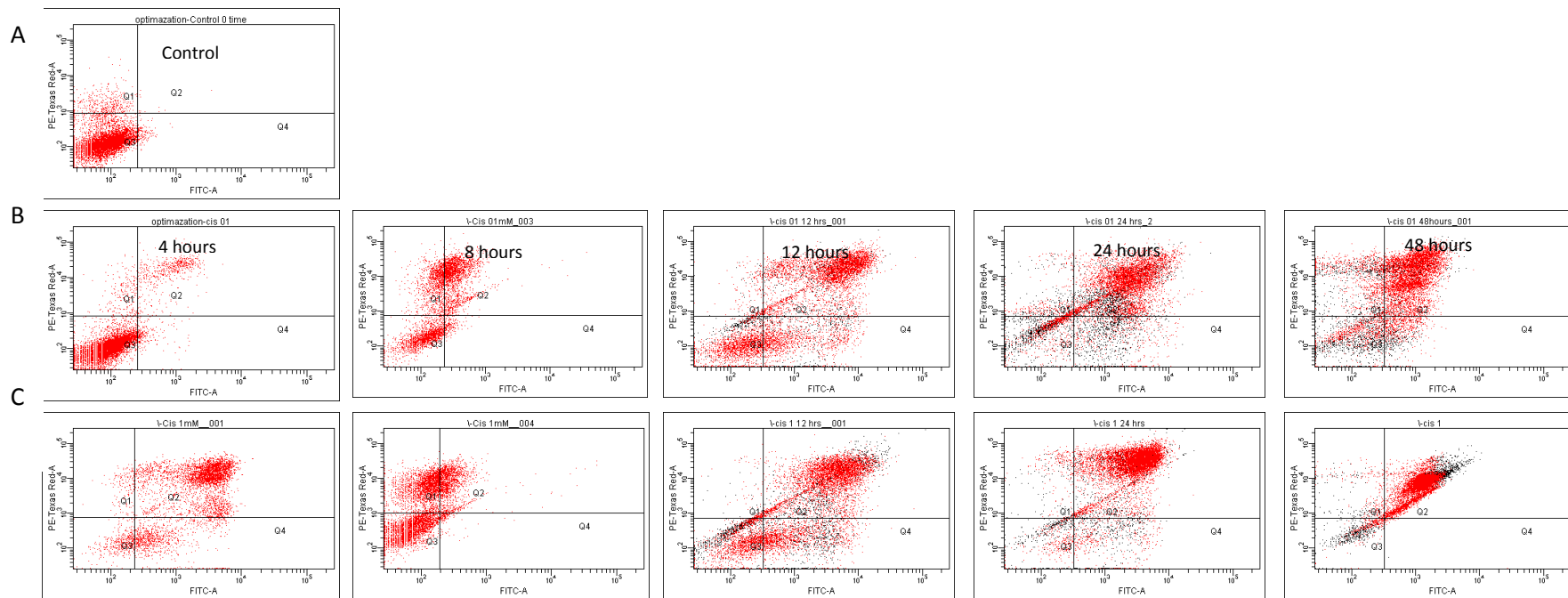


Figure 10.8 Representative dot plots illustrating the induction of apoptosis following cisplatin treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in 1321N1 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with cisplatin at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

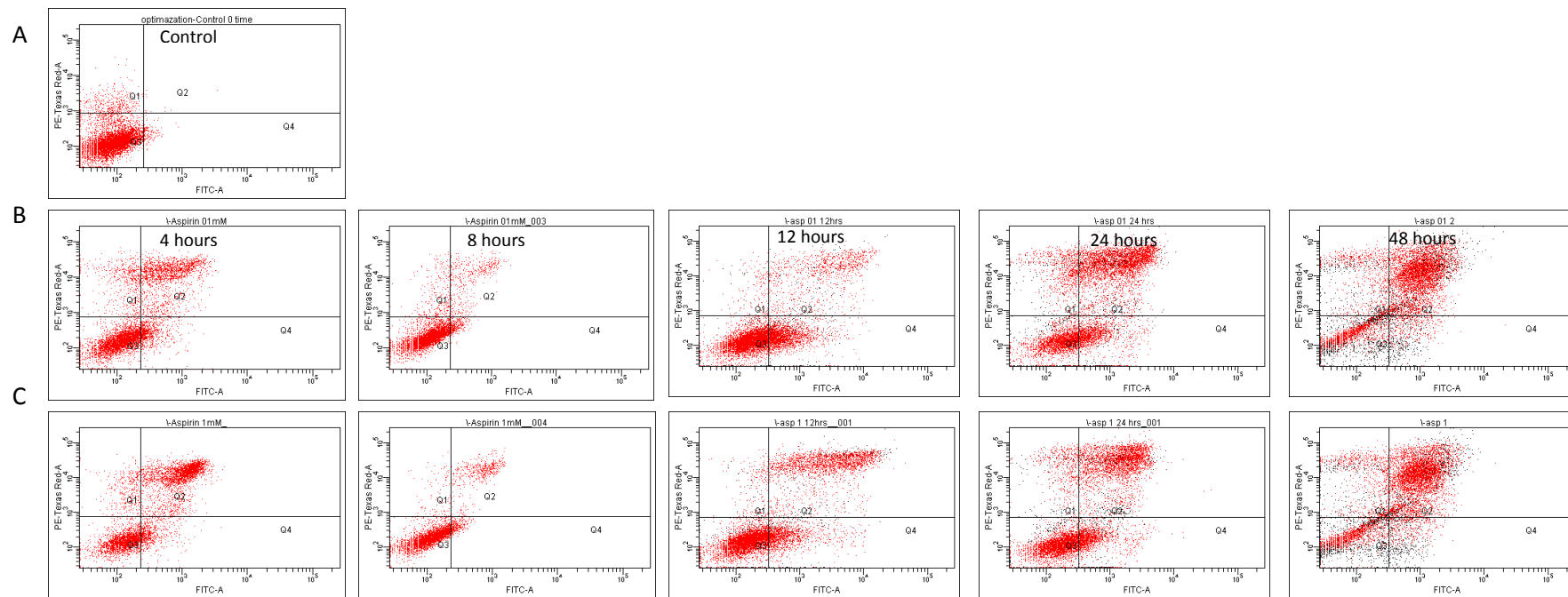


Figure 10.9 Representative dot plots illustrating the induction of apoptosis following aspirin treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in 1321N1 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with aspirin at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

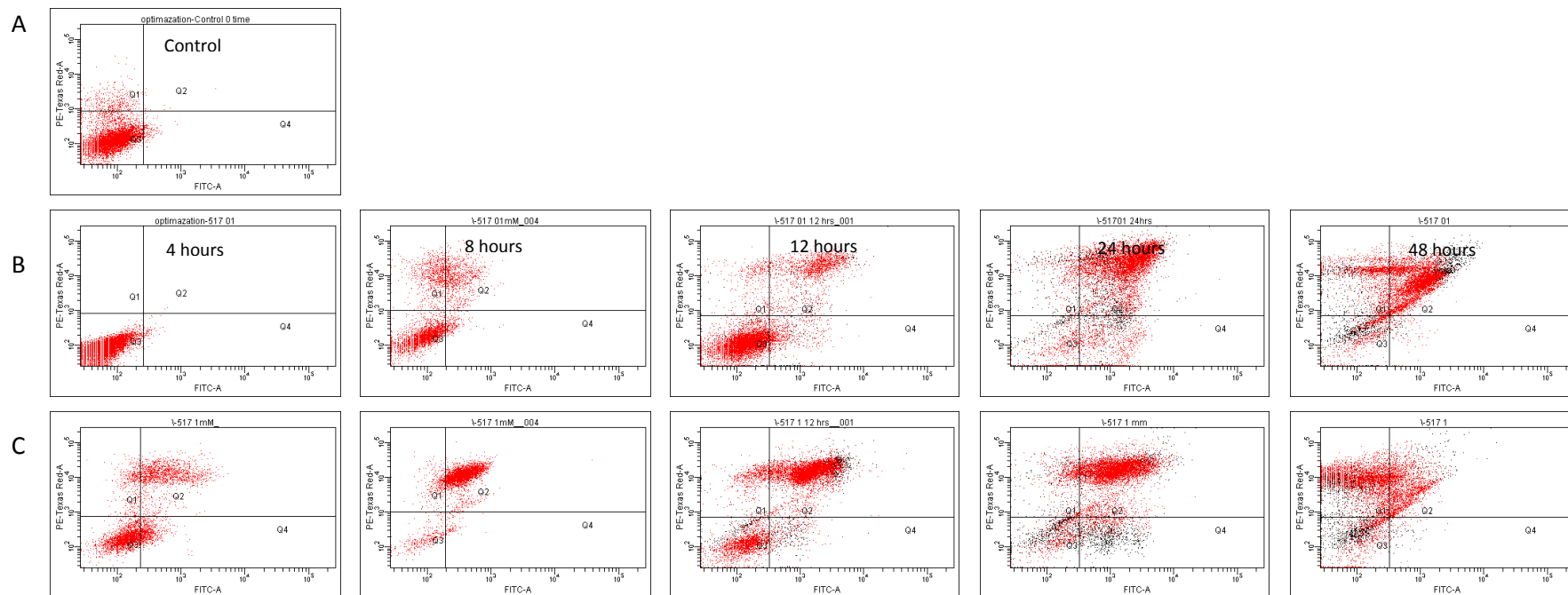


Figure 10.10 Representative dot plots illustrating the induction of apoptosis following PN517 treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in 1321N1 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with PN517 at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

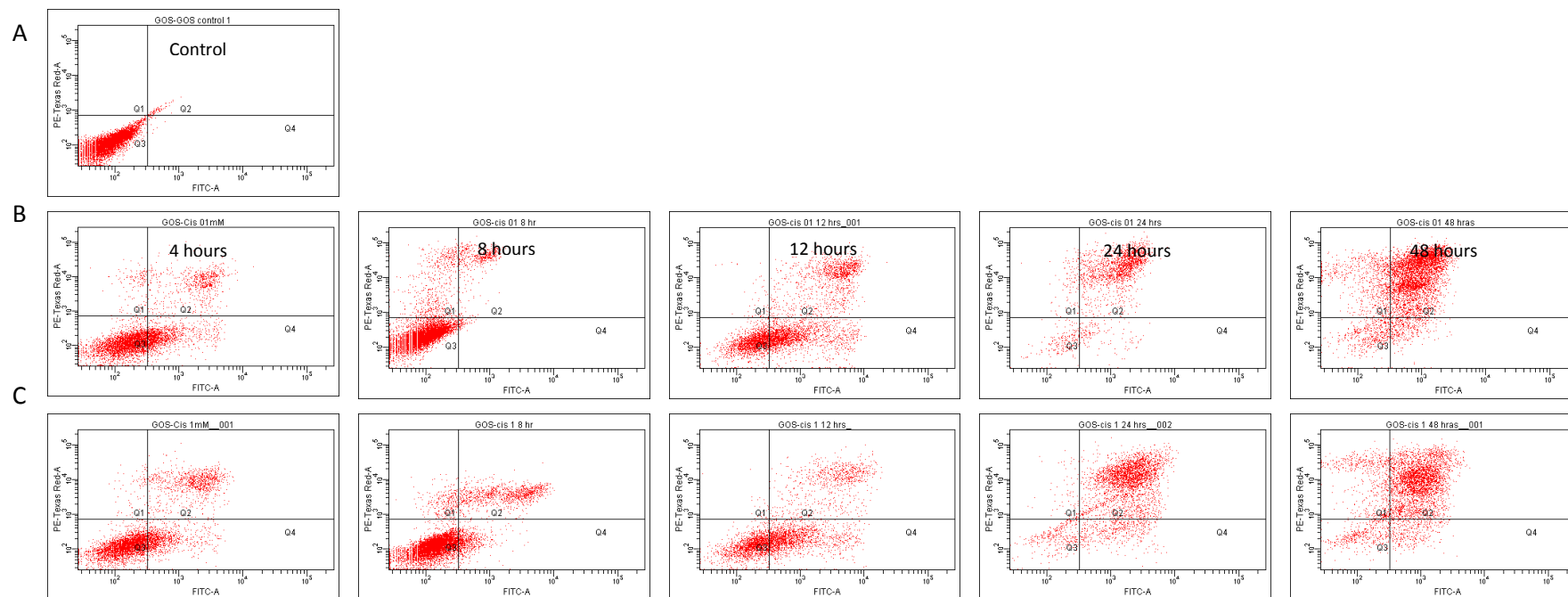


Figure 10.11 Representative dot plots illustrating the induction of apoptosis following cisplatin treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in Gos-3 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with cisplatin at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

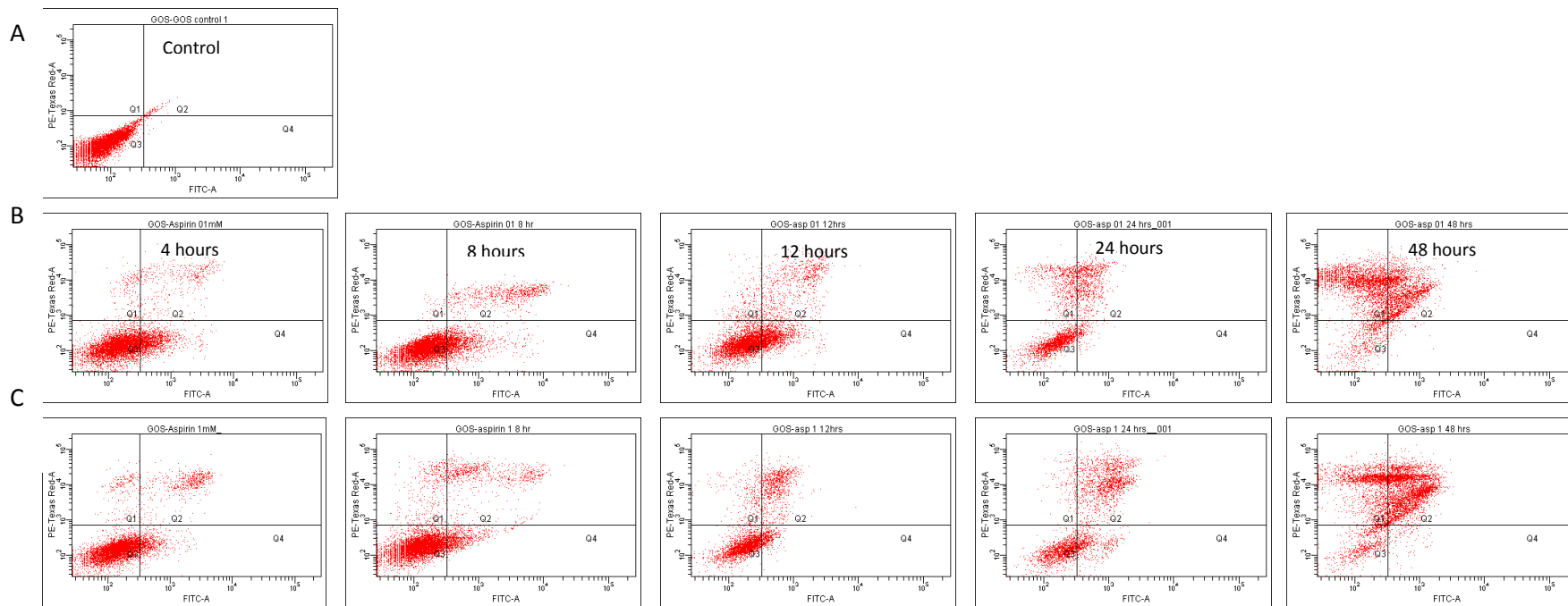


Figure 10.12 Representative dot plots illustrating the induction of apoptosis following aspirin treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in Gos-3 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with aspirin at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

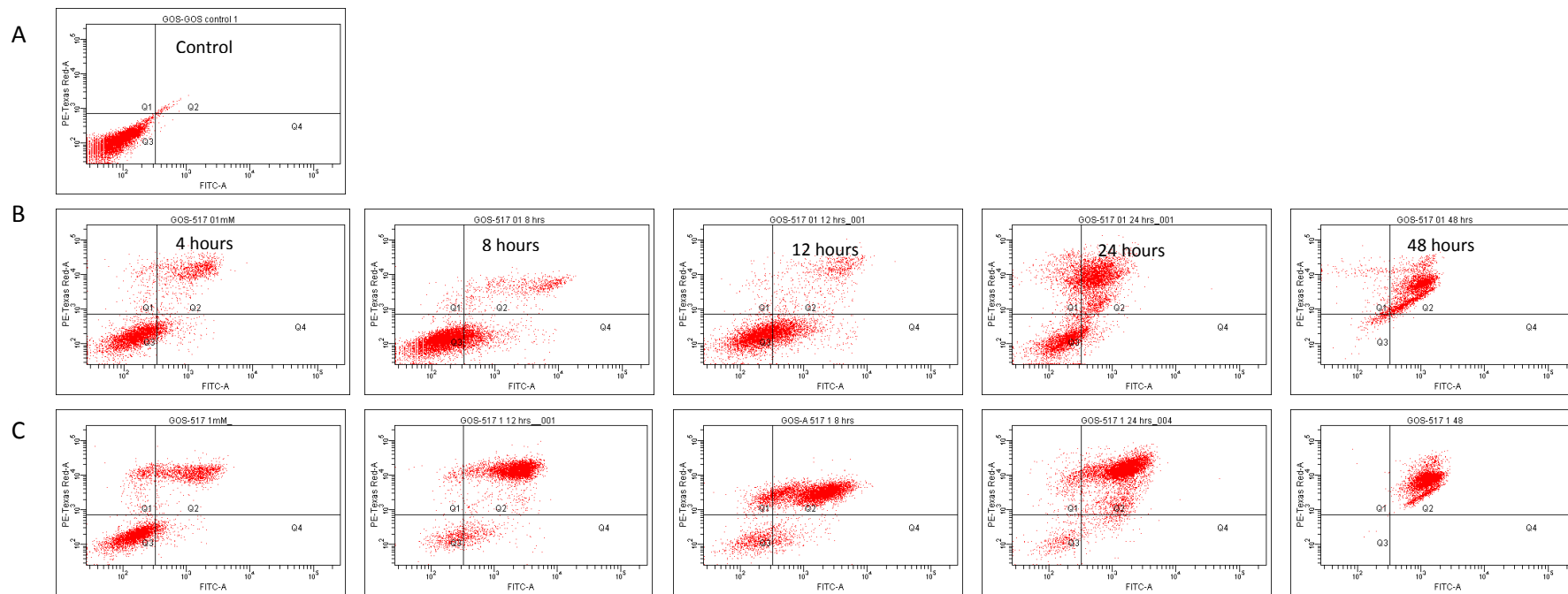


Figure 10.13 Representative dot plots illustrating the induction of apoptosis following PN517 treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in Gos-3 cell line. Panel A illustrates the control untreated sample, panel B drug treatment with PN517 at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

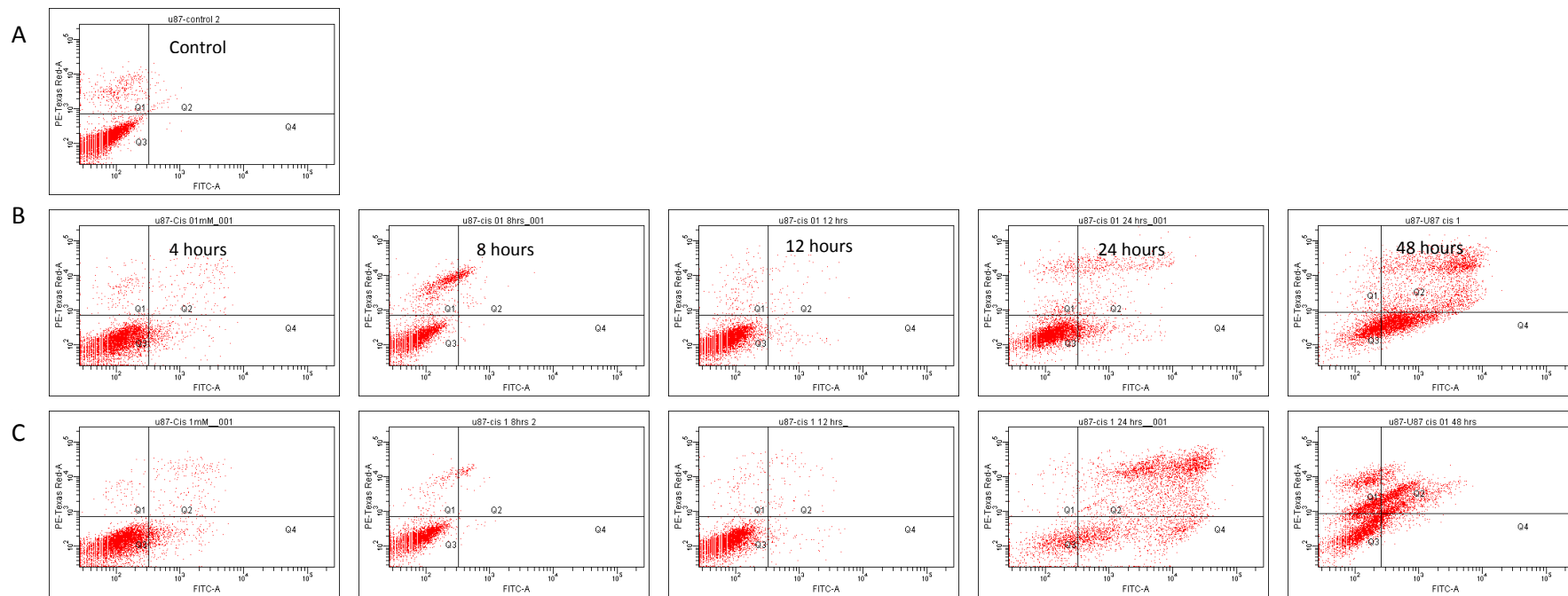


Figure 10.14 Representative dot plots illustrating the induction of apoptosis following cisplatin treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in U73 MG cell line. Panel A illustrates the control untreated sample, panel B drug treatment with cisplatin at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

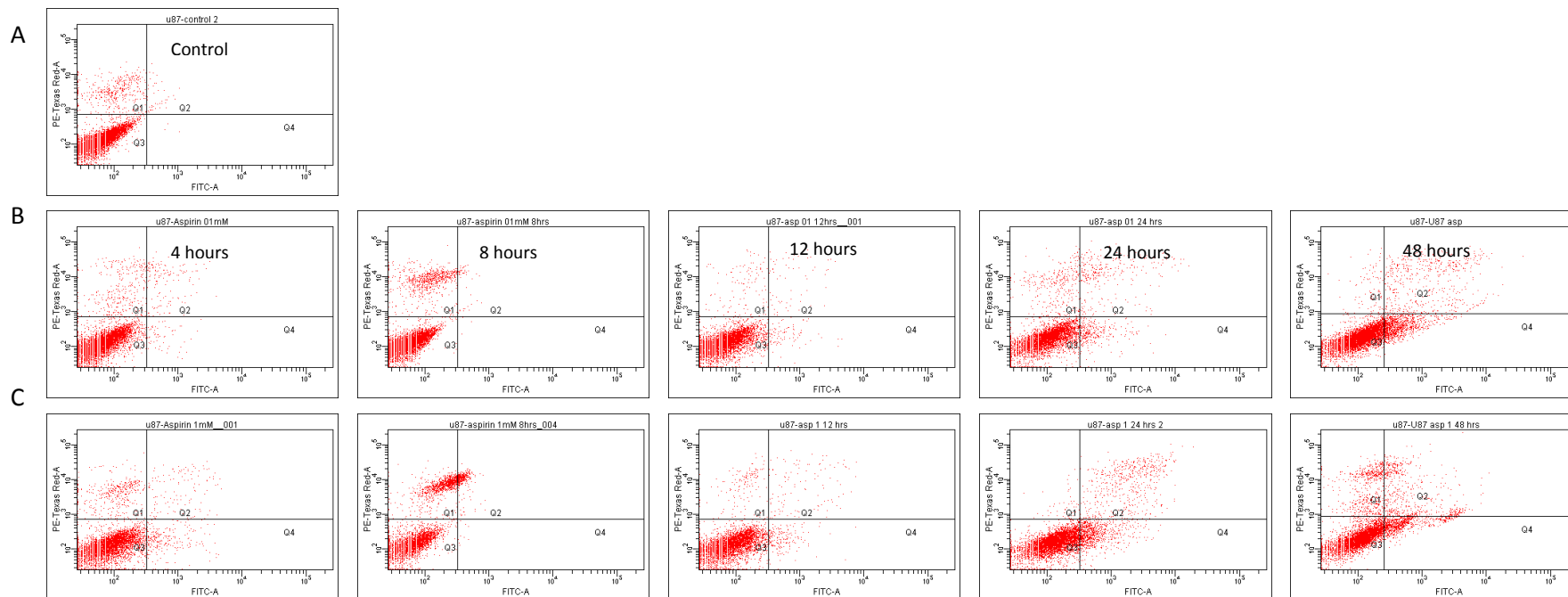


Figure 10.15 Representative dot plots illustrating the induction of apoptosis following aspirin treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in U87 MG cell line. Panel A illustrates the control untreated sample, panel B drug treatment with aspirin at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

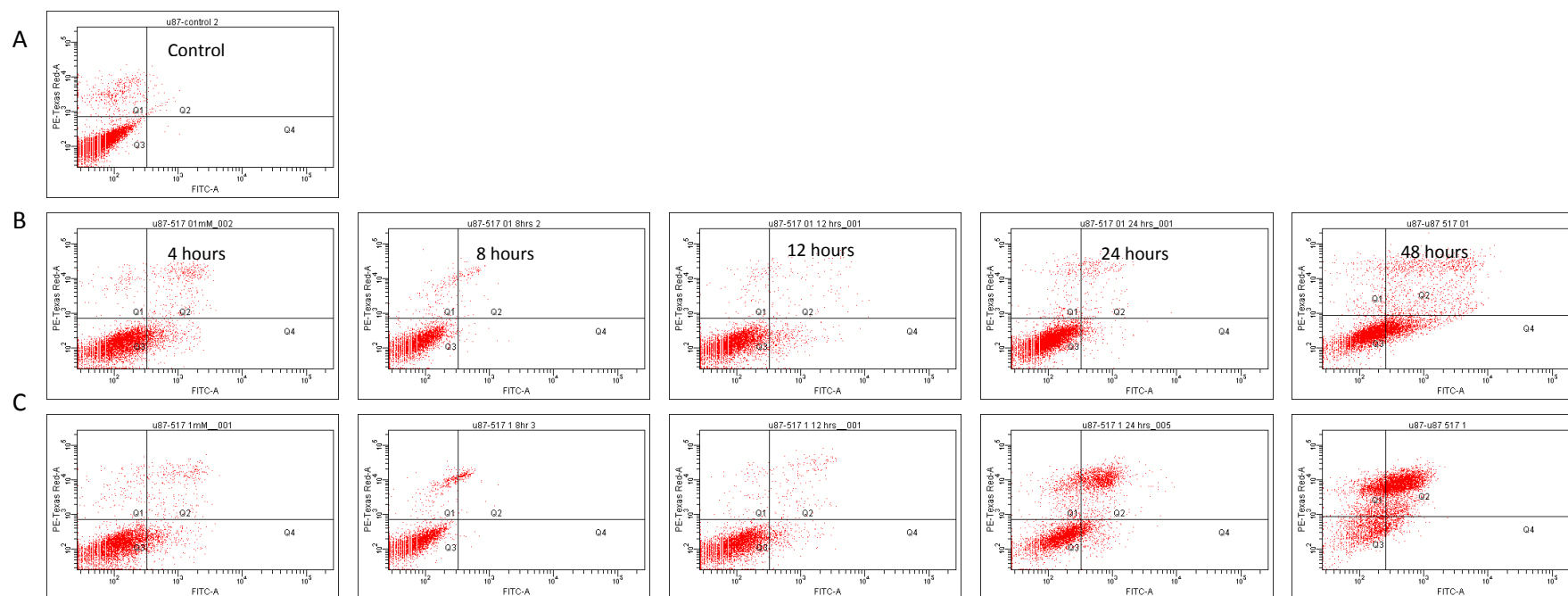


Figure 10.16 Representative dot plots illustrating the induction of apoptosis following PN517 treatment for 4, 8, 12, 24 and 48 hrs at 0.1 mM and 1 mM in U87 MG cell line. Panel A illustrates the control untreated sample, panel B drug treatment with PN517 at 0.1 mM, and panel C 1 mM treatment over time with flow cytometric analysis of FITC-annexin V and PI staining. Annexin V+/PI- (lower right quadrant) represents early apoptotic cells, and annexin V+/PI+ (upper right quadrant) represent late apoptotic cells.

Short Term Culture Supplementary Data

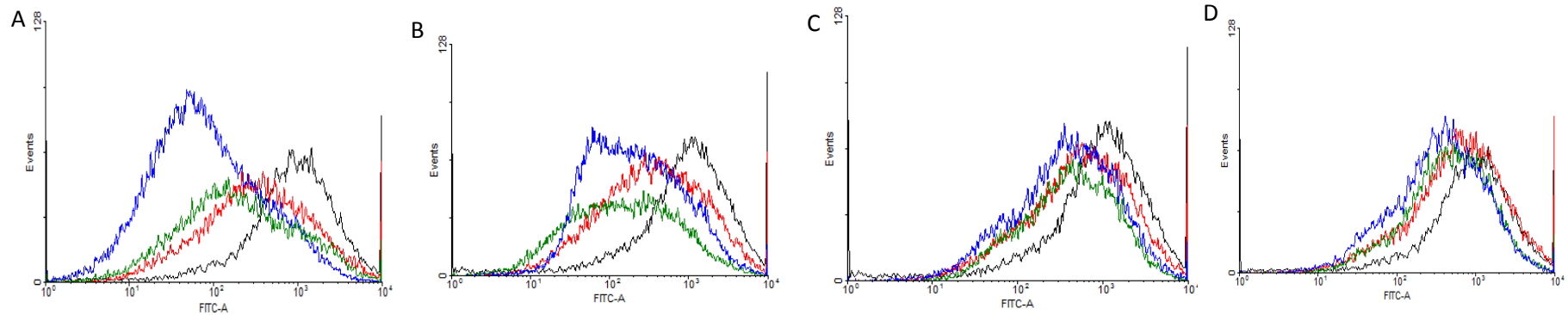


Figure 10.17 Cell proliferation measured by flow cytometry following drug treatment in BTNW911 short term culture. The figure illustrates the effect of drug treatment on proliferation over three days where the black peak corresponds to day 2, red to day 4, and green to day 6 and blue to day 8. A leftward shift indicates a decrease in fluorescence indicating proliferation. Panel A illustrates representative results for control treatment; panel B cisplatin; panel C aspirin; panel D PN517.

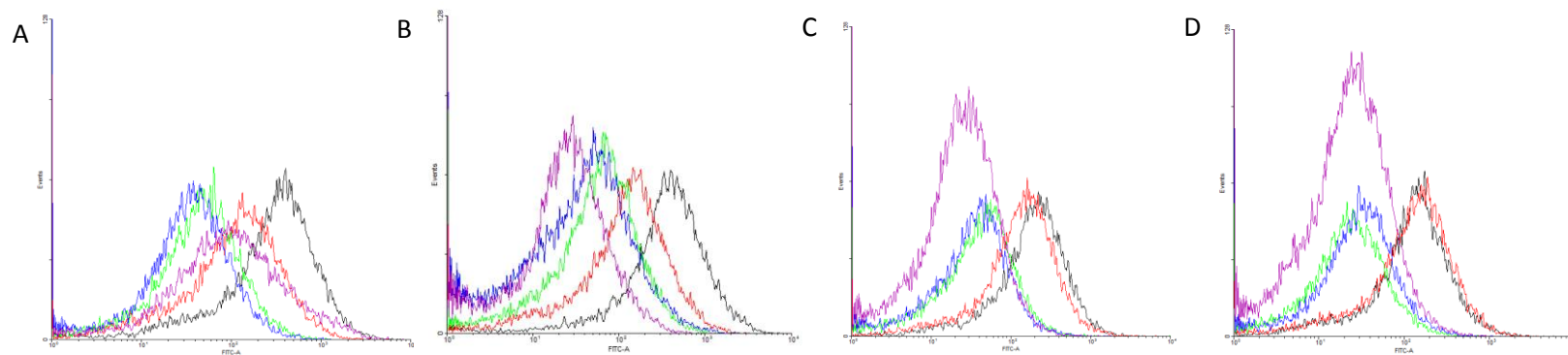


Figure 10.18 Cell proliferation measured by flow cytometry following drug treatment in BTNW914 short term culture. The figure illustrates the effect of drug treatment on proliferation over three days where the black peak corresponds to day 2, red to day 4, and purple to day 6, green to day 8 and blue to day 10. A leftward shift indicates a decrease in fluorescence indicating proliferation. Panel A illustrates representative results for control treatment; panel B cisplatin; panel C aspirin; panel D PN517.