Evaluation of the Therapeutic Potential of Red Clover Extract and Red Grape Seed Extract on Human Adult Malignant Brain Tumours *In Vitro*

A thesis submitted by

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For My Nanaji
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Abstract

Gliomas are rare intrinsic brain tumours which account for 2% of all cancers. Glioblastoma multiforme is the most malignant malignant glioma form and remains incurable. The biological features which preclude successful therapy include heterogeneity, diffuse invasive patterns and angiogenesis. Despite, advances in current conventional treatments the median survival time is only 14 months. Hence there is a need to investigate novel therapeutic approaches which can be included alongside conventional treatment. One such approach is the use of micronutrients in the management of glioblastoma multiforme. This study evaluated the affects of two micronutrient extracts, red clover extract (RCE) and red grape seed extract (RGSE), on human adult malignant brain tumours *in vitro.*

Four primary (or short-term) cell cultures derived from human brain tumour biopsies, an established cell line and normal human brain cells from an epileptic pateint were used to measure the cell viability, anti-invasive, anti-angiogenic and pro-apoptotic potentials, following 48-hour treatment with the IC_{50}s of either micronutrient extract.

Both RCE and RGSE exhibited similar affects on the glioma cell cultures. They both appeared to reduce cell viability, invasive potential and angiogenesis potential though did not appear to have any significant affect on the apoptotic portential of the glioma cultures. For example, incubation with 0.007-1ug/ml RCE caused a significant ($p<0.05$) reduction of in viability of glioma cells but did not affect viability of normal astrocytes. Similar results were obtained for RGSE. These doses also resulted in a significant decrease in invasion and angiogenesis ($p<0.05$). Effects varied between cell lines but in general decreased by 50-60%.

This suggests that both RCE and RGSE do affect the development of glioma cell cultures *in vitro* and warrant further study into the pathways in which this may occur.

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List of Abbreviations

CAM	Complementary and alternative medicine
CD44	Cluster of differentiation 44
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
ECM	Extra celluar matrix
FCS	Foetal calf serum
GalC	Galactocerebroside
GB	Glioblastoma multiforme
GD3	Ganglioside
GFAP	Glial fibrillary acidic protein
HS	Human serum
MMP	Matrix metalloproteinases
NCAM	Neural cell adhesion molecules
NCS	Newborn calf serum
NG2	Neural Glial 2
RCE	Red clover extract
RGSE	Red grape seed extract

THESIS

CHAPTER 1: Introduction

1.1 Epidemiology of Brain Tumours

Intrinsic brain tumours account for only 3% of all adult cancers with an incidence of approximately 14-15 per 100,000, in each gender. They are rare when compared to other somatic cancers such as lung cancer, which is almost 11 times more prevalent. However, in the UK alone, 9,365 new patients were diagnosed with this fatal disease in 2011 (Fig 1) (Office for National Statistics 2012, ISD Scotland 2014, Welsh Cancer Intelligence and Surveillance Unit 2014, Northern Ireland Cancer Registry 2012, Cancer Research UK 2014) with this number rising each year.

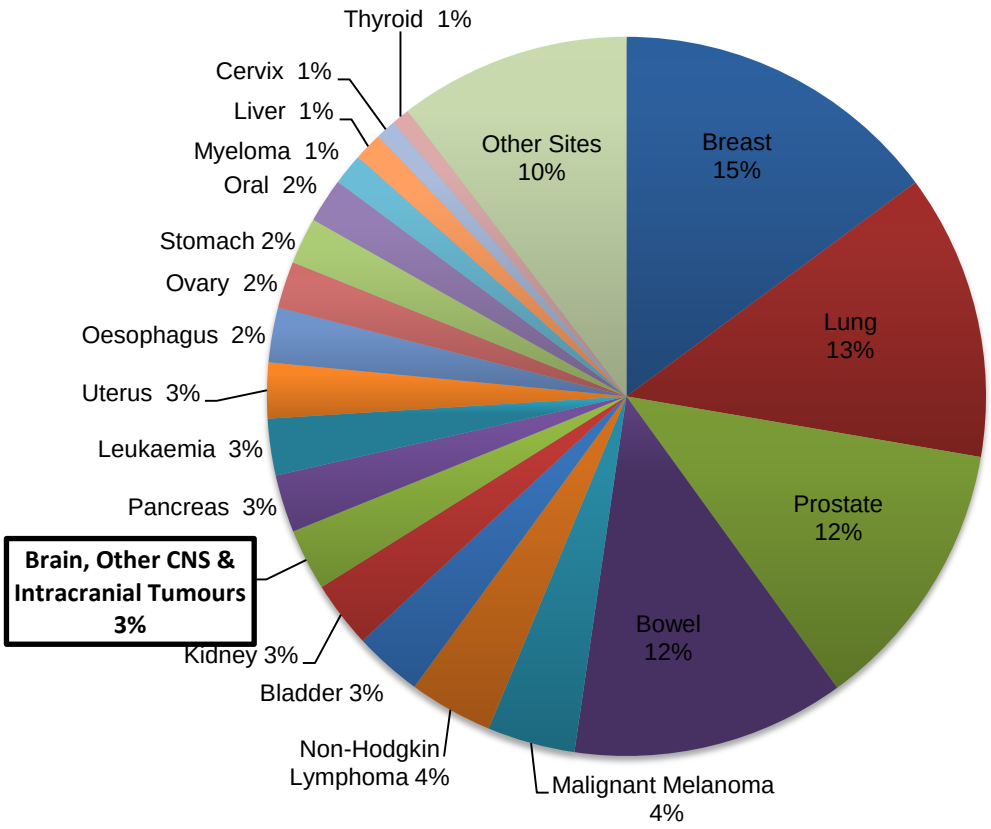


Figure 1 The approximate percentage incidence of various cancers in 2010 including the low incidence of brain and CNS tumours. Adapted from cancer research UK website (2014)

Over the past three decades the incidence in developed countries has rapidly risen increasing by between 35% - 45% (Inskip 2010; Hoffman 2006 p27; Pilkington 2001 p408; Radhakrishnan 1995 p 67). This is probably due to a number of factors including, improved diagnostic and imaging techniques, better documentation of diagnosis, environmental factors and an increasingly elderly population. Differences in incidence can also be seen across ages, gender and ethnic groups in developed countries.

An advanced age positively correlates with a greater risk of being diagnosed with a brain tumour (Fig 2). The incidence peaks between six decades of life. Beyond this point both the incidence and rate decline, either due to lack of investigation and detection of neoplasms in the elderly (McKinney 2004, p12), alternatively due to a number of other mortality factors encountered by this population.

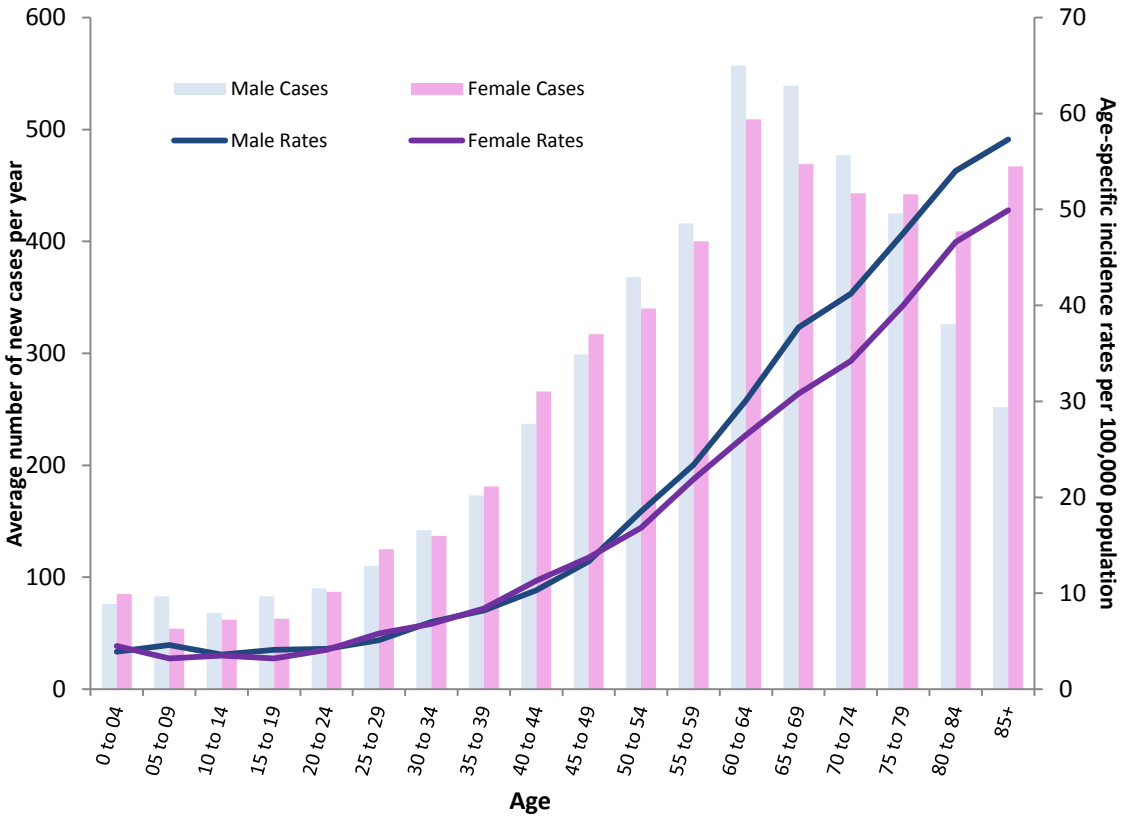


Figure 2 The incidence of brain tumours over the average adult lifespan, in the UK, and the rate per 100,000 capita in males and females between 2008 and 2011. Adapted from Cancer Research UK website (2014)

Figure 2 also illustrates a general male predilection, most significantly beyond the fifth decade. Gliomas in particular have a male: female ratio of 1.5:1.0. Although this is not representative of the greater tendency of females to be diagnosed with meningiomas, it has been suggested that this inclination could be a result of socio-economic factors such as occupation (McKinney 2004, p12).

As well as a gender partiality, Caucasians have an increased risk of being diagnosed with brain tumours compared to Asian or African origins, in the same country. *Glaser et al.* (1996) demonstrated that non-Hispanic whites, in the San Francisco Bay Area have a higher frequency of brain malignancies than white Hispanics, Japanese, African Americans, Chinese, and Filipinos. This data is mimicked when examining global incidences with American Indians and Asians being the groups with the lowest risk between 2002 and 2006 (National Center for Health Statistics). Unfortunately, unlike ethnicity, global differences are usually cautiously interpreted, as around, a four-fold difference is observed in incidence of malignant brain tumours when comparing countries with high incidences such as Australia, Canada, Denmark, Finland, New Zealand and the US to countries with a low incidence such as the Philippines and India. This difference is probably due to inconsistancies in registration and criteria for diagnosis but also too many socio-economic factors which result in brain tumours being undiagnosed in many patients (Lo 2012; Wiemels 2010, p308; Schwartzbaum 2006, p501).

1.2 Aetiology of Brain Tumours

Despite improvements in diagnosis and registration, to date, the causes of brain tumours remain unknown. Speculation and the current school of thought suggests that brain tumour development results from an accumulation of genetic alterations which occur as a result of physical, chemical and/or biological damage. (McKinney 2004, p14). Very little is known about the genetic risk factors for brain tumours, though this may be attributed to the few factors identified within this field (Reilly 2009, p2). Of the identified inherited

syndromes, brain tumours are associated with several familial cancer predispositions including Li-Fraumeni, neurofibromatosis type 1 and 2, tuberous sclerosis, and syndromes involving adenomatous polyps lead to a genetic predisposition to the occurrence of brain tumours. This development only accounts for around 1-2% of brain tumours. Other genetic abnormalities thought to be associated with the development of brain tumours involve mutations of genes associated with basic cellular metabolic pathways for example pathways involved in DNA repair and stability, immune responses, oxidation and detoxification. Published data into the risk associated with genetic mutations is very limited and further study is required to identify any specific polymorphisms related to brain tumour growth.

This however is not considered the sole causative agent and in addition a number of other environmental risk factors are being investigated including ionising radiation, extremely low frequency electromagnetic fields, mobile phones, infections and chemical agents (McKinney 2004, p14).

High frequency radiation, involved in radiotherapy, can result in the breaking of molecular bonds, thus causing DNA damage. Therefore, patients suffering from paediatric cancer or leukaemia, receiving radiation therapy, have an increased risk of developing brain tumours in adulthood despite radiation being administered at therapeutic doses (McKinney 2004, p6). It has also been reported that survivors of the Hiroshima atomic bomb have a greater risk of developing meningiomas (Shintani 1999) though the radiation did not seem to increase the risk of brain tumours to developing foetuses.

Extremely low frequency electromagnetic fields (ELF-EMF) (30-300 Hz) are present in domestic and industrial electricity supplies including but not limited to pylons, energy plants, radio, TV, public transport vehicles and video terminals. The effects of ELF-EMFs depended on their intensity and time of exposure. Investigation and published research into the risk of developing brain tumours due to ELF is very sparse and methodologies are inconsistent. *Kheifets et al* (2010) suggested inconsistent results may be attributable to

numerous shortcomings in the studies, notably assessment of exposure. Despite this various *in vitro* studies have indicated ELF-EMFs lead to DNA breaks of double and single strands (Lai 1996), DNA-protein and DNA-DNA crosslinks (Singh 1998), and increased apoptosis (Lai 2004) in rat brain cells. These effects were reported to be mediated by free radicals (Simko 2007). Currently, conclusions concur that ELF-EMF has a weak link to the risk of developing brain tumours, though research is still ongoing (Gadhia 2004, p65; Kheifets 1995). Conversely, recent studies have investigated the therpeutic effects of ELF-EMF such as improvement and facilitation of bone healing, increasing neurogenesis and in the treatment of cancer (Cuccurazzu 2010, Masutani 1995, p462; Robles 1998, p1113). Due to the inconsistent nature of current conclusions the need to better assess exposure, including development of a more complete job-exposure matrix combining job title, work environment, and exposure to electric fields, magnetic fields, contact current, spark discharge, and other chemical and physical agents (Khiefets 2010; Mee 2009; Estécio 2002) is warranted.

Similarly mobile phone handsets emit radiofrequency signals from the microwave spectrum. Though the energy levels of these waves are insufficient to damage DNA, public concern over the possible health effects of using mobile phones has resulted in various investigations into the relationship between mobile phones and brain tumours. *Hardell et al*. (2013; Carlberg 2012) reported an association, clearly indicating an increased risk for both malignant brain tumors and acoustic neuroma after long-term use of wireless phones and mobile phones. Other studies have shown leakage across the blood brain barrier (BBB) (Salford 1994; Persson 1992) and data suggesting indirect damage to neurons (Salford 2003; Hassel 1994). Despite this, the risk associated with mobile phones is controversial as the studies published either reported on small sample numbers or have allowed for short latent periods. The general consensus, currently, is that mobile phone use presents no risk, but this requires further investigation (Kundi 2010; Lahkola 2005).

A number of viruses, including retroviruses, papovaviruses, and adenoviruses have been found to induce brain tumours in experimental animal models. However, this has not been shown in humans. Various studies have suggested that live polio vaccines contaminated with SV40 might increase the risk of brain tumours (Pepper 1996; Martini 1996) though further studies did not support this evidence. In contrast atopic diseases, for example asthma and eczema, have been found, in a number of independent studies, to be 'protective' against the development of gliomas. Limited research has been done to identify possible mechanisms related to how infections may initiate alignancy or play a protective role remains unknown and further study is required.

A variety of both beneficial and harmful chemicals may be found in various foods and within the environment. One such example, found both the environment and foods, such as some vegetables and cured meats, are the N-nitroso compounds. These compounds are able to cross the blood brain barrier (BBB) and have mutagenic tendencies, enabling them to potentiate the carcinogenic process. N-nitroso compounds both environmental and dietary have been studied as potential carcinogens (Lewin 2006; Tricker 1991). In contrast to these studies N-nitroso compounds, are involved in chemotherapy regimens given to patients subsequent to diagnosis of any cancer, therefore suggesting that some N-nitroso compounds may be protective and not harmful.

Another chemical studied for its potential carcinogenic effects is aspartame, a low calorie sweetener found in various food products and fizzy drinks like diet coke. It was suggested that aspartame might be involved in the aetiology of some brain tumours though it was concluded in a study, that aspartame was not a risk factor (Mallikarjun 2015). However, if there was a pre-existing tumour, it has been suggested that it progresses rapidly in the presence of aspartame (Olney 1996; Pilkington and Rooprai personal communication).

Tobacco has been found not to cross the BBB and therefore, though carcinogenic to other organs of the body, studies are inconsistent with only some populations being linked to development of brain tumours (Hou 2015). Alcohol similarly does not seem to be related to brain tumour development. Other chemicals including hair dyes and hair sprays, pesticides and traffic pollution, which have been linked to other cancers, have yielded inconclusive results though are all still undergoing further study (Khanolkar 2016).

A variety of occupations have been linked to an increased risk of developing brain tumours due to exposure to carcinogenic or neurotoxin compounds such as polycyclic aromatic hydrocarbons, phenols, organic solvents, and lubricating oils. Farmers, for example, are 30% more prone to brain tumours (Khuder 1998, p252) probably due to their exposure of pesticides and other specifics related to farming (Viel 1998; Bohnen 1995). Apart from workers associated with farming and the petrochemical and oil industries, studies within other occupations, investigating this risk factor remains inconsistent and no specific chemical has been associated with the risk of petrochemical and oil industry workers though multiple exposures have to be considered (McKinney 2004, p16).

1.3 Classification of Gliomas

1.3.1 WHO Classification

Today the most widely accepted classification scheme of CNS tumours is that of the World Health Organisation (WHO) (Table 1) (Louis 2007; Kleihues 2002). The WHO system, developed by Zulch, was first published in 1979, as a result of a series of meetings over the period of the 1970s between various neuropathologists, neuro-oncologists, radiation oncologists and various other health professionals. Revision thereafter of this original WHO system, by Kleihues and Cavanee, occurred in 1993, 2000 and most recently in 2007 (Louis 2007; Kleihues 2002; Kleihues 2000; Kleihues 1993). This scheme

sought to classify all CNS tumours using a system based on the histopathologic features of the tumour, as described in the Daumas-Duport scheme, and survival rates.

Daumas-Duport et al. (1988) developed a classification based on the presence of cellularity and the presence or absence of four histopathological features; presence of mitoses, nuclear atypia, endothelial cell proliferation, and the presence and extent of necrosis (Daumas-Duport 1988). In this system, one point was given for each malignant feature thus increasing points correlated with increasing malignancy accordingly Grade I tumours display cellularity only, grade II tumours display mitosis as well, grade III tumours display grade II characteristics and nuclear atypia and finally grade IV tumours display all 4 histological features including anaplasia and necrosis. This scheme is now referred to as the St Anne-Mayo classification system (Doolittle 2004). The WHO classification is based on cell of origin, proportion of malignant features, and tumour grade (I-IV). A brief description of glial tumours is illustrated in Table 1.

1.3.2 The Future of Classification

New techniques of classifying brain tumours may lie in gene expression analysis and molecular genetics. Techniques such as these invite the possibility of improved tumour classification thus resulting in patient stratification for treatment and prognosis (Caskey 2000). An example of such a study has profiled the gene expression, using cDNA array technology of human primary glioma tissue samples. A good correlation was reported for survival against molecular classification (Fuller 2002). Another new method includes the use of support vector machines (SVM), creating a model to predict astrocytoma grades following MRI to identify common features of the neoplasms (Milchenko 2016). The diagnostic performance of SVM was reported to be significantly better than clinician diagnosis for higher grade tumours.

Table 1 Who classification of gliomas adapted from *Louis 2007*

Tumour classification	Histological grade of malignancy
Astrocytoma	
Pilocytic Astrocytoma	I
Pilomyxoid astrocytoma	II
Pleomorphic xanthoastrocytoma	I
Subependymal giant cell astrocytoma	I
Astrocytoma (Low-grade, diffuse)	I-II
Anaplastic astrocytoma	III
Glioblastoma multiforme	IV
Giant cell glioblastoma	IV
Gliosacrcoma	IV
Oligodendroglioma	
Oligodendroglioma (low grade)	I-II
Anaplastic oligodendroglioma	III
Mixed gliomas	
Oligoastrocytoma	II
Anaplastic oligoastrocytoma	III
Ependymoma	
Subependymoma	I
Myxopapillary ependymoma	I
Ependymoma	II
Anaplastic ependymoma	III
Glioblastoma	
Giant cell glioblastoma	IV

1.4 Gliomas

Gliomas are thought to be derived from glial cells or neural progenitor cell and are the most commonly diagnosed CNS tumour accounting for approximately 86% of diagnosed tumours (McKinney 2004, p12). Gliomas include astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas. Astrocytomas are the most commonly occurring glioma with grade IV astrocytomas or glioblastoma multiforme being the most difficult neoplasm to treat.

1.4.1 Astrocytomas: The Most Common Glioma

Astrocytomas are the most commonly occurring neuroepithelial tumours and may be described as well-differentiated diffusely infiltrating tumours predominately comprising neoplastic astrocytes. They may be characterised into two groups; those that diffusely infiltrate and those that do not. Diffuse astrocytomas account for at least 75% of astrocytic tumours. They have a poorer prognosis and a higher risk of recurrence as there is no discernible boundary between the tumour and the normal tissue, thus during resection many residual cells in the brain tissue lead to recurrence.
Location of the tumours varies greatly and is largely dependent upon age though they are most commonly located in the frontal, temporal and parietal lobes of the brain.

Low grade tumours are graded WHO grades I or II, thus presenting only one or two malignancy features. These tumours exist as either non-diffusely infiltrating, such as pilocytic astrocytoma (WHO grade I), pleomorphic xanthoastrocytoma (WHO grade II) and subepndymal giant cell astrocytoma (WHO grade I), or as diffusely infiltrating tumours. The former tumour type occurs in paediatric patients and young adults which, are all generally slow growing, well differentiated and circumscribed with a lack of anaplastic progression due to slow limited invasion thus the prognosis of these tumours

is more favourable. The later tumour types occur mainly in adults and are capable of undergoing malignant transformation, therefore carrying a more worrying prognosis. These tumours may be categorised into one of three variants which are identified upon resection, using histological techniques, in accordance to the most predominant cell type, specifically fibrillary astrocytomas which are by far the most common, gemistocytic astrocytomas and protoplasmic astrocytomas (Kleihues 2000).

Malignant tumours are graded WHO grades III or IV. These tumours display three or more histological characteristics and are all poorly differentiated with a more marked malignant progression. Unlike most solid tumours, these neoplasms rarely metastasize outside the brain instead they undergo a 'go or grow' phenomemnon (Bolteus 2001). This theory describes the mutually exclusive nature of brain tumour cells to either proliferate or diffusely infiltrate the contingent brain. Invasion is perhaps the most significant biological feature that precludes successful treatment. Other biological features exhibited by malignant neoplasms that obstruct treatment are cellular heterogeneity and angiogenesis. Evasion of treatment therefore leaves patients diagnosed with malignant neoplasms with a bleak prognosis.
Anaplastic astrocytomas are diffusely infiltrating astrocytomas with proliferative potential and either focal or dispersed anaplasia and a display of hypercellularity which is often important in diagnosis. They have a tendency to infiltrate without tissue destruction thus often leading to enlargement of adjacent structures. The tumour mass frequently appears granular and opaque with a soft consistency. Anaplastic astrocytomas have a poor prognosis with tumours undergoing malignant progression, though progression of recurrent tumours is more rapid that those that arise *de novo*.

Glioblastoma multiforme (GB) is the most malignant form of astrocytoma, at WHO grade IV this neoplasm portrays all the features of malignancy and is amongst the most difficult of astrocytomas to treat effectively and remains incurable. GBs account for approximately 15-20% of all intracranial neoplasms and 50% of all gliomas (Glantz 1991, p1741). Morphology of GBs

varies greatly, due to its heterogeneous nature. Cells appear a number of different shapes with marked invasive cell populations budding of the tumour mass thus invading both hemispheres of the brain and characteristically displaying the 'butterfly' effect. GBs are also associated with oedema, degeneration and occasional haemorrhaging. Molecular studies support two pathways of occurrence of GBs, they can arise both *de novo* and as a result of malignant progression from anaplastic astrocytomas. Prognosis of GBs is poor with most cases leading to mortality with a year of diagnosis (Hottinger 2014, p32; Stupp 2005, p988; Grossman 2004; McKinney 2004, p13;).

1.4.2 Oligodendrogliomas

Oligodendroglial tumours can be either well-differentiated, low grade tumours (WHO grade I or II) or malignant tumours (WHO grade III). Unlike astrocytic tumours the incidence for oligodendrogliomas is far less. Traditionally these were thought to comprise 2% to 5% of primary brain tumors however recent studies have indicated these account for around 4% to 15% of gliomas (Engelhard 2003, p444; Paleologos 2001, p59). Oligodendrogliomas are localized to the white matter of the cerebral hemispheres. Macroscopically the tumours mass may appear soft and gelatinous and can be associated with infiltration of the surrounding cortex and adjacent leptomeninges. The tumour cells are monomorphous and in areas of increased cellularity the tumour mass may display circumscribed nodules. Morphologically they have uniform round nuclei slightly larger than those of normal cells. During degeneration the mass creates a distinctive 'honey-comb' effect which is used as a useful diagnostic feature. Survival rates of oligodendrogliomas are more favourable than diffuse astrocytomas specifically post-operative median survival time is 3-5 years (Chinot 2001, p13; Sun 1998, p886; Shaw 1996, p288). Also, in contrast to astrocytomas, recurrence and malignant progression occurs less frequently.

1.4.3 Progenitor Cells

The presence of a population of cancer stem cells has been discovered, over the years, as a result of research into the existence of cancer progenitor cells in cancers such as leukemia (Lapidot 2001; Reya 2001; Bonnet 1997). Normally progenitor cells or stem cells are cells that indefinitely self-renew and give rise to differentiated cells (Watt 2000). Pluripotent stems cells, have the ability to give rise to multipotent or tissue-specific stems cells, which in turn have been identified in various organs including bone marrow, breast, intestine, lung, skin and brain (Shackleton 2006; Dekaney 2005; Kim 2005; Alonso 2003; Krause 2001; Uchida 2000). Though dormant under normal physiological conditions, these, may also, be stimulated, during homeostasis and injury repair, into simultaneous self-renewal, maintaining a stem cell population, and differentiation, giving rise to a new population of all the cell types of a given tissue (Shackleton 2006; Krause 2001), accountable for tissue function and homeostasis (Weissman. 2001) for example stem cells of neural tube origin give rise to neurones, astrocytes, oligodendrocytes and ependymal cells (Fig 3). Such a phenomenon is known as trans-differentiation.

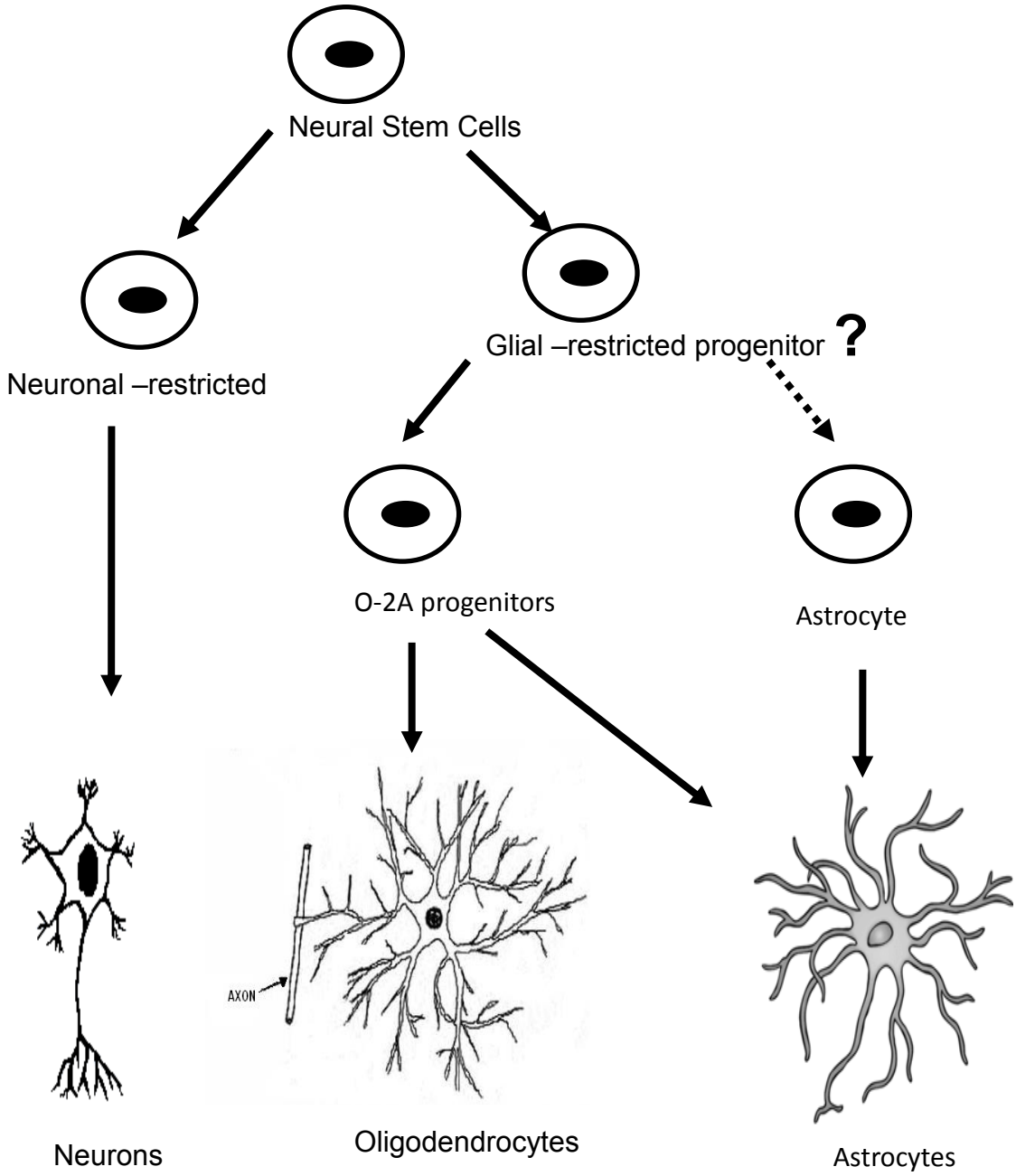


Figure 3 Differentiation of progenitor cells in the neural tube adapted from *Fan et al.* (2007)

Cancerous stem cells however continually clone tumour cells thus are responsible for maintenance of the tumour mass which ultimately leads to increasing malignancy (Singh 2003, p5822).

1.5 Clinical Presentation of Gliomas

Patients with primary neoplasms usually present with drowsiness, headaches, vomiting/nausea and/or papilloedema as a result of increased pressure and neural tissue compression due to small increases in the tumour mass. Cerebrospinal fluid (CSF) pathways may also be blocked as a consequence of raised pressure preventing drainage thus causing hydroencephalus. Personality & cognitive changes, focal neurological deficit or seizures may also present if increased compression, direct infiltration or neuronal destruction occurs.

Presentation varies according to location of the tumour and whether or not it is deep seated. Table 2 illustrates the symptoms associated with some of the various regions, of the brain. The most common primary symptoms will be a seizure or epileptic fit, upon which hospitalization and emergency surgery is required.

Table 2 Symptoms that can be caused by tumours in different parts of the brain adapted from Cancer Research UK website 2014

Position of the tumour	Symptoms
Frontal lobe	Personality changes Loss of inhibitions Apathy Difficulty with planning and organising Being irritable or aggressive Weakness in part of the face, or on one side of the body Difficulty walking Loss of sense of smell Problems with your sight or speech
Temporal lobe	Forgetting words Short term memory loss Seizures déjà vu or phantom smells
Parietal lobe	Speech difficulty Problems with reading or writing Loss of feeling in part of the body
Occipital lobe	Visual impairment

1.6 Diagnosis and Classification of Gliomas

Advances in diagnostic tools over the years have lead to successful diagnosis of brain tumours. Examples of such include better visualisation of the tumours using computed tomography (CT) and magnetic resonance imaging (MRI) scanning and better diagnostic conclusions using microscopy and Immunohistology.

1.6.1 Imaging

CT scanning was first introduced in the 1970s by Sir Godfrey Hounsfield. This technique is an advancement of the X-ray machine though unlike an X-ray machine a CT scanner emits multiple beams to examine a cross-sectional image of the body thus allowing a 3-dimensional visualisation of the tumour. This scan is usually accompanied by iodine based compounds which are absorbed by the tissues and detected by the CT scanner. Unfortunately, the resolution of soft tissues, compared to bony tissues is poor therefore CT scans are better used for bony tissues. *McCormack et al.* (1992) showed that the seven times increase in the risk of recurrence of low grade astrocytomas was associated contrast enhancement on a CT scan. Reduced integrity of the BBB increases enhancement in CT scans rather than the presence of the tumour itself.

MRI uses the presence of strong magnetic fields to visualise the soft tissues of the body and is based on the alignment of hydrogen atoms in the body in response radio waves of the magnetic fields. Images are produced on a computer to visualise not only 2-dimensional slices of a 3-dimensional image but also different planes of the image. Contrast enhancements agents or paramagnetic compounds, such as gadolinium are not normally required though on occasion they are administered intravenously to increase image sensitivity.

Positron Emission Tomography (PET) measure cerebral blood flow & metabolic activity. A variant of PET, Single Photon Emission Computerized Tomography (SPECT), also functions using the same principles. PET scans are effective in identifying the progression of low grade tumours to high grade tumours. They are also able to identify recurrent tumours. PET scans visualise the low grade tumours as 'cold' as they are hypometabolic and malignant areas are perceived as 'hot' or hypermetabolic (Francavilla 1989, p1). Unfortunately as it is an expensive procedure it is very rarely used except in the case of private patients.

1.6.2 Immunohistology

Following initial diagnosis during surgery, a clinical diagnosis is usually determined as a result of histological examination of biopsy derived paraffin sections. This technique is used to investigate and conclude cell lineage, histological type and malignancy. This technique was first discovered in the late 1970s when *Kohler and Milstein* reported immortal/transformed cells developed monoclonal antibodies (Kohler 1975). These cells gave rise to cell populations that divided indefinitely and secreted the antibody. These antibodies were used to identify surface antigens, their receptors or cytoplasmic and nuclear antigens of both normal and neoplastic cells. A number of antigenic markers may be related to the detection of astrocytic tumours some of which include glial fibrillary acidic protein, cluster of differentiation 44, neural cell adhesion molecules and integrins.

Glial fibrillary acidic protein (GFAP) is an intermediate filament thought to retain the mechanical strength and structure of astrocytes (Eng 1971) it is a distinguished marker of normal, reactive and neoplastic astrocytes. Extended studies of astrocytomas have proved this marker to be invaluable for both diagnosis and research. It is not however, thought to be exclusive to astrocytomas alone, instead it is expressed, to some extent, in all gliomas apart from meningiomas, medulloblastomas and brain metastasis (Oh 1999; Fischer 1989). The origin of such tumours may be misinterpreted as

astrocytic. Instead they are more likely to display a plastic phenotype *in vitro* when the microenvironment is changed (Tenenbaum 1996).

Cluster of differentiation 44 (CD44) is an adhesion molecue and a marker of invasion and progenitor cells. It is up-regulated in brain tumours as a result of involvement of hylauronic acid (HA) expression increasing in the extracellular matrix (ECM), during invasion, regardless of tumour grade (Delpech 1993). CD44 is involved in this invasive process and is commonly studied as a marker of astrocytic invasion. *Ranuncolo et al*. (2002) suggested that overexpression of CD44 could be relevant in determining the highly invasive behaviour of gliomas, though it does not behave as an independent prognostic factor for survival

Neural cell adhesion molecules (NCAMs) are members of the immunoglobulin super family. They are cell surface glycoproteins that mediate cell-cell adhesion independent of Ca^{2+} (Keilhauer 1985), play an important role in neurite outgrowth and fasciculation and are involved in migration/invasion (Appel 1993). Expression of NCAM has been shown to reduce migration/invasion of glioma cells both *in vitro* as well as *in vivo* (Owens 1998; Gratsa 1997; Edvardsen 1994; Edvardsen 1993). In addition to cell-cell adhesion, signal transduction is induced by hemophilic interaction of NCAM, resulting in neuronal differentiation (Kolkova 2000; Walsh 1996) and inhibition of cell proliferation (Edvardsen 1993; Edvardsen 1993). Additionally *Prag et al.* (2002) reported exposure to NCAM strongly affected the motile behaviour of glioma cells independently of homophilic NCAM interactions, probably through interference with factors regulating cellular attachment.

The fundamental cellular function of *integrins* is their adhesive properties, which mediate extensive and important cellular functions by interacting with the extracellular matrix (Palecek 1997; Condic 1997; Gumbiner 1996; Miyamoto 1995;). The interactions of integrins with the ECM also activate signal transduction (Clark 1995; Zachary 1992). Many known ECM molecules that interact with integrins, including fibronectin, collagen, and vitronectin,

contain an RGD consensus sequence (D'Souza 1988; Ruoslahti 1986). As such integrins are an attractive target for the treatment of several cancers (Xiao 2016). Several factors expressed in glioma cells have been found to regulate integrin expression, particularly, urokinase, a plasminogen activator (uPA), secreted by glioma cells. uPA has been shown to upregulate integrin $\alpha v\beta 3$ expression by autocrine mechanism (Pedroja 2009, p20708).

Galactocerebroside (Gal C) belongs to the family of cerebrosides. It is a common cell surface glycolipid marker for mature oligodendrocytes and appears to regulate myelin formation (Marcus 2006; Dupree 1998; Bosio 1996; Coetzee 1996). *Tenebaum et al.* (1996) identified the loss of this marker in their studies, identifying the changes in antigenic expression in the presence of different serum supplementation.

Neural glial 2 (NG2) a transmembrane chondroitin sulphate proteoglycan, is expressed on oligodendrocyte progenitor cells (OPCs) (Chekenya 2002). OPCs or NG2 cells terminally differentiate into oligodendrocytes and NG2 cells, thus the number of NG2 cells is comparable to the number of mature oligodendrocytes in the gray matter (Dawson 2000). Unlike astrocytes and oligodendrocytes, mature cell markers such as GFAP and Gal C are not expressed on NG2 cells (Butt 1999 Reynolds 1997; Levine 1993; Levine 1987) demonstrating their distinct nature.

Ganglioside GD3 belongs to a family of complex acidic glycolipids. Despite poor documentation it is thought they are functionally diverse in the normal brain and are also expressed and play a role in brain tumours (Yates 1995; Wikstrand 1994; Fredman 1994; Pilkington 1993; Shinoura 1992). *GD3* is not specific to oligodendrogliomas it is reported to be down-regulated in gliomas (Gratsa 1997).

1.7 Biological Features Evading Treatment

1.7.1 Heterogeneity

A predominant challenging feature of astrocytic tumours is their cellular heterogeneity or complexity (Fig 4). When compared to the single sub cell population of liver cells of a hepatoma, gliomas can result in up to 14 different sub populations. Differing processes of differentiation and anaplasia conceive a variety of morphologies with cells displaying antigenic difference. This therefore results in sub populations which are not only phenotypically different but also genotypically different (Marusyk 2010; Pilkington 1992). This characteristic cellular heterogeneity is seen in early passages and is important in tumour growth and maintainance. Later passages see retention of fewer cell sub populations (Pilkington 1992).

Development of cell culture techniques such as cloning individual cell populations, *in vitro* growth of neurospheres and multicellular tumour spheroids, and identification of new markers including nestin, Mushashi-1 and CD133 confirm the existing heterogeneity of gliomas and are also suggestive of the existance of a stem cell population within gliomas. The presence of stem cells in the CNS strongly suggests a role for these cells in tumour initiation and resistance to current therapeutic strategies.

Differential resistances to radiation and drug therapies makes gliomas more complicated to treat. One study cloned 16 sub populations from an early passage (P3-10) human glioma cell culture and exposed each to radiation. Of the 16 populations two populations remained completely resistant to the radiation and were employed for further study (Yang 1992). An eariler study reported a similar resistance to chemotherapy (Yung 1982). Identification of properties of the individual cell populations opens new and exciting paramaeters of study, providing vectors in novel therapeutic strategies for these tumours.

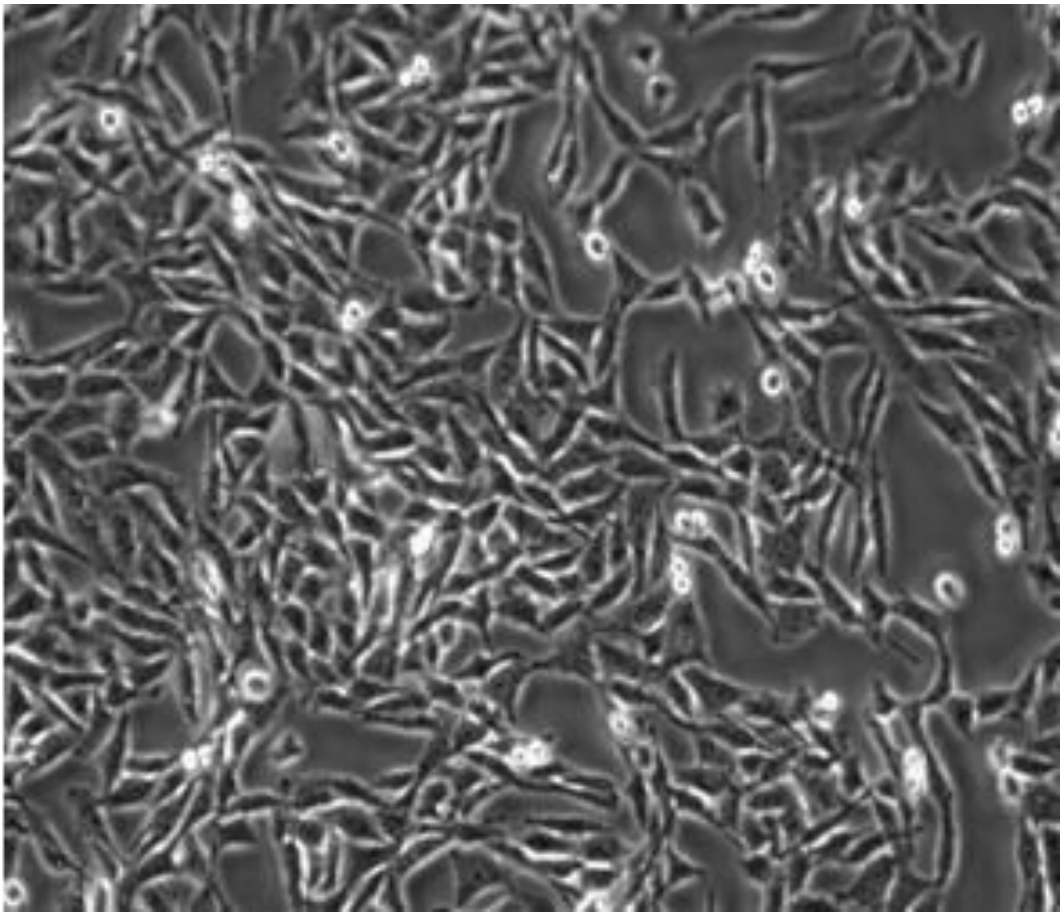


Figure 4 Phase contrast micrograph of an anaplastic astrocytoma (III) primary cell culture IPSB-18. Cells of different morphologies are depicted in particular elongated and partial star shapped cells are illustrated.

1.7.2 Invasion

Perhaps the most significant biological feature which precludes successful therapy is invasion. Invasion is a complex pathway, which involves the interaction of various components (Liotta 1986) including cell adhesion molecules (e.g. Cluster of differentiation 44, CD44), proteases (e.g. matrix metalloproteinases – MMPS), extracellular matrix (ECM) components and growth factors enabling the attachment of invading tumor cells to extracellular matrix (ECM), disruption of ECM components, and subsequent cell penetration into adjacent brain structures.

In non- CNS tumours, metastasis is the major cause of morbidity and death, however, in primary brain tumours, there is no metastasis. Invasion allows the formation of secondary tumours within the brain. Under normal conditions, a negative feedback mechanism may limit the behavior of normal cells, such as endothelial cells, and lymphocytes. Although tumor cells use the same mechanism as normal cells, they seem to lack the appropriate feed back mechanism during invasion.

One of the most plausible reasons for the failure of brain tumours to metastasize lies in the interaction of neoplastic glia with the non-brain endothelial cells. For extravasation of glioma cells at a distant site they must possess the complement of adhesion molecules necessary to facilitate adherence to the endothelial wall. Cluster of differentiation 15 (CD15), is an adhesive oligosaccharide epitope which facilitates neoplastic cell/endothelial cell adhesion and confers high metastatic potential in non-neural tumours (Dejana 1992; Matsusako 1992). Human brain tumours show little or no CD15 expression (Martin 1995; Reifenberger 1992) while the chemically induced rat glioma cell lines, A15A5, F98 and C6, show strong expression of the CD15 antigen (Martin 1995). These latter experimental rat tumours, unlike their human counterparts, show a propensity for metastatic behaviour. Furthermore, in vitro adhesion of metastatic carcinoma cells and brain tumour cells to vascular endothelium correlates with the level of CD15 expression (Martin 1995).

Invasive ‘guerrilla’ cells display the ‘go or grow’ theory (Bolteus 2001) whereby the cells are either growing and proliferating within the tumour mass or the cells enter G_0 phase and bud away from the tumour mass and migrate across the brain cortex by primarily digesting the ECM with the aid of elevated levels of glycosidases, which break down the glycosaminoglycan chains that form the mesh of the ECM, and proteases that break down protein structures with the ECM. Elevated levels of hyluronic acid (HA) also digest the ECM. It is unknown how HA facilitates tumour cell *in vitro* (Nakagawa 1996) though various possibilities have arisen; 1) it may provide a lead for the tumour cells

to follow across the ECM 2) it may provide immunity for the cells.

The sequence of events, originally described by *Liotta*, during tumour cell invasion of the extracellular matrix are described as a three-step hypothesis (Onishi 2011). The first step is tumour cell detachment from the primary tumor mass and attachment via cell surface receptors which specifically bind to components of the extra cellular matrix (ECM) such as laminin and fibronectin. The anchored tumour cells then secrete hydrolytic enzymes which can locally degrade the matrix. And finally tumour cell locomotion into the region of the matrix modified by proteolysis.

Detachement of the cells involves various events. Firstly destabilization of cadherin-mediated junctions that hold the primary mass together must occur. Cadherins (E-, P-, and N-cadherin) form calcium-dependent, transmembrane, cell–cell adherent junctions. Cadherins may function as suppressors of tumor growth and invasion (Demuth 2004). Decreased cadherin function in carcinoma progression, is correlated with poor prognosis (Bremnes 2002).

Subsequently a decline in the expression of connexin 43, a component of gap junctions, leads to a reduction in junction formation. Fewer inhibitory signals may be resulted due to decreased gap junction formation, facilitating uncontrolled cell division and de-differentiation (Ruch 1994). Connexin 43 is the most abundant gap junction protein in CNS and is expressed primarily in astrocytes (Dermietzel 1993). *McDonough et al.* (1999) reported that reduced gap junction formation correlates with increased motility of glioma cells *in vitro*. Clevage of CD44, which anchors the primary mass to ECM, by the metalloproteinase ADAM is the final part of the process. CD44 is cleaved by ADAM 10 and 17. Both the intracellular and extracellular cleaved components of CD44 promote cell migration (Okamoto 1999, p 25530).

Adherence to the ECM is most commonly facilitated by integrins. They interact with two groups of ligands including a variety of ECM proteins, such as fibronectin, fibrinogen and vitronectin, and cell surface molecules, that are

members of the immunoglobulin supergene family, such as vascular cell adhesion molecule (VCAM-1) and intracellular adhesion molecules (ICAM-1, ICAM-2). In particular, the integrin αvβ3, which binds to fibronectin, vitronectin, and tenascin-C in ECM, is thought to play a central role in glioma invasion (Leavesley 1993, p165). Up regulation of αvβ3 expresion leads to increased motility of human glioma cells and a decrease in apoptosis sensitivity (Platten 2000).

The most common proteases involved in the degradation of the ECM are MMPs. The first experimental evidence for a role of MMPs in tumor development was discovered in the 1980s. It was reported that a type IV collagenase was demonstrated to be involved in melanoma invasion and metastasis (Liotta 1980). These are a family of zinc dependent enzymes which all pave a pathway for the malignant tissue to migrate or invade along. 23 members of the MMP gene family have been identified to date (Nagase 2006) the most commonly noted in GBs as being up-regulated, are MMP-2, MMP-7, MMP-9, MMP-14 and MMP-15, these can be down-regulated in the presence of micronutrients such as selenium (Rooprai 2007).

Regulation of MMPs occurs at many levels: transcriptional activation of MMP genes can occur in response to agents such as growth factors, oncogene expression and phorbol esters (Matrisian 1985; Kerr 1988; Matrisian & Hogan, 1990). TGFβ upregulates the expression of MMP-2 and MMP-9 (Wick 2001; Salo 1991; Overall 1991) while EGF and PDGF were shown to induce stromelysin gene expression in fibroblasts (Matrisian 1985).

Integrins have also been shown to induce MMP expression. The ligation α5β1 integrin, reported by *Werb et al.* (1997), showed that, very late antigen-5 (VLA-5) on fibroblasts induced collagenase and stromelysin gene expression. Another study reported that binding of the αvβ3 integrin up-regulated the expression of MMP-2 in melanoma cells (*Seftor* 1992, p1557). The αvβ3 integrin was also capable of directly binding to the active MMP-2 enzyme inhibiting adhesion to vitronectin (Brooks 1996, p687). From studies in glioma

cells *Deryugina et al.* (1997, p2474) showed that αvβ3 binding to MMP-2 occurred through the C-terminal portion of the enzyme. Treatment of GB cell lines with α3β1 antibodies increased MMP-2 activity and invasion in matrigel assays (Chintala 1996).

Interleukin-1β (IL-1β) has also been shown to induce stromelysin gene expression in fibroblasts (Frisch & Ruley, 1987; Quinones 1989).The conversion of latent proenzyme to its active form is another level of regulation via plasmin or active stromelysin. Although neither plasmin nor stromelysin appear to activate MMP-2 (Okada 1990) *in vitro*, they have been shown to activate MMP-2 by means of MMP-14 (MT-MMP) (Strongin 1995).

A variety of zymographic & enzyme-linked immunosorbent assay (ELISA) studies have revealed increased activity of MMPs in GBs and anaplastic astrocytomas when compared to low grade astrocytomas and normal brain, particularly for MMP-9 (Nakagawa, 1994; Rao 1993, 1996). Glioma cells *in vitro* have been shown to secrete many MMPs including MMP-1, MMP-2, MMP-9, MMP-3 and MMP-7 (Nakano 1993). The amounts secreted into the culture medium are often higher than those extracted from tissue (Woessner, 1995). In GBs and anaplastic astrocytomas immunoreactivity to MMP-9 and MMP-2 has been reported in endothelial and tumour cells (Rao 1996; Sawaya 1996).

Secreted MMPs, from glioma cells have the ability to degrade the surrounding brain tissue (Lund-Johansen 1991; Bjerkvig 1986), and MMP-2 & MMP-9 can degrade collagen IV a component of the ECM. *Liotta* (1986) proposed that such proteolytic enzymes were involved in invasion. Indeed, the highly invasive astrocytoma cell line, SF-188 expressed MMP-2 & MMP-9 (Rutka, 1995) also GB cell cultures have been shown to secrete MMP-9 during *in vitro* 2-dimensional invasion studies using Matrigel (Rao 1994). Upon treatment with the appropriate inhibitors the ability to invade was reduced (Pan 2015).

Upregulation of MMP-2, and MMP-9, in particular, has been associated with a high degree of maglinancy and enhanced glioma invasion in several *in vitro* and *in vivo* model systems (Wild-Bode 2001). The transition of tumour cells towards the invasive phenotype, involves gene products such as MMP-2, though the mechanisms of initiation and maintenance of glioma invasiveness remain unknown.

Inhibition of MMPs could aid a reduction in invasion ultimately preventing recurrence of tumour growth. The natural tissue inhibitors of MMPs (TIMPS), these are currently being studied for their anti-invasive and anti-angiogenic properties. The classical notion of TIMPs in tumour biology is rather narrow, however, and there is a growing appreciation that these proteins.

In vivo, TIMPs, play a major role in the regulation of MMP activity. (Liotta, 1986; Ennis & Matrisian, 1994; Matrisian, 1990). TIMPs are secreted proteins that consist of a family of inhibitors that is comprised of 4 gene products (TIMPs 1–4) which inhibit secreted MMPs with similar potencies (Apte 1995; Greene, 1996; Blavier 1999). Individually, TIMPs differ markedly in their affinity for MMP interactions and gene regulatory mechanisms. Specifically TIMP-1 interacts with pro-gelatinase-B (MMP-9) and is subject to tight control at the transcription level (Phillips 1999), whereas TIMP-2 binds pro-gelatinase A (MMP-2) and shows constitutive gene expression (Blavier 1999).

Existance of differences also occurs with regard to biochemical properties: TIMP-3 is itself an ECM-associated protein but TIMP-1, -2 and -4 are freely diffusible (Leco 1994). Moreover, TIMP-1 is ineffective as an inhibitor of MT1-MMP, whereas TIMP-2 and TIMP-3 are both functional in this regard (Will 1996). The general notion is that TIMPs act as the ‘brakes’ of the malignant process. Thus, antisense-mediated down-regulation of TIMP-1 can induce tumorigenic and migratory behavior of cells in mouse fibroblasts (Khokha, 1989). Overexpressed or exogenous TIMP-1 and TIMP-2 reduce tumour invasion and metastasis *in vivo* (DeClerck 1991; DeClerck 1992). Additionally, TIMP-3 down-regulation has been noted at the invasive edge of highly

aggressive colorectal carcinomas (Powe 1997), and overexpressed TIMP-4 resulted in decreased invasive capacity of breast carcinoma cells (Wang 1997).

The uses of TIMPs lay across a broad spectrum.TIMP-1 can stimulate the proliferation of erythroid precursors (Gasson 1985), and alongside TIMP-2 can positively influence the proliferation of numerous cell types (Hayakawa 1992; Hayakawa 1994; Wingfield 1999). Additionally, TIMP-2 is able to inhibit *in vitro* proliferation of human microvascular endothelial cells stimulated with bFGF (Murphy 1993), and TIMP-3 promotes apoptosis (Ahonen 2003, p2122; Baker 1998), possibly through stabilization of TNF alpha receptors (Smith 1997).

There is a wealth of evidence showing that MMPs are overexpressed in malignant gliomas but studies of TIMPs in gliomas give conflicting results. High TIMP levels have been linked with increased malignancy. The suggestion of a balance between MMPs and TIMPs to increase the activity of MMPs in culture may be due to reduced TIMP levels rather than elevated enzyme secretion. Notably, lesser amounts of TIMP would be secreted by tumour cells having an invasive phenotype compared to tumour cells with a non-invasive phenotype (Halaka 1983). Reduced expression of TIMP-1 and -2 with increasing glioma grade has been reported, suggesting that a lack of inhibitor expression may contribute to a more aggressive glioma phenotype (Mohanam 1995; Kachra 1999). In contrast other studies have reported an upregulation of TIMP-1 or -2 expressions in malignant tumours (Nakano 1995; Saxena 1995; Lampert 1998). Recently the expression of TIMP-4 was described as a marker of gliomas (Rorive 2010) and has also been shown to be upregulated with increasing malignancy.

1.7.3 Angiogenesis

Angiogenesis, another hallmark of malignant gliomas, is the formation of new blood vessels from the pre-existing microvasculature thus supporting the tumour mass (Auerbach 2003). The formation of new blood vessels is attributed to either vasculogenesis or arteriogenesis. Vasculogenesis is the embryonic process, however was also identified in tumours in the novo production of blood vessels from circulating marrow derived endothelial progenitor cells. Arteriogenesis refers to enlarged arteriolar networks produced to sustain increased oxygen demands and this process doesn't play significant role in tumour biology (Tate 2009).

Angiogenesis is a critical process for cell survival and development (Arias 2009) in particular during the development events in the female reproductive organs and wound healing. However, aberrant angiogenesis in the pathogenesis of numerous diseases including rheumatoid arthritis, diabetic retinopathy, Parkinson's disease, Alzheimer disease and cancer is also associated with excessive angiogenesis. In contrast, insufficient angiogenesis can be linked to stroke, infertility and heart disease. Normally endothelial cells, which line all blood vessels and constitute virtually the entirety of capillaries, divide rapidly in response to a physiological stimulus like hypoxia.

Tumour blood vessels differ from those formed in wound healing and from those in normal organs. The structure of these new blood vessels is abnormal with an irregular or increased diameter, thickened basement membrane, random branching, elevated permeability, lack the defining structural features of arterioles, capillaries or venules, and highly proliferative endothelial cells (Tate 2009). Additionally, it has been shown that abnormal blood vessels in gliomas create a vascular niche that houses glioma stem cells capable of giving rise to an entire tumour (Tate 2009). Unusually, despite the large size of some vessels, blood flow is typically poor and can change directions or even stop. The 'leaky' nature of these tumour vessels allows macromolecules to leave and may be the cause of metastasis by facilitating the movement of

tumour cells into the bloodstream. Though this is a rare consequence and may be attributed to the lack of Cluster of differentiation 15 (CD15). Blood vessel permeability can also lead to the accumulation of fibrin in the extracellular matrix thus creating a favourable environment for angiogenesis (Tuettenberg 2006).

Induced by hypoxia, a cause of oedema, and oncogenic mutations, vascular endothelial growth factor (VEGF) is one of the many factors involved in angiogenesis and is a primary stimulant (Brekken 2000). Some MMPs are thought to be mediators of both invasion and angiogenesis (Nuttall 2003). Other stimulants of anigiogenesis are summarised in Table 3.

Table 3 Some stimulants and inhibitors of angiognenesis.

Proteins	Inhibitors
Proteins	
	TIMP-1 (tissue inhibitor of metalloproteinase-1)
Vascular endothelial growth factor	
Angiogenin	TIMP-2 (tissue inhibitor of metalloproteinase-2)
Accidic fibroblast growth factor	
Basic fibroblast growth factor (bFGF)	TIMP-3 (tissue inhibitor of metalloproteinase-3)
Interleukin 8	
Hepatocyte growth factor	Angiostatin
Epidermal growth factor	Endostatin
Placental growth factor	Thrombospondin
Platelet-derived growth factor	Interferons
Transforming growth factor alpha	Platelet factor 4
Tumour necrosis factor alpha	
Scatter factor	
MMPs	
Small molecules	
Prostaglandins E1 and E2	
1-Butyryl glycerol	
Adenosine	
Nicotinamide	

VEGF increases vascular permeability leading to extravasation of plasma proteins and dissociation of pericyte coverage. VEGF is a great deal more active than histamine in enhancing vascular permeability (Nagy 1989). Studies have shown antibodies specific to VEGF reduced angiogenesis and almost completely inhibited the growth of human tumours in mice (Kim 1993; Asano 1995). Colorectal cancers have been known to produce VEGF this therefore may be an indication that other cancers also secrete the protein (Nakata. 1998). Studies have suggested the use of VEGF as a marker of tumour growth or recurrence (Fuhrmann-Benzakein 2000).

There are four main steps involved in angiogenesis (Krishna 2016; Paper 1998; Denekamp 1993) angiogenic factors are secreted by cancer cells (or adjacent tissues) which instigates the growth of a bud from the dissolved basement membrane. The basement membrane is a layer of specialized connective tissue that encircles capillaries and serves as the connection point between the extracellular matrix (ECM), it also provides structural support to the capillary. Vascular (endothelial) cells proliferate and migrate from the bud toward the angiogenic stimulus for example a low-oxygen (hypoxic) environment. Finally the sprout eventually forms a hollow tube (lumen) and joins its end with another sprout to form a new capillary vessel.

The key components of the first step of angiogenesis, the breaking down of existing blood vessels, are the Angiopoietin-1 and its receptor Tie-2, which in normal brain are bound together resulting in a close association between pericytes and endothelial cells that stabilise the vasculature. In hypoxic conditions Ang-2 is up-regulated in endothelial cells whereas Ang-1 is increased in tumour cells. Ang-2's increased expression seems to lead to the initial regression of blood vessels seen in early tumour angiogenesis. Tie-2 signalling up-regulates MMP-2 expression and in conjunction with VEGF promotes continuation of angiogenesis (Tate 2009) it is at this stage the vessel basement membrane and surrounding ECM are degraded allowing endothelial cell migration and proliferation (Tate 2009). Angiogenic stimuli

cause increased permeability through dissolution of adherens junctions (Haas 2012) between endothelial cells. Proliferation of these cells occurs early in angiogenesis and continues when the new capillary sprouts elongates. The survival and proliferation of endothelial cells is promoted by activation of PI3K/Akt through modulation of numerous cell cycle regulators like: cyclin D1, p27 and Bcl-X2. The MAPK signalling pathways (ERK1/2, p38 and JNK) mediate growth factor and mechanical force-induced proliferation. The proteolysis of basement membrane is necessary to promote invasion into the surrounding matrix and is controlled by proteolytic enzymes and its inhibitors. The MMP-2 and MMP-9 play major synergistic role in basement membrane degradation they are also promoting pro-angiogenic signalling by exposing endothelial cells to molecules such as VEGF and fibroblast growth factor (FGF). (Tate 2009) MMPs have a great affinity for fibronectin, elastin, laminins and collagens which are the major extracellular matrix components found in endothelial cell basement membrane and interstitial space. Following regression of existing vessels and breakdown of the basement membrane, endothelial cells proliferate and begin migrating toward tumour cells that express pro-angiogenic compounds. Activation of endothelial cells upregulates cell surface adhesion and migration molecules such as integrins and CD44. Upregulation of cell surface receptors, transmembrane heterodimer proteins integrins αvβ3, α5β1 and αvβ5 in addition to cell migration increases cell adhesion and survival (Tate 2009) Activated endothelial cells secrete platelet-derived growth factor (PDGF) that ‘recruit’ smooth muscle cells and pericytes to the site of newly sprouting vessels to aid in establishment of a new basement membrane (Tate 2009). Pericytes play a major role in vascular stability by inducing the deposit of a matrix and initiating signals allowing cell differentiation and quiescence. The complex final steps of tumour angiogenesis involve a dramatic changes in extracellular environment and are not well understood (Tate 2009). Formation of new blood vessel is accomplished by alignment of EC in bipolar mode, then tubular morphogenesis and finally the lumen formation. In the following step the vascular loops are formed from connected sprouts and blood begin to flow through the new vessel loop.

Judah Folkman (1971) introduced the concept of angiogenesis in 1971. He postulated that if a tumour was deprived of new blood vessel formation the tumour would only grow a few centimeters in diameter, thus reducing malignant progression and the problems faced by current treatment. Consequently an anti-angiogenic therapy would manage tumour development and aid current treatment.

1.8 Apoptosis

A high rate of angiogenesis in brain tumours is accompanied by a severe reduction in apoptosis (programmed cell death) a pathway which ideally would be activated during treatment. However, apoptotic resistance is a classic feature of gliomas which result in accumulation of these mutations. Apoptosis was discovered in the early seventies, before this necrosis was thought to be the only form of cell death. The importance of studying apoptosis in cancer remained unappreciated for nearly 2 decades. Now it is well documented that most cytotoxic anti-cancer agents induce apoptosis (Boik 2001). Apoptosis is mediated by a family of cysteine aspartic acid-specific proteases or caspases (Fig 5). Caspases exist within the body, as inactive precursors of their active forms (zymogens). Upon activation these zymogens engage in various pathways resulting in apoptosis. They are divided into three groups; apoptosis effectors (including 3, 6 and 7), apoptosis initiators (including 2, 9, 8 and 10) and cytokine maturation (1, 4, 5, 11, 12, 13 and 14) (Strasser 2000; Cryns 1998). Activation of the apoptosis pathways may be initiated in one of two modes; mitochondrion (intrinsic) pathway and Death Receptor (extrinsic) pathway (Fig 5).

The intrinsic pathway involves the disruption of the mitochondrial membrane. Consequently a number of mitochondrial proteins are released including cytochrome c. Cytochrome c in conjunction with apoptotic protease activating

factor -1 (Apaf-1) and zymogen procaspase 9, promote the assembly of apoptosomes; cytochrome c, Apaf-1 and caspase 9 complex, ensuing the activation of caspase 9 which consequently initiates the apoptotic caspase cascade.

The extrinsic pathway employs a death receptor; a transmembrane receptor (including Fas, TNF and TRAIL) which upon ligation with its respective ligand (FasL, TNF and TRAIL) activates caspases 8 and 10. These subsequently cleave and activate effector caspases 3 and 7 resulting in apoptosis. Unlike the intrinsic pathway the extrinsic pathway is independent of the p53 protein. It has been reported that glioma cell cultures do not appear to activate the extrinsic apoptotic pathway in response to irradiation or cytotoxic drugs (Steinbach 2004), instead they display a high expression of inhibitor proteins, which may be responsible for the failure of glioma cells to activate caspases in response to apoptotic stimuli.

There have been tremendous advances in the understanding of apoptosis, particularly, in the identification of molecules involved and understanding of function and pathways (Bogler, 2002; Lowe, 2000). The protein p53, is commonly associated with defects in cancerous tissue. It is an example of a molecule implicated in regulation of apoptosis (Pucci, 2000; Smith 2000). p53 acts as a scanner for proliferating cells. Minor damage to DNA is repaired and cells with major DNA destruction are destroyed by apoptosis. It also facilitates the process of differentiating cells to leaving the cell cycle. Many brain and systemic tumours have shown mutation and/or loss of the p53 gene suggesting that cycling cells accumulate defective DNA material. p53 is implicated in the intrinsic pathway of apoptosis. Increasing activation of the p53 molecule could induce a pro-apoptotic effect.
It has been reported that the protective gene p53 is activated by progesterone therefore redudcing the risk of cancer. As RCE mimics the structure of progesterone it would be assumed to induce activation of p53 and as such apoptosis

Tumour cell death by cytotoxic approaches such as anticancer drugs, γ irradiation, suicide genes or immunotherapy, is predominantly mediated through induction of apoptosis (Boik 2001; Fulda 1998; 2001; 2002; Hengartner 2000; Kaufmann 2000; Herr 2001; Debatin 2002; Johnstone 2002). Despite the concept that anticancer drugs act by inducing cell death, defects in apoptosis programs may contribute to tumour progression and treatment resistance (Cory 2002; Igney 2002).

Resistance to apoptosis is seen in the normal brain, where differential resistances exist (Salgado 2007) and increases during malignancy. Migrating cells in the marginal zones of the GBs do appear to be more sensitive to apoptosis (Lefranc 2005) illustrating an inverse correllation between resistance to apoptosis and migration (Sarafian 2009). Such a phenomenon is attributed to the increased expression of $\alpha V\beta 3$ in the marginal infiltrating zones where it acts as a regulator of invasion, proliferation and angiogenesis (Bogler 2005), confirming the inverse relationship between apoptosis and angiogenesis.

There is little evidence that the current modes of coventional treatment mediate their effects via induction of apoptosis (Steinbach 2004). Thus pro-apoptotic novel therapies are increasingly studied. One such therapy is Clomipramine Hydrochloride (CLOM), a tricyclic antidepressant. In use for over thirty years, it has the ability to induce apoptosis in malignant glioma cells *in vitro* (Daley 2005). The induction of apoptosis when using CLOM is associated with the intrinsic pathway (Daley 2005; Levkovitz 2005; Ekert 2004).

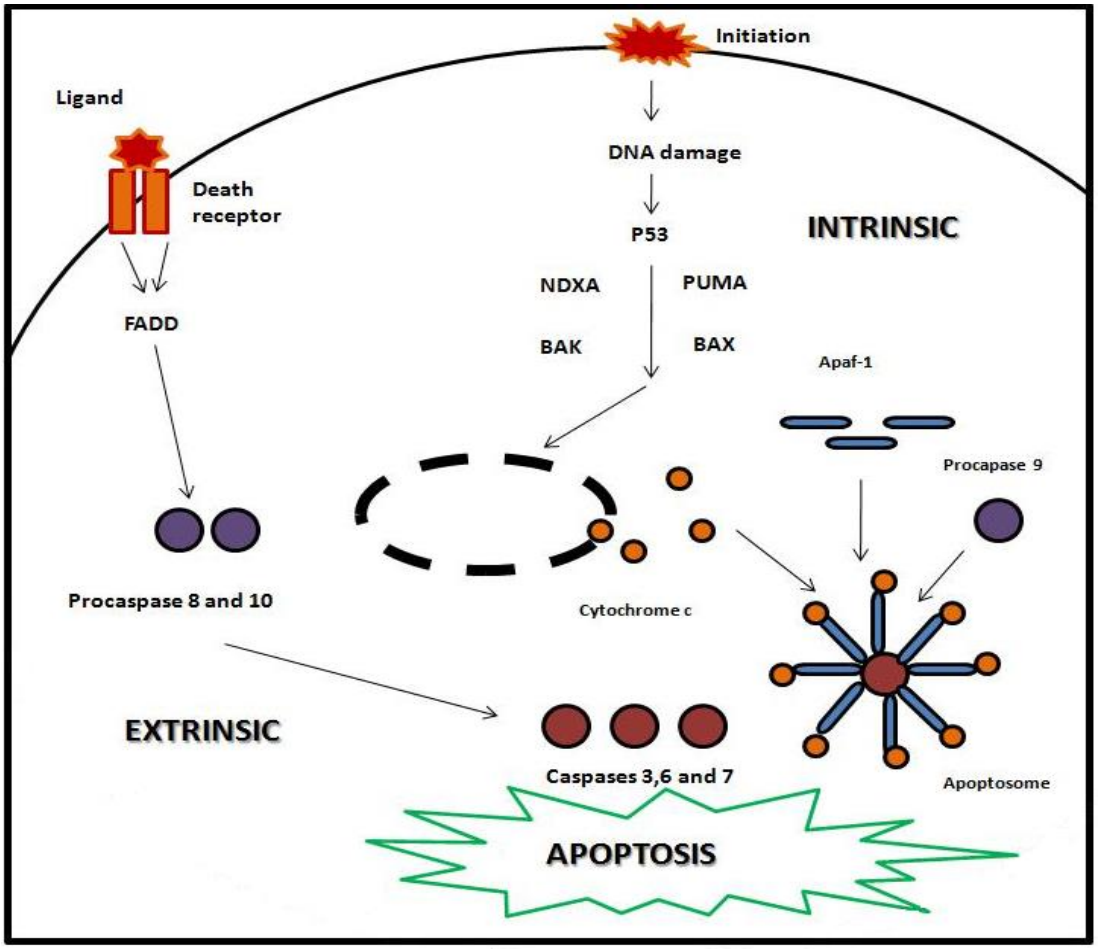


Figure 5: Apoptosis initiation pathways both extrinsic and intrinsic including caspases involved.

1.9 Conventional Treatment

Current conventional therapies for malignant gliomas include neurosurgery followed by radiotherapy. Previously, chemotherapy was only offered to these patients at recurrence to provide adjuvant and palliative support (Grant 2004; Grossman 2004; Rampling 2004), though it is now offerefed concomitantly with radiotherapy. Provided as a combination these therapies are known as the gold standard treatment.

1.9.1 Neurosurgery

Neurosurgery is not curative as local recurrence occurs due to the ability of such tumours to invade the contiguous nervous tissue instead surgery aims to histologically diagnose, debulk or resect as much of the tumour mass as possible to relieve symptoms created by the presence of the tumour. Risks associated with neurosurgery include post operative paralysis, brain damage, infection, psychosis and even death.

However, over the years new sophisticated developments in surgery have reduced the potential risks associated with neurosurgery. For example, the availability of corticosteroids in the 1960s reduced pressure in the brain thus easing the debulking process. Sterotactic frames (Kelly 1986) allowed a greater precision in locating exact sites of tumours thus reducing the space operated on. Stereotactic surgery is commonly used to operate of ill-defined tumours, small tumours or those that exist in locations which are difficult to operate in and to map and define expressive tissue (Salcman 1990; Moore 1989). Stereotactic surgery is also favoured when deriving biopsies for diagnosis purposes.

The use of real time MRI (RT-MRI) and micro cameras have allowed greater precision allowing the surgeon to identify not just the location of the tumour but also the quantity of tumour mass removed thus avoiding unnecessary

removal of brain tissue (Carpentier 2008). The invention of microsurgical tools have also allowed for greater accuracy at the tumour border. Various other surgical techniques using laser beams have recently been produced these are less invasive processes.

Complete surgical resection of the tumour with sufficient margins of adjacent normal tissue cannot be achieved due to the diffuse mode of local spread so most gliomas are reported to re-grow (Berger 1996). As such radiotherapy is considered.

1.9.2. Radiotherapy

Radiotherapy, although considered to be aggressive, is the most effective of the three therapies. It is give to patients with malignant brain tumours, when the cels are rapidly dividing. Though it is most commonly administered in conjunction with neurosurgery with the intention of killing any residual cells, it is only admistered once, due to the resitrait of the body against radiation. Radiotherapy damages the DNA using the cytotoxic affects of X-rays or γ–rays, produced by megavoltage linear accelerators or nuclear particles such as cobalt^{60} and caesium^{137}.

Randomized studies have shown 50 to 60 Gy post operative radiation of the whole brain increases median survival time from 14 weeks to 36 weeks (Shapiro 1976; Walker 1980; Walker 1978) however these quantities have been associated with radiation injury (Walker 1979; Bleehen 1980; Chang 1983) various studies are continually being carried out to reduce radiation injury and neurotoxicity however results represent selected patients with local diseases have a significant impact as shown in a study by *Sarkaria et al* (1995) who underwent a clinical trial of a novel interstitial radiation device placed after debulking. Further studies in this field are required to reduce toxicity and unnecessary necrosis. Also, new approaches to improve treatment of brain tumours, incorporating radio-chemotherapy after surgery are being investigated.

1.9.3 Chemotherapy

Chemotherapy is an adjuvant therapy which was given to patients with high risk of recurrence, though now is commonly concomitant with radiotherapy. Chemotherapy may improve survival albeit slightly, but is associated with significant toxicity, however important advances in the development and use of cytotoxic drugs have been made recently including the use of Temozolomide (an oral alkylating agent) (Gilbert 2013; Stupp 2005), carmustine (gliadel wafers) (Westphal 2003; Westphal 2003) and Avastin (Junck 2011). The advantage of chemotherapy over other therapies is that it is administered when the cells are at their most vulnerable; after surgery.
Success with chemotherapy relies on three fundamentals; debulking must have occurred, the chemotherapy agent should be available and chemotherapy should be administered immediately after surgery at the maximum dose tolerated and for a limited time only (Grossman 2004). Unfortunately, these prerequisites are not always achieved. For example, during the therapy of brain tumours, patients are put on adjuvant trials after partial resections and stereotactic biopsies the cytotoxic drug is required to act upon a larger number of impervious cells. Chemotherapy can be administered either individually or as a combination of cytotoxic drugs. Some of which include carmustine, lomustine, Irinotecan, Methotrexate, Carboplatin, Vincristine, Procarbazine and the newly developed Temozolomide.

Having demonstrated the ability to cross the blood-brain barrier (Ostermann 2004; Patel 2003), temozolomide, was considered for glioma therapy, after phase I and II clinical trials indicated that temozolomide has considerable potential in treating gliomas and improving the quality of life of patients (Bower 1997; Newlands 1996; Paulsen 1999). *Professor Malcolm Stevens et al.* introduced this novel molecule in the late 1980s.

Temozolomide, is a new class of second-generation imidazotetrazine prodrugs, it undergos spontaneous conversion under physiological conditions to the active alkylating metabolite methyltriazeno-imidazole-arboxamide

(MTIC) (Stevens 1987) and as such, does not require hepatic metabolism for activation (Clark 1995). These agents are structurally unique as they contain three adjacent nitrogen atoms that confer unique physicochemical properties and much greater antitumor activity (Stevens 1987). The principle mechanism responsible for temozolomide cytotoxicity seems to be the methylation of DNA. The active alkylating agent MTIC, degrades to the methyldiazonium cation, and transfers the methyl group to DNA (Spassova 1985; Denny 1994). The methylation of the O^6 position of GMP in DNA, although relatively infrequent (6-7%), is usually regarded as the lethal lesion (Zhang 2006; Danson 2001; Newlands 1996; Friedman. 2002; Nagasubramanian. 2003). *Hegi et al*. (2005) did a correlative study on specimens from EORTC 26981/NCIC CE.3 to examine the association of O^6-methylguanine-DNA methyltransferase (MGMT) gene promoter methylation status with survival. MGMT is cytoprotective, it repairs methyl adducts at the O^6 position on guanine in DNA, and transfers the methyl group to an internal cysteine acceptor residue. This reaction results in irreversible inactivation of MGMT, requiring increased *de novo* protein synthesis to restore repair activity. Restoration of MGMT activity is a relatively rapid event, usually occurring within several hours, in human malignant brain tumors (Schold 2004). The effectiveness of temozolomide, therefore, is only assumed to be significant with daily repetitive dosing schedules predicted to be most successful at depleting MGMT and thereby enhancing its cytotoxicity. As such it was reported the effect on survival of combining radiation and temozolomide was most marked in glioblastoma patients whose tumors had MGMT promoter methylation, those without did not appear to receive significant benefits of the combined therapy.

The methyldiazonium cation can also react with RNA and with soluble and cellular protein (Bull 1987). However, the methylation of RNA and the methylation or carbamoylation of protein do not appear to have any known significant role in the antitumor activity of temozolomide (Friedman. 2000).

Studies into the efficacy of temozolomide as an adjuvant to radiotherapy (Stupp 2005; Stupp 2002) to improve survival time by 14.6 months after primary surgical resection. When comparing the effects of temozolomide as adjuvant therapy with radiation and radiation alone, adjuvant therapy improved survival rates with approximately 9 % of patients surviving 5 years (Stupp 2009). In addition, temozolmide taken with other chemotherapies has been shown to be efficacious (Vredenburgh 2009), however may also exhibit increased adverse effects (Saran 2016).

Studies into administrating the chemotherapy systemically has led to the production of carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea) wafers or Gliadel wafers which are inserted after debulking into the surgical bed with the aim of destroying any residual cells in the vicinity of the surgical bed. Gliadel wafers are increasingly a popular approach for delivery of chemotherapy. These biodegradable, impregnated polymers are implanted in the tumor bed at the time of resection, providing a controlled release of local chemotherapy for approximately 3 weeks (Brem 1996), while minimizing systemic adverse effects (Tamargo 1989; Fung 1998; Ewend 1996; Ewend 1998; Brem 1992; Withrow 2004). Many studies on the use of Gliadel wafers for the treatment of both primary and recurrent gliomas have shown improved survival with no marked increase in adverse effects (Brem 1991; Brem 1995; Sipos 1997; Olivi 2003; Westphal 2003), though concern about associated complications have arisen, both in published case reports and in a small case series (Weber 2005; McGirt 2002; Engelhard 2000; Subach 1999) . These reported complications include malignant cerebral edema, resection cavity cyst formation, cerebrospinal fluid (CSF) leak, wound healing abnormalities, and increased perioperative seizure activity (Engelhard 2000). Gliadel wafers were reported to increase median survival of >13 months in primary malignant glioma patients (Westphal 2003). Recent studies reported Gliadel wafers with concomitant temozolomide was not only well tolerated by patients but also resulted in a median survival of >18 months (McGirt 2009; Larocca 2005).

1.9.4 Avastin: An Anti-Angiogenic Treatment

Another mode, of increasing interest within the field of conventional therapy, are molecular targets, in particular the drug Avastin (Bevacizumab). Avastin targets the molecule vascular endothelial growth factor A (VEGF-A). Malignant gliomas are highly vascularized tumors. Angiogenesisis is one of the pathological hallmarks of GBs. One of the difficulties of developing effective treatments for gliomas has been poor drug penetration through the blood-brain barrier. By targeting tumor vasculature, theoretically it may be possible to bypass this dependence of drugs to cross the blood-brain barrier to reach their target. Both experimental (Kamoun 2009; Wong 2010) and clinical (Friedman 2009; Kreisl 2009) evidence have reported anti-angiogenic drugs decrease vasogenic edema and patients' requirement for corticosteroids which are a significant cause of morbidity.

Avastin is a humanized monoclonal antibody that binds to, a stimulant of angiogenesis, VEGF-A, and stops tumor growth by preventing the formation of new blood vessels. Avastin may work by causing regression of existing microvessels, helping arrest tumor growth and reduce tumor size, ‘normalization’ of surviving mature vasculature, making the tumor vasculature more conducive to effective anti-cancer therapy and inhibition of neovascularization (Mukherji 2010).

Avastin was first approved in combination with chemotherapy in the treatment of lung (Johnson 2004), breast (Miller 2007), renal (Yang 2003) and colorectal cancers (Berretta 2008; Hurwitz 2004). Investigation into the use of Avastin in the treatment of GBs has yielded positive results and in 2009 the FDA granted approval for Avastin to be used in the treatment of GBs following prior therapy (Cohen 2009). Studies combining Avastin and chemotherapy drugs, in particular irinotecan and temozolomide reported improved overall survival times.

Despite initial reluctance to investigate Avastin in patients with brain tumors owing to concerns of intracranial haemorrhage, a series of 29 patients with recurrent malignant gliomas treated with Avastin and irinotecan showed no significant haemorrhage (Stark-Vance 2005). *Schiff et al*. (2008) reported Avastin in combination with irinotecan, when compared to Avastin alone not only reduced progression but also improved survival rates. However, irinotecan had been previously tested as a single agent in phase 2 trials and was found to have low levels of progression free survival. These results led to investigation of the use of Avastin alongside temozolomide. Although this combination was also well tolerated, it did not improve median overall survival or progression free survival (Verhoeff 2010, p1723). Investigation into combinations with Avastin and other chemotherapy drugs is on-going.

1.10 Conventional Medicine Clinical Trials.

Conventional medicine is continually being investigated and evaluated. Globablly clinical trials into the efficacy of various drug combinations are being performed. Currently in the UK alone there are approximately 20 clinical trials recruiting adults for glioma therapy research. Table 4 summaries a few of the trials currently recruiting.

Gliomas display several biological features that succesfully evade conventional therapy. Due to these complications, despite continuing clinical trials and experimentation, conventional medicine has only made two novel advances in the past three decades, namely Temozolomide and Avastin.

Table 4 A brief summary of the top 10 clinical trials being performed for the use of conventional medicine as treatment for brain tumours, in the UK in 2016 (Clinicaltrials.gov).

Trial therapy	Phase
Olaparib and Temozolomide in Treating Patients With Relapsed Glioblastoma	I
Cediranib Maleate With or Without Gefitinib in Treating Patients With Recurrent or Progressive Glioblastoma	II
Erlotinib Compared With Temozolomide or Carmustine in Treating Patients With Recurrent Glioblastoma Multiforme	II
Acridine Carboxamide in Treating Patients With Recurrent Glioblastoma Multiforme	II
Bevacizumab in Recurrent Grade II and III Glioma	II
Temozolomide Compared to Procarbazine, Lomustine, and Vincristine in Treating Patients With Recurrent Malignant Glioma	III
Imatinib Mesylate in Treating Patients With Gliomas	II
Enzastaurin and Temozolomide in Treating Patients With Primary Gliomas	I
Study of Nivolumab Versus Temozolomide, Given With Radiation Therapy, for Newly-diagnosed Patients With Glioblastoma (GBM, a Malignant Brain Cancer)	III
Study of Temozolomide Plus Radiation Therapy With Nivolumab or Placebo, for Newly Diagnosed Patients With Glioblastoma (GBM, a Malignant Brain Cancer).	II

1.11 Requirements of New Therapies

Treatment for GBs to date, regrettably has not been successful in curing these debilitating neoplasms, though a number of possible therapies have been investigated, for their management, in both the conventional (including temozolomide, gene therapy and stem cells) and complementary (including flavonoids) fields. For an agent to be of therapeutic value for brain tumour management it should not only be cytotoxic, anti-invasive, anti-angiogenic or pro-apoptotic but most importantly it should be able to cross the blood-brain barrier (BBB).

1.12 Use of Complementary and Alternative Medicine

Complementary and alternative medicine refers to those health care practices and products that are not considered part of conventional medicine. The words 'complementary' and 'alternative' are used in conjuction, suggesting they mean the same thing. Although they are a combined phrase i.e. complementary and alternative therapies' (CAM), but there is an important difference. It is not always easy to decide whether something is a complementary or alternative therapy.

Originally described by *Eisenberg et al*. (1998) as interventions neither taught widely in medical schools nor generally available in US hospitals, CAM has undergone a radical change of thought and has recently been described by *Ernst et al* (2008) as diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole, by satisfying a demand not met by orthodoxy or by diversifying the conceptual frameworks of medicine.

A complementary therapy is a therapy which can be used alongside conventional medical treatment. They aim to decrease morbidity and help cope with treatment. An alternative therapy is generally used instead of conventional medical treatment. The main difference between alternative and

conventional treatments is all conventional cancer treatments have to go through rigorous testing by law in order to prove that they work though most alternative therapies have not been through such testing. As such it some alternative therapies may cause previously unknown side effects.

There are many different types of complementary therapy, including aromatherapy, acupuncture, massage therapy, visualization, yoga and herbal medicines. Health care professionals are generally supportive of the use of complementary therapies, though some can be reluctant of their use as many have not been scientifically tested in the same way as conventional treatments. Various research trials have been performed though more studies and consistency of testing is needed to help develop knowledge on the optimum function and use of complementary therapies. Unlike complementary therapies, alternative therapies are used instead of conventional medical treatment. People with cancer have various reasons for wanting to try alternative therapies, some may not start conventional treatment and may choose to use an alternative therapy instead and others stop conventional cancer treatment and switch to an alternative therapy. Currently investigation on complementary and alternative medicines (CAM) in the management/treatment of, not only brain tumours but also many other aliments including other cancers, heart disease, asthma and many more, is actively being performed.

The prevalence of CAM is increasing every year; one or more CAM therapies are used by 46% of the UK population in their lifetime and 10% annually visit a CAM practitioner (Thomas. 2004; Thomas 2001). CAM, in the UK, has been divided into three groups by the government; professionally organised alternative therapies, complementary therapies and alternative disciplines which is sub divided into; Long-established and traditional systems of healthcare and other alternative (House of Lords; Science and Technology, sixth report, 2001).

CAM has widely been investigated as part of a therapeutic regime against cancer. Up to 90% of cancer patients use one or more forms of CAM,

including relaxation and meditative techniques, vitamins and herbal medicines and physical or movement therapies (Shen 2002; Richardson 2000; Navo 2004; Swarup 2006; Vapiwala 2006). Specifically, evaluation of the primary brain tumour patients found that approximately 40% use one or more forms of CAM (Armstrong 2006; Heese 2010) in particular 39% of patients chose to employ homeopathy (Heese 2010). Another common CAM modality, chosen by patients, is Chinese herbal medicine. Chinese herbal medicine has long been used for treating malignancies, side effects of conventional treatment and as adjuvant therapies (Hedigan 2010; Chiaramonte 2010).

Despite the current popularity of CAM, most mainstream oncologists have very little understanding of the therapies (Newell 2000). CAM is also not aways encouraged due to a lack of herbal standardization, quality control issues, safety and toxicity concerns, interactions with existing therapeutic modalities, a lack of proven efficacy by standard clinical trials and a lack of mechanistic details, to name a few (Azaizeh 2006) this therefore disheartens patients who believe a greater interest and understanding, in CAM, should be offered by doctors (Gray 2002).

The increased interest in CAM can be attributed to several factors including the suggested benefits of CAM for cancer patients, the desire to take control of their illness and feel hopeful, the notion that CAM modalities are safe and nontoxic, the ease with which they may be obtained and the decreased faith in conventional medicine to meet patient needs. (Balneaves 1999; Henderson 2004; Munstedt 1996; Navo 2004; Ponholzer 2003; Richardson 2000; Richardson 2002; Sparber 2000; Verhoef 1999; Verhoeff 2010; Hilsden 1999; White 2002). Expectations for CAM are quite high with patients expecting CAM to improve their quality of life, alleviate symptoms, prolong life and cure their disease. Those with incurable diseases, particularly value CAM for its medicinal potential (Richardson 2000; Ernst 1998; Eisenberg 1998; Kaptchuk 1998).

Health professionals in general do express positive views when CAM is used complementarily and not as an alternative. Results so far published have

shown that CAM can play a role in improving the quality of life and general well-being (Adams 2004; Adams 2011). CAM modalities are also involved in supportive care (Leis 2007). In recent years, naturally occurring compounds present in diet and beverages, known as nutraceuticals, have gained considerable attention because of their beneficial effects on health as cancer chemopreventive or cardioprotective agents. Nutraceuticals play an important role, in CAM, as a source of effective anti-cancer agents currently over 62% of currently used anti-cancer agents are derived in one way or another from natural sources (Gonzales 2006), including phytochemicals

1.13 Phytochemicals and Cancer

Phytochemicals are nutraceuticals which derive from plants products. Successful studies are being conducted *in vitro* to show a wide range of antioxidant phytochemicals, (including flavonoids, polyphenols and carotenoids) are radioprotective in experimental systems (Paul 2010; Weiss 2003; Borek 2001). Some phytochemicals induce apoptosis in cancer cells (Banjerdpongchai 2016; Mehdinezhad 2016) such as, including prostate cancer cells and breast cancer cells (Pinto 2001), colon cancer cells (Zheng. 2003) and ovarian cancer (Zhang 2016). Additional research is needed to establish the role of phytochemicals in conjunction with cancer treatment by radiation as antioxidant supplementation during radiation therapy may protect cancer cells, in conjunction to normal cells, and reduce the efficacy of treatment (Borek 2004).

There are also reasons for thinking that some herbal supplements might biochemically interfere with chemotherapy as well as radiation treatments, (Labriola 1999) alter treatment compliance (Durant 1998). Patients taking chemotherapeutic drugs and CAM are at least 27% at risk of developing clinically relevant CAM–drug interactions (Welder 2006). As such health professionals are reluctant to recommend CAM. An increasing number of CAM practitioners, though, aid understanding and knowledge of any drug interactions between CAM and conventional therapies.

1.13.1 Flavonoids

Flavonoids are commonly found in foodstuffs including vegetables, fruit, nuts, seeds, wine and tea, and it is estimated that the daily intake in the western diet ranges from 200mg/day to 1gram (Hertog 1995; Pierpoint 1990).

The basic structure comprises two benzene rings linked by heterocyclic pyran or pyrone ring in the middle. These agents display many therapeutic properties including anti-inflammatory, antioxidant, antiviral, and anticarcinogenic activity. Certain flavonoids have been found to exhibit anti-proliferative properties against some cancers and are known to cross the BBB (Zbarsky 2005; Datla 2001).

1.14 CAM: A Thearpy for Gliomas

1.14.1 CAM in clinical trials

The rising use of CAM has also had an impact on the number of clinical trials performed. Clinical studies can provide CAM modalities the necessary scientific accreditation for use as therapies. Table 5 summaries the limited clinical trials currently being performed in brain related therapies.

Table 5 A brief summary of a fraction of clinical trials currently being performed for the use of CAM in oncology and brain related therapy in 2016.

Trial	Phase
Complementary and Alternative Medicine Usage Among Participants Enrolled in Phase I Oncology Clinical Trials	I
Measuring Concerns of Cancer Patients Referred to Complementary Medicine Treatment Integrated Within Oncology Service	
Oregon Center for Complementary and Alternative Medicine in Neurological Disorders	
Improving Hand Use in Multiple Sclerosis	II
Nutritional Supplements & Complementary/Alternative Medicine by Prostate & Breast Cancer Patients	
Dietary, Herbal and Alternative Medicine in Glioblastoma Multiforme	II

1.15 Evaluation of Micronutrients in Glioma Therapy

The current study is part of a clinical trial which involves the use of micronutrients (vitamins, minerals, flavonoids and other agents found in food), in brain tumour management (Rooprai 2003). The research is based upon the original hypothesis and studies within other groups, which was that naturally occurring compounds such as flavonoids may effectively serve as anti-glioma agents (Dissanayake 2016; Li 2016; Kiekow 2016; Aroui 2016; Wang 2015). This efficacy is based on the ability of citrus flavonoids (particularly tangeretin) to interfere with underlying mechanisms of tumour invasion *in vitro* (Rooprai 2001). The clinical trial involves a combination of 7 micronutrients; tangeretin, chokeberry extract, lycopene, selenium, turmeric, red grape seed extract and red clover extract. Earlier *in vitro* studies have indicated that chokeberry extract, tangeretin and selenium have promising therapeutic potential for malignant gliomas *in vitro*.

1.16 Red Clover Extract

Red clover extract is a green powder extracted from the red clover plant (appendix). Its two main constitutes are the isoflavonoids, commonly also found in soya are genistein and daidzein. In addition, red clover extract also contains the precursors of the above named isoflavonoids; Biochanin A and formononetin respectively. The two precursors have a methylated structure, which must be demethylated to become the active forms. This is a successful process, *in vivo*, as demonstrated by *Setchell et al* (2001).

Genistein and daidzein mimic oestrogen by binding to the oestrogen receptor in cells (Adams 2012) and been shown not only have ability to cross the blood brain barrier (Moskot 2014; Upadhyay 2014) but also to exhibit the same the neuroprotective actions and functions in the brain (Kostelac 2003).

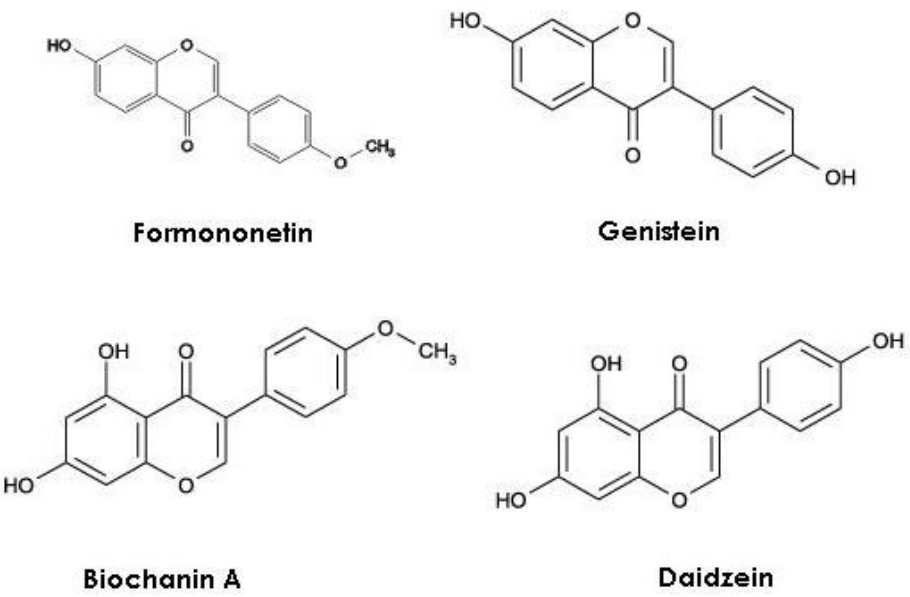


Figure 6 The isoflavonoids of Red Clover Extract. Genistein and Daidzein are the active components and Formononetin and Biochainin A are their respective precursors**.**

In addition to affecting the pathways effected by activation of the oestrogen recepetor, genistein in particular, has been shown to inhibit the nuclear protein topoisomerase II (Okura 1988), platelet-activating factor- and epidermal growth factor-induced expression of c-fos (Tripathi 1992), diacylglycerol synthesis (Akiyama 1987), and tyrosine kinases (Dean 1989). Additionally, it inhibits microsomal lipid peroxidation (Jha 1985) and angiogenesis (Fotsis 1993).

1.16.1 Red Clover Extract and Cancer

Due to the isoflavonoids being structurally related to oestrogenic steroids and thus having the ability to mimic oestrogen and bind to its receptor sites, this provide the rationale for lower rates of hormone related cancers such as

breast, prostate and ovarian cancers(Zhao 2016; Marcela 2016; Yang 2016; Kang 2016; Wang 2015)..

The active isofalonioids found in red clover extract have also been shown in a vast number of studies to inhibit a number of non-hormone dependent cancers including leukaemia (Zhang 2012), liver cancer (Zhou 2016), lung cancer (Yang 2016) and stomach cancer (Huang 2014).

Kang et al (2016), demonstrated that the consumution of genistein, through soy milk, decreased the proliferation of human prostate cancer cells by regulating p21 (component of cell cycle regulation). Other studies like *Shen et al* (2000) also found that genistein was also anti-proliferative in prostate cancer. The study found the cells became arrested in the G_1 phase of the cell cycle thus ceasing proliferation, however unlike in breast cancer; it did not promote a pro-apoptotic effect in prostate cancer.

Similar effects on cancer cell proliferation have also been noted in non-hormone related cancers in humans such as osteosarcomas (Hu 2015), lung cancer (Liu 2015), pancreatic cancer (Han 2012) and colon cancer (Yoo 2015).

In a 2008 study, *Li et al* (2008) illustrated that genistein inhibits cell proliferation and promotes apoptosis via the intrinsic pathway in breast cancer. An earlier study carried out in 2003 by *Gong et al.* noted a similar finding. A study analyzing testicular cancer cells demonstrated an enhanced expression of caspase 8, caspase 3 and PARP, suggesting genistein exhibited a pro-apoptotic effect on these cells (Al-Maghrebi 2016). Laryngeal cancer cell lines have also exhibited pro-apoptotic effects when treated with genistein (Du 2016).

Fortunately, in 2008, *Zhang et al*, found that genistein was anti-invasive, on prostate cancer. Genistein has also been found to induce apoptosis via the extrinsic pathway in ovarian cancer cells (Thasni 2008).

As the effects of the isoflavonoids, found in RCE, have been increasingly positive when looking the anti-invasive and pro-apoptotic properties in other cancers, though few studies have been carried out on the effects of genistein or daidzein on brain tumours and no studies have been performed on the effect of red clover extract on brain tumours this warrants further investigation.

1.17 Red Grape Seed Extract

Red grape seed extract is a red powder (appendix) containing a number of active flavonoids including catechins, epicatechins and epicatechin (-3-0-) gallate or collectively polyphenols (Polyphenolics.com). *In vivo* these flavonoids exist as monomers, dimmers and/or oligomers. They are active in conjunction with one another rather than as separate entities i.e. in oligomeric form.

Polyphenols are important components of dietary agents that have been demonstrated by epidemiological, preclinical, and clinical studies to have various health promoting benefits, including neuroprotective (Malhotra 2016), cardioprotective (Zhang 2015), and chemopreventive effects. These regulate various cellular processes, such as cell proliferation, cell cycle, cell survival, and apoptosis that have been implicated in cancer prevention and treatment.

1.17.1 Red Grape Seed Extract and Cancer

Its active ingredients, oligomeric proantho-cyanidins (OPCs) are known to be anti-angiogenic (Boik 2001) and have also been shown to inhibit growth of cancerous cells.

Eng et al (2003) suggested the use of RGSE as a chemo protective agent for breast cancer as it arrests the cells in the G_1 phase of the cell cycle. Similarly prostate cancer also undergoes a block in tumour cell growth when treated

with RGSE (Singh 2003). *Yao et al* (2016) analyzed the effect of proantho-cyanidins in nasopharyngeal carcinoma. This study found not only a reduction in the viability of cells but also the induction of cell cycle arrest at the G2/M phase causing apoptosis. Furthermore, upon examination of the mechanism of apoptosis they noted a increased expression of the pro-apoptotic protein Bax, cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PRAP) protein, along with a decrease expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL. Other examples of studies with demonstrate the increase in apoptotic potential following treatment with either grape seed extract or proantho-cyanidins (Zhou 2016; Kumar 2015; Chen 2014;Parsad 2012) illustrated similar mechanisms of action in other cancer types.

In 2004 *Singh et al* also found that grape seed extract induced apoptosis and reduced angiogenesis in prostate cancer cell. Much like *Singh et al*, *Zheng et al* (2015) demonstrated a reduction in angiogenesis following rtreatement with an OPC. They looked at human astrocytoma cells and human hepatoma cells following treatment with oligomer procyanidins (F2), a fraction extracted from grape seeds. They noted that EGFR-PI3K-AKT-mTOR and MAPK-ERK1/2 pathways were inactivated and vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP)-2 expressions were supressed, ultimately reducing the presence of angiogenesis. Other studies also demonstrated a reduction in the expression of VEGF and MMPs (Stagos 2015; Kanavi 2014; Kuroyanagi 2014).

Metastatic cancers, when treated with a form of grape seed extract also tend to have similar outcomes; anti-proliferative effects and pro-apoptotic effects (Rivera 2016; Martinez 2005).

Much like RCE, although components of grape seeds have been reviewed for their effects on brain tumours, RGSE has not yet been investigated for its anti-glioma effects. As grape seed extracts and their components have anti-cancer effects RGSE warrants investigation into it’s anti-glioma effects. Preliminary studies have indicated that this extract has got anti-invasive potential in brain tumours (Rooprai et al. personal communication).

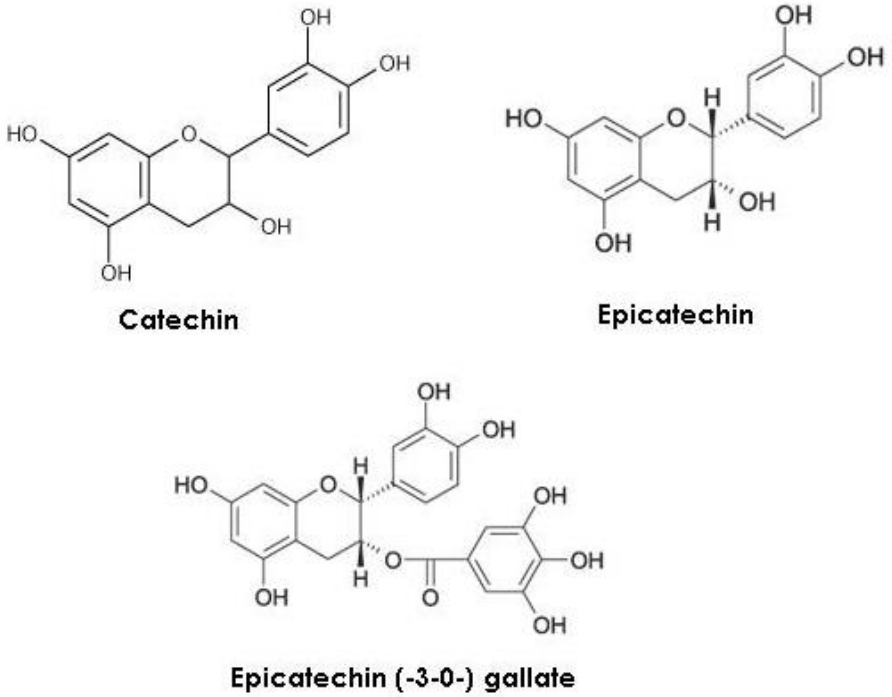


Figure 7 The isoflavonoids of Red Grape Seed Extract.

1.18 Aims

The current study is part of a clinical trial which involves the use of micronutrients (vitamins, minerals, flavonoids and other agents found in food), in brain tumour management (Rooprai 2003). The research is based upon the original hypothesis which was that naturally occurring compounds such as flavonoids may effectively serve as anti-glioma agents. This efficacy is based on the ability of citrus flavonoids (particularly tangeretin) to interfere with underlying mechanisms of tumour invasion in vitro (Rooprai 2001). The clinical trial involves a combination of 7 micronutrients; tangeretin, chokeberry extract, lycopene, selenium, turmeric, red grape seed extract and red clover extract. Earlier in vitro studies have indicated that chokeberry extract, tangeretin and selenium have promising therapeutic potential for malignant gliomas *in vitro* (Personal communication; Rooprai 2001).

The aims of the proposal are to evaluate the anti-invasive, pro-apoptotic and anti-angiogenic potential of red clover extract and red grape seed extract on a number of biopsy-derived GBs by answering the following questions;

- At what concentration is the viability of the cells affected?
- Do the micronutrients affect cell invasion?
- Is the expression of cell adhesion molecules impacted by the micronutrients?
- Do the micronutrients affect apoptosis?
- Can changes in microenvironment affect antigenic expression?
- Do the micronutrients affect angiogenesis?

1.19 An Overview of the Thesis

Chapter 2 outlines the methods used as part of this study to address the research questions set out. Chapter 3 is a small study undertaken to determine if changing the microenvironment, in which the cells are cultured, changes the phenotypic properties of glioma cells. The phenotypic changes of these cells were determined by investigating their antigenic expression. Results of experimentation are highlighted in chapter 4 and are discussed in chapter 5. Following this are the references and appendicies.

CHAPTER 2: Materials and Methods

2.1 Source of Cell Cultures

Specimens provided from the Neurosurgical staff at King's College Hospital, London, obtained from epileptic patients at temporal lobectomy, served as normal astrocytic cultures and four biopsy-derived malignant cell lines at low passages were also studied. Ethical approval for this study has been granted by King's; LREC protocols 02-056 (Do flavonoids cross the human Blood brain barrier) and 00-173 (Brain tumour invasion: *in vitro* studies). Table 6 summaries all the biopsy cultures used as part of this study.

Table 6 Four biopsy-derived malignant cell lines and 1 normal astrocytic culture

Cell line	Sex of Patient	Passage number	Biopsy grade
MUAB-C	F	4-6	Normal astrocytic cell line
MUMG-GM	M	4-6	Grade IV GBM
MUTC-GM	M	5-7	Grade IV GBM
MUIH-GM	M	4-6	Grade IV GBM
MUPK-GM	M	3-5	Grade IV GBM

Biopsies provided by staff at King's College Hospital. Following initial culture, these cultures were investigated at low passage.

Human umbilical vein endothelial cells (HUVECs), used as part of angiogenesis assays, were provided by Dr Alan Cooper, University of Portsmouth. The passage of these cells is unknown, though experimentation was carried out after 3-5 passages. Existing cultures of IPSB-18 (P26-30), for all other studies were provided by Dr Timothy van Meter, Virginia Commonwealth University, though were originally cultured from biopsy by other members of the neuro-oncology group. Cell lines employed for micro-environment studies were provided by collaborators in Ireland and Norway.

2.2 Micronutrients

The red clover extract (RCE), donated by Linnea, in Switzerland is a green coloured powder constituting a mixture of four isoflavones of which Formononetin and Biochanin A are precursors of Genistein and Daidzien, respectively. Linnea performed HPLC yielding a total quantity of isoflavones found in the extract, to be 40.4%, specifically 17.8% biochanin A, 22.1% formononetin, 0.4% genistein and 0.1% daidzein (appendix).

The red grape seed extract (RGSE) supplied by Polyphenolics, USA is a fine red/brown coloured powder. The main constituents of the red grape seed extract are; catechin, epicatechin and epicatehin (-3-0-) gallate, of which 10% (min) are monomers, 60-80% oligomers and 25% (max) polymers as illustrated by HPLC analysis by Polyphenolics (appendix). These individual constituents undergo condensation reactions thus elongating to form oligomeric proanthocyanidins (personal communication)

Initially the micronutrients were dissolved in Dimethyl sulfoxide (DMSO – Acros Organics 414885000) (100mg/ml), and filtered. Though further dilutions employed Dulbecco's modified Eagle's medium without phenol red (clear DMEM – GIBCO 31053-028). A working solution of 1mg/ml was prepared, from which subsequent dilutions were formulated. At a later date it was discovered that the DMSO had a direct effect on the cells, resulting in anomulus results. Subsequent adaptation of the methodology meant the micronutrients were dissolved in clear DMEM instead, before being filtered and diluting further.

2.3 Cell Culture techniques

2.3.1 Setting up Primary Human Astrocytic Culture

Upon resection the neurosurgeon immediately placed the biopsy into DMEM supplemented with 1% antibiotic/antimycotic solution and then treated as *Freshney et al.* (2006, p 5; 2005) describe in the protocol for setting up a primary explants culture. The tissue was rinsed in Hanks balanced salt solution (HBSS – GIBCO 14170070) in a petri dish and unwanted tissue, such as blood vessels or necrotic areas were dissected and removed. Remaining tissue was finely chopped with scalpels into pieces, approximately < $1mm^3$. This was diluted in DMEM supplemented with 10% heat-inactivated foetal calf serum (FCS) and 1% antibiotic/antimycotic solution (complete medium). The mixture was transferred to culture flasks, which were placed in the incubator at 37^oC and 5% CO_2. The cultures were maintained for 1-2months (dependent upon the individual cultures) to achieve confluence.

2.3.2 Maintenance of Glioblastoma and Astrocytic Cultures

The astrocytic cultures were routinely cultured as monolayers at physiological pH by equilibration with 5% CO_2 in air in a standard humidified incubator at 37^oC, in either $25cm^2$ or $75cm^2$ culture flasks using complete media; constituting 10% foetal calf serum (FCS – Sigma F-7524), unless stated otherwise and 1% antibiotic/antimycotic solution (penicillin/ streptomycin – GIBCO 15240-062) in Dulbecco's modified Eagle's medium (DMEM – GIBCO 61965-026), unless otherwise stated. Prior to use the FCS was heat inactivated a procedure which involves immersing the serum into a 60^oC waterbath for five minutes, to inactivate the complement. The medium was changed whenever it was required, usually between 3-4 days, dependent upon the culture. Once confluent cells were passaged or harvested for experimentation.

Cells involved in the evaluation of microenvironment changes (chapter 3) were cultured for 48hr in complete medium supplemented with 10% FCS, newborn calf serum (NCS – Biowest S0450) or human serum (HS – Biowest S4190), as required.

Cultures involved with all other experiments were treated with the micronutrients 48hrs prior to experimentation unless stated otherwise.

2.3.3 Maintenance of HUVEC Cultures

HUVEC cultures were maintained in a similar manner to the astrocytic cultures with the exception of the media used. Prior to culture the 25 mm^2 flasks were coated in 1% gelatin for 1 hour and the excess removed the cells were then seeded in a media specific to HUVEC cells; large vessel endothelial cell basal medium (TCS cellworks) constitutes 10% foetal calf serum, 0.1% antibiotic supplement (Gentamicin/Amphotericin), and 0.1% of each of the following beta fibroblast growth factor (bFGF), epidermal growth factor (EGF) and hydrocortisone.

2.3.4 Mycoplasma Testing

Mycoplasma staining is important in the use of cell culture, as contamination of cultures is unaffected by ordinary antibiotics (Hay 1989; Harlin 2008). The procedure was followed in accordance to the instructions of the mycoplasma kit (GIBCO M7006).

2.4 Cell Viability Techniques

2.4.1 Phase Contrast Microscopy

Phase contrast microscopy is a technique to obtain micrographs of the morphology of the cells. Briefly cultures were maintained to around 80% confluence upon which they were examined under a phase contrast microscope. Micrographs were also taken after treatment of flasks with concentrations of the micronutrients to observe any cell death, if any, and to what extent angiogenesis occurred during experiments involving HUVEC cultures.

2.4.2 Population Doubling Time

Population doubling time (PDT) is important to measure, to determine whether any abnormalities in growth periods occur at any point during experimentation. For example as mycoplasma contamination is undetectable to the naked eye, contamination may be detected indirectly via the culture growth patterns.

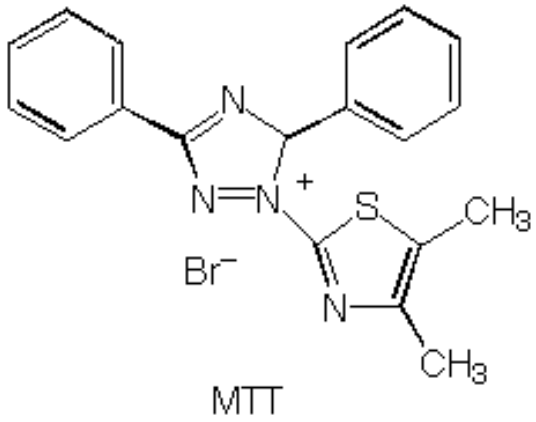
To calculate the population doubling time, the cells were harvested and counted, in order that a population of 100,000 cells could be seeded into a 6 well plate. Cell count was achieved using the trypan blue method. Cells were initially harvested and resuspended in 1ml of complete media, from which 20μl was combined with 20μl of trypan blue stain. 20μl of this were then placed on a haemocytometer. Live cells, those that were not stained blue, were counted in the central square. Both chambers of the haemocytometer were counted and the cell count deduced by inserting the two counts (α and β) into the below equation:

$$\text{Number of cells/ml} = \frac{(\alpha + \beta)}{2} \times 2 \times 10{,}000$$

Each cultured well was counted at timed intervals and the total number of cells, plotted against time.

The lag phase of this curve may be due to a period of adaptation to the *in vitro* environment thus there are only a few cells present. As the cells enter the log phase they begin to replicate and grow. It is from this region of the graph that we may calculate population doubling time. The plateau phase represents depletion in environmental conditions such as space and nutrients and therefore the number of cells begin to settle and die.

2.4.3 MTT cytotoxicity assay



MTT

Figure 8 MTT structure which reacts with active mitochondrial proteins to form a soluble blue/purple salt which may be dissolved**.**

MTT, a quantitative, colorimetric assay for mammalian cell survival and proliferation, is a tetrazolium salt, which is converted from yellow and soluble

to a blue/purple, insoluble formazan salt, by the succinate-tetrazolium system active only in viable cells. This insoluble compound is unable to be measured by normal absorbance methods; therefore it must be solubilised using DMSO. The intensity of the colour change indicates the enzymatic activity of viable cells. Optimized for glioma cells this protocol is based on the experiment described by *Nikkah et al* (1992) and adapted due to apparent biphasic results.

Cell were harvested and plated in 96 microtitre plates in clear complete medium, as the phenol red may interfere with the final reading. They were plated at a density of 10,000 cells per plate in 200µl clear complete medium. Cells were left overnight to adhere after which the medium was replaced with fresh medium (without foetal calf serum) containing a range of concentrations of the micronutrients dissolved in clear DMEM (initially DMSO) and incubated for 48hr at 37^{o}C and 5% CO_2. To ensure consitancy between experimentation and indication of inconsistencies, a positive and negative control were concurrently observed in each plate.

Subsequent to incubation the media was replaced with 100 µl MTT solution (1mg/ml) for 4 hours after which it was discarded and the formazan crystals dissolved in 200 µl DMSO. The solution was gently pipetted up and down to attain homogeneity and then placed in an incubator at 37°C for 5 minutes to let the air bubbles that may have been generated disappear. Absorbance was measured at 570 nm on a FLUOstar Optima plate reader.

2.5 Charcterisation of Astrocytic Cell Cultures

2.5.1 Analysis of Antigenic Expression Using Flow Cytometry

This semi-quantitative method characterises the antigenic expression of cells using fluorescently labelled antibodies. This procedure will allow for the comparison of untreated tumour cells against either untreated normal

astrocytes or tumour cells treated with micronutrients. Once confluent the cells were harvested from flasks using tryple express (trypsin) for 10min. The cells were then neutralised and washed with phosphate buffer solution (PBS) supplemented with 5% FCS. All washes were performed using PBS supplemented with 5% FCS and then centrifuged for 5 mins at 1000rpm. The cells were resuspended in 200μl of for 10 mins the appropriate primary antibody (Table 7) washed and resuspended in the appropriate secondary streptavidin fluorescein (FITC); for example, if the host species was mouse the secondary adopted would be anti-mouse.

Table 7 Primary antibodies were used to characterise cell cultures. An illustration of the dilutions used and the host species the antibody was cultured in.

Antibody	Dilution	Host species	Monoclonal or Polyclonal	Source
GFAP	Neat	Mouse	Monoclonal	CRUK
CD44	1:100	Mouse	Monoclonal	CRUK
β1	Neat	Mouse	Monoclonal	CRUK
αV	Neat	Mouse	Monoclonal	CRUK
NCAM	1.1000	Mouse	Monoclonal	Abcam

The cells were then washed and resuspended in PBS (2% FCS). Prior to analysis 5μl of propidium iodide (PI) was added and analysis was performed using a FACs Calibur against a suitable control.

2.5.2 Immunocytochemistry

Complementing flow cytometry, this qualitative method of characterisation will allow for the detection of the antigens GFAP, CD44, β1, αv, and Eric-1 (NCAM) using the biotin streptavidin FITC protocol. Cells were harvested and resuspended in either complete medium or various concentrations of the micronutrient, onto 24x24 mm glass coverslips, previously autoclaved, and

placed inside 35mm sterile plastic petri dishes. These were then incubated for 48hrs. The cells were then rinsed in PBS and incubated for one hour with the primary antibody at the appropriate dilution (Table 8).

Table 8 Primary antibodies used to characterise cell cultures. An illustration of the dilutions used and the host species the antibody was cultured in.

Antibody	Dilution	Host species	Monoclonal or Polyclonal	Source
GFAP	Neat	Mouse	Monoclonal	CRUK/Abcam
CD44	1:100	Mouse	Monoclonal	CRUK/Abcam
β1	Neat	Mouse	Monoclonal	CRUK
αV	Neat	Mouse	Monoclonal	CRUK
NCAM	1.1000	Mouse	Monoclonal	Abcam

Coverslips were washed three times in PBS and incubated with biotinylated secondary antibody specific to the host species of the primary antibody (DAKO monoclonal (anti-mouse) - F0313 polyclonal (anti-rabbit) - E0354) for 30 mins. Consequently the coverslips were washed in HBSS and incubated in the dark with fluorescein-streptavidin conjugate (FITC). The cells were washed, again, three times and fixed with acid alcohol (1ml concentrated HCl in 90 ml of 70% ethanol) for two mins after which they were rinsed three times in HBSS or until discolouration of the phenol red indicator ceases. It is important to remember, for internal antigen detection such as GFAP fixation occurs prior to incubation with primary antibody. Counterstaining with propidium iodide (PI) followed for 10 sec, only, then immediately flushed with HBSS. The coverslips were rinsed in HBSS and then mounted on to glass slides in Citiflour anti-fadent.

2.6 Invasion Assays

2.6.1 2-dimesional Invasion Assay using Boyden Chambers

Boyden or Transwell TM chambers measure the ability of the cells to invade the surrounding matrix. Prior to performing the invasion assay, cells were required to be gradually weaned down to serum free media as abruptly depriving cells of serum is not always well received by many cell types. To achieve this, cells were first cultured in the presence of 10% FCS and 1% antibiotic for approximately 24hrs or until the cells were sub-confluent, after which, the medium was replaced with complete media supplemented with 5% FCS and antibiotic and incubated for a further 24 hrs. Subsequent to this incubation the serum supplemented media was once again decreased this time to 2.5% FCS for 24 hrs. Finally the media was replaced after 24hrs with serum free media.

Four hours prior to seeding, the chamber is coated in 100μl of ECM component proteins routinely human placental collagen type IV (10μg/ml. Sigma no. C5533). The polycarbonate filter divides the well into an upper and lower compartment, the cells are seeded on the upper surface of the filter in serum-free medium and the lower chamber is filled with complete medium, a chemo attractant. Upon incubation at 37^{o}C for 48hrs the cells were washed and stained with a crystal violet stain diluted 1:100. The cells were then counted in both the upper and lower chambers and the percentage invasion is calculated using the equation below;

$$\text{Percent invasion} = \frac{\text{mean cells in the lower compartment}}{\text{mean total cell number}} \times 100$$

2.7 Detection of Apoptosis

2.7.1 Annexin V Assay

Cells were plated in 25 cm^2 tissue culture flasks at 1 × 10^5 cells per flask, incubated overnight, and treated with micronutrients or DMSO control for 48hr. Subsequent to incubation, media from the flasks wasa collected as they could contain dead or dying cells that had detached from the flasks. Adherent

cells were harvested and added to the respective media. Cells, at a concentration of 1 × 10^5 cells per 100 µl, were washed with ice cold phosphate-buffered saline and resuspended in binding buffer (140 mM NaCl, 10 mM HEPES, 2.5 mM CaCl, pH 7.4) and stained using annexin V-FITC followed by incubation at room temperature in the dark 15 min. Before analysis a few drops of PI was added and measured by flow cytometry on a Beckman Coulter Epics XL-MCL Flow Cytometer within one hour of annexin V/PI staining. A minimum of 10,000 events was collected for each sample.

2.7.2 TUNEL Assay

The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed in order to detect apoptotic cells to confirm annexin V results. Beyond treatment of cells with the micronutrients, the cells were fixed in 4% paraformaldehyde for 10 min. TUNEL staining for detection of apoptotic cells was done using the TUNEL Apoptotic Detection kit (Promega) as per the manufacturer's instructions. Briefly, the fixed cells were washed in PBS and then incubated with 0.2% Triton x 100 in PBS for 5 min at room temperature. After washing the cells were then incubated in 50 µL of terminal deoxynucleotidyl transferase end-labeling cocktail with FITC for 60 min in the dark at room temperature. The reaction was terminated, with 2% SSC solution for 15min and washed in PBS. The cells were then mounted with antifading DAPI mount (Vecter Labs). Slides were allowed to dry in the dark, observed under a fluorescent microscope.

2.8 Detection of Angiogenesis

2.8.1 Tubule formation assay

HUVEC cells were cultured in the presence of the IC_{50} of the micronutrients or in complete media in 24 well plates coated in 1% gelatine. These were then incubated for 48hrs. Assessment of tubule formation involved fixing the cell preparation in 70% ethanol at 4°C for 15 min, rinsing in PBS, and staining with

calcein. Tubule formation was assessed by counting the number of tubule branches.

Upon reduction of tubule formation in the presence of the micronutrients, tumour cells will undergo experimentation further angiogenesis experiments.

2.8.2 Co-Culture: Glioma Cells and HUVEC

Initially 10,000 HUVECs were seeded on one half a sterile coverslip and left overnight. Subsequent to this 5000 tumour cells were seeded on the other half of the coverslip and left to incubate for four hours (Fig 11). Subsequently the coverslips were treated with IC_{50}s of RCE or RGSE and micrographs were taken at 6, 24 and 48 hours respectively and the number of tubules counted.

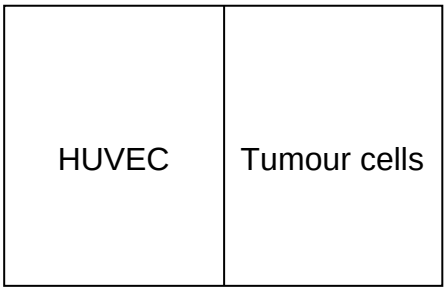


Figure 9 Illustration how coverslips were seeded with both HUVECs and brain tumour cells. 5000 cells of each, HUVECS and glioma cells were seeded on either side of the cover slip and cultured together.

2.9 Statistics

All studies analysed were performed in triplicate with the average of three experiments being statistically analysed as required. In particular statistical analyses were carried out on the MTT results using Stats direct software, to determine the mean and standard deviation. Any outliers, outside three standard deviations, were excluded.

Analysis on the flow cytochemical data was examined using ANOVA analysis.

CHAPTER 3: Microenvironmental Changes of Growth Sera

Preliminary investigations into the effects of microenvironmental changes, of growth sera, on astrocytic cultures were performed. This was achieved through the evaluation of antigenic expression, commonly associated with gliomas.

3.1 Introduction

Heterogeneity is regarded as a prominent feature of gliomas due to the divergent processes of differentiation and anaplasia giving rise to a mixture of different cellular appearances. It is likely that this phenomenon may give rise to differing degrees of malignancy being manifest in different regions of the same tumour. The cellular heterogeneity seen histologically is, to some extent, retained *in vitro* during early passages. Histological recognition *in vitro* generally includes the expression of common differentiation markers such as glial fibrillary acidic protein (GFAP) for astrocytes and galactocerobroside (Gal C) for oligodendrocytes. Indeed glioblastoma (GB) is generally believed to arise from cells of astrocytic lineage, although oligodendroglial or progenitor cell derivation is also possible (Pilkigton 2001). It has also been proposed that a rare sub-population of cells within the tumour mass has the ability to self re-new, differentiate and sustain tumour growth (Bruce 1963)**.**

Recent studies have shown that individual cells within these tumors are capable of giving rise to cells of multiple lineages (Hemmati 2003; Ignatova 2002; Kukekov 1997). This could be explained by cells undergoing trans-differentiation; manipulation of tissue-specific stem cells into tissue specific cells that they would not normally develop into (Fan 2007) for example

hematopoietic stem cells giving rise to neural cells or neural stem cells giving rise to blood cells (Mezey 2000; Bjornson 1999). Another example of this was seen when dorsal domains of the spinal cord and telencephalon, which were thought to give rise to astrocytes, were also found to produce oligodendrocytes (Kessaris 2006; Richardson 2006; Cai 2005; Fogarty 2005; Vallstedt 2005). One study reported that glia that express NG2, which have been shown previously to be progenitor cells for glia during development, in the adult mouse are capable of producing both oligodendrocytes and protoplasmic astrocytes *in vivo* (Zhu 2012). However, two independent studies found that adult NG2-positive glia gave rise to oligodendrocytes and neurons, but not astrocytes, *in vivo* (Rivers 2008; Menn 2006). Despite controversy, positive evidence surrounds the study of manipulation of tissue-specific stem cells into tissue specific cells that they would not develop into (Fan 2007).

A similar phenomenon was reported in earlier studies by *Raff et al.* (1983) who explored the influences of external factors the cellular environment, such as the presence or absence of foetal calf serum in the culture medium, on the developmental pathways of astrocytes and oligodendrocytes. They assessed morphological and imunocytochemical criteria of the divergent pathways of cell development in the immature glia of the rat optic nerve. They used A2B5 antibody for the detection of Type 2 astrocytes (fibrous) in this study and proposed that both fibrous astrocytes and oligodendrocytes may be derived from a primitive precursor cell (the so-called “O-2A” or oligodendrocyte Type 2 astrocyte progenitor cell), dependent upon the influence of the environment (Temple 1985; Raff 1984; Raff 1983). This observation provided the first evidence for developmental plasticity and environmental influence in the differentiation of glial cells in the central nervous system (CNS). Subsequent studies showed that the presence of GFAP-negative, A2B5-positive cells and GFAP-positive, A2B5-positive cells in a malignant astrocytoma culture (IPSB-18) which provided evidence that the O-2A progenitor and Type 2 astrocyte phenotypes occur both *in vitro* in human neoplastic as well as in normal developing rat neural tissue (Pilkington 1992; Knott 1990).

Furthermore, *Tenebaum et al*. (1996) identified the plasticity of oligodendroglioma cells by successfully showing that when taken from a rat brain, they could be manipulated in the presence of serum supplementation, into losing the expression of oligodendrocytic antigenic markers and acquiring astrocytic markers. Upon re-introduction into the rat, these cells were able to revert back to their original antigenic expression. They also confirmed that human oligodendroglioma cells had a similar culmination being induced, by the absence or presence of serum, into oligodendrocytes or Type 2 astrocytes, respectively.

Various antigenic markers characteristically expressed by gliomas, aid identification of tumour phenotypes *in vitro*. Such markers include cluster of differentiation 44 (CD44), neural cell adhesion molecules (NCAM), GFAP, ganglioside (GD3), Gal C and Neuron-Glial 2 (NG2).

Astrocytic marker CD44 is a cell surface adhesion molecule (Ariza 1995; Li 1995; Radotra 1994; Pilkington 1993) and a principal receptor for hyaluronan (HA) (Aruffo 1990), a glycosaminoglycan found to be highly expressed in the extra cellular matrix (ECM). Brian tumours are highly diffuse and are difficult to target because of this invasive behaviour. Involvement of HA in this invasive process results in its increased ECM expression, regardless of tumour grade (Delpech 1993). CD44 is as a result also up-regulated in brain tumours. As CD44 is involved in this invasive process and is commonly studied as a marker of astrocytic invasion.

Members of the immunoglobulin super family, NCAMs are cell surface glycoproteins that mediate cell-cell adhesion independent of Ca^{2+} (Keilhauer 1985), play an important role in neurite outgrowth and fasciculation (Appel 1993) and are involved in migration/invasion (Gratsa 1997). *Gratsa et al* (1997) showed that during migration the NCAM expression is down-regulated.

GFAP an intermediate filament protein constituent thought to retain the mechanical strength and structure of astrocytes (Eng 1971) and is a valuable marker of normal, reactive and neoplastic astrocytes (Pilkington 2001).

GD3 is a ganglioside belonging to a family of complex acidic glycolipids. It is thought they are functionally diverse in the normal brain and are also expressed and play a role in brain tumours (Yates 1995; Fredman 1994, Wikstrand 1994; Pilkington 1993; Shinoura 1992). Earlier studies have reported that malignant gliomas contain a higher concentration of GD3. Its expression has been shown to correlate with the degree of malignancy (Berra 1985).

Gal C belongs to the family of cerebrosides. It is a common cell surface glycolipid marker for mature oligodendrocytes and appears to regulate myelin formation (Marcus 2006; Dupree 1998; Bosio 1996; Coetzee 1996). *Tenebaum et al.* (1996) identified the loss of this marker in their studies when the microenvironment was altered.

NG2 a transmembrane chondroitin sulphate proteoglycan, is expressed on oligodendrocyte progenitor cells (OPCs) (Chekenya 2002; Chekenya 2007) though it is not expressed by multipotent stem cells. It is however up regulated once stem cells begin to differentiate. NG2 is then strongly expressed whilst cells undergo proliferation and retain a certain degree of developmental plasticity. Upon termination of this, NG2 expression is down regulated. However NG2 remains up regulated in tumours, due to continual proliferative activity (Stallcup 2008).

CD73 is known to be expressed in oligodendrocytes (Maienschein 1996), its function. It is known to be involved in various signalling pathways in the body including extracellular dephosphorylation of nucleoside monophosphates to their corresponding nucleosides and cell– matrix interactions (Stochaj 1992).

As an extension of previous work performed by both *Raff et al.* (1983) and *Tenenbaum et al.* (1996), and with the aim to investigate the currently debated use of sera to supplement growth media, this study will aim to identify if both astrocytic and oligodendrocytic cultures are influenced by a change in microenvironment by a change in antigenic expression.

It is hypothesized that despite the general consensus, it may be advised to supplement growth sera with human serum to best mimic *in vivo* conditions.

3.2 Materials and Methods

3.2.1 Cell Cultures

Established glioma cells (Table 9) were cultured as monolayers in plastic culture flasks, in a standard humidified incubator at 37^{o}C and 5% CO_2. Cells were grown in Dulbecco's modified eagles medium (DMEM) supplemented with 10% inactivated foetal calf serum (FCS), new born calf serum (NCS) and 1% AB or 10% Human serum (HS) and 1% AB (pen/strep) for 48hr.

Table 9 Summary of the cell cultures used as part of this study including the source

Cell line	Tumour type
IPSB-18	Astrocytoma III
U251	Glioblastoma multiforme
A172	Glioblastoma multiforme
HF66	Glioblastoma multiforme
U373	Glioblastoma multiforme

3.2.2 Population Doubling Time

A population of 100,000 cells was seeded into a six well plates and maintained in DMEM supplemented with the appropriate medium. At timed intervals namely 4hr, 6hr, 12hr, and every six hours subsequent until a population doubling time could be achieved.

The cell count was achieved using the trypan blue method. Cells were initially harvested and resuspended in 1ml of complete media, from which 20μl was combined with trypan blue stain.and counted using a haemocytometer.

3.2.3 Immunocytochemistry (ICC)

Cells were maintained on sterile microscope glass cover slips in DMEM supplemented with the appropriate serum, as described above. Once confluent, the cells were washed in Hank's balanced salt solution (HBSS). Qualitative analysis of the antigens NG2, GD3, NCAM, CD44, CD73, Gal C and GFAP was carried out.

Table 10 Primary antibodies used to characterise cell cultures. An illustration of the dilutions used and the host species the antibody was cultured in.

Antibody	Dilution	Host species	Monoclonal or Polyclonal	Source
GFAP	Neat	Mouse	Monoclonal	CRUK
CD44	1:100	Mouse	Monoclonal	CRUK
NCAM	1.1000	Mouse	Monoclonal	Abcam
Gal C	Neat	Rabbit	Polyclonal	Millipore
GD3	Neat	Mouse	Monoclonal	CRUK
CD73	1.1000	Mouse	Monoclonal	Abcam

For cell surface staining fixation was performed after incubation with the primary and secondary antibodies for two mins with acid alcohol (1 vol 1% HCL: 99 vol 70% ethanol), whereas fixation for cytoplasmic staining was carried out with acid alcohol for one min prior to incubating with the primary antibody. Upon incubation with both antibodies the cells were washed and counterstained with Hoechst Blue. The monolayer of cells were then mounted in Citifluor and analysed with a confocal microscope.

Each micrograph was scored by identifying the intensity/quantity of fluorescence. An average score of three micrographs was taken.

3.2.4 Flow Cytometry

When confluent the cells were harvested from flaks using cell dissociation solution (GIBCO) for 10min. The cells were then neutralised with phosphate buffer solution (PBS) supplemented with 5% appropriate serum and washed. All washes were performed using PBS supplemented with 5% appropriate serum and then centrifuged for 5 mins at 1000rpm. The cells were resuspended in 200μl of for 10 mins the appropriate primary antibody (NG2, GD3, NCAM, CD44, GalC, GFAP and CD73) washed and resuspended in streptavidin fluorescin (FITC). The cells were then washed and resuspended in PBS (2% appropriate serum). Prior to analysis 5μl of propidium iodide (PI) was added and analysis was performed using a Beckton-Dickenson FACS Calibur against a suitable control.

3.3 Results

The standard morphology of malignant glioma cells cultured in different microenvironments, exhibit differing growth patterns. Illustrated here, the IPSB-18 cell line (Fig 12) develops in a monolayer formation in the presence of FCS though when cultured in NCS and HS the pattern becomes ‘patchy’ and the cells begin to grow as clumps.

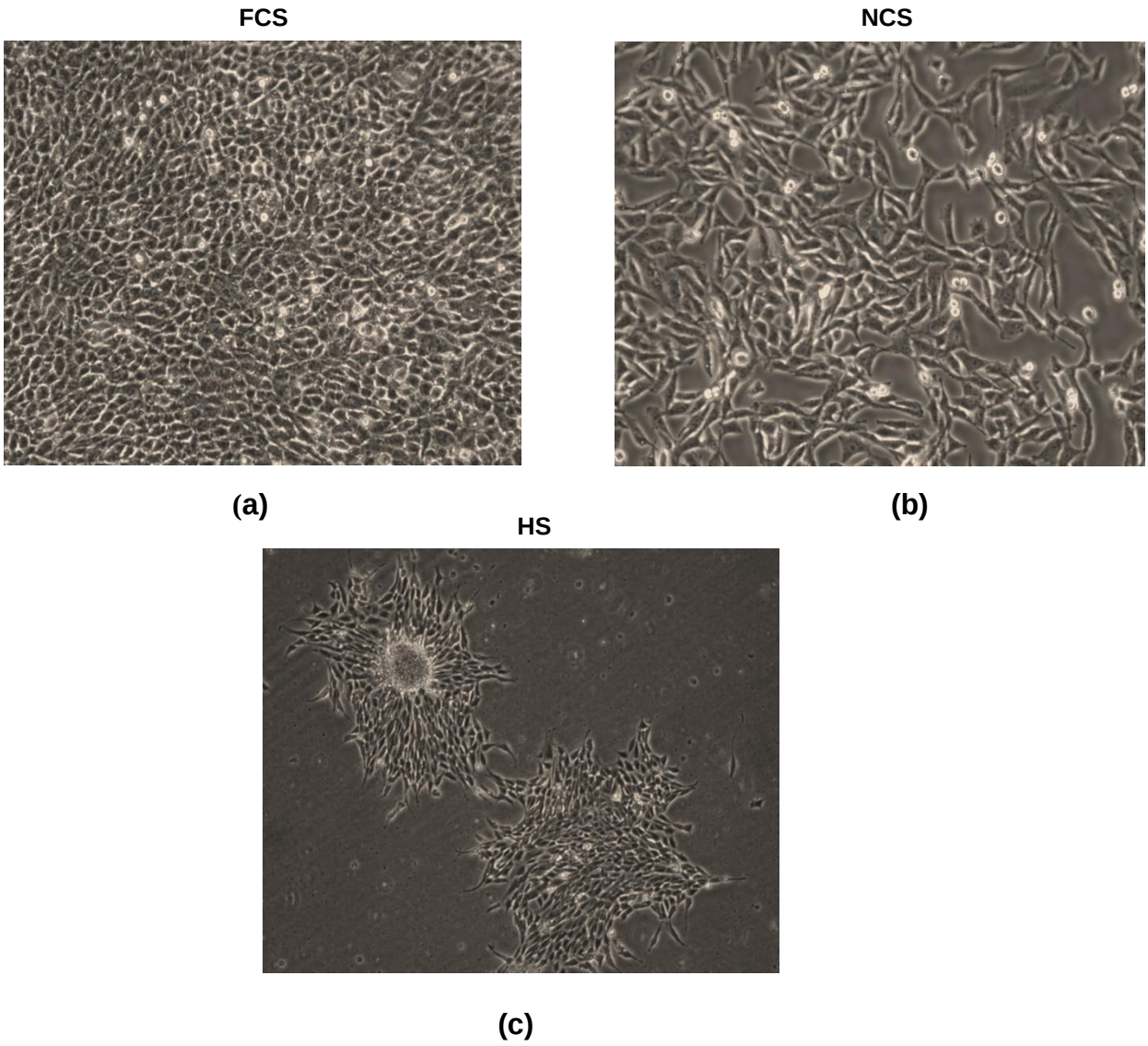


Figure 10 Phase contrast micrographs of the IPSB-18 cell line when cultured in the presence of (a) FCS (b) NCS and (c) HS. Cells grown in the presence of FCS grew in a consistant monolayer, whereas in the presence of NCS or HS the cells grew in patches and the morphology of the cells differed in each microenvironment.

The population doubling times (PDTs) supported the growth rates seen in the micrographs of the indiviual cultures when grown in the presence of differing growth media.

Table 11 Summary population doubling times (PDTs) of the cell cultures in this study

Cell line	**PDT**		
	FCS	*NCS*	*HS*
IPSB-18	48	72	102
U251	26	29	63
A172	37	49	76
HF66	32	56	93
U373	20	42	72

Table 11 summarises the PDTs of all the cultures. When the microenvironment was supplemented with NCS, in all cultures, the PDT was approximately double that of cultures grown in FCS. Cultures supplemented with HS grew slower still with most PDTs being more than double those cultured in FCS. This initial morphological change is mimicked in the apparent phenotypical change of antigenic expression.

Representative micrographs depict dual staining of the changes seen in Gal C and GFAP when cultured in differing serum supplementation (Fig 11). Evaluation of the antigenic expression of Gal C and GFAP, the two prominent markers for olidendrocytes and astrocytes respectively illustrated staining intensity of glioma cell cultures varies when the serum supplementation is altered, when the microenvironment is changed such that there is no antigenic expression of Gal C seen when the cells are cultured in FCS. However when cultured in NCS or HS there is a slight indication of fluorescence.

Table 12 and 13 summarise the expression of GFAP is down regulated, in cultures grown in FCS compared to those grown in NCS and HS. This is commonly seen across the remaining cell cultures also.

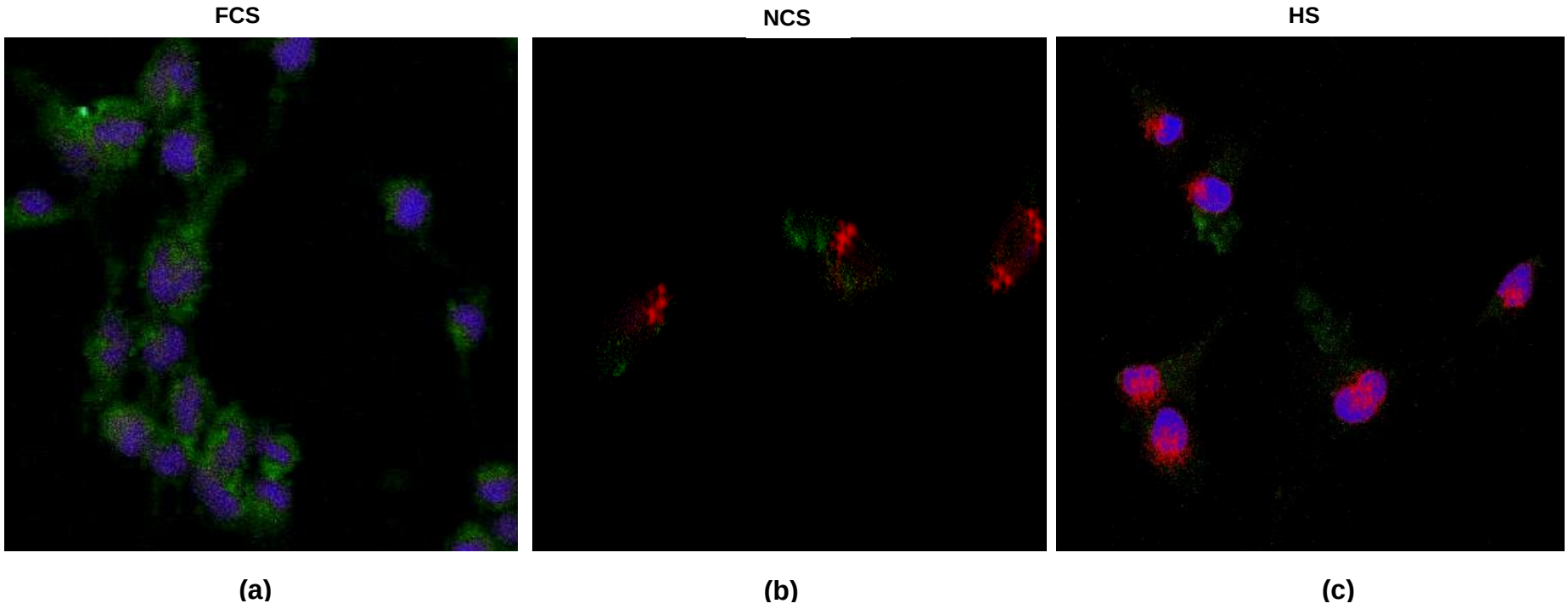


Figure 11 Micrographs of the changes in antigenic expression of Gal C (red) and GFAP (green) when cultured in the in the presence of (a) FCS (b) NCS and (c) HS for the cell line IPSB-18 (an established anaplastic astrocytoma). The nuclei are counterstained with DAPI (blue).

Table 12 Summary of the immunocytochemical results of the presence of six prominent markers found in oligodendrogliomas and astrocytomas when grown in media supplemented with foetal calf serum (FCS), Newborn calf serum (NCS) and Human serum (HS)

Cell line	Serum used to culture	Antibody Expression %						
		CD44	NCAM	GFAP	GD3	Gal C	NG2	CD73
IPSB-18								
	FCS	+++	+	+++	+++	±	-	+
	NCS	+	±	+	±	-	-	+
	HS	±	±	+	±	+	++	+
U251								
	FCS	+++	+	+++	+++	-	+	-
	NCS	+	+	++	++	++	+	-
	HS	-	±	+	+	++	+	-
A172								
	FCS	+++	+	+++	+++	-	±	+
	NCS	+	±	++	+	±	+	-
	HS	+	-	++	±	+	++	+
HF66								
	FCS	+++	+	+++	+++	-	±	+
	NCS	++	±	++	+	±	+	+
	HS	+	±	++	±	+	+	+
U373								
	FCS	+++	+	+++	+++	-	±	-
	NCS	++	±	++	+	+	+	+
	HS	±	±	++	±	±	++	+

Where + = positive expression ± = some expression and - = little or no expression

When investigating Gal C an opposite phenomenon occurred namely when the microenvironment was changed from FCS to NCS or HS, in all the cell lines Gal C appeared to be up regulated. The down regulation of Gal C expression in the IPSB-18 cell line when cultured in NCS could be assume an anomaly (Table 12), as Fig 11 clearly depicts the presence of Gal C expression. Table 12 also illustrated an apparent down regulation of CD44, NCAM, GD3 and NG2. The expression of CD73 remained fairly consistent.

Flow cytometry semi-quantitatively compliments the immunocytochemical data. The plots illustrated fluorescent intensity against cell count and all plots are compared to the no primary antibody, negative control. The significance of the variation in expression of the different antigens after flow cytometry, as calculated by the t-test when compared back to cells cultured in FCS, is summarised in Table 13.

The t-test confirmed significant changes in expression of the antigenic markers across all the cell line for CD44, NCAM, GFAP and NG2. Gal C and CD73 were only depicted to illustrate significant changes in cultures grown in HS not those in NCS.

Table 13 Summary of the significant difference between the median flow cytometry results of the presence of prominent markers when grown in media supplemented with Newborn calf serum (NCS) and Human serum (HS) compared with foetal calf serum (FCS

Cell line	Serum used to culture	P value of t-test						
		CD44	**NCAM**	**GFAP**	**GD3**	**Gal C**	**NG2**	**CD73**
IPSB-18								
	NCS	0.14	0.006*	0.07	0.048*	0.08	0.4	0.13
	HS	0.013*	0.006*	0.06	0.0005**	0.02*	0.02*	0.06
U251								
	NCS	0.6	0.05*	0.64	0.01*	0.1	0.01*	0.006*
	HS	0.3	0.0068*	0.3	0.5	0.004*	0.003*	0.09
A172								
	NCS	0.21	0.07	0.04*	0.09	0.9	0.01*	0.29
	HS	0.5	0.089	0.09	0.08	0.06	0.5	0.002*
HF66								
	NCS	0.002*	0.001**	0.048*	0.01*	0.0009**	0.000**	0.003*
	HS	0.009*	0.008*	0.029*	0.11	0.0001**	0.000**	0.005*
U373								
	NCS	0.006*	0.37	0.02*	0.11	0.13	0.001**	0.009*
	HS	0.025*	0.49	0.02*	0.5	0.01*	0.000**	0.001**

* < 0.05, ** <0.001 A p value of below 0.05 is significant.

3.4 Discussion

A change in serum supplementation impacted antigenic expression in both original studies performed by *Tenenbaum et al* (1996) and *Raff et al* (1983)**.** More recent studies (Brewer 2007) also depicted similar variations in cell differentiation under different serum supplements. Serum supplementation of the culture media is required to provide the cells with basic nutrients, hormones and growth factors. These constituents promote growth and potentiate specialised cellular functions. Although different sera constitute similar components, differences are identified in the percentage of these constituents as in the comparison of foetal calf serum, newborn calf serum and human, particularly in the protein content. Serum supplementation of the culture media is required to provide the cells with basic nutrients, hormones and growth factors. These constituents promote growth and potentiate specialised cellular functions. Although different sera constitute similar components differences are identified in the percentage of these constituents as Table 14 illustrates in the comparison of foetal calf serum, newborn calf serum and human serum.

The major differences between the three sera are the percentages of haemoglobin and total protein. Previous studies have found haemoglobin had no effect on cell growth (Rooprai et al. personal communication), therefore the key to trans-differentiation suggestively lies in the differing content of the total protein. The choice of sera used in this study was initially based on previous studies in Norway where normally newborn calf serum is used to supplement media (personal communication).

Table 14 Similarities and differences in three different sera; foetal calf serum, newborn calf serum and human serum

Component	Foetal calf serum	Newborn calf serum	Human serum
Total protein (g/100ml)	3 - 4.5	4.5 – 6.0	5.7
Haemoglobin (mg/100ml)	$\leq$ 25	20	6.21
pH	6.8 – 8.1	7 – 8	7.62
Osmolarity (mOsm/kg)	260 - 330	240 – 340	325
Endotoxin (EU/ml)	$\leq$ 10.0	100	9
Mycoplasma	none	none	none

The results of this study found that there were differences in the antigenic expression using both flow cytometry and immunocytochemistry as a result it was suggested cells should be cultured in media supplemented with human serum. However, due to the cost and impracticality of obtaining human serum it was suggested use of foetal calf serum as a media supplement should continue until such time, human serum becomes readily available.

Such differences may be responsible for the differing growth patterns exhibited by the IPSB-18 culture (Fig 10), specifically the difference in percentage protein content. This most immediate difference illustrated, upon changing the microenvironment, cells cultured in foetal calf serum normally grow as a monolayer, however on changing to human sera the cells began to grow in patches. We may therefore assume the ability to manipulate characteristically astrocytic cultures, to differentiate away from the norm resulting trans-differentiation. This does suggest the presence of a potential cell type with the ability to become manipulated by varying the microenvironment.

In introducing changes in the growth sera, a phenotypic change was not just illustrated in the morphological growth patterns but also in the antigenic expression of characteristic markers. Clear distinctions in change of antigenic expression were predominately seen when investigating GFAP, for all the cell lines. Normally associated with astrocytic cultures, the expression of this antigen was markedly down regulated in immunocytochemical micrographs, when cells cultured in the presence of FCS were compared to those cultured in the presence of NCS or HS (Table 12). Upon comparison of this data to flow data the t-test analysis showed significant differences in the data (Table 13).

Similarly Gal C, which is specifically, associated with oligodendrocytes, in the presence of NCS and HS, the astrocytic glioma cultures were seen to express Gal C (Fig 11). The differences in this data however were only noted as significant when comparing the FCS cultures to the HS cultures (Table 13).

CD44 was another antigen which showed significant changes in the differences between antigenic expressions in the cultures. CD44 is normally up-regulated in astrocytomas as a result of involvement of hylauronic acid (HA) expression increasing in the extracellular matrix (ECM), during invasion, regardless of tumour grade (Delpech 1993). *Hagel et al* (1999) showed in characterisation of CD44 positive cells in oligodendrogliomas, reactive astrocytes were revealed, at the invasive margin of the tumour. Therefore a down regulation of CD44 could indicate a change in the phenotype, from astrocytic to another.

NG2 was found to be up regulated in the cell cultures. This marker is associated with an up-regulation during proliferation and differentiation. As such the significant differences depicted could indicate a change in differentiation and indeed increased proliferation. This may also offer an explanation for why differences are seen in growth patterns, the cultures grown in HS grew in clumps.

NCAM also showed significant differences in expression (Table 13) depicting an up regulation upon change. This supports the changes seen in NG2 as the change in proliferation and differentiation may also be associated with there invasive potential. NCAMs are also seen to be up regulated in cultures grown in HS where the cells grew in clumps.

A consistency in GD3 expression suggests the malignancy of the tumours did not vary. Though a significant difference in CD73 when cells were cultured in HS compared to cells cultured in FCS, did suggest a greater presence of oligodendrocytic phenotypes.

Additional experimentation was also carried out on some cell lines using serum free medium (SFM) with and without platelet derived growth factor (PDGF). This data showed a similar occurrence. The Gal C expression of two established oligodendrogliomas cell lines was down-regulated in the presence of NCS and HS and the GFAP expression was up-regulated.

These dramatic indications, suggested trans-differentiation in astrocytic glioma cultures when their microenvironment is altered. As such, a cell type with the ability to change phenotypically.

3.5 Conclusion

The suggestion of trans-differentiation indicates the importance of the microenvironment in which the cells are cultured. Whilst evaluation of the changing environments would suggest the optimum serum supplement is human serum, it is not readily available. It is therefore favourable to continue using FCS as the chosen serum supplement.

CHAPTER 4: Results

4.1 Morphology

Heterogeneity is a feature of malignant brain tumours, each unique in morphology with up to 14 different sub populations of cells, including progenitor cells. The classic shape associated with astrocytic cells, ‘star’ or ‘spindle’, was seen in micrographs of the control cell cultures (MUAB-C) (Fig 12f). When compared to the control culture the glioma cells illustrated a differing morphology, with large spindle like extensions, each cell growing separately from one another and consistently across a monolayer. The cell shape seen in IPSB-18, (Fig 12a) however, were smaller in size and initially grew in small patches, overlapping one another, eventually spreading across, and growing as a consistent monolayer.

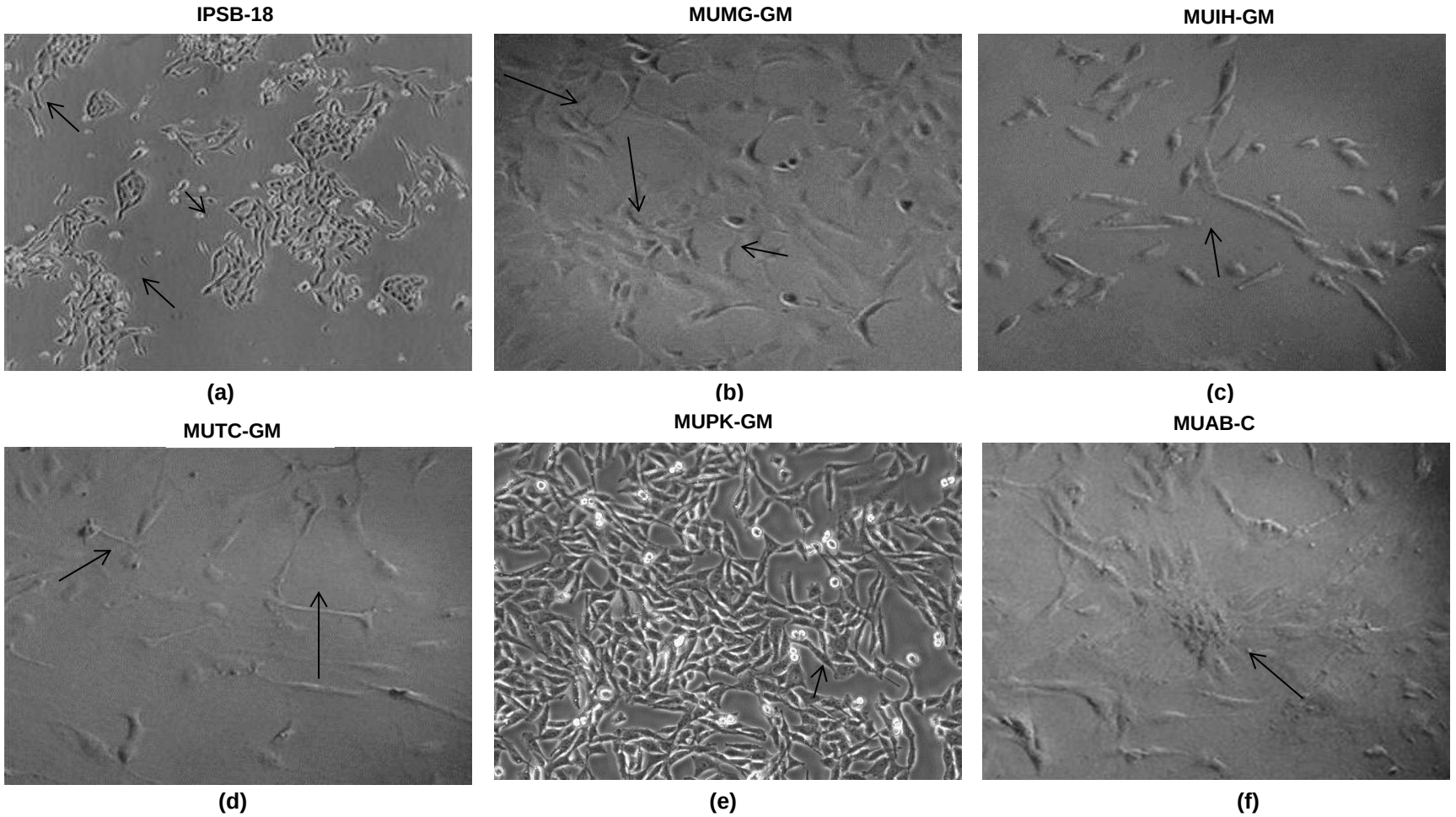


Figure 12 Phase contrast micrographs of the cell lines involved in this study including (a) IPSB-18, (b) MUMG-GM, (c) MUIH-GM, (d) MUTC-GM, (e) MUPK-GM and (f) control cell line MUAB-C (where possible arrows indicate examples of individual cell morphologies). Magnification x 40. Differences between the cell cultures are clearly visible. The most noted difference includes, that between MUAB-C, (f) the control cell culture with clear star shaped cells and the remaining cultures which have spindle shaped cells.

4.2 Population Doubling Times of Giloma Cultures

Table 15 Population doubling times (PDT) of glioma cell lines and the control cell line (MUAB-C)

Cell Line	Average PDT (hr)	SD	Tukey's HSD (p)
IPSB-18	36	5.57	<0.01
MUMG-GM	165	7.81	<0.01
MUIH-GM	210	7.81	<0.01
MUTC-GM	197	2.65	<0.01
MUPK-GM	347	9.54	<0.01
MUAB-C*	72	2	N/A

* Control astrocytic cells

Cultured in DMEM culture media supplemented with 10% inactivated foetal calf serum and 1% antibiotics. The PDT results of the glioma cultures were statistically different to one another and to the control culture MUAB-C with a $p<0.05$ (ANOVA and Tukeys HSD).

Summarised in Table 15, the IPSB-18 glioma cell culture was observed to have the shortest PDT (Fig 13). Within as little as six hours this population increased from 10,000 cells to approximately 12,000 cells with a doubling time of around 36 hours. The remaining glioma cultures had a much longer PDT with MUPK-GM having the longest time of approximately two weeks (Fig 13). The control cell line (MUAB-C) had a PDT of around 72 hours. This remained fairly consistent with increasing passages. The rate at which the glioma cell cultures doubled, however, decreased with increasing passage.

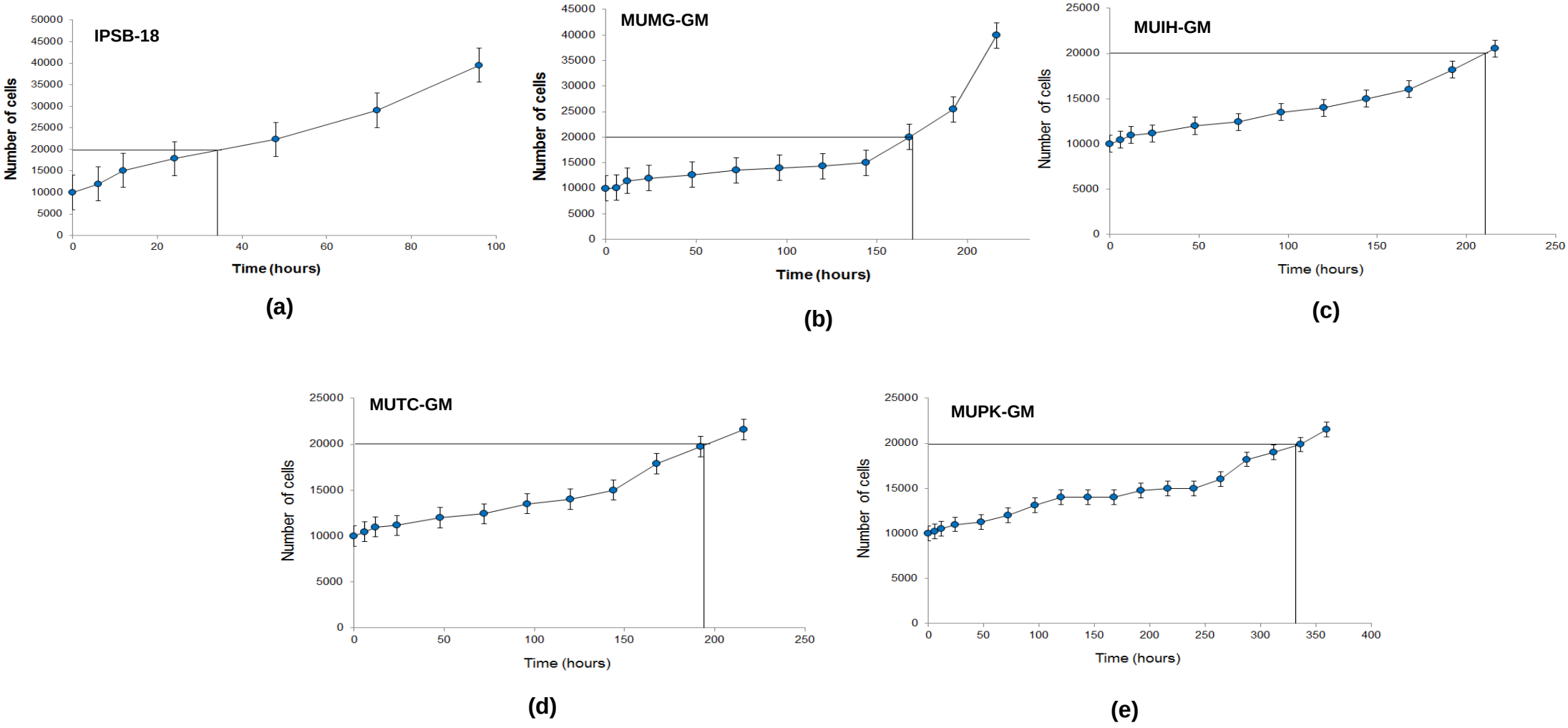


Figure 13 Population doubling time of glioma cell line IPSB-18 (a), MUMG-GM (b), MUIH-GM (c) MUTC-GM (d) and MUPK-GM (e). Readings were taken wherever possible at least at 12 hourly intervals. Identification of the population doubling time (i.e. increase from 10,000 cells to 20,000 cells) has been indicated by the black line.

4.3 The Effect of Micronutrient Extracts on Cell Viability

4.3.1 The Toxic Effects of DMSO

Optimized for glioma cells the MTT protocol is based on the experiment described by *Nikkah et al* (1992), this protocol was adapted due to apparent biphasic results. Despite being a simple, well documented and widely used assay, errors of analysis have been reported as a result of various problems ranging from differing responses within various cell lines (Nikkhah 1992; Nikkhah 1992; Chen 1990) to interactions with various compounds (Wang 2010; Young 2005; Ngai 1998), the use of micronutrients being one such hindrance. Undesirably the use of micronutrients has been shown to reduce the MTT salt in the absence of cells (Peng 2005; Bruggisser 2002) at fairly high concentrations.

Initial experiments using the IPSB-18 primary cell culture (grade III) yielded inconsistent and rarely reproducible results with large standard deviations.

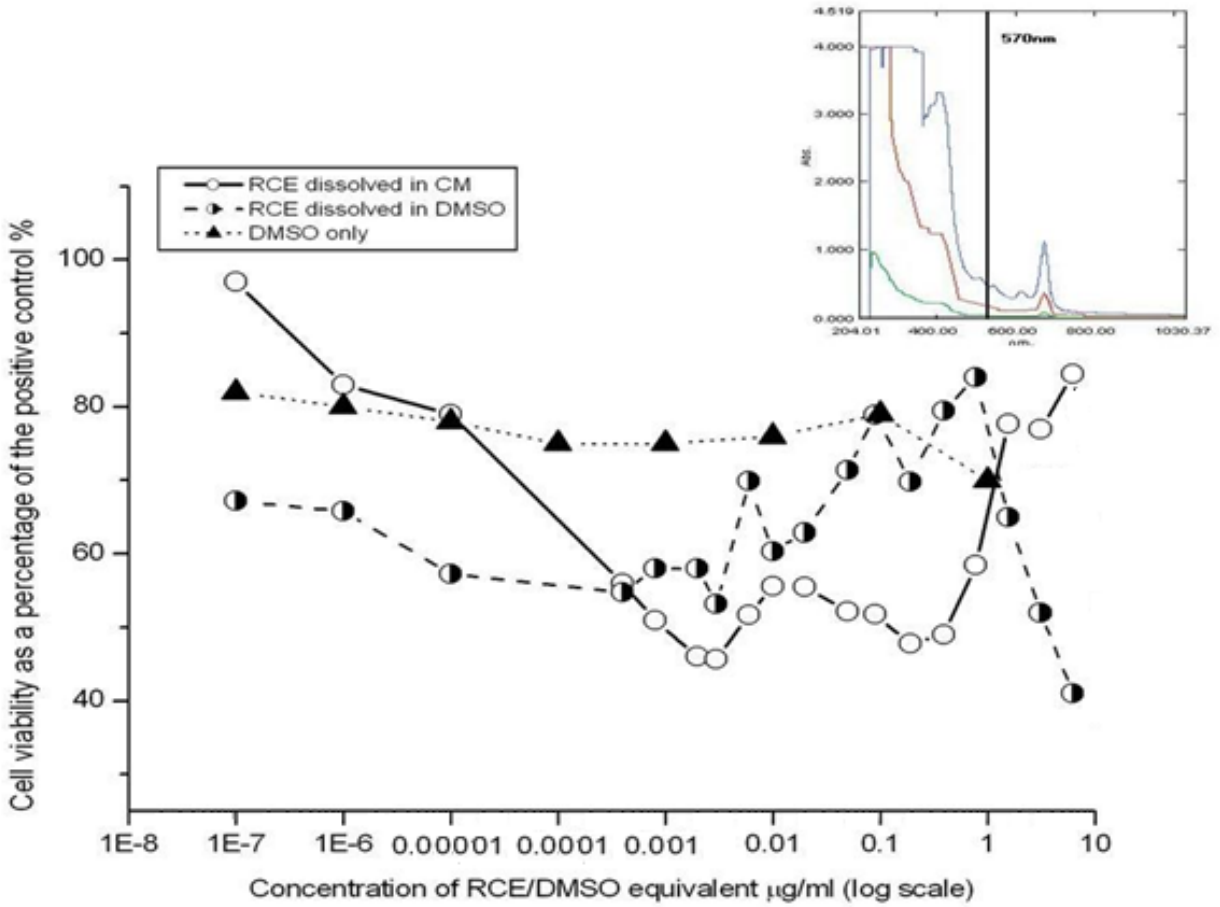


Figure 14 The mean cell viability of IPSB-18 (an established anaplastic astrocytoma cell line), as a percentage of the positive control (clear DMEM) against the concentration of (a) RCE dissolved in clear DMEM, (b) RCE dissolved in DMSO and (c) DMSO only on a log scale. Indicated is a decrease in cell viability upon treatment of cell cultures with DMSO thus impacting the cell viability of the treated cells. The inserted absorbance spectra indicate upon a concentration of RCE greater than 1μg/ml (blue and red) the colour of the micronutrient affects the absorbance reading at 570nm.

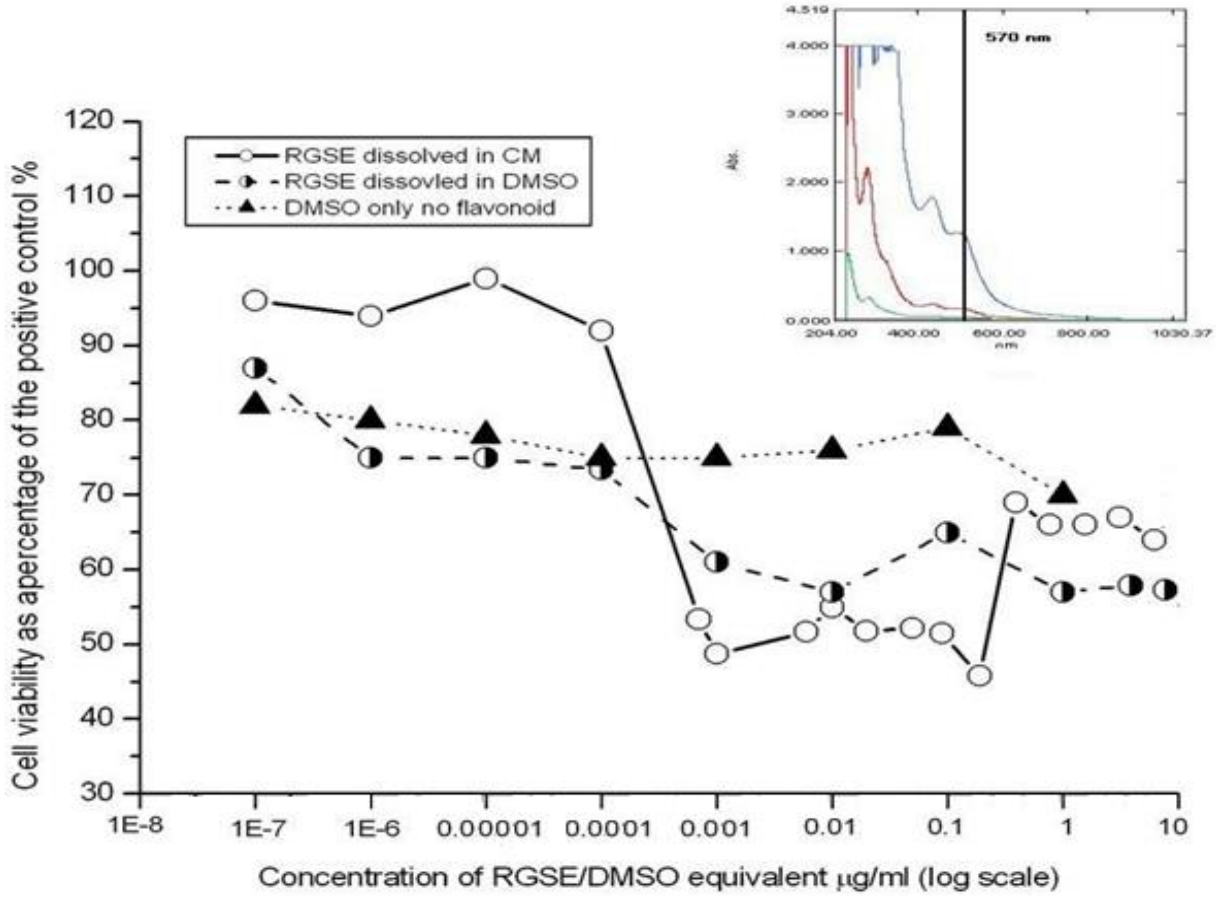


Figure 15 The mean cell viability of IPSB-18 (an established anaplastic astrocytoma cell line), as a percentage of the positive control (clear DMEM) against the concentration of (a) RGSE dissolved in clear DMEM, (b) RGSE dissolved in DMSO and (c) DMSO only on a log scale. The insert represents the absorbance spectra of three concentrations of RGSE dissolved in clear media ; 10μg/ml (blue), 1μg/ml (red) and 0.1μg/ml (green) upon a concentration of RGSE greater than 1μg/ml (blue and red) the colour of the micronutrient affects the absorbance reading at 570nm.

DMSO, often used to aid solubilisation of micronutrient extract, has a significant toxic effect on the IPSB-18 astrocytoma cell line (Fig 14). Results using the MTT viability assay on glioma cells exposed to DMSO used to dissolve the micronutrients showed at least a 20% decrease in viability when compared to cells treated with micronutrient extracts dissolved in clear DMEM only. When this DMSO toxicity his combined with high concentration deposition of RCE, evaluation of cel viability is anomulus. An almost identical pattern is seen with RGSE treatment of the IPSB-18 as RGSE also has a significant absorption at 570nm (Fig 38). This suggested the DMSO used to dissolve the micronutrient extracts may be having a toxic effect on the delicate brain tumour cells. This affect may be attributed to the mode of action of DMSO.

4.3.2 Cell viability following treatment with micronutrient extracts

Initial studies treated glioma cell cultures with RCE and RGSE over a wide range of concentrations. In particular the IPSB-18 cell line was treated using a range of concentrations between 250mg/ml to 0.04μg/ml. When performing the MTT assay, concentrations <20μg/ml appeared to decrease cell viability of glioma cultures, whereas concentrations >20μg/ml appeared to increase viability. Each of the assays was compared with phase contrast micrography. The reduction in cell viability was seen in all the respective analyses.

However, following further investigation it was found that the pigmentation of the micronutrients at higher concentrations was interfering with the absorbance reading of the assay. Therefore, subsequent evaluation of the cell viability of RCE and RGSE was carried out with micronutrient concentrations below 10μg/ml and 20μg/ml, respectively. These analyses suggested that both micronutrient extracts had an effect on cell viability. To ensure the effect of the micronutrient extracts was specific to glioma cells, experimentation on the control culture (MUAB-C) was performed. Neither micronutrient appeared

to affect the viability of normal brain tissue following 48 hours of treatment at

concentrations of between 0.001μg/ml and 10μg/ml. These were the same concentrations at which the glioma cells were investigated to determine the IC_{50} (Fig 16).

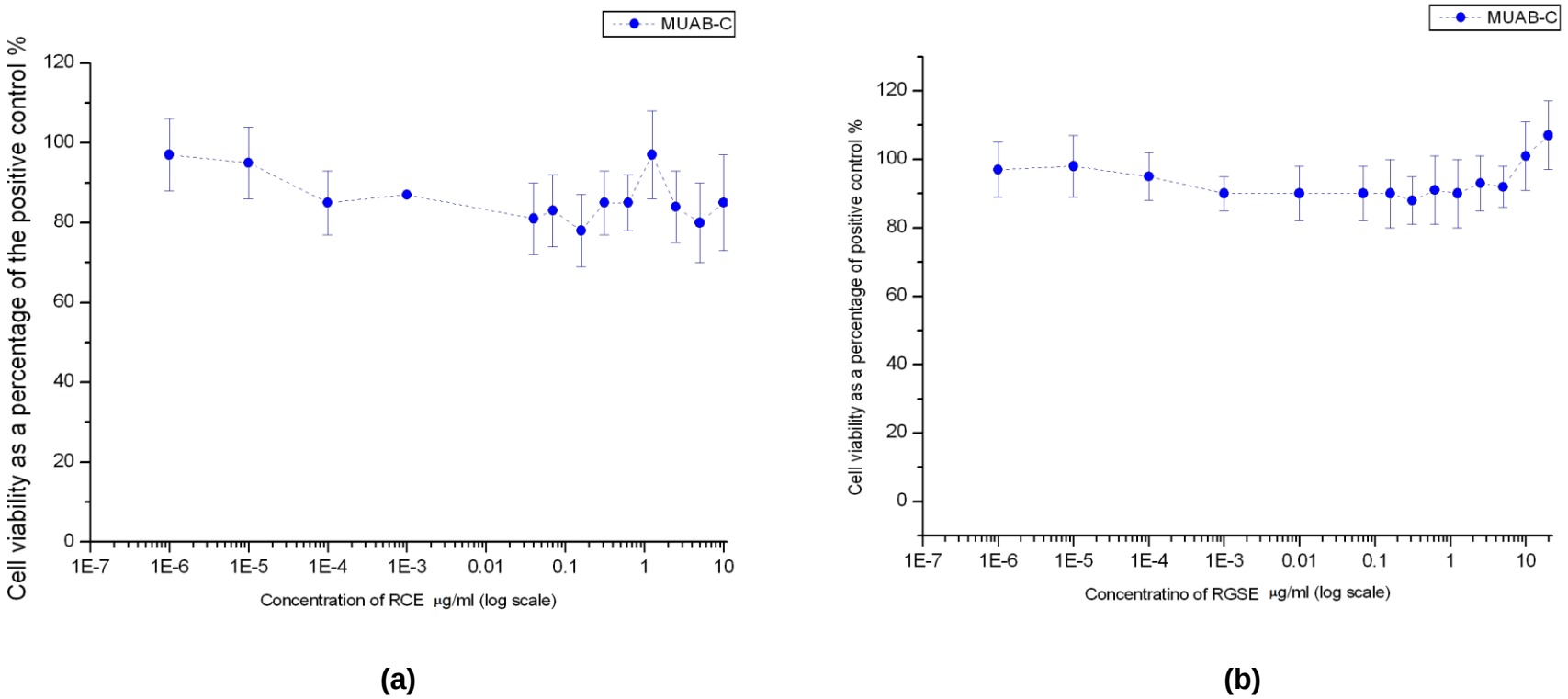


Figure 16 Mean percentage viability of MUAB-C using a) RCE and b) RGSE. The percentage viability was calculated as a percentage of the positive control (point 0 – assumed100% viability). The standard deviation is displayed as error bars. No significant differences were noted in the viability of MUAB-C following ANOVA analysis (p= 0.13 and 0.33 respectively)

4.3.3 Interference of Micronutrient Pigments on Cell Viability Assay

Initial studies on the IPSB-18 glioma cell culture found that, at concentrations of between 20μg/ml and 250μg/ml, incubation with RCE resulted in an initial decrease in cell viability at the lower concentrations of the micronutrient, followed by an apparent increase at the highest concentrations (Fig 17). This apparent increase in viability was also seen when IPSB-18 was treated with the same concentrations of RGSE (Fig 18).

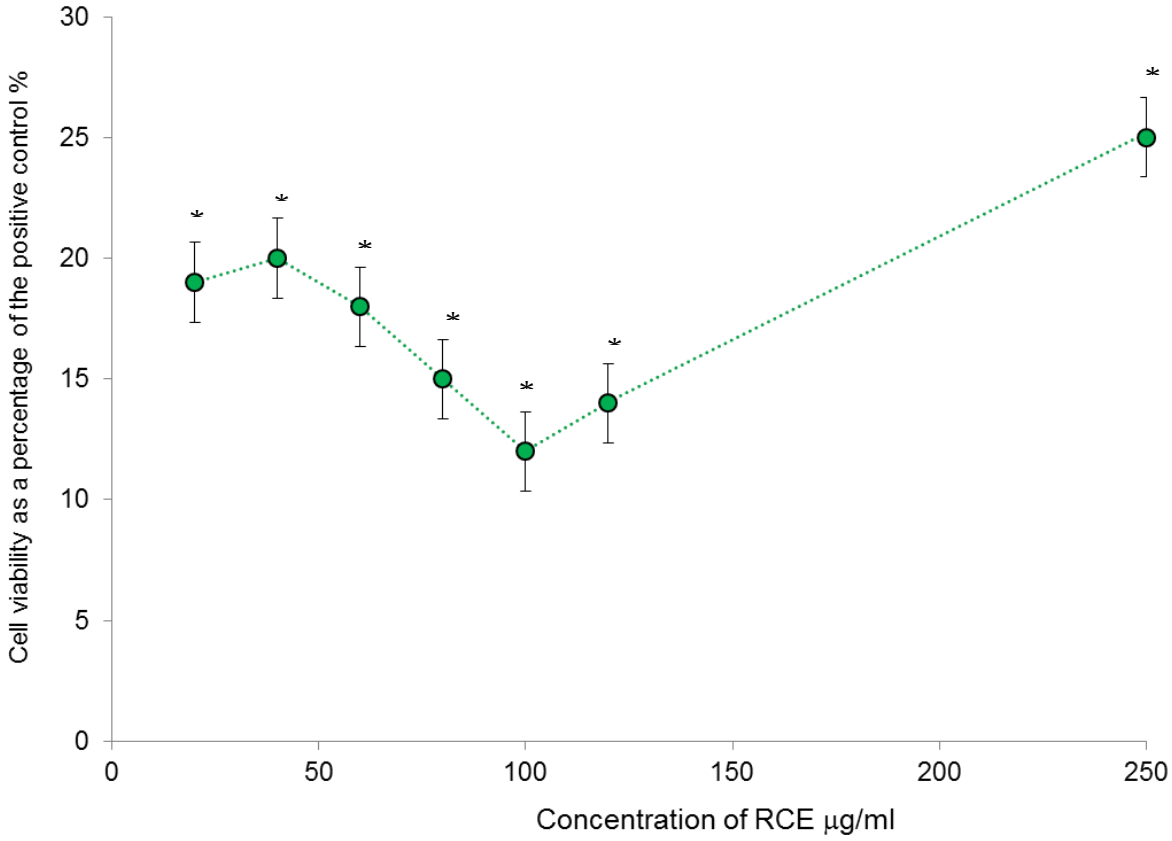


Figure 17 Mean percentage viability of IPSB-18 using RCE at concentrations between 250μg/ml to 20μg/ml. The percentage viability was calculated as a percentage of the positive control (untreated cells - point 0 is assumed 100% viability). The standard deviation is displayed as error bars. Significant difference between untreated and treated cells, following a Tukey's HSD test is displayed (* $p<0.01$)

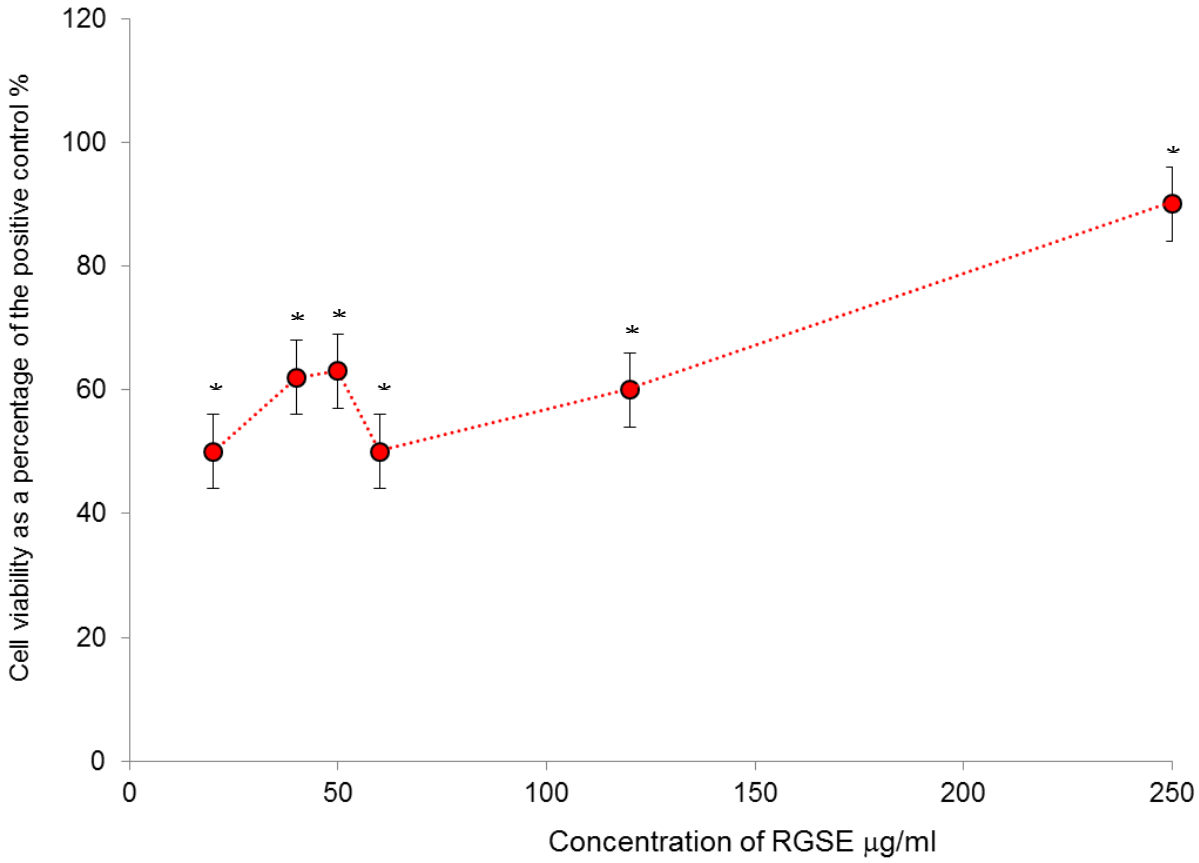
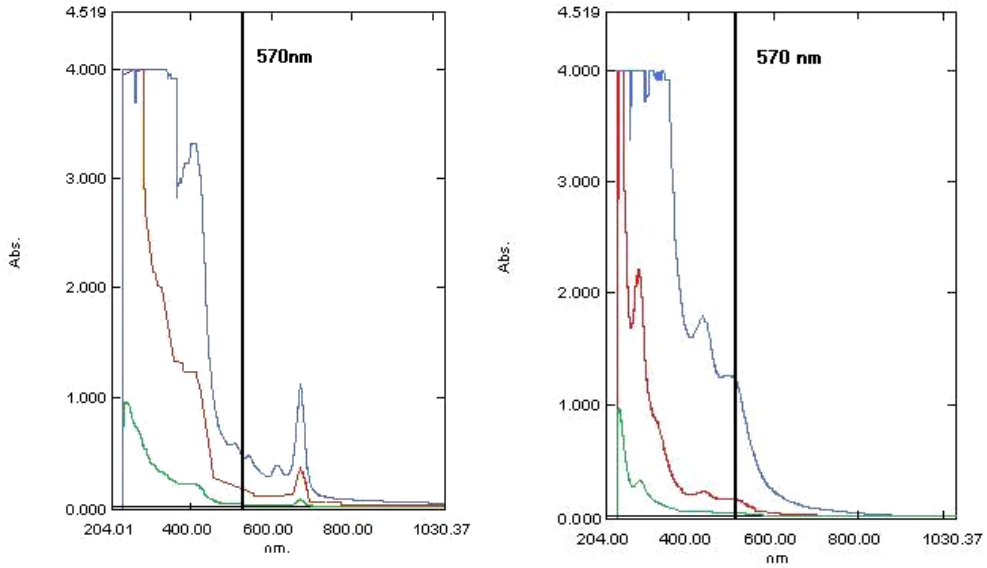


Figure 18 Mean percentage viability of IPSB-18 using RGSE at concentrations between 10µg/ml to 250µg/ml. The percentage viability was calculated as a percentage of the positive control (point 0 – assumed 100% viability). The standard deviation is displayed as error bars. Significant difference between untreated and treated cells, following a Tukey's HSD test are displayed (* $p<0.01$)

The apparent increase in cell viability of the glioma culture, IPSB-18, was more prominent at the highest concentrations. However, phase contrast micrographs showed no viable glioma cells following treatment with concentrations of >60µg/ml of either micronutrient extract. This suggested an anomalous result and therefore warranted further investigation.

The mean viability, as a percentage of the positive control, of the IPSB-18 (astrocytoma) cell line when treated with RCE shows a dose dependant decrease up to approximately 1μg/ml of RCE. Beyond this a variable and general increase in viability appears. Following washing of the wells during viability assays, it was noted that, despite copious washing, it was not possible to remove all of the extract. The absorbance of treated plates without the presence of the MTT assay components added was examined to determine whether any residual extract may be contributing to the unusual result found with increasing concentration of the micronutrients. Despite increased washing of the wells (> 4 times) it was confirmed that the extract was resulting in the biphasic anomaly.

Supplementary studies confirmed that the natural colour of the extracts interfered with the optical density/absorbance readings (Fig 19). Although RCE and RGSE has absorption maxima at between 200 and 400nm it still has significant absorption at 570nm which rises dramatically as we move to concentrations greater than 0.1μg/ml. Thus, any deposited RCE at such concentration can significantly contribute to absorption at this wavelength. making, concentrations of >1μg/ml of both micronutrients were unsuitable for use in the MTT viability assay.



(a) **(b)**

Figure 19 Absorption spectra of three concentrations read at 570nm; 10μg/ml (blue), 1μg/ml (red) and 0.1μg/ml (green) for (a) RCE and (b) RGSE. Both RCE and RGSE indicate an absorbance at 570nm (vertical black line), the absorbance at which the MTT assay is read.

4.3.4 Red Clover Extract and Cell Viability

The IC_{50} of RCE was found to be in the μg/ml range for all of the glioma cell cultures. The MUTC-GM and IPSB-18 cultures were more sensitive to the micronutrient, with a lower IC_{50} (Fig 20).

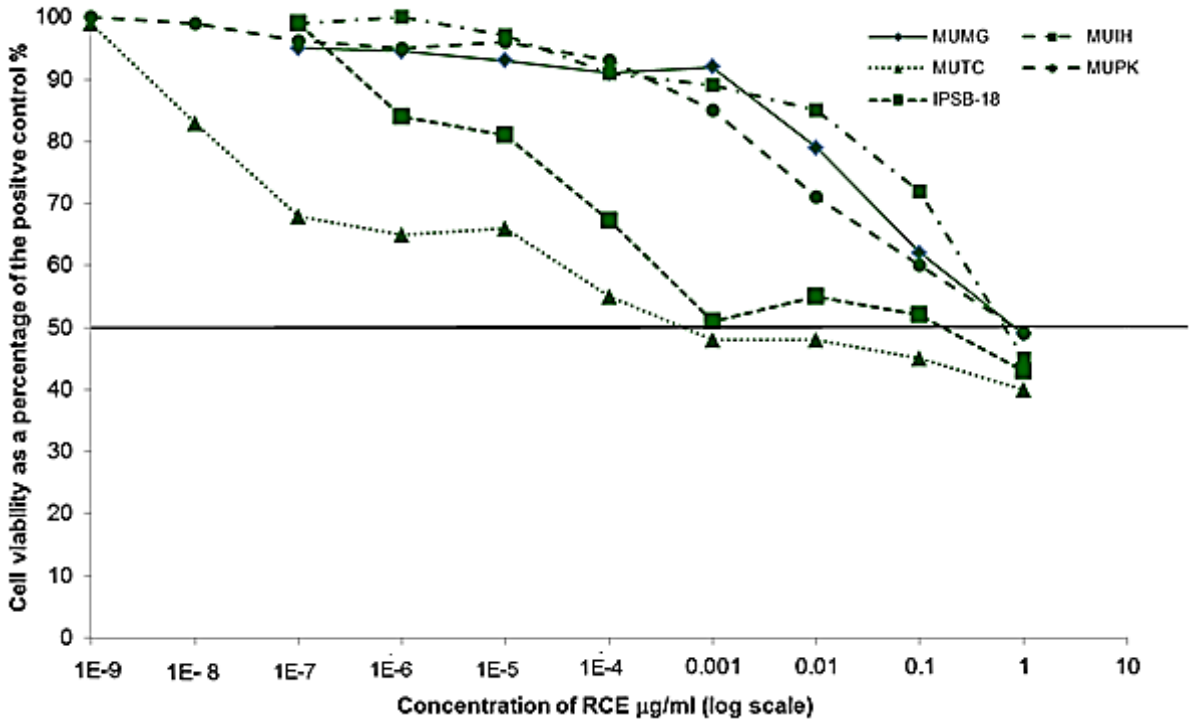


Figure 20 Cell viability of glioma cell cultures when treated for 48 hours with red clover extract (n=3). The three primary glioma cultures namely, MUMG-GM, MUIH-GM and MUTC-GM depicted IC_{50}s in the µg/ml range. MUPK-GM and IPSB-18 illustrated much lower IC_{50}s. Error bars are illustrated in Figures 22 and 23

IPSB-18 was the only established cell culture to be used in the evaluation of the affects of RCE and RGSE on human adult glioma cells. The increased passage number of the IPSB-18 is suggestive of decreased heterogeneity of the cells (Pilkington 2001), as such the IC_{50} was expected to be lower than the other glioma cultures.

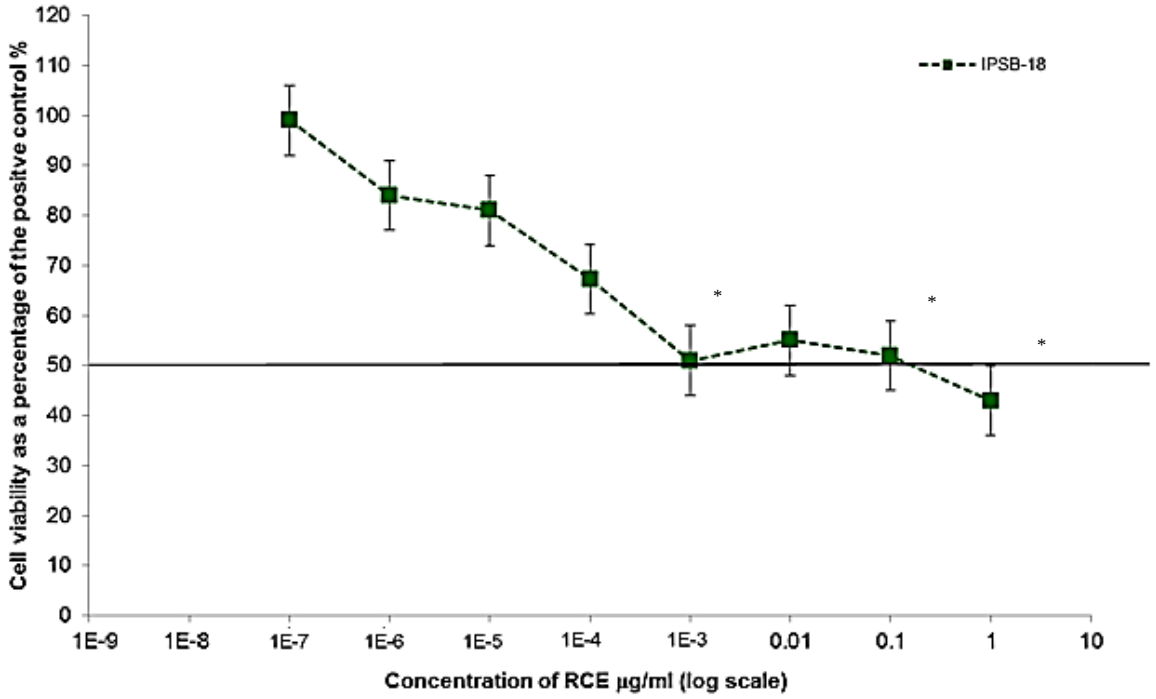


Figure 21 Scatter plot illustrating the cell viability of IPSB-18 (an established anaplastic astrocytoma cell line) as a percentage of the positive control (clear DMEM) against the concentration of RCE on a log scale. The error bars represent the standard deviation. A significant difference is seen in the change in viability of treated cells when compared to untreated (*p <0.001 following Tukey's HSD test)

The IC_{50} of RCE on IPSB-18 was found to be 400ng/ml (Fig 21). Due to the increased heterogeneity indicated in low passage cultures, the IC_{50}s of the other glioma cultures were expected to be higher than 0.001µg/ml. The IC_{50} of RCE for MUMG-GM (p5) was found to lie around 1µg/ml (Fig 22).

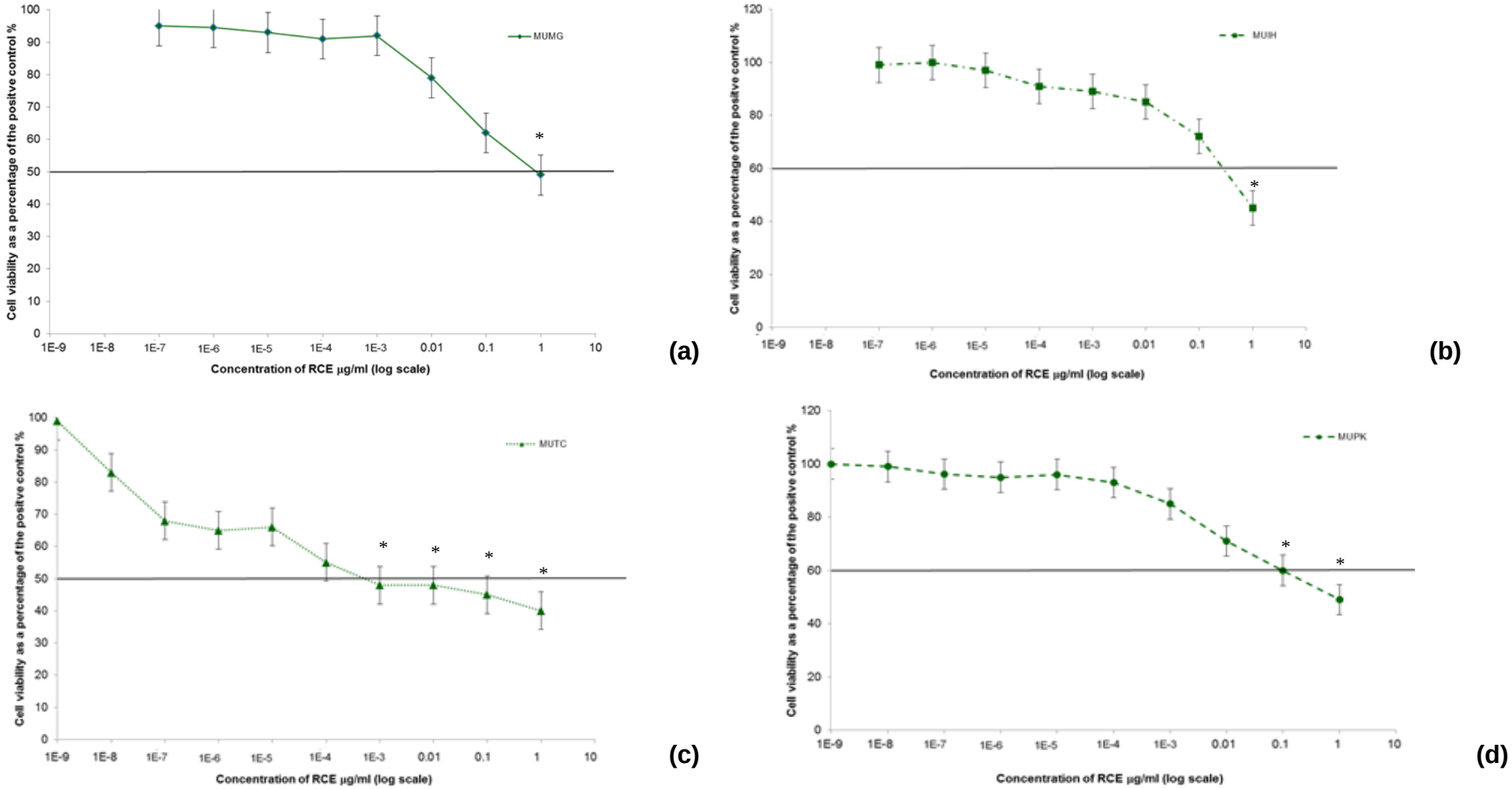


Figure 22 Mean percentage cell viability of (a) MUMG-GM, (b) MUIH-GM (c) MUTC-GM and (d) MUPK-GM following incubation with RCE for 48hrs at concentrations between 0 and 1µg/ml. The percentage viability was calculated as a percentage of the positive control (point 0 – assumed 100% viability). The standard deviation is displayed as error bars. A significant difference is seen in the change in viability (*p <0.001 following Tukey's HSD test for all glioma cell lines)

Similarly, to MUMG-GM, the remaining primary cultures, MUIH-GM, MUTC-GM and MUPK-GM (p4), were also investigated following incubation with RCE at concentrations below 1μg/ml. The IC_{50} values illustrated in figure 22 have been summarised in Table 16. The IC_{50} of MUTC-GM was found to be the lowest, followed by IPSB-18. The other glioma cultures indicated higher IC_{50} values when compared to IPSB-18, as expected.

Table 16 Summary of the IC_{50} of RCE with various cell cultures including biopsy derived primary cultures (MUMG, MUTC, MUIH and MUPK) and an existing low passage cell line (IPSB-18).

Cell Culture	**Passage Number**	**IC_{50}** *RCE*
IPSB-18	29-30	0.4μg/ml
MUMG-GM	4-5	1 μg/ml
MUTC-GM	3-4	0.007μg/ml
MUIH-GM	3-4	0.98μg/ml
MUPK-GM	3-4	0.80μg/ml

4.3.5 Red grape seed extract

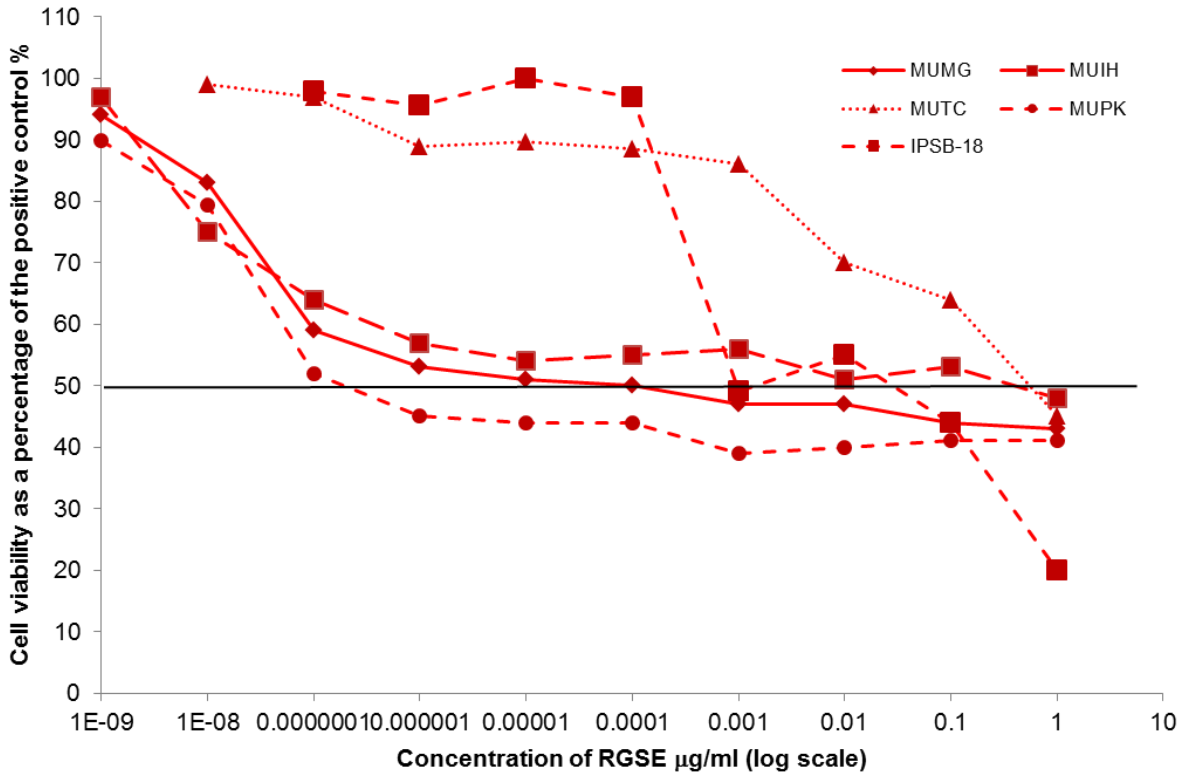


Figure 23 Cell viability of glioma cell cultures when treated for 48 hours with red grape seed extract (n=3). The IC_{50} for red grape seed extract was at a low concentration (ng/ml) for all cell cultures.

Incubation with red grape seed extract resulted in a similar reduction in viability of the glioma cell cultures as that seen in response to incubation with RCE. Figure 24 illustrates the mean percentage cell viability of IPSB-18 following 48 hr treatment with RGSE and the IC_{50} for RGSE was found to be 0.0008µg/ml, lower than the IC_{50} of RCE (0.4µg/ml). The remaining glioma cultures were also found to be more sensitive to RGSE (Figure 25 and Table 17).

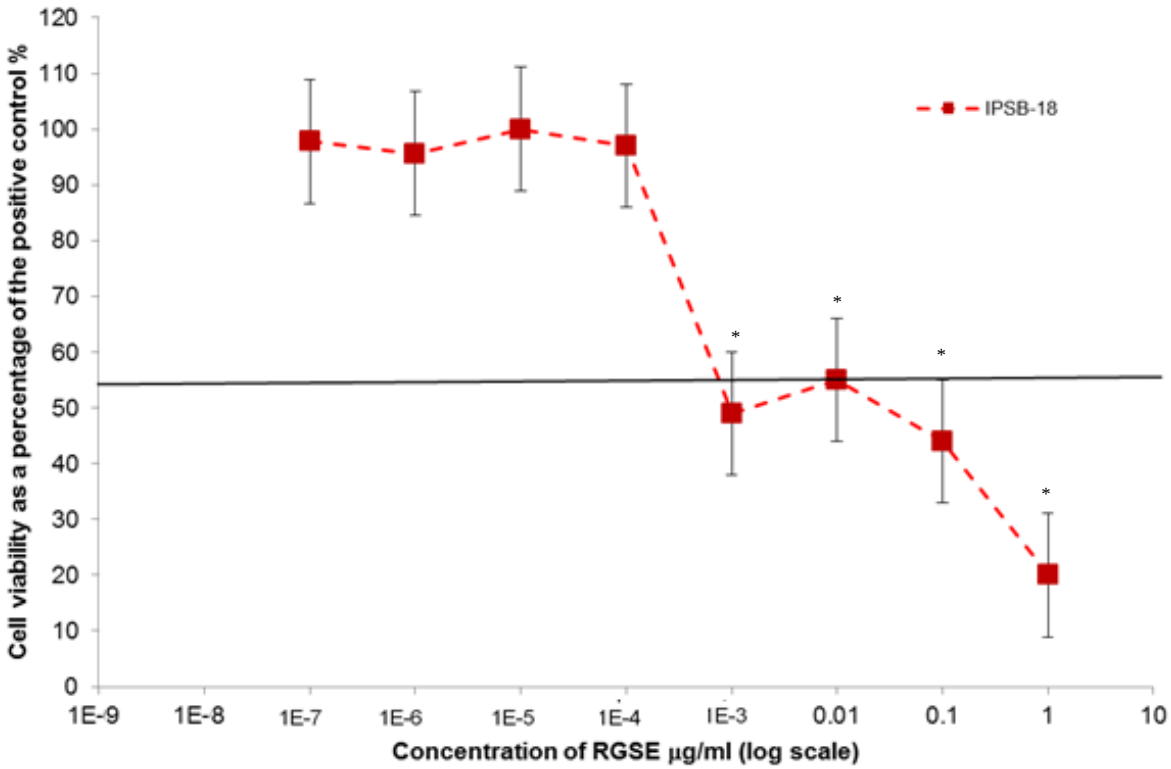


Figure 24 Mean percentage cell viability of IPSB-18 following 48 hr treatment with RGSE. The percentage viability was calculated as a percentage of the positive control (point 0 – assumed 100% viability). The standard deviation is displayed as error bars. A significant difference is seen in the change in viability of treated cells when compared to untreated (*p <0.001 following Tukey's HSD test)

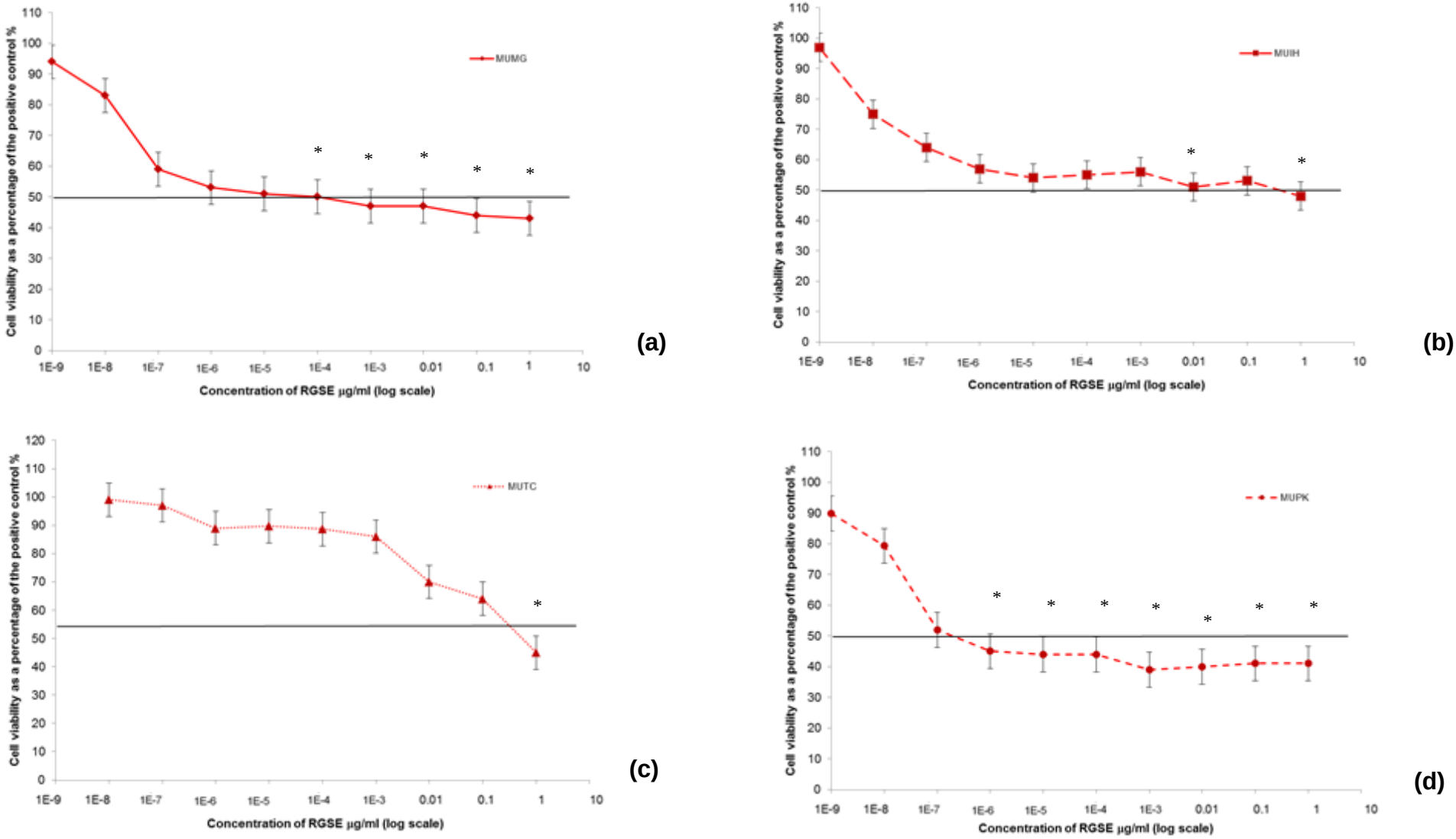


Figure 25 Mean percentage viability of (a) MUMG-GM, (b) MUIH-GM (c) MUTC-GM and (d) MUPK-GM following incubation for 48hrs with RGSE. The percentage viability was calculated as a percentage of the positive control (point 0 – assumed100% viability). The standard deviation is displayed as error bars. A significant difference is seen in the change in viability when compared to the untreated control (*p <0.001 following Tukey's HSD test for all glioma cell lines).

The IC_{50} of RGSE for the biopsy derived primary cell cultures was generally lower than those of RCE. The IC_{50} values for MUIH-GM, MUTC-GM and MUPK-GM (p4) are shown in Table 17.

Table 17 Summary of the IC_{50} of RGSE for various cell cultures including biopsy derived primary cultures and an existing low passage cell line (ISPB-18)**.**

Cell Culture	Passage Number	IC_{50} *RGSE*
IPSB-18	29-30	0.001μg/ml
MUMG-GM	4-5	0.0001μg/ml
MUTC-GM	3-4	0.5μg/ml
MUIH-GM	3-4	0.4μg/ml
MUPK-GM	3-4	0.0000001μg/ml

4.4 Antigenic Expression of Glioma Cultures using Flow Cytometry

Following the suggestion of a reduction in glioma cell viability, the evaluation of RCE and RGSE was extended to determine whether other characteristic biologic features of glioma cells, including invasion and angiogenesis, were affected following 48-hour treatment *in vitro* with the respective IC_{50} of either micronutrient. The antigenic expression of a cell can provide an indication of the possible biologic features affected within the cell cultures. The antigens chosen for investigation, therefore, are those involved in the pathways that govern invasion, and/or angiogenesis.

Cells were investigated for changes in expression of six antigens namely GFAP, CD44, Eric-1 NCAM, β1 integrin and αv integrin. Representative plots are shown in Figure 26. Statistical analysis of all the plots is illustrated in Table 18.

Plots were produced showing only cells which were PI negative and FITC positive; these are live cells expressing the antigen. Cells were excluded if propidium iodide (PI) positive (indicating a permeable nuclear membrane and therefore dead) and if PI negative but also FITC negative (indicating no expression of the antigen).

The expression of GFAP in the IPSB-18 culture was found to be as high as that of the biopsies in this study, in particular MUTC-GM (Table 18). When compared to the untreated cells GFAP did not appear to change in the micrographs. CD44 did not exhibit a significant up or down regulation in any of the cultures. Though a slight up regulation was illustrated following treatment with RCE, when compared with the untreated culture, this was not marked enough to be statistically significant. Eric-1 NCAM did not indicate a significant difference in expression following treatment with either micronutrient extract when compared to the untreated cultures. Both β1 and αv integrins demonstrated marked up regulation in the flow plots and a significant difference was found between the treated and untreated cultures suggesting an effect of RCE and RGSE on the invasive properties of the glioma cells.

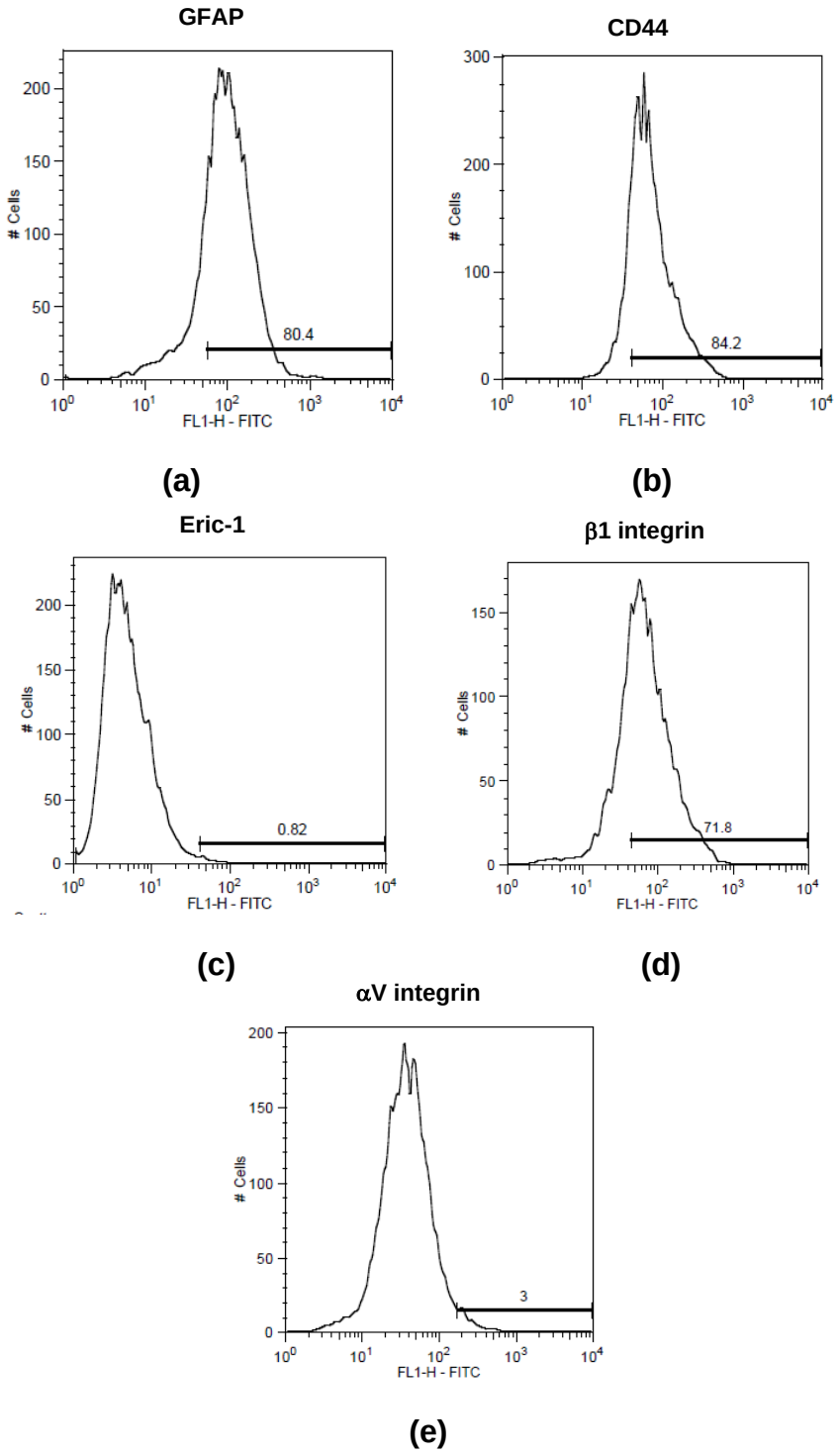


Figure 26 Representative plots of the percentage of fluorescent cells, indicating the median expression (FL-1H-FITC – intensity of fluorescence) of (a) GFAP, (b) CD44, (c) Eric-1 NCAM, (d) β1 integrin and (e) αv integrin in a treated glioma cell culture (IPSB-18), following 48 hour treatment with the IC_{50} of RCE. The expression of CD44, Eric-1 NCAM and both integrins was expected to be up or down regulated upon treatment with the IC_{50} of RCE. The median percentage expression is indicated on the plot.

Table 18 Mean antigenic expression (%), standard deviation (SD) and *p* values (calculated using a unpaired t-test) of GB cell lines following 48hr treatment with RCE or RGSE at the respective IC_{50}, when compared to the antigenic expression of the respective untreated glioma culture.

CELL LINE	RCE					RGSE				
	GFAP	*CD44*	*Eric-1 NCAM*	*β1 integrin*	*αv integrin*	*GFAP*	*CD44*	*Eric-1 NCAM*	*β1 integrin*	*αv integrin*
IPSB-18										
mean	83.6	1.88	4.39	86.2	87.2	79.1	3.24	5.67	98.2	72.4
SD	11.3	7.5	7.9	4.5	5.5	13.4	5.3	8.2	7.6	2.8
p	0.015	0.109	0.98	0.0007	0.0008	0.0439	0.08	0.89	0.0006	0.0017
MUMG-GM										
mean	61.2	5.76	23.6	78.6	51.2	85.6	4.30	28.9	69.1	76.5
SD	2.6	5.2	6.7	7.9	6.4	2.1	2.5	1.2	8.88	8.4
p	0.003	0.23	0.068	0.0006	0.0006	0.00002	0.073	0.097	0.0013	0.0008
MUIH-GM										
mean	75.3	2.98	16.5	65.3	65.8	83.4	2.57	11.9	64.8	61.9
SD	5.3	7.8	5.9	7.4	9.2	2.1	6.1	2.3	6.6	8.7
p	0.008	0.093	0.07	0.0012	0.0005	0.0013	0.059	0.11	0.001	0.0005
MUTC-GM										
mean	83.9	4.15	6.34	96.8	53.8	87.9	7.27	4.59	95.4	69.8
SD	3.6	10.8	3.4	2.9	7.4	7.5	6.9	8.7	1.5	8.9
p	0.0002	0.192	0.0693	0.0001	0.0019	0.062	0.175	0.078	0.0001	0.0009

4.6 Analysis of Antigenic Expression using Immunocytochemistry

To verify flow cytometry data, the expression of the same six antigens was investigated using immunocytochemistry. Upon comparing the immunocytochemical micrographs against the flow cytometry plots, it was found that the same trends in antigenic expression changes were seen. Of the six antigens measured on the IPSB-18 glioma cell culture, the most discernable differences in expression, following treatment with either RCE or RGSE, were seen with the α and β integrins (Fig 27). The change in expression of GFAP, following treatment, was not as clearly seen in the micrographs (Fig 27a). Table 19 summaries the intensity of fluorescence visualized following 48hr treatment of the glioma cell cultures with the IC_{50} of RCE or RGSE. The expression was measured over three fields under the microscope.

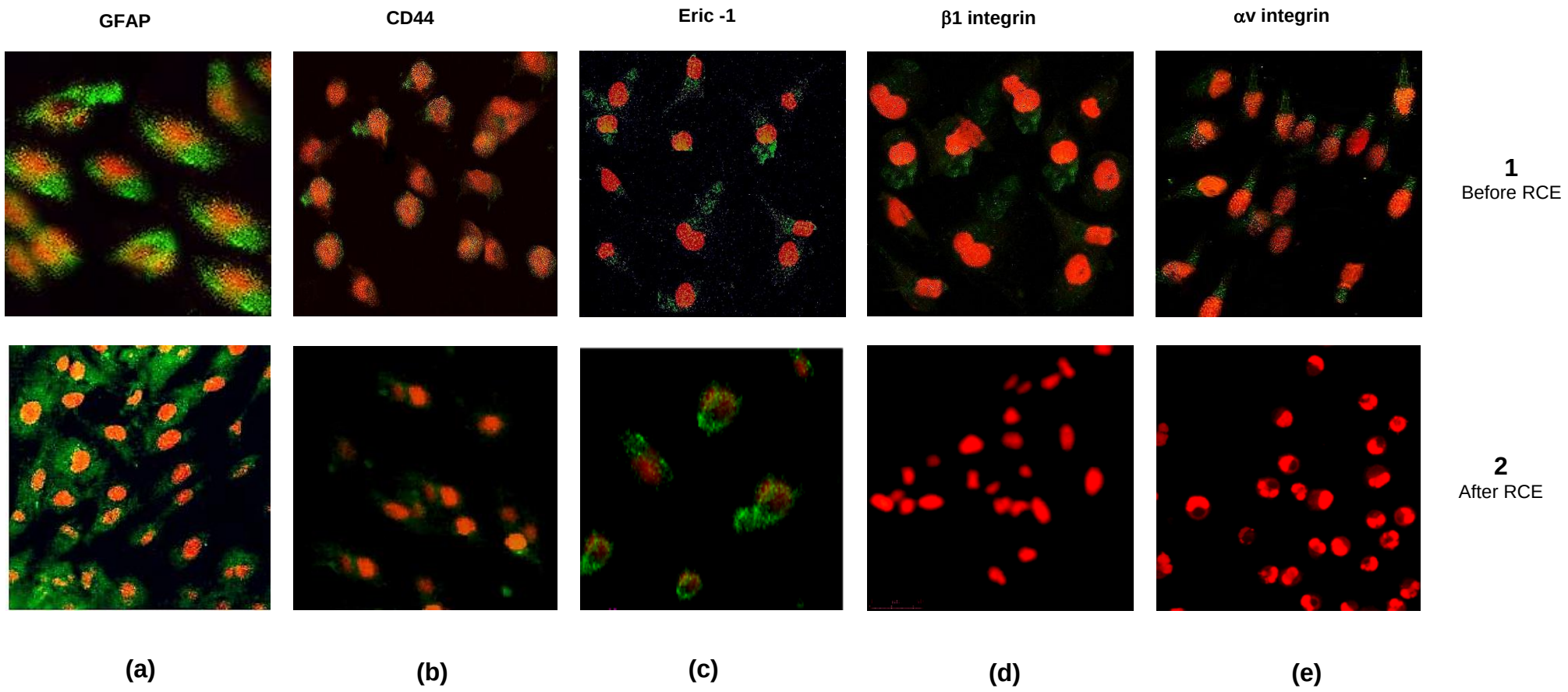


Figure 27 Representative micrographs of the expression of (a) GFAP, (b) CD44, (c) Eric-1 NCAM, (d) β1 integrin and (e) αv integrin, without treatment of RCE (1) and following 48 hr treatment of the IC_{50} of RCE on IPSB-18 cells (2). Magnification x40

Table 19 Antigenic expression in glioma cell lines incubated with the respective IC_{50} of either RCE or RGSE for 48 hours, of five antigens were visualized over three fields and compared to an untreated control (assumed 100%) (+ = positive expression, ± = some expression and – little to no perceived expression).

CELL LINE	RCE					RGSE				
	GFAP	*CD44*	*Eric-1 NCAM*	*β1 integrin*	*αv integrin*	*GFAP*	*CD44*	*Eric-1 NCAM*	*β1 integrin*	*αv integrin*
MUAB-C	+++	±	±	++	±	+++	±	-	++	±
IPSB-18	+++	±	++	±	-	+++	±	±	±	-
MUMG-GM	+++	-	++	±	-	+++	±	±	±	-
MUIH-GM	++	-	++	±	-	++	-	+	±	-
MUTC-GM	+++	-	+	±	-	+++	-	++	-	-

4.5 2D Change in Invasive Potential of Glioma Cell Cultures

Significant changes in the expression of the integrins suggested that there would be a change in the invasive potential of the glioma cells subsequent to treatment with either micronutrient. All the cultures were analysed in transwell chambers (Chapter 2.7) after treatment with either micronutrient extract (at the IC_{50}), for 48 hours, including the control cell culture (MUAB-C). Three fields were counted using a haemocytometer and each analysis was repeated three times, resulting in a total of nine field counts per experiment.

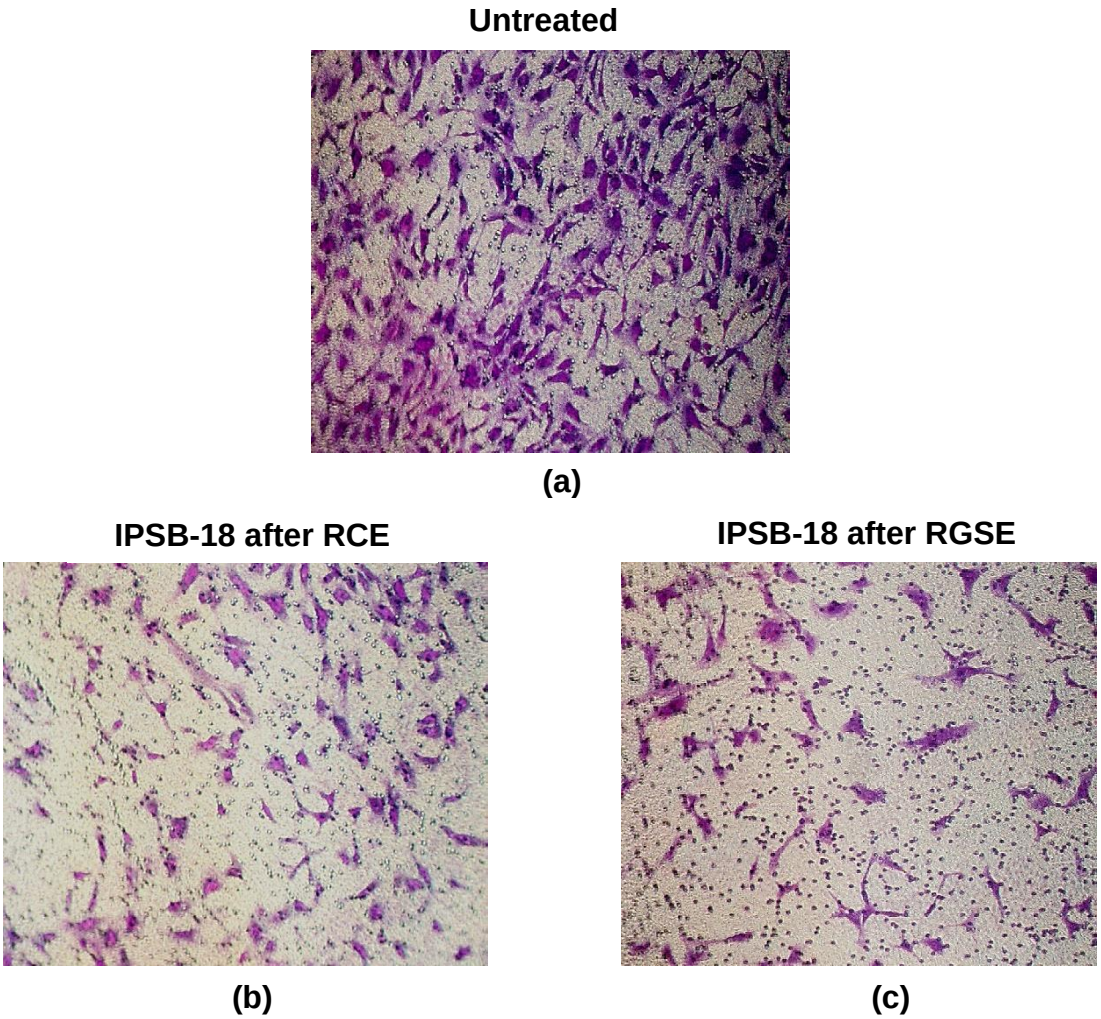


Figure 28 Representative micrographs of the invasive potential of GBs (IPSB-18) untreated (a) and after treatment with the respective IC_{50} of micronutrient extracts; (b) RCE and (c) RGSE. Following treatment in the presence of either micronutrient fewer cells diffused across the chamber indicating a reduction in invasive potential. Micrographs illustrate cells which have invaded. Magnification x40

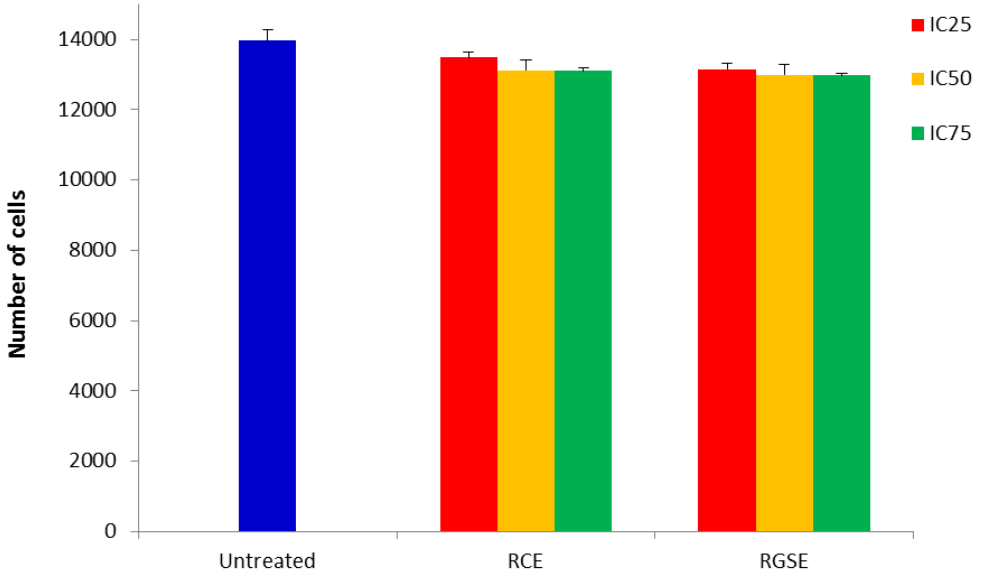


Figure 29 The mean number (three repetitions) of MUAB-C control cells which have invaded across the ECM in the Boyden chamber. Invasion of cells treated with the IC_{50} of RCE or RGSE were compared to untreated cells. The standard deviation is displayed as error bars. Following an ANOVA analysis no significant difference was found between the untreated and treated cultures

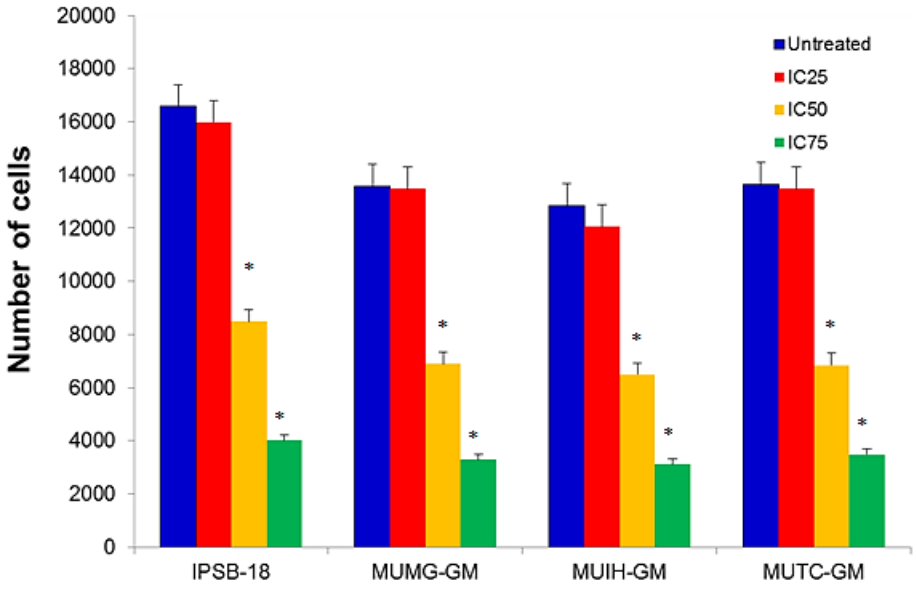


Figure 30 The mean number (three repetitions) of glioma cells invading across the ECM of the Boyden chamber following 48 hour treatment with RCE as a percentage of the positive control (clear DMEM). The standard deviation is displayed as error bars. Significant differences calculated using Tukey HSD test are indicated (* p<0.05,

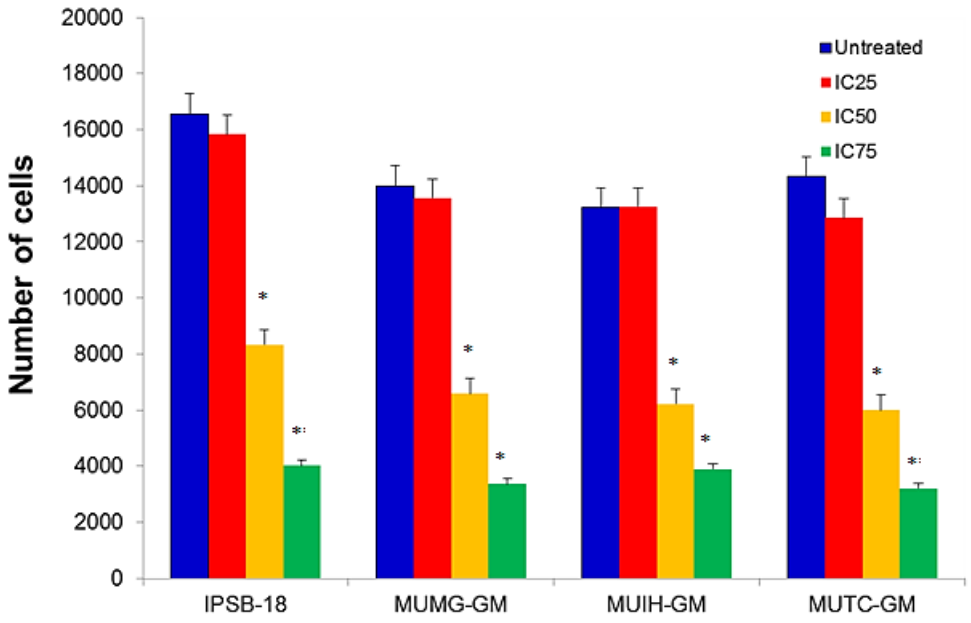


Figure 31 The mean number (three repetitions) of glioma cells invading across the ECM of the Boyden chamber following 48 hour treatment with RGSE as a percentage of the positive control clear DMEM. The standard deviation is displayed as error bars. Significant differences calculated using Tukey HSD test are indicated (* p< 0.05,

4.6 Apoptosis Assays – Annexin V

The reduction of cell viability as a result of exposure to the micronutrients seen earlier (section 4.3) could also be attributed to a pro-apoptotic effect. Two assays were used to investigate the apoptotic potential of the two micronutrients; the annexin V assay and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

Annexin V flow plots distinguish between live, dead and apoptotic cells. Figure 33 represents data for the IPSB-18 cell culture, an established glioma cell line, prior to any treatment and following treatment with either RCE or RGSE. The plots illustrate three foci; live cells on the lower left, apoptotic cells on the lower right and necrotic cells at the top. While a small number of apoptotic cells can be seen in the untreated samples, upon treatment with either RCE or RGSE no apoptotic were detected were detected in the lower right. This was true across all the cell lines.

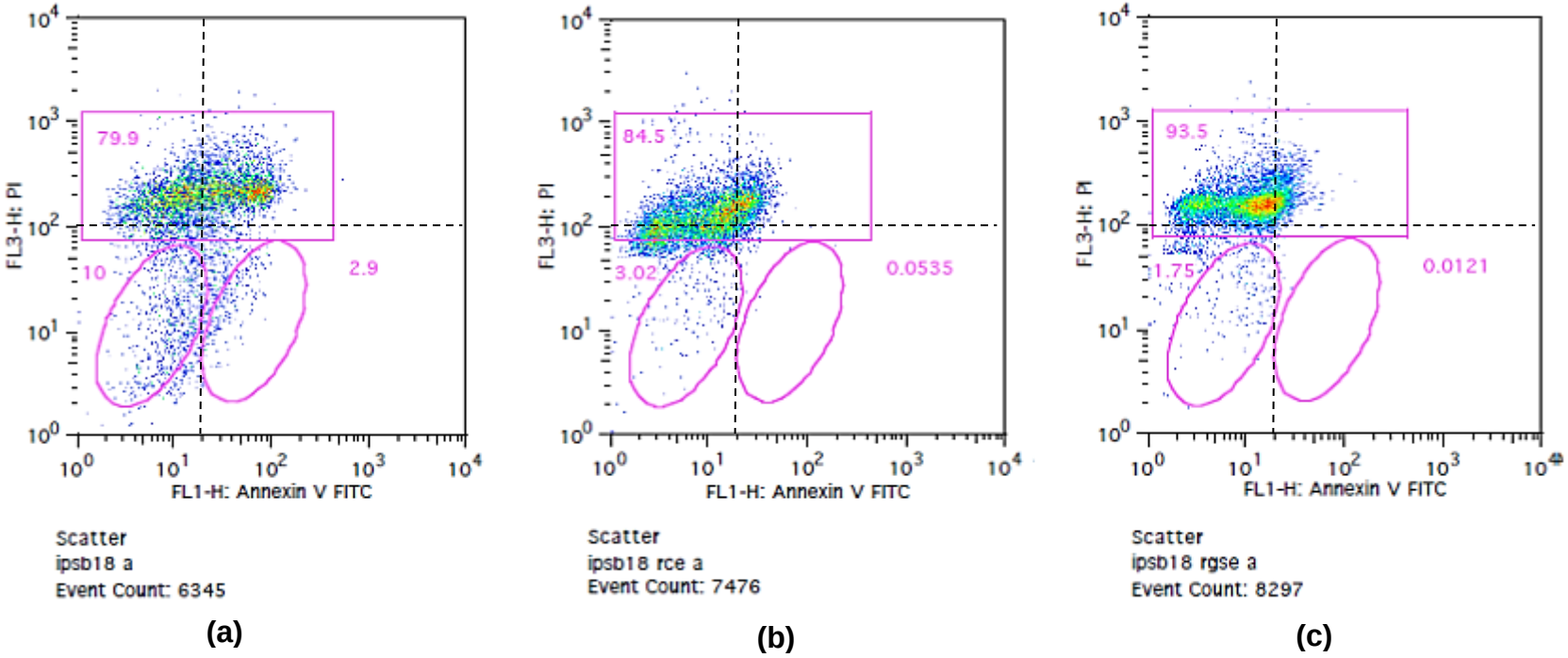


Figure 32 Apoptosis in the IPSB-18 glioma cell culture before (a) and after 48hr treatment with the IC_{50} of RCE (b) or RGSE (c). Data highlighted in the square are dead cells. Data highlighted by the ovals shown live cells (left) and apoptotic cells (right).

Table 20 Summary of the percentage of live, apoptotic and necrotic cells after 48 hour treatment with either micronutrient extract (at the IC_{50}) (n=3). Change was measured in comparison to the respective untreated cell culture, using a t-test. No significant differences when compared to untreated cells were noted.

CELL LINE	RCE			RGSE		
	Live	*Apoptotic*	*Necrotic*	*Live*	*Apoptotic*	*Necrotic*
MUAB-C*	52.8	10.5	29.7	67.3	12.8	32.4
IPSB-18	3.02	0.05	84.5	1.75	0.01	95.5
MUMG-GM	2.04	0.03	79.9	1.25	0.02	89.4
MUIH-GM	2.14	0.08	85.7	2.01	0.01	91.6
MUTC-GM	1.95	0.04	90.3	0.95	0.01	89.1

Note: percentages do not add up to 100%. Cells not included were not viable
*Control cell line
IC_{50} of RCE - IPSB-18, 0.4μg/ml; MUMG-GM, 1 μg/ml; MUTC-GM, 0.007μg/ml; MUIH-GM, 0.98μg/ml; MUPK-GM, 0.80μg/ml
IC_{50} of RGSE - IPSB-18, 0.001μg/ml; MUMG-GM, 0.0001μg/ml; MUTC-GM, 0.5μg/ml; MUIH-GM, 0.4μg/ml; MUPK-GM, 0.0000001μg/ml

No early pro-apoptotic effect was noted for any cell lines when compared to MUAB-C. Instead, a mass of dead cells were visible in the uppermost quadrants and some cells remained in the lower left quadrant (live cells). The cells indicated in the uppermost right hand quadrants suggest perhaps the occurance of late apoptosis. This indicated that, RCE and RGSE were indeed cytotoxic, and perhaps a shorter exposure to the respective IC_{50}s either micronutrient extract would illustrate an early pro-apoptotic effect. To ensure that this result was not a false negative an additional assay, the TUNEL assay, was performed to support the analysis.

4.7 Apoptosis Assays - TUNEL

The TUNEL assay results suggested a similar outcome to that seen in the annexin V plots. Apoptotic potential was indicated by the visualization of fluorescence in three different fields of the cultures analysed. The micrographs illustrated in Figure 34 illustrate that there were no visual differences between the untreated glioma cells, which indicated little to no fluorescence, and those treated with either RCE or RGSE. The lack of fluorescence suggests very little or no apoptotic potential. This result was seen across all the glioma cell cultures and the MUAB-C control culture. This result suggested that neither RCE nor RGSE affect the apoptotic potential of the glioma cells. Furthermore, due to the limited apoptotic potential seen in the untreated cultures it may be speculated that these cell cultures do not undergo apoptosis as their major pathway of cell death.

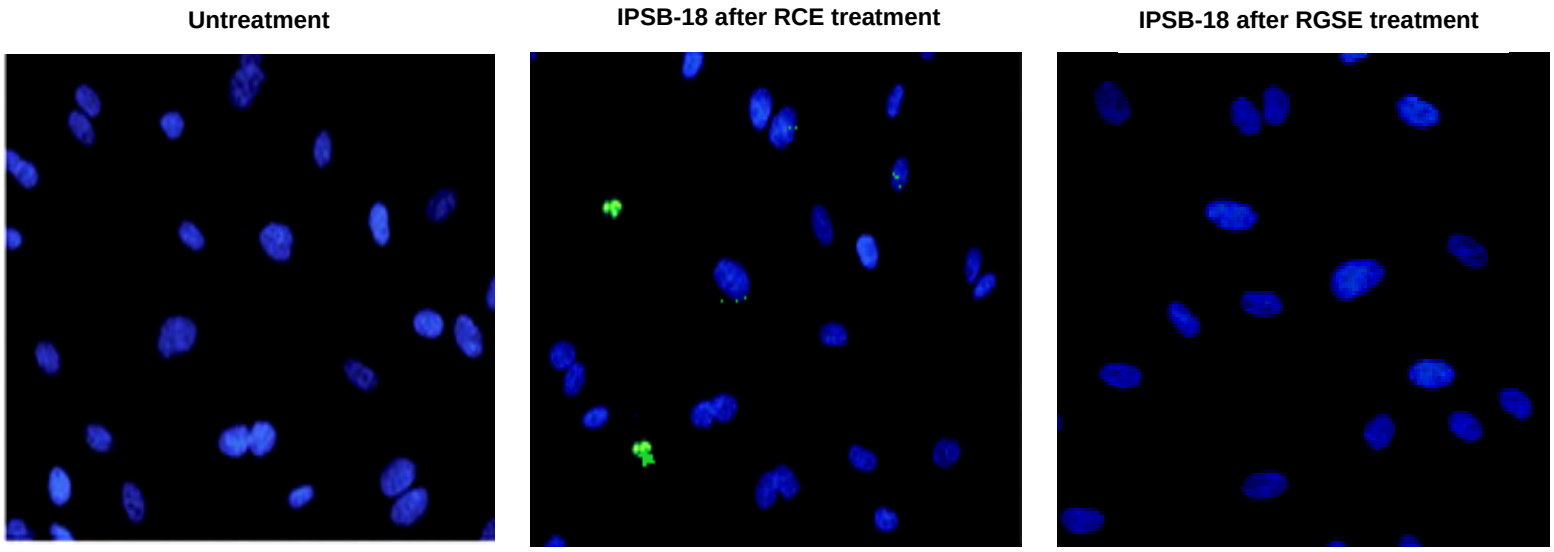


Figure 33 Micrographs representing apoptosis following the TUNEL assay in (a) untreated cells and following treatment with (b) RCE or (c) RGSE for IPSB-18 (nuclei = blue, apoptotic cells = green). Magnification x40

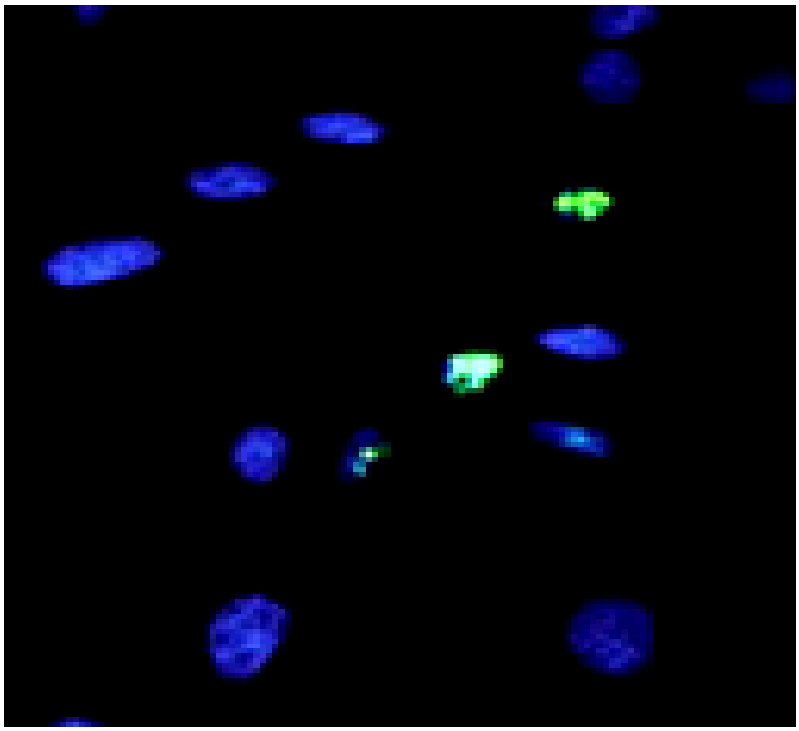


Figure 34 Micrograph representing apoptosis using the TUNEL assay in untreated MUAB-C control cells (nuclei = blue, apoptotic cells = green).

The suggestion that neither RCE nor RGSE induce apoptosis, at concentrations tested, in GB cell cultures was indicated by the results of the two independent assays.

Table 21 Mean number of fluorescent cells counted

CELL LINE	Untreated	RCE	RGSE
MUAB-C*			
Mean	4	3	1
SD	3	2	1
IPSB-18			
Mean	2	1	0
SD	1	1	0
MUMG-GM			
Mean	1	0	0
SD	1	0	0
MUIH-GM			
Mean	1	1	0
SD	1	1	0
MUTC-GM			
Mean	3	0	0
SD	2	0	0

Counts across three fields in an average of three experiments totaling nine counts, following 48hr treatment with the respective micronutrient at the IC_{50}.
*Control cell line
IC_{50} of RCE - IPSB-18, 0.4μg/ml; MUMG-GM, 1 μg/ml; MUTC-GM, 0.007μg/ml; MUIH-GM, 0.98μg/ml; MUPK-GM, 0.80μg/ml
IC_{50} of RGSE - IPSB-18, 0.001μg/ml; MUMG-GM, 0.0001μg/ml; MUTC-GM, 0.5μg/ml; MUIH-GM, 0.4μg/ml; MUPK-GM, 0.0000001μg/ml

4.8 Angiogenesis Assays – Tubule Formation Assay

This initial angiogenesis assay was carried out to determine whether either micronutrient could be shown to have an anti-angiogenic effect. This was achieved by culturing HUVEC cells and incubating these in the presence of either RCE or RGSE. During the angiogenesis process HUVEC cells normally exhibit tubule formation. In this assay the number of tubules formed after 48 hour treatment with either of the micronutrients were counted and plotted on a histogram. Figure 36 illustrates the findings and suggests a propensity for both micronutrient extracts to decrease tubule formation. In particular RCE was found to have a greater anti-angiogenic effect when compared to RGSE.

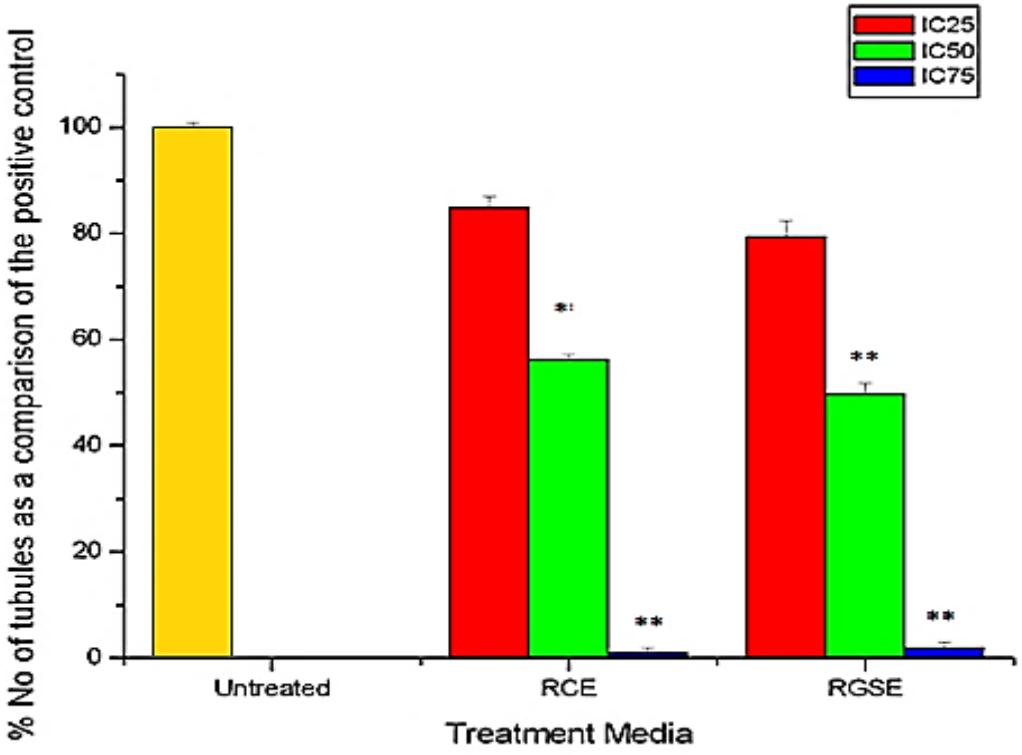
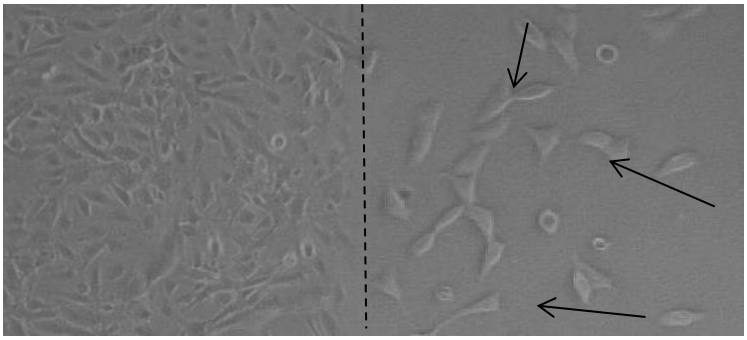


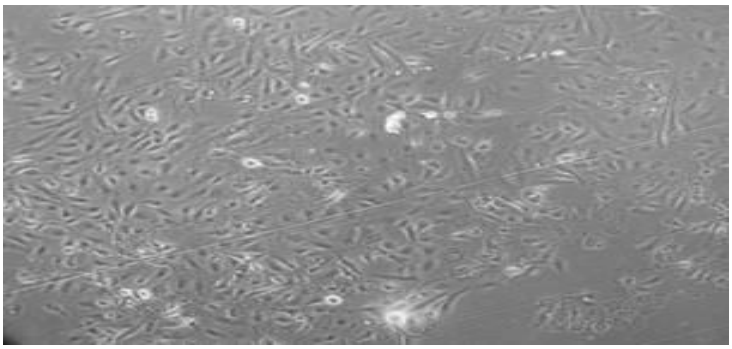
Figure 35 The average numbers of tubules formed by HUVEC cells were counted in three different fields, to examine the extent of the affect of RCE and RGSE on tubule formation. The graph illustrates the counts taken as an average of three repetitions of the experiment. Significant differences calculated using ANOVA are indicated (* $p<0.05$, ** $p<0.001$).

4.9 Angiogenesis Assays – Co-Culture Assay

The number of tubules counted upon co-culturing HUVECs with each of the glioblastoma cell lines were found to increase with time and could be seen to protrude towards the GB cells (Fig 37). It was also visually noted that the cell viability and the rate of growth of the HUVEC cells also increased upon introduction of GB cells. When treated with either of the micronutrient extracts, it was found that the number of tubules counted were fewer than those counted in the untreated cultures.



(a)



(b)

Figure 36 Representative micrographs illustrating tubule development of HUVEC cells (a) co-cultured with glioblastoma cells (the dotted line illustrates the delineation of the GBs on the left and HUVECs on the right) and (b) not co-cultured with GBs, without treatment with RCE or RGSE. Arrows indicate tubule formation. Cells were cultured for 48 hours

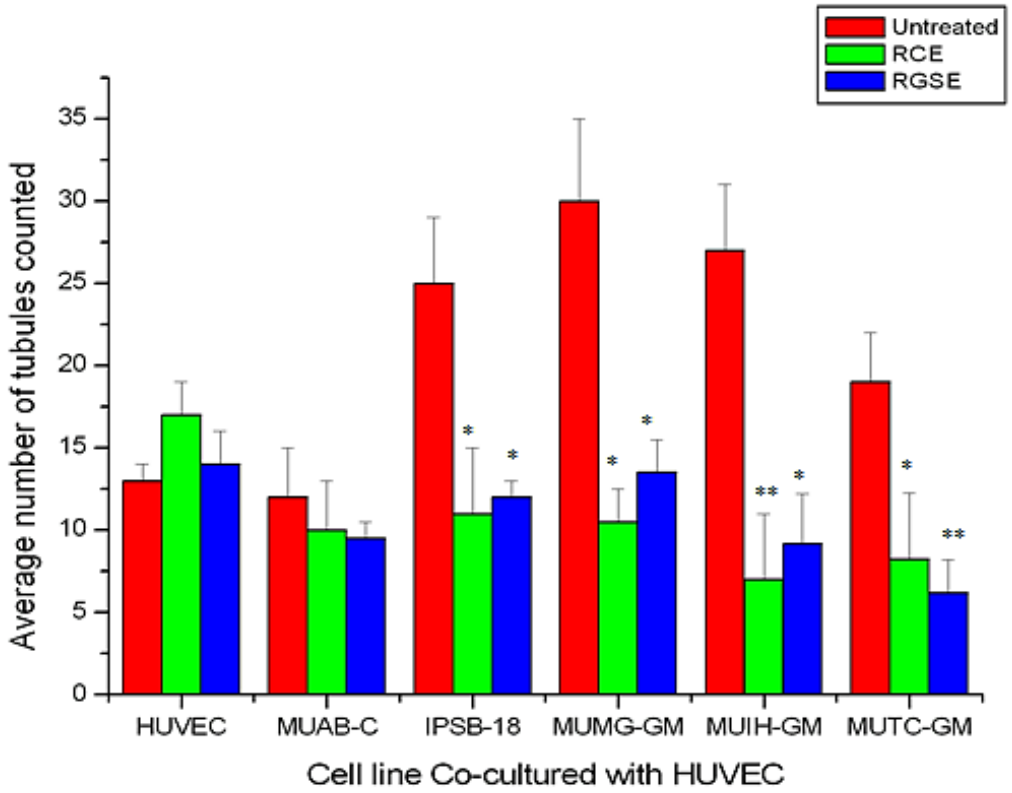


Figure 37 The average number of tubules counted in three different fields, of three separate experiments, of treated and untreated HUVEC cells co-cultured and not co-cultured with GBs. Cells were treated with the IC_{50} of the either micronutrient. Significant differences were measured in comparison to the untreated control using a Tukey HSD test (*p<0.05, **p <0.001)

4.10 Summary of Results

Analysis of both red clover extract (RCE) and red grape seed extract (RGSE) found both micronutrient extracts had an effect on the glioma cell cultures investigated, but not the control astrocytic cell culture MUAB-C. It can be seen that both micronutrients reduce viability, decrease invasive potential and decrease angiogenesis. However this study did not illustrate an effect on the apoptotic potential of the glioma cultures or the control cell culture.

CHAPTER 5: Discussion

High grade gliomas, in particular glioblastoma multiforme (GB), continue to have a poor prognosis. Conventional therapies such as radiotherapy and chemotherapy have been largely unsuccessful in curing malignant brain tumours (Biasoli 2014; Farah 2016). Survival rates for grade IV tumours are as little as six to nine months (McKinney 2004; Ho 2014; Farah 2016), and significant advances in conventional therapies are also limited (Weathers 2014 p.1; Tseng 2016). This has encouraged patients to investigate the benefits of complementary and/or alternative medicines (Eisele 2014), which could potentially alleviate the trauma associated with disease progression and side effects of radiation and chemotherapy.

This study investigated the therapeutic effect of two micronutrient extracts, specifically red clover extract and red grape seed extract in adult malignant brain tumours *in vitro.* Both these extracts and their individual active compounds are known to exhibit anticancer effects and and in some cases the individual active compunds have been known to show to affect various aspects of brain tumour development and growth, however, to the best of our knowledge neither extract has been reported for their to affect biopsy derived glioma cell cultures. The investigation of these two extracts analysed their effect on cell viability, invasion and angiogenesis. The study also investigated both micronutrient extracts for their effect on apoptosis. Both red clover extract (RCE) and red grape seed extract (RGSE) were found to affect the invasive and angiogenic potential of the glioma cultures but did not appear to exhibit early apoptosis in the cells.

5.1 The Effect of Glioma Cell Heterogenity on Cell Culture Survival

Initially the study evaluated four biopsy glioblastoma multiforme (GB) cultures (grade IV), a primary cell culture (anaplastic astrocytoma grade III), a control astrocytic cell culture and a human umbilical vein endothelial cell culture. Biopsy cultures were chosen for experimentation as they offer several advantages over autopsy-derived materials and animal models. Being relatively fresh, the cell viability of the extracted and cultured biopsy cells is high, in the range of at least 90% (Totowa 2001), thus providing cultured cells of a similar phenotype as those *in vivo. In vitro*, however, unpredictability of biopsy derived cultures is seen with increasing passage (Janus 1992). Therefore, biopsy derived glioma cultures at no greater than passage 10 were used as part of this study to maintain integrity.

As low passage cultures are extremely sensitive to the freezing and resurrection process, unfortunately, the biopsy derived culture MUPK-GM, initially used in this study was unable to be sustained. This failure to resurrect might be due to the DMSO found in freezing media. DMSO when used as part of freezing medium partially solubilizes the membrane of the cells (Yamashita 2000). It may be that the particular biopsy derived culture (MUPK-GM) was particularly sensitive to DMSO in the freezing medium explaining the failed resurrection. Further study on the affects of DMSO in freezing medium on biopsy derived glioma cells is warranted.

The primary cell culture, IPSB-18 was evaluated at passage 20. IPSB-18 has historically been used to assess the affect of other micronutrients on characteristic biologic features such as cell viability and invasion (Rooprai 2001; Rooprai 2007; Rooprai personal communication). To retain consistency with previous studies, and allow future comparisons of the various micronutrient affects on glioma cell cultures, the therapeutic affects of both RCE and RGSE were examined on this primary culture also.

5.2 Morphological Heterogeneity of Glioma Cell Cultures

Gliomas are highly heterogeneous and can consist of up to 14 different cell sub-populations (Pilkington 2001; Marusyk 2010; Sottoriva 2013; Inda 2014; Crespin 2016). *Potter et al* (2009) reported low passage cultures retain the global tumour profile *in vivo* when compared to higher passage cultures. As a result, each tumour is genotypically and phenotypically different from any other, thus presenting the initial hurdle in developing a common treatment for brain tumours (Rao 2003; Louis 2006; Mason 2008; Sathornsumetee 2008; Wen 2008; Weathers 2014). Within this study heterogeneity of the glioma cell cultures was seen using phase contrast microscopy.

The IPSB-18 primary GB culture exhibited small cells which grew rapidly in small clumps. The biopsy derived cultures; MUMG-GM, MUIH-GM, MUTC-GM and MUPK-GM exhibited larger, longer spindle like cells, with clear differences in the morphology between cell cultures. The differences in the morphology of the GB cultures may be assumed to be related to the heterogeneous nature of the GB tumour (Bozkurt 2014, p1021). Further study into the various cell types found within each of the biopsy cultures is required. One such technique to investigate the different subpopulations within the cultures is flow cytometry. Studies have shown by measuring the antigenic expression of glioma cell cultures a number of different populations may be identified (Prestegarden 2010). Flow cytometry assays in this study do indicate differing percentages of antigenic expression between the glioma cultures (Table 18), though further investigation would be required to investigate the different subpopulations which exist between the cultures.

The control astrocytic cell culture, MUAB-C, was the only culture which depicted the classical astrocytic ‘star shape’. This result was expected of the normal astrocytic cell culture. MUAB-C cells were found to develop at a slower rate when compared to the biopsy derived GB cultures, which not only grew at a faster rate but only partially presented the classic ‘star shape’

illustrated by the control cell culture. Cancer cells are known to divide and grow indefinitely though normal cells will only grow when required, this may explain the differences seen between the GB cultures and normal astocytic cultures. This was also illustrated by the population doubling times (PDTs).

5.3 Differences between Population Doubling Times of Glioma Cultures

Most normal cell lines are only able to undergo a few subcultures, or passages. These are called finite cell lines. The limited number of passages is dictated by the population doubling time of a particular cell population, before it stops growing due to aging (Hayflick 1985; Hayflick 1994).

The process of doubling the cell population can be described by growth curves which show the population doubling time (PDT). The PDT indicates the period of time during which the number of cells double in number. The traditional curve illustrates three growth phases (as shown in Figure 36). Initially the cells enter a latent period which shows no growth, called the lag phase. This period allows the cells to recover from trypsinisation, reconstruct the cytoskeleton, secrete matrix to aid attachment, and spread out across the surface, thus re-entering the cell cycle (Figure 36). Subsequently an exponential growth is noted known as the log phase. In this period the cell population doubles over a definable period, known as the doubling time and characteristic for each cell line. Increased growth results in eventual overcrowding such that the entire surface is occupied. Overcrowding eventually withdraws the cells from a growth cycle. This is illustrated as a plateau or stationary phase. Some cells may differentiate during this phase; others simply exit the cell cycle and return to G0 but retain viability. A reduction in proliferation in the stationary phase can be attributed to a high cell density and exhaustion of growth factors in the growth medium (Stoker 1973; Westermark 1975).

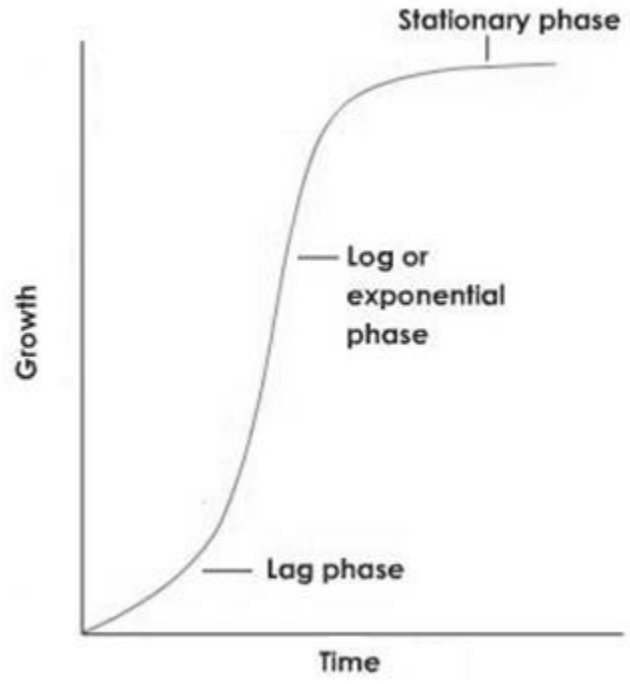


Figure 38 Growth Curve: Cell growth follows a three phase pattern. Initially the cells the cells enter a lag phase during which no growth occurs this is followed by the log phase. Here the cells increase exponentially. Finally, the cells enter the latent phase where cells are unable to growth and develop any further (Laird 1965, p282).

MUAB-C was found to have a PDT of 72 hours and unlike the biopsy cultures this time remained consistent with increasing passage number. IPSB-18 (passage 29-30) indicated the shortest PDT at 36 hours, when compared to the longer PDTs of the lower passage biopsy derived cultures in particular MUPK-GM took around two weeks to double its population. The population doubling times (PDT) of the glioma cell cultures were statistically analysed to compare each glioma culture with the control cell culture (MUAB-C). The ANOVA analysis yielded a p value of 0.000 and as such a Tukey's test was performed to identify where the significant difference lay. As summarised in Table 23 there appeared to be a statistically significant difference between the PDT of each glioma culture compared to the normal astocytic cell culture, MUAB-C ($p=<0.01$) (Table 23).

As previously discussed the differences between the PDTs may be due to the greater heterogeneous nature of the biopsy derived cultures (Pilkington 1992).

Different cells, with differing phenotypes, may exhibit unique periods of growth, which may be affected with increasing passage number. The cultures were therefore only investigated at specific passages, to best retain an assumed phenotypic consistency, throughout evaluations (Table 22).

Table 22 Summary of population doubling times of the cell cultures used in this study. A p value following a Tukey test illustrates the comparison between the glioma cultures and the control culture (MUAB-C – PDT = 72hr)

Cell Line	Population doubling time (hr)	p value (when compared to MUAB-C)	Passage investigated
IPSB-18	36	<0.01	29-30
MUMG-GM	165	<0.01	4-6
MUIH-GM	210	<0.01	5-7
MUTC-GM	197	<0.01	4-6
MUPK-GM	347	<0.01	3-5

5.4 Cell Viability: The Toxic Effects of RCE and RGSE

5.4.1 DMSO Alters the Effects of Micronutrient Extracts

DMSO is known to permeate the cell membrane and interact with the cell metabolism (Violante 2002; He 2012). *Peng* (2005) has suggested that DMSO has no direct effects on the MTT assay and in fact DMSO is used to solubilise the deposited tetrazolium salt prior to microtitre plate reading. It was therefore assumed these results occurred due to DMSO being used to dissolve the flavonoid extracts. DMSO has been known to display a variety of therapeutic effects and cytotoxic effects (Santos 2003) and thus it is reasonable to suggest DMSO does have an affect on cell viability of brain tumour cell cultures investigated as part of this study. As this study showed DMSO had a cytotoxic effect on glioma cultures, it is therefore suggested that, prior to dissolving any extract in any solvent, the cells being treated should be initially investigated for any toxicity the solvent may display towards the cell culture, using a suitable viability assay.

5.4.2 Pigmentation of Micronutrient Extracts

Despite removing DMSO from the assay and replacing this with clear DMEM, the results remained confusing; there was an apparent reduction in viability following 48-hour treatment with low concentrations of either extract, followed by an apparent increase in viability at higher concentrations, depicting a biphasic curve (Figs 18 and 19). This increase in percentage viability at higher concentrations, for both RCE and RGSE, suggested that both micronutrients were encouraging cell growth and maintenance of cell populations between concentrations of 50μg/ml and 250μg/ml. Similar results were also reported in other studies (personal communication) and remain unexplained. Some groups suggest a lag within the cell cycle (personal communication) though this did not seem plausible as the investigation yielded an end-point result rather that a continuous result.

Further analysis into the occurrence of the biphasic curve analysed phase contrast micrographs of glioma cells following 48-hour treatment with either of the micronutrient extracts. The micrographs have shown that at concentrations greater than 10μg/ml for RCE and 20μg/ml for RGSE, more than 90% cell death was induced. The biphasic curve therefore appears to be an anomaly. Such a phenomenon has previously been reported using concentrations of anthocyanidins (Elisia 2008). In this study, *Elisia et al* (2008) showed that when determining the differences between various viability assays, at concentrations of anthocyanidins between 0.4 to 9.4 mg/mL, the results yielded were inconsistent.

As reported (Chapter 2) further analysis of the treated 96 well plates found residues of the extracts remaining, which despite copious washing, were not being entirely removed from the wells. The interference of the pigments in the extracts on the viability assay absorbance value was determined by measuring the optical density of RCE and RGSE. It was noted that RCE and RGSE both interfere with absorbance values at 570nm at concentrations higher than 1μg/ml, as demonstrated by the peaks on the optical density

graphs at 570nm (Fig 24). As the residual colour of both RCE and RGSE interfered with the final absorbance at 570nm it was determined that RCE and RGSE should not be analysed at high concentrations using the MTT assay. It is therefore recommended that prior to any viability investigation, the chosen compound be tested for interference, at the desired concentrations, with the appropriate wavelength.

Viability assays read at a wavelength of lower than 570nm, such as the MTS assay (470nm), should be replaced by viability assays read at higher wavelengths. An alternative to both the MTT and MTS assays, is using DRAQ7™. This flow cytometry technique accurately differentiates and quantifies live and dead/dying cells in a given cell sample, by using the fluorescent dye DRAQ7™ (Biostatus). DRAQ7™ is a far-red emitting DNA-specific dye, having its Exλmax at 488 nm especially for detection by flow cytometry. It is impermeant to live cells but rapidly enters any leaky or dead cell and stains its nuclear DNA. Unlike some nuclear dyes like PI, DRAQ7™ does not stain cytoplasmic RNA of viable cells and thus avoids false positive results (Rieger, 2010). The low wavelength at which it is read also does not interfere with micronutrient dyes thus alleviating the problem of interference with absorbance readings, in the MTT assay.

As a reduced viability had been demonstrated by concentrations of RCE and RGSE lower than 10μg/ml it could be inferred the IC_{50} of each micronutrient extract may be found at lower concentrations which would not interfere with the use of the MTT assay. Table 24 summarizes the IC_{50} values for all the glioma cultures.

Table 23 Summary of the IC_{50}s of the micronutrients using various cell cultures including primary cultures set up from biopsies, an existing low passage cell line.

Cell Culture	Passage Number	IC_{50}	
		RCE	*RGSE*
IPSB-18	29-30	400ng/ml	1ng/ml
MUMG-GM	4-6	1 μg/ml	0.1ng/ml
MUTC-GM	5-7	7ng/ml	500ng/ml
MUIH-GM	4-6	0.98μg/ml	400ng/ml
MUPK-GM	3-5	0.80μg/ml	0.1pg/ml

The IC_{50} for RCE and for RGSE for the glioma cultures all differ from one another. This may reflect the previously discussed, heterogeneous natures of these cultures. Previous researchers have reported that glioma cultures differ phenotypically (Pilkington 2001, Sottoriva 2013). Each sub-cell population within each culture may respond to a different concentration of micronutrient extract. As such, differences in the glioma culture IC_{50}s were expected to be seen. This study showed significant differences in viability were seen within the glioma cultures, as the concentration or RCE or RGSE was increased.

The glioma culture, IPSB-18 was found to have one of the lowest IC_{50s}, for RCE (400ng/ml). *Knott et al.* (1990) showed IPSB-18 consisted of a low number of different cell populations. This reduced heterogenous nature of the culture may explain the lower IC_{50}. The effect of the micronutrients on each cell type will be reacted upon in a different way so it can be speculated that the more cell variations within a culture, more micronutrient is required to target the cells. This is supported by the higher IC_{50}'s seen for some of the biopsy cultures which are assumed to be more heterogeneous.

Interestingly MUPK-GM and MUTC-GM did display increased sensitivity to RGSE and RCE respectively, when compared to the other cell cultures (Table 24). Given the decreased cell heterogeneity found within IPSB-18, it may be assumed MUPK-GM and MUTC-GM also exhibit a low number of different cell

populations. Further characterization of the sub populations within these glioma cultures, using flow cytometry is warranted.

Despite limited analysis on the MUPK-GM cell culture this assay indicated a reduction in cell viability following treatment with RCE or RGSE. This suggested that MUPK-GM was likely to have yielded similar outcomes as the other biopsy derived GB cultures. Given the reduction in cell viability of all the glioma cultures, further investigation into why this occurred was required.

5.5 Changes in Environment Effect Antigenic Expression of Glioma Cultures

Routinely cell biopsies and established cultures are characterised by examining the antigenic expression of the cells. In this particular study flow cytometry and immunocytochemistry were used to analyse the antigenic expression of the glioma cultures and whether treatment with either of the micronutrient extracts affected expression.

5.5.1 Changes in Microenvironment affect the Phenotype of the Glioma Cells

Prior to evaluating the antigenic profile of the biopsy derived GB cultures, this study appraised the microenvironment in which the cells were cultured; specifically, serum supplement. Globally, the controversial topic of debate around the choice of serum used to supplement growth media has resulted in differing use of serum supplementation (Chimenti, 2014). Personal communication with labs in Norway, who have adopted the use of newborn calf serum instead of foetal calf serum to culture glioma cells *in vitro*, encouraged the investigation into whether the microenvironmental properties, of the culture media have an affect on glioma cells.

This study assessed the antigenic expression of seven antigens in five

established glioma cell cultures, with particular focus on glial fibrillary acidic protein (GFAP) and galactocerbrosicide (Gal C) (Chapter 3). These two indicative markers of cells of astrocytic and oligodendrocytic origin respectively, were investigated specifically to support studies performed by *Raff et al.* (1983) and *Tenenbaum et al.* (1996). The culture media in which glioma cells were grown, were supplemented with either foetal calf serum (FCS); the standard serum supplementation adopted, newborn calf serum (NCS); the serum adopted by colleagues in Norway (personal communication) and human serum (HS); to mimic glioma cell growth *in vivo*.

The change of antigenic expression found in this study can be seen in the immunocytochemical micrographs of GFAP expression (Fig 13). In particular, the expression of GFAP was shown to be down regulated with differing sera supplementation, specifically FCS<NCS<HS. Upon analysis of the flow cytochemical data (Table 13) statistically significant differences were seen in three of the glioma cell cultures when compared to cells cultured in FCS, namely A172 (NCS $p=0.04$), HF66 (NCS $p=0.048$; HS $p=0.029$) and U373 (NCS $p=0.02$; HS $p=0.02$).

A similar but opposing, result was seen when analyzing the expression of the oligodendrocytic marker galactocerobroside (Gal C). Immunocytochemical micrographs indicated altered expression with the antigens being up regulated, specifically FCS>NCS>HS. Flow cytochemical results showed significant differences in four of the five glioma cell cultures evaluated; IPSB-18 (HS $p=0.02$), U251 (HS $p=0.004$), HF66 (NCS $p= 0.0009$; HS $p= 0.0001$) and U373 (HS $p=0.01$).

These findings support similar occurrences reported in previous studies by *Raff et al* (1983) and *Tenenbaum et al* (1996), reinforcing the notion that microenvironmental changes manipulate cells to change phenotype. Studies by *Raff et al.* (1983) explored the influences of external factors the cellular environment, such as the presence or absence of foetal calf serum in the culture medium, on the developmental pathways of astrocytes and

oligodendrocytes. They assessed morphological and immunocytochemical criteria of the divergent pathways of cell development in the immature glia of the rat optic nerve. They used A2B5 antibody for the detection of Type 2 astrocytes in this study and proposed that both fibrous astrocytes and oligodendrocytes may be derived from a primitive precursor cell (the so-called "O-2A" or oligodendrocyte Type 2 astrocyte progenitor cell) dependent upon the influence of the environment. Subsequent studies showed that the presence of GFAP-negative, A2B5-positive cells and GFAP-positive, A2B5-positive cells in a malignant astrocytoma culture (IPSB-18) which provided evidence that the O-2A progenitor and Type 2 astrocyte phenotypes occur both in human neoplastic cells *in vitro* and in normal developing rat neural tissue (Knott 1990; Pilkington 1992).

Furthermore, *Tenebaum et al.* (1996) identified the plasticity of oligodendroglioma cells by successfully showing that, when taken from a rat brain, they could be manipulated in the presence of serum supplementation, into losing the expression of oligodendrocytic antigenic markers and acquiring astrocytic markers. Upon re-introduction into the rat, these cells were able to revert back to their original antigenic expression. They also confirmed that human oligodendroglioma cells had a similar fate being induced, by the absence or presence of serum, into oligodendrocytes or Type 2 astrocytes, respectively.

Conclusions of these studies suggested the optimal use of serum supplementation with the growth media for GB cultures was human serum as this mimicked conditions *in vivo*. Unfortunately, as human serum is not readily available and/or produced, studies may be subject to delay and it is not an economically viable option. As such, to maintain consistency with previous studies, investigation into the effects of other micronutrient extracts on IPSB-18 and other glioma cultures (Rooprai 2007), the remainder of this study grew the glioma cells in growth media supplemented with 10% FCS, unless otherwise indicated. Further investigation into trans-differentiation of glioma cells, cultured in the presence of FCS as the growth supplement of choice, is

warranted, with the aim to develop a media supplement able to imitate conditions *in vivo*.

5.5.2 Micronutients Alter Antigenic Expression of Glioma Cell Cultures

Evaluation of the glioma cell cultures investigated the expression of five antigens; GFAP, CD44, NCAM eric-1, αv and β1. These antigens were chosen to retain uniformity with studies performed previously on glioma cell lines, assessing the effect of other micronutrient extracts (Rooprai, 2007). Flow cytochemical data compared the antigenic expression of untreated glioma cultures vs. glioma cultures following 48-hour treatment with either RCE or RGSE. The immunocytochemical data illustrated the approximate percentage expression based on the amount of fluorescence visualized.

GFAP expression

Glial fibrillary acidic protein (GFAP) was first described in 1971 by Eng et al. (1971) and is a member of the cytoskeletal protein family and is widely expressed in astroglial cells and in neural stem cells (Eng 1971; Doetsch 2003). It is also expressed in astroglial tumours, such as astrocytomas and GB (Jacque, 1978; Hamaya, 1985; Abaza, 1998). The expression of GFAP provided a positive control confirming the integrity of the cultures used as astrocytic in origin. Flow cytochemical data indicated that GFAP expression in IPSB-18 cells treated with RCE was found to be significantly down-regulated compared to untreated IPSB-18 cells ($p = 0.015$). When treated with RGSE however, although the expression of GFAP appeared to be down regulated, this change did not reach significance ($p=0.0439$). This change in antigenic expression was mimicked by the other glioma cultures (Table 18).

Immunocytochemical data did not however confirm a down regulation in the expression of GFAP.

CD44 expression

CD44, a cell surface adhesion molecule (Pilkington 1993; Radotra 1998; Ariza 1995; Li 1995) and a principal receptor for hyaluronan (HA) (Aruffo 1990), is a glycosaminoglycan found in the extra cellular matrix (ECM). CD44 has been implicated in the invasive process (Merzak 1994) and has been suggested as a biomarker of cancer stem like cells (Yan 2015). As part of the evaluation of the effects of RCE and RGSE, investigation into the expression of CD44 by gliomal cells following treatment with the micronutrients was carried out.

Neither flow cytochemical nor immunocytochemical data indicated a significant change in expression of CD44 by the cell lines under investigation following treatment with RCE and RGSE (Table 18 and 19). CD44 is overexpressed in brain tumours and is involved in the invasive process and cell-cell adhesion (Merzak, 1994). Therefore, a down regulation would have indicated a possible anti-invasive effect. A slight decrease in CD44 was seen in the immunocytochemical micrographs of glioma cultures following treatment with RCE and RGSE when compared to the untreated glioma cultures, however, analysis of the flow cytochemical data found that this was not statistically different ($p=>0.05$). RCE and RGSE do not appear to affect the pathways underlying the invasive process within GBs as indicated by expression of this antigen. Although CD44 is known to mediate invasion in gliomas (Merzak 1994; Yoshida 2012) and has previously been shown to be down regulated following treatment with micronutrient extracts (Rooprai 2007). Additionally, grape seed extract and its individual components have been shown to downregulate CD44 in other cancers, including colon cancer (Kumar 2014) and breast cancer (Ouhtit 2014). Curcumin is also amongst one of the other micronutrients which has demonstrated a clear down regulation of CD44 (Chung 2015). Further investigation into the expression of CD44 is required.

Eric-1 NCAM expression

Eric-1 NCAM is a cell surface glycoprotein that also mediates cell-cell adhesion independent of Ca^{2+} (Keilhauer 1985), and plays an important role in neurite outgrowth and fasciculation (Appel 1993). Eric-1 NCAM has also been found to be involved in migration/invasion (Gratsa 1997). Although no significant difference was found in the expression of Eric-1 NCAM between the treated and untreated cultures ($p=0.06$-0.11) (Table 18), immunocytochemical micrographs indicated a down regulation. This therefore suggested further study may be warranted.

As both CD44 and Eric-1 NCAM exhibited only a slight down regulation in expression in immunocytochemical data but analysis of the flow cytochemical data did not indicate a significant change, further investigation into the anti-invasive potential of both micronutrient extracts was required.

αV integrin expression

In malignant gliomas, the expression of many integrins, including αv and β1 are reportedly increased, relative to their levels in normal brain tissue (Gingras, 1995; Rooprai, 1999; Paulus, 1993).

The expression of αv intergrin was found to be down regulated in all the IPSB-18 cell cultures following treatment with RCE ($p<0.001$) and RGSE ($p<0.001$) when compared to the untreated cell culture. A similar result was found for the biopsy derived glioma cultures ($p<0.001$) (Table 18). Immunocytochemical data also depicted a clear down regulation in the expression of αV when compared to untreated cell (Fig 28).

αv is unique in that it can pair with multiple β subunits to form five recognized receptors, αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8 (Nemeth 2007). Integrins αvβ3 and αvβ5 are consistently overexpressed in GBs in comparison, other integrins and play an important role in gliomagenesis, progression, invasion

and angiogenesis (Brooks 1994; Friedlander 1995; Hynes 2002; D'Abaco 2007). Integrin αvβ3 in particular, is necessary for the formation, survival, and maturation of newly formed blood vessels (Brooks 1994). Therefore, a down regulation in αv suggests that RCE and RGSE have anti-invasive and anti-angiogenic potential. *Blandin et al* (2016), suggested a downregulation of αv integrin may be useful to reduce invasion of GBs.

β1 integrin expression

β1 integrin is also highly expressed in glioma cell lines, when compared to normal brain tissue (Gingras 1995; Paulus 1993). It has been shown that blocking the β1 integrin can reduce invasive potential in glioma cell lines (Tysnes, 1996; Friedlander, 1996; Tonn, 1998).

Following 48-hour treatment of IPSB-18 with RCE or RGSE a down regulation of β1 integrin was seen. Following comparison of this against the untreated cells, the down regulation was found to be statistically significant for both RCE ($p<0.001$) and RGSE ($p<0.01$) (Table 18). Down regulation of β1 integrin expression was also found following treatment with RCE or RGSE in all the biopsy derived cultures ($p<0.001$). Immunocytochemical micrographs also illustrated a clear down regulation in expression of β1 integrin (Fig 28).

β1-integrin initiated invasion is likely mediated by the α3β1 integrin receptor, although several subunits can pair with β1 integrin (Tysnes 1996; Knott 1998; Fukushima 1998). The data for the expression of integrins supported the anti-invasive capabilities of both micronutrients.

5.6 Diffuse Invasion Reduced After Micronutrient Treatement

Various molecules are implicated in the invasive potential of gliomas, including CD44, NCAM and integrins. Investigation into the antigenic expression of these molecules may support the suggestion both RCE and RGSE may exhibit anti-invasive potential, however the difference in expression of CD44 and NCAM were not found to be statistically significant. Therefore, to investigate the anti-invasive potential of both micronutrient extracts further, modified Boyden chambers were used. This 2-dimensional study was performed using all the glioma cultures and the control cell line MUAB-C. Cells were seeded into the modified Boyden chamber above a layer of extra cellular matrix (ECM). The cells which migrated through the chamber and across the ECM were then counted.

When analysed, the normal cell culture MUAB-C, very little invasion. Examination of data found that this was based on only 1 or 2 cells migrating through the chamber. Given MUAB-C was assumed to have 'no' invasive potential, incubation with RCE or RGSE would determine if either micronutrient promoted the invasive process. Neither of the micronutrient extracts was found to have an effect on invasive potential of MUAB-C, with the average invasion remaining at 0%. This indicated RCE and RGSE do not promote invasion in MUAB-C.

The glioma cultures (Fig 29) did initially display invasive potential. The anti-invasive effects of both micronutrient extracts are visible in Figures 29, 31 and 32 where upon treatment with RCE and RGSE, the number of invading glioma cells reduced dramatically. Invasion is a multi process biological feature, and it may only be speculated which pathway may be affected. Recent research has identified several key molecules that regulate GB cell migration and invasion, and may serve as attractive targets. Similarly, *Zhou et al (2016)*, found that grape seed extract also demonstrated a reduction in invasion in luncg cancer.

One such explanation for the anti-invasive potential may lie in the down regulation of integrins and their interaction with the cells migratory processes. Integrins, in particular β1 subunit, play an important role in glioma biology and its expression has been correlated with the invasive behavior of glioma (Paulus, 1996) It is supported by the observation that inhibition of β1 integrin leads to decreased motility while inhibition of αv integrin results in increased motility. This correlates with data previously depicted by flow cytochemical and immunocytochemical data.

Another possible explanation may lie in the effect of the micronutrients on matrix metalloproteinanses (MMPs). MMPs play a vital role in invasion by paving a pathway for the malignant tissue to migrate or invade along. Down regulation of various MMPs in the presence of micronutrients has previously been reported (Rooprai 2007). As such, inhibition of MMPs could aid a reduction in invasion ultimately preventing recurrence of tumour growth. Preliminary flow cytochemical data illustrated a slight down regulation of MMP 2 and 9 following treatment of glioma cell cultures with either of the micronutrients. Initial findings indicated MMP2, an initiator of both invasion and angiogenesis, was reduced in treated glioma cells, when compared to untreated cells. Recent studies in to the 'knock-out' of MircoRNAs for membrane type MMPs found that this downregulation suppressed the malignancy of gliomas cells *in vitro* (Sakr 2016), supporting the suggestion that targeting MMPs could reduce the likelihood of both invasion and angiogenesis.
Grape seed extract has been shown in breast cancer (Dinicola 2014) to downregulate the expression of both MMP2 and MMP9. Further investigation into the expression of MMPs could provide an explanation into the pathways interrupted by RCE or RGSE during invasion.

Other possible targets to investigate the affect of the invasive potential include tGLI1 (a novel splice variant of GLI1), SDF-1, Pin1, OPN, MGP, ADAM19,

VAT-1, Sema3A-neuropilin-1, and AJAP1. (Nakada, 2010; Mertsch 2009; Lo 2009; Kenig 2010; Bagci 2009; Atkinson 2009; Lamour 2010; Qi 2009; Mertsch 2009).

5.7 Late Pro Apoptotic Potential: A Qualitative Indication

In the normal brain, cells of the CNS exhibit differences in their resistance to the automated process of apoptosis, the most resistant of cells being microglia and the most vulnerable, neurons. The sensitivity of glial cells lies between the two extremes (Salgado 2007). During malignant progression, tumour cells acquire an increased resistance to apoptotic agents and procedures. In particular the invading cells of GBs are less sensitive to apoptosis, resulting in a high frequency of relapse (LeFrank 2005). Two assays were performed to determine whether either of the micronutrients displayed pro-apoptotic characteristics, annexin V and the TUNEL assay.

The control cell line (MUAB-C) displayed a small number of apoptotic cells when analysed with the annexin V assay (an average of 10%). This remained consistent following treatment with RCE (10.5%) or RGSE (12.8%). The TUNEL assay illustrated apoptotic cells as fluorescent. The number of fluorescent cells was then counted in an average of three fields. The results were similar to those in the annexin V assay with only three or four fluorescent cells in each field counted (Fig 35).

Untreated IPSB-18 glioma cells displayed a small proportion of apoptotic cells (2.9 %) (Fig 33). Following treatment with RCE and RGSE the percentage of apoptotic cells decreased (0.05% and 0.01% respectively), though this change was not significant. Instead, the majority of cells appeared in the necrosis quadrant of the annexin V plot. The TUNEL assay micrographs also showed an average of 0-1 fluorescent cells in each field (Fig 34). This result was seen across all the biopsy derived glioma cultures (Table 20).

Although a slight reduction in apoptotic potential was illustrated in treated glioma cells when compared to untreated cells, this was not a significant change with only a difference of around one or two cells. It can therefore be suggested that glioma cells do not exhibit an apoptotic affect following treatment with either RCE or RGSE, though cell death had occurred.

Apoptosis is rarely seen in glioma cultures, possibly as a result of the relationship between rates of apoptosis and tumor grade, not being straight forward, though one explanation could be attributed to integrins, in particular the expression of $\alpha v\beta 3$. As more cells enter an invasive state their sensitivity for apoptosis decreases. This phenomenon can be attributed to the enhanced expression of $\alpha v\beta 3$, in the marginals infiltrating zones (Sarafian 2009). $\alpha v\beta 3$ serves as a basic regulator of proliferation, invasion and angiogenesis (Bogler 2005). Integrins bind to a variety of ECM ligands including vitronectin, fibronectin, fibrinogen, thrombospondin, proteolyzed collagen, von Willebrand factor and osteopontin. Such ligation activates intracellular signaling pathways that promote cell survival, proliferation, motility and inhibit apoptosis (Bogler 2005; Horton 1997; Stupack 2002).

As discussed earlier, the expression of the αv was found to be down regulated in all treated glioma cultures, when compared to the control cell culture MUAB-C. Preliminary investigation of the $\beta 3$ integrin also exhibited a very slight upregulation of antigenic expression when compared to the normal brain tissue *in vitro* (Fig 39).This would suggest more cells should be sensitive to the apopotic pathway, however, the limited apoptotic potential displayed when analysed using both annexin V and TUNEL assays was not significant and therefore does not suggest that RCE or RGSE induce apoptosis.

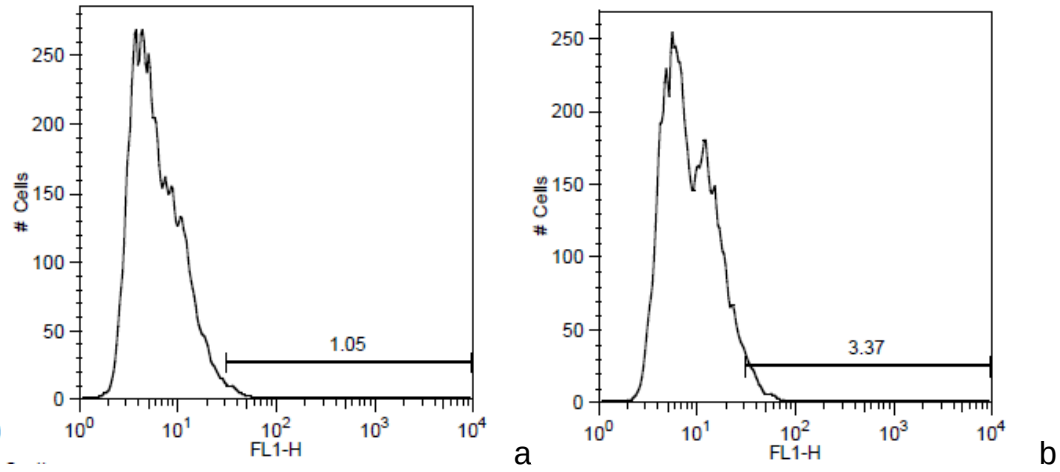


Figure 39 The expression of β3 integrin in a) normal brain tissue and b) glioma cultures. β3 expression is increased in malignant tissue. When in compound form, αvβ3, thus resulting in a resistance to apoptosis and a vulnerability to invasion and angiogenesis.

These findings suggest the dominant form of cell death in glioblastoma is necrosis rather than apoptosis, and this histological feature is part of the definition of this tumor type (Kleihues 2000). This has been verified in recent literature which states that despite apoptosis being caspase dependent and necrosis caspase independent, both these pathways share common starting points such as death domains like p55 TNF receptor (TNF-RI) and Fas and the adapter protein FADD. Both micronutrients in particular RGSE exhibit antioxidant properties and are thus are known to reduce free radicals (Bagchi 2000) and induce apoptosis mainly by targeting Fas death domain. However, prolonged activation of Fas without phagocytosis of apoptotic cells is seen to induce cell death via necrosis *in vitro* in cell lines (Eisele 2011). In the annexin V plots the majority of cells lay in the live or dead quadrants, with one or two cells in the apoptotic quadrant, suggesting RCE and RGSE exhibit a possible necrotic effect.

An explanation for such an occurrence can be related to defective apoptosis signaling. This may be caused by increased expression of anti-apoptotic molecules or by impaired function of pro-apoptotic molecules (Igney and

Krammer 2002). Examples of such mechanisms are illustrated by inhibitor of apoptosis proteins (IAPs) such as survivin. This is expressed at high levels in many tumours and has been reported to be associated with noncompliant disease and poor prognosis (Salvesen 2002; Altieri 2003). It has been reported that glioblastoma cultures are positive for survivin expression (Chakravarti A, 2002). A downregulation of survivin combined with administration of the chemotherapeutic agents temozolomide or etoposide was has been shown to exhibit a synergistic cytotoxic effect on GBs (Cruz 2016). Such IAPs can inhibit effector caspases, including caspase 3, 6 and 7, at the core of the apoptotic pathways. Therefore, therapeutic modulation of IAPs could target a key control point in cancer resistance (Goyal 2001).

Although neither RCE nor RGSE instigated apoptosis alone, it could be postulated, in the presence of another factor, either micronutrient could potentially exhibit pro-apoptotic features. Apoptosis is rarely seen in glioma cultures though some studies have increased the affinity for glioma cells to undergo apoptosis. It was reported by *Gondi et al.* (2010) that in the presence of human umbilical cord cells, enriched in CD44 and CD133, gliomas cultures, underwent apoptosis. This suggests glioma cells have the ability to demonstrate apoptotic behavior. Further investigation, therefore, is warranted into adjuvant compounds which together with the chosen micronutrients could induce apoptosis.

Alternatively, autophagy of glioma cells could have occurred. Autophagy is accepted as the mechanism by which glioma cells undergo programmed cell death when the apoptotic pathway is inhibited (LeFrank 2005). It operates in stress conditions such as low oxygen, commonly found in GBs. Proautophagic drugs block the mechanistic target of rapamycin (mTOR) a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription (Hay 2004; Beevers 2006; Fulda 2015). mTOR belongs to the phosphatidylinositol 3-kinase-related kinase protein family. Expression of of mTOR in GBs is up regulated and results in a resistance to apoptosis and autophagy. Further

study is warranted into the effects of RCE or RGSE on the expression of mTOR and their ability to induce autophagy.

5.8 Anti-angiogenic potential of the micronutrients

In addition to being highly invasive, GBs are extremely vascular. Angiogenesis plays an important role in GB growth. Anti-angiogenesis based therapies have been shown to be effective in reducing vascularization in GB patients. However, therapeutic resistance can develop in patients, suggesting that GB cells are capable of altering their signaling pathway (Soda 2013). Therefore, it is important to identify novel angiogenic factors that play essential roles in tumor angiogenesis and GB progression.

This study found that RCE and RGSE both exhibit anti-angiogenic activity. Though the mechanisms by which they do this, have not been confirmed. It is clear that when human umbilical vein endothelial cells (HUVEC) are treated with either micronutrient the number of tubules produced decrease when compared to untreated HUVECs (Fig 36). The reduction in the percentage number of tubules reduced from 100%, in untreated cells to 55% ($p=<0.05$) following treatment RCE and 50% ($p=<0.001$) when treated with RGSE. This suggested both micronutrients do exhibit anti-angiogenic properties.

This phenomenon was also depicted when HUVECs were co-cultured against glioma cells (Fig 38). The percentage number of tubules reduced significantly ($p=<0.05$), following treatment with either RCE or RGSE.

These results supported studies by *Su et al* (2005), who illustrated a reduced number of blood vessels in bladder cancer cells both *in vitro* and *in vivo*, after cells had been treated with genistein, the active compound found in RCE. Additionally grape seed extracts have been reported to reduce angiogenesis in breast cancer cells and prostate cancer cells *in vitro* (Kaur 2009). Furthermore this study suggested the mechanism by which grape seed

extracts inhibit this biological feature, is by inhibiting vascular endothelial growth factor (VEGF) (Wen 2008; Kanavi 2014). It is possible, therefore, for RCE and/or RGSE to affect any number of factors in the angiogenesis pathways including VEGF and MMPs.

In brain tumors, VEGF was shown to be over expressed in gliomas compared to normal tissue (Osterberg 2016; Li 2009; Plate 1993; 1992). VEGF has been implicated as a central mediator of angiogenesis in GBs. *In vitro* and *in vivo* studies have confirmed a correlation between tumor grade and VEGF expression in gliomas (Kawai 2014). Additionally, studies using animal models have shown that inhibiting VEGF function also inhibits growth of glioma cells *in vivo* and causes regression of blood vessels (Maity 2000). *Osterberg et al*. (2016) illustrated myeloid cell-restricted VEGF-A deficiency led growth delays in gliomas. VEGF is known to enhance vascular permeability (Nagy 1989) and studies have shown that antibodies specific to VEGF reduced angiogenesis (Alishekevitz 2014).

In addition some MMPs are thought to mediate angiogenesis (Nuttall 2003). MMP-2 and MMP-9 are highly expressed in astrocytic tumours and their expression positively correlates with tumour grade, especially those of MMP-9 (Kargiotis 2006). MMPs are involved in proteolytic degradation of ECM proteins to facilitate invasion and angiogenesis (Binder 2002). MMP-9 in particular, has also been shown to activate TGF-β (Yu 2000) and to increase availability of VEGF (Bergers 2000), in turn promoting angiogenesis.

As this study has shown that both micronutrients have anti-invasive and anti-angiogenic properties it is possible they are acting through down-regulation of MMPs. Another MMP, which belongs to the membrane-type MMPs (MT-MMPs), MT1-MMP is up-regulated in malignant gliomas with it's expression is associated with tumour invasion and angiogenesis (Forsyth 1999; Nakada 1998). MT-MMPs play a part in both invasion and angiogenesis (Hotary 2000; Hiraoka 1998) by cleaving ECM substrates and cell surface receptors, including integrins. MT1-MMP mediates activation of MMP2, which binds to

integrin αvβ3 that promotes MMP2 maturation (Deryugina 2001). Interestingly, it has been shown that two growth factor-cytokine dependent pathways of angiogenesis exist that are promoted by distinct integrins one dependent on αvβ3 and the other dependent on αvβ5 (Friedlander 1995). It has been reported, that the key role of αvβ3 integrin is in the survival and maturation of newly formed blood vessels (Ucuzian 2010). By using antagonists of this integrin, after angiogenesis is initiated, such antagonists induce apoptosis of the proliferating vascular cells without affecting pre-existing quiescent vessels (Brooks 1994), probably because αvβ3 is shown to be selectively up-regulated in proliferating vascular cells (Brooks 1994). Thus the down regulation of αv could suggest decreased maturation of MMP2 and thus a decrease in the survival of newly formed blood vessels and thus angiogenesis. Additionally over expression of MT1-MMP in glioma cells results in up-regulation of VEGF secretion and consequently increased invasion of endothelial cells and angiogenesis (Deryngina 2002).

There are many mechanisms and pathways by which RCE and RGSE display anti-angiogenic potential, which warrant further investigation. As has been illustrated in this study invasion and angiogenesis are both affected by both micronutrients and therefore it is probable the mechanism by which they are both inhibited relies on the same molecules including integrins, MMPs and growth factors such as VEGF.

Conclusion

This study examined the effect of red clover extract and red grape seed extract on cell viability, invasive potential, apoptotic potential and angiogenic potential in malignant human adult brain tumours *in vitro*. The evaluation of both micronutrient extracts indicated that both micronutrient extracts appear to display anti-cancer potential.

It was reported that both micronutrients affect glioma cell viability, although it was identified that neither micronutrient exhibits pro-apoptotic properties implying cell viability may be resultant of an alternative pathway. The study also indicated both micronutrients affect the ability of glioma cells to invade across an extracellular matrix *in vitro*, thus suggesting both micronutrients have an effect on invasive potential. Examination of tubule viability and growth following treatment with either micronutrient resulted in a reduction of tubules when compared to untreated glioma cells, suggesting both micronutrients display an anti-angiogenic affect. This study has provided a firm foundation for further investigation into, the underlying mechanisms in which both micronutrient extracts affect cell viability, invasion and angiogenesis in human adult brain tumours.

This novel find strongly suggests the vital need to investigate natural compounds such as the micronutrients within red clover extract and red grape seed extract for therapeutic roles for brain tumour therapy.

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APPENDIX

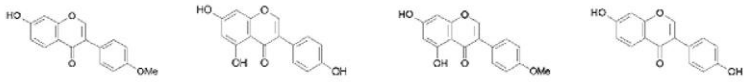
HPLC RED CLOVER EXTRACT

LINNEA
QUALITY ASSURED

RED CLOVER EXTRACT IFL 40 (USP)

NAME OF THE PLANT: Red clover, Trifolium pratense
PART OF THE PLANT USED: Aerial parts of the plant
CHEMICAL DEFINITION: Mixture of isoflavones: Biochanin A, Formononetin, Daidzein and Genistein
SYNONYMS: Pavine clover, Cow grass

STRUCTURAL FORMULA:



Formononetin Genistein Biochanin A Daidzein

TECHNICAL DESCRIPTION:

Appearance:	Dark green amorphous powder
Solubility:	Slightly soluble in methanol and organic solvents; insoluble in water
Identity:	HPLC TLC
Loss on drying:	Max 3 %
Sulphated ash:	Max 4 %

Assay: Not less than 40% total isoflavon-aglycones as: Biochanin A, Formononetin, Daidzein and Genistein (*in which Biochanin A and Formononetin predominate*)

Possible carrier: Maltodextrin

Microbiology: Complies EP

Storage: Sealed container and protected from light and moisture

PRODUCT APPLICATIONS: Red clover isoflavones act as phytoestrogens and are used primarily in the management of menopausal symptoms and to promote cardiovascular health in women. In men Red clover isoflavones are used to support prostate health.

THERAPEUTIC CATEGORIES: Menopause, prostate health, cardiovascular health, breast health, anti-aging.

Linnea SA informative document Last revision: 01.09.2010

RED GRAPE SEED EXTRACT

Polyphenolics
Realizing the health benefits of the grape

MegaNatural® - BP™ Grape Seed Extract contains over 90% natural proanthocyanins - key active ingredients found in red grape juice that have been shown to provide a variety of health benefits.

MegaNatural® - BP™ Grape Seed Extract is made from pure grape seeds - no skins or stems - and is made by a patented water extraction process (US Patent No. 5,544,581). This patented process provides a consistently high grade extract with a low molecular weight for potentially greater absorption and a unique patented (US Patent No. 7,767,235 B2) structure.

Polyphenolics
A Division of Canandaigua Wine Company, Inc.
Realizing the health benefits of the grape

12667 Road 24
Madera, California 93637
Telephone: (559) 661-5556
Fax: (559) 661-5630

CERTIFICATE OF ANALYSIS

PRODUCT: MegaNatural™
Kosher Grape Seed Extract, Gold
"Triangle K Kosher"
CODE: PIN KO7000
FORM: POWDER
PATENT: U.S. PATENT #4,698,360

LOT NO.: 18132501-02
MANUFACTURE DATE: June 30, 2003
CERTIFICATE DATE: July 23,2003
EXPIRATION DATE: June 30, 2006

This product is certified Non-GMO and Non-Irradiated

ANALYSIS	RESULTS	SPECIFICATIO
DESCRIPTION		
Appearance	Conforms	Rose Beige Powde
Flavor Evaluation	Conforms	Bitter and Astringe
CHEMICAL		
Total Phenolics (gallic acid equivalents, dry basis)	90.4	≥ 90g GAE/100 g
Relative HPLC phenol profile	Conforms	Typical of Standar
pH (4% in water)	3.0	2.0 to 5.5
PHYSICAL		
Moisture (by vacuum oven)	4.8	≤ 8.0%
Insoluble Substances (1% in water)	Conforms	< 5%
Free Flow Density (compliant with USP specification <616>)	Conforms	0.25 to 0.50 g/mL
Tap Density (compliant with USP specification <616>)	Conforms	0.40 to 0.65g/mL
Particle Size	Conforms	+ 35 mesh none + 80 mesh 20% m - 200 mesh 20% m
MICROBIOLOGICAL		
Total Plate Count	Conforms	< 1000 CFU/g
Yeast & Mold	Conforms	< 100 CFU/g
Coliform	Conforms	< 10 CFU/g
Salmonella (per 30 g)	Negative	Negative
E. coli (per 10 g)	Negative	Negative

MegaNatural

Steve Kupina
Manager Technical Services