Sensitivity to and functional effects of tricyclic agents on glioma: an immunohistochemical and *in vitro* study

Thesis Submitted By:

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I Abstract

The current outlook for patients suffering malignant glioma is poor, with sub-optimal delivery of agents across the blood-brain barrier, migration of tumour cells to areas that are not exposed to chemotherapeutic assault and the ability of tumour cells to repair DNA damage caused by anti-proliferative agents. The objectives were to demonstrate the potential of targeting the mitochondria of glioma cells through the use of tricyclic antidepressants and to show that the success of this approach is largely dependent on the metabolic capacity of the patient and the expression of the norepinephrine transporter on glioma cells. A range of tricyclic agents were screened against a panel of tumours using MTT, ATP-TCA and Annexin-V assays; the metabolic capacity of seventeen glioma patients was assessed through HPLC-MS/MS sampling of plasma Clomipramine concentrations and cytochrome P450 drug metabolism enzyme genotyping via real-time PCR; the presence of the norepinephrine transporter was elucidated through immunohistochemical, immunocytochemical, Western blotting and real-time PCR techniques and finally apoptotic potential was determined by screening tumours (retrospectively and prospectively) via real-time PCR. Chemosensitivity results show that Clomipramine (the range for five tumours tested was 42.57 ± 16.58 µM) and Nortriptyline (the range for four tumours tested was 30.22 ± 14.81 µM) were the most effective agents when tested in the MTT assay and Norclomipramine (the range for five tumours tested was 7.65 ±3.53 µM) and Nortriptyline (the range for five tumours tested was 33.15 ± 13.72 µM) were the most effective agents when tested using the ATP-TCA assay. Annexin-V flow cytometry supported these results and further evidenced that Clomipramine induces apoptosis in malignant glioma.

*The genotypic status of CYP2D6 and CYP2C19, combined with plasma levels of Clomipramine/Norclomipramine achieved in vivo, showed that mutations in the CYP2D6*2X3 allele significantly affect the metabolism of Norclomipramine ($p<0.05$). Immunohistochemical, immunocytochemical and Western blotting techniques demonstrated the presence of the norepinephrine transporter (encoded by the SLC6A2 gene) on glioma cells, however real-time PCR results suggest that the exons within the SLC6A2 gene contain splice variants. Taqman low density array of retrospective tumour samples revealed variation in the expression of apoptotic genes, with no discernable pattern, and that it is possible to modulate the expression of these genes by exposing SNB-19, DK-MG and UPAB glioma cells to tricyclic antidepressants, Procarbazine, Dexamethasone and Valproic acid. Evidence from this study demonstrates that tricyclic antidepressants provide a new approach to mitochondrially-mediated therapy for malignant glioma that express the Norepinephrine transporter, which overcomes the resistance to targeting proliferation and growth factors.*

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Finally to my husband Michael and son Thomas, this is for you.

III Declaration

I declare that whilst studying for the degree of Doctorate of Philosophy at the University of Portsmouth I have not been registered for any other award at another University. The work undertaken for this degree has not been submitted elsewhere for any other award. The work contained in this thesis is my own with the following exceptions:

The immunohistochemistry was performed by Mrs P. Johnson

The HPLC-MS/MS was performed by Dr Richard Walker.

The MTT assay using IPSB-18 was performed by Mr T. Ahmed.

IV Abbreviations

AA	Anaplastic Astrocytoma
ACNU	Nimustine
AGT	O6-alkylguanine- DNA alkyltransferase
AIF	Apoptosis inducing factor
AMIT	Amitriptyline Hydrochloride
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocation
AO	Anaplastic Oligodendroglioma
AOI	Area of interest
AR	Adrenergic receptor
ATP	Adenosine triphosphate
ATP-TCA	Adenosine triphosphate-tumour cell assay
B-BB	Blood-brain barrier
BCNU	Carmustine; 1,3-bis(2-chloroethyl)-1-nitrosourea
BTB	Brain tumour barrier
cAMP	cyclic Adenosine monophosphate
CAR	Coxsackie virus-adenovirus receptor
CCNU	Lomustine;1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
cDNA	Complementary DNA
CED	Catheter enhanced delivery
CHLORPRO	Chlorpromazine
CLOM	Clomipramine Hydrochloride
CNS	Central nervous system
CR	Complete response
CSF	Cerebrospinal fluid
CT	Chemotherapy
CV	Coefficient of variation
CYP	Cytochrome p450
DF	Degrees of freedom
DIABLO	Direct inhibitor of apoptosis binding protein with low pI
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DOX	Doxepin
DR	Death receptor
DTIC	Dacarbazine; 5-(3,3-Dimethyl-1-triazenyl)imidazole-4-carboxamide
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EIAC	Enzyme inducing anticonvulsants
EIAED	Enzyme inducing antiepileptic drugs
EM	Extensive metabolisers
FAD	Flavine adenine dinucleotide
FBS	Foetal bovine serum

FFPE	Formalin fixed paraffin embedded
FGF	Fibroblast growth factor
FITC	Fluoroscein isothiocyanate
GBM	Glioblastoma multiforme
GCV	Ganciclovir
GD3	Ganglioside 3
gDNA	genomic DNA
GP	General practitioner
GSH	Glutathione
GSSG	Glutathione reductase
H&E	Haematoxylin and eosin
HBSS	Hanks balanced salt solution
HGG	High grade glioma
HIF	Hypoxia inducible factor
HPLC	High pressure liquid chromatography
HSP	Heat shock protein
HSV	Herpes simplex virus
IAP	Inhibitor of apoptosis protein
IHC	Immunohistochemistry
IM	Intermediate metaboliser
IMIP	Imipramine hydrochloride
IMM	Inner mitochondrial membrane
IV	Intravenous
LOH	Loss of heterozygosity
LRP	Lung-related resistance protein
MAO	Monoamine oxidase
MAPK	Mitogen activated protein kinase
MCJ	Methylated j-protein
M-CSF	Macrophage-colony stimulating factor
MGMT	O(6)-methylguanine-DNA methyltransferase
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilisation
MPTP	Mitochondrial permeability transition pore
MRI	Magnetic resonance image
MS	Mass spectrometry
MTD	Maximum tolerated dose
MTIC	3-methyl-(triazen-1-yl)-imidazole-4-carboxamide
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NABTC	North american brain tumour consortium
NAD	Nicotine adenine dinucleotide
NB	Neuroblastoma
NE	Norepinephrine
NET	Norepinephrine transporter
NF1	Neurofibromatosis-1
NICE	National institute for clinical excellence
NIH	National Institute for Health

NO	Nitric oxide
NORCLOM	Norclomipramine Hydrochloride
NORTRIP	Nortriptyline Hydrochloride
ODC	Ornithine decarboxylase
OG	Oligodendroglioma
OMM	Outer mitochondrial membrane
OS	Overall survival
p53	Protein 53
PCD	Programmed cell death
PCR	Polymerase chain reaction
PCV	Procarbazine, CCNU, Vincristine
PDGFR	Platelet derived growth factor receptor
PFS	Progression free survival
PKC	Protein kinase c
PM	Poor metaboliser
PROMETH	Promethazine
PS	Phosphatidyl serine
PT	Permeability transition
PTCH	Patched homologue
PTEN	Phosphatase and tensin
QC	Quality control
qRT-PCR	quantitative Real time PCR
RCCFM	Recovery cell culture freezing medium
RISC	RNA induced silencing complex
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
RT	Radiotherapy
RTK	Receptor tyrosine kinases
RTOG	Radiation Therapy Oncology Group
SD	Stable disease
SEM	Standard error of the mean
SERT	Serotonin receptor transporter
SMAC	Second mitochondria derived activator of caspase
SNP	Single Nucleotide Polymorphism
SSRI	Selective Serotonin Re-uptake Inhibitor
TCA	Tricyclic antidepressant
TDC	Test dose concentration
TGF	Transforming growth factor
TLDA	Taqman low density array
TMZ	Temozolomide; Temodar®
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis inducing ligand
TTP	Time to progression
VDAC	Voltage dependent anion channel
VEGF	Vascular endothelial growth factor
WHO	World health organisation

1 General Introduction

1.1 Background to Brain Tumours

Brain tumours are classified into primary and secondary tumours whereby the former originate from the brain itself while the latter metastasise from another location in the body. Histologically, primary tumours can be either malignant or benign, whereas secondary tumours are, by definition, always malignant. The intracranial tumours can be further subdivided into extracerebral and intracerebral; extracerebral tumours are generally Meningioma, intracerebral tumours are either intrinsic lesions arising from the substance of the brain, the majority of which are generically referred to as glioma, or extrinsic lesions such as metastases or lymphoma. There are also primary neoplasms considered to be neuronally-derived tumours such as the supratentorial primitive neuroectodermal tumour (sPNET), medulloblastoma and central neurocytoma but these are less frequent than the glial-derived neoplasms and are predominantly seen during childhood.

Primary brain tumours represent over one hundred different tumour types with a wide divergence of biologies and clinical prognoses, but despite the heterogeneity of these neoplasms they all challenge the field of neuro-oncology in the same manner. Glioma, so called due to the striking histological similarity of their component cellular elements to glial cells (astrocytes, oligodendrocytes and ependymal cells), includes Astrocytoma, Oligodendrocytoma and Ependymoma. Astrocytomas are the most frequently occurring of the glioma.

Malignant or high-grade glioma include Anaplastic glioma (Anaplastic Astrocytoma, Anaplastic Oligodendroglioma and Anaplastic mixed Oligoastrocytoma) designated World Health Organisation (WHO) Grade III and Glioblastoma multiforme (GBM) and Gliosarcoma designated WHO Grade IV (Louis, Ohgaki et al. 2007).

Until the development of *in vitro* and *in vivo* models, to further elucidate the mechanisms underlying central nervous system (CNS) malignancies, little information was available regarding their behaviour *in situ*. The process of obtaining human biopsy tissue following surgical resection is not possible in all cases due to location of the tumour, for example the brainstem glioma. Because of the delicate nerve connections to the motor and sensory systems and its role in the regulation of cardiac and respiratory function, tumours of the brainstem are usually inoperable.

Performing experiments *in vitro* provides limited information due to the failure to replicate the delicate microenvironment encompassed by the intricacies of the blood-brain barrier (B-BB). This is one reason why the formidable clinical challenge that malignant brain tumours and brain metastases present has meant that no really significant therapeutic advances have been made in the last decade.

This study focuses solely on glioma, for which the prognosis remains poor. Unlike the non-CNS cancers, where survival rates generally continue to increase, primary brain tumours remain largely incurable and highly lethal.

1.2 Classification and Grading of brain tumours

Comprised of both brain and spinal cord tumours, the CNS malignancies represent a vast array of tumour types, classified according to perceived lineage, histological diversity and prognosis. The WHO classification schema recognises intrinsic lesions from each type of neuroepithelial cell, as well as tumours of the specialised cells of the pineal gland, those derived from embryonic brain precursor cells and tumours of mixed origin including those arising from blood vessels.

In addition, extrinsic tumours arise from the meningeal coverings of the brain and spinal cord or from the sheaths of the cranial and spinal nerves and secondary tumours may also arise from outside the CNS.

The WHO classification of tumours of the nervous system contains more than fifty clinico-pathological entities, plus many variants within a particular subtype (for example the Meningioma has twenty-two variants), with a great variation in biological behaviour, response to therapy and clinical outcome (Louis, Ohgaki et al. 2007), however this system (unlike the St. Anne Mayo system) is not criteria-based.

Of all intracranial CNS tumours, approximately 60% are of neuroepithelial origin (glioma), 28% are derived from the brain coverings (Meningioma) and 7.5% are located in cranial and spinal nerves. Lymphomas and germ cell tumours account for 4% and 1% respectively (Louis, Ohgaki et al. 2007).

Four different malignancy grading systems are in current use for tumours of astrocytic lineage (WHO, St. Anne/Mayo, Ringertz, Kernohan); the Astrocytoma, Anaplastic Astrocytoma and Glioblastoma. The grades are assigned on the microscopic appearance of the tumour, however the numerical grade can vary depending on which system is referred to. It is important, for this reason; to specify which grading system is being used.

The St. Anne/Mayo system (Daumas-Duport, Scheithauer et al. 1988) has proved to correlate better with survival than the Kernohan system (Kernohan 1938); it is similar to the WHO system (Louis, Ohgaki et al. 2007) but it is criteria-based and can only be applied to invasive tumours of astrocytic lineage. The Ringertz system (Ringertz 1950), superseded the Kernohan system in 1950, is a three-tier system that was prompted by the lack of clear differences in survival times between grade III and IV tumours. Interestingly, the St. Anne Mayo system, which is the most commonly used grading system, does not take into account local infiltrative invasion and location in the brain and yet these features can significantly determine outcome.

	WHO classification
Juvenile Pilocytic Astrocytoma	Grade I Score 0
Diffuse, fibrillary, protoplasmic and gemistocystic Astrocytoma	Grade II Score 1
Anaplastic Astrocytoma	Grade III Score 2
Glioblastoma multiforme & variants	Grade IV Score 3 or 4

Table 1.2.1 Grading of astrocytic tumours according to WHO and St. Anne/Mayo systems

The grading system is based upon the recognition of the presence or absence of four morphologic criteria: nuclear atypia, mitoses, microvascular proliferation (endothelial hyperplasia), and necrosis. The method results in a summary score which is translated into a grade as follows: 0 criteria = grade 1, 1 criterion = grade 2, 2 criteria = grade 3, 3 or 4 criteria = grade 4. This is based upon the number of the four histological features identified in a biopsy by the neuropathologist. A grade I tumour, for example, would have none of the histological criteria and the diagnosis of grade I would be based upon hyper-cellularity alone. A grade IV neoplasm would display microvascular proliferation and/or necrosis as well as the other four criteria (which arise in lower-grade tumours). Unfortunately two important features which exert a major influence over prognosis/survival i.e. location within the brain and diffuse invasive behaviour, are ignored from these 'histological criteria' diagnostic systems. The histology and clinical data were derived from a previously reported series of patients with astrocytomas, treated with radiotherapy at Mayo Clinic between the years 1960 and 1969.

1.3 Pathology and Genetics of tumour types included in this study

1.3.1 Astrocytic Tumours

Tumours of astrocytic origin constitute the largest proportion of glioma. The characteristics and pathogenesis of astrocytic tumours is described in Figure 1.3.1.1 and they vary greatly in morphology, genetic profile and clinical behaviour. The Pilocytic, or juvenile, Astrocytoma (PA; WHO Grade I) is the most frequent CNS neoplasm in children, and is predominantly located in the cerebellum and midline structures, including the optic tract, brain stem and spinal cord. It infiltrates adjacent brain structures but grows slowly and usually has a favourable prognosis with five-year survival rates of more than 85% (WHO Grade I). Some PAs occur in the setting of neurofibromatosis type 1 (NF1), particularly those of the optic nerve (optic glioma).

Other astrocytomas usually develop in the cerebral hemispheres of adults and diffusely infiltrate adjacent brain structures. Low grade diffuse astrocytomas (WHO grade II) occur in young adults and grow slowly. However, they diffusely infiltrate the brain and cannot generally, therefore, be completely surgically resected. Morphologically, tumour cells resemble differentiated astrocytes. Mutations in protein 53 (p53), a transcription factor that regulates the cell cycle and hence acts as a tumour suppressor, are found in two-thirds of astrocytomas and are considered an early event. The five-year survival rate of low-grade diffuse Astrocytoma is greater than 60%.

Anaplastic Astrocytoma (AAs; WHO grade III) often develop from low-grade Astrocytoma, grow relatively fast and typically progress to Glioblastoma within two to three years, accompanied by genetic alterations, including loss of heterozygosity (LOH) on chromosome 19.

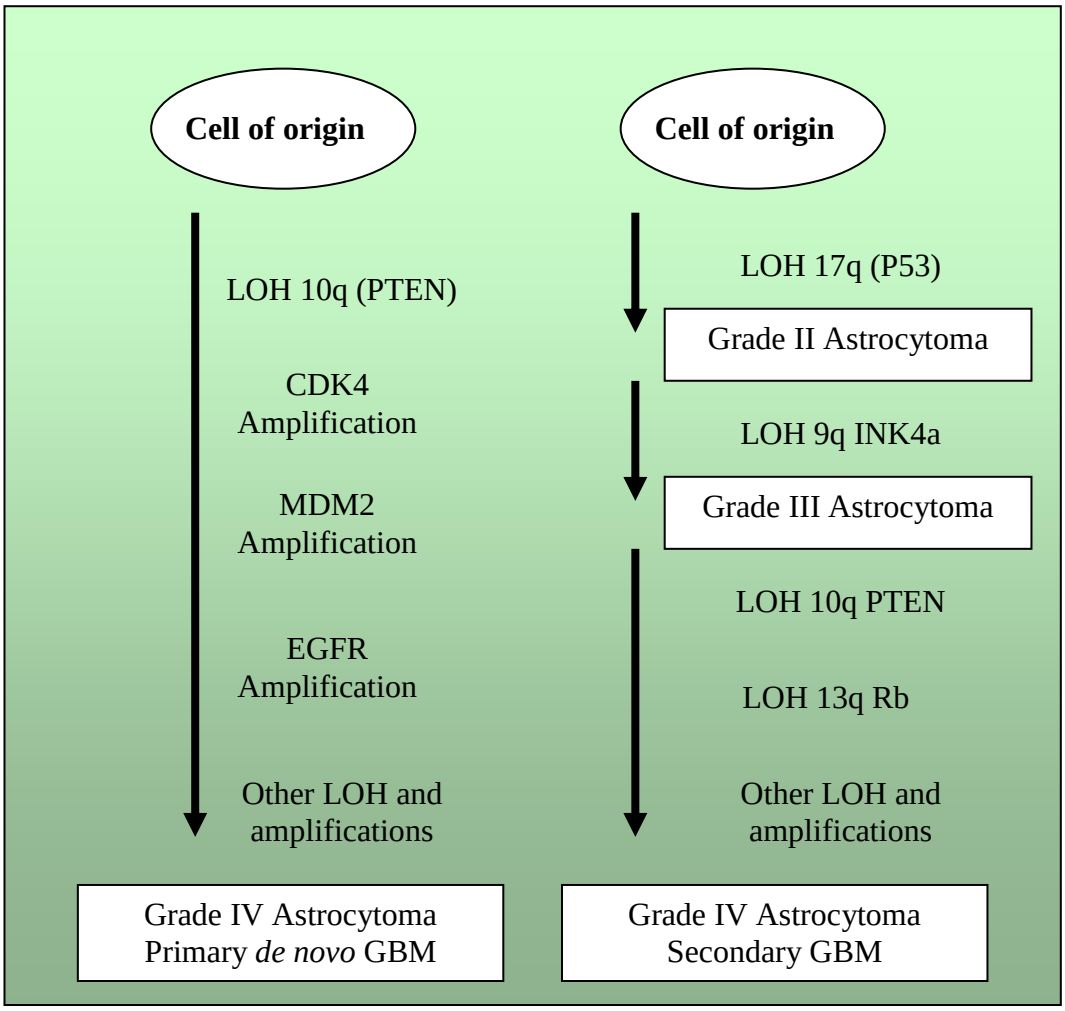


Figure 1.3.1.1 Flow chart showing the genetic pathogenesis of astrocytic tumours

Figure adapted from (Ohgaki, Dessen et al. 2004), there are two distinct pathways that lead to primary *de novo* Glioblastoma multiforme and secondary Glioblastoma multiforme, with progressive loss of heterozygosity and amplifications.

1.3.2 Glioblastoma multiforme (WHO grade IV)

This is the most frequent and most malignant nervous system tumour. Secondary GBMs develop by malignant progression from low-grade and Anaplastic Astrocytoma and are characterized by p53 mutations and LOH on chromosome 10q (see 1.3). Primary GBMs are more frequent (>80% of cases) and develop rapidly in an older age group (mean age, 55 years), with a short clinical history of less than three months. Their genetic profile includes amplification and overexpression of the epidermal growth factor (EGF) receptor gene, phosphatase and tensin homologue (PTEN; a tumour suppressor gene) mutations, $p16^{INK4A}$(which regulates the cell cycle by binding and deactivating various cyclin-cyclin dependent kinase complexes) deletions and loss of chromosome 10. Both GBM types diffusely infiltrate the brain, including the opposite hemisphere and show high cellularity and large areas of necrosis despite excessive vascular proliferation. Characteristic morphology of these tumour slides is shown in Figure 6.2.2.1.

1.3.3 Oligodendroglioma

These neoplasms develop from myelin-producing oligodendroglial cells or their precursors and are typically found in the cerebral hemispheres of adults, often including the basal ganglia. Histologically, they are isomorphic, with a typical honeycomb pattern and delicate tumour vessels (“chicken wire” pattern). Characteristic morphology of these tumours can be seen in Figure 6.2.2.1. Anaplastic Oligodendroglioma (AOs; WHO Grade III) show features of anaplasia and high mitotic activity and carry a less favourable prognosis.

Genetic hallmarks of Oligodendroglioma (OGs) are LOH on chromosomes 1p and 19q (see 1.3). OGs that carry these genetic alterations show a marked sensitivity to deoxyribonucleic (DNA)-based chemotherapy.

1.3.4 Meningioma

These slowly growing, usually benign, neoplasms develop from arachnoidal cells in the meninges. There is a prevalence in post-menopausal women, thought to be linked to the progesterone receptor (Lusis, Chicoine et al. 2005), particularly in respect to those located in the spine. Meningiomas do not infiltrate the brain but may cause symptoms of intracranial pressure due to compression of adjacent brain structures (WHO Grade I). Preferential sites are the cerebral hemispheres. Meningioma can often be cured by surgical resection. Malignant Meningioma are much less frequent; they may infiltrate the brain and often recur locally. Characteristic morphology of these tumours can be seen in Figure 6.2.2.1.

1.3.5 Paediatric medulloblastoma and supratentorial primitive neuroectodermal tumours

The medulloblastoma and supratentorial primitive neuroectodermal tumour (sPNET) are rare paediatric embryonal tumours, around which controversy exists regarding their origin. These two tumour types were studied during the course of the project, to provide an insight as to their origin, biological behaviour and pathogenesis, and the data was published via peer-reviewed journal (see appendix 9.3).

1.4 Biology of brain tumours

Tumour recurrence, high morbidity and high mortality, despite many technological advances associated with malignant brain tumours, may be attributed to a number of distinct biological features unique to tumour cells including proliferation, angiogenesis (formation of new blood vessels), cellular heterogeneity (including differential response to therapeutic agents by various sub-populations of cells) and local invasion.

Cell division, proliferation and apoptosis are essentially similar in both normal and tumour cells; the tumour cell, however, differs from its normal counterpart in that these processes are aberrantly regulated.

Cell proliferation is a key element of cell growth. A number of genetic aberrations, such as mutations in the p53 or PTEN tumour suppressor genes or amplification of oncogenes such as EGFR, allow tumours to circumvent the cell cycle checkpoint and cell arrest signals, blocking the apoptotic pathways and allowing the unregulated division of brain tumour cells (Merzak, Raynal et al. 1994).

Angiogenesis is another essential component of many physiological and pathological conditions including proliferation and local spread of brain tumours. The neovascularisation of high grade glioma is a characteristic inherent to these devastating tumours, and is mediated via physiological substances secreted by the brain tumour itself (Plate and Risau 1995).

Angiogenesis involves three main stages: blood vessel breakdown (through angiopoeitin-1 and its receptor Tie-2), breakdown of extra cellular matrix (ECM) and migration of cells to form new blood vessels (which involves matrix metalloproteinases; MMPs) and migration of endothelial cells and formation of new blood vessels (involving CD44 and integrins) (Tate and Aghi 2009).The formation of abnormal, dysfunctional tumour vasculature and glioma cell invasion along white matter tracts are major components of the difficulty in treating brain tumours effectively and hallmarks of glioma. As a result of angiogenesis, tumour vessel structure is markedly abnormal and this causes difficulty in delivering chemotherapy (Sathornsumetee and Rich 2007). Vessel changes also cause tumour hypoxia and oedematous areas in the brain (Jain, di Tomaso et al. 2007).

Finally, one of the most important features of human brain tumours is cell invasion. There is increasing evidence that invasive cells transiently arrest from the cell cycle during the migratory phase; therefore they are refractory to radiotherapy and many chemotherapy regimens. Moreover, invading neoplastic glia are protected from a number of cytotoxic drugs by virtue of being located in brain areas with an intact B-BB. Invasion is a complex, multi-step process modulated by a variety of factors (Bolteus, Berens et al. 2001). Firstly glioma cells detach from the primary mass (involving neural cell adhesion molecule downregulation and CD44 cleavage) and then mediators, such as integrins, bind to the ECM. Glioma cells secrete proteases (similar to MMPs) which degrade the ECM and finally cytoplasmic mediators (such as myosin) allow the invading glioma cells to alter their shape and become motile (Tate and Aghi 2009).

Invading cells frequently migrate away from the main tumour bed, where chemo- and radio-therapy are directed, and so are a highly lethal cell population. Overall, despite the advances brought about in neurosurgery and neuroradiology, such as non-invasive gamma knife and stereotactic radiosurgery, the outcome for patients remains dismal due to the complex biology of brain tumours.

1.4.1 Glioma angiogenesis and Vascular Endothelial Growth Factor-A

There are five members of the mammalian vascular endothelial growth factor family: VEGF-A, -B, -C, -D and P1GF (placental growth factor). An additional ligand, -E, is encoded by the Orf-virus genome. These ligands bind to cell surface receptors such as VEGF receptor tyrosine kinases (VEGFR1, -2 and -3) and co-receptors (heparin sulphate proteoglycans, neuropilins -1 and -2). VEGF-A (often simply referred to as VEGF in the literature, and will be referred to as such in this thesis) is a pro-angiogenic factor that binds to VEGFR-1 and VEGFR-2 receptors, stimulating intracellular signalling, gene expression, cell migration and new blood vessel sprouting (Latham, Molina-Paris et al.).

Neuronal cells, astrocytes and tumour cells secrete VEGF (-A), whereas the receptors are specifically expressed in vascular endothelial cells (Plate, Breier et al. 1994; Plate and Risau 1995; Kargiotis, Rao et al. 2006). Malignant glioma cells secrete a variety of angiogenic factors such as VEGF and fibroblast growth factor (FGF), which helps them co-opt blood vessels and it is VEGF-A that is thought to have a significant role in brain tumour angiogenesis (Jain, di Tomaso et al. 2007).

The upregulation in gene expression by these tumours has at least four mechanisms (Shibuya 2009):

1. a hypoxia/HIF-related mechanism related to low oxygen concentration in tumour tissue
2. stimulation via the epidermal growth factor receptor (EGFR) signalling pathway (HIF-independent)
3. the FoxMIB transcription factor stimulates VEGF in GBMs (HIF-independent)
4. the HuR protein is upregulated in hypoxic conditions and suppresses the post-transcriptional degradation of VEGF-A mRNA contributing to increased VEGF levels

The ability of brain tumours to upregulate the expression of VEGF and VEGFR plays a major role in tumour angiogenesis and tumour growth. Because of the importance of VEGF signalling in angiogenesis, a process whereby tumours form blood vessels and undergo vascularisation, an antibody was developed to block this signalling pathway (Sathornsumetee and Rich 2007). Based on the results of a phase III clinical trial, Bevacizumab, an anti-VEGF-A neutralising IgG has been developed (Avastin™). In combination with agents such as irinotecan, it has been shown through phase II clinical trial to be beneficial in patients with malignant glioma (Vredenburgh, Desjardins et al. 2007; Norden, Young et al. 2008).

Problems with anti-angiogenesis strategies such as Avastin are that whilst the reduced angiogenesis kills many 'standard' brain tumour cells, it does not kill cancer stem cells. This population of cells gain energy through anaerobic respiration as they thrive under low oxygen conditions. Therefore stem cells are able to invade post-Avastin therapy and lead to dissemination of the disease throughout the brain.

1.5 The Blood-Brain Barrier

The B-BB is formed by brain endothelial cells lining the cerebral microvasculature and the association via tight junctions with astrocytes. Astrocytic feet, which project from the astrocyte cell body, and are also known as '*glia limitans*' surround the endothelial cells of the B-BB and provide biochemical support to them. It is an important mechanism for protecting the brain from fluctuations in plasma composition, and from circulating agents such as neurotransmitters and xenobiotics capable of disturbing neural function (Abbott and Romero 1996). The B-BB allows only low molecular weight lipophilic agents to penetrate by passive diffusion. Efflux pumps, such as P-glycoprotein, remove the drug from the CNS and thereby reduce the cellular drug accumulation (Tews, Nissen et al. 2000). Drugs that are highly protein bound have a decreased penetration of the CNS. Genetic instability and innate chemoresistance of tumour cells that are common to all malignancies further complicate the issue. The planning of treatment modalities for patients with high grade glioma (HGG) is a challenge harder than that of any other cancer due to the extreme vulnerability of the CNS.

The invasive nature of these highly malignant tumours precludes a surgical cure; macroscopically the tumour can appear completely resected when in reality there are aberrant cells that have migrated through the parenchyma to establish themselves in a new location. The tight junctions of the endothelial and astrocytic cell interaction at the B-BB limit the effectiveness of the currently available chemotherapeutic agents that otherwise have the potential to target invading cells. The B-BB is more effective at restricting the passage of substances than endothelial cells in capillaries elsewhere in the body; the transmembane proteins constituting the tight junctions, such as *occludin* and *claudins* dimerise and anchor to the endothelial cells through *zo-1*. The growth of a tumour can disrupt the B-BB which in theory would enable chemotherapeutic agents to reach the tumour, but tumour cells tend to migrate away from the main tumour site into an area of intact non-neoplastic B-BB and protect themselves from chemotherapeutic assault.

In addition, glioma exhibit diffuse infiltration into the normal brain parenchyma and the tumour cells often show morphological features similar to reactive glia cells, making it difficult to discriminate tumour cells from other neural cell populations both *in vitro* and *in vivo*. Despite the fact that these malignancies are highly invasive in the brain, they rarely metastasise out of the CNS (Thorsen and Tysnes 1997). Invasion is the most significant biological feature of intrinsic brain tumours that precludes successful treatment (Pilkington 1997; Pilkington, Bjerkvig et al. 1997; de Ridder 1999).

The potential of Clomipramine hydrochloride (a tricyclic antidepressant traditionally used to elevate serotonin levels in the brain and treat depression) and the other tricyclic agents, that are the subject of this study, to treat intrinsic brain tumours lies within their innate ability to cross the B-BB and, unlike any other modality, reach these 'guerrilla' cells.

1.6 Tried and tested approaches for malignant glioma

Most HGG patients undergo a combination of treatment modalities, including surgical resection, radiotherapy (RT) and chemotherapy (CT) following histological diagnosis. There exists conflicting data regarding survival and extent of surgical resection due to treatment bias in younger patients, or those with less eloquent tumour location (Stummer, Reulen et al. 2008). More extensive resections often provide higher grade diagnoses (Glantz, Burger et al. 1991), due to the large degree of heterogeneity of tumours, however maximal surgical removal improves survival in most studies. If greater than 98% of tumour volume can be removed there is a significant survival advantage of 13 months, whereas is less than 98% tumour volume is removed the survival is 8.8 months (Lacroix, Abi-Said et al. 2001).

1.7 Traditional chemotherapy targets glioma cell proliferation

Most current chemotherapeutic agents work by preventing replication and transcriptional activities through blockade of DNA-helix unwinding or re-coiling, a fundamental step in the process of proliferation. This study will examine some current agents alongside experimental tricyclic drugs that target the mitochondrion, to compare sites of action.

	Mode of action	Administered	CNS Penetration
BCNU	Alkylators of DNA & cross-linking at DNA, RNA and proteins.	Intravenously	Good
CCNU		Orally	Good
Procarbazine		Orally	Good
Temozolomide		Orally	30% of serum level
Vincristine	Microtubule inhibitor	Intravenously	Poor
Irinotecan **Topotecan** **Etoposide**	Inhibition of topoisomerase	Intravenously	Good
Cisplatin **Carboplatin** **Oxaliplatin**	Platinum analogues; cause aquation in DNA	Intravenously	Poor (water soluble)

Table 1.7.1 A table comparing the mode of action, administration route and CNS penetration of the main chemotherapy agents used to target DNA and proliferation in malignant glioma.

Details on the efficacy of these agents can be found in sections 1.7.1 to 1.7.4.2. Temozolomide is thought of as the best current treatment option for first-line chemotherapy, Irinotecan has limited efficacy as a monotherapy but shows promise in combination with Temozolomide and Avastin (section 1.7.3.1) whereas Topotecan is generally used in combination with radiotherapy (Sasine, Savaraj et al.)

1.7.1 Nitrosoureas agents

The nitrosoureas, predominantly 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; Carmustine) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU; Lomustine) were, until recently, the chemotherapeutic agents of choice in the treatment of HGG. They are lipophilic, non-ionised agents with good CNS penetration. They act as DNA-alkylating agents, cross-linking guanine nucleobases in DNA double-helix strands, directly attacking DNA. The DNA strands are unable to uncoil and separate, the result is that the cells are unable to proliferate. BCNU, CCNU and their active metabolites are excreted in the urine and few drug interactions are known (Levin, Stearns et al. 1979).

Historically BCNU monotherapy is the gold standard in the treatment of purely astrocytic tumours, whereas CCNU combination therapy has been extensively studied in AAs and tumours of oligodendroglial origin (Cavaliere and Newton 2006). The nitrosoureas were the first single agent therapy used to treat glioma because they readily cross the B-BB due to their small size, non-ionized state and high lipid solubility (Gumerlock 1989). BCNU became the most commonly used single-agent CT for recurrent disease and was moved to the adjuvant setting after a randomised trial (BTSG 69-01) demonstrated BCNU and RT superior to RT alone (Gehan and Walker 1977). Similar results were obtained in studies using CCNU (Walker, Green et al. 1980; Chin, Young et al. 1981). One meta-analysis suggests that nitrosourea-based adjuvant CT has a modest benefit for patients with Anaplastic glioma and a smaller benefit for those with GBM (Levin, Silver et al. 1990).

1.7.2 Procarbazine, CCNU (Lomustine) and Vincristine (PCV)

Procarbazine and its metabolites also cross the B-BB, with rapid equilibrium between plasma and cerebrospinal fluid (CSF) (Newton, Turowski et al. 1999). Metabolism in the liver yields azo-procarbazine and hydrogen peroxide which results in the breaking of DNA strands. As a single agent, Procarbazine has been compared to intravenous (IV) BCNU, Methylprednisolone, and IV BCNU with Methylprednisolone, in patients with malignant glioma. The overall median survival was similar for the Procarbazine and BCNU groups, but long-term survival at up to 24 months was superior in the Procarbazine group (Green, Byar et al. 1983).

Vincristine is a water-soluble molecule that penetrates the B-BB poorly (Jackson, Sethi et al. 1981). Despite its limited CNS penetration, Vincristine is said to show activity as part of multi-agent regimens against low-grade glioma, Oligodendroglioma, and Anaplastic Astrocytoma (Evans, Jenkin et al. 1990; Levin, Silver et al. 1990; Glass, Hochberg et al. 1992). However, Vincristine has never been rigorously evaluated as a single agent for primary brain tumours. Vincristine binds to tubulin dimers, inhibiting assembly of microtubule structures. Disruption of the microtubules arrests mitosis in metaphase and prevents cells from proliferating.

Nitrosourea-based combination chemotherapy regimens were developed in the 1980s and showed promise. PCV was compared to single-agent BCNU as post-radiation therapy in a randomised trial (Levin, Wara et al. 1985). There was no significant difference in overall survival (OS) or time-to-tumour progression (TTP) for the group as a whole. However, in the subset of patients with Anaplastic glioma (rather than GBM), there was a significant increase (46 weeks) in TTP and survival for the group receiving PCV. A recent retrospective analysis of the Radiation Therapy Oncology Group (RTOG) database, including more than four hundred patients with Anaplastic glioma, revealed no survival advantage for patients treated with post-radiation PCV versus BCNU (Prados, Scott et al. 1999).

Conventional PCV chemotherapy for CNS tumours offers limited anti-tumour activity. Randomised trials on malignant glioma in the 1970-90s failed to demonstrate a significant increase in survival by the addition of chemotherapy to a treatment regimen (Walker 1978; Walker, Green et al. 1980).

PCV is generally palliative and has significant side-effects and limited efficacy. Although the benefits of adjuvant CT are modest compared with similar therapy for the non-CNS cancers recent findings have shown that there may be a future in modalities such as ‘salvage agents’ (agents administered in the hope that they may extend survival by a few extra months).

1.7.3 Topoisomerase inhibitors

1.7.3.1 Irinotecan

Irinotecan, also known as CPT-11, is a camptothecin derivative. It is a prodrug that requires de-esterification by carboxylesterases to yield its active metabolite, SN-38, which is 1,000 times more potent at inhibiting topoisomerase I (Newton, Turowski et al. 1999). The target of drugs of the camptothecin family is DNA topoisomerase I, a nuclear enzyme involved in the relaxation of the DNA double helix required for replication and transcription activities. They stabilise the enzyme-DNA complex and prevent the re-ligation of the single-strand breaks created by the enzyme, which are converted to double-strand breaks upon the collision with a replication fork during the S-phase.

Topoisomerase I plays a critical role in DNA transcription, replication and repair. In early trials of this agent, some responses were documented in patients with progressive, persistent, or recurrent malignant glioma (Colvin 1998; Friedman, Petros et al. 1999). The ability to selectively target tumour cells lies in the contact inhibition phenomenon inherent to ‘normal’ cells, whereby they cease cell division upon contact with other cells.

Preclinical studies demonstrated that the efficacy of CPT-11 against CNS tumours is enhanced when combined with alkylators such as BCNU in a schedule dependent manner (Coggins, Elion et al. 1998). It was therefore sought to determine whether the anti-tumour activity of CPT-11 could be enhanced by BCNU in patients with malignant glioma.

In phase I trials, a radiographic response was achieved in five newly diagnosed patients (14%) whilst a similar response was seen in five patients with recurrent disease. In more than 40% of both newly diagnosed and recurrent patients a stable disease (SD) state was achieved. Median TTP was 11.3 weeks for recurrent GBM patients and 16.9 weeks for recurrent AA/AO patients (Reardon, Quinn et al. 2004). A synergistic effect of CPT-11 combined with TMZ has also been reported in preclinical studies (Patel, Elion et al. 2000).

1.7.3.2 Topotecan

Topotecan is a water-soluble small molecule camptothecin analogue with excellent CNS penetration that binds directly to topoisomerase I without activation (Newton, Turowski et al. 1999). A phase II trial for adults with newly diagnosed or recurrent malignant glioma has demonstrated three partial responses (PR; 12%) and 10 with stable disease (SD; 40%) in twenty five newly diagnosed patients and three PRs (8%) and ten SDs (26%) in thirty eight patients with recurrent disease (Friedman, Kerby et al. 1999). Activity in paediatric patients with low- and high-grade glioma, medulloblastoma, and brainstem glioma was not demonstrated in a phase II study (Blaney, Phillips et al. 1996).

1.7.3.3 Etoposide

Etoposide is highly lipophilic but does not readily cross the B-BB due to its large size. Etoposide has been demonstrated to have minimal activity as a single agent in some patients with malignant glioma (Fulton, Urtasun et al. 1996; Chamberlain 1997). Etoposide (phosphate) directly inhibits topoisomerase II and, like CPT-11 selectively targets dividing tumour cells. The use of oral Etoposide at 50 mg/m²/day for twenty one days, followed by a fourteen day rest period and an additional twenty one days of oral Etoposide (50 mg/m²/day)was demonstrated effective and feasible (Chamberlain 1993). Of twelve evaluable patients with recurrent brainstem glioma, one presented complete response (CR), three PRs and two SDs, for a median duration of response of eight months.

1.7.4 Platinum analogues

1.7.4.1 Cisplatin and Carboplatin

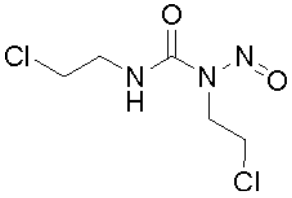
Cisplatin and Carboplatin are water-soluble and penetrate the B-BB poorly. These DNA-damaging agents cause tumour DNA to undergo ‘aquation’ and form cross links that displace a chloride ligand in the double helix. This directly interferes with mitosis and the subsequent DNA repair is unable to take place, thus triggering apoptosis. Like the other ‘cell-cycle specific’ agents they do not affect normal cells because whilst the duration of the cell cycle is identical in both normal and tumour cells, the proportion of cells undergoing division in tumour cells is much higher.

Cisplatin demonstrated variable activity against a wide range of tumours, including AA, GBM, medulloblastoma/sPNET, CNS lymphoma, and germ cell tumours (Kornblith and Walker 1988; Kyritsis 1993; Kim, Hochberg et al. 1996). In combination with CCNU or cyclophosphamide plus Vincristine, Cisplatin is also effective against high-risk and recurrent medulloblastoma (Packer, Sutton et al. 1991). Carboplatin is a cisplatin analogue with a similar activity profile and comparable cytotoxicity *in vitro*. As a single agent, Carboplatin has demonstrated efficacy in paediatric patients for both low-grade glioma and recurrent malignant primary brain tumours (Allen, Walker et al. 1987; Friedman, Krischer et al. 1992). In adults, single-agent carboplatin has shown minimal activity for the treatment of recurrent malignant glioma (Yung, Mechtler et al. 1991).

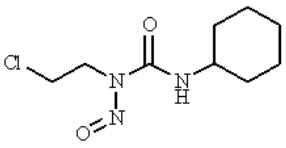
1.7.4.2 Oxaliplatin

Oxaliplatin, like Cisplatin and Carboplatin, is a platinum-based cytotoxic agent. However, unlike other platinum-based agents it does not appear to cause ototoxicity, nephrotoxicity, cardiac toxicity or alopecia in adults. Data for malignant glioma are limited to nine patients with recurrent GBM (Soulie, Raymond et al. 1997).

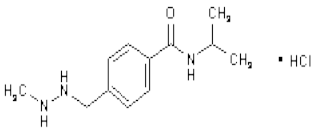
1.7.5 The structures of traditional glioma chemotherapy agents that target proliferation



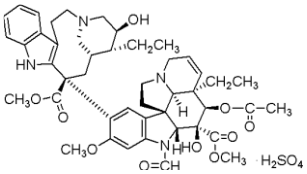
BCNU



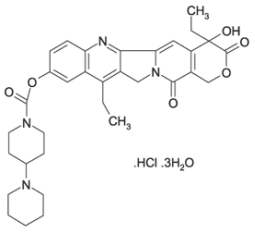
CCNU



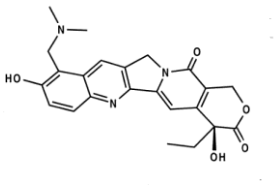
Procarbazine



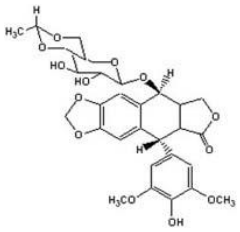
Vincristine



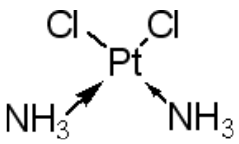
Irinotecan



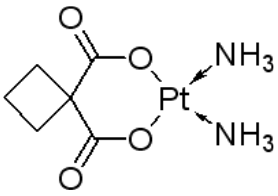
Topotecan



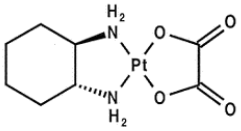
Etoposide



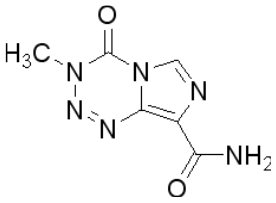
Cisplatin



Carboplatin



Oxaliplatin



Temozolomide

Figure 1.7.5.1 Traditional chemotherapy agents currently approved for use in glioma

BCNU, CCNU, Procarbazine, Vincristine, Cisplatin, Carboplatin, Oxaliplatin and Temozolomide are *reactive* chemotherapy agents that interfere with normal cellular processes and enhance cell death once they have reacted with their target molecule. They are, however, fairly indiscriminate and have powerful side effects. Irinotecan, topotecan and Etoposide are *interactive* agents that depend on lock-and-key recognition processes to interfere with cellular pathways. This makes them very specific agents but limited in efficacy due to the existence of parallel pathways for most critical processes. The most effective interactive drugs exhibit long-term high-affinity biological activity so that they can reach their target before being degraded and block the normal molecule from binding.

1.8 Recent approaches that have shown modest results

1.8.1 An oral agent to target proliferation

Temozolomide (Temodal®; TMZ), now approved by the National Institute for Clinical Excellence (NICE) for use in both newly diagnosed and recurrent glioma, is an orally-administered DNA alkylating agent. The results of the first clinical trial, published in 2001, showed it to exert a modest effect both as a single approach and adjuvant to radiotherapy (Brada, Hoang-Xuan et al. 2001; Lanzetta, Campanella et al. 2003). The potential for this licensed drug is high given the convenience of an oral preparation that can be taken at home; by combining it with other clinically significant agents it may be possible to optimise its anti-tumour activity.

Temozolomide is an oral agent, which is reported to, at least partially, cross the B-BB. It is an imidazotetrazine derivative of 5-(3,3-Dimethyl-1-triazenyl)imidazole-4-carboxamide(Dacarbazine; DTIC), which spontaneously hydrolyses to its biologically active form 3-methyl-(triazen-1-yl) imidazole-4-carboxamide (MTIC). The mechanism of anti-tumour actions is the disruption of DNA replication via methylation at specific residues including 06 position of guanine, N7 position of guanine and N3position of adenine.

Temozolomide is now being evaluated in multiple new clinical trials in combination with wide variety of additional therapeutic agents including other chemotherapeutic agents, cell signalling inhibitors, anti-angiogenic agents and therapeutics designed to overcome mediators of chemotherapy resistance.

1.8.2 Targeting the platelet-derived growth factor receptor

Gleevec (STI571, Imatinib mesylate; Novartis) is an oral, small-molecule adenosine triphosphate (ATP) mimetic that inhibits the kinase activity of several oncogenes, including BCR-ABL, c-ABL, c-KIT and PDGF receptors. It selectively inhibits the catalytic activity of the platelet derived growth factor receptor (PDGFR) and c-kit tyrosine kinases (Holdhoff, Kreuzer et al. 2005).

Gleevec did not possess any significant anti-tumour activity when studied in glioma by Wen et al. (2006), and was highly unsuitable for use in patients taking enzyme-inducing anti-epileptic drugs (EIAEDs) as they required far higher doses than patients not taking an EIAED, suggesting that the metabolism of Gleevec was severely impaired (Wen, Yung et al. 2006). Conversely Reardon et al. (2005) found that combination of Gleevec with a ribonucleotide reductase inhibitor was well tolerated and produced some anti-tumour activity (Reardon, Egorin et al. 2005). Gleevec was also found have anti-tumour activity and act synergistically with Clomipramine in C6 glioma cells (Bilir, Erguven et al. 2008). Anti-tumour activity of Gleevec was also observed in a subset of HGG, and the results showed that sensitivity correlated with PDGFR expression (Hagerstrand, Hesselager et al. 2006). Since PDGFR-alpha and –beta were only expressed in 25 and 19 cases respectively in a series of one hundred and one brain tumours (Haberler, Gelpi et al. 2006) this finding would account for the low response rate in clinical trials.

1.8.3 Bypassing the blood-brain barrier with impregnated wafers

Gliadel (polifeprosan 20 with Carmustine implant) wafers, inserted into the resection cavity at recurrence of tumour, can be used following TMZ therapy (Brandes, Tosoni et al. 2004). Adjuvant systemic CT increases survival of primary malignant glioma patients beyond 12-18 months.

The results of a multi-institutional phase I trial evaluating the safety of surgically implanted biodegradable impregnated polymers, as the initial therapy for malignant brain tumours, were first reported in 1990. The first study combined locally delivered BCNU and concurrent standard external beam radiation therapy. Median survival in this group of older patients (mean age = 60) was forty two weeks, eight patients survived one year, and four patients survived more than eighteen months. This interstitial chemotherapy with Gliadel appeared to be safe as an initial therapy (Brem 1990). Gliadel is the only interstitial CT treatment approved for malignant glioma. Analysis of a large trial by Westphal and colleagues (2006; n = 240) showed that malignant glioma patients treated with Gliadel wafers at the time of initial surgery in combination with RT demonstrated a survival advantage at two and three years follow-up compared with placebo (Westphal, Ram et al. 2006).

1.8.4 Blocking the vascular endothelial growth factor receptor with a monoclonal antibody

Avastin® or bevacizumab is a humanised monoclonal antibody targeted against VEGF (-A). A phase II trial of Avastin co-administered with Irinotecan (see section1.7.3.1) produced a radiographic response and survival benefit (Vredenburgh, Desjardins et al. 2007; Vredenburgh, Desjardins et al. 2007). A trial of Avastin in fifty-five glioma patients, who were taking various other forms of chemotherapy alongside, showed a complete response in 2.3% of patients, partial response in 31.8% of patients, minimal response in 29.5% of patients and stable disease in 29.5% of patients. The agent was well tolerated and showed long term disease control (Norden, Young et al. 2008).

1.8.5 Modulating the immune response to tumours with antibodies and cytokines

Immunotherapy with monoclonal antibodies targeting tumour-specific antigens has emerged in the last two decades as a novel potential adjuvant treatment for all types of neoplasia. Results from ongoing Phase I/II clinical trials are encouraging, as disease stabilisation and patient survival prolongation have been observed (Reardon, Akabani et al. 2006). Advances in preclinical and clinical research indicate that treatment of brain tumours with monoclonal antibodies can be increasingly adjusted to the characteristics of the targeted tumour and its environment. This aspect relies on the careful selection of the target antigen and corresponding specific monoclonal antibody, and antibody format (size, class, affinity), conjugation to the appropriate toxin or radioactive isotope (half-life, range), and proper compartmental administration.

Following the discovery in 1989 that the radioiodinated anti-tenascin monoclonal antibody 81C6 was observed to have selective uptake by tumour cells (Zalutsky, Moseley et al. 1989), one group has further established the efficacy and tolerance of81C6 (131I-m81C6) when injected into the surgically created resection cavity of patients with newly diagnosed and recurrent malignant brain tumours.

Their current approach involves administering a ^{131}I-m81C6dose to deliver 44Gy to the 2-cm resection cavity perimeter and is based on dosimetry analyses demonstrating that a 44 Gy boost dose by 131I-m81C6achieved optimal tumour control while minimizing toxicity (Reardon, Akabani et al. 2006).

In the phase II study, 20 patients with newly diagnosed malignant glioma (14 GBM, 6 AA) received ^{131}I-m81C6 administered to achieve a 44 Gy boost. Grade 3/4 toxicities were limited to haematotoxicities with three leucopoenia, one neutropoenia and one thrombocytopenia. A median survival of 93.6 weeks was observed for GBM, it has not been reached yet for AAs.

Transforming growth factor-beta (TGF-beta) is a pro-invasive and immunosuppressive cytokine that plays a major role in the malignant phenotype of glioma. One novel strategy of disabling TGF-beta activity in glioma is to disrupt the signalling cascade at the level of the TGF-beta receptor I (TGF-betaRI) kinase, thus abrogating TGF-beta-mediated invasiveness and immune suppression.

One study, which involved the implantation of SMA-560 cells (derived from a spontaneous murine Astrocytoma) into the striatum of VM/Dk mice (albino, inbred strain containing Prn-i locus) and subsequent administration of SX-007, an orally active, small-molecule TGF-betaRI kinase inhibitor, showed twenty five percent of the animals to be disease-free at the end of the study (Tran, Uhl et al. 2007). The data suggests that SX-007 exerts a therapeutic effect via the reduction of TGF-beta 1 mediated invasion and reversal of TGF-beta 2-mediated immune suppression. By reversing immune suppression immune surveillance is restored and hence the tumour is ablated.

Promising results were also seen when the antifibrotic drug 5-methyl-1-phenyl-2-(1H)-pyridone (pirfenidone) elicited a growth-inhibitory effect and reduced TGF-beta2 protein levels in human glioma cell lines (Burghardt, Tritschler et al. 2007).

1.8.6 Delivering chemotherapy directly to the tumour site

One of the latest advances in the treatment of brain tumours is the trial of convection enhanced delivery (CED), which overcomes the need for selection of agents that can cross the B-BB. This method was originally introduced and refined in the early 1990s by researchers at the National Institute of Health (NIH) and involves drug infusion under high pressure using intracranial catheters. CED allows for delivery of high concentrations of therapeutic agents directly into brain tumours and surrounding parenchyma. This method eludes the B-BB and allows the use of regional drug therapy, while at the same time limiting systemic toxicity (Ferguson and Lesniak 2007).

Catheter enhanced delivery has been improved with the emergence of patient-specific algorithms, according to target-tissue anatomy and patient physiology, whereby the trajectory of the catheter is determined using magnetic resonance diffusion tensor imaging (Sampson, Raghavan et al. 2007). By using ^{123}I-labeled human serum albumin, high-resolution single-photon emission computed tomographic images were obtained at and coregistered with magnetic resonance imaging scans to determine the distribution according to catheter site (Sampson, Brady et al. 2007). Intra-tumoural infusions were anisotropic and resulted in limited coverage of the enhancing tumour area and adjacent peritumoral regions, whereas intracerebral infusions resulted in broad distribution across the parenchyma. A necrotic core is a prevalent occurrence in a tumour and the results from Smith and Humphrey (2006) present findings that show it can profoundly alter the interstitial fluid pressure and velocity distributions in its vicinity.

Because the necrotic core does not have a functional vasculature there can only be interstitial fluid transport in that region. The pressure decrease is markedly less in the necrotic core because no fluid can be reabsorbed into the vasculature there, thus the interstitial fluid velocity is significantly higher than when there is no necrotic core. Ironically the presence of a necrotic core is thought advantageous to convection-enhanced delivery because the convective flow reaches a larger region of the tumour (Smith and Humphrey 2006). By placing the infusion site proximally to the tumour the flow of drug is propelled outward. This is in contrast to the findings of Baxter and Jain who showed that the necrotic core had little or no impedance on reducing the elevated tumour interstitial fluid pressure (Baxter and Jain 1990).

The convective fluid velocity is not high over a large region but the advantage is that it can be used to deliver macromolecules, proteins and other high molecular weight compounds that are normally denied access to the brain parenchyma by the B-BB. Therefore CED shows huge potential in the delivery of chemotherapeutic agents, perhaps with an intra-tumoural catheter plus a number of proximal catheters, designed to target both the tumour and the invading cells.

1.8.7 Reprogramming glioma cells with gene therapy

Gene therapy is a particularly attractive and novel approach towards the treatment of single-mass brain tumours due to their lack of propensity to metastasise. Despite the strong resistance towards systemic therapies, due to the B-BB, it is possible to inject therapeutic adenoviruses intra-tumourally using safe and fast procedures. Oncolytic adenoviruses are genetically manipulated human adenoviruses that acquire a replication phenotype in cancer cells, but show a more restrictive phenotype in normal cells. Several features of wild-type adenoviruses can be modified to acquire tumour replication properties (Chiocca 2002).

In the 1990's, retroviral vectors were found to give suboptimal delivery of therapeutic genes to cancer cells, however when substituted with adenoviral vectors it was found that they were not able to transduce a high enough number of tumour cells to result in a dramatic therapeutic effect. By directly injecting the tumour with a cell line actively producing a adenoviral vector carrying a gene conferring drug sensitivity to the tumour, mammalian cells can be 'infected' and cause the cell to incorporate the new genetic material into their genome (Culver 1993). One pro-drug study employed NIH 3T3 cells and the herpes simplex virus enzyme thymidine kinase. HSV-tk sensitised the tumour cells to an antiviral drug (Ganciclovir, GCV) which is a natural substrate for HSV-tk (Oldfield, Ram et al. 1993). The enzymatic process induced by GCV leads to death of the cell expressing the herpes TK activity, i.e., death of the tumour cells. Since the HS-tk enzyme which is normally present in mammalian cells has very low affinity for GCV, systemic toxicity related to this mechanism is not observed.

Gene therapy has some striking advantages (see table Table 1.8.1), firstly the adeno-viral vectors selectively target cells which are actively synthesising DNA. Thus surrounding normal, and hence non-proliferating, cells will remain unaffected. Secondly the transduced tumour cells will be destroyed by the host immune system and GCV treatment. This also eliminates the potential for mutagenesis initiated by the insertion of foreign genetic material.

Target	Advantages	Disadvantages
Tumour suppressor genes	• Most tumours have one or more inactivated tumour suppressor genes • Restoration of tumour suppressor gene function can correct tumour phenotype • Restoration of certain genes, such as p53, has a bystander effect (the inhibition/killing of non-transduced cells) • Apoptotic pathways are restored resulting in greater sensitivity	• Poor penetration and delivery in solid tumour mass • Limited use as a single agent • Efficacy is not universal • Leaky replication observed in normal cells
Suicide genes	• Non-toxic prodrugs can be administered in high doses without significant effects to normal cells • Toxic drug transfers to neighbouring tumour cells via gap junctions, causing bystander effect	• Drug-induced premature cell death (following chemotherapy) results in impaired viral replication • Timing, if used in combination with chemotherapy, is critical
RNA Interference (RNAi)	• It is both an endogenous regulatory mechanism and a tool to therapeutically suppress over-expressing genes • Vector-based approaches have high transfection efficiency, continuous production of siRNA in cells and sustained protein suppression	• Delivery of double-stranded RNA is inefficient • Cell type specificity • High cost of double-stranded RNA
Angiogenesis	• Tumours are dependent on angiogenesis for growth and metastasis	• Efficacy is limited due to redundant signalling pathways
Cytokines & dendritic cells	• Cytokines activate responder cells • In combination, cytokines synergise to facilitate tumour regression • Adenoviral-transduced dendritic cells can exhibit adjuvant effects and induce dendritic cell activation and maturation • *Ex vivo* treatment of dendritic cells circumvents pre-existing antiviral immunity	• Cytokines have a short *in vivo* half-life and sub-therapeutic levels at the tumour site • Transduction rates of adenoviruses to dendritic cells is low resulting in low efficacy

Table 1.8.1 Potential targets of gene therapy

The advantages and disadvantages of five targets of gene therapy are detailed, showing the huge potential of adenoviral therapy in treating malignant glioma. The most promising results have been found when using gene therapy in combination with chemotherapy, however the dosing schedule is critical for optimal adenoviral delivery (Folkman 1990; Heise, Sampson-Johannes et al. 1997; Addison, Bramson et al. 1998; Emtage, Wan et al. 1999; Narvaiza, Mazzolini et al. 2000; Okada, Tsukada et al. 2001; Liu, Huang et al. 2002; El-Deiry 2003; Milhavet, Gary et al. 2003; Aghi and Martuza 2005; Boucher, Im et al. 2006; Lumniczky and Safrany 2006; Iorns, Lord et al. 2007; Sharma, Tandon et al. 2009).

The drawbacks of the HSV-tk/GCV study published in 1993 are that the studies were performed in rats for only twenty-eight days (Ram, Culver et al. 1993). The proliferative nature that rat brain tumours display shows that they possess differing characteristics to their invasive human counterparts and so are not an ideal model for such experiments.

Studies have also been performed using the native HSV in GBM. The replication-competent HSV mutant HSV1716 was injected directly into the tumours of high-grade glioma patients. Because replication is a cytolytic process, cell death is brought about once the adenovirus has been incorporated into cells. Of twelve patients studied in this manner, all tolerated the therapy well with no side effects. The study showed the potential for gene therapy using native HSV (Rampling, Cruickshank et al. 2000; Papanastassiou, Rampling et al. 2002).

Adenovirus transduction is dependent on the coxsackie virus-adenovirus receptor (CAR) expression in cells. Low uptake efficiency is seen when using native adenoviral vectors; it is possible to increase the uptake in endothelial cells by re-targeting the adenovirus with a fibroblast growth factor 2 (FGF-2) ligand (Gupta, Wang et al. 2006). This means that gene therapy using native HSV could have an antiangiogenic effect in brain tumours. The latest studies using adenoviral vectors involve the deletion of viral genes that interact with tumour suppressor genes, the modification of the ability to infect cancer cells with more potency, and the inclusion in the viral genome of elements of transcription that are sensitive to transcription factors upregulated in cancer cells (Alemany, Balague et al. 2000).

Although they occur at a relatively low incidence, brain tumours contribute significantly to morbidity, often affect children and overall have a poor prognosis. Due to marked resistance to RT and CT, the prognosis for patients with GBMs is very poor. The majority of patients die within nine to twelve months and less than 3% survive more than three years. Many genetic alterations involved in the development of nervous tissue tumours have been identified and may lead to novel therapeutic approaches, including gene therapy.

There have also been advances contributing to the increased knowledge surrounding molecular abnormalities and resistance to radio and chemotherapy has led to a new approach of targeting aberrant signal transduction pathways (Sathornsumetee and Rich 2006). Targeting the underlying pathogenesis, via a small-molecule inhibitor or monoclonal antibody, may provide the way forward in treating this devastating, and largely incurable disease.

1.9 Approaching the mitochondrion as a new target for malignant glioma

Mitochondrial membrane permeabilisation is the critical event in the process leading to physiological or chemotherapy-induced apoptosis. This event is governed, in part, by the mitochondrial permeability transition pore (MPTP). The composition of the MPTP differs in normal and malignant cells; ANT2 (adenine nucleotide translocator; gene the expression of which is repressed in quiescent cells) is transcribed in proliferating tumour cells (Giraud, Bonod-Bidaud et al. 1998; BarÃ¡th, Albert-Fournier et al. 1999), the peripheral benzodiazepine receptor (PBR) and mitochondrial creatine kinase are overexpressed in some tumours (O'Gorman, Beutner et al. 1997; Kanazawa, Tanaka et al. 1998; Schiemann, Schwirzke et al. 1998; Venturini, Zeneroli et al. 1998; GaliÃ¨gue, Jbilo et al. 1999). Overexpression of PBR confers a relative resistance to oxidative stress (Carayon, Portier et al. 1996). More importantly functional Bax is frequently reduced in cancer cells (Rampino, Yamamoto et al. 1997; Brimmell, Mendiola et al. 1998; Ouyang, Furukawa et al. 1998; Yagi, Akiyamaâ¡ et al. 1998) and Bcl-2 is overexpressed in a large percentage in neoplasms (Reed 1994; Kroemer 1997; Reed 1997), strengthening the hypothesis that the control of the MPTP can be altered in tumours.

It is increasingly evident that DNA-targeted agents are effortlessly circumvented by sophisticated DNA repair mechanisms inherent to brain tumours (Pilkington, Parker et al. 2008). Therefore the specific delivery of drugs to mitochondria may provide the foundation to treat these devastating tumours.

A recurring problem with conventional chemotherapy is that they exploit endogenous apoptotic pathways that are frequently subject to alterations such as mutations (p53), blockade of death receptors (CD95/CD95L pathway) and over expression of Bcl-family members. A solution would be to target cell death further downstream of events that are commonly aberrated.

1.10 The role of mitochondria in determining the fate of glioma cells

Mitochondria exert dual functions in the regulation of cell survival and cell death. They are the powerhouse of the cell for the production of energy, and are therefore vital for the survival of the cells in addition to them being the gateway of the intrinsic pathway of apoptosis (Kroemer, Galluzzi et al. 2007). In light of the critical regulatory role of mitochondria in the control of cell death and the fact that mitochondrial functions are frequently altered in malignant glioma, agents that target the mitochondria are considered promising in the elimination of tumour cells that are resistant to conventional therapies (Gogvadze, Orrenius et al. 2009). Whilst the mitochondrion is largely-dependent on nuclear-encoded factors, some functional independence remains, which means that mitochondria-targeting drugs offer the advantage to act independent of upstream events that are often blocked in cancers. Thus, mitochondriotoxic compounds are likely to be effective in otherwise refractory tumours and may overcome problems of multi-drug resistance (Fulda 2010).

The mitochondrial membrane consists of phospholipid bilayers (the exact composition of which is detailed in table Table 1.10.1 below), termed the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM). The OMM contains an array of protein and lipid complexes including translocases of the outer membrane (TOMs), adaptor proteins, antioxidants, lipid transferases, ion channels and members of the Bcl-2 family either as resident integral proteins (Bak) or as cytosolic translocates (Bax or tBid) (Colombini 1980; Thomas, Gebicki et al. 1989; Fisk and Yaffe 1997; Colquhoun 1998; Santel and Fuller 2001; James, Parone et al. 2003). The OMM is responsible for fatty acid degradation, tryptophan metabolism and monoamine neurotransmitter metabolism (via monoamine oxidase).

	Inner mitochondrial membrane (%)	Outer mitochondrial membrane (%)
Phosphatidyl choline	38.4	45.6
Phosphatidyl ethanolamine	24.0	32.6
Phosphatidyl inositol	16.2	10.2
Phosphatidyl serine	3.8	1.2
Phosphatidic acid	1.5	4.4
Cardiolipin	16.1	5.9

Table 1.10.1 The phospholipid compositions of the inner and outer mitochondrial membranes

The inner membrane contains a higher proportion of cardiolipin where it supports the function of a large number of enzymes involved in the electron transport chain (Lomize 2008).The IMM contains proteins with five different functions: redox proteins associated with oxidative phosphorylation, ATP synthases which generate ATP, transport proteins that regulate metabolites to and from the matrix, protein import machinery and fusion and fission proteins (Raff, Barres et al. 1994). The high proportion of cardiolipin (which contains four fatty acids rather than two) makes the IMM impermeable, unlike the OMM it does not contain porins and virtually all molecules require specific transporters to enter the matrix (Raff, Barres et al. 1994). The IMM contains coenzyme Q – cytochrome c reductase, cytochrome c, NADH dehydrogenase and succinate dehydrogenase which are all enzymes involved in oxidative phosphorylation (the product of which is ATP, produced in the matrix) and also means that the IMM is the site of production of reactive oxygen species (ROS) superoxide and hydrogen peroxide (Lomize 2008). The IMM is also the site responsible for the electron transport chain (see 1.12), the energy released from which drives the flow of protons through the membrane in a process called chemiosmosis and results in a membrane potential across the membrane (Raff, Barres et al. 1994).

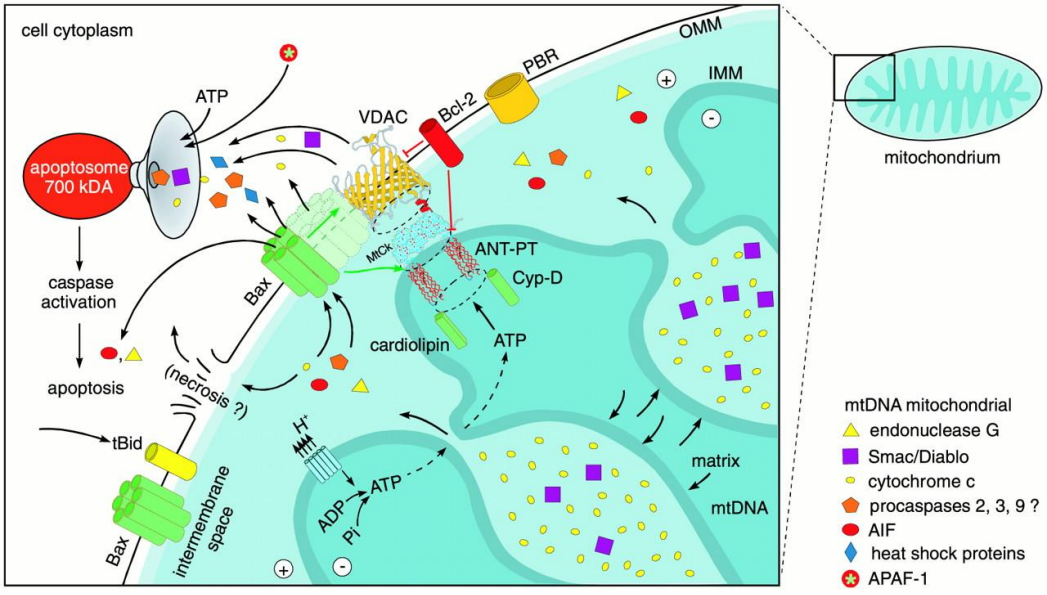


Figure 1.8.7.1 Mitochondrial regulation of apoptotic cell death in glioma (Mayer and Oberbauer 2003)

The main components of the permeability transition pore are the VDAC and the adenine nucleoside translocator (see 1.18) which are adjacent in the outer and inner mitochondrial membranes, respectively. Besides these abundant proteins, peripheral benzodiazepine receptor and Bax/Bcl-2 in the outer mitochondrial membrane and cyclophilin D and cardiolipin in the inner mitochondrial membrane complete the PT complex. The big apoptogenic proteins such as cytochrome c (12 kDa), second mitochondria-derived activator of caspases/direct inhibitor of apoptosis (IAP)-binding protein with low pI (smac/Diablo; 100 kDa), AIF (57 kDa), and others are predominantly stored in vesicles created by infolding of the IMM (intercristal space). During apoptosis induction, the ANT conductance and permeability increase to 700 pS and 1.5 kDa, respectively. This leads to osmotic swelling of the mitochondrial matrix by water influx succeeded by compression of the intercristal space. Cytochrome C, Smac/DIABLO and AIF are released from their intercristal storage into the intermembrane space. The ejected proteins cytochrome c, caspases, ATP, and the cytoplasmic apoptosis protease activating factor-1 (APAF-1) associate in the cytosol to huge apoptosome complexes (700 kDa), which cleave downstream caspases.

The mitochondrion is however considered an 'innocent bystander' during permeabilisation of the outer membrane because the mechanism is entirely controlled by the Bcl-2 family of proteins. Pro-apoptotic Bcl-2 family members (see 1.23) Bid, Bax and Bak mediate the permeabilisation of the OMM. Apoptotic signalling cleaves Bid (via caspase 8 or granzyme B) which activates a highly truncated form (tBid) which localises to the OMM. There, tBid induces the oligomerisation and insertion of Bax into the OMM or the oligomerisation of Bak, and subsequent of cytochrome c (Wei, Zong et al. 2001). Rupture of the OMM is essential for mitochondrial demise and apoptosis will not be triggered simply by virtue of damage to either membrane or damage targeted to mitochondrial DNA (but may signal other forms of cell death such as necrosis) (Chipuk, Bouchier-Hayes et al. 2006). The mitochondrial membrane potential of tumour cells is reduced in comparison to non-neoplastic cells, providing a window of opportunity for mitochondria-specific agents to enter the cell, disrupt ATP turnover and initiate cancer cell-specific apoptosis (Pilkington, Parker et al. 2008).

Mitochondria have long been known to be sensitive to a large variety of drugs, nutrients and hormones. Investigations into the disruption of oxidative phosphorylation, changes in mitochondrial gene expression, alterations in membrane function or induction of free radical damage have shown that a number of drugs, originally developed for other purposes, act via the mitochondrial DNA or membrane (Fulda 2010).

Mitochondria have significant roles in bioenergetics and vital signalling of the mammalian cell. Consequently, these organelles have been implicated in the process of carcinogenesis; the alterations of cellular metabolism and cell death pathways. Multiple molecular routes of malignant transformation appear to result in the common ability of many tumours, especially the fast-growing Glioblastoma and Astrocytoma (Lowry, Berger et al. 1983; Dastidar and Sharma 1989), to take up large amounts of glucose.

Defects in the execution of the apoptotic programme enable neoplastic cells to survive beyond their intended lifespan, allowing them time to accumulate genetic alterations that affect the mitochondrial role in apoptosis. Virtually all-known cytotoxic anticancer drugs, when effective, induce apoptosis of malignant cells so unravelling the pathways leading to mitochondrial membrane permeabilisation thus offering new targets for drug development. Most tumours carry defects in their apoptotic pathways and should, therefore, be more poised for cell death so the mitochondrion of tumour cells is an attractive target.

1.11 The generation of adensine triphosphate provides a selective target in tumour cells

Adenosine triphosphate is generated in two ways in normal cells: oxidative phosphorylation in mitochondria and glycolysis in the cytoplasm. All cells use both pathways but rely overwhelmingly on oxidative phosphorylation, switching to glycolysis at times of oxygen deprivation. A key biological difference between normal differentiated cells and tumour cells is altered metabolism, whereby tumours cells acquire a number of stable genetic and epigenetic alterations to retain proliferation and survive in hypoxic conditions (Wolf, Agnihotri et al. 2010). A classic adaptation is a shift to aerobic glycolysis from mitochondrial oxidative phosphorylation. Aerobic glycolysis in tumour cells characterised via a high uptake of glucose and low oxygen consumption, despite the availability of oxygen was first described in 1930 by Otto Warburg, and is called 'The Warburg Effect'. Warburg noticed that whereas normal cells generate 10% of their total cellular ATP through glycolysis, tumour cells generate up to 50% of their ATP through the same process even when oxygen is present (Wolf, Agnihotri et al. 2010). Targeting key metabolic enzymes involved in modulation of the 'Warburg' effect and characterisation of glycolytic metabolism within glioma tissue therefore may be novel and lead to therapeutic approaches for the treatment of these tumours (La Schiazza, Lamari et al. 2008).

1.12 Oxygen sensing by mitochondria at complex III

The catabolic pathways of the cell (glycolysis, citric acid cycle, fatty acid oxidation and amino acid oxidation) all lead to the production of two high-energy electron donors, nicotine adenine dinucleotide (in the reduced form, NADH) and flavine adenine dinucleotide (in the reduced form, $FADH_2$). Electrons from NADH and $FADH_2$ are passed through the electron transport chain to oxygen, which is further reduced to water. During electron transport, the enzymes of the electron transport chain create a proton gradient across the inner mitochondrial membrane. This gradient is used by the enzyme ATP synthase to produce ATP (Lambert and Brand 2009). The electron transport chain is comprised of four enzymes (complexes I, II, III and IV), which are all present on the inner mitochondrial membrane. Complex I (NADH dehydrogenase) removes two electrons from NADH and transfers them to the electron carrier, ubiquinone. The reduced product is called ubiquinol. Complex II (Succinate dehydrogenase) removes electrons from succinate and transfers them to ubiquinone via FAD. Complex III (Cytochrome bc_1 complex) removes two electrons from the reduced-state electron carrier ubiquinol and transfers them to two molecules of the electron carrier cytochrome C. Complex III also moves four protons across the inner mitochondrial membrane. Complex IV (Cytochrome C oxidase) removes two electrons from the two molecules of cytochrome C and transfers them to molecular oxygen, producing water (Alberts, Johnson et al. 2007).

During the process of electron transport across the four complexes, protons (H+ ions) are released into the intermembrane space between OMM and IMM. A total of six protons, two each from complexes I, III and IV, travel across the membrane to the intermembrane space and down an electrochemical gradient to complex V (ATP synthetase) whereby they pass back through to the matrix. This process is known as chemiosmosis (Mitchell 1961) and the energy produced from the flow of protons through complex V is sufficient to produce three molecules of ATP.

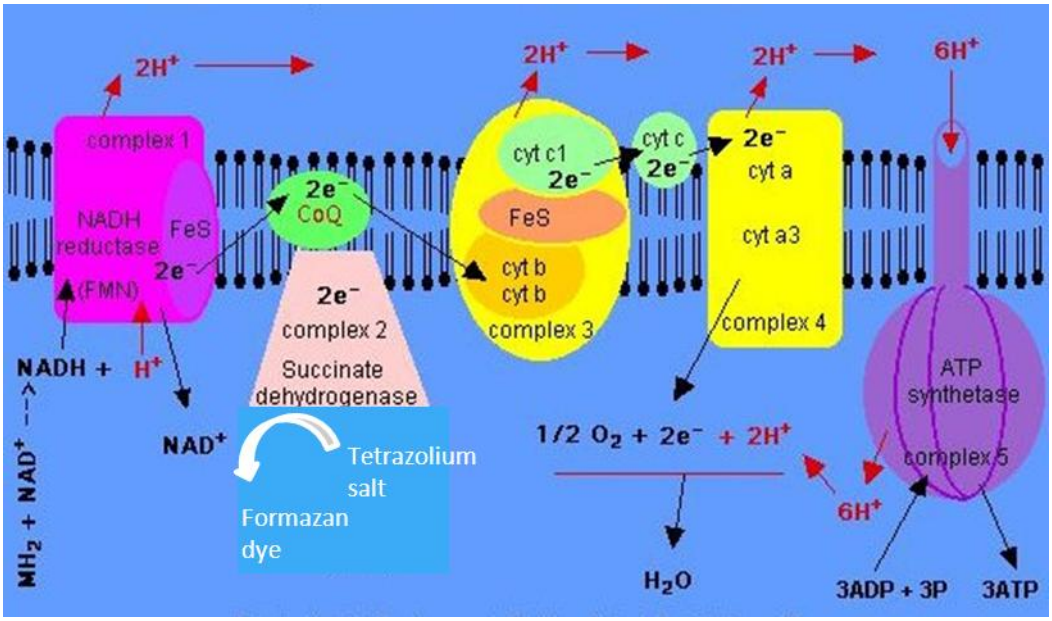


Figure 1.8.7.1 Schematic representation of the electron transport chain showing five complexes within the mitochondrial membrane (Ophardt 2003)

The transport of electrons across the chain is initiated via the reaction of an organic metabolite (from the mitochondrial matrix) with coenzyme NAD+ (nicotinamide adenine dinucleotide). Of relevance to this study is complex III which, in normal cells, would sense that conditions are becoming hypoxic (a sign that a cell is nearing the end of its life) and release reactive oxygen species to trigger apoptosis. It may be that the process of oxygen sensing is deviated in tumour cells but Clomipramine hydrochloride, which exerts its effects via complex III (Daley, Wilkie et al. 2005), is able to trigger the release of ROS by a different mechanism. The conversion of MTT tetrazolium salt to formazan by complex II, using electrons generated from the reduction of NADH by complex I, is shown in white.

In eukaryotes, complex III of the electron transport chain consists of an assembly of 11 proteins encoded by nuclear and mitochondrial genes The function of complex III is to accept electrons from ubiquinol, to transfer them to cytochrome C, and to translocate protons across the inner mitochondrial membrane (Iwata, Lee et al. 1998).

It has also been suggested that the electron transport chain; and complex III in particular, acts as an oxygen sensor in the mitochondria by releasing ROS in response to hypoxia. The increase in ROS production is paradoxical since the concentration of oxygen, a substrate for ROS production is decreased under hypoxia.

Oxygen sensing via complex III and the triggering of apoptosis via ROS release is important near the end of life, since tumour growth requires angiogenesis which is triggered by the onset of hypoxia within the tumour (Chandel, McClintock et al. 2000; Chandel and Schumacker 2000; Srinivas, Leshchinsky et al. 2001; Chandel 2002; Bell, Emerling et al. 2005; Brunelle, Bell et al. 2005; Guzy, Hoyos et al. 2005; Guzy and Schumacker 2006).

1.13 Tricyclic agents with known Blood-Brain Barrier penetration as candidates for targeting mitochondria

There are a number of antidepressant and antipsychotic agents that are known to cross the B-BB, so the screening of such agents to assess their potential as anti-tumour agents in glioma would seem to merit attention.

1.13.1 Antidepressants have an affinity for the serotonin and norepinephrine transporters

Clomipramine is a tertiary amine antidepressant that has been shown to selectively kill tumour cells *in vitro*. Traditionally, it is prescribed for depression with agitation, obsessive compulsive disorder, panic attacks, narcolepsy and chronic pain.

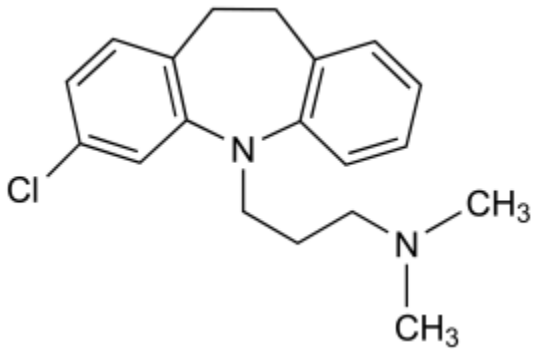


Figure 1.13.1.1 Chemical structure of Clomipramine Hydrochloride

Clomipramine is the 3-chloro derivative of Imipramine. Clomipramine is a strong, but not completely selective serotonin reuptake inhibitor, as the primary active metabolite Norclomipramine acts preferably as a norepinephrine reuptake inhibitor (Stahl 2000).

Amitriptyline (sold as Triptafen®) is a TCA drug. It inhibits serotonin and noradrenaline reuptake almost equally. Amitriptyline is approved for the treatment of endogenous depression and involutional melancholia. Adult typical dosages are 75 to 200 mg daily, with half this initially for elderly or adolescents. It may also be used to treat nocturnal enuresis (bed wetting).

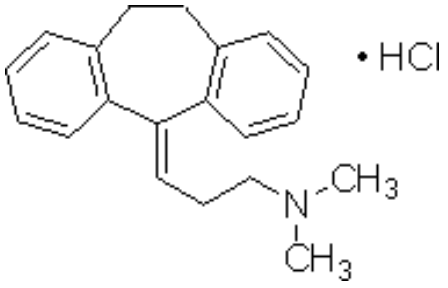


Figure 1.13.1.2 Chemical structure of Amitriptyline Hydrochloride

Nortriptyline is a second generation tricyclic antidepressant marketed as the hydrochloride under the trade name Allegron®. It is used in the treatment of depression and childhood nocturnal enuresis. In addition it is sometimes used for chronic pain modification, migraines, and labile affect in some neurological conditions.

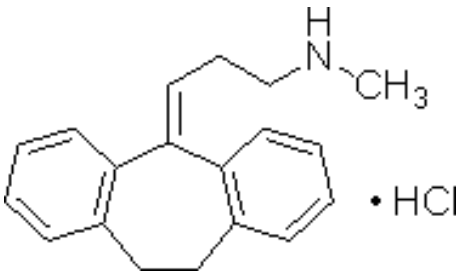


Figure 1.13.1.3 Chemical structure of Nortriptyline Hydrochloride

Doxepin is a psychotropic agent with tricyclic antidepressant and anxiolytic properties, known as brand-name Sinequan® (Pfizer Inc.). Doxepin inhibits the reuptake of serotonin and noradrenaline from the synaptic cleft (dual action).

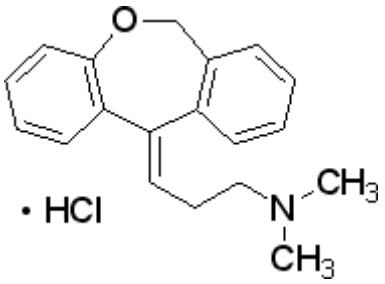


Figure 1.13.1.4 Chemical structure of Doxepin Hydrochloride

Imipramine (sold as Tofranil®) is an antidepressant medication, a TCA of the dibenzazepine group, mainly used in the treatment of clinical depression and enuresis. Imipramine, a tertiary amine, inhibits the reuptake of serotonin more so than most secondary amine tricyclics, meaning that it blocks the reuptake of neurotransmitters serotonin and noradrenaline almost equally. Imipramine is converted to Desipramine upon metabolism.

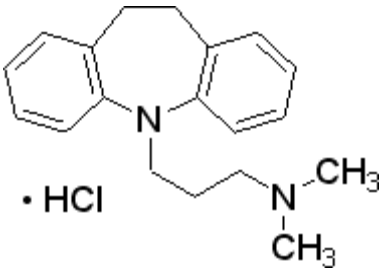


Figure 1.13.1.5 Chemical structure of Imipramine Hydrochloride

1.13.2 Other tricyclic compounds in clinical use that may show cytotoxic activity against malignant glioma

Promethazine is a first-generation H1 receptor antagonist antihistamine and antiemetic medication. It is available over the counter in the United Kingdom, Switzerland, and many other countries (Phenergan®). Promethazine also has strong anticholinergic and sedative effects. Previously it was used as an antipsychotic, although it is generally not administered for this purpose now; Promethazine has only approximately 1/10 of the antipsychotic strength of chlorpromazine.

Chlorpromazine was the first antipsychotic drug, used during the 1950s and 1960s. Used as chlorpromazine hydrochloride and sold under the trade name Largactil®, it has sedative, hypotensive and antiemetic properties as well as anticholinergic and antidopaminergic effects. It also has anxiolytic (alleviation of anxiety) properties. Today, chlorpromazine is considered a typical antipsychotic.

1.14 Clomipramine hydrochloride: a tricyclic antidepressant with the potential to selectively trigger apoptosis in tumour cells

Previous research into Clomipramine hydrochloride, a tricyclic antidepressant (TCA) which has been used to competitively inhibit the serotonin and norepinephrine transporters for over forty years, has shown that it has the ability to induce apoptosis in malignant glioma and Neuroblastoma cells *in vitro* (Daley, Wilkie et al. 2005; Levkovitz, Gil-Ad et al. 2005; Parker and Pilkington 2006). Clomipramine is demethylated by the liver to its active metabolite Norclomipramine (Desmethylclomipramine). In the 1970s, it was found that TCAs showed selective inhibition of mitochondrial activity in yeast cells (Wilkie 1979). It was surmised that the wide ranging actions of the TCAs *in vivo* was due to interactions with membranes and membrane-bound enzymes, in particular the mitochondrial membrane (Linstead 1971).

These lipophilic compounds, which were first shown to block oxygen consumption in yeast and human fibroblasts (Linstead 1971; Wilkie 1979), exert their effect via the inhibition of complex III of the mitochondrial respiratory chain (Daley 2001; Daley, Wilkie et al. 2005). By blocking the mitochondrial electron transport chain, the tricyclics are thought to initiate an increase in superoxide production and hence hydrogen peroxide production, decreased membrane potential and possibly mitochondrial permeability transition pore (MPTP) formation (Pilkington, Parker et al. 2008). As a consequence, cytochrome C is released and activation of pro caspase 9, caspase 3 and endonuclease G results in DNA degradation and apoptotic death.

It has been demonstrated that the tricyclics, in particular Clomipramine, initiate the intrinsic pathway of caspase-3-dependent apoptosis (Daley 2001; Daley, Wilkie et al. 2005). Here, Clomipramine initiates stress-induced apoptosis through its effect on phosphorylated proto-oncoprotein c-jun (p-c-jun, part of the JNK-AP-1 signal transduction pathway) and causes it to translocates to the nucleus (Behrens, Sibilia et al. 1999; Levkovitz, Gil-Ad et al. 2005).The intrinsic pathway of cytochrome C is triggered which activates caspase 9 and converges on executioner caspase 3 leading to apoptotic cell death (Daley 2001).

The differential effect seen, whereby Clomipramine initiates apoptosis in neoplastic glial cells whilst leaving normal glial cells and neurones unaffected, can be attributed to the capability of normal cells to recover (Wilkie 1979). Other mechanisms responsible for the selective killing of tumour cells by clomipramine might be the higher number of mitochondria in tumour cells, so uptake of Clomipramine would be greater, or the altered pH of tumour cells.

Clomipramine has also been observed to have potential for use in B-cell lymphomas. When tested in seventeen derived B cell lines, it was more effective than the selective serotonin reuptake inhibitor (SSRI) Fluoxetine in all but one case. Exposure of cells to Clomipramine resulted in rapid and concentration dependent increases in non-viable cells and caspase activity. In eight of the cell lines tested >50% antiproliferative response was seen at a concentration of 20µM (Meredith, Holder et al. 2005). Normal B cells, derived from tonsil tissue, assayed in parallel were unaffected by Clomipramine. Thus Clomipramine offers anti-tumour potential in both CNS tumours and B-cell lymphoma.

Recently Clomipramine has been studied in rat C6 glioma cells, in combination with Gleevec (see 1.8.2). Through Annexin-V FITC flow cytometry and chemosensitivity testing in both monolayer and spheroid cultures it was possible to show synergism between the two agents and the mechanism of action was elucidated to be through inhibition of DNA synthesis and inhibition of cyclic adenosine monophosphate (cAMP) (Bilir, Erguven et al. 2008).

Many *in vitro* studies show that antidepressants have potent anticancer properties, with all agents in this class differing in potency according to mechanism of action and cell type in which they were studied (Volpe, Ellison et al. 2003; Levkovitz, Gil-Ad et al. 2005; Arimochi and Morita 2006). Irrespective of their mechanism of action, the antidepressants have shown the ability to kill neoplastic cells (Levkovitz, Gil-Ad et al. 2005; Arimochi and Morita 2006), halt proliferation (Volpe, Ellison et al. 2003) and drive previously chemotherapy-resistant cells to be sensitive (Peer and Margalit 2006).

1.15 Drawbacks when selecting tricyclic antidepressants for use in patients with malignant glioma

There are many established and new documented problems associated with administration of tricyclic antidepressants. These compounds lack specificity and therefore exert many unpleasant side-effects. They also have many interactions; a particular problem seen in patients with brain tumours (who are likely to suffer seizures) is the interaction with anticonvulsants such as Carbamazepine. A high proportion of brain tumours present in elderly patients (Pilkington 2001) and therefore contraindications such as congestive heart failure, myocardial infarction and hypotension are more prevalent.

Observations in tricyclic antidepressants	
Contraindications	MAOIs, acute intoxication, anxiety, epilepsy, liver disease, kidney disease, hypotension, cardiovascular dysfunction, pre-existing bone marrow depression, hyperthyroidism and paediatric patients under the age of 18
Side effects	Drowsiness, anxiety, emotional blunting (apathy/anhedonia), confusion, restlessness, dizziness, akathisia, hypersensitivity, changes in appetite and weight, sweating, sexual dysfunction, muscle twitches, weakness, nausea and vomiting, hypotension, tachycardia, and rarely, irregular heart rhythms. Twitching, hallucinations, delirium and coma are also some of the toxic effects caused by overdose
Interactions	• Anticholinergics: TCAs increase the anticholinergic effects • Antihypertensives: TCAs antagonise clonidine and guanoethidine • Carbemazepine: TCA levels are decreased due to induction of hepatic enzymes by Carbamazepine • Cimetidine: TCA levels are increased due to inhibition of hepatic enzymes by Cimetidine • Lithium: increases risk of neurotoxicity (tremor, ataxia, seizures) • MAOIs: additive serotenergic effects • Sympathomimetics: enhances response to norepinephrine and potentiates arrhythmia • SSRIs: TCA levels increased due to inhibition of microsomal enzymes by SSRIs • Thyroid medication: increases risk of arrhythmia if given in combination with levothyroxine
Specificity	Not very specific (hence large number of side effects), inhibition of: • Na channels • Ca channels • 5HT-2, 2A, 2C, 6, 7 • D1, D2 & D3 • Alpha-1 & alpha-2 adrenergic receptors • NMDA receptors

Table 1.15.1 Established and new problems when choosing an antidepressant for use in malignant glioma

There are a number of contraindications, side effects and interactions listed for the tricyclic antidepressants; however close-monitoring of patients on these drugs can alleviate any long-term problems.

1.16 The norepinephrine transporter (NET): the link between tricyclic antidepressants and apoptosis in glioma?

The NET was the first of the monoamine transporters to be cloned (Pacholczyk, Blakely et al. 1991) and since then is has been discovered that such sodium and chloride ion-dependent transporters use the electrochemical gradient of sodium between the inner and outer leaflets of the cell membrane to pump neurotransmitters from low concentrations outside the cell to much higher concentrations inside the cell. From previous studies it is clear that the uptake and subsequent metabolism of neuronally-released NE by glial cells is crucial in maintaining and regulating extracellular NE concentrations (Inazu, Takeda et al. 2003). They may exert significant control of noradrenergic activity by inactivating NE that escapes neuronal re-uptake in sites distant from terminals, and thus are cellular targets for the antidepressant drugs that inhibit NE uptake.

The neuronal NET (also known as the noradrenaline transporter; NAT) is a member of a family of twelve trans-membrane sodium- and chloride-ion dependent neurotransmitter transporters that aid in the termination of noradrenergic neurotransmission by the reuptake of NE into the pre-synaptic terminals from which it was released (Bonisch, Boer et al. 1990; Blakely, De Felice et al. 1994; Bonisch and Bruss 1994). NETs are molecular targets for several psychoactive agents, including cocaine, amphetamines and the tricyclic antidepressants (which act as NE reuptake inhibitors) (Axelrod, Whitby et al. 1961; Iversen 1965; Ritz, Lamb et al. 1987).

The NET transporter is also expressed in non-neuronal tissues where it is responsible for the removal of circulating catecholamines; these sites include the lungs (Bryan-Lluka, Westwood et al. 1992; Tseng and Padbury 1998), placenta (Ramamoorthy, Prasad et al. 1993; Bzoskie, Yen et al. 1997; Padbury, Tseng et al. 1997), adrenal medulla (Bonisch, Martiny-Baron et al. 1990; Bonisch, Paulus et al. 1991; Cubells, Kim et al. 1995) and PC-12 cells (Bonisch, Boer et al. 1990; Bonisch, Paulus et al. 1991; Bruss, Porzgen et al. 1997).

It has been shown that normal brain astrocytes contain transport systems capable of removing various neurotransmitters from the synaptic cleft by transporters present in the plasma membrane. Because of their location around synapses and at the periphery of capillaries their uptake systems are important for regulating the neuronal environment. Reports on the uptake of [^{3}H]NE by astrocytes involving an active mechanism suggested a functional uptake, RT-PCR demonstrated that astrocytes expressed mRNA for the cloned NET protein and Western blots indicated that anti-NET polyclonal antibody recognised a major band of 80kDa in rat astrocytes which all suggest that neuronal NET is functionally expressed in cultured rat astrocytes (Inazu, Takeda et al. 2003).

1.17 Apoptosis

In theory, apoptosis can be divided into two main pathways, both being defined by the biochemical action of proteases called caspases, which lead to eventual cell death. The 'extrinsic' pathway is driven by the activation of extracellular death receptors located on the cell surface, such as Fas (CD95; Apo1), TNFR1 (p55; CD120a), DR3 (Apo3, WSL-1, TRAMP, LARD), DR4, DR5 (Apo2, TRAIL-R2, TRICK2, KILLER) and CAR1, involving the recruitment and activation of initiator caspase-8 and –10. The apoptotic signal is initiated by notable ligands (FasL, TRAIL and TWEAK) recognised specifically, and in most cases exclusively, by their corresponding death receptors (Fas, DR4 or 5 and TweakR respectively) (Wiley, Cassiano et al. 2001).

Conversely the 'intrinsic' pathway, which occurs at the mitochondrial membrane, is initiated by a disruption in the mitochondrial membrane potential, followed by the release of cytochrome C into the cytosol which, in turn, contributes to the formation of a protein complex, namely an apoptosome.

This multimeric complex, formed from the interactions of cytochrome C, protease-activating factor-1 (Apaf-1), pro-caspase-9 and dATP eventually allows the activation of caspase-9 (Zou, Henzel et al. 1997; Li, Zhu et al. 1998; Polster and Fiskum 2004).

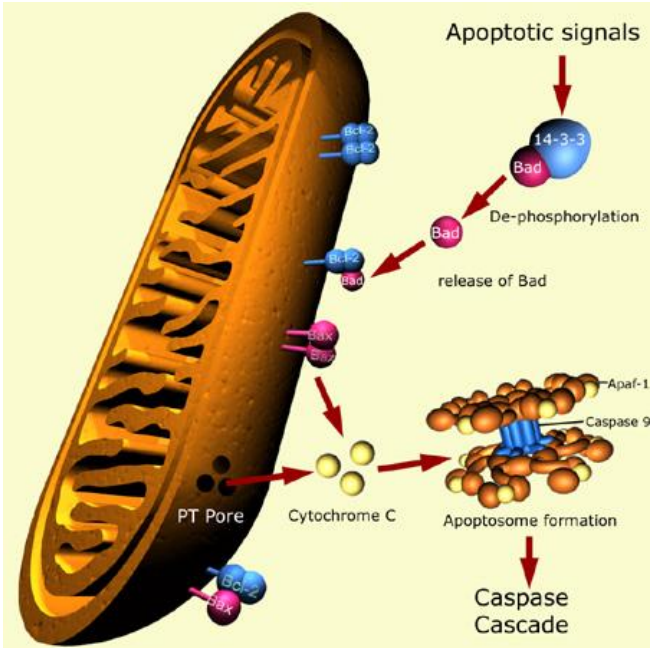


Figure 1.13.2.1 The multimeric apoptosome complex(Dash 2009)

The pro-apoptotic (indicated in red) and anti-apoptotic (indicated in blue) proteins involved in the regulation of apoptosis. Formation of the PT pore and subsequent release of cytochrome C initiates formation of the apoptosome and triggers caspase activation.

The common downstream pathway for both intrinsic and extrinsic activation involves effector caspases, adaptor molecules, such as Apaf-1 and regulatory molecules such as Bcl-2 proteins and inhibitors of apoptosis (IAPs) (Eldadah and Faden 2000). Both the intrinsic and extrinsic pathways converge downstream on 'executioner' caspases, mainly caspase-3, which is responsible for the cleavage of structural cytoplasmic and nuclear proteins, with consequent cell death and collapse. Both the extrinsic and intrinsic pathways are calcium-dependent and the stimulation of calcium-dependent proteases can result in a disruption of cytoskeletal organisation and the formation of 'blebs' (surface protrusions).

Central to apoptotic death is the interchange of cycling calcium ions between the endoplasmic reticulum and the mitochondria controlled by co-ordinated activity of ion channels (Taylor, Lawrence et al. 1993). Calcium-mediated phospholipase activation can result in the impairment of mitochondrial function with the collapse of membrane potential and cessation of ATP synthesis. The activation of a calcium and magnesium dependent nuclear endonuclease (DNA fragmentation factor 40) is associated with chromatin cleavage and appears to play a crucial role in apoptosis (Orrenius, McCabe et al. 1992).

During apoptosis the nucleus breaks up into DNA fragments of 180-200kD and the endoplasmic reticulum transforms into vesicles, which are released as apoptotic bodies into the extracellular space. The apoptotic bodies, containing cell organelles and nuclear fragments, are phagocytosed by surrounding microglia and astrocytes (Haanen and Vermes 1996).

1.18 The Mitochondrial Permeability Transition Pore in apoptosis

From a molecular perspective, the MPTP consists of the voltage-dependent-anion-channel (VDAC; also known as mitochondrial porin) in the OMM and the adenine-nucleotide-translocator (ANT) in the IMM (Grimm and Brdiczka 2007). Permeability transition is a sudden and sustained increase of the permeability of the IMM for solutes less than 1.5kD. The result is the breakdown of the mitochondrial membrane and cessation of ATP synthesis. The blockade of the respiratory chain results in the formation of ROS.

The second effect is an osmotic one, with the high concentration of solutes in the mitochondrial matrix drawing in water molecules from the cytosol (Grimm and Brdiczka 2007). Subsequent to this factors are released (cytochrome C and Smac) that execute the suicide programme of apoptosis. The proteins associated with permeability transition are the link between the proteins that regulate apoptosis and the major effectors of apoptosis, the caspases. Therefore they represent an excellent therapeutic target.

1.19 Mitochondrial Outer Membrane Permeabilisation in apoptosis

The activation of caspases in the mitochondrial pathway require mitochondrial outer membrane permeabilisation (MOMP), an event that is considered to be the 'point' of no return' during apoptosis as numerous proteins diffuse from the matrix between the OMM and IMM to the cytosol. In normal cells, which undergo apoptosis, MOMP occurs through the formation of a channel through which large protein molecules (such as cytochrome C and Direct Inhibitor of Apoptosis-Binding protein with Low pI; DIABLO) can escape into the cytosol. The channel is thought to be comprised of Bax or Bad (because they lack the BH-4 homology domain) in combination with Bid (which is activated by caspase-8 cleavage to its active form, tBid) (Antonsson, Conti et al. 1997; Schendel, Xie et al. 1997).

1.20 Extrinsic and intrinsic pathways of apoptosis

The two pathways of apoptosis (extrinsic pathway, triggered when an extracellular ligand such as TNF-α, Fas and TRAIL binds to a death receptor such as CD95/Fas/Apo1, TNFR1, TNFR2 and DR3-6; intrinsic pathway, triggered by any stimuli that signal cellular damage, such as ionising radiation, cytotoxic agents, hypoxia, DNA damage and growth factor withdrawal) (Degterev, Boyce et al. 2003) are irreversible once triggered, so it is important that the apoptotic pathway exists in all human cells as an endogenous, heavily regulated mechanism by which cells essentially self-destruct (Degterev, Boyce et al. 2003). The ability to undergo programmed cell death has a role in several normal physiological processes such as embryogenesis and immunity. Whereas the expression of anti-apoptotic proteins is an important physiological safeguard to prevent cellular destruction, these proteins play a pathologic role in many malignancies, in glioma in particular. High levels of anti-apoptotic proteins have been shown to both result in resistance to chemo- and radio-therapy and to cancer progression (Schmitt 2003).

1.21 The role of inhibitor of apoptosis-inducing proteins in apoptosis

The IAPs represent the last cellular safeguard against apoptosis and block both the extrinsic and intrinsic pathways by inhibiting caspase activity (Degterev, Boyce et al. 2003). There are eight members of the IAP family of proteins that have been identified and their principle role is to bind to the caspases. XIAP, c-IAP1, c-IAP2 and Bruce (Apollon) have been shown to be highly expressed in a number of malignant glioma cell lines (Chen, Naito et al. 1999; Wagenknecht, Glaser et al. 1999). During MOMP the IAPs are activated by the release of second mitochondria-derived activator of caspase (Smac)/DIABLO and Omi from the mitochondria, which work to prevent the binding of IAPs to the caspases and thus allow the cascade to proceed (Kim, Emi et al. 2005).

1.22 The role of caspases in apoptosis

Caspases, cysteine proteases, are present in all cells as latent enzymes. They are recruited to receptor-associated complexes whose formation is initiated via oligomerisation (for example TNF receptors, FAS and Apaf-1). Activation via dimerisation or dimerisation accompanied by proteolytic cleavage generates a cascade that cleaves proteins critical for cell survival. Caspases -8, -9 and -10 (and sometimes -2 and -5) are termed initiators and are located upstream of the effectors, namely caspases -3, -6 and -7.

Research in apoptosis has established a central role for the caspase enzymes. The determination of the structures of caspase-1, caspase-3 and caspase-8 has greatly enhanced the knowledge surrounding the function and specificity of the whole caspase family. This provides a starting point for elucidating the role of the caspase enzymes and the design of selective inhibitors to treat caspase-mediated diseases (Grutter 2000).

1.23 The role of Bcl-2 Family in apoptosis

Bcl-2 is the prototype for the family containing pro-apoptotic (Bax, Bak and Box) and anti-apoptotic (Bcl-2, Bcl-xL and Bcl-w) proteins that govern mitochondrial membrane permeabilisation and determine commitment to apoptosis (Zamzami, Brenner et al. 1998). The Bcl-2 family exert their effects on apoptosis via surveillance of mitochondrial membrane potential and govern cytochrome C release into the cytosol by binding to a mitochondrial porin channel(also known as voltage dependent anion channel; VDAC), which once there activates caspase-9 and caspase-3.

Zamzami et al (1998) also suggest that the release of cytochrome C is mediated by the effects of the mitochondrial permeability transition (PT) pore located on the inner leaflet of the mitochondrial membrane. This work is further supported by the theory that the Bcl-2 gene family activate or deactivate the PT pore through regulation of mitochondrial ATP and calcium flux (Zamzami, Brenner et al. 1998; Weeber, Levy et al. 2002).

The Bcl-2 homology domains are known to be crucial, as deletion affects apoptosis and survival rates. The structure of the Bcl-2 family is a hydrophobic helix surrounded by amphipathic helices, some of the members also possess transmembrane domains (Oltersdorf, Elmore et al. 2005).

1.24 Interactions between extrinsic and intrinsic pathways

In some cells, notably hepatocytes, the extrinsic pathway can intersect the intrinsic pathway through caspase-8 cleavage mediated activation of the pro-apoptotic protein Bid (Yin, Wang et al. 1999; Kaufmann, Tai et al. 2007). The C-terminal truncated form of Bid (tBid) translocates to the mitochondria and promotes further caspase activation (caspase-9, -3, -6 and -7) through the intrinsic pathway. In these situations, loss of Bid or overexpression of Bcl-xL inhibits cell death (Yin, Wang et al. 1999).

Certain cell types (such as some neuronal populations) can survive the cytochrome C release step, at least for a limited amount of time (Martinou, Desagher et al. 1999; Potts, Vaughn et al. 2005). In such cells the activity of caspases is stringently regulated by caspase-inhibiting IAPs. In these cases apoptosis requires the release of DIABLO from the mitochondria to counteract the IAP inhibition and thereby allow caspase activation (Youle and Strasser 2008).

1.25 The regulation of apoptosis

Intact apoptosis pathways protect cells against transformation to a neoplastic state and provide the mechanisms by which cytotoxic agents exert their effects (Goldsmith and Hogarty 2005). Deregulated proliferation alone is not sufficient for tumour formation but when these pathways are disrupted through alterations in the cell death machinery, they act alongside deregulated oncogenes, such as Ras, to promote tumour initiation and resistance to therapy (King 2009).

Tumour cells possess a number of mechanisms for evading normal programmed cell death (PCD). The Bcl-2 family (see 1.23) are able to manipulate cell death via the MPTP, which is involved in the regulation of the matrix, calcium ions, pH and voltage within the mitochondria. Some members can induce (pro-apoptotic; Bax, Bak, Diva, Bcl-Xs, Bik, Bim, Bad, Bid and Egl-1) or inhibit (anti-apoptotic; Bcl-2, Bcl-XL, Mcl-1, CED-9, A1 and Bfl-1) cytochrome C release from the mitochondria, which then initiate caspase-9 and -3 activity. Neoplastic glial cells express a much higher proportion of anti-apoptotic proteins from the Bcl-2 family which protect from caspase activation. Progression of tumours and grade of malignancy are linked to the degree of expression of Bcl-2 family proteins (Deininger, Weller et al. 1999). Other mechanisms possessed by tumour cells via which they can inhibit apoptosis are the expression of inhibitor of apoptosis proteins (IAPs) (LaCasse, Baird et al. 1998). The IAP Survivin is highly tumour-specific and in Neuroblastoma tumours interacts with Smac/DIABLO by binding to caspase-9 which inhibits apoptosis (Igney and Krammer 2002).

1.26 Detection of apoptosis

The Annexin-V assay is able to detect the degree of apoptosis occurring in a cell population and is based upon the presence of phosphatidylserine (PS), made by the two PS synthases PSS1 and PSS2, which is normally located on the inner leaflet of the plasma membrane, but undergoes transbilayer movement during apoptosis and becomes exposed on the cell surface (Grandmaison, Nanowski et al. 2004).

Annexin-V FITC binds to the exposed PS residues in a calcium dependent manner, after a rise in nuclear calcium concentration that causes the translocation of the calcium regulated proteins to the nuclear envelope (Raynal, Kuijpers et al. 1996). This mechanism, usually a pivotal step in the recognition and removal of apoptotic cells by phagocytes (Zwaal, Comfurius et al. 2005), allows the attachment of the Annexin V-FITC probe and allows the visualisation of apoptotic cells via flow cytometry. Annexin V-FITC has an affinity for PS and attaches in a calcium-dependent manner.

1.27 Caspase-independent programmed cell death

As with caspase-dependent cell death, caspase-independent cell death relies on MOMP taking place. The process may utilise less tractable mechanisms, such as loss of mitochondrial function or release of inter-membrane space proteins to catalyse death but it still results in reduced energy production and cell death (Chipuk, Bouchier-Hayes et al. 2006).

The identification of five isoforms of apoptosis inducing factor (AIF) has allowed the characterisation of the nuclear functions of this protein. The mitochondrial release, nuclear translocation and DNA fragmentation associated with AIF have been extensively demonstrated in several systems and cell lines to date (~ 900 Medline references to date, 2005). Expressed as a precursor of 67kDa, AIF is placed within the N-terminal prodomain of the protein (Susin, Lorenzo et al. 1999) and once inside the mitochondrion is processed to a mature form of 62kDa by proteolytic cleavage (Otera, Ohsakaya et al. 2005).

AIF is required for the maintenance and maturation of the electron transport chain, specifically complex I (Vahsen, Cande et al. 2004). After a cell death insult, AIF translocates from the mitochondria to the cytosol and nucleus (Susin, Lorenzo et al. 1999; Mate, Ortiz-Lombardia et al. 2002; Ye, Cande et al. 2002). Once in the nucleus, AIF interacts with DNA and/or RNA to cause caspase-independent chromatin condensation and large scale DNA fragmentation (Susin, Lorenzo et al. 1999; Ye, Cande et al. 2002).

Apoptosis inducing factor does not display intrinsic endonuclease properties, its DNA-degrading activity appears to depend on downstream nuclease Endo G, a DNase of mitochondrial origin released in a caspase-independent manner in pro-apoptotic conditions (Wang, Yang et al. 2002). Other proteins, such as heat-shock protein 70 (Hsp70), regulate the pro-apoptotic function of AIF (Ravagnan, Gurbuxani et al. 2001; Gurbuxani, Schmitt et al. 2003; Ruchalski, Mao et al. 2006). In fact, Hsp70 is the main inhibitor of caspase-dependent and caspase-independent PCD (Beere, Wolf et al. 2000; Ravagnan, Gurbuxani et al. 2001; Daugaard, Rohde et al. 2007).

The expression of AIF is inhibited by Bcl-2 19KDa interacting protein 3 (BNIP3) whereby it associates with PTB-associating splicing factor (PSF) and HDAC1 (histone deacetylase 1) contributing to transcriptional repression of the AIF gene. The reduction in AIF expression results in decreased apoptosis in glioma cells, and therefore increased resistance to cell death (Burton, Eisenstat et al. 2009).

One of the pivotal events in programmed cell death is the release of proteins AIF, cytochrome C, Endo G, Omi/HtrA2 and Smac/DIABLO, yet how they exit the mitochondria remains unresolved (Hengartner 2000; Zamzami and Kroemer 2001; Kuwana, Mackey et al. 2002; Henry-Mowatt, Dive et al. 2004; Uren, Dewson et al. 2005; Gogvadze, Orrenius et al. 2006; Munoz-Pinedo, Guio-Carrion et al. 2006). It is possible that the proapoptotic proteins Bax and Bak participate in this process (Letai, Bassik et al. 2002), through allosteric changes brought about in response to death signals (Gross, McDonnell et al. 1999).

It is also known that AIF requires proteolytic cleavage to yield a 57kDa form that can be released from the inner mitochondrial membrane (Otera, Ohsakaya et al. 2005; Uren, Dewson et al. 2005), this is achieved though the activation of calpains and cathepsins (Polster, Basanez et al. 2005; Yuste, Moubarak et al. 2005). Calpains (non-lysosomal) and cathepsins (lysosomal) are cysteine proteases that control AIF cleavage in a calcium-dependent (Polster, Basanez et al. 2005; Takano, Tomioka et al. 2005; Yuste, Moubarak et al. 2005; Cao, Xing et al. 2007) and calcium-independent manner (Yuste, Moubarak et al. 2005; Yacoub, Park et al. 2006), respectively.

1.28 The regulation of apoptosis by tumour suppressor p53

p53 is one of the most frequently mutated of tumour suppressors in human tumours and has been intensely studied for years(Lane and Crawford 1979; Oren and Levine 1983; Chumakov 1987; Kinzler and Vogelstein 1989; Lane 1992). It is a major orchestrator of the cellular response to stress through its regulation of apoptosis (Fridman and Lowe 2003), cell cycle arrest (Wagner, Kokontis et al. 1994), senescence (Wynford-Thomas 1996), DNA repair (Marx 1994) and genetic stability (Achanta, Sasaki et al. 2005). For many years it was thought that p53 relied upon its function as a transcription factor to regulate these stress types (Levine 2001), however in the last eight years it has been revealed that it directly participates in the intrinsic apoptosis pathway by interacting with members of the Bcl-2 family to induce MOMP (Erster and Moll 2004; Vaseva and Moll 2009).

The first studies to reveal a transcription-independent function of p53 on apoptosis showed that p53 regulates caspase activity and upon receiving a stress signal, translocates from the cytoplasm to the mitochondria where it interacts with members of the Bcl-2 family to inhibit or activate them. MOMP then takes place which in turns triggers the caspases and chromatin degradation (Ding, McGill et al. 1998; Gottlieb and Oren 1998).

1.29 Molecular alterations in brain tumours

Brain tumours arise from the accumulation of several genetic and epigenetic events over a long time period. Identifying the molecular alterations that distinguish cancer cells from normal cells will ultimately help to define the nature and pathology of tumours. Two different approaches of biomarker discovery have been employed: the first approach looks at markers from specific pathways one-by-one and the second approach is that of a high-throughput screening method, however molecular alterations add complexities to either approach.

1.29.1 Single nucleotide polymorphisms and their effect on response to therapy

A SNP is a DNA sequence variation occurring when a single nucleotide (A, T, C or G) in the shared sequence differs between paired chromosomes in an individual. The two alternate forms are termed ‘alleles’. Since most DNA does not encode proteins the majority of SNPs cause only ‘silent’ variation which has no visual phenotypic effect. However some SNPs on an allele can have an effect, in this case drug response and distribution. Single nucleotide polymorphisms are highly abundant, and are estimated to occur at one out of every one thousand bases in the human genome (Sachidanandam, Weissman et al. 2001). In addition to diagnostic applications SNPs are useful markers in population genetics.

1.29.2 Splice variants and their effect on response to therapy

Pre-mRNA splicing is an essential, precisely regulated process that occurs following gene transcription and before mRNA translation. Spliceosomes recognise the boundary between exonic and intronic sequences in pre-mRNA and catalyse the cut-and-paste reaction that removes introns and joins exons. Once exon recognition is completed, the connected exons are exported out of the nucleus as a mature mRNA that can be translated into protein. Alternative pre-mRNA splicing is responsible for the production of multiple mRNAs from a single gene, subsequently multiple protein isoforms, and this process is essential for the generation of protein diversity.

Up to 60% of all human genes present at least one alternative splice variant, and alternative splicing could potentially generate more transcripts from a single gene than the total number of genes in an entire genome (Black 2003). The physiological activity of splice variant products compared to the wild-type protein may be completely different. Whole functional domains may be added or deleted from the protein coding sequence. The apoptosis genes Bcl-(x)L, Caspase-9, and Caspase-2 encode both pro- and antiapoptotic variants (Syken, De-Medina et al. 1999; Wu, Tang et al. 2003). It is also possible that splice events transform membrane-bound proteins into soluble proteins, as is the case for the fibroblast growth factor receptors (Jang 2002). For the vast majority of alternative splice events, their functional significance is still unclear. However identifying the presence of new splice variants in diseases such as cancer may lead to a clearer understanding the differences observed in response to therapy.

1.30 Rationale for targeting the mitochondria with tricyclic antidepressants

Traditional chemotherapy approaches currently offer little long-term benefit for most malignant glioma and is frequently associated with adverse side-effects that diminish the quality of life. Hence, new approaches are needed that can provide long term management for malignant brain tumours, whilst permitting a decent quality of life (Fisher and Buffler 2005).

Targeting DNA directly is a difficult area because tumour cells possess so many DNA-repair mechanisms and no two tumours have the same method of circumventing cell death, this is evident in the poor results seen in clinical trials for DNA-acting agents such as Temozolomide and Gliadel. An alternative approach is to indirectly cause cellular degradation by disrupting the normal mitochondrial balance of Bcl-2 proteins and caspases and evidence in support of this hypothesis will be presented in this study.

Despite the vast array of research carried out in the field of neuro-oncology the incidence of brain tumours continues to rise and the prognosis remains dismal. It is well known that heterogeneity of both tumours within patient cohorts and the cells within a single tumour, the presence of the blood-brain barrier and the invasive phenotype of tumours are the main contributing factors that preclude successful treatment. The application of a 'one fits all' approach to the treatment of brain tumours clearly does not work, with only subsets of patients responding in clinical trial. To remove the problem of B-BB penetration from the equation it is necessary to employ agents that are known to have good B-BB penetration (see table Table 1.7.1). In this context the use of archival agents with the propensity to cross the B-BB may be of significance. An added, possible advantage is that this approach is far less costly than the continual development of expensive new agents that fall at the first hurdle.

Moreover, B-BB disruption in the disease state cannot be relied upon for entry of drugs into the non-neoplastic CNS which harbours invasive populations of unseen tumour cells. Thus, the screening of tricyclic agents is deemed a viable study. The tricyclic agents are known to access to the CNS, and are also capable of targeting aberrant ‘guerrilla’ tumour cells that have invaded the normal brain parenchyma and thus survive chemotherapeutic assault.

1.31 Hypotheses, aims and objectives of this study

Hypotheses

1. that tricyclic antidepressants are able to induce cell death effectively in glioma

2. that P450 genotype influences chemosensitivity in malignant glioma

3. that the norepinephrine transporter expression in glioma influences sensitivity to tricyclic antidepressants

4. that mitochondria are a potential target for therapy in malignant glioma

Aims

1. To compare drug efficacy and potency of different tricyclic antidepressants and demonstrate the differences in sensitivity between glioma cell lines and early passage cultures

2. To analyse paired plasma and whole blood samples derived from glioma patients taking Clomipramine and demonstrate that mutations in the CYP450 enzymes responsible for metabolising Clomipramine affect response to tricyclic antidepressant therapy

3. To demonstrate the presence of the human norepinephrine transporter in malignant glioma

4. To examine the expression of a number of pro- and anti-apoptotic genes in malignant glioma

Objectives

- To investigate the efficacy and potency of eight compounds with tricyclic structure on established cell lines SNB-19 and DK-MG, early passage cultures UPAB, UPMC and UPJM and normal human astrocytes CC-2565 using the MTT, ATP-TCA and Annexin V assays

- To obtain clinically relevant Clomipramine concentration values using repeat plasma samples from seventeen glioma patients via HPLC-MS/MS and, using paired blood samples, prepare extracts of genomic DNA to analyse for mutations in the CYP450 enzymes (2D6 and 2C19) responsible for metabolising Clomipramine using a multiplex real-time PCR assay

- To demonstrate the presence of the norepinephrine transporter on normal human astrocytes, Glioblastoma multiforme, Astrocytoma, Oligodendroglioma, Meningioma and Schwannoma tumours using immunohistochemical, immunocytochemical, Western blotting and real-time PCR techniques

- To screen cDNA synthesised using mRNA extracted from a retrospective panel of formalin-fixed paraffin-embedded glioma samples for the presence of pro- and anti-apoptotic markers and examine patterns of expression using micro fluidic Taqman low density array cards pre-loaded with primers

2 General materials & methods

2.1 Materials

Reagents	Company	Code
Adenosine Triphosphate	Sigma Aldrich	FLAAS-1VL
Amitriptyline Hydrochloride	Sigma Aldrich	A-8404
Annexin V-FITC Kit	BD Biosciences	556419
Chlorpromazine	Sigma Aldrich	C-8138
Clomipramine Hydrochloride	Sigma Aldrich	C-7291
DAB Liquid Chromogen	Biogenex	HK130-5K
Dilution buffer	DCS, Hamburg	In house
Dimethyl Sulphoxide	Sigma Aldrich	414885000
DMEM:F12 media	Invitrogen	12634-010
Doxepin Hydrochloride	Sigma Aldrich	D-4526
Dulbecco's Modified Eagle's Medium	Invitrogen	61965-026
Foetal Bovine Serum	Sigma Aldrich	F-7524
Glutamax	Invitrogen	VX35050038
Hanks Balanced Salt Solution with phenol red	Invitrogen	14170-070
Hanks Balanced Salt Solution without phenol red	Invitrogen	14175-046
Imipramine Hydrochloride	Sigma Aldrich	I-0899
Luciferase	Labtech International	LAB-LA-12
Luciferin	Labtech International	LAB-BT11-100
MTT Powder	Sigma Aldrich	M-5655
Mycoalert Kit™	Cambrex Biosciences	LT07-318
Norclomipramine Hydrochloride	Sigma Aldrich	N-1280
Norepinephrine Transporter Antibody	Chemicon	MAB5620
Nortriptyline Hydrochloride	Sigma Aldrich	N-7261
Nuclease-free water	Promega	P-1193
Penicillin/Streptomycin	Sigma-Aldrich	P-0187
Plasmocin™	Invivogen	Ant-mpt
Power Block™	Biogenex	BS-1310-25
Promethazine Hydrochloride	Pharmacy	-
Recovery Cell Culture Freezing Media	Invitrogen	12648-010
Sodium Hydroxide Pellets	Sigma Aldrich	S-5881
Staurosporine	Sigma Aldrich	S-6942
Supersensitive™ Link-label IHC detection system	Biogenex	QD-200-0XE
Taqman drug metabolism genotyping reaction mixture	Applied Biosystems	-
Taqman universal master mix (no UNG)	Applied Biosystems	4364341
Taqman universal master mix	Applied Biosystems	4364338
TCER	DCS, Hamburg	In house

TE-Buffer	Promega	V-6231
Trypan Blue	Sigma Aldrich	T-8154
Tryple™ with phenol red	Invitrogen	12605-028
Tryple™ without phenol red	Invitrogen	12604-043
Plasticware		
1.5ml sterile eppendorf tubes	VWR	306030806
14.0 ml Corning PP Centrifuge Tube	Fisher Scientific	FB55950
25 cm^2Corning tissue culture flasks	Fisher Scientific	TKT500010Q
7.0 ml Sterile PP Bijou	Fisher Scientific	FB51523
75cm^2Corning tissue culture flasks	Fisher Scientific	TKV123031L
FACS Tube	BD Biosciences	352003
Greiner barcoded cryovial 1.0 ml internal thread	Greiner	123279-250
Mr Freezy Cryopreservation vessel	Fisher Scientific	CRY120010T
PP 96-well u-bottom microplates	Marathon	353072
96-well PCR microplates	Abgene	AB-0900
0.2ml PCR Tubes	Sigma Aldrich	P-3114
Other		
Ethanol	Sigma Aldrich	459844
Barricidal™	Fisher Scientific	CMD040020G
DNA-Zap	Ambion	9890
Industrial Methylated Spirit	Sigma Aldrich	458600
Whatman FTA® Cards	Fisher Scientific	FDA367005P
Whatman FTA Cards	Fisher Scientific	FDU367010W
Whatman Micropunch	Fisher Scientific	FDU365040U
White 96-well plates	Thermo Electron	7905

2.2 Methods

2.2.1 Tissue Culture

All cell culture was performed in a laminar flow hood under sterile conditions. Barricidal™ was used to sterilise all surfaces that came into contact with biopsy material or cell cultures. The cells, both early passage cultures and established cell lines (see Table 2.3.1), were initiated in-house from brain tumour biopsy tissue (LRECs 00-173, 02-056 and 04-066) were cultured initially in Plasmocin™ for two weeks and tested using a Mycoalert Kit™ to ensure they were free of mycoplasma and bacterial or fungal infection. All cells were then cultured in Dulbecco's Modified Eagle's Medium (DMEM; with the addition of Glutamax) and 10% heat inactivated foetal bovine serum (FBS; necessary to establish reactive astrocytes in culture) without the addition of antibiotic/antimycotic. Antibiotics and antimycotics were omitted from all tissue culture reagents apart from transport media because brain tumours are 'sterile' tumours due to their CNS location (unlike, for example, a colorectal tumour) and as long as a high standard of aseptic technique is maintained contamination is not an issue. Since compounds such as these are unable to cross the B-BB (except in certain cases of meningitis) it ensures a more accurate *in vitro* environment. All tissue culture reagents were aliquoted into sterile duran bottles or sterile bijou bottles and labelled for use with one cell culture only to avoid cross-contamination. Cells were maintained and propagated in a humidified tissue culture incubator, designated specifically for decontaminated human brain tumour cells only, at 37^{o}C with 5% CO_2/95% air atmosphere.

2.2.2 Biopsy Tissue

Glioma cells were derived from primary cultures of human biopsy material taken at the time of surgery in the neuro-surgical theatres of Hurstwood Park Neurological Centre, Haywards Heath. All biopsied glioma tissues, from which the early passage cultures were derived, underwent histological confirmation by a neuropathologist and were classified according to standard WHO criteria. Samples were transported in transport media (DMEM with 1% antibiotic-antimycotic) in Royal Mail 'safeboxes' immediately following surgery; processing took place within forty-eight hours of surgical resection. The surgical biopsies were manually disaggregated with two scalpels under sterile conditions to form suspensions, from which primary monolayer cultures were established. Cells were routinely propagated in DMEM with the addition of FBS. Tissue culture medium was maintained at physiological pH by equilibration with 95% air/5% CO_2.

2.2.3 Biopsy tissue that has previously been exposed to radiotherapy and chemotherapy

Frequently glioma biopsies are derived from patients that have already received radio- and/or chemo-therapy which causes them to be resistant to culture *in vitro*. The cores of such biopsies are largely necrotic and selecting tissue for disaggregation is difficult. However, the surgeon was able to dissect areas of 'infiltrative edge' which results in a higher chance of success. Biopsies that had previously been exposed to therapy were disaggregated as described in 2.2.2 and incubated at 37^oC/5% CO_2 for 5 days.

Following the initial settling period, 1.0 ml of the culture medium in which the cells were growing was removed every 24 hours and replaced with 1.0 ml fresh culture medium, thereby avoiding removal of essential growth factors and cellular messengers. Where possible, cyst fluid, taken alongside the biopsy, was added to the culture medium to ensure the growth media mimicked the *in vivo* conditions as closely as possible. As a result, a number of recurrent Glioblastoma multiforme tumours were established in culture.

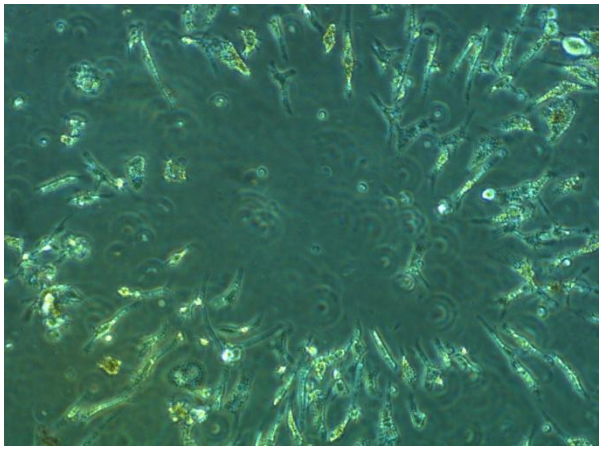


Figure 2.2.3.1 Recurrent Glioblastoma multiforme UPRH tissue taken from a right temporal lobe excision

Recurrent GBM UPRH was taken from a 39-year old male following excision biopsy from the right temporal lobe in Feburary 2005. Biopsies had been taken from this tumour on two previous occasions, the first in March 2005 and a subsequent biopsy had been taken (date not listed on report) and a Gliadel wafer had been inserted (see 1.8.3). The patient had received post-operative radio- and chemo-therapy following the initial de-bulking surgery in 2004. The biopsy report for this sample confirms large amounts of necrotic tissue and some fields of high-grade mixed Astrocytoma/Oligodendroglioma.

2.2.4 Passaging Cells

The cultured cells were routinely sub-cultured (passaged) when they reached 'confluency' (which means the surface of the flask is completely covered with cells. Cells should not be allowed overgrow as they lose their contact inhibition and enter lag phase of growth. The DMEM was aspirated using a sterile pipette and discarded and cells were washed using 2.0mls of Hanks Balanced Salt Solution (HBSS) to remove any residual FBS-containing media and cell debris. Cell-cell adhesion is mediated by a series of homotypic interacting glycoproteins (cell adhesion molecules), some of which are calcium dependent and hence sensitive to dissociation agents which remove the calcium ions. Thus if subsequently using the cells in a study that requires the presence of calcium, for example the Annexin-V assay, it was necessary to equilibrate the cells in a calcium buffer prior to analysis. Tryple (Tryple™; a recombinant-enzyme-based cell dissociation alterative to crude trypsin which bypasses the need for inactivation using FBS) was used to harvest the cells without damaging the membrane integrity or 'strip' the receptors from the cell surface (1.0 for a 75cm^3 flask; 0.5ml for a 25cm^3 flask). During the one minute dissociation period the flasks were placed into the incubator to maintain the optimum temperature. The cell suspension was aspirated into a sterile 14.0ml centrifuge tube and the flasks rinsed with 2.0ml DMEM twice to ensure all cells were removed. The cell suspension was then divided equally between flasks and fresh media added. Cells were not centrifuged into a pellet unless subsequently cryopreserving them; centrifugation was avoided at all times when working with early passage cultures to minimise damage to the cell.

2.3 Glioma cells used in the study

Name	Designation	Population Doubling Time (hrs)	Sex (Age) of patient	Origin	Passage obtained at (if not 'in-house)
SNB-19*†	Glioblastoma multiforme (Grade IV)	18	Male (47)	DSMZ	p20
DK-MG*	Glioblastoma multiforme (Grade IV)	20	Female (67)	DSMZ	p23
UPAB	Glioblastoma multiforme (Grade IV)	21	Male (73)	In-house	N/A
UPMC	Glioblastoma multiforme (Grade IV)	24	Female (69)	In-house	N/A
UPJM	Anaplastic Astrocytoma (Grade II/III)	N/A	Male (42)	In-house	N/A
CC-2565	Normal human astrocytes	31	Male (18)	Cambrex Biosciences	p1
SH-SY5Y	Neuroblastoma	28	Female (4)	ECACC	p8

Table 2.3.1 Cells used in the study

The prefix UP denotes the University of Portsmouth where the early passage cultures were initiated from biopsy tissue. The * denotes cells donated by Dublin City University)which were established cell lines from the DSMZ cell bank. †SNB-19 has been shown to be identical to U251MG (Garraway, Widlund et al. 2005) SH-SY5Y was purchased from the ECACC cell bank and is a thrice-cloned derivative of SK-N-SH (Biedler, Helson et al. 1973). Population doubling times were generated from growth studies over 120 hours (see 2.3.1). Established cell lines are tumours that have been serially passaged twenty times in culture.

2.3.1 Establishing population doubling times of cells used in the study

To establish population doubling times for cells used in the study, growth patterns were monitored over 5 days. In brief, 100,000 cells were seeded into 25cm^{3}flasks (in triplicate) then harvested (according to 2.2.4) and counted (see 2.3.5) every 24 hours. The growth of UPJM was inconsistent and the effect of harvesting cells frequently diminished cell numbers and resulted in no data for this tumour.

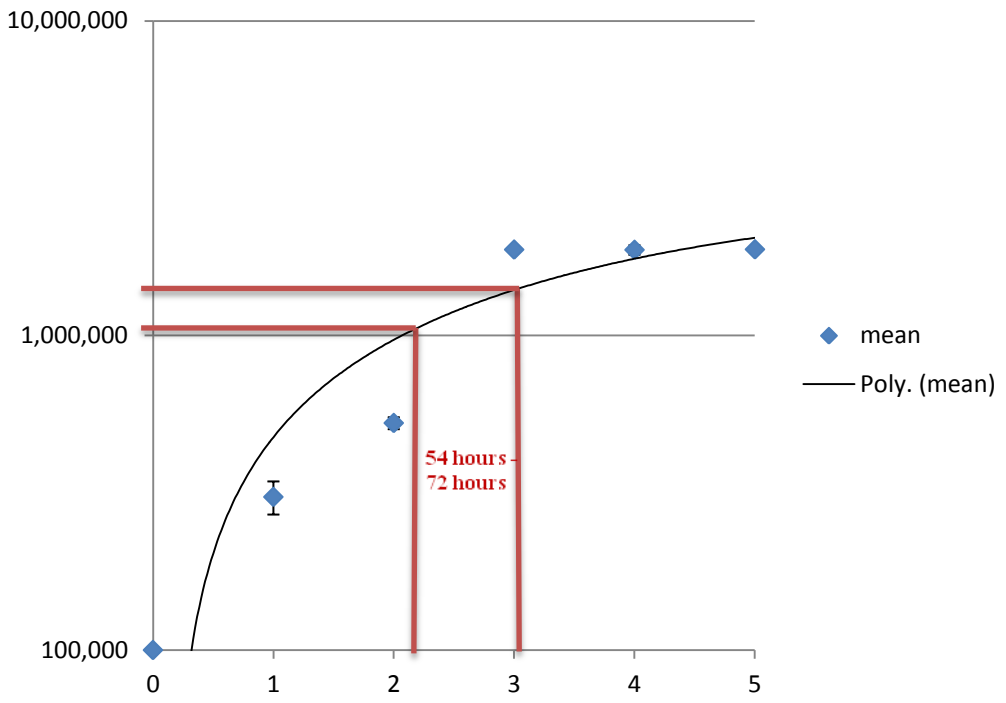
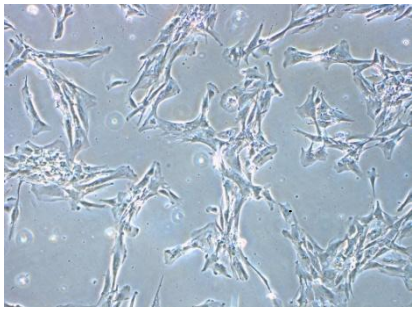


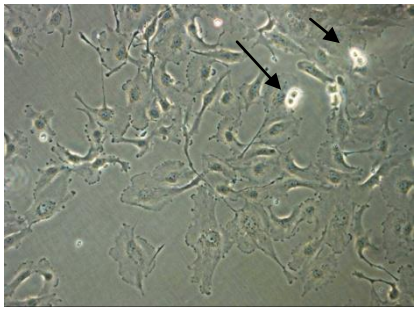
Figure 2.3.1.1 A representative plot showing population doubling data for SNB-19

The red bars show that by selecting values from the y-axis (number of cells) it is possible to highlight a period of exponential growth and extrapolate values on the x-axis (time in days) to give the number of hours in which the population doubled. In this instance the time taken for the population to double was from 54 – 72 hours, therefore the population doubling time is 18 hours.

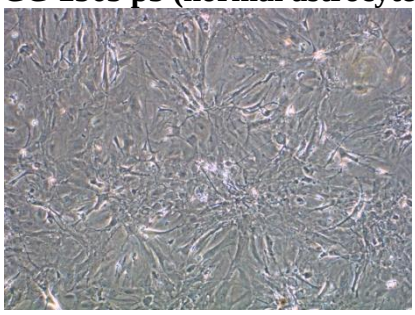
2.3.2 Phase contrast pictures of cells used in the study



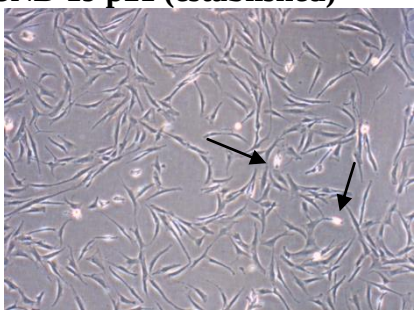
A: CC-2565 p3 (normal astrocytes)



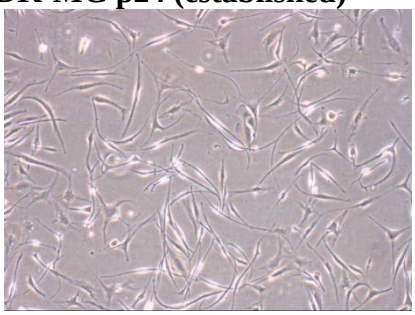
B: SNB-19 p21 (established)



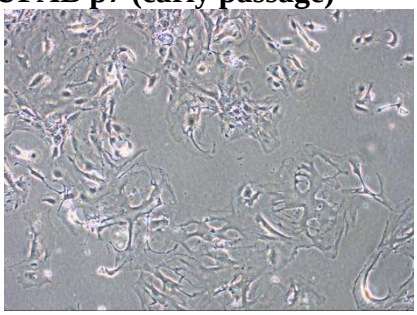
C: DK-MG p24 (established)



D: UPAB p7 (early passage)



E: UPMC p5 (early passage)



F: UPJM p4 (early passage)

All phase contrast pictures were taken at 200X magnification on a Zeiss phase contrast microscope. A) Normal human foetal astrocytes (cerebral, non-neoplastic) grown to 50% confluence in a tissue culture flask. The cells were grown as an adherent monolayer; these cells had been passaged three times. B) Tumour cell line SNB-19 was grown to 75% confluence in an adherent monolayer, the cells had been passaged twenty-one times. This cell line was actively dividing (mitotic bodies are indicated by arrows), the cells are flattened with ruffled edges and other cells had long processes. C) Tumour cell line DK-MG grown to confluence, in an adherent monolayer, the cells had been passaged twenty-four times. The cells are closely packed and display heterogeneous morphology. D) Early passage culture UPAB (not established) shown here at 50% confluence after seven serial passages. There are a number of mitotic f visible (shown by arrows); the cells appear homogeneous and have long, slender extending processes. E) Early passage culture UPMC (not established) shown here at 50% confluence after five serial passages. The cells are actively diving (mitotic figures are visible), and there are cells present with multiple processes. F) Early passage culture (UPJM) shown at 50% confluency, these cells are heterogeneous in nature. Some cells appear flattened whilst others show long, fine processes. The cell sizes also vary.

2.3.3 Cryopreservation

Cells previously harvested according to the procedure described above were centrifuged for five minutes at 1000rpm/200g. The supernatant was aspirated and the cell pellet re-suspended in 1.0ml of recovery cell culture freezing medium; RCCFM Gibco). The addition of 10% glycerol to RCCFM, which is the main difference from traditional freezing media, enables a much higher percentage of viable cells to be resurrected. The cell suspension was transferred into a barcoded cryovial (Greiner Bio-one) and all tubes placed in a 'Mr Freezy' at -80^{o}C for twenty-four hours to allow the temperature to decrease slowly, buffered by the isopropanol surrounding the tubes.

2.3.4 Thawing Cells

The cryovial containing the required culture was taken from liquid nitrogen storage, placed in a clean glass beaker (one cryovial per beaker to ensure no cross-contamination) and left to warm in a 37^{o}C incubator for up to five minutes. The cell suspension was then transferred to a flask containing the appropriate volume of pre-warmed media (5.0 mls for a 25cm^{3} flask and 12.0 mls for a 75 cm^{3} flask) and left to settle overnight.

2.3.5 Cell Counting

In order to plate cells for a cytotoxicity assay or seed the correct number into flasks for flow cytometry it was necessary to quantify the concentration of cells. The monolayers were harvested as previously described and centrifuged for five minutes at 1000rpm/200g to form a pellet. The pellet was re-suspended into 1.0ml media and from this 100µl was placed into a cuvette, 500µl plain media added and analysed as a 1:6 dilution using the trypan blue dye-exclusion assay.

The cell concentration (cells per millilitre) was calculated by the Vi-Cell XR and by using this number; it was possible to calculate how much of the cell suspension should be added to fresh media for downstream experiments:

$$\text{Vol.} = \frac{\text{Volume of cell suspension (µl) x Number of cells required for assay}}{\text{Number of cells/ml}}$$

Figure 2.3.5.1 The Vi-Cell XR (Becton Dickinson)

Automated trypan blue dye exclusion testing apparatus with cell size gating and ‘Bioprocess’ function which plots growth curve and enables population doubling time evaluation

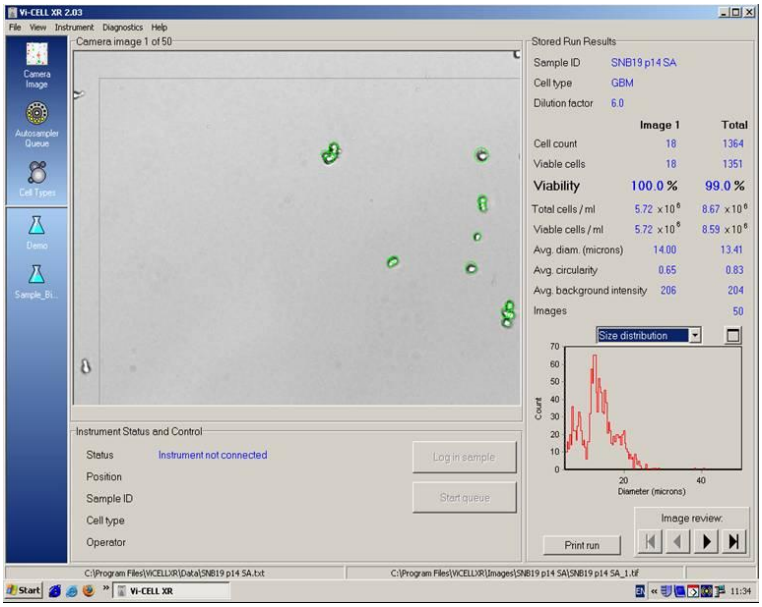


Figure 2.3.5.2 A screen capture taken during analysis of SNB-19

The Vi-Cell XR has the capability to distinguish individual cells within a cell clump. It circles viable cells in green (as seen above) and non-viable cells in red. It is not possible to remove dead cells from the cell suspension so only the viable cell number contributed to the final concentration.

2.3.6 Annexin V/Propridium Iodide Flow Cytometry

Cells grown to 90% confluence in $25cm^3$ flasks were incubated with varying concentrations of drug for up to six hours and harvested by firstly removing the complete media to centrifuge tubes (to ensure that all cells were subject to analysis), before using 5.0mls of clear Tryple™. During the two-minute dissociation period, flasks were placed in the incubator (37^oC, 5% CO_2) to maintain the optimum temperature. The Tryple™ was removed by centrifugation at 1000rpm/200g after the addition of DMEM with 10% FBS added.

The cell pellet was re-suspended in 1.0ml of calcium binding buffer (according to the manufacturer's kit instructions), used to replace the calcium ions lost during dissociation with Tryple™ (which are essential for the attachment of the probe), before removing 500µl of the single cell suspension to a FACS tube.

Controls used in the analysis were performed by omission of the drug incubation in one flask (negative control), and addition of 1µM Staurosporine to one flask (positive control). Five micro litres of Annexin V FITC (proprietary solution, concentration not provided) and 5µl of Propridium iodide (50 µg/ml) were added to all tubes, by placing a drop of the fluorochrome on the side of the tube and inverting it. The tubes were incubated for 15 minutes at room temperature, in the dark. Subsequent to this, 400µl of calcium binding buffer was added to each tube and analysed by the BD FacsCalibur (Becton Dickenson, UK) within 1 hour.

2.3.7 Staurosporine as an apoptosis stimulus positive control

Staurosporine, which is traditionally used for its antibiotic properties, has the ability to inhibit protein kinases through the prevention of ATP binding to the kinase. This is achieved through the stronger affinity of Staurosporine to the ATP-binding site on the kinase. Staurosporine is a prototypical ATP-competitive kinase inhibitor in that it binds to many kinases with high affinity, though with little selectivity (Karaman, Herrgard et al. 2008). This lack of specificity has precluded its clinical use as an anticancer agent, but has made it a valuable research tool. In research, Staurosporine is used to induce apoptosis. The mechanism of how it mediates this is not well understood, however it has been found that one way in which Staurosporine induces apoptosis is by triggering Bax-activated caspase-3-dependent apoptosis(Chae, Kang et al. 2000; Zhang, Gillespie et al. 2004).

2.3.8 Cell density seeding titration

To ensure that exponential growth was maintained for the duration of the assay, and avoiding cells becoming fully confluent and entering senescence, a cell number titration was performed. Cells were seeded in 96-well tissue plates and propagated in DMEM with the addition of 10% FBS. The titration was performed before the first MTT experiment was carried out on any new tumour, and then always plated at exactly the same density.

Cells were harvested as outlined in section 2.2.4 and seeded into a 96-well flat bottomed plate (see plate layout 2.3.9). Cells were seeded in eight rows from A-H at the following densities: 250, 500, 750, 1000, 2000, 3000, 4000 and 5000 cells/well. Cells were incubated and left to grow for 96-hours (the same time as the MTT assay takes). After a 96-hour period the wells were examined under phase contrast microscope to see which density was most appropriate for the individual cells.

2.3.9 MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, or MTT assay measures continued metabolic activity of cells over a fixed time period. The reduction of the tetrazolium salt to formazan (via the succinate dehydrogenase enzyme present in cells) is measured to calculate metabolic viability or proliferative ability after the removal of a cytotoxic agent.

MTT is cleaved to formazan by the 'succinate-tetrazolium reductase' system which belongs to the mitochondrial respiratory chain and is active only in viable cells. The reaction involves a direct transfer of electrons from reduced pyridine nucleotide to the respective diaphorase which in turn presumably transfers the electrons to the tetrazolium salt, reducing it to the insoluble formazan (shown in Figure 1.8.7.1). Recent evidence suggests that mitochondrial electron transport may play a minor role in the cellular reduction of MTT.

Since most cellular reduction takes place in the cytoplasm, and probably involves the pyridine nucleotide cofactors NADH and NADPH, the MTT assay can no longer be considered strictly a mitochondrial assay. There are a number of concerns surrounding the MTT assay, regarding the possible interference of the MTT dye with the test compounds and the fact that cells deemed viable by trypan blue dye-exclusion are not detected by the MTT assay (Ahmad, Ahmad et al. 2006).

Cells were seeded into 96-well tissue culture plates, at between 750 – 4000 cells/well according to population doubling time (the longer the population doubling time, the higher the density).They were harvested before becoming 100% confluent (completely covering the surface area of the tissue culture flask) so that they were in exponential growth phase. The cells were left to settle for 24-hours to ensure they adhere and had resumed exponential growth before exposing to varying concentrations of the test agent for, in this particular assay, 48 hours (to allow maximal cell kill and to ensure that any cell kill via second messengers could occur). It was important that cell seeding density was titrated upon first use of any new tumour so that the cells did not become confluent, and hence stop proliferating, during the assay. After a 24-hour recovery period (to allow the discrimination of cytotoxic agents from agents that are cytostatic) the MTT dye (1 mg/ml; Sigma Aldrich) was added, incubated for four hours and then analysed by spectrophotometry at 570nm.

Cells were harvested by adding 1.0 ml Tryple™ to all wells for one minute then removal of the cell suspension and the two subsequent 5.0 ml DMEM washes of all wells (to ensure all cells have been recovered). The cell suspension was centrifuged, the supernatant removed and the pelleted cells resuspended in fresh clear DMEM (DMEM without the addition of phenol red is required to ensure no background absorbance wavelength when reading plates) and plated in 96-well microtitre plates (see Figure 2.3.24.1) according to the density titration, seeded in 200µl of DMEM (without phenol red). Test agents were assayed in triplicate, whereby each of four agents were tested per plate.

Cells were left for 72 hours to adhere and reach exponential growth phase, after which the medium was replaced by fresh medium containing a range of concentrations of the test agent and incubated for 48 hours at 37^{o}C. Drug solutions were subsequently removed and replaced with 200 µl fresh plain DMEM for 24 hours.

To analyse the plates the DMEM was removed from all wells and replaced with 100µl MTT solution (1mg/ml) for 4 hours before the addition of 100µl dimethyl sulphoxide (DMSO). The plates were then placed on a shaker for 30 minutes to allow the DMSO to dissolve the formazan crystals before reading the absorbance of the plates at 570nm on a Polarstar Optima Plate Reader. One row was left untreated with the drug, and one row was treated with 0.5µM NaOH, these served as positive and negative controls respectively. All results from the assay were automatically transferred to Excel and the appropriate statistical tests applied.

2.3.10 ATP-TCA Assay

The Adenosine Triphosphate-Tumour Cell Assay (ATP-TCA) involves plating out primary cell cultures or cell lines in the presence of single agents or a combination of two or more agents, and measuring the residual cellular ATP concentration after a 6-day incubation period. Six days is the required incubation time when assaying primary cells obtained directly from surgical biopsy to allow all cell types apart from the tumour cells of interest to die, i.e. lymphocytes and neurons. It is necessary therefore to run the assay in exactly the same manner for all cells regardless of passage number to allow a true comparison to be made and for good laboratory practice to be maintained. Cellular ATP is one of the most sensitive end-points for measuring cell viability because ATP levels decrease markedly immediately upon cell death (Maehara, Anai et al. 1987). ATP determination is measured by bioluminescence using the luciferase 'firefly' reaction and can measure both proliferating and non-proliferating malignant cells. The luciferase firefly reaction takes place with oxidation of luciferin to oxyluciferin and light (luciferin + O_2→ oxyluciferin + light), catalysed by the firefly luciferase enzyme. ATP acts as a co-factor in this reaction, the levels of which determine the levels of light emitted as a result. The reaction takes place in the following two stages:

- luciferin + ATP → luciferyl adenylate + pyrophosphate
- luciferyl adenylate + O_2 → oxyluciferin + AMP + light

The reaction is very efficient, converting virtually all energy input to light. The resulting light (luminescence) is detected using a luminometer (Gould and Subramani 1988). Multi-centre studies performed on the ATP assay have demonstrated a high evaluability rate of >93% with different tumour types (Cree and Kurbacher 1999). An ATP luminescence assay was used to measure chemotherapeutic drug sensitivity and resistance of SNB-19, DK-MG, UPAB, UPMC and UPJM (see Table 2.3.1 for six days in serum-free media (DMEM:F12 in a 1:1 ratio) and 96-well polypropylene microplates. Row A acted as a negative control whereby a maximal inhibitor (a 1:5000 dilution of triton-X 100 in plain serum-free media which inhibits all cell growth) was applied to the cells and Row H acted as a positive control whereby the test agent was omitted and substituted with plain DMEM:F12. In rows B-G the four test agents were applied in triplicate at 200, 100, 50, 25, 12.5 and 6.25% of the test dose concentration (TDC; 100µM, determined by the plasma levels achieved *in vivo*[1]). The plates were incubated for six days at 37 ^{o}C with 5% CO_2, and the remaining cellular ATP extracted with tumour cell extraction reagent (TCER; a proprietary reagent from DCS, Hamburg, which contained ammonium meta-vanadate, hepes buffer and triton-x 100).

[1] *The 100% TDC value for any agent to be tested using the ATP-TCA is routinely determined from the cMax values obtained through phase I clinical trial. Since the tricyclic antidepressants have been through clinical trial for depression and not brain tumours the cMax values were checked against the data obtained in chapter 4, whereby plasma levels were measured in patients taking Clomipramine hydrochloride on a long-term basis for brain tumours. Te TDC of 100µM is at the low end of the range of concentrations that were achieved *in vivo*.

Aliquots of the extract were transferred to a white 96 well polystyrene plate to which an equal amount of luciferin-luciferase (both from Labtech International; a buffered solution containing both compounds and buffered with trizma-succinate and HEPES prepared in-house) was added. The resulting luminescence was read in a luminometer (MPLX, Berthold Diagnostic Systems, Germany) and the data transferred to an Excel (Microsoft) spreadsheet for analysis. The results were expressed as the percentage inhibition at each concentration tested.

2.3.11 Constructing a standard curve for the ATP-TCA assay

An ATP standard curve was run before reading the adenosine triphosphate-tumour cell assay (ATP-TCA) plates, to ensure that all reagents and equipment were working correctly. For this protocol 50µl dilution buffer (a proprietary buffer from DCS, Hamburg, comprising sterile water with 1% HEPES added) was added to nine wells of a white 96-well microplate.

Then 25µl of previously diluted ATP (Sigma Aldrich; 10µl of 100mcg/ml solution added to 4.0ml dilution buffer to give a 250ng/ml stock) was added to the first well and then serially diluted in the eight successive wells by transferring 25µl each time and mixing. The final resulting ATP concentrations were 83.33, 27.76, 9.253, 3.084, 1.028, 0.342, 0.114, 0.038 and 0.012 ng/ml ATP. To these nine wells 50µl luciferin-luciferase reagent was added and then the plate was placed into a luminometer (MPLX, Berthold Diagnostic Systems, Germany) for the light output, which is directly proportional to the ATP concentration, to be measured.

2.3.12 Drug concentrations used in this study

Drug	Source	FW	Mass (mg)	Moles	Volume (µl)	Molarity	TDC (µM)	800% TDC (µM)	Vol req'd for 800% TDC	Total vol CAM (ml)	Solvent
Clomipramine	Sigma; C-7291	351.3	1000	2.8466	10000	284.657	100	800	14.052	5	Water
Norclomipramine	Sigma; N-1280	337.29	25	0.0741	1000	7.412019	100	800	53.9664	0.5	Ethanol
Amitriptyline	Sigma; A8404	313.86	1000	3.1861	10000	318.6134	100	800	12.5544	5	Ethanol
Nortriptyline	Sigma; N-7261	299.84	500	1.6676	10000	166.7556	100	800	23.9872	5	Ethanol
Doxepin	Sigma; D-4526	315.84	1000	3.1662	10000	316.616	100	800	12.6336	5	1M HCl/Water
Chlorpromazine	Sigma; C-8138	355.33	500	1.4071	10000	140.7143	100	800	28.4264	5	Water
Promethazine	NHS Pharmacy	320.88	25	0.0779	1000	7.791075	100	800	51.3408	0.5	N/A
Imipramine	Sigma; I-0899	316.87	1000	3.1559	10000	315.5868	100	800	12.6748	5	Water
Procarbazine	NHS Pharmacy	257.76	50	19.39	10000	34.14	4.26	44	8.8	5	Water
Dexamethasone	NHS Pharmacy	516.40	4	7.74	1000	7.7459	0.415	8	5.14	5	Water
Valproic Acid	NHS Pharmacy	144.21	100		1000		346.72	2773.76	20	5	Water

The range of concentrations used in the MTT assay and ATP-TCA assay were 6.25 – 200 µM and the range for flow cytometry was 20-100 µM. All drugs were made up in the appropriate solvent, aliquoted and stored at -20^{o}C.

Since there is no existing data concerning *in vivo* human brain levels of tricyclics it was extremely difficult to determine the concentrations at which to test the tricyclic agents. The MTT and ATP-TCA assay concentrations were formed following examination of the plasma levels taken from a series of anecdotal patients (values can be found in Table 4.3.1) and the cyst fluid level taken from a brain tumour patient, 100 µM was deemed the test dose concentration that could easily be achieved *in vivo*.

2.3.13 Eluting gDNA samples for CYP450 genotyping of patients taking Clomipramine hydrochloride

Whatman FTA® Cards were used for the long-term storage of biological samples, in this case whole blood. The cards reduce the possibility of sample degradation due to temperature variation, leakage and spoilage. Extracted DNA may also degrade, and as such samples are unable to be re-analysed. These commercially available cards impregnated with reagents designed to lyse cells, prevent growth of bacteria and protect DNA.

The Whatman FTA cards were suitable for use in this study as they were easy to send in the post, the patients only had to provide a small amount of blood and it could be stored and transported easily. Whatman FTA cards have been designed for use by either adding a 2.0mm punched disc directly to a PCR reaction mixture or alternatively eluting the DNA from the paper matrix before use.

In this assay it was necessary to quantify the DNA before adding to the 96-well plate as all samples needed to contain the same amount of material (40ng) for optimisation with the primer probe genotyping assay.

The FTA cards had been loaded with finger prick whole blood samples, taken by a trained phlebotomist, from eighteen brain tumour patients who were taking Clomipramine. After ensuring the blood had been loaded uniformly onto the card (allowing the lysing and antimicrobial agents to work to full capacity) 2.0mm punches were taken using a Whatman Harris micro-punch, with care being taken in between punches to decontaminate the blade and cutting mat with DNA-Zap. The discs were placed into sterile 1.5ml eppendorf tubes and then washed three times with 100µl Whatman FTA Purification Reagent to remove any lysed erythrocytes and any other debris. The discs were then washed twice in 100µl TE-buffer before being dried in a heating block, with the tube lids open, for ten minutes at 50^{o}C. The DNA was then eluted by placing each dried disc into a fresh eppendorf tube and adding nuclease-free water for 10 minutes at 95^{o}C. The DNA was quantified using the ND-1000 Nanodrop Spectrophotometer.

2.3.14 Using the Nanodrop Spectrophotometer

Purity and quantity of nucleic acids (previously extracted as described in 2.3.13) was checked by spectrophotometry at 260 nm and 280 nm using a Nanodrop ND-1000 spectrophotometer. By loading 1.4µl of the sample onto the pedestal it was possible to quantify the concentration of genomic DNA (gDNA), complementary DNA (cDNA) or ribonucleic acid (RNA).

2.3.15 Dewaxing tissue sections

To enable the staining of tissues preserved in formalin-fixed paraffin-embedded (FFPE) blocks, 4 µm sections were cut on a microtome and processed using a Leica XL Stainer (Leica Microsystems, Germany) according to the manufacturer's instructions to remove the paraffin wax. In brief, slides were immersed three times in Clearene® (a selected blend of terpenes formulated specifically to replace xylene and toluene in histology laboratory procedures; NHS Supplies) and then immersed twice with fresh 100% industrial methylated spirit (IMS). IMS is miscible with Clearene and two repeat incubation steps (both with fresh IMS) ensured the complete removal of Clearene and also the rehydration of the tissue section.

2.3.16 Dehydrating tissue sections

To enable visualisation of tissue sections subsequent to any staining procedure it is necessary to remove all water from a tissue section and mount it in a xylene-based resin. In brief, slides were immersed three times in 100% IMS (to remove any water) and then immersed five times with Clearene (to clear the slides). Five Clearene steps are necessary to ensure complete removal of any residual IMS.

2.3.17 Haematoxylin and Eosin Staining

A haematoxylin and eosin stain was carried out on all FFPE tissue sections to visualise the morphology of the tumour, this enabled the selection of an area of interest (AOI) from which two 0.6 mm punches could be extracted. A 'regressive' method was employed whereby the tissue was deliberately over-stained and then differentiated in acid alcohol until only the nuclei were stained with good chromatin detail. The slides were subsequently 'blued' in tap water to give a good contrast to the eosin counter-stain which stained non-nuclear tissue components varying shades of red and pink. Slides containing one 4 µm section were taken to water on a Leica XL Stainer as described in section 2.3.15. The automatic staining programme selected consisted of two five minute incubations with Harris' Haematoxylin, a wash step with alkaline tap water to 'blue' the tissue and a brief rinse step with 1% acid alcohol. The slides were then checked microscopically to ensure only the nuclei were stained. The sections were returned to the machine, washed in alkaline tap water, stained with eosin for five minutes and washed a final time with tap water. The slides were then dehydrated and cleared according to the method in section 2.3.16.

2.3.18 Immunoperoxidase staining

In order to visualise the presence of antigens, in this case the NET protein, on tissue samples, immunoperoxidase staining can be carried out. Antibodies corresponding to the NET protein are conjugated to a colour-producing peroxidase enzyme and reveal the distribution and localisation of differentially expressed proteins in fixed tissue.

Due to the cross-linking present in tissues that have been fixed with formalin, it was necessary to pre-treat the sections to 'unmask' or retrieve' the antigens. The immunoreactivity was restored via a thirty minute incubation with pH 6.0 citrate buffer in a pressure cooker. In addition to antigen retrieval it was also necessary to block non-specific staining in the tissue.

To visualise the presence of the norepinephrine transporter (NET) on human brain tumour cells, 4 µm tissue sections cut from FFPE blocks were stained using an immunoperoxidase technique. A ready-to-use Supersensitive™ kit from Biogenex was used to stain the slides, and manufacturer instructions followed at all times. In brief, the first stage was the application of a primary antiserum (150 µl of 1:500, determined by in-house antibody titration) onto the section (previously cut from FFPE tissue blocks) which contained the NET antibody (raised in a mouse). The site of interaction between primary antibody and specific antigen was then detected by first applying a mixture of biotin-labelled anti-mouse immunoglobulins (from the ready to use multilink kit) and then applying a pre-formed complex of streptavidin and horseradish peroxidase (HRP). Due to the strong affinity that streptavidin has for biotin, the two stages bind and so the peroxidase was then visualised by way of the DAB-H_20_2 reaction. The bound HRP catalysed the oxidation of DAB by $H_20_{2,}$and consequently there was accumulation of insoluble brown product at the site of the antigen. Three blocking steps were used in this technique:

1) Endogenous and naturally occurring peroxidase was blocked by placing the slides in a mixture of alcohol and H_20_2 (ready to use, RTU, proprietary formulation supplied with kit) after dewaxing
2) Non-specific staining during the primary antibody incubation was blocked by incubating the slides with Powerblock™ (Launch Laboratories) subsequent to peroxidase blocking and prior to application of the primary antibody
3) Non-specific strept(avidin) and biotin binding of the primary and secondary antibodies was blocked by incubating the slides with a few drops of an avidin blocker and then a biotin blocker (both from Vector Laboratories) subsequent to the Powerblock™ step and prior to applying the primary antibody. The procedure ensures all endogenous biotin, biotin receptors, or avidin binding sites present in tissues are blocked prior to the addition of the labelled avidin reagent

2.3.19 IHC Antibodies & Reagents

Antibody	Company	Type		Dilution
Norepinephrine Transporter	Chemicon	Ascites	Monoclonal	1:1000
Power Block™	Biogenex	Universal blocking reagent	-	-
Supersensitive™ Link-label IHC Detection System	Biogenex	Multilink kit	-	-
DAB Liquid Chromogen	Biogenex	Chromogen	-	-

2.3.20 Immunocytochemical staining of the norepinephrine transporter in glioma cells

Where fixed biological tissue is not available for IHC staining, immunocytochemical (ICC) staining can be performed. The ICC technique is used to stain fresh cells grown in culture and differs from IHC because the cells lack the extra cellular matrix (ECM) which would normally surround them *in vivo*. The advantages to this technique are that it can stain tumours for which no FFPE tissue has been stored; the protocol takes around three hours. Cells are first fixed and then blocked with serum, the same species as the secondary antibody was raised in, to stop non-specific binding. Like IHC, this method is indirect and a primary antibody binds to the NET protein antigen via a specific epitope. A secondary antibody, with Alexafluor 488 covalently linked, binds to the primary antibody and an immunopositive signal can be visualised via confocal microscopy. The cells were fixed using 1.0 ml of 4% paraformaldehyde solution for two minutes at room temperature before washing with PBS. Potential non-specific binding was blocked with 10% goat serum (one hour at room temperature) before washing with PBS.

The first stage was the application of a primary antibody for one hour at room temperature before washing with PBS. The site of interaction between primary antibody and specific antigen was then detected by application of an Alexafluor 488-conjugated secondary antibody for 30 minutes (in the dark). After washing cells with PBS the nuclei were counterstained with hoescht blue before washing one final time with PBS. The coverslips were then mounted onto slides using vectashield and visualised using a Zeiss LCM710 confocal microscope with Zen 2009 acquisition software.

2.3.21 Detection of norepinephrine transporter proteins via western blotting

SDS-page electrophoresis allows the separation of sample proteins for western blotting. The proteins are electrophoretically separated based upon weight and electrical properties as they migrate through a polyacrylamide gel matrix. Once separated, proteins can be electrophoretically transferred to a membrane for immunoblot assay.

2.3.22 Preparing the SDS gel

An 8% gel was prepared according to Table 2.3.2 below and poured into a 1.0 mm glass chamber, which had previously been cleaned with detergent and 100% ethanol. The gel was left to cast for 20 minutes. Gel were prepared and stored overnight in wet tissue paper and foil, at 4^{o}C, for use the following day.

	Volume (mls)
Water	4.6
30% acrylamide	2.7
1.5M tris (pH 8.8)	2.5
10% SDS	0.1
10% ammonium sulphate	0.1
TEMED	0.006

Table 2.3.2 The reagents required to prepare an 8% SDS gel

The first four reagents listed in the table are mixed together, swirled and placed inside a fume cabinet before addition of the final two reagents. The gel mixture is swirled and poured into the glass slide immediately as cross-linking occurs as soon as TEMED in added.

2.3.22.1 Quantifying protein levels in glioma cell lysates using the Bradford Assay

The cell lysates were measured for protein content using the Bradford Assay. In brief, 1 µl cell lysate was mixed with 150 µl Bradford dye and left to stand for three minutes before the optical density was measured at 595nm. A standard curve (previously constructed using known concentrations of bovine serum albumin) was used to calculate protein concentrations. The final amount of sample per well was adjusted to 20 ug.

2.3.22.2 Protein detection via western blot technique

Protein samples were mixed with loading buffer (0.5% beta-mercaptoethanol added to 15 µl Laemmli buffer; Bio-Rad, Hemel Hempstead, UK) and heated at 96 ^{o}C for four minutes. The samples were then separated for 90 minutes at 140V in an electrophoresis chamber containing 1X buffer (see Table 2.3.3below). The samples were then electroblotted onto Hybond Polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia, Little Chalfont, Bucks, UK) at 370mA for 120 minutes (at 4^{o}C).

The blots were placed in a 5% non-fat milk solution (in TBST, see Table 2.3.3) for one hour before probing with the primary antibody overnight at 4^{0}C. Following three washes with TBST, the membrane was incubated with a secondary horseradish peroxidise (HRP) conjugated antibody for 3 hours. The specific signal was visualised using the ECL Plus kit (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) and captured using the chemiluminescence imaging station Chemi-Genius 2 (Syngene, Cambridge).

	Reagents
Electrophoresis buffer (5X)	For 500ml: • 7.55g tris base (128 nM) • 47g glycine (1.25M) • 2.5g SDS (17.3mM-0.5%) • adjust to pH 8.3 with HCl
Transfer buffer (5X)	For 1000ml: • 15.14g tris base (125mM) • 72g glycine (0.96M) • 2.5g SDS (8.7mM-0.25%) • adjust to pH 8.3 with HCl For 1X: • 200ml of 1X transfer buffer • 200ml of methanol • 600ml water
Tris buffered saline (TBS; 10X)	For 500ml: • 24.23g tris base • 40/03g sodium chloride • adjust to pH 7.6 with HCl For 500ml 1X TBS tween-20 (TBST): • 50ml TBS (10X) • 0.5ml Tween-20 (0.05%) • 450ml H2O
Loading buffer (Laemmli; 2X)	• 125mM tris base • 20% glycerol • 4% SDS • 0.0002 bromofenol blue • 5% fresh beta-mercaptoethanol

Table 2.3.3 The composition of buffers used for the Western blotting technique

To preserve the quality of buffers, they are made up at 5X concentration and diluted to 1X working concentrations immediately prior to use, as required.

2.3.23 Quantitative Real Time Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a process whereby nucleic acid, in this case, DNA is amplified many thousand-fold, thus generating sufficient material for experimental analysis. For genotyping, genomic DNA is used to determine any polymorphic alterations and for gene expression studies, messenger RNA (mRNA) is detected using primers that span an exon. Quantitative real time PCR (qRT-PCR) has been developed from basic PCR, to allow the measurement of PCR products as they accumulate. Detection of a fluorescent signal, generated each time the target sequence is amplified, i.e. one cycle, allows quantification. The cycle number at which the fluorescence reaches a threshold value of ten-times the standard deviation of baseline fluorescence emission is used for the quantitative measurement. This cycle number is termed the threshold cycle (C_t) value and is defined as the fractional PCR cycle number at which the fluorescent signal is greater than the minimal detection level.

2.3.23.1 Genotyping via quantitative polymerase chain reaction

Genomic DNA, obtained by the method described in section 2.3.13 was amplified in 96-well plates by qRT-PCR on a Biorad iCycler instrument (Biorad Laboratories, UK). Each PCR reaction had a final volume of 25µl comprising 11.25µl DNA (40ng per well or nuclease-free water if a no-template control), 13.75µl of Taqman Drug Metabolism Genotyping Reaction Mixture (Applied Biosystems, UK) diluted ten-fold in Taqman Universal Master Mix (without the addition of Amperase UNG). Each two-step PCR cycle comprised of denaturing (15 seconds at 92^{o}C) and annealing/extending (90 seconds at 60^{o}C).

At the end of each run a final melt curve cycle was performed (cooling to 50^{o}C and then increasing stepwise 1^{o}C to 95^{o}C) was performed to exclude the presence of primer-dimer artefacts. The results were analysed and statistical tests carried out according to the methods described in section 4.2.4.

2.3.23.2 Extraction of ribonucleic acid for use in gene expression studies via Quantitative Real Time Polymerase Chain Reaction

RNA extraction was performed using an Ambion Recoverall kit™. Briefly, the sample lysates were slow thawed at +4˚C and gently vortexed prior to the addition of 550 µl of 100 % ethanol in each tube. Debris was removed from the lysate/ethanol mix by pre-filtering with a Nucelospin® filter spin column with collection tube. 700 µl of each sample lysate/ethanol mix was loaded into corresponding labelled filter column, sealed and microfuged at 11,000rpm (11 000g) for 1 minute. The remaining lysate/ethanol mix was loaded into the corresponding filter tube and microfuged again as described above.

The 700µl of filtrate was loaded into an Ambion Recoverall kit™ cartridge for RNA isolation, previously inserted in collection tubes, and the lysate/ethanol mix pipetted onto the centre of each cartridge. The cartridges were centrifuged at 10,000rpm (10 000g) for 60 seconds, the flow through discarded and the filter cartridge re-inserted into the same collection tube. This was repeated two additional times until all the lysate had been processed for each sample. The cartridges were washed according to the manufacturer's instructions and the flow through discarded.

DNase treatment of the immobilised nucleic acids was performed by the addition of 60µl of DNase mix to the centre of each filter cartridge. After thirty minute incubation at room temperature, the cartridges were washed as previously described. Finally, the filter cartridge was placed into a fresh collection tube and 50 µl of heated (95 ºC) nuclease free water placed into the centre of the filter. Following sixty seconds incubation at room temperature, the filters were microfuged for 60 seconds at 13,000rpm/13 000g. This was repeated to give a final volume of 100 µl total RNA.

Purity and quantity was checked by spectrophotometry at 260 nm and 280 nm by placing 1.4 µl of eluent on the sampling pedestal of a scanning NanoDrop ND-1000 spectrophotometer (see 2.3.14). Aliquots of each sample were stored at -80ºC or reverse transcribed to produce cDNA in a two step RT-PCR reaction.

2.3.23.3 Reverse Transcription of extracted RNA for gene expression studies via Quantitative Real Time Polymerase Chain Reaction

Reverse transcription was performed using an ABI High-Capacity cDNA Archive Kit according to the manufacturer's instructions. Briefly, a 75 µl aliquot of master mix was added to a 0.2 ml PCR tube, to which an equal volume of purified RNA, diluted in nuclease free water, was added. For RT negative wells, a 15 µl master mix aliquot was prepared where the multiscribe RT enzyme was substituted with nuclease-free water. To this was added 15 µl of diluted RNA in nuclease-free water. The final RNA concentration in the RT mix was 1-20 ng/µl. The tubes were kept in a chilled cooling block until ready to load into the thermal cycler (Hybaid Omn-E).

Cycling conditions were step 1, 25 °C x 10 min, step 2, 37 °C for 120 min. After removal from the thermal cycler, the tubes were pulse spun in a microfuge at 12,000rpm (12 000g) for 30 seconds and stored overnight at +4 ºC. Synthesised cDNA content was measured using a NanoDrop spectrophotometer prior to use.

2.3.24 Statistical analyses

All raw data was transferred into Microsoft Excel and statistical analyses were performed using Graphpad Instat 3 and Graphpad Prism 5. Please see individual chapters for further details of the exact tests performed.

3 Comparing chemosensitivity assays for measuring the efficiency of agents with potential for killing glioma cells

3.1 Introduction

Data available from previous research has shown Clomipramine to successfully induce apoptotic cell death in malignant glioma cells using the oxygen electrode (Hansatech Instruments), DNA laddering, caspase assays and mitochondrial membrane potential assays. It has also been observed that Clomipramine exerts its effects independently of p53 regulation (Daley 2001). There exist a number of antidepressant and antipsychotic agents that are known to cross the B-BB, some of which have been suggested as having potential as anti-tumour agents. By comparing three methods for chemosensitivity testing, namely the MTT assay (method adapted from (Freshney 2005), ATP-TCA assay (Cree 1998) and the Annexin-V Assay (protocol developed in-house) it might be possible to identify the most sensitive method for rapidly assessing the potential of new agents. The published data on the ATP-TCA describe it to be a superior method to the MTT assay based upon sensitivity, reproducibility, linearity, precision to measure as few as 39 cells per well (Andreotti, Cree et al. 1995). The inclusion of foetal bovine serum in assay media increases growth and survival of both normal and malignant cells, whereas the serum-free system of the ATP-TCA assay supports only malignant cells via endogenous cell factors (Andreotti, Linder et al. 1994). Traditional assays can deliver slightly ambiguous results as homogenous high passage cell lines provide little insight into the mechanism of cell kill (and whether indeed cells die via apoptosis or necrosis) as the apoptotic threshold is lowered with increasing passage number (Parker and Pilkington 2006).Alongside the viability assay it is important to consider whether the agent is causing apoptosis or necrosis and this can be done rapidly using the Annexin-V assay with Staurosporine as a control.

Previous research in our laboratories, and others, has shown that Clomipramine hydrochloride, a TCA in use for over forty years, has the ability to induce apoptosis in malignant glioma cells *in vitro* (Daley 2001; Daley, Wilkie et al. 2005; Levkovitz, Gil-Ad et al. 2005; Meredith, Holder et al. 2005; Parker and Pilkington 2006).Thus, following the initiation of a clinical trial of Clomipramine in patients with primary GBM and grade III Astrocytoma, based at King's College Hospital, London (LREC 01-235), it was important to attempt to predict the efficacy of a given drug treatment, especially in the case of glioma which are often characterised by a poor prognosis.

The complexity of the mechanisms behind invasion of neoplastic cells into the brain has been an encumbrance to the development of new therapeutic modalities for malignant brain tumours. Current drug strategies are found to be ineffective in targeting the invading cell population, since they are prevented from entry to the normal brain by the B-BB. They are able to reach the tumour site due to local disruption of the blood-brain-tumour-barrier (BBTB), due in part to the secretion of angiogenic factors during co-option of blood vessels, however guerrilla cells are protected by virtue of their position in an area of intact B-BB. Radiotherapy affects dividing cells but has only been shown to improve survival time in primary adult brain tumours – it is not generally used at all in children under the age of three years.

Inexpensive archival drugs such as Clomipramine and Amitriptyline, which we know can cross the B-BB, have hitherto been overlooked as potential anti-glioma agents. These mitochondrially-acting agents show great potential as they do not suffer chemoresistance in the same manner as the DNA-acting agents, which are compromised via the innate propensity of neoplastic glial cells to rapidly and effectively repair DNA damage.

Prior to clinical trial the general way to assess if a new agent has potential to kill tumour cells *in vivo* in man is to carry out *in vitro* studies on human brain tumour cells. This gives an indication of the efficacy of the drug should it reach the tumour bed. The *in vitro* assays do not take into consideration the B-BB or the blood brain tumour barrier (BBTB), which are the major reasons for the failure of CT, but it is known that the B-BB can be disrupted by the growth of a tumour (Galanis and Buckner 2000), thus allowing the drug to reach the CNS. Research has also been carried out into increasing the permeability of the B-BB, via osmotic induction (combined with pre-treatment of calcium and sodium channel blockers)(Neuwelt, Frenkel et al. 1979; Neuwelt 1980; Gumerlock, Belshe et al. 1992; Siegal, Rubinstein et al. 2000), mannitol (Neuwelt, Frenkel et al. 1979; Zunkeler, Carson et al. 1996) and bradykinin (Raymond, Robertson et al. 1986; Bartus, Elliott et al. 1996; Elliott, Hayward et al. 1996; Koga, Inamura et al. 1996; Matsukado, Inamura et al. 1996; Emerich, Snodgrass et al. 1999; Bartus, Snodgrass et al. 2000; Borlongan and Emerich 2003).

Once biopsy tissue has been surgically dissected from its *in vivo* environment therein follows the question of viability. It is fundamental to ascertain the viability of cells derived from biopsy tissue so that reliable cytotoxicity testing can be performed. The development of a reliable method for chemosensitivity testing *in vitro* has been a longstanding objective in cancer research. Early attempts to stratify patients for chemotherapy were based upon clonogenic assays, the ability of single cells to clone themselves into a colony. These assays were successful only when culturing high-passage cell lines, the plating efficiency of primary tumour cells derived from patients was only around 1-2% (Salmon 1987).

Toxicity is a complex event *in vivo* where there may be direct damage to cells by a cytotoxic anticancer agent, such as neurotoxicity in the brain, inflammatory events and systemic effects. Currently it is not possible to assess the extent of any systemic and physiological effects *in vitro* as available assays generally act at the cellular level. The definition of cytotoxicity varies depending on whether cells are killed or their metabolism is altered. In the case of anticancer agents the focus is on the ability of the test agent to induce cell death, preferably via apoptosis.

Cell growth is generally defined as the regenerative potential of cells; this can be determined by clonal growth, net change in population size and change in cell mass or metabolic activity. It is important, when measuring the effect on cell growth, to remember the differences that exist between the *in vivo* and *in vitro* measurements.

Currently there is no *in vitro* model that takes into account the presence of the B-BB, and for this reason inhibitory concentrations achieved *in vitro* cannot be directly extrapolated to determine the efficacy of a drug regimen. It is possible to measure the plasma concentrations of cytotoxic anticancer agents and even though it is tempting to postulate how much of the active metabolite travels across the B-BB into the tumour site, it is not currently possible to measure the exact perfusion of an agent to the tumour site.

A study is underway currently, however, to determine the concentration of Clomipramine at the brain tumour site, using patients who receive the drug orally. Through the use of microdialysis electrodes implanted both intra-tumourally and in the adjacent normal brain it is hoped that levels that cross the B-BB and sequester into the tumour and non-neoplastic cells, respectively, can be determined (Cruickshank, G; Birmingham, UK). Regional distribution concentrations of Clomipramine in the rat brain have been determined via HPLC following administration of 15mg/kg; the data showed that the cerebral cortex had the highest concentration (2.9 µg/mg), with successively lower concentrations in the hypothalamus, striatum, cerebellum, hippocampus and brainstem (Aitchison, Datla et al. 2009).

The MTT assay measures continued metabolic activity of cells over a fixed time period. The reduction of the tetrazolium salt to formazan (via the succinate dehydrogenase enzyme present in cells) is measured to calculate metabolic viability or proliferative ability after the removal of a cytotoxic agent.

The assay used in this study was adapted from Freshney (Freshney 2005) and is relevant for the assessment of cytotoxicity of Clomipramine which exerts its effect through the mitochondria. The cells were exposed to Clomipramine and then after washing with warm phosphate buffered saline (PBS) the cells were allowed to recover for 24 hours.

Washing the plates is essential to allow the recovery of cells, simply removing the media and replacing with fresh media is not sufficient to ensure the complete elimination of any test agent. Any cell debris is washed away, leaving adhered cells attached to the bottom of the plate wells, to allow any cytostatic or senescent cells to recover. By including a recovery period the method encompasses both the antimetabolic and antiproliferative capabilities of the test agent, neither of which can be demonstrated by senescent or cytostatic cells.

Cells require a continual input of free energy for three major purposes: two of which are the active transport of molecules and ions and the synthesis of macromolecules and other biomolecules from simple precursors. The free energy used in these processes, which maintains a cell in a state that is far from equilibrium, is derived from the surrounding microenvironment. In most processes, the carrier of free energy is ATP. ATP is an energy-rich molecule because its triphosphate unit contains two phosphoanhydride bonds. The turnover of ATP is very high because motility, active transport, signal amplification, and biosynthesis (needed for cell proliferation) can occur only if ATP is continuously regenerated from adenosine diphosphates (ADP).

Therefore, measurement of ATP can serve as a marker for cell proliferation. The determination of ATP using bioluminescence is a well established technique. It uses the ATP dependency of the light-emitting, luciferase-catalysed oxidation of luciferin for the measurement of extremely low concentrations of ATP (Cree 1998).

The serum-free growth conditions of the ATP-TCA assay facilitate selective growth of malignant cells and the results are reported to give good correlation with clinical outcome in ovarian carcinoma (Andreotti, Cree et al. 1995; Kurbacher, Bruckner et al. 1997; Kurbacher, Cree et al. 1998), breast carcinoma (Cree, Kurbacher et al. 1996; Kurbacher, Cree et al. 1996) and melanoma (Cree, Neale et al. 1999).

The test dose concentrations (TDCs) used in both assays are traditionally taken from data available through phase I clinical trial, however these levels for use in brain tumours (rather than depression) were established from levels measured in plasma (see chapter 4) and the concentrations seen in cyst fluid. All tricyclic agents for MTT and ATP-TCA assay were deemed to have a TDC (100%) of 100μM. Traditional assays utilising extremely homogenous high passage cell lines provide little insight into the mechanism of cell kill or the true IC_{50} or IC_{90} as the apoptotic threshold is lowered with increasing passage number (Parker and Pilkington 2006). Alongside the viability assay it is important to consider whether the agent is causing apoptosis or necrosis and this can be done rapidly using the Annexin-V assay with Staurosporine as a control.

Clomipramine (50µM) has previously been reported to exert an apoptotic effect on human myeloid leukaemia HL-60 cells (Xia, DePierre et al. 1998), C6 glioma cells (25µM) on and human Neuroblastoma SH-SY5Y cells (20µM) (Levkovitz, Gil-Ad et al. 2005). It has also been demonstrated that synergism can be achieved by applying Gleevec and Clomipramine in combination (Bilir, Erguven et al. 2008). Significant morphological changes following incubation with 12µM Clomipramine, represented by red (Propridium iodide) fluorescence of fragmented apoptotic nuclei, were observed by Levkovitz et al. (Levkovitz, Gil-Ad et al. 2005) when compared to blue (Hoechst) fluorescence of the intact nuclei treated with vehicle (saline). Similar morphological findings were presented by Daley when human malignant glioma cells were incubated with Clomipramine (maximum incubation period of four hours) and subsequently stained with ethidium bromide and acridine orange (Daley 2001).

Internucleosomal DNA fragmentation measured by electrophoresis in glioma cell lines was also demonstrated by Daley confirming DNA laddering and hence condensation of chromatin, the ‘classic’ hallmark of apoptosis (Daley 2001). These findings, from the literature on Clomipramine, confirm the potent apoptotic effect that Clomipramine has on tumour cells. They also observe the higher resistance of primary cell cultures (Gil-Ad, Shtaif et al. 2004; Parker and Pilkington 2006) which can be accounted for, in part, by the high proportion of non-neoplastic cells maintained in these short-term, low passage, cultures but may also reflect the presence of particularly resistant sub-populations of neoplastic cells in early passage culture, such as cancer stem cells.

Apoptosis after Clomipramine treatment is associated with the intrinsic pathway of mitochondrial cytochrome C release (Daley, Wilkie et al. 2005; Levkovitz, Gil-Ad et al. 2005), following a rapid increase in p-c-Jun (Levkovitz, Gil-Ad et al. 2005), and activation of caspase 9 (Ekert, Read et al. 2004).

The two main pathways of apoptosis can be identified in mammalian cells, both controlled by caspases and eventually converge on 'executioner' caspase 3 (Cohen, Wilson et al. 2004), which is responsible for the cleavage of structural cytoplasmic and nuclear proteins, with consequent cell death and collapse (Ceruti, Mazzola et al. 2005).

The capabilities of chemosensitivity are far-reaching when screening new agents, and by using assays that analyse the activity of the succinate dehydrogenase enzyme, or ATP activity, of the electron transport chain, in individual tumours, it is possible to correlate an *in vitro* response with the preclinical effect *in vivo* and tailor the chemotherapy to suit the individual (Blumenthal 2005). Chemosensitivity is an *ex vivo* means of determining the cytotoxic, cytostatic or apoptotic potential of anticancer agents. It is imperative that all agents undergo rigorous *in vitro* testing and *in vivo* animal model testing before being subjected to clinical studies in man. In addition to the classic DNA-damaging agents such as the PCV chemotherapy triad many new classes of drug exist such as kinase and non-kinase enzymes, transcription regulators, agents targeting growth factors, chemokines, angiogenic regulators and protein-protein interactions (Blumenthal 2005).

The difficulty when prescribing a chemotherapeutic regimen is that patients with similar tumour histology may display different responses due to differences in age, metabolic capacity, liver function, diet (vegetarians have a higher blood pH extending the half-life of some compounds), concurrent or prophylactic medication such as a corticosteroid or anticonvulsant and differences in the ratio of component cell sub-populations within heterogeneous glioma.

The aim of this chapter was to compare efficacy and potency of eight tricyclic agents using *in vitro* chemosensitivity screening, thus demonstrating the potential of such agents for glioma therapy. Using a range of cell lines and early passage cultures would highlight the differing sensitivities and heterogeneity between cell lines and early passage cultures. By contrasting and comparing the three *in vitro* assays the study could go some way towards designing an optimum chemosensitivity assay.

3.2 Methodology

The MTT assay was chosen for its reproducibility as well as for the fact that the assay measures mitochondrial activity, this is pertinent for the tricyclics which act via complex III of the electron transport chain (see 1.12). The MTT assay was carried out as previously described in section 2.3.9 using Clomipramine hydrochloride, Norclomipramine hydrochloride, Amitriptyline hydrochloride and Nortriptyline hydrochloride on four early passage cell cultures/cell lines and one normal human astrocyte early passage culture. All the concentrations were tested in triplicate, within one plate and co-efficient of variations calculated.

The ATP-TCA assay was carried out as previously described in section 2.3.10 using all eight tricyclic agents on five early passage cell cultures/cell lines. In addition to those, three 'traditional' agents, namely BCNU (see 1.7.1 for mechanism of action), DTIC (active form of Temozolomide; see 1.8.1 for mechanism of action) and AMN107 were tested in parallel on three tumours to enable a comparison of the efficacy of DNA-targeting agents to the mitochondrially-targeting tricyclics. AMN107, a potential successor to Gleevec, is a potent inhibitor of the leukaemia protein Bcr-Abl and, unlike Gleevec, has the added advantage of inhibiting both wild-type and mutant forms of the chimeric protein (Golemovic, Verstovsek et al. 2005; O'Hare, Walters et al. 2005; O'Hare, Walters et al. 2005; Weisberg, Manley et al. 2005). All the concentrations were tested in triplicate, within one plate and co-efficient of variations calculated.

The purpose of the Annexin V assay was to ascertain at what relevant concentrations apoptosis occurs so the concentrations of Clomipramine used in the assay were based upon the data generated from the MTT and ATP-TCA assays. The concentrations chosen for use, in this assay, are very low compared to the levels seen in plasma. However it has not been fully established what levels are achieved at the tumour site, although Clomipramine has been shown to be measurable in the cyst fluid from one anecdotal patient (unpublished results).

When designing treatment regimens it is important to cause as little disturbance to the CNS as possible, as necrotic cells are not cleared by macrophages in the same way that suicidal apoptotic cells are and hence leave caseous (a form of coagulative necrosis in which the necrotic cells clump together to form a focus within an injured tissue) or even calciferous necrotic debris.

The Annexin V Assay was carried out as previously described in section 2.3.6 using five cell cultures/cell lines and one normal human astrocyte early passage culture. Staurosporine, a protein kinase inhibitor capable of inducing apoptosis via the prevention of ATP-binding, was used as a positive control so that the quadrants on the flow cytometer could be correctly assigned. In order to identify where apoptotic cells fall on the histogram (which differs between cell lines according to cell size) a positive control is required for each individual sample.

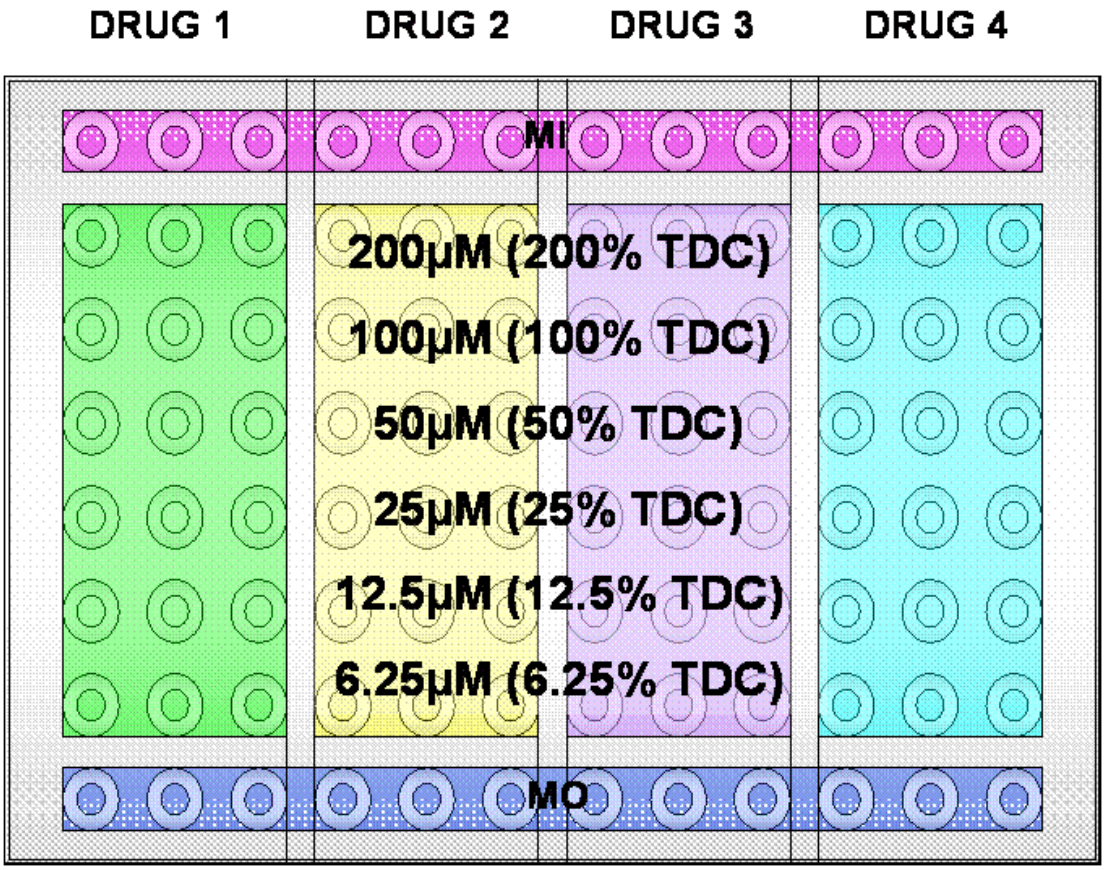


Figure 2.3.24.1 Layout of the 96-well plate used for MTT and ATP-TCA chemosensitivity assays

The top row of wells contained the triton-x 100 maximal inhibitor (positive control), the bottom row of the plate contained only cells and plain media to act as a positive control. Rows B to G contained the test agent, in triplicate, which was serially diluted as indicated. Four agents were tested per plate. For the MTT assay, flat-bottomed wells were used to allow the cells to adhere whereas u-bottomed plates were used for the ATP-TCA assays to encourage spheroidal growth. The test plate in which the MTT assay was run contains flat-bottomed wells to encourage two-dimensional monolayer growth. By propagating an adherent culture it was possible to perform 'washing' steps in the protocol, where a buffered saline solution was added to all wells to remove any remaining test agent. Fresh media was then be added to allow a 24-hour recovery period. It had been reported that following exposure to Clomipramine, some brain tumour cells were capable of recovery within twenty-four hours (Daley 2001), hence this recovery period was incorporated into the assay both to ensure that the actions of the test agent were in fact cytotoxic, not cytostatic, and to allow any viable cells to proliferate.

Since the cells were not adherent in the ATP-TCA assay the protocol was slightly different as the cells needed to be lysed with tumour cell extractant reagent before an aliquot of the extract transferred to a white 96-well plate for luminometry readings to be taken. White plates were used to measure bioluminescence because of their reflective properties and the background readings (taken automatically by the luminometer) were subtracted from all values.

3.3 Results

3.3.1 Calculating MTT & ATP-TCA Results

Data from the plate reader/luminometer was transferred automatically to an Excel spreadsheet where the results were expressed as a percentage inhibition at each of the six TDCs tested. Inhibition was calculated using the equation:

Inhibition = 1 – (test – MI)/(MO–MI)_100

The maximum inhibition (MI) data is obtained from row A of the test plates, in which TCER has been applied to cells to ensure 100% cell kill. The zero inhibition (MO) data is obtained from row H of the test plates in which cells have been incubated with media only to ensure maximum viability. The results are interpreted and compared using four parameters. The drug concentrations that achieve 50 and/or 90 % growth inhibition (IC_{50} and IC_{90} respectively) are calculated by interpolation using Excel and a macro in appendix 9.2 which compares each inhibitory value to the ones either side of it. The 'area under the concentration–inhibition curve' (IndexAUC) values were calculated from the data using the trapezoidal rule, and represent the total amount of drug uptake by the tumour cells in the assay. In simple terms; the higher the AUC value, the higher the sensitivity of the tumour. A sensitivity index (Index SUM) is calculated as the sum of the percentage inhibition at each concentration tested (SI = 600 - Σ %Inhibition at 200,100,50,25,12.5 and 6.25% TDC). Previous ATP-TCA studies have found that this natural logarithmic Index SUM calculated by direct addition of the percentage survival at each concentration tested provides a better indication of sensitivity or resistance to different drugs in different tumour types (Hunter, Sutherland et al. 1993; Konecny, Crohns et al. 2000).

The total inhibition of growth resulted in an index of 0 and no inhibition of growth at any concentrations produces an index of 600. Any Index SUM value falling between 0 and 300 is deemed as ‘sensitive’ to the test agent, between 300 and 350 is deemed as ‘equivocal’ and 350 – 600 is deemed ‘resistant’. Statistical tests, specifically coefficient of variation, were performed using non-parametric methods which did not rely on Gaussian distribution.

Although there is no distinct pattern between tumour grade or passage number of cells and the sensitivity of cells to Clomipramine hydrochloride, the results are promising and further investigation is warranted. It is interesting to note that UPMC, the cells with least sensitivity, appears to be an aggressive tumour that presented in accident and emergency and the acutely ill patient did not survive surgery.

	Clomipramine				Norclomipramine				Amitriptyline				Nortriptyline			
	AUC	**IC 90**	**IC 50**	**Index**	**AUC**	**IC 90**	**IC 50**	**Index**	**AUC**	**IC 90**	**IC 50**	**Index**	**AUC**	**IC 90**	**IC 50**	**Index**
UPAB	20357	40.18	26.12	**205**	7244	12.26	9.57	**229**	17745	37.55	21.57	**204**	17895	49.50	20.59	**225**
SNB-19	19645	40.06	22.40	**162**	19192	20.99	3.47	**38**	16590	45.30	24.72	**302**	15616	54.67	20.53	**283**
DK-MG	14197	23.07	12.05	**207**	23599	18.11	11.50	**2**	14685	41.65	21.93	**305**	3404	43.77	19.63	**297**
UPMC	15391	93.79	54.62	**270**	14069	152.96	58.97	**242**	12167	194.61	67.40	**304**	13436	203.76	49.85	**314**
UPJM	-	-	17.40	**-**	-	-	-	**-**	-	-	-	**-**	-	-	-	**-**
CC-2565	-1290	3628.65	1266.07	736	-1671	5533.10	3073.95	755	-2791	8986.03	4992.24	807	-2685	3047.92	1693.29	821

Table 3.3.1 A summary tableof AUC, IC_{90}, IC_{50} and $INDEX_{SUM}$ values for four tricyclic antidepressants analysed using the MTT assay in glioma cells

The data emboldened in grey are values <300 indicating sensitivity in the four tumours tested. Four cell cultures (UPAB, SNB-19, DK-MG and UPMC) are sensitive to Clomipramine and Norclomipramine. UPAB is sensitive to Amitriptyline and UPAB, SNB-19 and DK-MG are sensitive to Nortriptyline. CC-2565 is not sensitive to any of the four antidepressants tested. UPJM senesced after completing a proportion of experiments and so data is not available for all agents tested using the MTT assay, however a prelimary MTT was carried out on UPJM cells using Clomipramine Hydrochloride and the IC_{50} has been included in the table. The data demonstrates the potential for using tricyclic antidepressants against malignant glioma and also the variability in IC_{50} values. The range and standard deviation for Clomipramine in five cell cultures is 42.57 ± 16.58 µM, the range and standard deviation for Norclomipramine in four cell cultures is 55.5 ± 25.62 µM, the range and standard deviation for Amitriptyline in four cell cultures is 45.83 ± 22.37 µM and the range and standard deviation for Nortriptyline in four cell cultures is 30.22 ± 14.81 µM. The IC_{50} values included for CC-2565 are not 'true' values as 50% cell kill was not achieved in the assay, they are forecasted values given the response that was seen at the concentrations tested.

	Clomipramine				Norclomipramine				Amitriptyline				Nortriptyline			
	AUC	IC 90	IC 50	Index	AUC	IC 90	IC 50	Index	AUC	IC 90	IC 50	Index	AUC	IC 90	IC 50	Index
UPAB	15245	90.72	52.92	**264**	19021	12.19	9.19	**96**	10476	174.67	86.00	415	18660	22.11	11.56	**137**
SNB-19	15410	97.32	35.09	**228**	19301	9.20	3.85	**19**	12284	171.69	66.32	337	18982	20.19	5.77	**72**
DK-MG	13460	163.91	48.48	**274**	18404	40.81	10.89	**115**	6671	192.26	130.65	584	16192	47.67	38.92	**288**
UPMC	14681	86.53	45.15	356	19327	5.83	3.24	**5**	15836	80.51	38.86	**274**	18965	15.30	5.92	**62**
UPJM	11386	171.96	85.60	349	18938	16.61	9.54	**105**	10318	173.07	83.12	444	18281	24.44	17.47	**142**

Table 3.3.2 A summary table of AUC, IC_{90}, IC_{50} and $INDEX_{SUM}$ values for four tricyclic antidepressants analysed using the ATP-TCAin glioma cells

The data emboldened in grey are values <300 indicating sensitivity in the five tumours tested. Two of the cell cultures are sensitive to Clomipramine, all cell cultures are sensitive to Norclomipramine, one cell culture is sensitive to Amitriptyline and all cell cultures are sensitive to Nortriptyline. The data for Norclomipramine agrees with that of the MTT (see Table 3.3.1) and all cell cultures are sensitive, whereas here UPMC and UPJM are shown to have equivocal values for Clomipramine. The data for Amitriptyline does not agree with that of MTT, here UPMC is sensitive. The MTT assay suggested UPMC was not sensitive to Nortriptyline whereas the ATP-TCA assay shows it has the lowest sensitivity index. CC-2565 was not included in the panel of established cell lines/early passage cultures assayed using the ATP-TCA due to the length of incubation period being 6 days. The very nature of the assay is that under serum-free conditions only neoplastic tumour cells are viable after six days (which means normal brain cells would not survive) and normal human astrocytes require the presence of serum to establish themselves as reactive astrocytes in culture. The data again demonstrates variability across the IC_{50} values; the range and standard deviation for Clomipramine was 50.51 ± 19.13 µM, the range and standard deviation for Norclomipramine was 7.65 ± 3.53 µM, the range and standard deviation for Amitriptyline was 91.79 ± 33.48 µM and the range and standard deviation for Nortriptyline was 33.15 ± 13.72 µM.

	Imipramine				Promethazine				Chlorpromazine				Doxepin			
	AUC	**IC 90**	**IC 50**	**Index**	**AUC**	**IC 90**	**IC 50**	**Index**	**AUC**	**IC 90**	**IC 50**	**Index**	**AUC**	**IC 90**	**IC 50**	**Index**
UPAB	10067	179.79	97.44	393	13324	159.18	55.53	311	13249	97.66	70.09	352	12696	96.98	62.95	434
SNB-19	15291	93.49	37.02	**279**	15319	89.42	44.81	**281**	14066	98.66	59.63	316	14768	89.91	46.54	330
DK-MG	12612	213.41	4.92	**209**	10921	181.91	18.285	**262**	4916	196.17	146.61	586	2082	192.04	158.40	599
UPMC	8565	181.38	106.39	467	15269	86.83	42.75	312	16683	82.04	21.75	**206**	16726	62.04	32.08	**234**
UPJM	13715	96.94	56.42	357	13748	170.93	27.39	**262**	15121	100.22	22.75	**271**	16403	89.16	20.43	**194**

Table 3.3.3 A summary table of AUC, IC_{90}, IC_{50} and $INDEX_{SUM}$ values for four additional tricyclic agents analysed using the ATP-TCA in glioma cells

The data emboldened in grey are values <300 indicating sensitivity in the five cell cultures tested. According to the index values SNB-19 is sensitive to Imipramine, and Promethazine, UPMC is sensitive to Chlorpromazine and Doxepin and UPJM is sensitive to Promethazine, Chlorpromazine and Doxepin. This demonstrates both the potential for use of other compounds with tricyclic structure (two are antidepressants, one a sedating antihistamine and one an anti-psychotic) against malignant glioma and also the variability in sensitivities displayed across tumours. This data is interesting and warrants further investigation. The area under the curve values represent the amount of drug taken up by the cells during the assay, and the lowest AUC values for all assays are seen here for DK-MG exposed to Chlorpromazine and Doxepin.

The index SUM values indicate sensitivity of an early passage or established culture to a test agent, whereas the IC_{50} value gives evidence of the degree of cell kill when cells are exposed to 50% of the test concentration. In some cases, for example UPMC tested via MTT assay, the sensitivity indices show that this early passage culture is most sensitive to Norclomipramine, however it is interesting to note that the IC_{50} value for Norclomipramine is higher than the IC_{50} values achieved by Clomipramine and Nortriptyline exposure. The index SUM values are based upon area under the curve (test concentration vs. Inhibition) and take into account the slope of the inhibitory curve, therefore are more accurate at predicting clinical response in patients than referring to a single IC_{50} value (Andreotti, Cree et al. 1995). An IC_{50} value would be a useful reference marker if there was evidence to show what concentration of test agent is present at the tumour site. It is difficult to compare the results from the MTT assay (Table 3.3.1) with those from the ATP-TCA assay (Table 3.3.2) because the MTT assay media contains serum and the ATP-TCA media is serum-free. The other main differences in the set up of the assays are the adherent growth of cells in the MTT assay and spheroidal growth that is encouraged in the ATP-TCA assay and the incubation times (2 days for MTT, 6 days for ATP-TCA). One interesting feature of the results of the two assays are that the parent drugs have higher IC_{50} values than the metabolites in the ATP-TCA assay and the reverse is seen in the MTT assay. This may be due, in part, to the half life of the compounds. The half-lives of Clomipramine and Amitriptyline are 21 and 15 hours (on average) respectively, whereas those for Norclomipramine and Nortriptyline can be up to 50 and 90 hours respectively (Nagy and Johansson 1977; Westenberg, De Zeeuw et al. 1977; Nagy 1980; Burch, Shaw et al. 1982).

Therefore the metabolites retain activity for a longer, and assays that last more than 48 hours would show them to be more potent. To accurately compare the assays, they would both need to be run over the same number of days. Although no discernable pattern of tumour sensitivity (according to tumour grade or type) was detected in these samples analysed using the MTT and ATP-TCA assays, the diversity of tumour types was small. Further investigation using a diverse range of tumour types may yield relevant information.

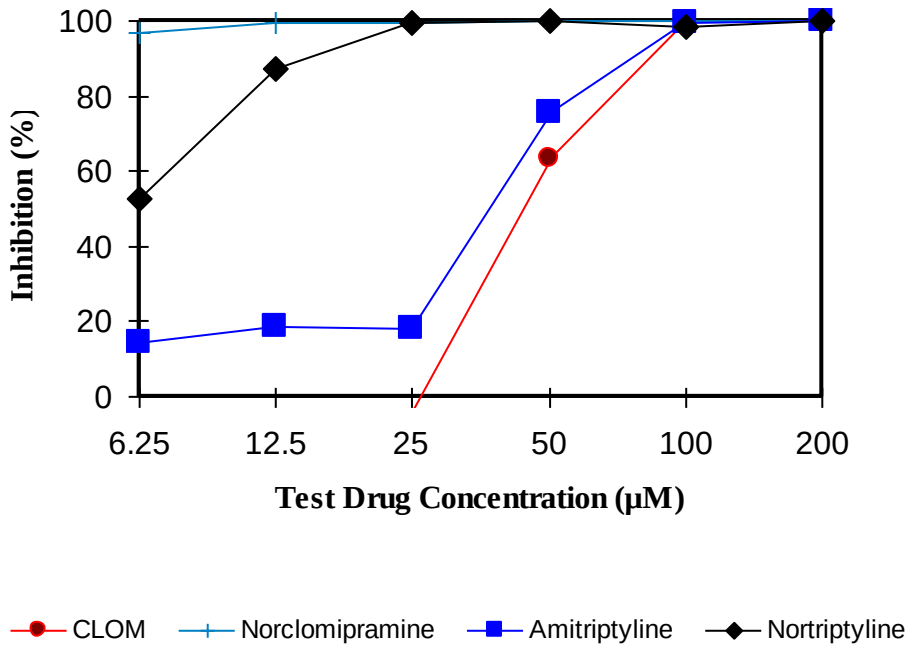


Figure 3.3.1.1 The ATP-TCA results for early passage culture UPMC

To illustrate the data obtained in Table 3.3.2 the results for four test agents have been plotted to demonstrate the dose-response curve. Clomipramine is the parent drug and Norclomipramine is the active metabolite of Clomipramine. Amitriptyline is the parent drug and Nortriptyline is the active metabolite. This graph represents one 96-well plate, in which each test drug concentration has been tested in triplicate. Error bars are smaller than the symbols and have been omitted; they do however demonstrate the reproducibility of the method. The coefficient of variation (CV) for this test plate was 10.59%. This value was calculated using the negative control wells and demonstrates the accuracy and reproducibility of the method. Any plates with CV's greater than 20% were discarded and repeated. Because the CV values would be adversely affected by clumping of cells or infection of primary cultures this is an accurate calculation of accuracy and a method of quality control. The most active tested was Norclomipramine, according to the Index SUM values. All tumours were sensitive to this agent. The values were the lowest of all the drugs tested. The agents with least efficacy across all cells tested were Imipramine and Amitriptyline, with only one cell culture demonstrating sensitivity in each case. There is no pattern of which cells will be sensitive to which agent; this further demonstrates the need for treating patients on an individual basis.

	SNB-19				UPAB				UPMC				DK-MG			
	AUC	**IC $_{90}$**	**IC $_{50}$**	**Index**	**AUC**	**IC $_{90}$**	**IC $_{50}$**	**Index**	**AUC**	**IC $_{90}$**	**IC $_{50}$**	**Index**	**AUC**	**IC $_{90}$**	**IC $_{50}$**	**Index**
AMN107	10580.99	199.97	80.82	403.99	11182.46	252.70	71.41	364.15	11384.20	138.28	78.71	418.96	9933	1518.16	13.61	303
BCNU	8038.34	216.74	114.37	428.29	1426.18	5272.39	2929.11	542.78	4658.61	456.03	253.35	476.23	9070	284.19	87.94	369
DTIC	1369.09	985.93	547.74	588.83	3398.34	848.02	471.12	512.59	5357.35	739.08	410.60	415.40	2184	725.66	405.14	583

Table 3.3.4 A summary table of AUC, IC_{90}, IC_{50} and $INDEX_{SUM}$ values for the data obtained via ATP-TCA for AMN107, BCNU and DTIC in four malignant glioma

Established cell lines SNB-19, DK-MG and early passage cultures UPAB and UPMC were plated individually for ATP-TCA analysis, each with three test agents AMN107, BCNU and DTIC. Two of these are the 'standard' chemotherapy agents administered for GBMs under NICE guidelines, namely BCNU (in the form of a Gliadel wafer, see 1.8.3) and DTIC (the active form of Temozolomide, see 1.8.1). AMN107 is a potential successor to Gleevec which has previously been tested in clinical trial for brain tumours (see 1.8.2). The reason for choosing these agents are that the TDCs have been calculated and tested for routine chemosensitivity testing in other types of tumour. None of the cells are sensitive to these agents. When compared to data from ATP-TCA analysis of the tricyclic agents it would appear, according the Index SUM values that the three cell cultures tested are sensitive to novel tricyclic agents and resistant to standard chemotherapy.

		SNB-19	DK-MG	UPAB	UPMC	UPJM	CC-2565
		Apoptosis (%)					
Control Sample		**1.25**	**2.05**	**2.27**	**10.77**	**3.03**	**0.36**
Staurosporine (6hr 1 µM control)		**21.47**	**71.56**	**5.04**	**47.43**	**38.94**	**1.94**
20µM	6h	1.25	1.87	2.35	9.96	1.01	0.29
	5h	0.92	1.50	3.21	10.81	3.20	4.68
	4h	0.95	1.38	2.11	12.74	0.50	3.21
	3h	1.29	1.18	3.00	11.72	0.88	2.51
	2h	0.71	1.40	1.87	10.99	0.24	4.12
	1h	1.45	1.03	1.46	10.13	1.31	2.98
40µM	6h	3.67	1.95	2.84	11.67	N/D	3.62
	5h	2.69	2.64	3.45	13.32	N/D	3.28
	4h	2.13	1.30	4.19	14.11	N/D	2.92
	3h	2.11	5.39	2.93	12.41	N/D	1.55
	2h	2.64	4.26	3.00	12.31	N/D	3.74
	1h	2.72	3.14	4.01	14.03	N/D	3.32
60µM	6h	4.84	**23.27**	3.86	14.26	N/D	2.86
	5h	5.14	**21.44**	3.40	12.16	N/D	0.03
	4h	**11.50**	**51.25**	2.99	12.80	N/D	5.65
	3h	**10.98**	**18.75**	3.71	13.62	N/D	0.32
	2h	**5.9**	**26.53**	3.66	12.33	N/D	4.49
	1h	**5.31**	**19.75**	3.08	13.47	N/D	3.52
80µM	6h	**5.89**	**27.13**	3.00	14.22	**12.32**	0.23
	5h	**15.91**	**20.86**	4.36	14.02	**10.00**	1.65
	4h	**10.55**	**38.92**	3.18	15.88	**10.47**	0.15
	3h	**4.98**	**42.25**	3.31	15.82	N/D	0.05
	2h	**9.18**	**23.76**	2.51	13.19	N/D	0.02
	1h	**10.27**	**23.41**	3.71	14.59	N/D	0.00
100µM	6h	**11.03**	**49.16**	3.91	10.97	**10.73**	0.36
	5h	**2.57**	**23.18**	2.77	12.91	**8.64**	0.01
	4h	**10.91**	**22.81**	2.97	11.64	**5.99**	3.21
	3h	**12.58**	**17.74**	4.98	11.94	**10.28**	0.04
	2h	**17.13**	**20.66**	4.68	10.76	**6.50**	0.05
	1h	**11.00**	**30.84**	2.79	9.41	N/D	0.38

Table 3.3.5 A summary of the apoptosis data obtained by Annexin V flow cytometry for Clomipramine

Highlighted in bold are the samples at which apoptosis (defined as a sample with greater apoptosis than the negative control) was achieved when compared to the negative and staurosporine controls, which were cells with no drug and cells with staurosporine added respectively. Results are expressed as a percentage of the total cell population analysed by flow cytometry. The aim was to examine which of the cell cultures were most sensitive to undergoing apoptosis and this table illustrates the differences between the cell cultures; with DK-MG being the most responsive when compared to the control values. In addition to the results seen in DK-MG there was some apoptosis observed in cell line SNB-19 at 60 µM and above, and in UPJM where data is available from 60 µM upwards (N/D indicates that there were not enough viable cells present, UPJM was an extremely slow-growing diffuse grade II/III astrocytoma that spontaneously senesced and so was difficult to propagate: the reason why established cell lines are easier to grow and the cells of choice for many researchers).

Cells undergoing apoptotic cell death expose their phosphatidyl serine residues and are detected by calcium-dependent binding of the Annexin-V reporter probe. By using a propridium iodide counter stain it is possible to further distinguish cells that are necrotic (or deemed ‘dead’ in this case) by virtue of their leaky membrane. Cells undergoing apoptotic cell death when exposed to the test agent bound to the Annexin-V probe in a non time-dependent manner, however without also measuring a marker of the end stages of apoptosis such as cytochrome C release it is not possible to determine if the suicide process was complete within the six hour experiment. Further work to design a multiplex assay, using a second phycoerythrin (PE) laser (in addition to the FITC one used for Annexin V) and a cytochrome C marker with a fluorescent PE tag might provide useful data.

3.4 Annexin-V Results

The MTT data indicates that there is considerable heterogeneity in the sensitivity of the cells tested to Clomipramine. The DK-MG cell line was the most sensitive to Clomipramine with an IC_{50} value of 12.05µM. This correlates with the data obtained by Annexin-V which also showed DK-MG to be the most sensitive to Clomipramine as determined by percentage of cells dying via apoptosis. The least sensitive neoplastic cell culture tested via MTT was UPMC with an IC_{50} of 54.62µM. This also correlates well with the Annexin-V data which would suggest that, at the concentrations tested, there was no apoptosis occurring when compared to apoptosis occurring in the untreated control sample. The results for UPMC obtained via the ATP-TCA assay would agree with the MTT and Annexin V results, indicating via the Index SUM that UPMC is not sensitive to Clomipramine, and that the IC_{50} is 45.15µM.

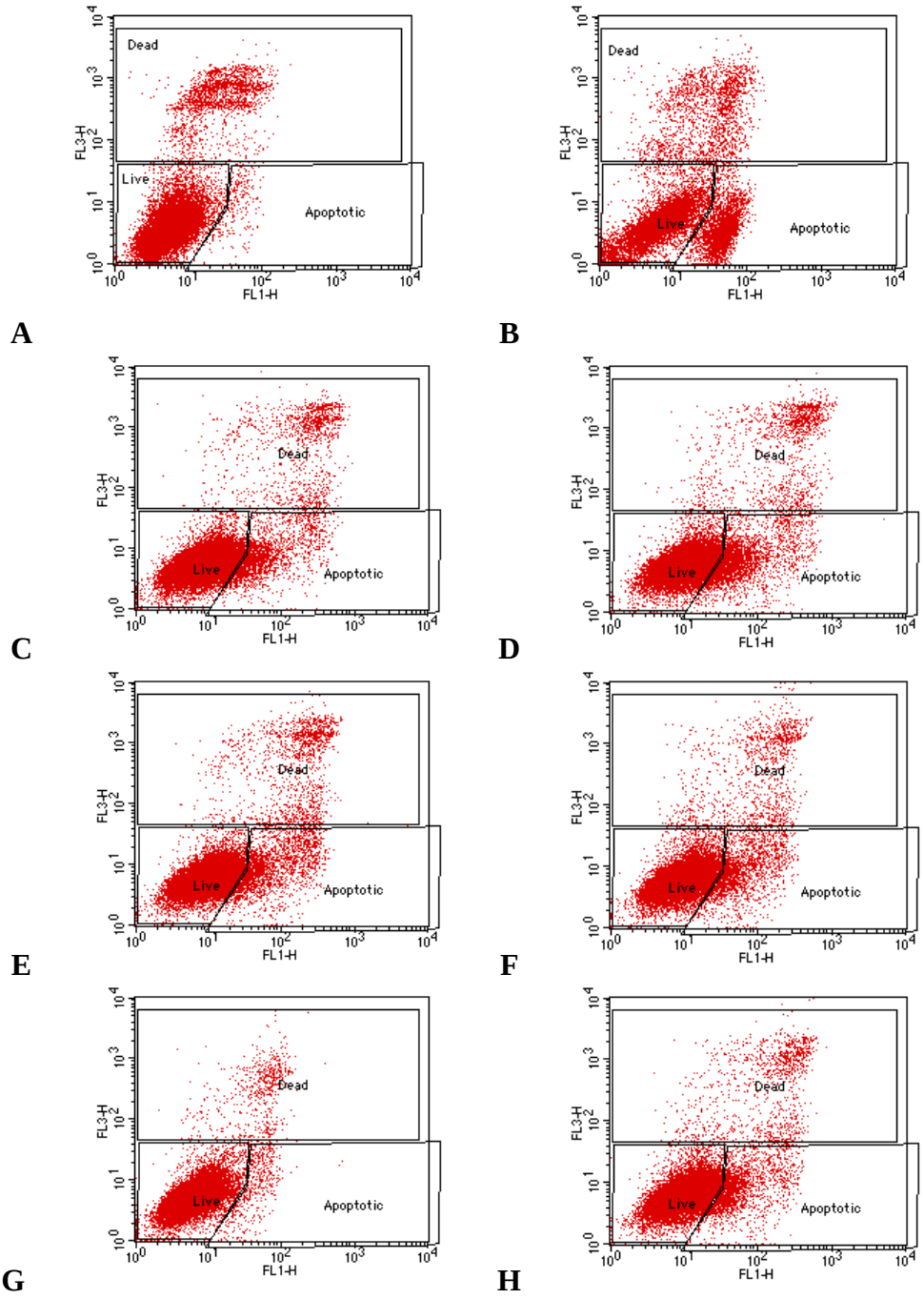


Figure 3.3.1.1 The Annexin-V flow cytometry data showing apoptosis occurring in tumour SNB-19 incubated with 100µM clomipramine

The flow cytometry plot consists of FL-1 data on the horizontal axis: the FL-1 detector shows cells that have the FITC fluorochrome attached and are undergoing apoptosis, against the FL-3 detector which represents leaky (dying cells) that have taken up propidium iodide. The graphs show that compared to the negative control (A) and the positive control (B) there is apoptosis in all samples (C-H respectively. The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

3.5 Discussion

Measuring the potency of chemotherapeutic drugs requires precise and accurate laboratory tests, with awareness of the limitations of the assay (Hanauske 1993). It is also important to choose a cytotoxicity assay suitable for the proposed cell death mechanism (Weyermann, Lochmann et al. 2005). Cytotoxicity assays possess some viability-related features, such as dehydrogenase activity or ATP amount, and they can be used to indirectly measure the metabolic activity. Amongst these, two of them are of highest importance. The MTT and ATP-TCA assays have been used to determine *in vitro* resistance and sensitivity (Wilson, Sargent et al. 1990; Sevin, Perras et al. 1993; Cree, Kurbacher et al. 1996; Kurbacher, Cree et al. 1998; Taylor, Sargent et al. 2001; Furukawa 2004; Nakamura, Saikawa et al. 2006).

Tetrazolium salts have become some of the most widely used tools in cell biology for measuring the metabolic activity of cells ranging from mammalian to microbial origin. With mammalian cells, fractionation studies indicate that the reduced pyridine nucleotide cofactor, NADH, is responsible for most MTT reduction and this is supported by studies with whole cells. MTT reduction is associated not only with mitochondria, but also with the cytoplasm and with non-mitochondrial membranes including the endosome/lysosome compartment and the plasma membrane. The net positive charge on tetrazolium salts like MTT appears to be the predominant factor involved in their cellular uptake via the plasma membrane potential.

However, second generation tetrazolium dyes that form water-soluble formazans and require an intermediate electron acceptor for reduction, are characterised by a net negative charge and are therefore largely cell-impermeable. Considerable evidence indicates that their reduction occurs at the cell surface, or at the level of the plasma membrane via trans-plasma membrane electron transport. The implications of these new findings are discussed in terms of the use of tetrazolium dyes as indicators of cell metabolism and their applications in cell biology (Berridge, Herst et al. 2005).

Cell lines DK-MG and SNB-19 cell lines appeared extremely homogeneous (based on cell size and shape) under phase contrast microscopy (see 2.3.2). A growing body of literature suggests that high passage cell lines and over sub-culturing of cells causes alterations in morphology, response to stimuli, growth rates, protein expression, transfection and signalling (Briske-Anderson, Finley et al. 1997; Chang-Liu and Woloschak 1997; Esquenet, Swinnen et al. 1997; Yu, Cook et al. 1997; Wenger, Senft et al. 2004; Sambuy, De Angelis et al. 2005). It has been proposed that cells in culture are stressed, because of the foreign environment, and immortalisation causes extra stress because artificially forcing cells to express a genetically engineered (possibly non-native) protein such as hTERT or SV40 equals extra energy expenditure and finite energy sources usually used for growth are depleted (resulting in a slower than normal growth rate) (Ryan 2007). Because of the lack of contact with the extra cellular matrix (ECM) vital characteristics such as the α5/β1-integrin and fibronectin interactions are lost (Schiller and Bittner 1995). Therefore it is very difficult to reflect the true *in vivo* conditions in an *in vitro* chemosensitivity assay.

In the MTT assay it was necessary to remove any residual test agent from the cells after the initial 48-hour incubation period and so, as such, there is the potential for removing any necrotic cells that have detached from the monolayer.

3.5.1 The importance of the concentration values depend on the levels of Clomipramine that can be achieved *in vivo*

Data from Weigmann et al. indicates that Clomipramine and its metabolite (Norclomipramine) are rapidly exchanged between blood and the brain. In a study carried out on Sprague-Dawley rats the mean brain concentrations were 12.5-fold higher for Clomipramine and 7.4-fold higher for Norclomipramine than in serum (brain Clomipramine and Norclomipramine levels were 5,593 ng/ml x h and 16,622 ng/ml x h respectively whereas serum Clomipramine and Norclomipramine levels were 447 ng/ml x h and 2,171 ng/ml x h respectively) (Weigmann, Hartter et al. 2000). This study highlighted the need for determination of a steady-state Clomipramine concentration rather than after a single dose, which could be misleading, and would involve administering Clomipramine each day at the same time over a number of days to weeks.

3.5.2 Application of the MTT assay in screening mitochondrially-acting drugs

The principle of the MTT dye is ideally suited when testing a drug such as Clomipramine, which exerts its effect via the mitochondria. However it may be useful with future studies to redesign the assay omitting as many washing stages as possible, because some of the cells which have not adhered may still be viable, but it is vital that the 24 hour recovery period is retained (to ensure that the anti-proliferative effect of the test agent can be determined). This stage also ensures that the test agent has had a true cytotoxic effect, and is not simply acting as a cytostatic agent.

3.5.3 Analysing the Index SUM values

The Index SUM is a natural logarithmic index, ranging from 0 – 600 for inhibition, with zero corresponding to complete cell kill, and 600 equating to no effect. Examination of the frequency histograms for each of the drugs tested shows considerable heterogeneity of chemosensitivity between individual tumours for all drugs tested, with the greatest activity (i.e. lowest Index SUM) seen for Norclomipramine, while the other test agents showed weaker but more variable activity. The ATP-TCA assay relates to cell proliferation via the amount of residual ATP that is quantified by luminescence following detergent lysis of the cells. Because ATP degrades rapidly it will not be detected in any dead cells, thus the measurements taken represent the degree of cell proliferation relative to the negative controls. Stable light-emitting reagents are the best reagents suitable for measuring ATP-converting reactions i.e. assays of metabolites or oxidative phosphorylation. Using ATP as an endpoint is extremely sensitive because measurements can be made down to 1 amol (Lundin 2000).

3.5.4 Comparative activity of agents in glioma cell preparations

Norclomipramine was universally the best test agent when assayed via ATP-TCA and MTT assays. It is the metabolite of Clomipramine; however it is not marketed or sold as a drug in its own right. Nortriptyline, which also showed considerable anti-tumour activity is manufactured and sold as Allegron® and, as such, could be considered as a putative chemotherapeutic option. UPMC was the most sensitive to Norclomipramine (Index SUM 5; IC_{50} 3.24μM) in the ATP-TCA assay which indicates considerable anti-tumour activity. However this tumour had an IC_{50} of 58.97 μM (Index SUM 242) when measured using the MTT assay, again this demonstrates the limits of the IC_{50} value in contrasting and comparing sensitivities.

The cell culture UPMC was derived from a biopsy, taken from a 68-year old female who presented in accident and emergency with left-sided hemi-plegia. The patient died twelve hours after an emergency craniotomy to remove a right front-parietal enhancing tumour. This would suggest an extremely aggressive tumour that suddenly presented. This may explain the lower sensitivity seen in the MTT assay which allows the recovery and proliferation of cells over a 24-hour period, an aggressive tumour such as this would rapidly proliferate within the test wells, and the sensitivity seen in ATP-TCA where cells are not allowed to recover before lysing the cells.

Amitriptyline was only observed to have anti-tumour activity in UPMC (Index SUM 274; IC_{50} 38.86) via ATP-TCA and UPAB (Index SUM 204; IC_{50} 21.57) via MTT assay. Again it is interesting to note that a high grade GBM is sensitive to agents. All other agents tested (Imipramine, Promethazine, Chlorpromazine and Doxepin) showed anti-tumour activity in some of the cell cultures with no pattern observed relating to passage number or histological classification. The data shows them not to be as effective as Norclomipramine and Nortriptyline at the concentrations tested, but Clomipramine, Amitriptyline, Promethazine, Chlorpromazine and Doxepin are parent drugs and as it will be predominantly the parent drug's metabolic product which will act *in situ* on the brain (as it does at a direct cellular level in the *in vitro* situation) it is more important to study the metabolic products. In no cell cultures did these drugs have no observed effect at all. This data shows the potential of all eight tricyclic test agents for use in the malignant glioma. In a clinical setting it would be clinically useful to test the patients' tumour cells with the panel of all eight drugs to ensure that the most effective agent is selected.

Of the five malignant glioma tested, the two established cell lines (SNB-19 and DK-MG) had the lower apoptotic threshold, with a significantly higher percentage of apoptotic cells present at 60µM Clomipramine and above. The three early passage cultures, developed 'in-house' from biopsy, had higher apoptotic thresholds, withstanding up to 100µM Clomipramine incubation for six hours.

The normal human astrocytes, tested in parallel, demonstrated that Clomipramine did not cause cell death at the concentrations tested. The neoplastic cells at the highest passage number (DK-MG) were responsive to Clomipramine in both the MTT and ATP-TCA assays; this could be due, in part, to the homogeneity of the sample population. Also, it is of interest to note that the Clomipramine was less effective at causing apoptosis in the CC-2565 non-neoplastic astrocyte cell line than Staurosporine (Table 3.3.5).

3.5.5 Results obtained for Amitriptyline Hydrochloride and Nortriptyline Hydrochloride by a supervised project student

During the course of the project, a Pharmacy Masters student (Ahmed, T: "The effect of Amitriptyline Hydrochloride on glioma cell line IPSB-18" 2005) was supervised in performing MTT assays on Glioblastoma multiforme (established) cell line IPSB-18. The results show that the IC_{50} for Amitriptyline was 71.04 µM and the IC_{50} for Nortriptyline was 61.25 µM. The values obtained by the student are higher than those obtained in this study on eatablished cell lines DK-MG and SNB-19, however IPSB-18 was serially passaged over two hundred times and this may influence the ability to undergo apoptosis. The values also agree that a GBM (IPSB-18) was sensitive to tricyclic antidepressants and show promise for the use of tricyclic antidepressants in glioma therapy. The drawbacks of this study were few repetitions of the drug concentrations (although they were assayed in triplicate) and different concentrations were used to those in this study (0.5 – 1000 µM). The results are extremely limited, however they do support the findings of this thesis.

3.5.6 Technical considerations and future directions of chemosensitivity testing

When designing an *in vitro* assay it is vital to ensure that the microenvironment replicates that of the tumour *in situ* as closely as possible. The principle behind the ATP-TCA assay is that by using a serum-free complete assay media only the growth of tumour cells, rather than non-neoplastic cells, will be supported after six days in culture. Normal human astrocyte growth is supported by the presence of serum (Freshney 2005), as is the growth of microglia when propagating early passage cultures or cell lines. When subsequently transferred to serum-free conditions both these cell types can survive for only a matter of days. Moreover, the use of human serum supplementation (rather than foetal bovine serum or foetal calf serum) not only alters growth rate but also changes the adhesive properties and antigenic expression of neoplastic cells (Vierck and Dodson 2000).

The TNF family of inflammatory mediators, in particular, can induce caspase-independent and caspase-dependent pathways to programmed cell death (Lange, Thon et al. 2005). The presence of inflammatory mediators in culture could potentially have a pro-apoptotic effect. It is for this reason that the ATP-TCA assay could be further developed with the addition of M-CSF to the serum-free culture media. This would support the survival of microglia and give a more accurate representation of induction of apoptosis *in vivo*.

Standard brain tumour cell culture conditions are not ideal (pH and D-glucose concentration in the culture medium) and the MTT response may vary greatly in viable cells due to the metabolic state of the cells (and the cellular concentration of the pyridine nucleotides). Furthermore it has been found that certain drugs, such as genistein, inhibit tumour cell growth but increase the production of formazan, giving false negative results so care must be taken when assaying new agents (Hanauske 1993; Pagliacci, Spinozzi et al. 1993).

Developing a 'gold standard' chemosensitivity assay for use in brain tumours needs to assess the effect on both metabolic activity and proliferation because of the ability of neoplastic glial cells to 'switch' phenotype from proliferative to invasive cell. A test agent that appears effective via ATP-TCA assay may have the ability to initiate cell death in an experimental plate but *in vivo* may in fact signal tumour cells to switch to an invasive phenotype and migrate away from the area of chemotherapeutic assault. Since it is impossible to determine the cellular proliferation of primary human tumour cells *in vivo* an appropriate biomarker would need to be employed.

Measuring the effect of a drug on cells *in vitro* is a tenuous issue due the number of events that take place. Traditional assays monitor one aspect of cellular function whereas the true picture *in vivo* encompasses many events taking place at the same time. The MTT and ATP assays are metabolic assays and do not reflect the ability of cells to proliferate, but it is possible to use a proliferative marker. By monitoring the metabolic status of cells, via ATP levels or succinate dehydrogenase activity, it is possible to determine cell viability.

Whilst ATP is a highly sensitive endpoint it does not reveal the degree of proliferation subsequent to drug exposure and a huge drawback is that dead cells and cell extracts can be metabolically active. This may explain, in part, the results from the MTT and ATP-TCA assays showing that the parent drugs were more potent than their metabolites in the MTT assay whereas the reverse was observed in the ATP-TCA assay. In contrast, by measuring a proliferative marker such as Ornithine decarboxylase (ODC) it is assumed that viable cells are actively proliferating which both *in vitro* and *in vivo* is not necessarily the case. Neoplastic glial cells are capable of senescing or even switching phenotype to that of an invading cell, in response to an external insult (Gratsa, Rooprai et al. 1997). Indeed this is one of the main reasons for the failure of chemotherapy in brain tumour patients, single cells are capable of migrating away from the main tumour site and establishing themselves in an area of intact B-BB. Many patients are treated with ineffective agents, exposed to unnecessary side-effects and confer no actual benefit because the resistance to chemotherapeutic agents poorly characterised (Jackson 2007). ODC is a universal marker expressed early in the cell cycle, with a short half-life that can be used to detect cell proliferation and would be useful as a supplementary marker in the MTT and ATP-TCA assays. This enzyme catalyses the conversion of Ornithine into the diamine putrescine, which is the precursor for the synthesis of the naturally-occurring polyamines (Russell 1985; Cohen 1998). Because of the ATP-TCA assay protocol, which lyses cells within the test plate and then takes an aliquot for analysis it is possible that the remaining cell lysate could be used to measure the levels of ODC.

The MTT and ATP-TCA assays were found to be significantly correlated by two groups (Hazary, Chaudhuri et al. 2001; Ulukaya, Ozdikicioglu et al. 2008) and conversely were reported to show no correlation (Kerokoski, Soininen et al. 2001). Moreover, it was reported that whilst ATP increased in the first three hours of analysis, the reduction of MTT decreased immediately (Liu, Song et al. 2003). Both assays reflect different aspects of the metabolic status of the cells but they can still report different outcomes depending on the time point because of the possibility of drug targets (energy sources, signalling molecules and DNA). According to the results of Ulukaya et al. cells are able to lose their ATP content but still retain some dehydrogenase activity (Ulukaya, Ozdikicioglu et al. 2008). This might further explain the observation that the parent drugs were more potent than their metabolites in the MTT assay. The ATP assay was found to be able to detect lower numbers of cells compared to the MTT assay (Petty, Sutherland et al. 1995; Eirheim, Bundgaard et al. 2004). The next steps in this research are to combine Clomipramine with other potentially synergistic agents to enhance the apoptotic effect. Indeed the relationship between Clomipramine and Dexamethasone in a dose-dependent manner have recently been reported in neoplastic glia (Higgins and Pilkington 2010).Moreover, it may also be possible to isolate cancer stem cells and/or other ‘clones’ from heterogeneous primary glioma to test the resistance of such subpopulations as well as to investigate the influence of hypoxia on drug activity.The difference in sensitivities between glioma, in this small study, indicates the importance of analysing early passage cultures, which retain original morphology and characteristics to a greater extent, alongside cell lines transformed for survival (Baguley and Marshall 2004).

4 The influence of cytochrome P450 genotype on chemosensitivity of glioma

4.1 Introduction

Individualising therapy for patients being treated with chemotherapeutic agents is now regarded as the most critical goal in cancer chemotherapy. Being able to stratify patients as to which agents will work to best effect in each individual will reduce the needless administration of broad-spectrum, highly toxic and debilitating drug regimens. The planning of treatment modalities for patients with high-grade glioma is a challenge harder than that of any other cancer due to the extreme vulnerability of the CNS.

Many sources of inter-individual differences exist in drug toxicity and efficacy, both pharmacokinetic and pharmacodynamic, as highlighted in Figure 3.5.6.1. These differences include body mass and composition, age, ethnicity and sex as well as disease state and organ function. Environmental factors include interactions with food, drinks, environmental toxins, other drugs (prescription, over the counter and herbal formulations), as well as interactions with drug formulation components (Deeken, Figg et al. 2007).

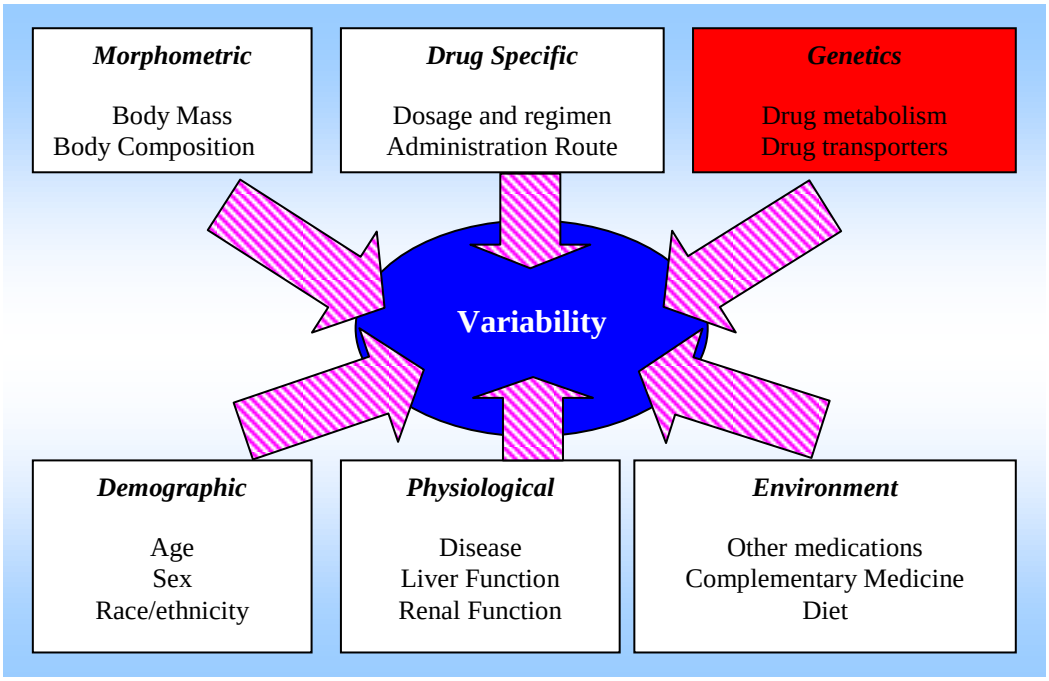


Figure 3.5.6.1 Sources of inter-individual variability in brain tumour patients

There are six main areas of variability in brain tumour patients which can affect the outcome of a treatment regimen. The factors involved in genetic variation, drug metabolism and drug transporters, are investigated in this chapter and chapter 6.

There are many factors affecting the efficacy of drug therapies, see Figure 3.5.6.1 Sources of inter-individual variability in brain tumour patients, however of particular relevance to this study are the genetic factors involved in heterogeneity, both drug metabolism (in particular the cytochrome p450 family of enzymes) and drug transporters (the SLC6A2 norepinephrine transporter; NET, see chapter 5). It is possible to examine specific mutations in the genes encoding the CYP enzymes that degrade tricyclic antidepressants, to see if there is loss or alteration in the drug metabolising capacity of a patient. Drug metabolising enzyme (DME) mutations in the genome can determine how well an individual patient will tolerate tricyclic antidepressant chemotherapy.

4.1.1 Genotyping CYP family enzymes responsible for tricyclic antidepressant metabolism in glioma patients

The Cytochrome P450 (CYP450) family of enzymes is expressed in many tissues, with the largest activities found in the liver. By conducting oxidative metabolism on many endogenous substrates and over ninety percent of drugs, their function is of vast clinical relevance and they have therefore been the focus of much research over the past decade (Deeken, Figg et al. 2007). Genetic variants, SNPs, exist in these enzymes, and significant alterations in drug metabolism, efficacy and toxicity can occur owing to these differences. The CYP superfamily consists of more than sixty enzymes that have been grouped into several families and subfamilies. Of interest to this particular study are the enzymes CYP2D6 and CYP2C19.

The isoenzymes which catalyse the polymorphic hydroxylations of debrisoquine and S-mephenytoin are cytochromes P450 2D6 and P450 2C19 (CYP2D6 and CYP2C19), respectively (Daly, Cholerton et al. 1993; Bertilsson 1995).

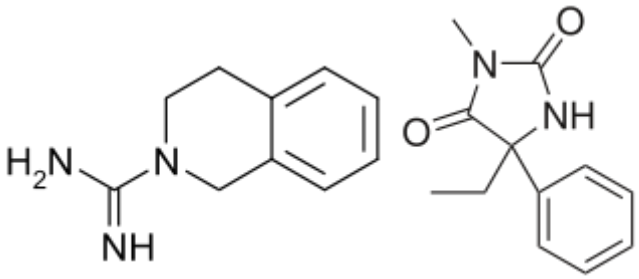


Figure 4.1.1.1 Debrisoquine (specific for CYP2D6) and S-mephenytoin (specific for CYP2C19) are reference compounds that define P450 active site specificity

CYP2D6 is involved in the stereospecific metabolism of several important groups of drugs, including the tricyclic antidepressants Clomipramine and Amitriptyline (Scordo, Caputi et al. 2004). CYP2D6 has a significant role in the metabolism of many lipophilic agents such as the common antidepressants (Clomipramine, Amitriptyline, Desipramine, Paroxetine and Fluoxetine) the antipsychotics (Haloperidol, Risperidone and Thioradazin), beta blockers (Carvedilol, s-Metaprolol, Propafenone and Timolol), antiarrhythmics (Flecainide, Lidocaine) as well as some antiemetics (such as Ondansetron).

Known inhibitors of CYP2D6 include Cimetidine, Fluoxetine, Haloperidone, Paroxetine, Methadone and Quinidine (Finn, Purnell et al. 1996). It is pertinent that Dexamethasone, which is used to treat oedema in patients with glioma, and Rifampin can induce enzymatic activity (Ingelman-Sundberg 2005). The gene encoding CYP2D6 is located on 22q13, thus far there have been fifty-eight allelic variants described. Including allelic subtypes there are over one hundred known variants, including twenty-two null alleles. Approximately 71% of the CYP2D6 alleles are functional in Caucasians, whereas 26% are non-functional. This is compared with Asians where only 50% of the alleles are functional. This may be due to the close proximity of the gene to two pseudogenes (CYP2D7 and CYP2D8), as well as the increase in relative activity of the locus with other CYP genes (Ingelman-Sundberg 2005). In addition to these variants CYP2D6 gene duplication, which results in ultra rapid metabolism, has been identified in different ethnic groups at high frequencies (Swedish 2%; German 3.6%; Spanish 7-10%; Italian 10%; Saudi Arabians 20% and Ethiopians 29%) (Smith 2004).

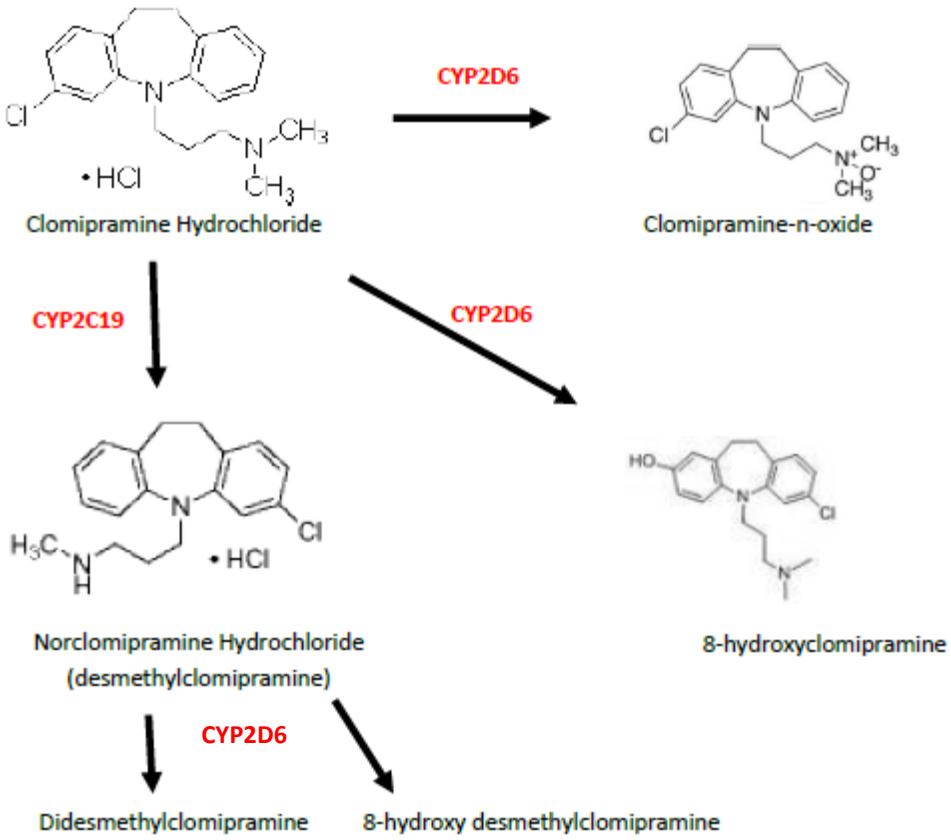
CYP2C19 is involved in the metabolism of some antidepressant and anti-anxiolytic drugs. The metabolism of Amitriptyline, Citalopram, Clomipramine, Diazepam, Imipramine and Moclobemide is catalysed by CYP2C19 (Carrillo, Dahl et al. 1996). CYP2C19 is expressed exclusively in the liver and duodenum (Totah and Rettie 2005). Commonly used acidic drugs that are metabolised by this enzyme include the proton pump inhibitors (Propranolol, Lansoprazole, Omeprazole and Pantoprazole), the antiseizure medications (s-mephenytoin and Diazepam) as well as Amitriptyline, Citalopram, Proguanil, Propranolol and Teniposide. Drugs known to inhibit enzymatic activity include Fluoxetine, Lansoprazole, Omeprazole and Ticlopidine (Flockhart 2007). When selecting agents such as the tricyclics to target the mitochondria of glioma cells, it is important to consider the interactions of any prophylactic agents that a person with a brain tumour may already be prescribed, such as Propranolol. Propranolol acts via the mitochondrial membrane, is an antagonist of norepinephrine and is metabolised via the same enzyme as Amitriptyline so, for example, should these two such agents be taken together might have reduced efficacy due to competition (Yoshimoto, Echizen et al. 1995; Naritomi, Teramura et al. 2004).

The gene encoding CYP2C19 is located on 10q24, combinations of twenty-three different single nucleotide polymorphisms (SNPs) in exon encoding regions comprise twenty-five allelic variants (Desta, Zhao et al. 2002). The CYP2C19*2 and *3 variants both notable for leading to premature stop codons and premature truncations of the protein, resulting in a completely inactive enzyme.

There is a high frequency of the CYP2C19*2 variant in African-Americans, Caucasians and Asians, whereas the CYP2C19*3 is less frequently observed. It is estimated that the CYP2C19*2 accounts for approximately seventy-five to eighty-five percent of poor metabolisers in Asians and Caucasians.

The CYP2C19*5 and CYP2C19*7 variants lead to translation of inactive enzymes, whereas the CYP2C19*6 and CYP2C19*8 variants have two and nine percent of the wild-type version, respectively (Goldstein 2001). The mutations seen in a population cause it to be polymorphic; the site on a chromosome at which an individual base differs is termed a SNP.

Plasma metabolite/drug ratios differ extensively according to metabolic phenotype and administration route. An example of this phenomenon is with the drug Risperidone where the plasma metabolite to drug ratio is thirty-fold lower in CYP2D6 poor metabolisers, compared to ultra rapid metabolisers, and four-fold lower after intramuscular compared to oral administration (Hendset, Haslemo et al. 2006). The major route of biotransformation of the parent drug Clomipramine is demethylation via the cytochrome P450 liver enzymes to active metabolite Norclomipramine (Desmethylclomipramine). Norclomipramine is then further hydroxylated to 8- hydroxyl Desmethylclomipramine and Di-desmethylclomipramine. Clomipramine is also hydroxylated to 8-hydroxy-clomipramine, via CYP2D6 (Yokono, Morita et al. 2001). The hydroxylation of secondary amine TCAs is catalysed by CYP2D6 (Sjoqvist and Bertilsson 1986) and the n-demethylation of Clomipramine is catalysed by several enzymes including CYP2C19 (Nielsen, Brosen et al. 1994).



4.1.1.2 The metabolism of clomipramine hydrochloride and CYP gene involvement

The parent drug, Clomipramine hydrochloride, is metabolised into three secondary amine structures. Norclomipramine is the most abundant of these and is further metabolised into two tertiary structures. Clomipramine is metabolised preferentially, but not exclusively by, CYP genes 2D6 and 2C19.

It may be possible to predict the capability to metabolise Clomipramine, and its more active metabolite Norclomipramine (because of the higher binding affinity for the norepinephrine transporter), via genotypic screening of the two CYP genes, CYP2D6 and 2C19. Whilst many studies have been carried out on screening the allele and genotype frequencies of the CYP2D6 and CYP2C19 genes in various populations (Wanwimolruk, Bhawan et al. 1998; Yamada, Dahl et al. 1998; Bathum, Skjelbo et al. 1999; Britzi, Bialer et al. 2000; Dandara, Masimirembwa et al. 2001; Tamminga, Wemer et al. 2001; Yokono, Morita et al. 2001; Bozina, Granic et al. 2003; Herrlin, Yasui-Furukori et al. 2003; Luo, Aloumanis et al. 2004; Scordo, Caputi et al. 2004; Halling, Petersen et al. 2005) only one study by Norguchi et al. (1993) reports that higher plasma concentrations of Clomipramine and lower plasma concentrations of Norclomipramine predict better clinical outcome in Japanese patients with depression despite the fact that Norclomipramine is the active metabolite of Clomipramine. This variation could be due to the Japanese diet which is richer in fish than red meats (possibly causing a more alkaline pH) and significantly differs from that of the Western continents.

Yokono et al. have also studied the metabolism of Clomipramine in a population of Japanese psychiatric patients (Yokono, Morita et al. 2001) but to date no studies have been performed on the correlation of CYP2D6, CYP2C19 and clinical effect of tricyclic antidepressants in cancer patients.

By screening a small group of glioma patients already taking Clomipramine and genotyping them for expression of the CYP2D6 and CYP2C19 alleles and pairing the data with that for concentration of Clomipramine (and its metabolite) in their plasma, it may be possible to see if genotype determines ability to metabolise Clomipramine. If the results show that genotype does correlate with the extent of parent drug metabolised then it might be possible, in brain tumour patients who would like to start taking Clomipramine, to predict how they will tolerate Clomipramine before it is prescribed and adjust the dose accordingly to minimise side effects. The side effects listed in the British National Formulary (BNF) are: arrhythmias and heart blockade, convulsions, hepatic and haematological reactions, drowsiness, dry mouth, blurred vision, constipation, urinary retention, hyponatraemia and neuroleptic malignant syndrome.

After oral administration some 50% of a dose of Clomipramine undergoes pre-systemic metabolism to Norclomipramine (Desmethylclomipramine) and secondary metabolite 8-hydroxy Desmethylclomipramine (see figure 4.1.1.2). The mean plasma elimination half-life of Clomipramine is twenty-five hours (range 20 – 40 hours). The half-life of Norclomipramine is somewhat longer (as discussed previously in 3.3.1) and thus this latter compound accumulates in plasma during chronic therapy.

When designing drugs for use in brain tumour patients, the primary consideration should be the pharmacokinetic profile because ultimately this contributes to the success or failure of a compound. As previously indicated in Figure 1.7.5.1, newer agents developed for use in glioma are interactive but the high specificity achieved by such compounds results in a lower efficacy. Thus, many drug companies are carrying out rational high-throughput drug metabolism and drug transport screening. Attention is now being focussed on optimising the pharmacokinetic profiles of drug candidates using transporter function (Ayrton and Morgan 2001; Mizuno and Sugiyama 2002).

4.2 Methodology

4.2.1 Clomipramine Questionnaire (LREC04-066)

This questionnaire was designed to obtain information on potential variations in chemotherapy, prophylactic medication, herbal supplements and lifestyle from the patients taking part in the study. It was important to know the body mass of the patient and the daily dose of Clomipramine in order to be able to correct the plasma concentrations of the antidepressant to a universal unit that can be compared between patients.

Other factors can affect the half-life and metabolism of an antidepressant, so information on age, other medication (anticonvulsants, steroids) and if they smoke or not was relevant to the study.

The patients were asked if they experienced any side effects, however this is a difficult factor to assess since something that is perceived as a side-effect by one person goes unnoticed in another. The other point to note is that in brain tumour patients the side effects could be due to any number of things (location of the tumour, other medication) and not a result of taking Clomipramine. Clomipramine, as recommended in the BNF, is taken via a dose escalation starting at 25mg/day. The recommended daily dose for depression is 30-250 mg/day (British National Formulary. Higher levels can be tolerated but Clomipramine has a narrow therapeutic range and when tested in 151 patients (at 25, 50, 75, 125, and 200 mg/day) the dose-effect curves were flattened, which suggests a disproportionate increase with dose (Gram 1999).

Please answer as many of the questions as you can, we want to account for all the differences between individuals.

Surname: **First Name:**

DOB: **Approx. Weight:**

Approx. Height: **Smoker?:** **YES/NO**
Ethnic Origin:
Units of alcohol per week:
(1 unit = half a pint of beer, 125mls wine, 25mls spirit)

How long have you been taking Clomipramine?

What dose are you currently on?

Have you always been on this dose? YES/NO

If no, please detail (if possible) when you last changed it, who recommended the change and why e.g. *Changed dose from 150mg/day to 100mg/day in July 2004 because of increase in seizures, recommended by GP*

Are you taking an anticonvulsant? If yes, please give details below

Are you taking any other medication? E.g. Dexamethasone

Do you currently experience any side effects? If yes, please give details below

Signature: Date:

4.2.2 High Pressure Liquid Chromatography – Mass Spectrometry (HPLC-MS.MS) analysis of the plasma concentration of Clomipramine and Norclomipramine

Plasma was obtained from whole blood collected into Lithium/Heparin sample tubes. Samples were stored at 4°C prior to analysis. If analysis was delayed then the plasma samples were stored at –20°C. Prior to analysis the plasma samples were defrosted and gently mixed before a 100µl aliquot was pipetted into an appropriately labelled microcentrifuge tube. Calibrators (Chromsystems calibrator standard for TCAs), quality controls (Chromsystems control for TCAs) and a blank (fresh frozen plasma) were prepared in the same manner. After adding 200µl working diluent/internal standard to each sample the tubes were vortexed, flicked to break up any clumps and then vortexed once again. All samples were then microcentrifuged for five minutes at 10,000 rpm. Subsequently 100µl supernatant was pipetted into appropriately labelled analysis vials with crimp caps. The samples were loaded onto the autosampler of a Waters Symmetry 300 C18 5µm 2.1 x 150mm column (WAT106172) with guard column (Phenomenex C18 Security Guard KJO-4282). The 'blank' was loaded into position one followed by the calibrant, controls and then patient samples. The analysis consisted of reverse-phase liquid chromatography, with detection by triple-quadrupole mass spectrometry. This is highly specific with the first quadrupole selecting ion masses equal to that of the ammoniated analyte ion ($M + NH_4^+$). Nitrogen as a collision gas produces daughter ions, which are selected in the second quadrupole. A third quadrupole selects only ions of the mass of a significant fragment of the analyte. Internal quality controls were analysed using QCPlus and deemed acceptable before the results were reported. The concentrations were reported to the nearest whole number.

4.2.3 Calibrating the BioRad iQ Real Time Detection System to detect a drug metabolism genotyping assay

To enable the detection of more than one fluorophore it was necessary to calibrate the i-Cycler using a standard kit, the calibration kit was used according to the manufacturer's instructions.

In brief, an external well factor plate was run (to calculate background) using the pure dye calibration protocol followed by a 96-well plate loaded with ten wells (B2-B11; C2-C11) of each of the two dyes (FAM™ and VIC™). The Genotyping Assay, for which this calibration was carried out, required multiplex PCR for two CYP genes using the fluorophore FAM™ (Excitation 490/20X; Emission 530/30M) and VIC™ (Excitation 530/30X; Emission 575/20M), however since a VIC™ calibrator solution was not available from the manufacturer it was recommended to use a fluorophore on the same excitation and emission spectra, in this instance JOE™.

4.2.4 A Taqman Drug Metabolism Genotyping Assay to detect mutations in the CYP2D6 and 2C19 alleles responsible for the metabolism of Clomipramine hydrochloride

The Taqman 5' nuclease assay from Applied Biosystems is a SNP genotyping system that enables rapid screening of patient samples via qRT-PCR. PCR amplification of genomic DNA with allele-specific primers enables rapid prediction of patient drug metabolising genotype and may lead to the adjustment of chemotherapeutic dose in the individual patient. Allelic discrimination using this method is based on the design of two TaqMan probes, specific for the wildtype allele and the mutant allele in a two-allele system. Each of the two probes is labelled with a different fluorescent reporter (in this case FAM™ and VIC™), and each is designed with the gene mutation affecting the middle part of the probe sequence. FAM™ is a green emitting fluorescent reporter tag that has an excitatory wavelength of 490nm and emission wavelength of 530nm, VIC™ is a yellow emitting fluorescent reporter tag that has an excitatory wavelength of 530nm and an emission wavelength of 575nm. When using two reporter tags it is important to choose two that emit at different wavelengths, so that they can be discriminated from each other. Well factors were collected before each run using iCycler iQ external well factor solution (Bio Rad) because collection of well factor data optimises fluorescent data quality and analysis in multiplex PCR.

The finger-prick blood samples of seventeen brain tumour patients (see Table 4.2.2 for tumour types and grades), who had been taking clomipramine for a minimum of two weeks, were processed as described in section 2.3.13 and the genomic DNA (gDNA) quantified as described in section 2.3.14. After vortexing all samples, the DNA was plated at a concentration of 40ng complementary DNA (cDNA) per well according to the worksheet and analysed via quantitative Real Time PCR qRT-PCR) (section 2.3.23).

Each PCR reaction had a final volume of 25µl comprising of 11.25µl of gDNA (diluted with the appropriate volume of nuclease-free water) and 13.75µl of the Applied Biosystems Taqman Drug Metabolism Genotyping Assay (CYP2D6*2X3 C_27102414_10; CYP2C19*2A C_1329163_10) to detect the SNPs involved with alleles 2X3 and 2A, respectively, which had been diluted tenfold with Taqman Universal Master Mix (without Amperase UNG).

The two assays chosen for preliminary analysis were for alleles CYP2D6*2X3 and CYP2C19*2A with minor allele frequencies in Caucasians of 0.46 and 0.15 respectively. Because readymade SNP assays are only recently available from Applied Biosystems there are a limited number of assays available, especially for 2C19. Because of the high cost involved these two were chosen to be the most relevant in this study. In a clinical setting, genotyping for one allele only would be of limited use. Instead one would need to screen patients for all alleles related to the CYP gene in question, taking into account ethnicity and minor allele frequencies.

The context sequence representation for the mis-sense mutation seen on allele CYP2D6*2X3 was AGCACAAAGCTCATAGGGGGATGGG[C/G]TCACCAG GAAAGCAAAGACACCATG and for the silent mutation on allele CYP2C19*2A was GGAGAGGAAAACTCCCTCCTGGCCC[C/T]ACTCCTCT CCCAGTGATTGGAAATA. This means that for CYP2D6 the SNP [C/G] can be read as C = wildtype allele 1 (VIC) and G = mutant allele 2 (FAM). For CYP2C19 the SNP [C/T] can be read as C = wildtype allele 1 (VIC) and T = mutant allele 2 (FAM). Forward and reverse primers were designed around the context sequence (the primer design information is not available to end-user), the forward primer meant that the probe bound to the same strand as the forward primer and the reverse primer caused the probe to bind to the same strand as the reverse primer.

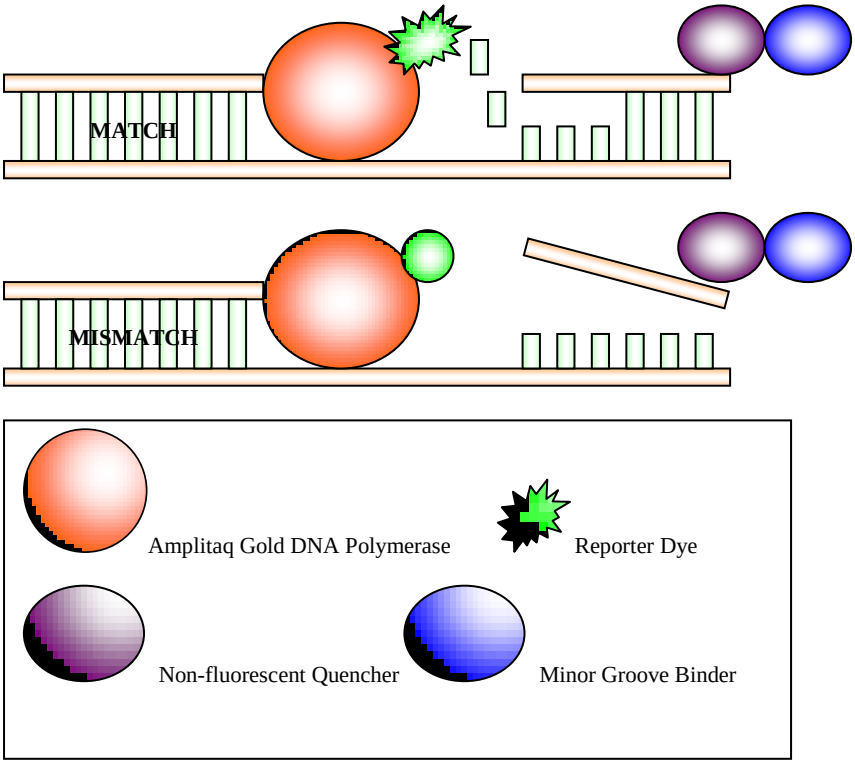


Figure 4.2.4.1 The principle behind the Taqman genotyping results for identifying mutations in the alleles of CYP2D6 and 2C19

The figure above shows results from matches and mismatches between target and probe sequences in TaqMan Drug Metabolism Genotyping Assays. In this case the target is mutations in CYPs 2D6 and 2C19. The major groove binder binds to its complementary sequence between forward and reverse primer sites. When the oligonucleotide probe is intact, the proximity of reporter dye to quencher dye results in quenching of reporter fluorescence by Forster-type energy transfer (FRET). The AmplitaqGold DNA Polymerase extends the primers bound to the genomic DNA template and cleaves only probes that are hybridised to the target. Cleavage separates the reporter from the quencher and results in a fluorescence increase;, this means the allele is present. Even a single nucleotide mismatch between the probe and target can reduce the efficiency enough to reduce the degree of reporter dye cleavage, and in turn the fluorescence.

The major groove binder increases the melting temperature (T_m) for a given probe length which allows the design of shorter probes. The greater difference in T_m values between matched and mismatched probes provides extremely robust allelic discrimination. Product amplification was performed up to forty cycles on a Biorad i-Cycler instrument (Biorad Laboratories, UK) after polymerase activation (10 minutes at 95^{o}C).

	Sample	cDNA ng/ µL [nano-drop]	cDNA ul/well	Total Vol µL / plate (a)	Promega water (b)
Control	**Control**	13.14	***3.0***	***21.3***	**57.4**
S1	**ANEC001**	9.37	***4.3***	***30.1***	**48.6**
S2	**ANEC010**	20.65	***1.9***	***13.6***	**65.2**
S3	**ANEC011**	7.84	***5.1***	***35.7***	**43.0**
S4	**ANEC012**	8.61	***4.6***	***32.5***	**46.2**
S5	**ANEC013**	28.57	***1.4***	***9.8***	**68.9**
S6	**ANEC014**	8.72	***4.6***	***32.1***	**46.6**
NTC CONTROL		0	***0.0***	***0.0***	**78.8**

Table 4.2.1 Calculation of the cDNA/nuclease free water dilution to ensure that all test wells had 40ng of template

The template was calculated for six wells plus one extra well to take into account any pipetting error. Samples were plated inside a dedicated class II laminar flow cabinet using DNAse/RNAse free consumables to ensure no genomic contamination occurred and a no-template control (mastermix, probe and nuclease-free water only) was used to double check that no contamination was present.

	Tumour Type	Age	Sex	Mass (kg)	Dose of Clom (mg/day)	Time on Clom	Other medication	Perceived Side Effects	Smoker	Ethnic Origin
ANEC001	Oligodendroglioma Grade II	39	M	78.00	150.00	2 years	Keppra, Tegretol	Lethargy, insomnia, weight gain	No	White British
ANEC004	Glioblastoma multiforme Grade IV	42	F	62.00	25.00	6 months	Keppra, Tegretol, PCV	Lethargy	No	White British
ANEC008	Oligodendroglioma Grade III	41	F	57.15	150.00	5 years	Tegretol	Dry mouth, hot flushes, low libido	Yes	White British
ANEC010	Oligodendroglioma Grade II	41	F	63.50	100.00	3.5 years	Tegretol, Keppra	Increase in partial seizures	No	White British
ANEC011	Glioblastoma multiforme Grade IV	54	F	55.00	150.00	4 years	Phenytoin	Low libido	No	White British
ANEC012	Metastatic breast adenocarcinoma	63	F	60.30	100.00	1.5 years	Epanutin, Dexamethasone, Ranitidine	Mild seizures	No	White British
ANEC013	Glioblastoma multiforme Grade IV	30	M	103.00	150.00	18 months	Dexamethasone, Phenytoin, Omeprazole	Shaking hand	No	White British
ANEC014	Astrocytoma Grade II	60	M	92.00	150.00	15 months	Epanutin, Dexamethasone, Simvastin, Ramipril	None	No	White British
ANEC015	Glioblastoma multiforme Grade IV	54	M	80.20	150.00	23 months	Phenytoin	Dry mouth, lethargy	No	White British
ANEC016	Glioblastoma multiforme Grade IV	45	F	45.00	100.00	3 weeks	Tegretol, Dexamethasone, Zoton, Domperidone	Dry mouth, constipation	No	White British
ANEC018	Oligoastrocytoma Grade III	36	M	82.50	150.00	8 months	Celebrex, Tamoxifen	None	No	White British
ANEC019	Glioblastoma multiforme Grade IV	59	F	60.00	75.00	18 months	Epilim	Shaking hands, impaired speech	No	White British
ANEC020	Gemistocystic Astrocytoma Grade IV	37	F	65.00	150.00	18 months	Tegretol, Iscador, Multivitamins	Dry mouth, lethargy	No	White British
ANEC021	Brain Stem Glioma Grade IV	40	M	76.20	75.00	18 months	None	None	No	White British
ANEC022	Gliomatosis cerebri	33	M	97.00	100.00	2.5 years	Tegretol, Dexamethasone, Glidazide, Pantoprazole	Slight twitching, weight gain	No	White British
ANEC024	Glioblastoma multiforme Grade IV	37	M	72.00	150.00	3.5 months	Phenytoin, Keppra, Dexamethasone, Temozolomide	Dry mouth, excessive sweating	No	White British
ANEC028	Glioblastoma multiforme Grade IV	73	M	80.00	150.00	4 weeks	Dexamethasone, Ranitidine, Simvastatin	None	No	White British

Table 4.2.2 Patient variability data obtained by questionnaire

4.3 Results

4.3.1 HPLC & CYP Genes in glioma patient plasma samples

The relationship between plasma Clomipramine and Norclomipramine concentrations and therapeutic effect have not previously been clearly defined, although for chronic antidepressant therapy a target range (Clomipramine + Norclomipramine) of 150 – 450ng/ml is used. After acute over dosage, plasma Clomipramine above 1000ng/ml (Norclomipramine 2000ng/ml) may be associated with serious toxicity in individual patients (Westenberg, De Zeeuw et al. 1977). The results in Table 4.3.1 below are the mean plasma concentrations calculated from four individual blood samples taken from each patient, once a month for four months expressed in ng/ml. The results show fluctuations in plasma levels of both parent drug and metabolite, and the degree of variation is represented by standard deviation values. Using ANEC008 and ANEC013 as an example, as they have particularly high standard deviation values for both Clomipramine and Norclomipramine, it is interesting to note that ANEC008 is taking Tegretol (Carbemazepine) and ANEC013 is taking a proton-pump inhibitor (Omeprazole). Carbemazepine and Omeprazole are both metabolised via CYP2C19 (also responsible for the major demethylation route of Clomipramine) and it might be possible that they are competing at the microsomal CYP enzyme level.

	Clomipramine (ng/ml)			Norclomipramine (ng/ml)			Total (ng/ml; 150-450ng/ml)	Total (µM)
ANEC001	67.84	±	12.47	150.80	±	30.78	218.64	0.64
ANEC004	155.00	±	25.52	239.50	±	97.66	394.50	1.12
ANEC008	111.83	±	45.23	290.25	±	122.97	402.08	1.14
ANEC010	26.00	±	3.40	67.50	±	9.65	93.50	0.27
ANEC011	104.00	±	38.70	229.00	±	88.40	333.00	0.95
ANEC012	129.33	±	16.29	57.63	±	10.19	186.97	0.53
ANEC013	77.40	±	46.48	43.70	±	28.86	121.10	0.25
ANEC014	127.98	±	26.02	206.00	±	44.65	333.98	0.95
ANEC015	54.48	±	17.95	154.75	±	50.05	209.23	0.60
ANEC016	69.00	±	21.64	235.25	±	69.47	304.25	0.87
ANEC018	96.10	±	30.14	241.33	±	70.22	337.43	0.96
ANEC019	22.67	±	2.50	135.00	±	26.85	157.67	0.45
ANEC020	60.78	±	32.12	212.25	±	31.70	273.03	0.78
ANEC021	87.57	±	21.60	77.17	±	13.12	164.73	0.47
ANEC022	205.67	±	78.08	119.33	±	30.09	325.00	0.93
ANEC024	25.60	±	11.90	39.53	±	6.67	65.13	0.19
ANEC028	165.00	±	18.50	199.00	±	38.70	364.00	1.04

Table 4.3.1 The plasma concentrations of Clomipramine and its metabolite in glioma patients as determined via HPLC-MS/MS please refer to Table 4.2.2 for daily dose

The data shows variability in the plasma concentrations achieved by the patients included in the study. It was necessary to correct these values for body weight and daily dose in order to carry out a comparative study because the patients were on different doses – the universal unit to which all patient samples are corrected is nanograms per millilitre, per milligram of Clomipramine taken each day, per kilogram body weight and these values can be seen in table Table 4.3.2. This allows comparison of all patients against each irrespective of weight or daily dose of Clomipramine. The values shown in µM are similar than those tested using the MTT and ATP-TCA (the lowest concentration was 6.25 µM). Without knowing the amount of Clomipramine and Norclomipramine that is able to effectively cross the B-BB it is difficult to suggest a daily dose for brain tumour patients.

Before carrying out the analysis of the qRT-PCR human CYP450 gene results the amplification plots were checked to confirm that the positive control had appeared as expected, and that the no-template controls had not appeared. The melt curves were checked to ensure that there was no non-specific amplification. The results produced by the Biorad i-cycler were copied into an Excel spreadsheet that had been set up to calculate the mean, standard deviation and coefficient of variation (CV) in the triplicate values. These values were reported if the CV was acceptable. The samples were then assigned to the homozygous or heterozygous categories depending on the presence of one or more fluorophore. The fluorophore JOE was always assigned to allele *1 (the major/wildtype allele) and fluorophore FAM was always assigned to allele *2 (minor/mutant allele). It is important to select two fluorophores, such as these, with differing excitation and emission wavelengths so that the alleles can easily be distinguished via PCR and there is no overlapping of wavelengths.

4.3.2 Analytical strategy of CYP450 analysis in glioma patient plasma samples

The significance of these alleles is that the presence of a mutated allele decreases the metabolism and distribution (biotransformation) of the drug, in this case Clomipramine. The results were then grouped into four categories, Clomipramine and 2D6, Clomipramine and 2C19, Norclomipramine and 2D6 and finally Norclomipramine and 2C19. Statistical analyses were performed using ANOVA to determine if there was any statistical relationship between either of the CYP genes and plasma levels of Clomipramine or its metabolite.

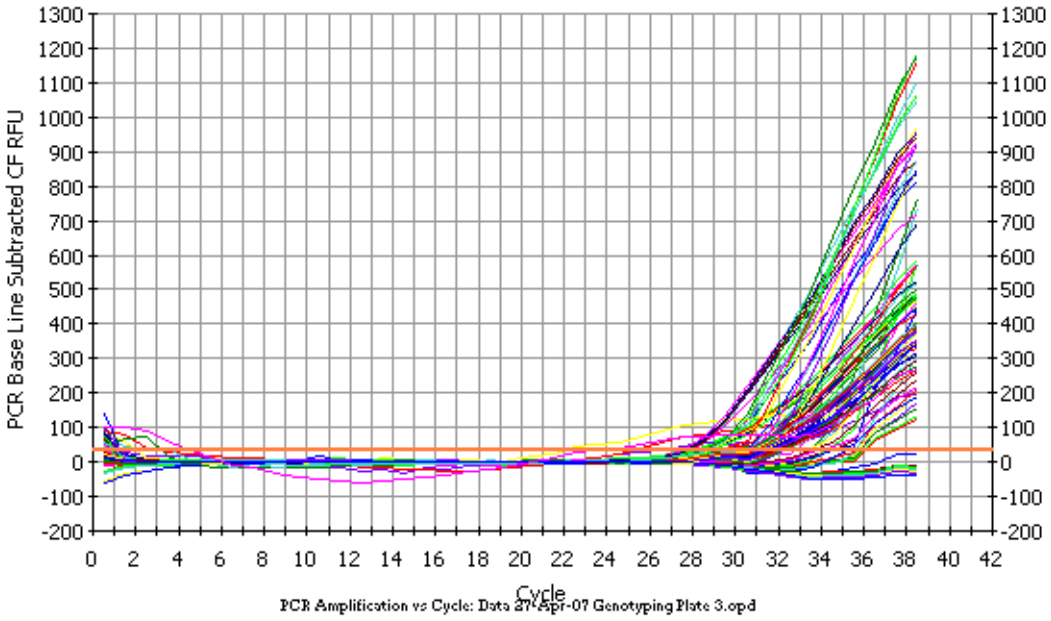


Figure 4.3.2.1 An example of qRT-PCR amplification vs. cycle data for VIC reporter dye in plate 1 using primers for mutations in CYP genes 2D6 and 2C19

On this logarithmic PCR trace the cycle number is shown along the X-axis and arbitrary fluorescence units are shown on the Y-axis. The different coloured lines represent each individual sample (see Table 4.3.1). The traces were checked to ensure that the fluorescence values were coming up and that there were no erroneous results, on this run the samples began to fluoresce around cycle threshold (Ct) 28 which shows that the concentration of template used the assay was correct. Any sample with a Ct value of 35 and upwards was regarded as negative when compared to the positive control and results are shown in Table 4.3.2.

There were pronounced inter-individual variations in the steady-state plasma concentrations of Clomipramine and its metabolites. The range of parent drug concentrations after correction with daily dose and body weight was 12.3 – 384.0 (75 ± 91) ng/ml mg^{-}1 kg $^{-}$1 and the range of metabolites was 19.0 – 594.0 (60 ± 128) ng/ml mg^{-}1 kg $^{-}$1. The seventeen subjects were divided into three groups for each of the parent drug and metabolite. These groups, determined by the reporter dye assignment to the alleles (i.e. JOE = wildtype major allele, FAM = mutant minor allele), were deemed homozygous major allele (Homo V), heterozygous (Hetero) or homozygous minor allele (Homo FAM). These groups were then numbered 1, 2 and 3 respectively. The table below shows the results for both corrected plasma concentration and genotype.

Case	CLOM	NOR CLOM	C/NC Ratio	Genotype	CYP 2D6*2X3	Genotype	CYP 2C19*2A
ANEC001	35.2768	78.416	0.45	Hetero	2	Hetero	2
ANEC004	384.4	593.96	0.65	Homo FAM	3	Hetero	2
ANEC008	42.60533	110.5853	0.39	Hetero	2	Homo FAM	3
ANEC010	16.51	42.8625	0.39	Homo V	1	Hetero	2
ANEC011	38.13333	83.96667	0.45	Homo V	1	Homo V	1
ANEC012	77.988	34.7529	2.24	Homo V	1	Homo V	1
ANEC013	53.148	30.00733	1.77	Homo V	1	Homo V	1
ANEC014	78.49133	126.3467	0.62	Homo V	1	Homo V	1
ANEC015	29.12597	82.73967	0.35	Homo V	1	Homo V	1
ANEC016	31.05	105.8625	0.29	Homo V	1	Homo V	1
ANEC018	52.855	132.7333	0.40	Homo FAM	3	Homo FAM	3
ANEC019	18.13333	108	0.17	Homo FAM	3	Homo FAM	3
ANEC020	26.33583	91.975	0.29	Homo V	1	Homo V	1
ANEC021	88.96773	78.40133	1.13	Hetero	2	Homo FAM	3
ANEC022	199.4967	115.7533	1.72	Homo V	1	Homo V	1
ANEC024	12.288	18.972	0.65	Homo V	1	Homo V	1
ANEC028	88	106.1333	0.83	Homo V	1	Homo V	1

Table 4.3.2 HPLC-MS/MS results corrected for clomipramine and norclomipramine dose (ng/ml mg^{-1} kg^{-1}) against genotype

The alleles selected for analysis were suitable for the group of patients selected for this study (see Table 4.3.1), with all three genotypes present. There were no patients with rare duplicated alleles; this is to be expected with only a small number of volunteers. The lower the Clomipramine/Norclomipramine (C/NC) ratio: the greater the ability of the patient to metabolise Clomipramine.

The results (Table 4.3.1) were then analysed by one way - Analysis of Variance (ANOVA) to determine the variance between plasma concentrations of Clomipramine and Norclomipramine when separated into three genotypes. This is a statistical analysis of individual parameters in one test, as a whole. It removes the need for single t-tests for each group to be carried out. The underlying assumption of the test is that there is equal variance. The mean, standard deviation and standard error of plasma levels of Clomipramine and Norclomipramine were calculated for each allelic group i.e. homozygous wildtype (Homo V), heterozygous (Hetero) and homozygous mutant (Homo FAM). From this source of variance could be determined via the sum of squares (SSq), degrees of freedom (DF) and mean of squares (MSq; SSq/DF). The variance ratio test provides an F value which, when applied to an 'F' table gives the significance value.

There is variation present in the C/NC ratios between patients in the study, this could possibly be attributed to the other prescription medicines that the patients are taking. For example, ANEC013 and ANEC022 both have the highest C/NC ratios and both take proton-pump inhibitors (Omeprazole and Pantoprazole). Regardless of the genotype of these patients and their genomic ability to metabolise Clomipramine, proton-pump inhibitors competitively inhibit CYP2C19 and can cause increased levels of Clomipramine. It might be advisable, in patients such as these two, to switch to an alternative gastric reflux medication such as ranitidine, which has no known CYP interactions (and is an H2-receptor antagonist).

Test	**1-way between subjects ANOVA**				
Comparison	**Clom by CYP 2D6 Group: Hetero, Homo FAM, Homo V**				
n	17				
Clom by CYP 2D6 Group	n	Mean	SD	SE	
Hetero	3	55.617	29.114	16.8092	
Homo FAM	3	151.796	202.188	116.7331	
Homo V	11	59.142	53.402	16.1014	
Source of variation	SSq	DF	MSq	F	p
CYP 2D6 Group	21585.866	2	10792.933	1.35	0.2911
Within cells	111973.179	14	7998.084		
Total	133559.045	16			

Table 4.3.3 1-way ANOVA analysis of the effect on Clomipramine metabolism by a mutation in one or more alleles of the CYP2D6*2X3 gene

Of the seventeen patients in this study, three have two mutated alleles (Homo FAM), three have two mutated alleles (hetero) and eleven have no mutations (Homo V) and patients have been divided into three groups of the basis of how many mutated alleles they carry for the *2X3 in the CYP2D6 gene. The results show that for the group of patients selected for study there is no significant ($p>0.05$) differences between number of mutated alleles and plasma concentrations of Clomipramine. In this group of patients, the genotype for CYP2D6*2X3 does not significantly affect the ability to metabolise Clomipramine.

Test	**1-way between subjects ANOVA**				
Comparison	**Clom by CYP 2C19 Group: Hetero, Homo FAM, Homo V**				
n	17				
Clom by CYP 2C19 Group	n	Mean	SD	SE	
Hetero	3	145.396	207.196	119.6249	
Homo FAM	4	50.640	29.412	14.7059	
Homo V	10	63.406	54.282	17.1655	
Source of variation	SSq	DF	MSq	F	p
CYP 2C19 Group	18584.184	2	9292.092	1.13	0.3504
Within cells	114974.861	14	8212.490		
Total	133559.045	16			

Table 4.3.4 1-way ANOVA analysis of the effect on Clomipramine metabolism by a mutation in one or more alleles of the CYP2C19*2A gene

Of the seventeen patients ten had no mutated alleles (Homo V), four had two mutated alleles (Homo FAM) and three had one mutated allele (hetero) and so have been grouped according to number of mutated alleles. The results show that for the group of patients selected for study there is no significant ($p>0.05$) difference between number of mutated alleles and plasma concentrations of Clomipramine. For this group of patients, a mutation in CYP2C19 does not affect ability to metabolise Clomipramine.

Test	**1-way between subjects ANOVA**				
Comparison	**NC by CYP 2D6 Group: Hetero, Homo FAM, Homo V**				
n	17				
NC by CYP 2D6 Group	n	Mean	SD	SE	
Hetero	3	89.134	18.577	10.7255	
Homo FAM	3	278.231	273.709	158.0258	
Homo V	11	76.307	38.006	11.4594	
Source of variation	SSq	DF	MSq	F	p
CYP 2D6 Group	98398.560	2	49199.280	4.18	0.0378
Within cells	164968.082	14	11783.434		
Total	263366.642	16			

Table 4.3.5 1-way ANOVA analysis of the effect on Norclomipramine metabolism by a mutation in one or more alleles of the CYP2D6*2X3 gene

Of the seventeen patients in this study, three have one mutated allele (hetero), three have two mutated alleles (Homo FAM) and eleven carry no mutated alleles (Homo V). The results show that for the group of patients selected for this study there is a significant ($p<0.05$) difference between number of mutated alleles and plasma concentration of Norclomipramine. The presence of two mutated alleles (Homo FAM) increases the metabolism of Norclomipramine, when you would expect a mutation to decrease the metabolism of Norclomipramine.

Test	**1-way between subjects ANOVA**				
Comparison	**NC by CYP 2C19 Group: Hetero, Homo FAM, Homo V**				
n	17				
NC by CYP 2C19 Group	n	Mean	SD	SE	
Hetero	3	238.413	308.426	178.0696	
Homo FAM	4	107.430	22.310	11.1550	
Homo V	10	79.651	38.318	12.1173	
Source of variation	SSq	DF	MSq	F	p
CYP 2C19 Group	58406.192	2	29203.096	1.99	0.1729
Within cells	204960.450	14	14640.032		
Total	263366.642	16			

Table 4.3.6 1-way ANOVA analysis of the effect on Norclomipramine metabolism by a mutation in one or more alleles of the CYP2C19*2A gene

Of the seventeen patients selected for study, three carry one mutated allele (hetero), four carry two mutated alleles (Homo FAM) and ten carry no mutated alleles (Homo V). The results show that there is no significant (>0.05) difference between number of mutated alleles and plasma concentration of Norclomipramine. The number of mutated alleles present does not affect ability to metabolise Norclomipramine.

4.4 Discussion

Individualising therapy for patients being treated with pharmaceutical agents is fast becoming the overarching goal of clinical research in the 21st century (Deeken, Figg et al. 2007). The pharmacogenetic differences seen in patients are multifactorial, such as the influences on the effect of the drug (receptors and target proteins) and the influences on pharmacokinetics (absorption, distribution, metabolism and excretion). The single most important factor is the presence of polymorphisms in the genes involved in drug pharmacokinetics, which can impact on the drug absorption, distribution, metabolism and excretion. The aim of this chapter was to demonstrate that mutations of the CYP450 enzymes responsible for metabolising Clomipramine affect response to tricyclic antidepressant therapy; however the principle is relevant to all chemotherapeutic agents and their corresponding CYP genes. The CYP genes 2D6 and 2C19 are responsible for the metabolism of Clomipramine and its metabolite Norclomipramine, respectively, and by selecting a small cohort of brain tumour patients it was possible to obtain plasma levels of Clomipramine (and Norclomipramine) and genomic DNA from fingerprick blood samples to examine inter-individual variation.

The HPLC-MS/MS is a clinically validated method for determining the concentration of Clomipramine and its metabolite in plasma. The daily dose was not identical in all patients, but was prescribed according to body mass and prophylactic medications, however by correcting the plasma concentrations for body mass and daily dose it is clear to see the vast differences in metabolic capacities.

The maximum corrected Clomipramine plasma level was 384 mg/ml mg^{-1} kg^{-1}(GBM, Grade IV) and the minimum was 12 mg/ml mg^{-1} kg^{-1}(GBM, Grade IV). Four of the patients included in the study (ANEC012, ANEC013, ANEC021, and ANEC022) had higher concentrations of Clomipramine than Norclomipramine but this did not correlate with genotype for the CYP genes tested, as three were Homo V and one was heterozygous.

The maximum corrected Norclomipramine plasma level was 594 mg/ml mg^{-1} kg^{-1} and the minimum was 19mg/ml mg^{-1} kg^{-1}. This shows a large variation in plasma levels, and information on the ability of the individual patient to metabolise Clomipramine is something that a general practitioner (GP) would not have access to when initially prescribing. It would, in theory, be possible to titrate the dose over a number of weeks (whilst monitoring side effects and adverse reactions) to see how much Clomipramine the patient can tolerate, however in patients suffering from malignant glioma there is a huge degree of urgency. There is now the worry of a spectrum of cognitive deficiencies which affect between 4-75% of cancer patients following chemotherapy (Staat and Segatore 2005; Raffa, Duong et al. 2006). This iatrogenic phenomenon is thought to be caused because the agents that are used to kill neoplastic cells may be equally (or more) harmful to the normal tissues of the brain (Jackson 2007). Thus the development of a selective and more targeted therapeutic approach or alternatively a selective protection of normal tissue is needed.

In previous studies, prior to invention of PCR-based techniques to analyse CYP enzymes, the method of calculating the Clomipramine/Norclomipramine ratio was utilised to give a finite value for the amount of metabolite present in the plasma, and hence the proportion of parent drug that has been metabolised. In past studies patients were also assigned to one of three categories, extensive metabolisers (EMs), intermediate metabolisers (IMs) and poor metabolisers (PMs) according to genotype. However since the discovery of many more allele variations it has become apparent that the presence of one or more mutated alleles does not necessarily mean a decrease in the CYP enzyme activity.

In CYP2D6*2X3 the presence of two mutated alleles (homozygous recessive) results in a functional gene and causes an increase in enzyme activity (Johansson, Lundqvist et al. 1993; Dahl, Johansson et al. 1995; Aklillu, Persson et al. 1996). In this study the Homo FAM group confers two mutated alleles and indeed this was the observation for this genotype, two mutated alleles resulted in an increased enzyme activity. As described in Figure 3.5.6.1 there are many factors affecting the metabolism of drugs in brain tumour patients, and any single one or combination of these could be affecting the plasma concentration at any one time.

In the present study, the HPLC-MS/MS data and PCR data was combined and then analysed with ANOVA to see if there was any differences between the genotype and individual plasma concentrations of both Clomipramine and Norclomipramine. This would indicate a role for that particular CYP gene in the metabolism of the drug.

The data analysis on page 205 shows statistical difference in groups for the role of CYP2D6*2X3 and the plasma levels of norclomipramine. CYP2D6 is responsible for metabolising Norclomipramine to its metabolites Di-desmethylclomipramine and 8-hydroxydesmethylclomipramine (4.1.1.2) and the presence of two mutated alleles results in a higher plasma concentration of Norclomipramine. The number of patients is so small that it would be impossible to draw any firm conclusions from the data obtained, however the higher concentrations of plasma Norclomipramine seen in patients with two mutated (but still functional) alleles agrees with the literature (Johansson, Lundqvist et al. 1993; Dahl, Johansson et al. 1995; Aklillu, Persson et al. 1996). Care should be taken when interpreting previous studies which designate patients as EM, IM or PM because a mutated allele does not necessarily correspond with a non-functional protein.

From several large studies in Caucasians (1456 Europeans) it can be estimated that for CYP2D6, 67% of all alleles encode for enzymes with normal activity (Homo V), 4% encode for enzymes with decreased activity, 27% lead to non functional enzymes and 2% encode for increased activity (Marez, Legrand et al. 1997; Sachse, Brockmoller et al. 1997; Griese, Zanger et al. 1998).The most common non-coding alleles are CYP2D6*4 (about 71% of null alleles), CYP2D6*5 (about 16% of null alleles), CYP2D6*3 (about 6% of null alleles) and CYP2D6*6 (about 4% of null alleles) all the other non coding alleles are rare and have a prevalence of 1% or lower of the non coding alleles.

Allelic variants that encode for enzymes with decreased activity are rare in Caucasians and the most common alleles are CYP2D6*9 and CYP2D6*10, each accounting for about 2% of all alleles. Two null-alleles in the CYP2C19 gene, *2 and *3, have been described to account for approximately 87% of all PMs in Caucasians and 100% of all PMs in Orientals (De Morais, Wilkinson et al. 1994; De Morais, Wilkinson et al. 1994). In addition, three non-coding alleles (CYP2C19*4, CYP2C19*5 and CYP2C19*6) have been described but the frequencies of these alleles are expected to be below 1% in Caucasians (Ibeanu, Goldstein et al. 1998). Deficiency of CYP2C19 occurs with a prevalence of PMs of 2-5% among white Europeans, Black Africans 4-5%, Black Americans 6% and 12% to 23% among Orientals (Bertilsson 1995).

4.4.1 The results of the CYP2D6 and 2C19 PCR for determining number of mutated alleles and the effect on metabolism of Clomipramine hydrochloride

The aim of the genotyping assay was to analyse whole blood samples (for which paired plasma samples had been obtained) to see if it were feasible to extract genomic DNA of high enough quality to demonstrate mutations of CYP450 enzymes. There are variations in the level of Clomipramine found in the plasma samples of patients with malignant glioma and the objective was to see if CYP genotype was one of the influencing factors.

The results of the CYP gene analysis are promising for the following reasons, firstly in terms of general genotyping this study shows that by taking a simple fingerprick blood sample it is possible to extract gDNA of suitable quality for RT-PCR CYP gene analysis. The protocol would need to be expanded in order to 'screen' for CYP gene and metaboliser status, as genotyping for only one or two alleles is of limited clinical use. Because of the large number of CYP2D6 and CYP2C19 variants that have been described to date it would be necessary to carry out the screening in 384-well plates using an automated robotic programme.

Further work to establish which of the CYP genes are responsible for metabolising chemotherapy used in glioma, might mean that an improved metabolic profile can be built up for each individual patient. It might also mean that more informed decisions can be made about which, for example, anticonvulsant or gastric reflux medication can be prescribed alongside glioma chemotherapy without the risk of competitive inhibition for the CYP enzymes. This technology would mean that drug administration can be tailored to the individual; this would cut costs when prescribing expensive novel agents, such as Gleevec, by avoiding unnecessarily high doses in patients with functional alleles and ensuring that the therapeutic window is reached in patients with non-functional alleles, thus rendering the regimen worthwhile.

The patients selected for the PCR study were on Clomipramine doses ranging from 25.0 – 150 mg/day showing a high degree of variation. ANEC004 was on a considerably lower dose of Clomipramine than any other patient because their GP felt this necessary whilst receiving chemotherapy. It is not clear whether there are any interactions from taking Clomipramine at the same time as standard chemotherapy, apart from the increase in apoptosis seen in Annexin-V flow cytometry between Clomipramine and Imatinib in rat C6 glioma cells and between Clomipramine and Dexamethasone in human glioma cells (Bilir, Erguven et al. 2008; Higgins and Pilkington 2010).

However in some reports of anecdotal patients taking the anticonvulsant Carbemazepine (Tegretol®) a higher frequency in seizures was seen when taking high doses of Clomipramine, this could be due to competitive inhibition of the CYP2D6 enzymes which are responsible for metabolising both Clomipramine and Carbemazepine (Ingelman-Sundberg 2005). Patients taking sodium valproate-based anticonvulsants such as Epilim® did not appear to experience any difference in frequency of seizures when taking Clomipramine. This could be due to the fact that sodium valproate and Valproic acid are metabolised by CYP2C9, 2A6 and 2B6 (Kiang, Ho et al. 2006). The suitability of the dose is difficult to determine as Clomipramine is prescribed alongside other medication, such as anticonvulsants, steroids, anti-emetics and other cytotoxics, however in an ideal situation patients would take as much Clomipramine as they could manage (the maximum we have observed so far is 200mg/day) without too many side effects.

It is possible to take into consideration the route of metabolism for each drug and, where known, the CYP enzymes involved, to ensure that there are no fundamental adverse effects such as competitive inhibition.

One difficulty in this cohort of patients is the presence of 'perceived' side effects. Patients may associate any adverse events with Clomipramine however the very nature of the disease, its location in parts of the brain that relate to memory, speech, balance and other vital functions, means that side effects such as impaired speech and partial seizures can be perturbed by surgical resection or tumour progression. There are a wide range of side effects seen in the patients studied, tiredness, dry mouth and lethargy are some of the milder, and more specific and readily identifiable, effects and tend to subside after two to four weeks of taking Clomipramine.

The observation of a reduction in the milder side effects tends to coincide with the point at which a steady state plasma concentration is achieved. The seizures and 'jerks' seen in other patients could be due to any number of factors, but it would be pertinent to check if these patients were on a Carbemazepine-based anticonvulsant (see Table 4.2.2), and the possibility of changing over to a valproate formulation. Where side effects such as weight gain have been reported (Table 4.2.2) this could be associated with adjuvant administration of a steroid (such as Dexamethasone) which is prescribed to brain tumour patients to reduce oedema (and intracranial pressure) prior to surgery.

5 Is the norepinephrine transporter, a molecular target for tricyclic antidepressants, expressed in glioma cells?

5.1 Introduction

In addition to examining the genetic variation in CYP450 genes responsible for the metabolism of Clomipramine and Norclomipramine, is also important to examine if there is genetic variation in the expression of drug transporter genes, see Figure 3.5.6.1, in this case the NET. The NET is thought to allow the tricyclic antidepressants entry to the tumour cell, and it is possible to examine protein and mRNA production of the NET SLC6A2 gene to see if they are expressed in an individual tumour or not. Knowledge of genotype corresponding to a particular chemotherapeutic drug is of limited use in a clinical setting without also knowing if the drug can gain entry to the tumour cells.

The presence of the NET on astrocytes (Inazu, Takeda et al. 2003) may indicate that glial cells are involved in the regulation of noradrenergic activity. Astrocytes contain two transport systems (a sodium-dependent neuronal transporter and a sodium-independent extra neuronal transporter, only an antibody against the neuronal NET is commercially available) that can remove monoamine neurotransmitters (serotonin, norepinephrine and dopamine) from the synaptic cleft by transporters present in the plasma membrane. Since glial cells contain monoamine oxidase, it is likely that astrocytes play an important role in regulating extracellular monoamine concentrations. This uptake system may play a part in removing excess monoamines and prevent uncontrolled spreading of nerve signals.

The fact that glial cells are able to remove excess norepinephrine and degrade it using monoamine oxidase (which is present on the mitochondrial membrane) might indicate that they exert significant control of norepinephric activity (Inazu, Takeda et al. 2003). To date, it has been demonstrated that both the neuronal and extra-neuronal forms of the NET are present in normal human brain astrocytes (Inazu, Takeda et al. 2003). It has not however been investigated if the neuronal NET is present on human malignant glioma cells, the presence of a cellular transporter known to be inhibited by the tricyclic antidepressants might go some way toward explaining the cytotoxic effect of antidepressants on brain tumour cells. While it has been demonstrated that upon entry to the cells Clomipramine exerts its effect via complex III of the mitochondrial respiratory chain (Daley 2001), it has not been discovered how Clomipramine hydrochloride enters the cell or the events taking place between the plasma membrane and the mitochondrial membrane. Because the presence of NET on human glioma cells has not been investigated it is unclear if the presence of NE affects glioma cell survival. It is known that normal human astrocytes are able to degrade NE via monoamine oxidase-B turnover (Pietrangeli and Mondovi 2004; Toninello, Pietrangeli et al. 2006), and it is also suggested that monoamine oxidase may play a role in cell signalling (Ou, Chen et al. 2006). It has been postulated that the serotonin transporter (SERT; solute carrier family 6 member 4; SLC6A4) may act not merely as a transport protein but also as a signal transducer (Fanburg and Lee 1997; Lee, Wang et al. 2001). However when investigated as an anti-tumour target it was shown that while it is present on B-cells, demonstrated via Western blotting, antidepressants affect malignant cells independently of this transporter (Meredith, Holder et al. 2005).

Because the tricyclic antidepressants act via inhibition of the SERT and NET, in particular Norclomipramine which preferentially blocks the NET transporter, it might be possible that if the NET was present on brain tumour cells and the tricyclic antidepressants inhibited the extra neuronal transporter in the same way they do the neuronal transporter, that by reducing the availability of NE to the tumour cell it alters the monoamine oxidase signal and triggers caspase-3 mediated apoptosis.

5.2 Methodology

5.2.1 Details of samples stained with the norepinephrine transporter antibody via immunohistochemistry

Patient Identifier	Age	Sex	Tumour Type
TORC07-0003	49	F	Normal human brain control
TORC07-0009	45	F	Oligodendroglioma (II)
TORC07-0010	43	M	Diffuse Astrocytoma (II/III)
TORC07-0011	82	F	Anaplastic Meningioma
TORC07-0012	43	F	Recurrent GBM (IV)
TORC07-0013	39	M	Schwannoma of spinal nerve root
TORC07-0014	41	M	Recurrent GBM (IV)
TORC07-0015	45	M	Anaplastic Oligodendroglioma (III)
TORC07-0016	72	F	GBM (IV)
TORC07-0017	52	F	Metastatic mammary ductal carcinoma
TORC07-0018	74	M	GBM (IV)
TORC07-0019	59	F	Recurrent GBM (IV)
TORC07-0020	48	F	Myxoid Degenerate Meningioma
TORC07-0021	66	F	GBM (IV)
TORC07-0022	73	F	Meningioma
TORC07-0023	72	F	Meningioma
TORC07-0024	69	M	GBM (IV)

Table 5.2.1 Details of the samples selected for IHC staining with the NET antibody

Sections were taken from one normal human brain control and sixteen tumour paraffin blocks (ten primary brain tumours, four Meningiomas, a secondary tumour and one cranial nerve tumour). The normal human brain control was non-neoplastic brain tissue removed during surgical resection of a brain tumour; it is not ethically possible to obtain normal brain tissue for research and it was not possible to access to post mortem tissue from a brain bank, so this was the best alternative control tissue available.

5.2.2 Immunoperoxidase staining of the NET

In brief, sections were dewaxed according to the method outlined in section 2.3.15 then placed straight into a mixture of alcohol and H_20_2 to block endogenous peroxidase. The slides were then incubated in pH 6.0 citrate buffer (25.0 ml citric acid, 175.0 ml sodium citrate and 1800 ml distilled water) and placed in a pressure cooker for thirty minutes. The sections were then covered with avidin blocker, rinsed and covered with biotin blocker prior to application of the primary antibody (as described in section 2.3.18). The slides were loaded onto a Dako Autostainer (Dako, UK). The programme consisted of thirty minutes incubation with the primary antibody, fifteen minutes incubation with the secondary antibody, fifteen minutes incubation with streptavidin-HRP and finally five minutes incubation with DAB. Once the programme had completed the sections were counterstained with eosin, dehydrated and cleared as outlined in sections 2.3.17 and 2.3.16.

5.2.3 Immunocytochemical staining of the NET

To visualise the presence of the norepinephrine transporter (NET) on human brain tumour cells, cultures of DK-MG, SNB-19, UPMC and UPAB were grown on glass coverslips, fixed and stained, CC-2565 normal human astrocytes were also included in the assay as a normal brain control and SH-SY5Y Neuroblastoma cells (which are known to express the NET) were used as a positive control. Monolayer cell cultures were harvested (as described in 2.2.4) and seeded into 6-well culture plates; each well contained a sterile coverslip onto which 2,000 cells pipetted and left to adhere for twenty-four hours.

The media from the wells was then removed, and the cells washed with sterile phosphate-buffered saline (PBS). The cells were stained according to the method outlined in 2.3.20 using the monoclonal NET antibody (MAB5620 Chemicon; 1:250 as determined by in-house titration) and Alexfluor488 conjugated secondary antibody (1:500, raised in a goat).

5.2.4 Isolation and visualisation of the norepinephrine transporter protein via Western blotting

Isolation and visualisation of the NET protein was carried out via western blotting according to the protocol outlined in 2.3.22.2. Briefly, cells lysates were prepared from CC-2565, SH-SY5Y, SNB-19, DK-MG, UPAB, UPJM and UPMC in 1.0 ml RIPA buffer containing 10 µl protease inhibitor cocktail (both from Thermo Fisher). The blots were blocked to prevent non-specific binding, probed with a monoclonal anti-mouse NET antibody at a dilution of 1:1000 (MAB5620, Chemicon) before incubating with a secondary HRP-conjugated anti-mouse antibody at a dilution of 1:5000. The resulting protein bands were visualised using a chemiluminescent ECL plus kit and imaging station.

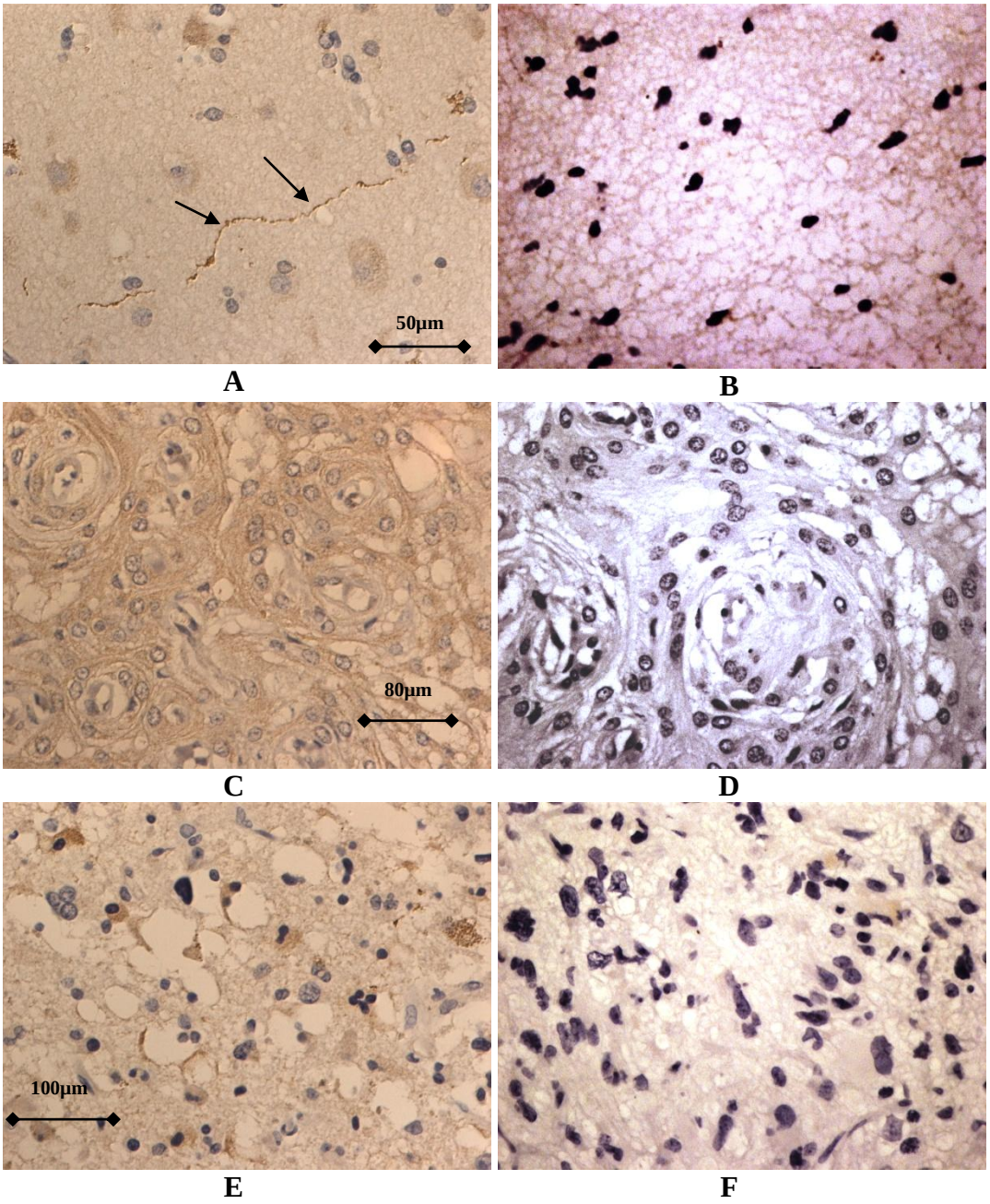
5.2.5 Isolation and qualification of messenger RNA encoding the SLC6A2 (Norepinephrine Transporter) Gene

To examine gene expression of the SLC6A2 gene, which encodes the neuronal NET, cell lysates were prepared from CC-2565, SH-SY5Y, SNB-19, DK-MG, UPAB, UPJM and UPMC in 1.0ml RA1 buffer (Machery Nagel) containing 10 µl beta-mercaptoethanol. The RNA was extracted from the cell lysates according to the method outlined in 2.3.23.2, quantified using a Nanodrop spectrophotometer (2.3.14) and reverse transcribed to cDNA according to the method outlined in 2.3.23.3. Complementary DNA, obtained by the method described in section 2.3.23.3 was amplified in 96-well plates by qRT-PCR on an Applied Biosystems 7500HT (Applied Biosystems, UK). All samples were run with a control GAPDH primer (a housekeeping gene ubiquitously expressed in all cells; Hs_02758991_g1 Applied Biosystems) and the test NET primer (Hs_01567442_m1, Applied Biosystems). Complementary DNA extracted from control Neuroblastoma cell line SH-SY5Y was run as a positive control, and wells run with the omission of template DNA served as a no-template negative control. Each PCR reaction had a final volume of 20µl comprising 9µl cDNA (100ng per well or nuclease-free water if a no-template control), 1µl of Taqman primer (Applied Biosystems, UK) diluted ten-fold in Taqman Universal Master Mix. Each two-step PCR cycle comprised of denaturing (15 seconds at 92^{o}C) and annealing/extending (90 seconds at 60^{o}C). At the end of each run a final melt curve cycle was performed (cooling to 50^{o}C and then increasing stepwise 1^{o}C to 95^{o}C) was performed to exclude the presence of primer-dimer artefacts. The results were analysed and statistical tests carried out according to the methods described in section 4.2.4.

5.3 Results

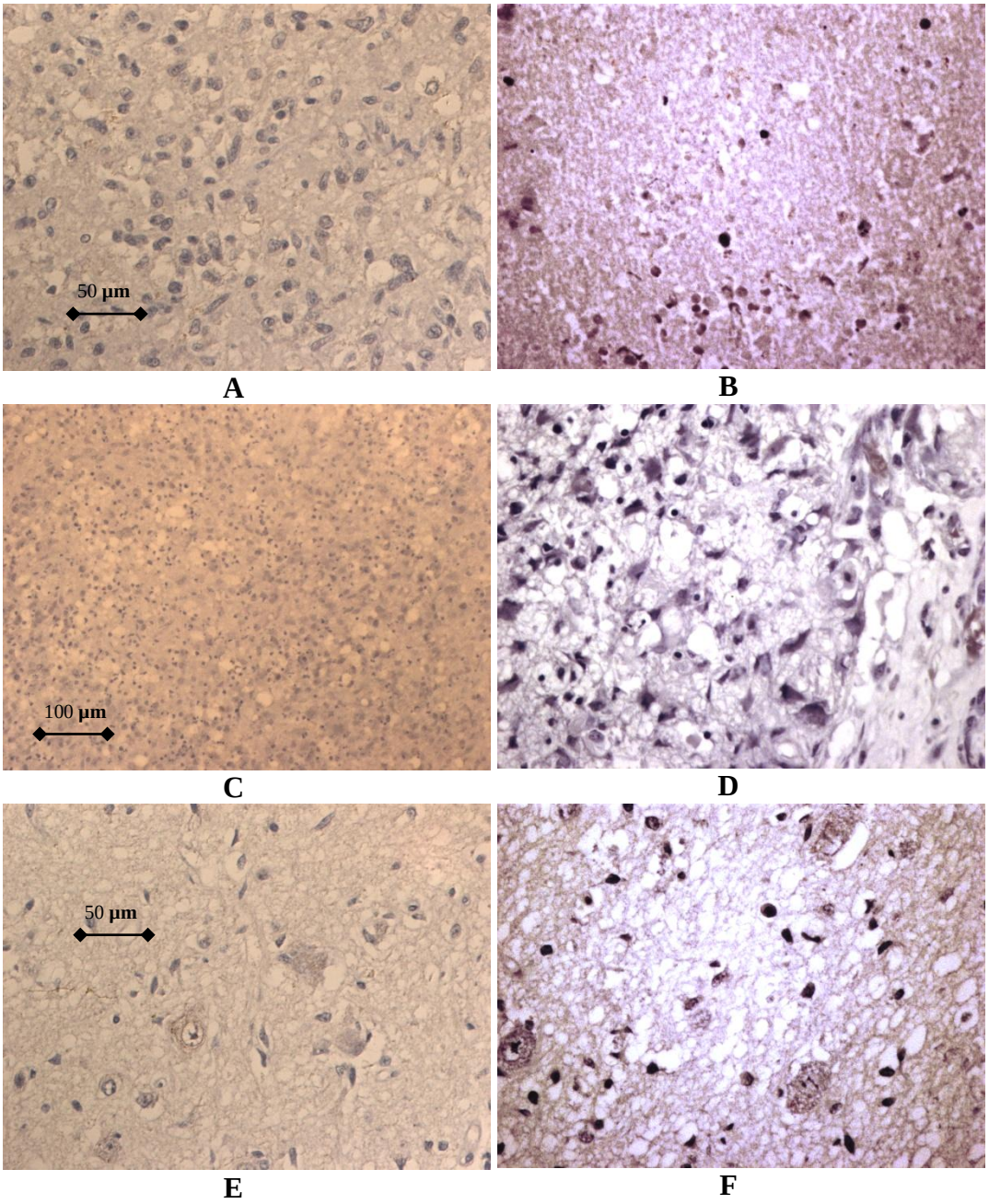
5.3.1 Norepinephrine Transporter IHC Staining

Of the sixteen tumours selected for study (alongside one normal human brain control, details of samples can be found on page 219, analysis by IHC of fifteen tumours showed positivity for the NET transporter and one tumour was negative. The normal human brain control showed positivity for the NET in neurones. Four of the seventeen tumours (TORC07-0009, Oligodendroglioma II; TORC07-0012, recurrent GBM IV; TORC07-0019, recurrent GBM IV; TORC07-0022, Meningioma) showed limited immunoreactivity, in some cases due to poor fixation (these samples had been delayed in transit from theatres and so fixation in formalin was not as rapid as for all other samples). One tumour (TORC07-0021, GBM grade IV) was negative for the NET transporter. Only one patient (TORC07-0019) was taking Clomipramine, adjuvantly to standard radiotherapy and chemotherapy, at the time that brain tumour tissue for this study was obtained. Clinical data showed that this patient was able to tolerate Clomipramine extremely well and was on a daily dose of 200 mg per day. There is no discernable pattern between tumour type or grade and presence of the NET protein. Expression of the NET is difficult evaluate via IHC due to differences in the quality of staining and the only way to quantify the expression of the transporter would be to perform absolute real-time PCR using primers for the NET gene.



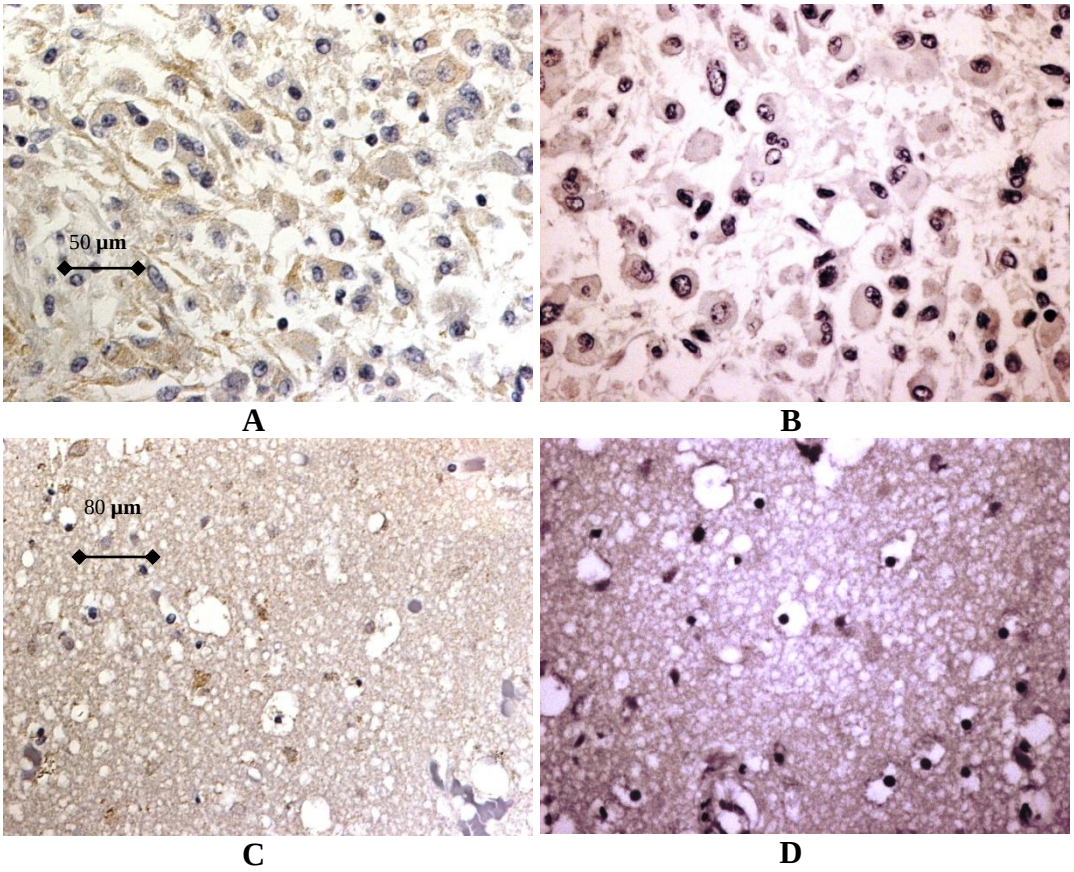
5.3.1.1 Results showing IHC staining of the NET in: TORC07-0003 (normal human brain control; A), TORC07-0024 (GBM; C) and TORC07-0016 (GBM; E).

Corresponding negative controls are on the right hand side (B, D & F). TORC07-0003 shows clear positive staining for the extra-neuronal NET on the neuronal processes (arrows) and this section shows the 'neuropil': an area of neurones with many overlapping processes. TORC07-0024 demonstrates immunoreactivity (brown staining) on glioma cell membranes and negativity on vascular endothelia and blood vessels (blue staining). This tissue shows enhanced cellularity and microvascular proliferation typical of a high grade tumour. TORC 07-0041 shows brown immunoreactivity present in the glioma cell cytoplasm. Vacuolation and nuclear atypia (again, typical in a GBM) are also present. All images were captured on a Zeiss Axiocam MRC using Axiovision Capture software and scale bars have been applied.



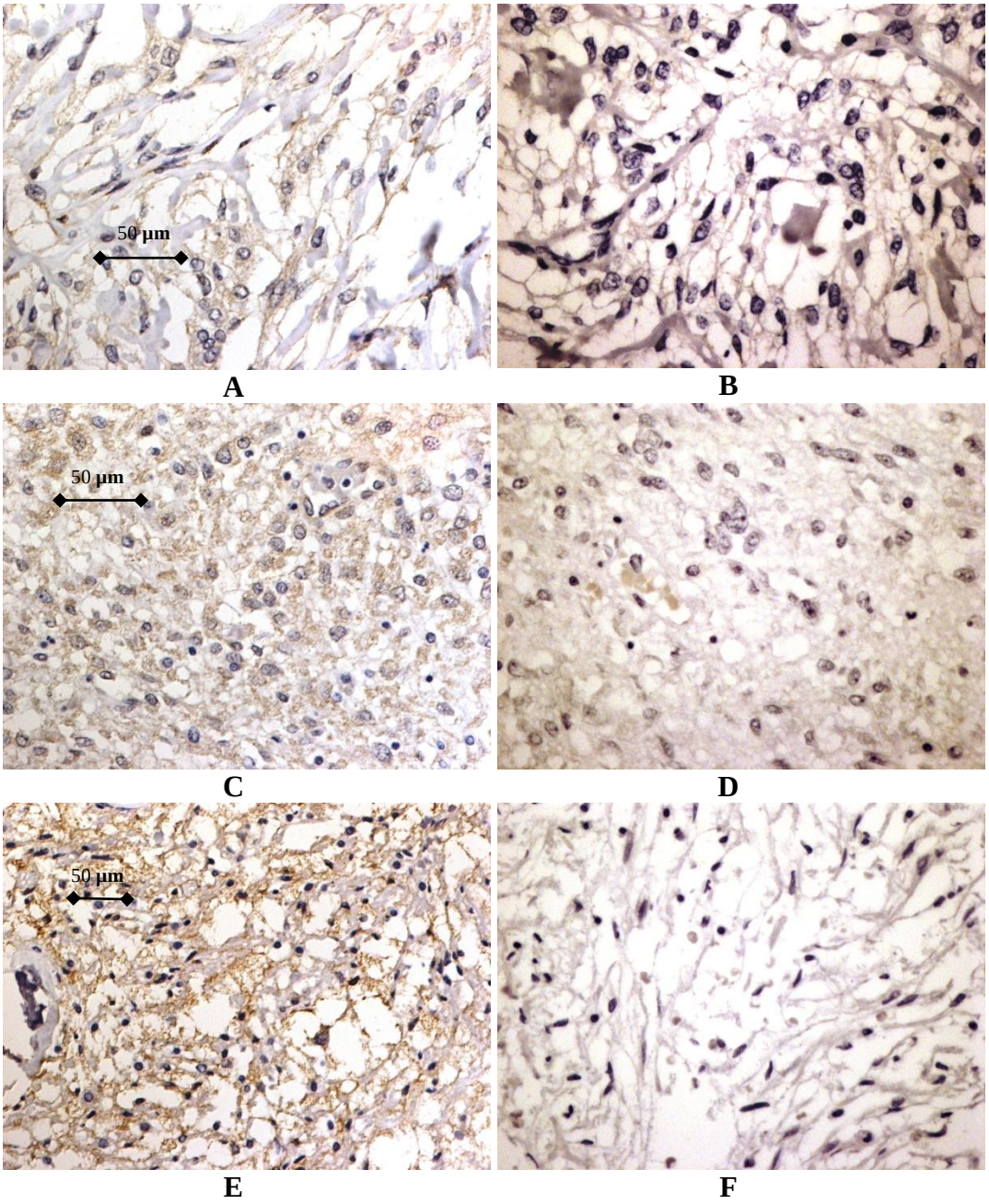
5.3.1.2 Results showing immunohistochemistry staining of the NET in: TORC07-0018 (GBM; A), TORC07-0012 (GBM; C) and TORC07-0021 (GBM; E).

Corresponding negative controls are on the right hand side (B, D & F).TORC07-0018 shows some positivity for the NET as demonstrated by brown stippled surface immunoreactivity. TORC07-0012 shows limited immunoreactivity for the NET, the large blood vessels suggest oedematous tissue and dark brown dots indicate infiltration of white blood vessel components, possibly microglia. There is some faint cytoplasmic staining, but due to fixation problems the quality is poor. TORC07-0021is negative for the NET on glioma cells. The immunoreactivity present is on neurones; there are no apparent mitoses or microvascular proliferation suggesting that this section has been taken from an area of lower grade within the tumour.



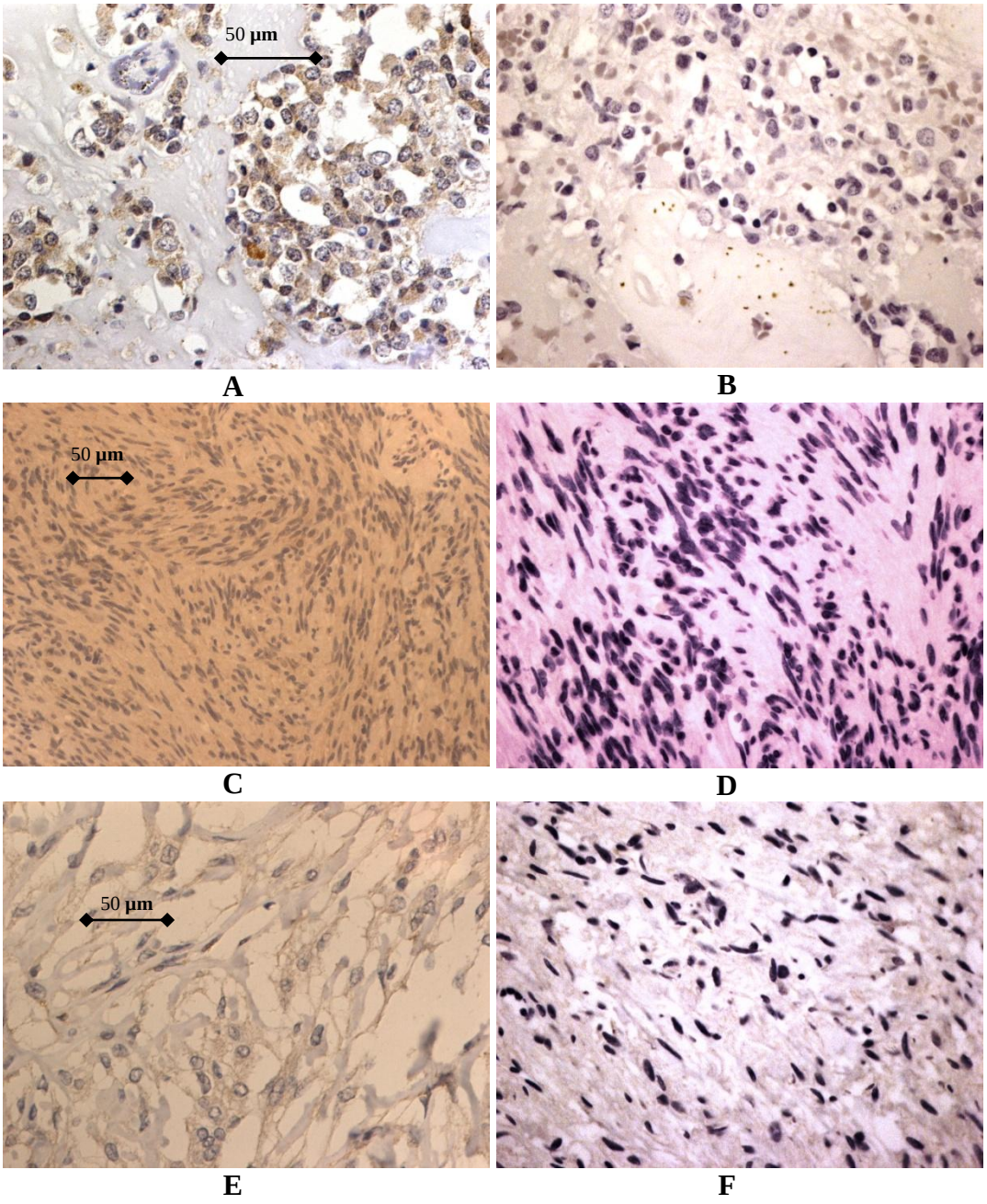
5.3.1.3 Results showing immunohistochemistry staining of the NET in: TORC07-0014 (GBM; A) and TORC07-0019 (GBM; C).

Corresponding negative controls are on the right hand side (B & D).TORC07-0014 shows positive cytoplasmic staining for the NET and eccentric nuclei with astrocytes. TORC07-0019 is a recurrent GBM that shows faint immunoreactivity, probably due to the poor quality of the FFPE block. This section also contains many blood vessels. All images were captured on a Zeiss Axiocam MRC using Axiovision Capture software and scale bars have been applied.



5.3.1.4 Results showing immunohistochemistry staining of the NET in: TORC07-0011 (Meningioma; A), TORC07-0020 (Meningioma; C) and TORC07-0023 (Meningioma; E).

Corresponding negative controls are on the right hand side (B, D & F).TORC07-0011 shows meningothelial cells positive for the NET and vascular endothelium negative for the NET. TORC07-0020 shows positivity for the NET on meningothelial cells, indicated by brown immunoreactivity. TORC07-0023 shows positivity for the NET on meningothelial cells. The dark spots are the cell nuclei and there are many blood vessels present (blue). All images were captured on a Zeiss Axiocam MRC using Axiovision Capture software and scale bars have been applied.



5.3.1.5 Results showing immunohistochemistry staining of the NET in: TORC07-0015 (Anaplastic oligodendroglioma; A), TORC07-0013 (Schwannoma; C) and TORC07-0022 (Meningioma; E).

Corresponding negative controls are on the right hand side (B, D & F). TORC07-0015 shows strong positivity for the NET, shown by the extensive brown immunoreactivity. The connective tissue has stained light blue. TORC07-0040 shows diffuse faint brown immunoreactivity. TORC07-0042 demonstrates clear positivity of meningothelial cells for the NET. All images were captured on a Zeiss Axiocam MRC using Axiovision Capture software and scale bars have been applied.

5.3.2 Norepinephrine transporter staining via immunocytochemistry

All cells selected for analysis via ICC, four GBM (two cell lines and two early passage cultures), the control SH-SY5Y Neuroblastoma and normal human astrocytes CC-2565 (see Table 2.3.1), showed immunoreactivity for the NET transporter. There is no discernable pattern between tumour type or grade and presence of the NET transporter. Expression of the NET is difficult to quantify via ICC due to differences in the quality of staining and however the results do confirm that the cells used in this study express the NET. UPJM was not available for ICC staining.

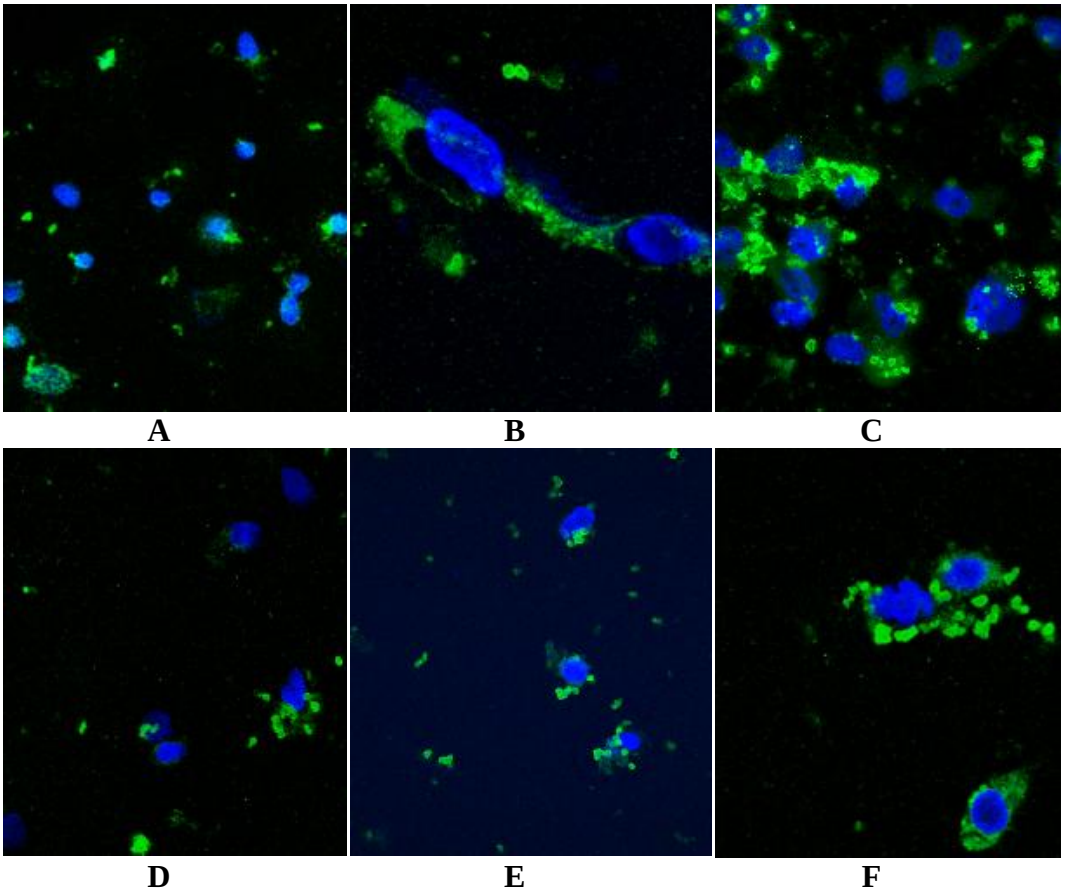


Figure 5.3.2.1 Immunocytochemical staining of the norepinephrine transporter in Glioblastoma multiforme, normal human astrocytes and Neuroblastoma

Neuroblastoma SH-SY5Y (A), normal human astrocytes CC-2565 (B), GBM established cell line DK-MG (C), GBM established cell line SNB-19 (D), early passage GBM UPAB (E) and early passage UPMC (F) were visualised using ZEISS LCM710 microscope and a X40 magnification oil immersion lens. The images were captured using ZEN2009 acquisition software and sixteen averages of fluorescence taken per picture. The Neuroblastoma SHSY-5Y acts as a control for NET staining, all cells show some degree of positivity for the NET and results correlate with the findings via IHC (see 5.3.1). The reaction product is dense and compact but cytoplasmic and probably overlying the nuclei in some cases (see bottom left of image A for an example of green stippled fluorescence overlying the nucleus).It is useful to stain established cell lines because only malignant cells are able to survive many serial passages *in vitro*. When staining primary cultures or some early passage cultures, there may be other cells types present (for example normal astrocytes, neurones and fibroblasts). It is impossible to quantify immunoreactivity via this staining method; however the findings qualify the presence of the NET on malignant glioma cells and provide further evidence that Clomipramine is a suitable candidate for inducing apoptosis in glioma cells.

5.3.3 Isolation and visualisation of norepinephrine transporter protein via western blotting

The evidence for the presence of the NET on glioma cells presented in sections 5.3.1 and 5.3.2 are further supported by the observation that all five glioma (SNB-19, DK-MG, UPAB, UPMC and UPJM, see Table 2.3.1) expressed the NET protein via western blot. The control Neuroblastoma (SH-SY5Y) and normal human astrocytes (CC-2565) also expressed the NET protein and these findings agree with the literature (Takeda, Inazu et al. 2002; Inazu, Takeda et al. 2003; Inazu, Takeda et al. 2003; Inazu, Takeda et al. 2003; Vaidyanathan, Affleck et al. 2007). Differences in the expression pattern were observed; all cell lysates probed for NET revealed a band with an apparent molecular weight of 46 kDa. This band is consistent with the weight of the unglycosylated form of the NET protein However, an additional band migrating at around 80 kDa was observed in DK-MG, SNB-19, UPAB and SH-SY5Y and this second band is consistent with the glycosylated form of the NET protein.

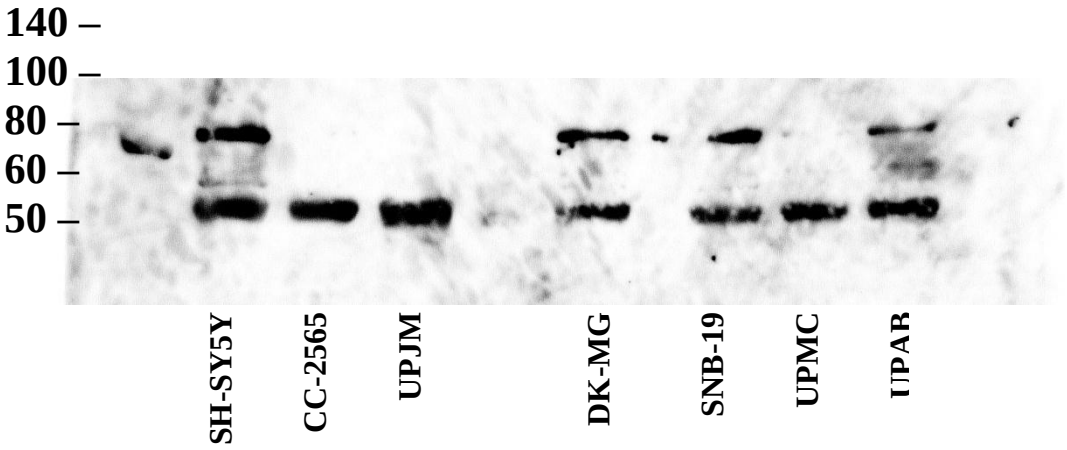


Figure 5.3.3.1 Western blot analysis of the norepinephrine transporter protein expression in five glioma samples (see Table 2.3.1)

Glycosylation of proteins is an enzymatic, post-translational, process whereby N-linked glycans are attached to the protein structure. The protein structure gains stability by the addition of the carbohydrate group and is able to fold correctly; therefore the N-glycosylated form of the NET protein is more stable. The unglycosylated form of NET protein degrades more rapidly. Unglycosylated proteins have reduced transport activity however ligand recognition is not affected. Tumour cells often carry an impairment in the processing of proteins to the fully glycosylated form (Melikian, Ramamoorthy et al. 1996). It is of interest the NET protein is expressed in all tumours analysed via Western blot, in addition to the normal human astrocytes and Neuroblastoma, however the presence of glycosylated and unglycosylated forms of the protein differed amongst these samples. It is difficult to say whether these findings support the hypothesis that expression of the NET influences sensitivity to tricyclic antidepressants, without exposing some tumours that do not express the NET to tricyclic antidepressants, however all tumours studied here tumours do have the potential to respond to norepinephrine.

5.3.4 Detection of mRNA encoding the Norepinephrine transporter SLC6A2 gene expression

Real-time PCR was used to assess the levels of the genes encoding the NET expressed in five glioma (SNB-19, DK-MG, UPAB, UPMC and UPJM), normal human astrocytes (CC-2565) and a Neuroblastoma (SH-SY5Y). The representative RT-PCR results indicate that the control Neuroblastoma cells express the NET whereas all other cells tested do not. The detection of NET in the control sample and detection of GAPDH housekeeping gene in all samples shows the assay and primers to be functioning correctly; however it does not correlate with the presence of functional NET protein demonstrated by the Western blotting technique. The NET primers used in this assay span exons 7 and 8 in the SLC6A2 gene however there might be one or more splice variants present in the RNA (see 1.29.2); the resulting mRNAs are different (and would not be detected by the primer chosen in this study) however proteins are still translated (albeit of differing isoforms) and identified via Western blot.

5.4 Discussion

5.4.1 The presence of the NET, and molecular alterations in the gene that encodes it, on human glioma cells

The IHC staining of sixteen tumours (and one normal human brain control) demonstrated immunoreactivity of the neuronal NET in thirteen cases (figures 5.3.1.1, 5.3.1.2, 5.3.1.3, 5.3.1.4 & 5.3.1.5). Meningioma TORC07-0022, Oligodendroglioma TORC07-0009 and Metastatic breast adenocarcinoma TORC07-0017 were negative for the NET, and did not demonstrate any immunoreactivity. The normal human brain control also shows immunoreactivity, in particular neuronal immunoreactivity. Evidence suggests that this application of the NET antibody (Chemicon, MAB 5620) in glioma IHC appears to be the first of its kind. The microscope used and type of staining performed provides limited information; it is not possible to see the exact location of immunoreactivity within the cell membranes or organelles. Verification that the neuronal NET is present on glioma cells has not been demonstrated before according to the literature. Given the small number of tumours in this study and the preparations used, there is no discernable link between tumour grade and degree of immunoreactivity for these samples. However a larger study, perhaps with the inclusion of one type of tumour only, might lead to a more definitive link between NET distribution and activity in glioma.

Immunocytochemistry (Figure 5.3.2.1) performed on the same cells that were exposed to tricyclic antidepressants in chapter three demonstrated presence of the NET on glioma, normal human astrocytes and Neuroblastoma cells. Western blotting, see Figure 5.3.3.1, performed using the same antibody as the IHC and ICC protocols, identified 80 kDa bands of glycosylated NET in all tumours and additional smaller bands of unglycosylated NET in four tumours (three glioma and one Neuroblastoma).

Mature mRNA encoding for the NET could not be detected via RT-PCR (see 5.3.4) despite the necessary control samples validating that the assay and primers were working correctly. As described in 1.29.2, splice variants in the SLC6A2 gene might be responsible for the expression of alternative mRNA which would not be identified by the primers.

The human NET gene (SLC6A2) is localized to chromosome 16q12.2 (Bruss, Kunz et al. 1993; Gelernter, Kruger et al. 1993), spans >45 kb and consists of 14 exons separated by 13 introns, with consensus sequences for RNA splicing at each exon–intron junction (Porzgen, Bonisch et al. 1995). In 1995 Porzgen et al. identified exon 14, which encodes the carboxy-terminal previously reported by Pacholczyk et al. in 1991 (Pacholczyk, Blakely et al. 1991). Many individual exons encode a single intra- or extracellular domain and transmembrane domain as a common feature with other members of the $Na+/Cl^-$-dependent neurotransmitter transporter family. Rapid amplification of cDNA ends (RACE), primer extension and ribonuclease protection experiments revealed a newly identified exon and multiple transcription start sites for the human NET gene at the 5V-flanking region (Kim, Kim et al. 1999).

The protein-coding region thus begins within exon 2. A number of splice variants in the NET gene have been identified: NET1a, NET1b, NET2a and NET2b. The former two cause intracellular retention of the protein (Bauman and Blakely 2002). Functional expression was only observed in NET1a and NET2a (Kitayama, Morita et al. 2001; Kitayama, Kumagai et al. 2002). At present, there is no useful probe (antibody) to specifically identify NET splice variant proteins (Burton, Kippenberger et al. 1998).

5.4.2 Future direction of studies on the norepinephrine transporter and tricyclic antidepressant activity

An ideal future study would be to quantify the expression of the NET transporter in a tumour (either via IHC or PCR) and to use the ATP-TCA to determine the IC_{50} and Index SUM for the same tumour in parallel, to see if there is a correlation between expression of the transporter and efficacy of the tricyclic antidepressants with higher NET-affinity.

5.4.3 Antidepressant activity and inhibition of neurotransmitters

The debate remains, after forty years of research, as to whether inhibition of noradrenaline or serotonin is the most important factor in conferring antidepressant efficacy and there is still no definitive answer. However, the inhibition of noradrenaline/norepinephrine does appear to be more important than inhibiting serotonin in conferring tumour cell death. When screening the antidepressant drugs in chapter three it was evident that Norclomipramine and Nortriptyline were the most effective of the eight agents tested at evoking tumour cell death *in vitro*.

When comparing equilibrium dissociation constant (K_d; binding affinity for SERT and NET) values for the agents tested (where available) it would appear that both Norclomipramine and Nortriptyline have a higher binding affinity for the NET than the SERT.

Generic Name	**Human SERT K_d(nM)**	**Human NET K_d(nM)**
Amitriptyline	4.3	35.0
Clomipramine	0.3	38.0
Desipramine	17.6	**0.8**
Doxepin	68.0	**29.5**
Imipramine	1.4	37.0
Norclomipramine	*40.0	***0.45**
Nortriptyline	18.0	**4.4**

Table 5.4.1 Antidepressant binding affinities for the serotonin transporter and norepinephrine: relevance to the ability of the tricyclics to induce apoptosis in glioma

Data taken from (Tatsumi, Groshan et al. 1997) and *(Hyttel 1994) showing the equilibrium dissociation constants (K_d) in nM. The drugs that are more selective for the NET than the SERT are highlighted in bold. The data shows that Norclomipramine and Nortriptyline are the most selective for the NET and this correlates with our observation *in vitro* that they are the most effective at causing tumour cell death. Although Desipramine was not screened in this study the data has been included to show the potential of this antidepressant in causing tumour cell death, if indeed the preference for NET over SERT is linked to ability to the initiation of apoptotic cell death.

5.4.4 Drawbacks of using tricyclic antidepressants as glioma therapy

A possible drawback of using antidepressants in brain tumour therapy is the apparent down-regulation of associated transporters following chronic administration. In a study performed using human embryonic kidney (HEK) -293 cells stably transfected with the human NET (hNET) it was shown that a 21-day incubation with Desipramine was sufficient to reduce the specific binding of [3H]nisoxetine in membrane homogenates in a concentration-dependent manner to 77% (Zhu, Blakely et al. 1998). These findings imply that the down-regulation is due to a selective reduction in hNET protein levels, presumably through a translational reduction or enhanced degradation of the hNET protein. Northern blotting indicated that exposure of the HEK-293 cells to Desipramine did not significantly alter mRNA levels. This data is however of limited clinical relevance since the cells used were from kidney and did not contain the native NET. Instead they were transfected with hNET cDNA under the control of the CMV promoter (Boshart, Weber et al. 1985). However it is possible that in a clinical setting down-regulation of the NET in brain tumour patients could pose a problem. An initial clinical trial may involve monitoring the expression of the NET during chronic administration of, for example, Nortriptyline. Indeed it is suggested that the expression of glial NET and SERT may be monitored via the expression on blood platelets (Uebelhack, Franke et al. 2006). This could provide a useful peripheral biomarker system for monitoring the transporter expression without the need for invasive procedures. There would be limited relevance for determining mRNA levels in platelets, since it is the protein levels that are affected, but binding ability could be monitored using radioactive ligands.

5.4.5 The significance of increased monoamine oxidase activity in glioma

In Parkinson's disease a genetically-based or environmentally-produced defect in mitochondrial respiration and the monoamine oxidase clearance of clearance of the neurotransmitter dopamine from the cytosol of neurons is thought to contribute to the development or progression of the disease (Cassarino and Bennett 1999). The increased activity of dopamine causes an increased flux of hydrogen peroxide (H_20_2), derived as a consequence of MAO activity, therefore H_20_2-mediated damage is a possibility (Cohen, Farooqui et al. 1997).

It is possible that a similar scenario exists for brain tumours, and that defects in the mitochondria of glioma cells, which prefer a hypoxic environment (Kaur, Khwaja et al. 2005), cause increased activity of MAO in clearing NE. It is known that MAO-generated H_20_2 oxidises glutathione (GSH) to glutathione disulfide (GSSG), and GSSG reacts spontaneously with thiol groups in proteins to form protein-mixed disulfides (Holmgren 1985; Rabenstein and Millis 1995). If the affected protein thiols are essential for a particular activity, that function is suppressed (e.g. succinate dehydrogenase, NADH dehydrogenase, ATPase, isocitrate dehydrogenase and succinate-supported mitochondrial electron transport) (Berridge and Tan 1993; Benard and Balasubramanian 1995). Therefore the thiol redox state is an important determinant of mitochondrial function, which, in turn, affects cellular viability (Reed 1990; Meister 1995).

Further studies are required before it can be postulated that the NET transporter provides the much sought-after link between the action of tricyclic antidepressants and apoptotic cell death. Whilst we know that Clomipramine exerts its effects through complex III of the mitochondrial respiratory chain, it is not known what events take place between the cell membrane transporter that allow the influx of the TCAs into the tumour cells and the outer mitochondrial membrane. However the fact that the intracellular concentration of biogenic amines, such as NE, determine whether a cell undergoes apoptosis is a key factor (Toninello, Salvi et al. 2004). It is tempting to postulate, since normal human glial cells have the ability to remove excesses of circulating neurotransmitters from the synaptic cleft (to protect surrounding neurons), that glioma cells (some of which prefer a hypoxic environment, particularly glioma stem cells) thrive on the high turnover of NE and ROS produced from MAO degradation of NE. There is evidence to show that apoptosis protection afforded by high concentrations of biogenic amines may be related to their scavenger effect on reactive oxygen species and interaction with the MPTP (Toninello, Salvi et al. 2004). The results of one study performed on rat glioma cells showed that norepinephrine may afford protection against apoptosis through interaction with beta adrenoceptors (Canova, Baudet et al. 1997). Catecholamines such as NE are able to modulate VEGF, an angiogenic cytokine, through ß-adrenergic receptors (ARs). Recently NE has been shown to upregulate VEGF, matrix metalloproteinases (MMPs) and interleukins -8 and -6 (IL8; IL6) in human melanoma cell lines (Yang, Sood et al. 2006; Yang, Kim et al. 2009). This is relevant to brain tumours as cells comprising the CNS and epidermal melanocytes have the same embryological origin, from the neural crest (Gilbert 2008).

Taking together the knowledge that oxygen sensing takes place at complex III of the electron transport chain (Chandel, Budinger et al. 1997; Chandel 2002; Bell, Emerling et al. 2005; Brunelle, Bell et al. 2005; Bell and Chandel 2007), and evidence that Clomipramine exerts its effect via complex III (Daley 2001), it might be possible that by inhibiting the entry of NE into the tumour cell (through the use of tricyclic antidepressants such as Nortriptyline and Desipramine) the hypoxic environment may be disrupted and apoptosis triggered due to subsequent protein-s thiolation of GSH in the tumour cell which suppresses one or more of the enzymes comprising the electron transport complexes, namely complex III (Cohen, Farooqui et al. 1997). There is also the possibility that removing NE from the tumour cell (through blockade of the NET) downregulates VEGF and angiogenesis, as has been shown in melanoma (Yang, Sood et al. 2006; Yang, Kim et al. 2009).

There is evidence to suggest that transformed cells use ROS signals to drive proliferation and other events required for tumour progression. This confers a state of increased basal oxidative stress, making them vulnerable to chemotherapeutic agents that further augment ROS generation or that weakens the antioxidant defences of the cell. In this respect, tumour cells may well die via apoptosis through the same systems that they require to thrive (Schumacker 2006).

5.4.6 Future studies & development strategies associated with the norepinephrine transporter

The next step in this research would be to investigate the effect of blockade or silencing of the NET. It is possible to design small interfering RNAs (siRNAs) against the NET that associate with the RNA-induced silencing complex (RISC) and cleave mRNA. If the NET was 'knocked out' then downstream experiments would reveal any biological impact and indicate whether the NET is involved in the action of the tricyclic antidepressants. It is also important to confirm that brain tumour cells generate more ROS than normal brain cells and if the thioredoxin antioxidant system is paradoxically amplified in malignant cells.

Finding new techniques for individualising patient therapy is a priority as the response to any given drug is highly variable across patient cohorts. This research is extremely relevant in oncology research as here, as in many other areas of therapeutics, chemotherapy needs to be performed within a narrow therapeutic index, balancing drug efficacy with significant drug toxicity.

6 The effects of tricyclic antidepressants on pro- and anti-apoptotic markers in glioma

6.1 Introduction

Malignant glioma are characterised by an intrinsic resistance to apoptosis, and the ability of component cells to efficiently repair DNA damage. Increasing evidence suggests that this is a fundamental mechanism by which gliomas evade elimination when treated with both conventional and targeted therapies. Increasingly, research is being focussed upon understanding the molecular mechanisms because a growing body of evidence would suggest that alterations in the apoptotic pathways are modulators of gliomagenesis and response to therapy.

Traditional cancer treatments, radiotherapy and chemotherapeutics, induce apoptosis only as a secondary effect of the damage they cause to vital cellular components. These treatments affect most proliferating cells without distinction between malignant or normal types. Therefore Clomipramine, and other tricyclics that exert an effect on tumour cells only, is an attractive candidate for glioma therapy. It has been shown in chapter 3 that tricyclic antidepressants have the ability to induce apoptosis in glioma cells (see 3.4) and previous studies indicate that Clomipramine has a direct effect on the mitochondrion (Daley, Wilkie et al. 2005). However it has not yet been shown whether Clomipramine is able to modulate the expression of apoptotic markers, which might further direct its use as a neo-adjuvant or adjuvant therapy. Tumour cells acquire specific anti-apoptotic mutations in the course of their malignant transformation, however they remain primed for apoptosis because of their underlying aberrant phenotype, therefore modulating the expression of apoptotic markers is a promising approach for glioma therapy.

6.1.1 A hypothesis-driven approach to design an apoptosis gene set

It has long been a goal of cancer research to produce predictive molecular assays capable of widespread use. Single genes are rarely useful, unless they happen to be the targets of the drugs concerned, but until recently it has been difficult to produce predictive multigene signatures. Currently, there are two possible approaches to the generation of multigene signatures for predictive chemosensitivity testing. The first is to screen very large numbers of genes using hybridisation arrays to generate signatures that correlate with clinical outcome (Wigle, Jurisica et al. 2002). The second is to take an hypothesis-driven approach to generate gene sets based on knowledge of the pathways involved in resistance and sensitivity to individual drugs (Kikuchi, Daigo et al. 2003). In this study the latter approach has been taken using a pre-designed apoptotic gene set loaded onto 384-well cards. The genes included in this study are: Akt, Bad, Bax, Bcl-xL, Bcl-2, Bid, c-FLIP, Fas, Fas-L, HIF-1α, Hsp60, Hsp70, Hsp90, IAP2, MCJ, NFkB, p53, Survivin and XIAP. This gene set was not intended to be comprehensive, but to include apoptosis-related genes.

6.1.2 The role of heat shock proteins and co-chaperones in apoptosis and cell death

Methylation-controlled J protein (MCJ) is a newly identified member of the DnaJ family of co-chaperones. Co-chaperones associate with the heat shock protein 70 (Hsp70) family of chaperones (Hsp90, Hsp70, and Hsp70) through these conserved domains and participate in protein folding and trafficking (Young, Hoogenraad et al. 2003).

Hypermethylation-mediated transcriptional silencing of the MCJ gene has been associated with increased resistance to chemotherapy in ovarian cancer. However, the biology and function of MCJ remains elusive. The MCJ protein, which localises to the mitochondria, has been shown to contribute to the chemosensitivity of tumours, and it stimulates Hsp70 ATPase activity (Hernandez Alvarado, Chien et al. 2006). MCJ was included in the apoptosis gene study because of its localisation to the mitochondria and association with Hsp70.

Heat shock or stress response is a cellular adaptive response, which helps maintain cellular homeostasis under stress. Among the many changes in cellular activity and physiology, the most remarkable event in stressed cells is the production of a highly conserved set of proteins, the Heat Shock or Stress Proteins (HSPs) (Schlesinger 1986).

Hsp90, which acts as a chaperone for unstable signal transducers to keep them poised for activation, interacts with phosphorylated Akt and promotes NFkB mediated inhibition of apoptosis; in addition it also blocks some steps in the apoptotic pathways. Interaction of Hsp90 and Akt leads to both promotion of cell survival through inactivation of Bad and caspase-9, and inhibition of apoptosis via NFkB (Ozes, Mayo et al. 1999).

Hsp70 is mostly anti-apoptotic and acts at several levels such as inhibition of translocation of Bax into mitochondria (Stankiewicz, Lachapelle et al. 2005), release of cytochrome C from mitochondria, formation of the apoptosome and inhibition of activation of initiator caspases (Tesniere, Panaretakis et al. 2008). When located inside cells Hsp70 inhibits apoptosis at multiple levels. Hsp70 may interact with Apaf-1 thereby inhibiting the formation of the apoptosome (the caspase-9-activating complex) that is required for post-mitochondrial activation (Beere, Wolf et al. 2000). Hsp70 also modulates JNK, NFkB and Akt signalling pathways in the apoptotic cascade. In contrast, Hsp60 has both anti- and pro-apoptotic roles. Cytosolic Hsp60 prevents translocation of the pro-apoptotic protein Bax into mitochondria and thus promotes cell survival but it also promotes maturation of procaspase-3, essential for caspase mediated cell death (Arya, Mallik et al. 2007).

6.1.3 The regulation of hypoxia-inducible factor in normoxic and hypoxic conditions

The principal regulator of transcriptional responses to hypoxia is the hypoxia-inducible factor (HIF) family of transcription factors. HIF is a heterodimeric complex comprised of an oxygen-regulated α subunit (HIF-α) (Semenza, Nejfelt et al. 1991; Semenza and Wang 1992). HIF-α is constitutively transcribed, and the protein continuously translated, however under normoxia it is rapidly degraded by the ubiquitin-proteasome system. Under hypoxic conditions HIF-α protein is not degraded and so accumulation allows it to heterodimerise and translocate to the nucleus (Huang, Arany et al. 1996; Huang, Gu et al. 1998).

The HIF-1α family member is an oxygen sensing transcription factor which requires the mitochondrial electron transport chain, in particular complex III, for stabilisation via an oxidant signal generation under hypoxic conditions (Talks, Turley et al. 2000; Sowter, Raval et al. 2003).

6.1.4 Cellular form of FLICE-inhibitory protein

The cellular form of FLICE-inhibitory protein (c-FLIP), which has also been analysed in this study, blocks death receptor-induced apoptosis and has been implicated in tumour progression. c-FLIP interacts with caspase-8, thereby preventing activation of the caspase cascade. A study of c-FLIP levels, alongside caspase-8 would be useful for agents that induce the extrinsic pathway of apoptosis.

6.1.5 Prophylactic medications that may influence the ability to induce apoptosis in glioma

As previously shown in chapter 4, see Table 4.2.2, patients with glioma frequently take prophylactic medications alongside chemo- and radio-therapy to reduce tumour-related side effects. Anticonvulsant drugs, such as Carbemazepine and Valproic acid, are taken to reduce the frequency of seizures and steroid drugs such as Dexamethasone are taken to reduce cerebral oedema. It is important to screen prophylactic agents for any influence they may have on the induction of apoptosis in tumour cells, to assess whether they would work synergistically or antagonistically with a tricyclic antidepressant, in addition to examining what effect they have on CYP450 enzymes as described in chapter 4.

6.2 Methods

In this study quantitative RT-PCR has been used to examine the expression of several apoptosis-related genes thought to be involved in resistance to some chemotherapeutic agents used in brain tumours. Three cohorts have been tested:

a) Seventeen FFPE samples were screened for apoptotic gene expression
b) SNB-19, DK-MG and UPAB (see Table 2.3.1) were incubated with 25% TDC (see 2.3.12) Clomipramine, Norclomipramine or Nortriptyline for 48 hours
c) DK-MG was incubated with 100% TDC Dexamethasone, Valproic acid or Procarbazine for 48 hours (see 2.3.12)

Firstly seventeen FFPE tumour samples were screened using the TLDA cards to establish if the technique was feasible in glioma, secondly three tumours were exposed to three tricyclic antidepressants to see if apoptotic gene expression changed over 48-hours (the number of test agents was limited to three due to the number of samples that could be tested in one card) and thirdly DK-MG was exposed to Dexamethasone, Valproic acid and Procarbazine (two prophylactic agents and one agent used as part of the PCV regimen, see 1.7.2). The number of samples that could be carried out were limited by the high-cost of the assay (to run each sample costs approximately £200.00).

6.2.1 Patients and Samples

6.2.1.1 Formalin-fixed paraffin-embedded tissue samples

A series of 17 brain tumour samples were obtained from surgical specimens, from Hurstwood Park Neurological Centre, with patient consent. All patients had formalin-fixed paraffin-embedded (FFPE) material taken for histology. This provided a source of material for qRT-PCR, and immunohistochemistry. The age, sex and tumour type for each patient studied are given in Table 6.2.1.

6.2.1.2 Exposing tumour cells to tricyclic antidepressants or prophylactic agents

Glioma cells SNB-19, UPAB and DK-MG were propagated in tissue culture flasks according to the method outlined in 2.2.1, harvested according to 2.2.4 and seeded into 6-well plates at a density of 10,000 cells per ml. Each well of the plate contained 2.0 mls of cell suspension, into which the relevant tested agent was added (see 2.3.12 for test concentrations). The plates were incubated at $37^{o}C/5\%$ CO_2 for 48-hours before harvested and lysing in 1.0 ml RA1 Buffer (Machery Nagel) containing 10 µl beta-mercaptoethanol.

6.2.2 Histology

Haematoxylin and eosin staining (2.3.17) was performed on the seventeen FFPE patient samples prior to analysis with qRT-PCR microarray (Figure 6.2.2.1). The stained slides were used to line up the FFPE blocks with the stylet in the manual tissue arrayer (see 6.2.3).

Patient Identifier	Age	Sex	Tumour Type
TORC07-0003	49	F	Normal human brain control
TORC07-0009	45	F	Oligodendroglioma (II)
TORC07-0010	43	M	Diffuse Astrocytoma (II/III)
TORC07-0011	82	F	Anaplastic Meningioma
TORC07-0012	43	F	Recurrent GBM (IV)
TORC07-0013	39	M	Schwannoma of spinal nerve root
TORC07-0014	41	M	Recurrent GBM (IV)
TORC07-0015	45	M	Anaplastic Oligodendroglioma (III)
TORC07-0016	72	F	GBM (IV)
TORC07-0017	52	F	Metastatic mammary ductal carcinoma
TORC07-0018	74	M	GBM (IV)
TORC07-0019	59	F	Recurrent GBM (IV)
TORC07-0020	48	F	Myxoid Degenerate Meningioma
TORC07-0021	66	F	GBM (IV)
TORC07-0022	73	F	Meningioma
TORC07-0023	72	F	Meningioma
TORC07-0024	69	M	GBM (IV)

Table 6.2.1 Age, Sex and Tumour Type for glioma included in the pro- and anti-apoptotic microarray study

Material was obtained from 12 out of 17 sample blocks. No mRNA could be retrieved from TORC07-0014 (recurrent GBM), TORC07-0016 (primary GBM), TORC07-0017 (metastatic breast cancer), TORC07-0019 (recurrent GBM) or TORC07-0022 (Meningioma). TORC07-0003 was a block of ‘normal’ human brain tissue taken from the periphery of a tumour during standard debulking surgery.

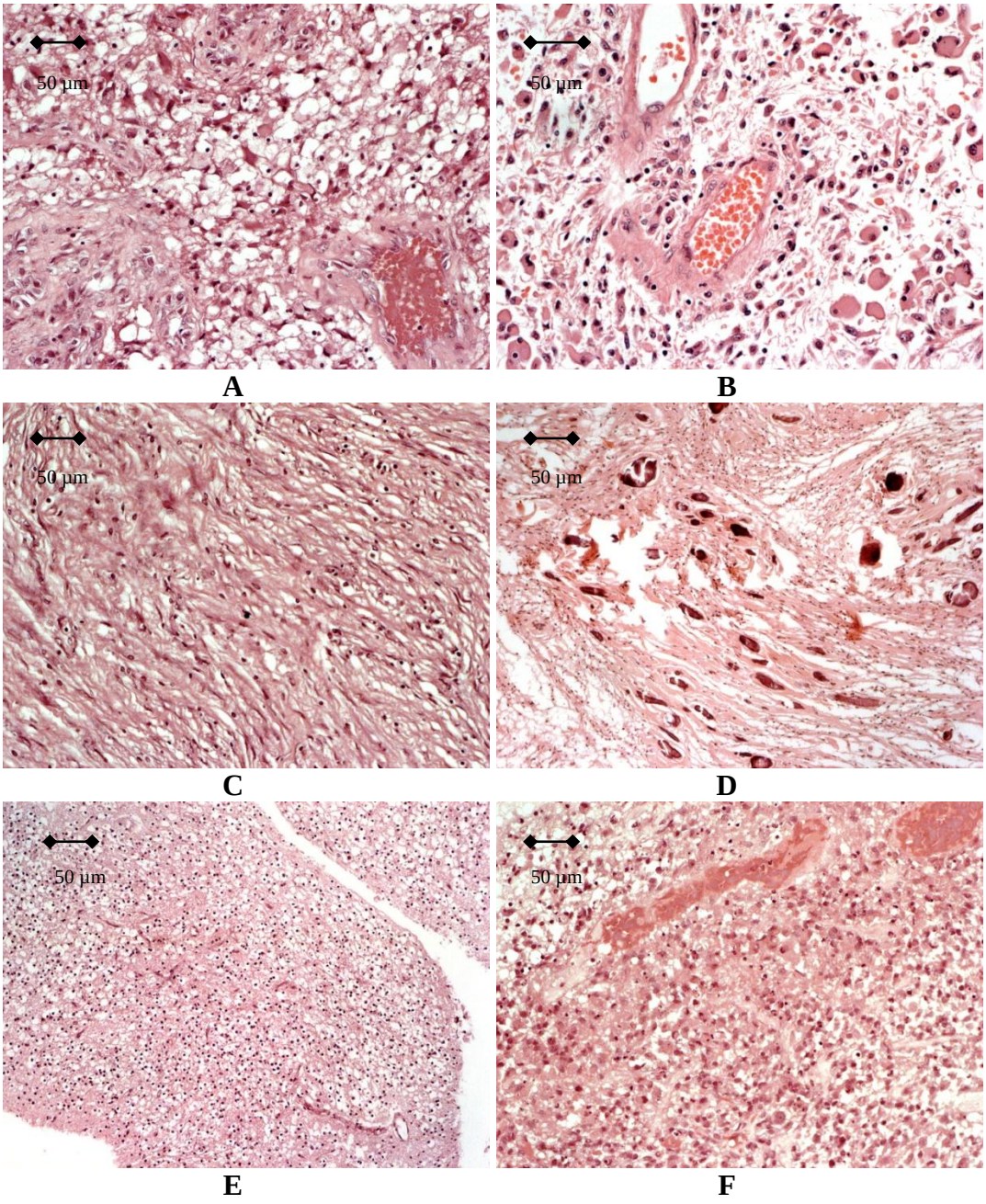


Figure 6.2.2.1 Haematoxylin and eosin staining of glioma included in the pro- and anti-apoptotic microarray assay

A selection of tumours, representing all types included in the study (see table 6.2.1): Glioblastoma multiforme - A shows microvascular proliferation, vacuolation an endothelial cells surrounding the blood vessels. Glioblastoma multiforme - B has large neoplastic glia with eccentric nuclei and extensive eosinophilic cytoplasm. Erythrocytes can clearly be seen within the two large blood vessels. Meningioma – C demonstrates parallel arrays of swirling meningothelial cells. Psammomatous meningioma – D has the same features as C but in addition has psammoma bodies scattered amongst the meningothelial cells. Oligodendroglioma – E comprises small cells with dark nuclei, the cells have a 'honeycomb' appearance and cytoplasmic vacuolation. Astrocytoma grade II/III – F is a highly cellular neoplasm, with large blood vessels present.

6.2.3 Extraction of mRNA from FFPE tumour tissue for use in the pro- and anti-apoptotic microarray study

The haematoxylin and eosin (H&E) stained sections of selected formalin-fixed paraffin-processed blocks of brain tumour core tissue, previously marked with tumour areas of interest (AOI) by a Neuropathologist, were matched and overlaid onto the corresponding FFPE block. The manual tissue arrayer (Beecher Instruments Inc, MTA1) fitted with a punch stylet 0.6 mm in diameter was aligned over the desired AOI, and punched out from the block. The stylet was decontaminated (Ambion DNA& RNA Zap) and cleaned (70% alcohol) between each FFPE block. A minimum of two 0.6 mm punches were obtained from each block and placed in a sterile labelled 1.5 ml microcentrifuge tube. The tubes were then heated at 70 ˚C in a Stuart SBH200D heating block for 20 minutes. Excess paraffin wax was subsequently removed from the tissue using a sterile fine-tip plastic Pasteur pipette.

Pre-warmed xylene (1.0 ml) was added to the tube and heated at 50 ˚C for 10 minutes. The microfuge tube was removed from the heating block, centrifuged at 12,000rpm (12 000g) for 2 minutes in a Sanyo MSE Microcentaur centrifuge. Waste xylene was then removed using a fine-tip pipette; the samples were washed two additional times with xylene. After the final wash the tissue had uncurled. Residual xylene was removed by the addition of 1.0 ml of 100% ethanol, and then the dewaxed sections were allowed to stand for 10 minutes at room temperature. Following centrifugation at 12,000 rpm (12000g) for 5 minutes the ethanol was removed by pipette, and the process repeated once again with 100% ethanol.

The microfuge tube lids were opened to allow the ethanol to evaporate completely at room temperature for 10 minutes, prior to protease digestion.

Protease digestion was performed by use of an Ambion Recoverall kit™. Digestion buffer (200 µl) was added to each tube tissue sample to rehydrate the tissue, followed by the addition of protease (4 µl) and incubated for 3 hours at 50˚C, with occasional flicking of the tube to encourage digestion. 240 µl of the Ambion RecoverAll™ Isolation Additive was added to the microfuge tube, which was then vortex mixed for 20 seconds and allowed to stand for 15 minutes at room temperature. The tubes were pulse spun in a microfuge at 12,000 rpm (12000g) for 30 seconds, after ensuring that the liquid was at the bottom of the tube, the lysate was then passed ≥ 5 times through a 0.8 mm needle (21 gauge) fitted to a 1.0 ml sterile polypropylene syringe to break up any large pieces of tissue. The resulting lysate were then stored at -20˚C for RNA extraction.

The stored lysate samples were thawed gently at $+4^0$C before the RNA was extracted according to the method described in 2.3.23.2 and reverse transcribed to synthesise complementary DNA according to the method outlined in 2.3.23.3.

6.2.4 Extraction of mRNA from fresh tumour cells for use in the pro- and anti-apoptotic microarray study

The RNA was extracted from the cell lysates (prepared according to 6.2.4) according to the method outlined in 2.3.23.2, quantified using a Nanodrop spectrophotometer (2.3.14) and reverse transcribed to cDNA according to the method outlined in 2.3.23.3.

6.2.4.1 Constructing a standard curve

To ensure reproducibility of the TLDA microarray card protocol a quality control tumour cDNA sample was made using fifty different tumours, both CNS and non-CNS. In brief, four 10 µm sections were cut from each of fifty tumour blocks and transferred into sterile eppendorf tubes. They were processed in the same manner as all other test samples according to the methods outlined in sections 6.2.3 and 2.3.23.3. The cDNA was then pooled and split into 40 µl aliquots and stored at -80^{o}C.

Prior to running the test tumour samples four dilutions of the QC cDNA were prepared, specifically 600, 300, 150 and 75 ng/µl of cDNA. The four samples were then pipetted into a TLDA card and run under a standard curve programme. The slope and R2 values for each gene were checked to ensure that the cards were operating correctly and that the fluorophore had not undergone any degradation during storage at +4^{o}C.

6.2.4.2 Polymerase Chain Reaction (PCR) using Taqman Low Density Arrays

TLDA cards are 384-well microfluidic cards containing four sets of 96 identical genes. The 96 genes are split into two rows of 48, each with a loading port. The card contains a control housekeeping gene, 18S. The genes of interest to this study were related to apoptosis, and details of those selected for analysis are detailed in Table 6.2.2 below. Samples can be pipetted into the ports and subsequent centrifuging loads 1µl per well. The cards were run according to the manufacturer's instructions. Each test sample (cDNA + mastermix) was made up with Taqman x2 Universal Master Mix with UNG Amperase (ABI, 4364338) and mixed with an equal volume of tumour cDNA (previously diluted with nuclease-free water to give 600ng/µl) to give a final concentration of 300 ng/µl. Four samples could be tested in one card (4 x 96 genes) by pipetting 100 µl into each of two ports (200 µl per sample required in total per card) of the 384 well cards.

Target	Alternative Name	Gene accession number	ABI Assay ID
APAF1	APAF1L	NM_181861	Hs00559421_m1
Bad	Bcl2 antagonist of cell death	NM_004322	Hs00188930_m1
Bax	Bcl2 associated X protein	NM_004324	Hs00180269_m1
Bcl2	-	NM_000633	Hs00153350_m1
Bcl-x(L)	Bcl2L1	NM_138578	Hs00236329_m1
Bid	-	NM_197966	Hs00609630_m1
c-FLIP	CFLAR	NM_003879	Hs00153439_m1
Fas	TNF receptor superfamily, member 6	NM_000043	Hs00163653_m1
Fas-L	FASLG	NM_000639	Hs00181225_m1
HIF1A	Transcription factor	NM_001530	Hs00153153_m1
HSP60	HSPD1 (Protein Folding)	NM_002156	Hs01866140_g1
HSP70	HSPA4 (Protein Folding)	NM_002154	Hs00382884_m1
HSP90	HSPCA (Protein Folding)	NM_005348	Hs00743767_sH
IAP2	BIRC2	NM_001166	Hs00357350_m1
MCJ	DNAJC15; Hsp50	NM_013238	Hs00387763_m1
NFkB	NFKB1	NM_003998	Hs00231653_m1
p53	TP53	NM_000546	Hs00153349_m1
Survivin	BIRC5	NM_001168	Hs00153353_m1
XIAP	BIRC4	NM_001167	Hs00236913_m1

Table 6.2.2 The apoptosis genes included in the study

To ensure quality control the master mix and nuclease-free water was aliquoted into sterile eppendorf tubes within a class II laminar flow hood designated for ‘clean’ work only, i.e. an environment where no nucleic acid work was carried out. The tubes were then closed and transferred to a class II laminar flow hood designated for cDNA work only prior to the addition of the relevant volumes of cDNA. To check the reproducibility of the assay, purity of samples and operator accuracy a positive control (QC cDNA) and a ‘blank’ (omission of cDNA and substitution with nuclease-free water) were included in the first card run. The loaded TLDA card was then placed, port upwards, into a balanced centrifuge (Sorvall Legend T, DJB Labcare) and spun at 2000rpm/380g to fill the card.

This was checked and the card spun again at 2000rpm/380g to remove any air bubbles. The card was then placed in a TLDA slide sealer, sealed, and the loading ports cut from the card before it was loaded into an AB 7900HT thermal cycler. PCR was performed for 90 min with the following conditions: AmpErase UNG Activation for 2 min at 50 °C; AmpliTaq Gold DNA Polymerase Activation for 10 min at 94.5 °C ; followed by 40 cycles each of Melt Anneal/Extend for 30 sec at 97°C and 1 min at 59.7°C The 'Auto Threshold Cycle' function was performed at the end of the run and resulting Ct data from the TLDA card was transferred to a Microsoft Excel spreadsheet, controls checked, and the data transferred to a Microsoft Access database for further analysis.

6.3 Results

Real time PCR is the most sensitive technique for detecting mRNA (an indication that transcription is taking place) compared to Northern blotting and the RNase protection assay (the two other commonly used assay for quantifying mRNA levels) and is sensitive enough to enable quantitation of RNA from a single cell (Dharmaraj 2011). Of the seventeen patient samples selected for the study, only twelve had detectable levels of housekeeping genes via qRT-PCR. Eleven of these were tumours and one was the normal brain control. In the five remaining samples, no housekeeping genes were detected so these samples were not analysed. It was assumed that this was due to the processing of these particular samples, whereby they had been stored in 10% buffered formalin for a number of months to years. Some of the samples had been transported to the laboratories in culture media before fixation in formalin (so a portion of the fresh tissue could be used for culturing) and this may have affected the quality of the nucleic acids. The presence of nucleases in all eukaryotic cells, particularly RNases, which are fairly small, compact proteins containing several cysteine residues that form numerous intramolecular disulfide bonds, causes problems when transporting biopsy tissue for analysis unless the RNases have previously been denatured (Srinivasan, Sedmak et al. 2002). Because of the delay in embedding the samples the RNA may have fragmented, due to the RNases ubiquitously found in fresh cells, so despite the qualification of both RNA and cDNA by the Nanodrop Spectrophotometer the quality of the cDNA template was too poor for analysis. In the twelve samples that were successfully analysed via qRT-PCR the housekeeping gene was present, 18S.

6.3.1 Constructing a standard curve to ensure efficiency of primers corresponding to pro- and anti-apoptotic genes of interest in glioma

Before running any tumour test cDNA samples the standard curves (Figure 6.3.1.1; Figure 6.3.1.2; Figure 6.3.1.3) were checked to ensure that slope and R2 values correlated with those of historical standard curves (Figure 6.3.1.4). This is a measurement of primer efficiency, and in turn the efficiency of identifying the genes of interest. If the fluorophore or primers are faulty then it can be picked up prior to running any valuable test cDNA. The plots generated are Ct values against log dilution and for relative quantification the amount of cDNA is not required. For a 10-fold dilution you should expect a shift of 3.33 cycles to the right because efficiency = 10^(-1/slope)-1. If the slope value is less than -4 (which equates to 77% efficiency) then there is an error that needs to be rectified before proceeding with test samples.

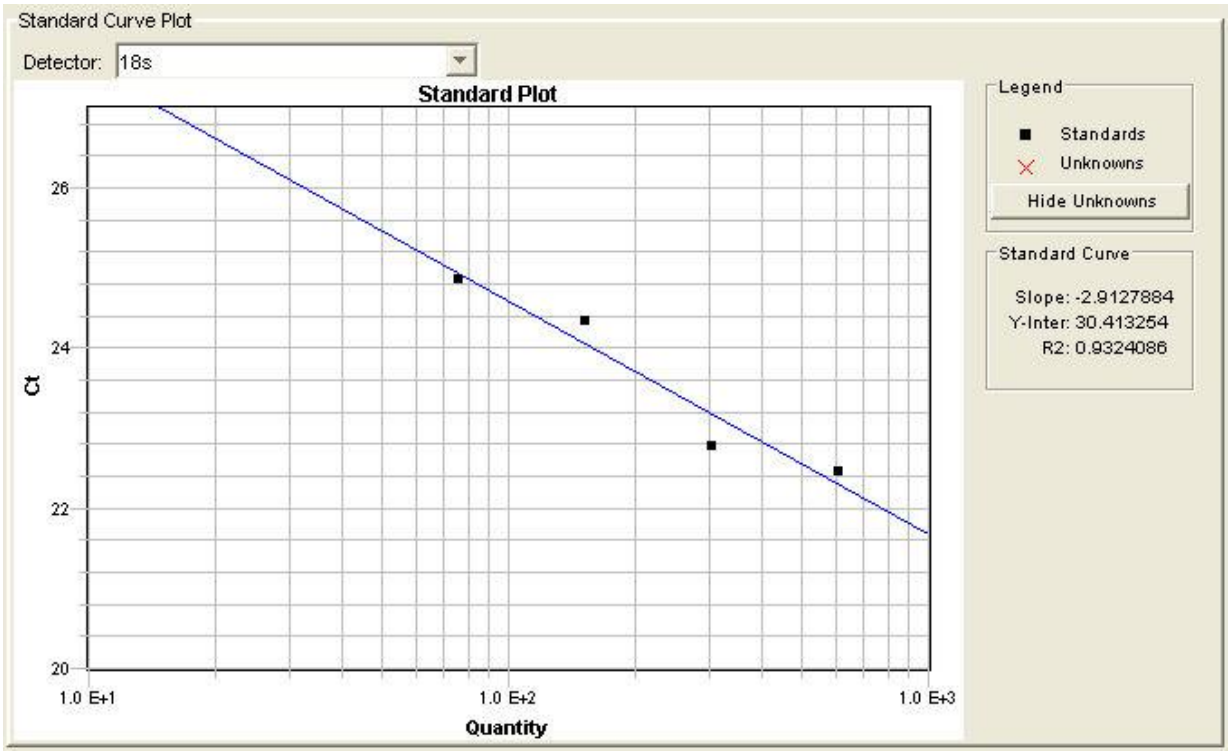


Figure 6.3.1.1 The standard curve for housekeeping gene 18S used to check primer efficiency of the pro- and anti-apoptotic gene set selected for glioma

The screen capture shows the slope value (-2.91) and R2 value (0.93) respectively. The slope value should be as near to -3.3 as possible because this represents 100% efficiency. The R2 value should be as near to 1 as possible, these two values and the plots themselves show that the standard curve was very accurate. There are no outliers and the values obtained are consistent with that of historical standard curves run on the 7900HT.

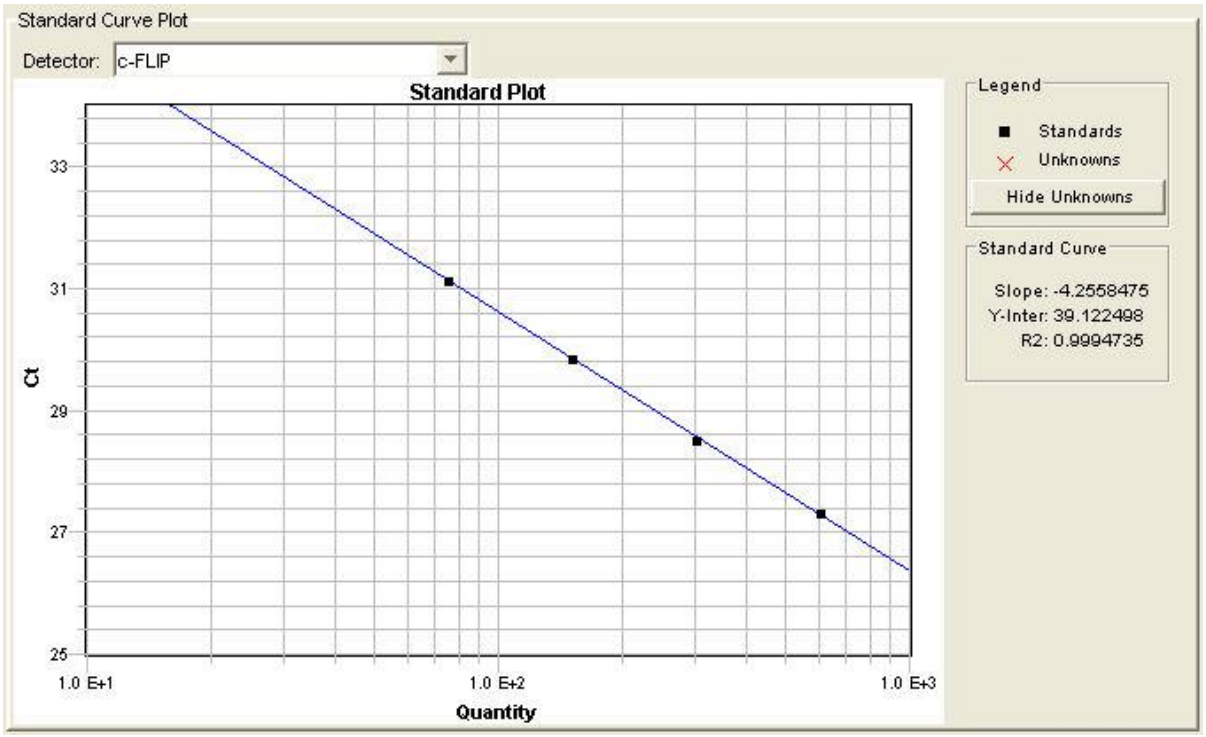


Figure 6.3.1.2 The standard curve for c-FLIP used to check primer efficiency of the pro- and anti-apoptotic gene set selected for glioma

The standard curve screen capture for c-FLIP shows a slope of -4.25 and an R2 value of 0.999, these are deemed accurate according to historical data obtained for the 7900HT.

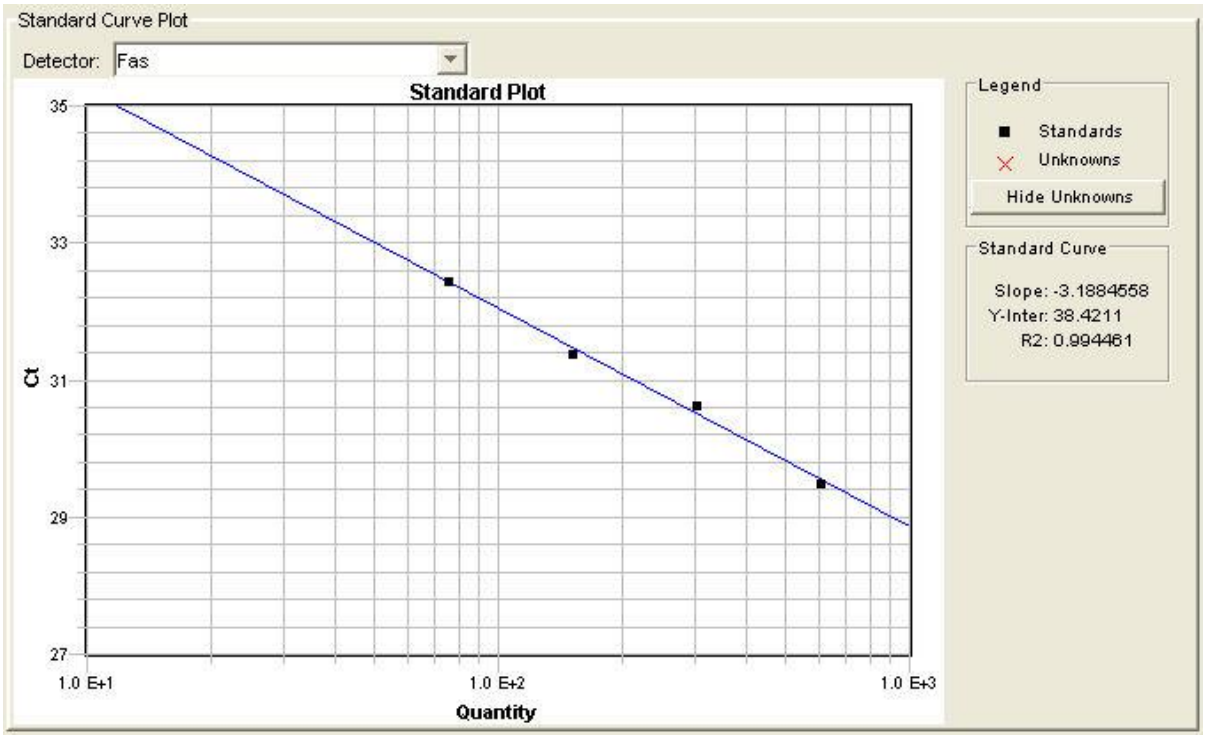


Figure 6.3.1.3 The standard curve for Fas used to check primer efficiency of the pro- and anti-apoptotic gene set selected for glioma

The screen capture for Fas shows a slope of -3.18 and an R2 value of 0.99 which is deemed accurate according to historical data obtained for the 7900HT.

To ensure the accuracy and reproducibility of the microarray cards and the efficiency of the housekeeping gene an audit was performed on QC data for housekeeping gene 18S. Each time the QC pooled cDNA sample was run on a card the values for 18S were stored and compared over 18 months.

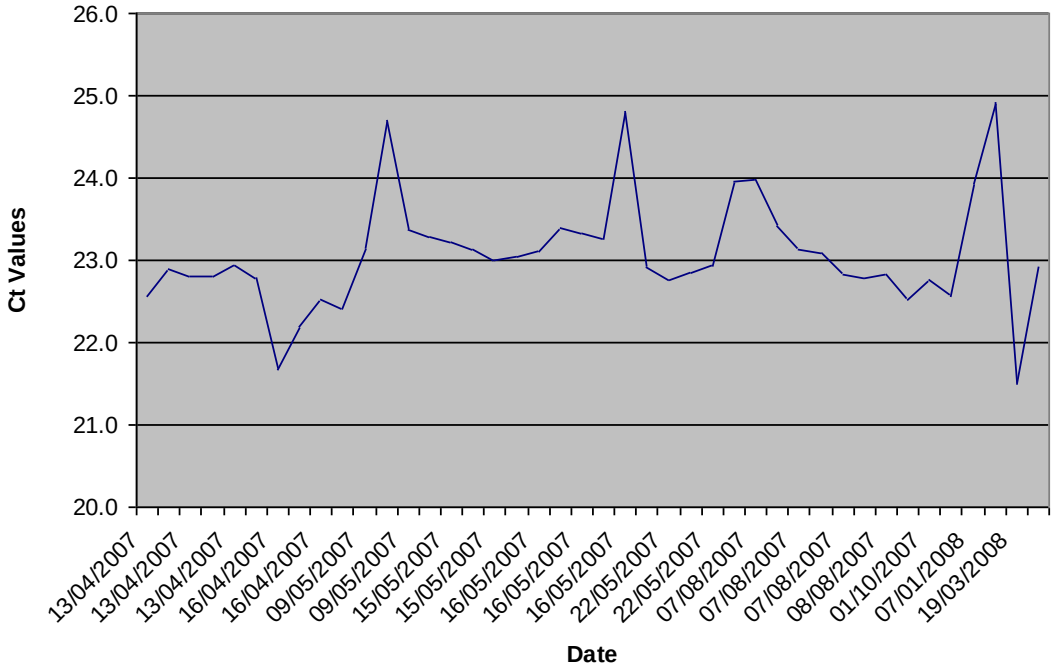


Figure 6.3.1.4 A timeplot of the Ct values for housekeeping gene 18S run using the QC pooled cDNA sample

The number of samples (n) was 42, the mean was 23.1, CV was 2.98% and the p-value (at 95% confidence interval) was 9.52. This shows that the variance in Ct values was low over an 18-month time period, and indicates that reproducibility of the assay is high.

6.3.2 Analysing pro- and anti-apoptotic gene expression data in seventeen formalin-fixed paraffin-embedded glioma test samples

To calculate relative gene expression of the test samples when compared to the housekeeping gene, the comparative method, which is based upon the exponential description of the PCR process assuming constant amplification efficiency equal to 1, was used. Data was first manipulated by calculating 2^-Ct for each gene and then standardised by dividing the 2^-Ct values by that for the housekeeping gene, 18S. The values were then transformed using the natural log of the standardised value, which assumes normal distribution, was then calculated to give relative gene expression.

To analyse the quality of the data obtained a whisker and box plot was constructed (see Figure 6.3.2.1), this is a histogram-like method for plotting data to show the accuracy of the values obtained and compare expression of the genes across all samples tested.

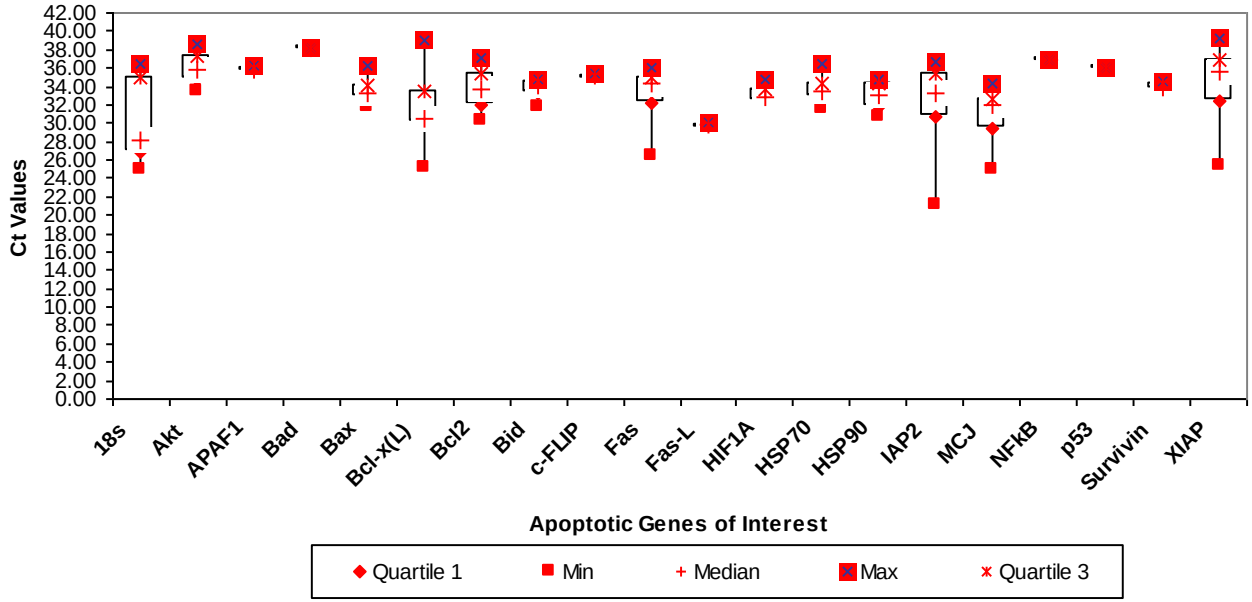


Figure 6.3.2.1 The box and whisker plot for pro and anti-apoptotic TLDA values obtained for all glioma test samples

The box represents 1/2 of the Ct values and is constructed using the first and third quartiles for each gene. The median value should intersect across the middle. The lines or 'whiskers' represent the minimum and maximum values (the range). The data shows that the Ct values obtained for the pro- and anti-apoptotic genes are skewed and there is variation in the Ct values obtained. This also indicates a large degree of heterogeneity in the samples tested because the range of expression varies greatly.

The values on page 268 were either negative (expressed at levels lower than the housekeeping gene, 18S) or positive (expressed at levels higher than the housekeeping gene). The normal brain sample expressed five genes (Akt, Bid, c-FLIP, Hsp70 and Hsp90) at levels lower than the housekeeping gene 18S and the remaining pro- and anti-apoptotic genes (Bcl-2, Bcl-xL, IAP2, Survivin, Bax, XIAP, Fas, Fas-L, HIF-1a and MCJ) came up as 'undetermined' meaning that the levels were so low they could not be detected by the assay. Genes that are undetected by the TLDA assay are expressed, but not upregulated. Of eleven tumours tested, only TORC07-0009, TORC07-0011, TORC07-0015, TORC07-0018 and TORC07-0021 had detectable upregulation of the pro- and anti-apoptotic genes. These are all chemotherapy naïve tumours; if these tumours had been exposed to chemo- or radio-therapy prior to analysis there might be acquired chemoresistance and upregulation of anti-apoptotic genes present and downregulation of the pro-apoptotic genes that were detected in this study (Bid and Bax) (Sarkaria, Kitange et al. 2008; Oliva, Nozell et al. 2010).

	TORC07-0009 Oligodendroglioma	**TORC07-0011** Anaplastic Meningioma	**TORC07-0015** Anaplastic Oligodendroglioma	**TORC07-0018** GBM	**TORC07-0021** GBM
Bcl-2					
Bcl-xL					
IAP2					
Survivin					
Bax					
Bid					
XIAP					
Fas					
Fas-L					
HIF-1a					
Hsp70					
Hsp90					
MCJ					

Table 6.3.1 A table of pro- and anti-apoptotic genes that were upregulated in five of the glioma tested via TLDA microarray

On the left hand side are the names of pro- and anti-apoptotic genes included in this study, across the top are the five tumours for which genes were detected. The genes that were upregulated in the five tumours presented here are shown in blue. Genes that are not relevant to the tumour are blacked out. TORC07-0009 displays the greatest number of upregulated anti-apoptotic genes, suggesting a resistant phenotype. The genes represent susceptibility to apoptosis, and therefore susceptibility to mitochondrially-targeting agents such as Clomipramine.

The data indicates that it is possible to extract mRNA, of high-enough quality for detection of nucleotide sequences specific to pro-and anti-apoptotic genes of interest, from FFPE brain tumour tissue. The quality of the material that can be obtained is largely dependent on the fixation process, in particular the time taken between surgery and placing the sample into buffered formalin.

Factors that affect mRNA retrieval and quality	Recommendations
Tissue autolysis **Nucleic acid degradation via endogenous nucleases**	• Cut samples into small pieces • Place fresh samples in DMEM + antibiotic for transport • Transport in cooled container • Start fixation within 24 hours of resection
Efficiency of cDNA synthesis	• Avoid over fixation – 24 hours maximum to avoid irreversible crosslinks • Store FFPE blocks at $4^{o}C$ – minimises fragmentation of RNA • Do not store pre-cut sections – sections should be taken from blocks as required and used immediately (exposure to light and air affects RNA quality)
RNA integrity	• Isolate RNA within a year after fixation and embedding for optimal nucleic acid retrieval

Table 6.3.2 A summary of factors that affect the quality of mRNA obtained from FFPE glioma biopsies for use in TLDA microarray

The time taken between steps to obtain fresh surgical biopsy tissue, fixation and extraction of mRNA determines the quality of cDNA that can be synthesised for examining the expression of pro- and anti-apoptotic genes in glioma(von Ahlfen, Missel et al. 2007). Looking at the number of samples in which housekeeping genes were detected, the most influential time period in this study appears to be between placing the samples in buffered formalin and embedding in paraffin, overfixation resulting in irreversible cross links and poor quality of nucleic acid retrieval.

If a large enough number of samples could be taken and studied it might be possible to create a signature of apoptosis genes that are upregulated or downregulated in certain tumour types. A larger sample group would yield better quality of data and potential statistical significances between gene expression and tumour type or tumour grade of malignancy. Because of the advances in oncology tumours are detected sooner and, as with somatic cancers, the amount of tissue available for researchers to perform chemosensitivity tests on will become smaller. This means that molecular tests, capable with only small amounts of nucleic acid from fresh or FFPE tissue need to be developed.

6.3.3 Analysing pro- and anti-apoptotic gene expression changes in cells incubated with tricyclic antidepressants

Each tumour had been exposed to Clomipramine, Norclomipramine, Nortriptyline alongside a negative control (untreated cells with omission of any test agents) to enable comparative expression to be calculated via the same method outlined in 6.3.2. The Ct values obtained for each test agent were first normalised against the housekeeping gene, then the values obtained for corresponding negative controls were subtracted from the values obtained for test samples to show change in expression of mRNA following drug exposure. The results shown are pro-apoptotic changes in expression following drug exposure compared to the untreated control, to evaluate if any of the three agents acts pro-apoptotically. For some genes a Ct value was ‘undeterminable’, and therefore these genes were not actively being translated into a functional product.

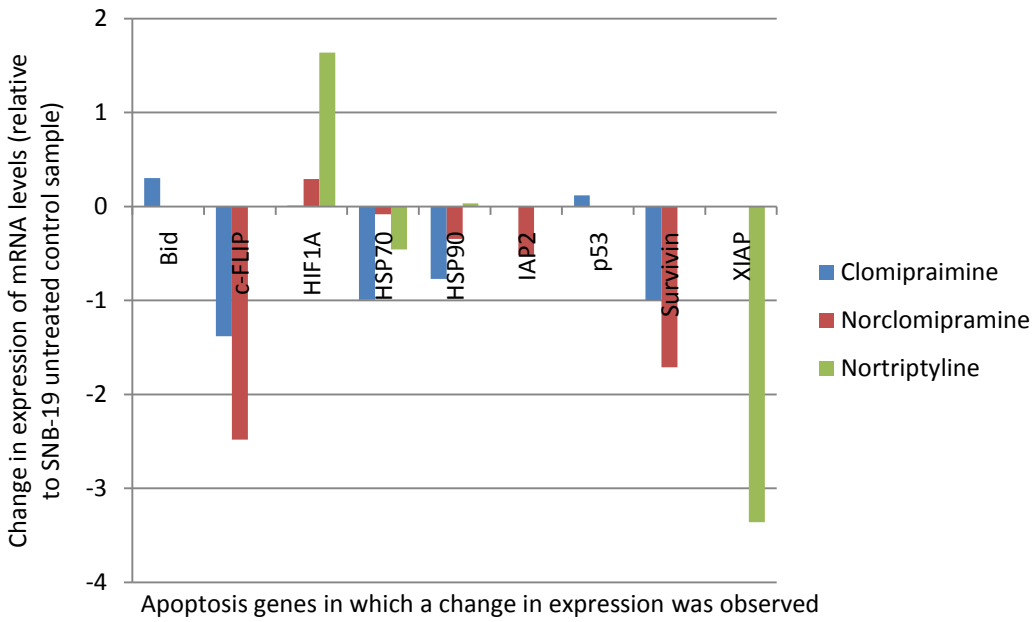


Figure 6.3.3.1 The change in expression of apoptosis-related genes following exposure of glioma cell line SNB-19 to three tricyclic antidepressants

The results shown are those in favour of the SNB-19 cells undergoing apoptosis; therefore they either represent an upregulation in pro-apoptotic genes or downregulation of anti-apoptotic genes. Bid and p53, the expression of which promotes apoptosis, were upregulated following exposure to Clomipramine. C-FLIP, Hsp70, Hsp90 and Survivin were downregulated following Clomipramine exposure, c-FLIP, Hsp-70, Hsp-90, IAP2 and Survivin were downregulated following Norclomipramine exposure and Hsp70 and XIAP were downregulated following Nortriptyline exposure. HIF-1α is involved in resistance to apoptosis and was upregulated following exposure to all three tricyclics and shows response, and adaptation, of the tumour to drug exposure.

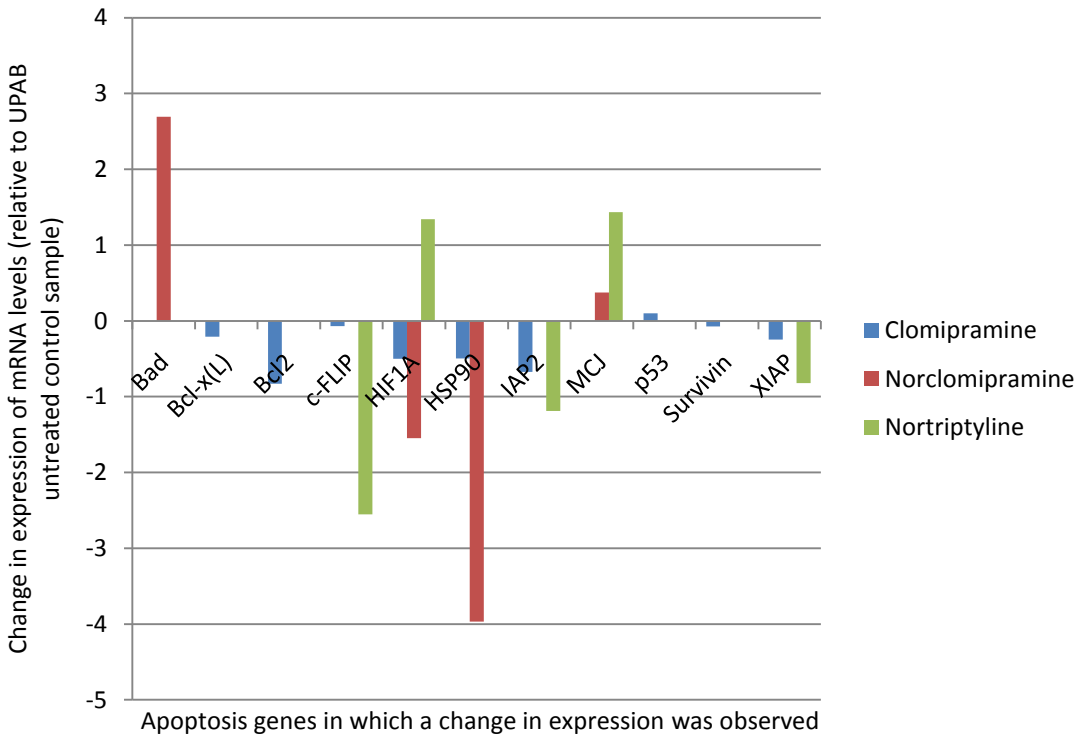


Figure 6.3.3.2 The change in expression of apoptosis-related genes following exposure of glioma early passage culture UPAB to three tricyclic antidepressants

The results shown are those in favour of the UPAB cells undergoing apoptosis; therefore they either represent an upregulation in pro-apoptotic genes or downregulation of anti-apoptotic genes. Bad and MCJ were upregulated following exposure to Clomipramine, p53 was upregulated following exposure to Clomipramine and MCJ was upregulated following exposure to Nortriptyline. Hsp90 and HIF-1α were downregulated following Norclomipramine exposure, Bcl-xL, Bcl-2, c-FLIP, HIF-1α, Hsp90, IAP2, Survivin and XIAP were downregulated following exposure to Clomipramine and c-FLIP and XIAP were downregulated following Nortriptyline exposure. When UPAB cells were exposed to Nortriptyline HIF-1α was upregulated, which is the same effect when SNB-19 was incubated with the same agent.

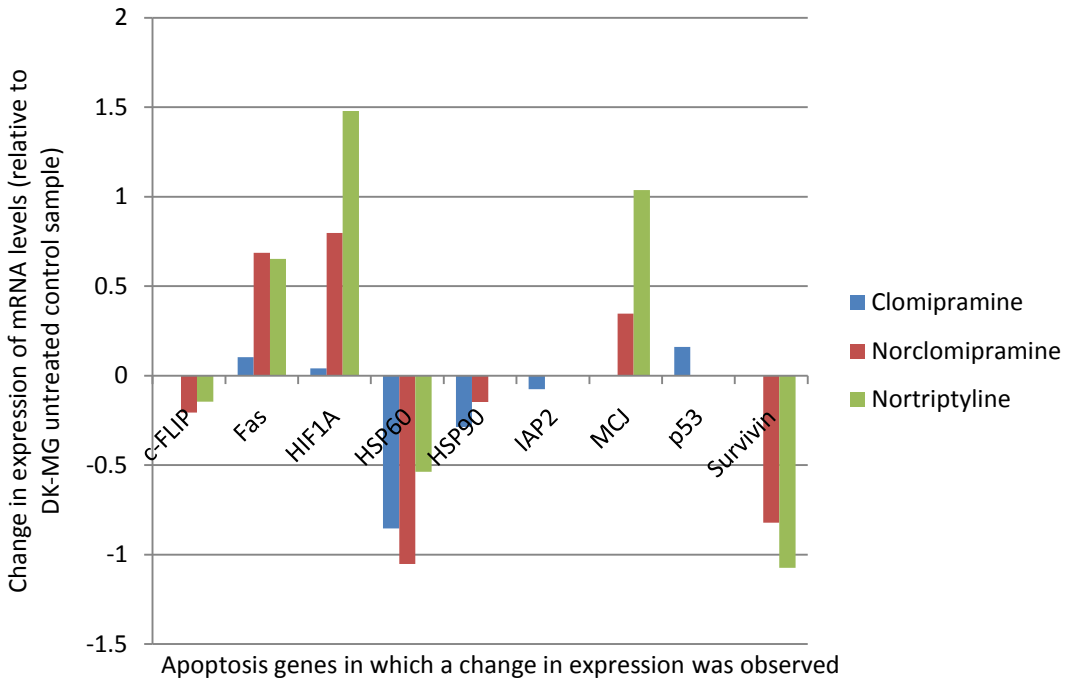


Figure 6.3.3.3 The change in expression of apoptosis-related genes following exposure of glioma cell line DK-MG to three tricyclic antidepressants

Following exposure to Clomipramine, Fas, HIF-1α and p53 were upregulated in DK-MG, whereas Hsp60, Hsp90 and IAP2 were downregulated. Following exposure to Norclomipramine, Fas, HIF-1α and MCJ were upregulated and c-FLIP, Hsp60, Hsp90 and Survivin were downregulated. Following exposure to Nortriptyline, Fas, HIF-1α and MCJ were upregulated whereas c-FLIP, Hsp60 and Survivin were downregulated. All changes, apart from those seen in HIF-1α, are in favour of apoptosis. The upregulation of HIF-1α is a response to drug exposure and indicates the tumour is resisting apoptosis.

The changes observed in all three tumours, when exposed to tricyclic antidepressants, show changes in expression which favours apoptosis. Even though the upregulation of HIF-1α does not pertain to increased susceptibility to apoptosis it was included in the data because it is a marker that requires complex III for stabilisation and was functionally expressed in all tumours and in the two cell lines was upregulated in response to cellular stress, i.e. drug exposure. In UPAB HIF-1α was downregulated, compared to the untreated control, following exposure to Clomipramine and Norclomipramine. It is difficult to isolate and explain the observations of a single gene, and further reinforces the need for studies on multiple genes of interest.

6.3.4 Analysing pro- and anti-apoptotic gene expression changes in DK-MG incubated with Dexamethasone, Valproic acid and Procarbazine

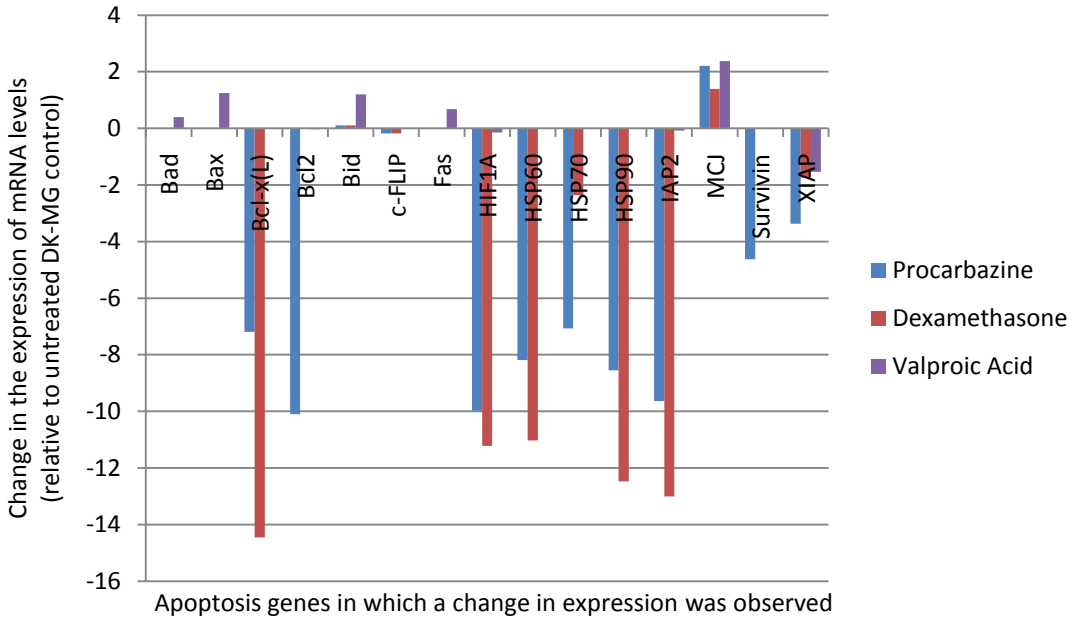


Figure 6.3.4.1 The change in expression of apoptosis-related genes in glioma cell line DKMG following exposure to procarbazine, dexamethasone or valproic acid

Incubating glioma cell line DKMG with Procarbazine for 24 hours resulted in downregulation of ten anti-apoptotic markers, of particular interest are Bcl-xL, Bcl-2 and XIAP which were not affected following incubation with tricyclic antidepressants (see 6.3.3). HIF1-α was downregulated following Procarbazine exposure, suggested reversal of drug resistance adaptation and MCJ was upregulated suggesting chemosensitivity of this tumour. Looking at this data, Procarbazine would be a useful agent to combine with a tricyclic antidepressant, particularly Clomipramine (incubation with which resulted in the lowest expression change for HIF1-α, see 6.3.3) therefore exposing DKMG to Procarbazine for 24 hours prior to Clomipramine might demonstrate a synergistic effect. The effect of DEX on DKMG cells is also quite evident, with a visible downregulation of Bcl-xL, IAP2 and XIAP; however unlike following Procarbazine exposure, Bcl-2 is not affected.

Like Procarbazine, DEX downregulated HIF1-α and upregulated MCJ, which indicates sensitivity to chemotherapy. Dexamethasone, unlike Procarbazine, is a prophylactic prescribed to many glioma patients and data showing its pro-apoptotic properties indicates that it needs further investigation. Valproic acid, like DEX, is a prophylactic anti-seizure medication prescribed to some glioma patients (see data in Table 4.2.2) and when DKMG was exposed to this agent the pro-apoptotic proteins Bad, Bax and Bid were upregulated whereas the anti-apoptotic proteins XIAP and Bcl-2 were downregulated compared to the untreated control cells. The modulation of these proteins is in favour of the cells undergoing apoptosis, and therefore represents a pro-apoptotic effect.

6.4 Discussion

Cancer is a complex process of changes in gene expression and altered regulatory pathways leading to severe changes as tumours develop. Mutations can act in different steps in cell division, motility, adherence or invasion, all of which contribute to cancer (Rempel 1998). A change in the splicing pattern of a gene (through the addition of extra base pairs) can affect different steps in the life of a cell. This can be cell growth, adhesion, migration, invasion and cell death. Additionally, the ways in which splicing is regulated can be altered leading to subsequent tumour formation. In normal human cells tumour suppressors keep cell growth in check and the proteins that regulate splicing are present in normal amounts. This chapter demonstrated the technical procedures required to examine apoptotic gene expression patterns in glioma and the effect that three tricyclic antidepressants, two clinically-relevant prophylactic medications and a traditional chemotherapy agent had on glioma cells.

6.4.1 The role of Survivin in apoptosis of glioma

Survivin, one of the apoptosis inhibitor proteins, has been detected in most cancers in humans (Grzybowska-Izydorczyk, Cebula et al. 2010; Kalliakmanis, Kouvidou et al. 2010; Kim, Kim et al. 2010; Rodriguez-Berriguete, Fraile et al. 2010; Small, Keerthivasan et al. 2010; Stenner, Weinell et al. 2010; Taubert, Heidenreich et al. 2010; Adamkov, Halasova et al. 2011; Bayram, Akkiz et al. 2011; Carrasco, Stamm et al. 2011; Jha, Shukla et al. 2011; Lechler, Renkawitz et al. 2011; Miura, Fujibuchi et al. 2011; Oliveras-Ferraros, Vazquez-Martin et al. 2011; Qian, Xi et al. 2011).

In addition, two splice variants (survivin-2B and survivin-deltaEx3) have been identified. The downregulation of Survivin was demonstrated in DKMG cells following exposure to Procarbazine, Norclomipramine and Nortriptyline (see 6.3), in UPAB following exposure to Clomipramine and in SNB-19 following exposure to Norclomipramine and Clomipramine. Therefore Survivin is a target for tricyclic antidepressant therapy in glioma. The results agree with one study where nine cell lines were tested, and Glioblastoma cell lines showed the highest levels of Survivin expression. The relative expression level of survivin-deltaEx3/Survivin was significantly higher in malignant than in benign brain tumour samples. Expression patterns were dominant for survivin-deltaEx3 in malignant brain tumours and dominant for survivin-2B in benign ones. Quantifying the levels of Survivin and its splice variants is useful for the prediction of the cell biological malignancy of glioma, independent of their pathological features (Yamada, Kuroiwa et al. 2003). It is also useful for demonstrating tumours in which a tricyclic antidepressant might be a useful therapy.

6.4.2 The role of Bcl-2 family proteins of modulating apoptosis in glioma

Because of their critical role in modulating the intrinsic apoptotic pathway, the Bcl-2 family of proteins and others have been studied in human glioma cell lines. Whilst both pro- and anti-apoptotic members have been shown to be expressed, the number of tumours upregulating genes is low. This might be due to the tumours being chemotherapy naïve.

Paradoxically, an overexpression of anti-apoptotic proteins has been found in lower-grade Astrocytoma, compared with GBMs (Steinbach and Weller 2004). This could be a sampling artefact, and difficulty in selecting an AOI has been demonstrated in this study (Figure 6.2.2.1) with tumours demonstrating heterogeneous cell populations or it may be a bad prognostic indicator for certain groups of lower grade glioma (therefore further cases are required). Recurrent GBMs have been shown to exhibit upregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL, and down regulation of pro-apoptotic Bax (Steinbach and Weller 2004). In this study the most compelling data on the Bcl-2 family results from exposing cells to tricyclic antidepressants, to drive a chemoresistant response. Slight changes were observed following exposure of UPAB to Clomipramine (Figure 6.3.3.2); however the most evident changes in expression were noted following exposure to Dexamethasone and Procarbazine (see Figure 6.3.4.1).

6.4.3 Upregulation of Bax, Bcl-xL and IAP2 suggests caspase blockade in TORC07-0021

There was upregulation of pro-apoptotic mRNA Bax and anti-apoptotic protein Bcl-xL (when compared to the normal brain sample). This would suggest that the ability to undergo MOMP in this tumour remained intact. Unfortunately no data was obtained for Bid in this tumour, but the data does show upregulation of IAP2 which is an inhibitor of caspase activation. In TORC07-0021 it could be postulated that resistance to apoptosis would be conferred by blockage of caspase activation by IAP2. It might be possible, in this instance, to use an agent that mimics the function of Smac and releases the IAPs from blocking caspase activation.

6.4.4 Upregulation of Bcl-2, Bcl-xL, IAP2 and Survivin suggests resistance of TORC07-0009 to undergo intrinsic pathway of apoptosis

Higher levels of Bcl-2 in HGG have been shown to correlate positively with survival (McDonald, Ironside et al. 2002). In TORC07-0009 (grade II Oligodendroglioma) Bcl-2 has been up-regulated (compared to normal brain) and as a single gene is impossible to discriminate between whether it is an anti-apoptotic marker or extended survival marker. Because this tumour has been analysed against a series of related apoptosis genes it is possible to build up a profile. The pro-apoptotic proteins Bax and Bid are not upregulated, suggesting the inability to undergo MOMP. There is upregulation of anti-apoptotic Bcl-xL and Bcl-2, and upregulation of IAP2 and Survivin (which are both IAPs). This would suggest that the balance tilts in favour of blockade of intrinsic apoptosis. There is upregulation of Fas and Fas-L suggesting that the receptor and ligand through which extrinsic apoptosis is mediated is intact. This represents a target in this tumour, if it were possible to induce extrinsic apoptosis while simultaneously activating Bid either through caspase-8 cleavage, or even directly, it may be possible to manipulate the intersection of the intrinsic pathway and bypass all the cellular blockades. It is, after all, generally accepted that the use of a single agent will confer little or no benefit because it is necessary to combine both an inducer and an inhibitor to steer the cascade in the direction away from blockade.

6.4.5 Detection of Bid in TORC07-0015 suggests proteins required for mitochondrial outer membrane permeabilisation are upregulated

TORC07-0015 (grade III Oligodendroglioma) showed an upregulation of pro-apoptotic Bid suggesting that MOMP could take place in this tumour, however the upregulation of XIAP and IAP2 show that apoptosis would be blocked at the caspases. Because Fas-L is up-regulated but the Fas receptor is not, it would not be possible to induce the extrinsic pathway of apoptosis through this particular death receptor. Both MCJ and Hsp90 are up-regulated in this tumour; MCJ is the co-chaperone protein for anti-apoptotic chaperone protein Hsp90, yet was found to be expressed in cells that are sensitive to chemotherapy (Hatle, Neveu et al. 2007). Again, this demonstrates the need for a multigene approach towards profiling tumours. As ‘stand alone’ markers proteins mean an entirely different interpretation to when they are up-regulated and analysed alongside other proteins. The MCJ protein is also up-regulated in TORC07-0021 (GBM) but alongside the up-regulation of Bax would appear favourable in terms of sensitivity to chemotherapy.

6.4.6 Upregulation of anti-apoptotic genes in TORC07-0018 suggest a resistance to undergo caspase-dependent apoptosis

There was an upregulation of the inhibitors of apoptosis IAP2 and XIAP in TORC07-0018 (grade IV GBM) and also an upregulation in the Fas receptor. This would suggest that if a stress signal was received, the intrinsic pathway of apoptosis could be triggered but the caspases are inhibited by the IAPs. Again, a Smac analogue might reverse the blocking action of the IAPs in this tumour.

6.4.7 Upregulation of MCJ in TORC07-0011 might indicate sensitivity to chemotherapy

TORC07-0011 (Anaplastic Meningioma) showed upregulation of just one gene, the MCJ protein. The MCJ protein was shown to be downregulated in a series of ovarian tumours that were resistant to standard chemotherapeutic agents (Hernandez Alvarado, Chien et al. 2006). This may indicate that TORC07-0011 is sensitive to chemotherapy, as it also has no upregulation of anti-apoptotic genes.

6.4.8 Exposure to tricyclic antidepressants, Procarbazine, Dexamethasone and Valproic acid modulates the expression of apoptotic genes in Glioblastoma multiforme

Following exposure to Clomipramine, Norclomipramine and Nortriptyline, three GBMs exhibited changes in the expression of pro-apoptotic genes and following exposure to Procarbazine, Dexamethasone and Valproic acid changes in pro-apoptotic genes were observed in DK-MG. Forty-eight hour exposure was sufficient to elicit changes in gene expression in the GBMs tested and the concentrations tested were clinically achievable. All agents tested showed modulation of apoptosis-related genes and should therefore be studied in more detail as to the influence they may have on the success or failure of a chemotherapy regime. Other novel agents that have been shown to modulate the expression of apoptosis-related genes are gamma linolenic acid (GLA), which upregulated Bax in C6 glioma cells (and also downregulated the DNA repair gene Ku80), and boswellic acid which showed a slight downregulation of Bcl-2 in LN-18 cells (Glaser, Winter et al. 1999; Benadiba, Miyake et al. 2009).

Boswellic acid was investigated for use as an alternative to Dexamethasone in the treatment of tumour-associated oedema in glioma, as Dexamethasone is reported to affect the cytotoxicity of traditional chemotherapy agents, however Dexamethasone might prove useful if given in combination with Clomipramine. The study showed that boswellic acid compared favourably with Dexamethasone (Kirste, Treier et al. 2011).

6.4.9 Using an antisense approach to target anti-apoptotic protein Bcl-2 in glioma

Several preclinical studies have demonstrated the feasibility of targeting Bcl-2 in glioma using an antisense approach. Antisense directed at Bcl-2 or Bcl-xL resulted in glioma cell death and sensitisation to the effects of chemo- and radio-therapy (Guensberg, Wacheck et al. 2002; Jiang, Zheng et al. 2003; Zhu, Li et al. 2003). Clinical trials of Oblimersen, an anti Bcl-2 agent, in melanoma have shown that whilst it can be administered with no significant toxicity but minimal antitumour activity was demonstrated (Bedikian, Millward et al. 2006). This lack of clinical activity observed *in vivo* reinforces the fact that single agents, both antisense and standard chemotherapy, generally confer little benefit because of the high degree of 'intelligence' of these aggressive tumours. Bcl-2 is only one member of a family of proteins that collectively impart a tumourigenic effect. This is especially true for the malignant glioma where no single anti-apoptotic protein is responsible for blocking the apoptotic cascade and data in 6.3 provides further evidence that Bcl-2 family proteins are upregulated and downregulated with no set pattern.

6.4.10 Other approaches to target the Bcl-2 family in glioma

A more feasible approach for malignant glioma would be an agent capable of targeting the Bcl-2 family in a broader manner. Walensky et al. demonstrated this approach by developing peptides with a hydrocarbon 'staple' that facilitated successful inhibition of Bcl-2 *in vitro* and in animal models (Walensky, Kung et al. 2004). HA14-1 represents the first small molecule inhibitor to be reported to possess anti-glioma activity in preclinical studies. It was demonstrated to enhance glioma cells to radio- and chemotherapy via inhibition of Bcl-2 binding to Bax (Manero, Gautier et al. 2006). This would allow the cell to undergo MOMP and allow the release of cytochrome C and Smac into the cytosol.

Several small molecule inhibitors are now in clinical development, the compound that has progressed furthest to date is ABT-737 (Abbott Laboratories, Abbott IL), a small molecule that simultaneously and efficiently inhibits Bcl-2, Bcl-xL and Bcl-w (Oltersdorf, Elmore et al. 2005). So far it has not been tested in glioma, but has activity against multiple haematological malignancies and small-cell lung cancer (Oltersdorf, Elmore et al. 2005; Konopleva, Contractor et al. 2006; Del Gaizo Moore, Brown et al. 2007; Kang, Kang et al. 2007; Kline, Rajkumar et al. 2007; Kohl, Hellinger et al. 2007; Trudel, Stewart et al. 2007). The difficulty in treating glioma, not necessarily seen in somatic tumours, is the heterogeneity of the subpopulations of cells comprising the tumours and the heterogeneity between patients. ABT-737 may work in patients with upregulation of Bcl-2, Bcl-xL and Bcl-w but not in patients with up-regulated IAPs. Therefore screening patients for their apoptotic gene signature would be of clinical value.

It would also be important to screen patients for apoptosis gene expression before and after treatment, since recurrent glioma are likely to have altered their expression and become resistant in order to establish an unbalanced apoptotic protein ratio for a second time. From the data obtained in this study, together with that reported by others, there is the suggestion that the general susceptibility of the cell to undergo apoptosis may be an important determinant of tumour chemosensitivity, outweighing more specific mechanisms (Cree, Knight et al. 2002). The qRT-PCR method can be performed with a few nanograms of RNA extracted from FFPE tissue.

6.4.11 Findings from the study of cell death-associated genes in glioma in relation to future developments

All of the samples used in this study were from surgical resection material, but in each case we have only used two 0.6mm punches from the FFPE blocks. Despite the small number of samples it was observed that many of the cell death-related genes contained within the card were expressed in brain tumours. The card, originally intended for use in non-CNS tumours, is not ideally tailored to the genetic profiling of brain tumours. Markers such as TGF-beta 1 and –beta 2 should be included in a card specific for brain tumours, along with invasion markers such as GD3.

6.4.12 Susceptibility to apoptosis *in vitro* as a marker for chemosensitivity

Bcl-2 expression has been studied extensively in various brain tumours (Krajewski, Krajewska et al. 1997; Watanabe, Tachibana et al. 1997) but no link to biological function or expression has been found. In one study of eight adult GBM patients Bcl-2 had no correlation with survival (Sano, Lin et al. 1999). So far Bcl-2 has no predictive value as a standalone marker, in a drug sensitivity study Bcl-2 lacked predictive value (Weller, Rieger et al. 1998) but all these studies have assessed the potential of Bcl-2 as a single gene. Its value in predicting drug sensitivity might be revealed when studied as part of the whole Bcl-2 family.

6.4.13 Technical limitations and practical considerations of profiling pro- and anti-apoptotic markers in glioma samples

From a technical point of view, this approach to sampling tumours for apoptosis profiling has limited uses. When designing a brain-specific TLDA card it is important to select the genes most relevant to the pathogenesis of the tumours. Investigations have recently been explored into the potential value of targeted therapy with Imatinib in recurrent Oligodendroglioma, based on observations of PDGFR overexpression and PDGF-alpha gene amplification in these neoplasms (Vredenburgh, Desjardins et al. 2007), coupled with the known inhibitory activity of Gleevec on the PDGFR-associated tyrosine kinase signalling pathways. It would, therefore, be of use to include PDGFR on a future TLDA card. This technique requires only a small amount of tissue to be able to extract enough mRNA for the TLDA card.

The quality of the nucleic acids extracted from FFPE samples was of poor enough quality in some tumours, due to both fixation issues and the necrotic nature of the tumour core, however the quality of nucleic acids extracted from fresh cells was high. While the size of other tumour types, and therefore tissue available for research, decreases as radio- and chemo-therapy approaches improve (they may be given before surgery is considered) this technique may prove useful. Certainly in lung tumours, where a trans-thoracic needle biopsy would be taken, a method for profiling many genes from a few nanograms of RNA would be useful. However for brain tumours debulking surgery is standard care, to relieve intracranial pressure and oedema, so there is no issue with the amount of tumour tissue available for testing. Perhaps a better method for brain tumours would be to extract nucleic acids directly from fresh tissue, rather than archival FFPE blocks. Future comparison of pre- and post-chemotherapy apoptotic gene expression might prove useful, if it were possible to get parallel samples from the same patient.

6.4.14 Individual response to therapy

Despite small numbers and limited data this study clearly demonstrates that no two tumours are the same, and therefore there is a case for not treating patients with 'standard' modalities. Hence, there should not be a 'one size fits all' approach to the treatment of brain tumours as they are individual in each patient. The mix of sub-populations of cells generates differing characteristics and the ratios of the various cell sub-types determines biological behaviour and response to therapy (Pilkington 2005).

In summary, this study suggests that future studies on the comparison of quantitative data from the ATP-TCA, with that from qRT-PCR, would have the advantage that relatively small numbers of tumours would be required to obtain data on the genes relevant to resistance and sensitivity to pro-apoptotic and other related agents which may show anti-glioma activity. This may prove to be particularly useful to investigate the mechanisms of sensitivity and resistance for drugs which are rarely used as single agents in specific tumour types, and for new drugs which have not yet entered the clinic. This approach spends less time and is less labour intensive than individually analysing single genes by real-time relative quantity PCR; it provides a significant advance for multivariate gene analysis, which can offer much more information about clinical outcome and chemosensitivity than examination of individual genes**.**

7 The potential applications of tricyclic antidepressants in glioma therapy

There are many chemotherapeutic agents available for the treatment of brain tumours, described in the introduction, some of which have been in use for many years and some that, although known and well studied, have only recently been approved by NICE. None of these new agents has however, made any significant impact on median survival times, with only modest improvements seen in concomitant radiotherapy and Temozolomide. Indeed, in the most recent clinical trials for radiotherapy plus concomitant and adjuvant Temozolomide versus radiotherapy alone in Glioblastoma showed that the addition of Temozolomide did not yield any advantage (Linz 2010). Personalised medicine uses information specific to each patient in order to optimise therapeutic regime. The goal is to use genomic, proteomic, transcriptomic, epigenomic and other comprehensive profiles to select the most suitable target for the most efficacious agents. Molecular diagnostics provide important information regarding specific mutations, such as the SNPs, and aberrant molecular pathways, such as those in the apoptotic cascade. Personalised medicine has had success in other cancers, for example HER2 overexpression in breast cancer to guide Herceptin™ therapy and the detection EGFR mutations to guide Tarceva™ in non-small cell lung cancer. As yet this type of approach does not exist for glioma, although tests for MGMT promoter methylation have been in use for clinical trials (to guide the use of TMZ) it is not yet the standard of care and genomic profiles do not form part of the decision-making process.

7.1 Tricyclic antidepressants are able to induce cell death effectively in glioma

Clomipramine is, in many ways, an ideal candidate for chemotherapy for brain tumours because of its lipophilic properties, which means it can cross the B-BB and has a twenty-four hour half life. Steady state concentrations can currently be achieved in man *in vivo* after a two week dose escalation. In addition, it sequesters at high levels in the brain and triggers apoptosis via the mitochondrial pathway. A major drawback of Clomipramine and indeed many other oral chemotherapeutic agents is non-compliance. Anecdotal data from patients taking Clomipramine shows that sometimes a dose is missed or patients attribute their perceived side-effects to Clomipramine and decide to reduce the dose without consulting their oncologist. In 1988 a Japanese research group implanted subcutaneous Clomipramine osmotic mini-pumps into nude male rats and measured both blood levels and brain levels of Clomipramine over seven days. A steady state concentration was achieved after only two days and regional brain distribution was observed (Kurata, Kurachi et al. 1988). A delivery system such as this might prove effective in brain tumour patients, to avoid such non-compliance.

The question when considering Clomipramine for brain tumour patients is; what level can be achieved at the tumour site? The plasma levels reported in chapter 4 demonstrate the metabolic capacity of the individual patient, but it is not known how these levels relate to those at the tumour site. The mean Clomipramine concentration was 69.7 µM ± 94.3 ng/ml mg^{-1} kg^{-1}, and the range was 12.29 – 384.4 ng/ml mg^{-1} kg^{-1}.

The mean Norclomipramine concentration was 107.9 ± 132.49 ng/ml mg^{-1} kg^{-1}, and the range was 18.97 – 593.96 ng/ml mg^{-1} kg^{-1}. Therefore the levels that can be achieved in plasma, when converted to micromoles range from 0.19 – 1.14 µM. The concentrations of Clomipramine tested in chapter three would appear high compared to the levels that can be achieved in plasma (the IC_{50}s for Clomipramine ranged from 15.79 – 46.66 µM, the mean concentration was 25.83 ± 11.61 µM) but evidence from Weigmann et al. suggests there may be a fold increase in Clomipramine concentrations crossing the B-BB (in rats there was a 12.5 fold increase in the concentration of Clomipramine and a 7.4 fold increase in the concentration of Norclomipramine) (Weigmann, Hartter et al. 2000).

When considering the plasma levels that were achieved in rats (converted to µM are 1.27 µM Clomipramine and 6.17 µM Norclomipramine), they are considerably lower than what was achieved in the rat brain (converted to µM are 15.92 µM Clomipramine and 47.32 µM Norclomipramine) (Kurata, Kurachi et al. 1988). So it may follow that the levels that can be achieved in the brain, in man, are a number of fold higher than those in plasma and therefore the levels tested in chapter three are clinically relevant. This evidence supports the concept of specific uptake into the brain.

It is tempting to speculate, in light of the recently presented research (Walker, Muir et al. 2009) that tricyclics such as Clomipramine may not only be used in treatment strategies but can actually protect against cancers including those of the brain. In this large retrospective cohort study of GP clinic data, patients on regular tricyclic drug treatment regimens were considerably less likely to develop tumours of the brain and colon. Therefore it would be scientifically relevant to propose a prospective, blind, controlled trial to test this hypothesis further.

Funding for clinical treatment studies, however, would be a huge challenge because the tricyclic antidepressants are generic drugs and industry support would be limited. It is commonly believed that trials of drugs such as Clomipramine cannot take place without industry support and because it is not financially attractive to the pharmaceutical industry the initial studies would need to be charity-funded, small-scale phase I and II trials. To date, Clomipramine (and Nortriptyline) has been offered as an additional treatment for some cancers, such as Glioblastoma, but the initiation of a small cohort dose-escalation study in patients with GBM should be considered as important in developing effective strategies in treating patients with this devastating and resistant malignancy.

7.2 Cytochrome P450 genotype influences chemosensitivity in malignant glioma

The outcome of glioma chemotherapy is determined by the interplay of several gene products that influence the pharmacokinetics and pharmacodynamics of agents, including drug disposition, specifically drug transporters and drug metabolism. The genotype of a patient needs to be determined only once for any given gene, since it does not change apart from in very rare somatic mutations, and if interpreted according to diagnosis and treatment options may provide clinical relevance. One of the biggest challenges in defining pharmacogenomic traits in malignant glioma is the need for patients who have the same diagnosis and have received the same treatment. As highlighted in this study, patient tumour samples are difficult to obtain and the degree of heterogeneity is large. A larger sample group is required to provide further insight into the prognostic value of CYP450 genotyping and drug metabolism.

In recent years, significant progress has been achieved in the individualisation of chemotherapy due to a plethora of new chemotherapeutic drugs and agents for targeted therapy (Morokoff and Novak 2004; Norden, Drappatz et al. 2008; Dai, Jiang et al. 2010; Krakstad and Chekenya 2010). However, response rates and overall survival rates have not improved significantly for brain tumours. This may be due at least in part to the enormous genetic heterogeneity within brain tumours, even between tumours of the same type (Pilkington 2005) and one hypotheses of this study was that the CYP2D6 and CYP2C19 genotype of brain tumour patients influences chemosensitivity in malignant glioma.

Moreover, besides individual somatic mutations or combinations of these in malignant brain tumours, the genetic background of each individual patient appears to have a major impact on treatment response and overall survival (Deeken, Figg et al. 2007).

It is now feasible to screen up to 1 million SNP genotypes at a time and the possibility of purchasing custom made micro arrays facilitates the screening of selected patient groups for susceptibility genotypes with the purpose of elucidating the question on finding the optimal individual treatment. Likewise, the next generation sequencing methodologies hold the power to allow selection of relevant SNP genotypes for each individual, thereby improving the cancer therapy considerably and diminishing the therapy side effects as much as possible. Establishment of a precise diagnosis based upon the individual genomic profile combined with histopathological tumour characteristics comprises important parameters for selection of optimal, individual treatment for cancer patients in which drug resistance and severe side effects are obstacles for a successful treatment and recovery. SNP genotypes have been associated with the patient's ability to metabolise chemotherapeutic drugs, optimize the cell DNA repair and escape severe side effects of the treatment. Today only a limited number of SNP genotype associations have been established but the technological advances will without doubt increase the number significantly over the next few years.

The future of research for malignant glioma requires focus on individualising treatment regimes for each patient; it is clear from the heterogeneity seen in CYP genes, metabolic capacities, expression of drug transporters and sensitivity of tumours to antidepressants (in this study) that these tumours are extremely heterogeneous and one single approach will not be effective. Because of the rapid cellular adaptation seen in fast-growing primary and recurrent brain tumours it is best to avoid monotherapies, and so developing combinations of synergistic agents would be the next stage in this research.

7.3 Norepinephrine transporter expression in glioma influences sensitivity to tricyclic antidepressants

The present study has demonstrated the presence of the NET transporter on human normal brain astrocytes (see 5.3.1.1), which supports the findings of others that glial cells assist in the degradation of excess catecholamines and protect surrounding neurones (Inazu, Takeda et al. 2003). It has also been shown that human brain tumour cells express the NET transporter (see 5.3.1.2, 5.3.1.3, 5.3.1.4 & 5.3.1.5), and so we can postulate they also possess active monoamine oxidase isoenzymes.

Glioma cells are able to inhibit antioxidant systems which remove ROS; when levels of ROS exceed the attenuation capacity of antioxidant enzymes such as superoxide dismutase (SOD) the proliferative rate exceeds, the reduction-oxidation (REDOX) balance of the cell is altered and can lead to ROS-induced changes in genomic DNA (Hodge, Peng et al. 2005).

7.3.1 Targeting reactive oxygen species and cellular oxidant stress through the Norepinephrine transporter

Reactive oxygen species and cellular oxidant stress has long been associated with cancer, however the nature of this association is complex and paradoxical:

(1) ROS and oxidant stress may induce glioma. According to the mitochondrial paradigm of cancer, mutations in mitochondrial genes encoding parts of the electron transport chain can lead to an increase in ROS generation. Mounting evidence supports the idea that ROS triggers malignant transformation by amplifying genomic instability (Wallace 2005) and might, therefore, mean that norepinephrine is an etiological factor in the development of glioma

(2) Tumour cells generate more ROS than normal cells, possibly due to higher metabolic rates, and some forms of cancer may be associated with increased ROS signalling (Szatrowski and Nathan 1991). This provides an attractive target for agents that target the electron transport chain

(3) The thioredoxin antioxidant system, responsible for maintaining the balance of ROS production, is overexpressed in malignant cells. Paradoxically thioredoxin reductase-1 (TR-1), which maintains an environment required for effective DNA binding of transcription factors and subsequent gene expression is over expressed in a variety of malignant tumours and a loss of TR-1 is associated with a reversal of tumour phenotype and a decrease in tumourigenicity (Hirota, Murata et al. 1999; Yoo, Xu et al. 2006). Silencing of the TR-1 gene in addition to use of a tricyclic antidepressant might prove an effective combination in glioma.

(4) Some chemotherapeutic agents may be selectively toxic to tumour cells because they augment oxidant stress and push these already stressed cells beyond their limit. This common effect suggests that neoplastic cells may be more vulnerable to oxidant stress because they function with a heightened basal level of ROS-mediated signalling, which is required for the increased rate of growth. If this is the case, then a chemotherapeutic agent such as Clomipramine that increases ROS generation may push a tumour cells beyond the breaking point of lipid peroxidation and the scavenging of electrons from the cell membrane lipids causes irreversible cell membrane damage (Adachi, Zhang et al. 2004).

Antioxidants, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, breakdown ROS produced by the electron transport chain and if it were possible to combine Clomipramine (or Nortriptyline) with an agent that inhibits the activity of one of the enzymes responsible for ROS breakdown it might further stress the tumour cell and initiate MOMP, leading to subsequent apoptosis. An alternative approach would be to silence the gene responsible for the production of the antioxidant enzymes, using small interfering RNAs (siRNAs) however this would need to be a tumour-specific effect. One study showed that phenylethyl isothiocyanate augments oxidant stress by decreasing oxidant scavenging and manifests in a decreased mitochondrial potential (Trachootham, Zhou et al. 2006); this may be a suitable candidate for combining with an antidepressant.

7.3.2 Targeting the norepinephrine transporter and mitochondrial superoxide dismutase

Reductions in the levels of SOD have been detected in tumours; however it is possible to restore antioxidant activity using a mimic such as Mangafodipir (Alexandre, Nicco et al. 2006). Combination of a tricyclic antidepressant, which may limit the entry of NE into the glioma cells that express the NET, with an agent that increases mitochondrial SOD (MnSOD; SOD-2) activity in glioma, might restore normal REDOX and antioxidant activity, and therefore susceptibility to apoptosis

7.3.3 Targeting the norepinephrine transporter and monoamine oxidase

It might be possible to combine PCZ with Clomipramine in the *ex-vivo* ATP-TCA study. PCZ inhibits monoamine oxidase (which in turn reduces NE degradation and ROS production) and the combination of this agent with one that blocks entry of norepinephrine into the tumour cell, i.e. Clomipramine, might cause greater disruption to cellular homeostasis and have a pro-apoptotic effect.

7.3.4 Targeting the Norepinephrine transporter and protein kinase C

Lithium carbonate is a mood stabiliser that is used to treat bi-polar disorder and augment antidepressant therapy. Apart from its main function in reducing norepinephrine levels and increasing serotonin levels, which it does via protein kinase C (PKC) suppression, lithium regulates the c-jun N-terminal pathway (Chen, Masana et al. 2000). In normal brain cells the suppression of PKC would simply reduce the levels of NE available to neurones but in tumour cells it causes cell cycle arrest. Because PKC is no longer activated, the levels of cAMP decrease and in turn so does the turnover of ATP (Chuang 2005).

It might be possible to combine a tricyclic antidepressant with lithium, the aim being that the tricyclic antidepressant would block the entry of NE into tumour cells that express the NET (thereby disrupting ROS production and oxygen sensing at complex III, possibly activating MOMP) and lithium would decrease the level of NE available. Studies by Bilir et al. have shown that a combination of Clomipramine and lithium chloride enhanced the effect of Vinorelbine in Neuroblastoma cell line SH-SY5Y (Bilir, Erguven et al. 2010). Because NE is transported into the cell by transporters other than the NE-transporter, reducing the availability of the neurotransmitter could potentially increase the degree of cell kill. Since lithium is usually effective after chronic administration, in a clinical setting it would be appropriate to administer lithium prior to an antidepressant in order for NE levels to be decreased effectively.

ROS and tumour biology are part of a complex equilibrium and it is not clear whether oxidants are required for tumour growth, and if oxidant stress can be exploited therapeutically. The heightened state of basal oxidative stress makes them vulnerable to chemotherapeutic agents, and it would seem that tumour cells may die by the same systems they require.

7.4 Mitochondria are a potential target for therapy in malignant glioma

Evasion of cell death is a hall mark of human cancers and a major cause of treatment failure (Hanahan and Weinberg 2000; Fulda 2009). The lack of efficacy of established therapeutic regimen is due, in part, to the blockade of cell death pathways (Fulda and Debatin 2006). Therefore drugs that activate cell death machinery and target one of the many cascades that converge on the mitochondria are attractive for use in glioma. On one hand mitochondria are critical for the survival of glioma cells, but also they govern the intrinsic pathway of apoptosis and are key regulators of cell death. Mitochondrial functions are frequently altered in glioma (Gogvadze, Orrenius et al. 2008) so mitochondrially-targeted compounds represent a promising approach to chemotherapy-refractive malignant glioma. Recently modifications in the generation of ROS have been linked to chemoresistance which further supports the theory that mitochondria are a target for glioma (Kroemer and Pouyssegur 2008; Bellance, Lestienne et al. 2009). Cancer cell mitochondria are structurally and functionally different to their normal counterparts, mutations in mitochondrial DNA (mtDNA) result in ROS overproduction and inefficient ATP production, moreover (Modica-Napolitano and Singh 2004; Bellance, Lestienne et al. 2009), tumour cells have extensive metabolic reprogramming that renders them more susceptible to mitochondrial perturbation than non-established cells (Gogvadze, Orrenius et al. 2008; Kroemer and Pouyssegur 2008). The reversal of apoptotic cascade blockade and activation of intrinsic cell death machinery such as MOMP represents a strategy to target malignant glioma (Galluzzi, Larochette et al. 2006; Armstrong 2007; Gogvadze, Orrenius et al. 2009).

Conceptually, gene expression profiles describe the transcriptional processes within a cell of a given type or state. It is this variation in transcription between a healthy and cancerous cell of the same type that provides useful information. The utility of this technique is that it does not typically rely on a single gene, but rather the clustering of several genes that highlight disease versus non-disease profiles. It has been shown in chapter 6 that a number of pro- and anti-apoptotic genes were expressed in malignant glioma samples, and that it is possible to modulate the expression using tricyclics and prophylactic agents. It is increasingly evident that the sophisticated cell death machinery present in normal, non-neoplastic, cells has the potential to function normally in tumour cells if the equilibrium between pro- and anti-apoptotic proteins could be restored through the use of agents such as those tested in chapters 3 and 6. The problem however is not simply as a result of overexpression of a single protein, rather the aberrant switching on and off of a series of related genes by the tumour itself and evidence in chapter 6 to show clusters of pro- and anti-apoptotic gene expression support this theory.

7.4.1 Potential applications of combined extrinsic and intrinsic pro-apoptotic therapy in glioma

Looking at the data in chapter 6, it might be feasible to twin one of the tricyclic antidepressants (see 2.3.12) with a recombinant ligand that activates the extrinsic apoptotic pathway via death receptors. Preclinical studies have shown that TRAIL has the ability to induce apoptosis *in vitro* with a subsequent anti-glioma effect. Some tumours remained resistant when it was trialled as a monotherapy but when used in combination with doxorubicin, Etoposide and TMZ an additive effect was noted (Fulda, Wick et al. 2002). Because of the efficacy of the tricyclics, which target the intrinsic apoptotic pathway, it might be possible to demonstrate an additive effect when combining them with a recombinant TRAIL ligand *in vitro*. So far there has been no clinical testing of death receptors (DRs) in glioma due to major concerns over toxicity, in particular the effect of TRAIL on human hepatocytes, but fortunately this effect is not universal to all DRs (Fulda, Wick et al. 2002).

Using a monoclonal antibody directed towards the DRs (Rieger, Ohgaki et al. 1999; Kaliberov, Stackhouse et al. 2004; Goldsmith and Hogarty 2005; Ricci and Zong 2006; Fiveash, Gillespie et al. 2008; Nagane, Shimizu et al. 2010) would be an option for future studies however it would need to be administered intra-operatively during surgery because it would not be able to cross the B-BB and it still may not reach the ‘guerrilla’ cells.

The Fas ligand and receptor were included in markers screened in glioma (see 6.3.2) and was only found to be present in two tumours, therefore using a monoclonal antibody targeted to a single DR would not be effective in all tumours and screening tumours for their apoptotic gene expression patterns to direct therapy might prove useful.

In addition to the clinical trial carried out using Oblimersen (see 6.4.9), work has been done on targeting various proteins that contribute to the apoptotic cascade. One group, using an adenovirus expressing-antisense Hsp70, found that by blocking the anti-apoptotic chaperone protein both triggered caspase-independent apoptosis and caused an immune response (Nylandsted, Wick et al. 2002). The drawbacks to this study were that they used a high-passage homogeneous cell line (which have been shown in chapter three to be under extra stress from immortalisation, see 3.5, i.e. more prone to undergo apoptosis and not as representative of *in vivo* apoptosis mechanisms as using primary cells). They also took a cell line cultured in serum back to a serum-free microenvironment and only targeted one protein. Without data showing the expression of Hsp70 in the xenografts following application of the adenovirus it is difficult to say whether or not Hsp70 was actually depleted. This could have been done via a TLDA microarray card, using either FFPE or collagenase-digested mouse brains.

7.4.2 Targeting the mitochondria with Clomipramine and a cathepsin inhibitor

Cathepsins are lysosomal cysteine peptidases and are part of a large family of lysosomal enzymes that traffick between various intra-cellular organelles and participate in general protein catabolism. They contribute to cancer progression and appear strongly associated with tumour cell invasiveness, through their proteolytic action on the extra cellular matrix (ECM). Both cathepsins –B and –L protect the cell from apoptosis through their action upstream of caspase-3, on Bcl-2. This has been demonstrated by the transfection of anti-sense oligonucleotides into IPTP cells, which resulted in induction of apoptosis (detected via caspase-3 activity) (Levicar, Dewey et al. 2003). In a clinical setting cathepsins are useful both as predictive markers for response to chemotherapy, tumours expressing high levels of cathepsins would not readily undergo apoptosis, and for designing inhibitors against them to be used as an adjuvant therapy. Moreover, Cathepsin L inhibitors such as stefin-A and stefin-B have been shown to increase apoptosis in malignant glioma cells treated with Clomipramine (Levicar, Dewey et al. 2003) by lowering the apoptotic threshold. The results shown in chapter 4 (Table 4.3.1), for the concentrations of Clomipramine in plasma samples, are much lower than the concentrations required to initiate apoptosis in the Annexin V assay (Figure 3.3.1.1) and therefore combining Clomipramine with an inhibitor that lowers the apoptotic threshold, such as one that targets cathepsin L, might prime the cells to undergo apoptosis more readily.

7.4.3 Targeting the mitochondria and gangliosides

Drug-based approaches to induce apoptosis in the mitochondrion carry huge potential for cancer therapy but there are also ways of triggering apoptosis by modulating endogenous processes. Gangliosides are acidic glyco-sphingolipids present during early foetal development of the brain, with GD3 being the predominant species (Kracun, Rosner et al. 1992). In neoplastic tissues gangliosides, including GD3, are overexpressed. They are located in the cell membrane and are responsible for adhesive, migratory and invasive properties in tumour cells (Merzak, Koochekpour et al. 1995). Normally in non-neoplastic tissue an overexpression of GD3 would result in endocytosis and subsequent mitochondrially-mediated apoptosis. However, tumour cells are able to acetylate GD3 into $GD3^{A}$ via sialate-9-*O*-transferase. This process is crucial to the survival of the tumour cell. $GD3^{A}$ expression represents a suitable target for glioma therapy because this ganglioside is not expressed in normal mature glial cells; the expression in mature glial cells is unique to tumours. When the GD3 acetylation is reversed, via the 9-*O*-acetylases, the restored balance of unmodified GD3 triggers the cell to undergo apoptosis (Malisan, Franchi et al. 2002).

It is possible to block GD3 production by targeting GD3 synthase; this can be with antisense oligonucleotides (Birkle, Gao et al. 2000), specific monoclonal antibodies (Hedberg, Dellheden et al. 2000) or imino sugars with structural similarity to N-butyldeoxynojirimycin (Kyriazis 2005).

The Annexin V assay is the most suitable method for visualising apoptosis and it might be possible to transfect glioma cells with antisense oligonucleotides, or expose cells for twenty-four hours to a monoclonal antibody or imino sugar before repeating the same experiment detailed in 2.3.6 to see if apoptosis occurs with lower concentrations of Clomipramine than was seen in Figure 3.3.1.1.

7.4.4 Targeting mitochondria through growth factor withdrawal

Growth factor withdrawal has been well described as a mechanism for triggering the intrinsic pathway and initiating apoptosis (Letai 2006). Several growth factor receptor tyrosine kinases (RTKs) are implicated in gliomagenesis, are thought to inhibit apoptosis and are a rational therapeutic target. Truncation of EGFR is the most common genetic abnormality in adult HGGs, whereas EGFR overexpression has been demonstrated in up to 85% of cases (Andersson, Guo et al. 2004). Other work that has been carried out recently involves the growth factors, such as EGF and VEGF as well as PTEN (Sano, Lin et al. 1999; Wedge, Kendrew et al. 2005).

GBMs often express EGFRvIII, a genomic deletion variant of EGFR which persistently activates the P13K pathway resulting in an anti-apoptotic blockade. This pathway is also regulated via the PTEN tumour suppressor gene. Multiple studies have shown that targeting of the EGFR constitutes possible successful anti-glioma strategies, and inhibition leads to activation of a pro-apoptotic pathway (Nagane, Coufal et al. 1996; Nagane, Levitzki et al. 1998).

However the first small-molecule inhibitors, such as Erlotinib and Gefinitib, have shown disappointing results when tested in HGG. Investigations into the poor response rate revealed that tumours appearing to express the EGFRvIII mutation did not necessarily transmit the anti-proliferative and pro-apoptotic EGFR signals because of an associated intact inhibitory PTEN signal (Mellinghoff, Wang et al. 2005). This again demonstrates the need to determine the expression of more than one protein at a time; single genes are rarely useful since they interact with many others.

Recently an oral, highly potent VEGF signalling inhibitor (Recentin™; Cediranib) has been trialled in patients with acute myeloid leukaemia. Unlike Avastin, Recentin has activity against VEGFR-1, -2 and -3 with additional activity against c-Kit (Wedge, Kendrew et al. 2005). This agent has potential for use in brain tumours and has the ability to block angiogenesis; however blockade of VEGF and angiogenesis would reduce oxygen levels and provide the perfect hypoxic environment in which stem cells can thrive. Recentin would need to be combined with a second agent, possibly one that targets the HIF pathways. Reducing HIF activity in cancer stem cells may promote their differentiation (see 6.1.3), thereby reducing their ability to repopulate tumours after chemo- and radio- therapies. To test this theory, it would be necessary to ablate the function of HIF-1α and HIF-2α in tumour models (Semenza 2003; Keith and Simon 2007).

The phase III randomised, active-controlled, clinical trial SAPPHIRE is currently underway in patients with recurrent or refractory Anaplastic Astrocytoma. Trabedersen (AP 12009), a novel synthetic antisense oligodeoxynucleotide that acts via inhibition of TGF-ß2 (Vallieres 2009) is used to treat patients via convection enhanced delivery directly to the brain. An antisense agent such as this, which reverses immune suppression and restores immune surveillance, could potentially be combined with an agent such as Clomipramine. Better still, if a similar antisense approach using TGF-β1, which modulates growth factor mediated glioma invasion (Merzak, McCrea et al. 1994) would be of value following Avastin® or Recentin® anti-angiogenesis therapy. Both could potentially be used in tandem with Clomipramine and this approach would support the hypothesis that mitochondria are a suitable target in glioma therapy.

7.4.5 Stem cell properties in glioma: implications for targeting the mitochondria

At present, tumour cells with stem cell-like characteristics have been described within several malignancies including brain tumours (Galli, Binda et al. 2004; Singh, Hawkins et al. 2004; Yuan, Curtin et al. 2004). The development of new cell culture methods, including the *in vitro* growth of neurospheres and multicellular tumour spheroids, and new antigenic markers of stem cells and glial/neuronal cell precursor cells, including nestin, Musashi-1 and CD133, have led to a reappraisal of the histological classification and origins of CNS tumours (Pilkington 2005).

These cancer stem cells generally constitute only a minor fraction of the bulk of the mass, but may have an essential role in tumour maintenance. This subset of cells, previously described in Glioblastoma, are capable of long-term proliferation, self-renewal and multi-potency *in vitro* under conditions normally used for neural cell growth (Galli, Binda et al. 2004; Yuan, Curtin et al. 2004). The marker CD133 distinguishes a sub-population of brain tumour cells showing stem cell-like characteristics. It was originally suggested that tumour formation *in vivo* can be solely attributed to a small fraction of CD133+ glioma cells (Singh, Clarke et al. 2003), however this has been subsequently refuted (Wang, Sakariassen et al. 2008). To determine whether CD133+ cells were chemo-resistant, CD133+ cells were isolated from primary cultures of Glioblastoma, previously exposed to Temozolomide (Liu, Yuan et al. 2006). The anti-apoptotic genes Bcl-2, Bcl-xL, c-FLIP, IAP2, XIAP, NAIP and Survivin were expressed at greater levels than autologous cells. Compared to the autologous cells, CD133+ cells expressed >30-fold high levels of the DNA repair protein MGMT, which is an acknowledged predictor of response to alkylating agents. Finally, when autologous primary and recurrent tumour tissues were compared, there was a significant increase in CD133+ expression observed upon tumour recurrence, suggesting that conventional chemotherapy may facilitate survival of the CD133+ cells.

Although additional markers in addition to CD133 are required to accurately recognise cancer stem cells in the brain, these cells form a major target for novel therapies. The finding is that CD133+ populations can be triggered under hypoxic culture conditions (Griguer, Oliva et al. 2008) and that anti-angiogenesis therapies such as Avastin® result in creating a hypoxic environment within glioma. This activates and disseminates CD133+ cancer stem cells suggesting that mitochondrial function in these cells is different from that of standard brain tumour cells. Exploiting MOMP and the NET, therefore, provide a better target for these cells than DNA-acting alkylating agents currently in use for the treatment of glioma (Pilkington, Parker et al. 2008).

The discovery of a chemo-resistant sub-population within Glioblastoma may indicate a novel target for therapy. Because of the adaptation of cancer stem-like cells following conventional chemotherapy to a more resistant phenotype, it is clear that a different approach would be required. Traditional agents target the bulk of the tumour but fail to eradicate the stem cell component; the tumourigenic stem-like cells then adapt, proliferate and up-regulate anti-apoptotic proteins. Therefore targeting the mitochondria of malignant glioma is a suitable approach and tricyclic antidepressants are suitable candidates for therapy.

There are many possible approaches to overcome the stem cell-like sub-population:

1. reverse drug/radiation resistance (see 1.8.7)
2. differentiate the cancer stem cells (see 1.4.1)
3. block the stem cell niche (see 1.8.6)
4. target cell surface antigens (see 1.8.5)
5. mitochondrial-based therapies (see 1.9)

The aspect analysed in this thesis, the intrinsic mitochondrial pathway associated therapies, specifically the tricyclic antidepressants, which may be used in various combinations, have recently been used to target CD133+ cells from malignant paediatric GBM under both hypoxic and normoxic culture conditions. Growth under hypoxic conditions was seen to protect against tricyclic-drug mediated apoptosis whilst CD133+ GBM-derived populations were also more resistant to Clomipramine than CD133- GBM-derived cells (Pilkington & Donovan, unpublished observations). In order to combat CD133+ cancer stem cells from glioma it might, therefore, be necessary to modulate oxygen conditions and then further investigate the mitochondria of these cells with regard to function of mitochondrial DNA mutations.

7.5 Future directions of research in malignant glioma

The search for an improved, more specific and less toxic approach to treating malignant glioma is being conducted at a remarkable pace, with strategies simultaneously being focussed upon angiogenesis, epigenetics, glioma stem cells and tumour immunology. There is substantial evidence from this study that Clomipramine (and indeed Nortriptyline) might be beneficial in human malignant brain tumours (see 3.3.1 & 3.4). There are forty years of pharmacokinetic and toxicity data from its use as an antidepressant, this would support an easy translation to early phase clinical trial. Clomipramine could be tested in both malignant glioma and malignant melanoma, which would further reinforce its evaluability as a potential chemotherapeutic agent. It is unknown whether previously studied dose ranges will achieve the necessary cytotoxic intra-tumoural concentrations of Clomipramine and, in addition, the nutritional and metabolic profile of patients with cancer differs from those in published Clomipramine papers for use in depression. It is for this reason that Clomipramine needs to be administered on an individual basis.

Current DNA microarrays allow us to simultaneously determine gene expression analysis of all known genes, and large-scale gene expression studies have provided new molecular classifications for certain brain tumours, for example Oligodendroglioma and the use of 1p19q. The results of the TLDA microarray are interesting since Oligodendroglioma are generally more sensitive to DNA-acting alkylating agents, where there is loss of heterozygosity (LOH) on 1p19q.

Based upon this co-deletion, Oligodendroglioma are treated with chemotherapy but astrocytic tumours without LOH on 1p19q are not. Clomipramine is a potential chemotherapeutic agent for use in these tumours without LOH on 1p19q.

Research into GBM should focus upon the mechanisms by which these aggressive tumours evade current treatment regimens, to develop novel agents and provide innovative therapeutic strategies. Increasing evidence would suggest that aberrations in the apoptotic pathway in the glioma are vital modulators of both gliomagenesis and response to therapy (Cavaliere and Newton 2006). The expression of anti-apoptotic proteins is an important physiological safeguard to prevent cellular destruction and the proteins that govern this phenomenon play a role in the pathogenesis of malignant brain tumours (Fulda and Debatin 2006; Fulda 2007; Fulda 2008; Fulda 2010; Fulda 2010; Fulda, Galluzzi et al. 2010).

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9 Appendices

9.1 Annexin V data showing apoptosis occurring in glioma cells treated with clomipramine hydrochloride

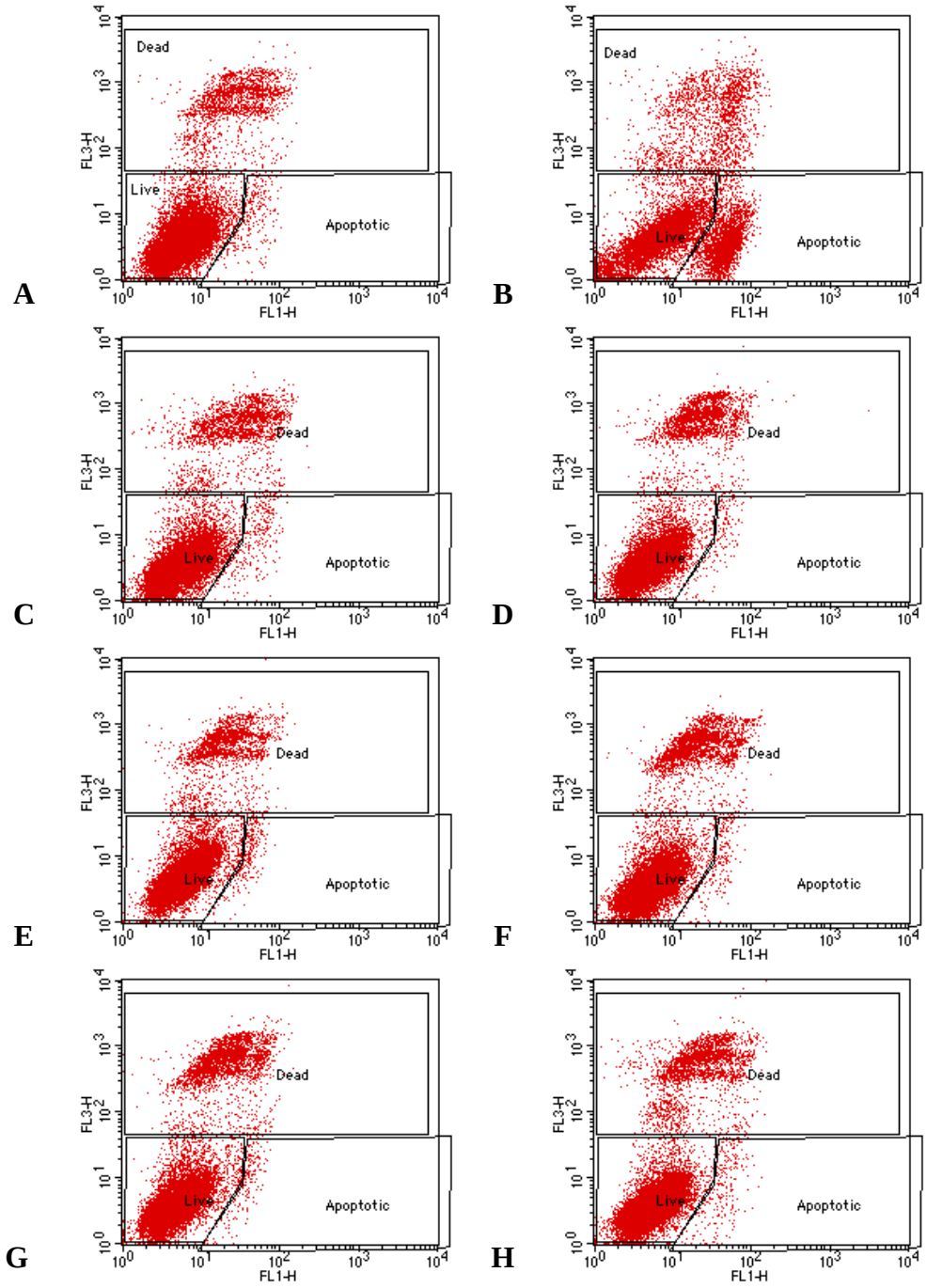

Figure 9.1.The Annexin-V flow cytometry data showing apoptosis occurring in tumour SNB-19 incubated with 20µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

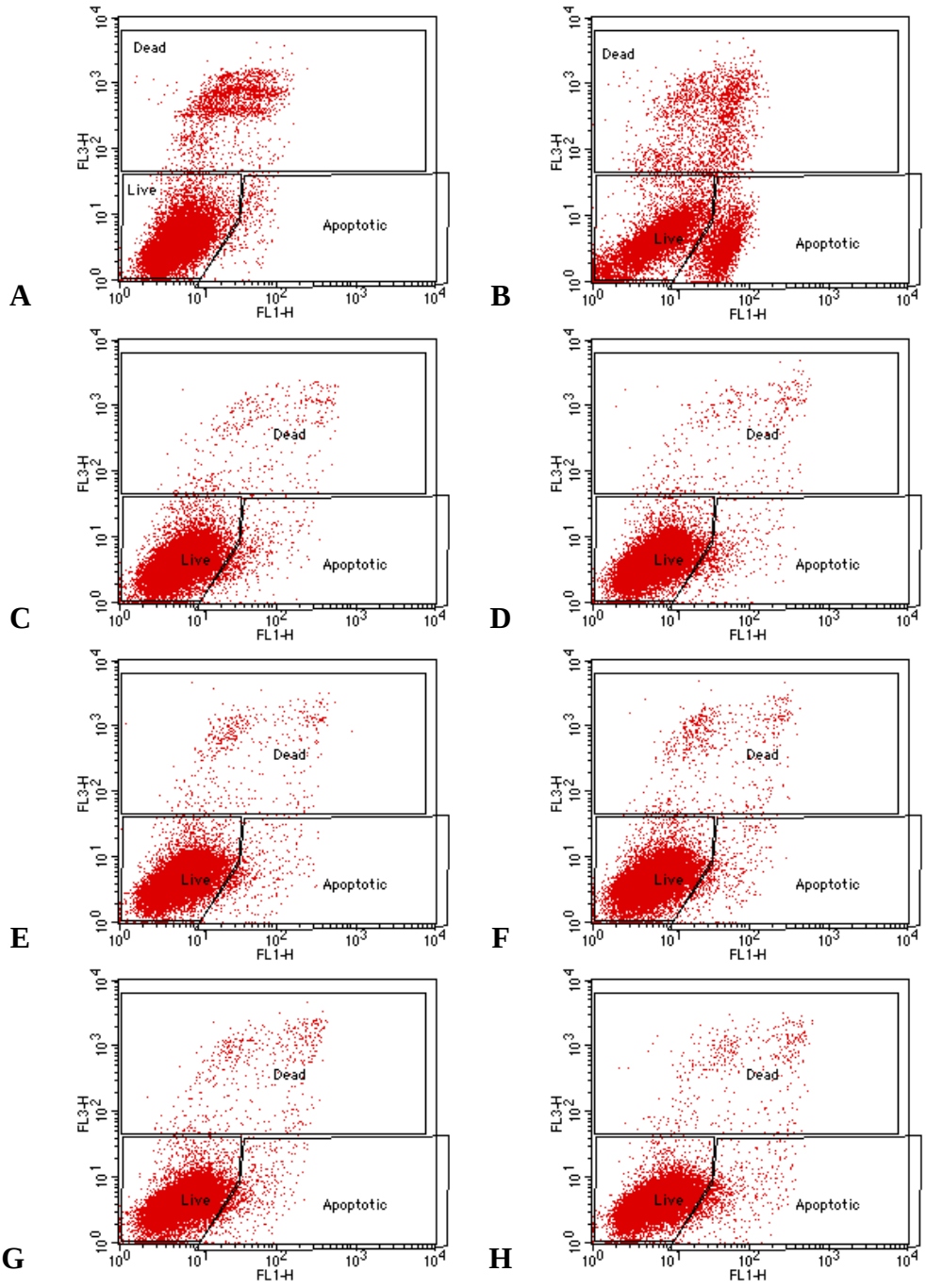

Figure 7.4.5.1The Annexin-V flow cytometry data showing apoptosis occurring in tumour SNB-19 incubated with 40µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

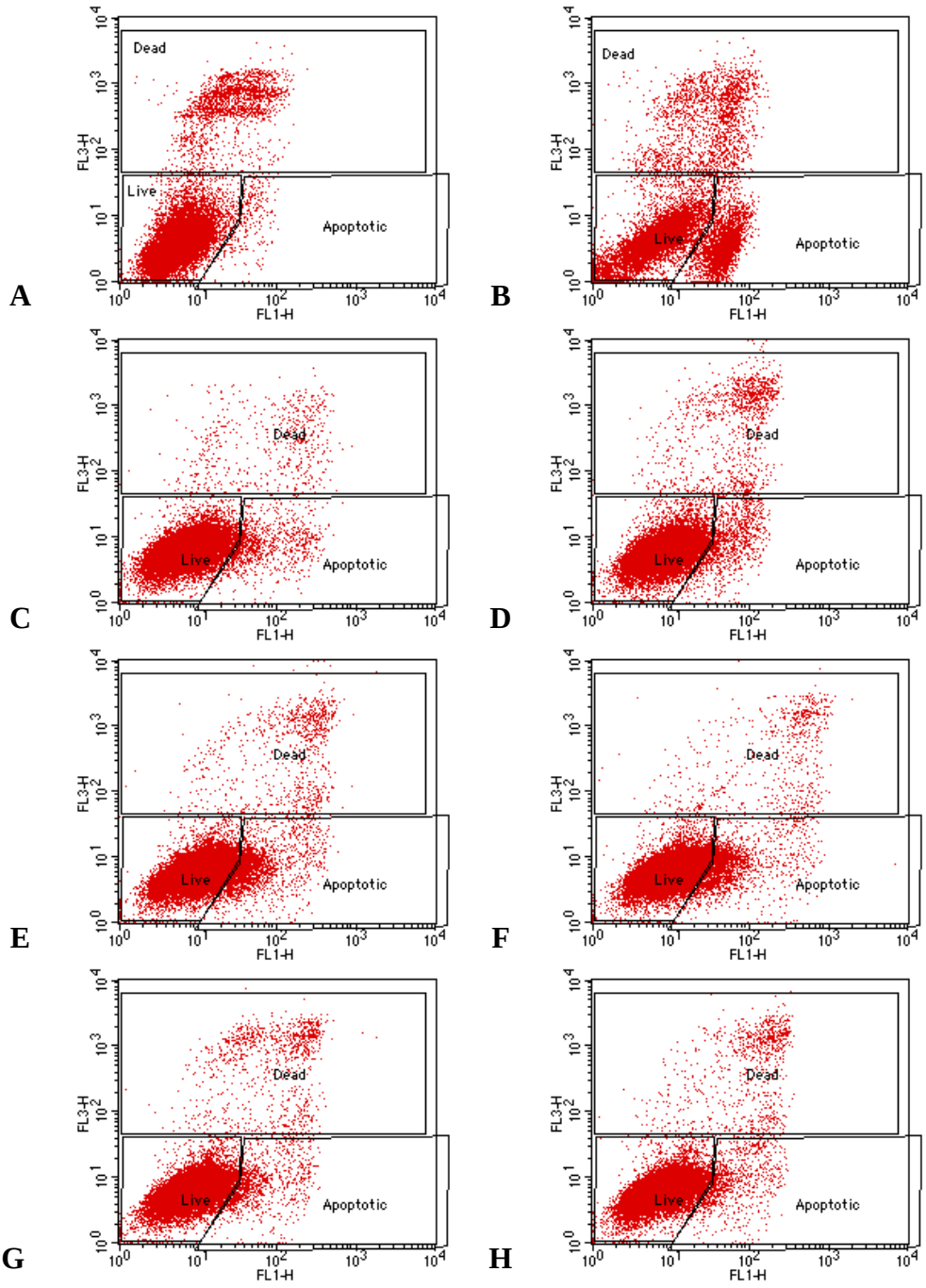

Figure 7.4.5.2The Annexin-V flow cytometry data showing apoptosis occurring in tumour SNB-19 incubated with 60µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-3 hour samples (C-E respectively), whereas apoptosis is starting to occur in the 4-6 hour samples (F-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

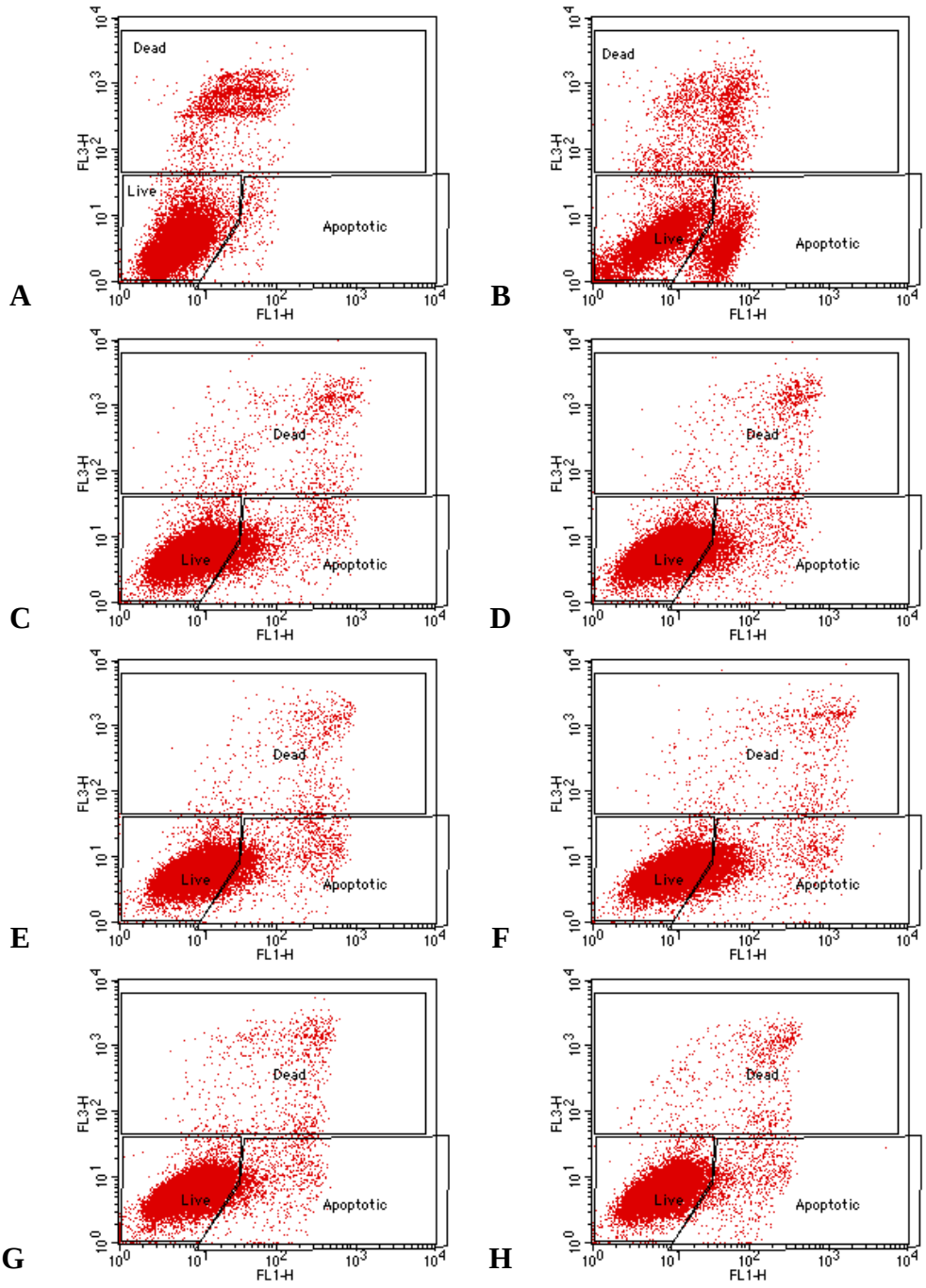

Figure 7.4.5.3The Annexin-V flow cytometry data showing apoptosis occurring in tumour SNB-19 incubated with 80µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is apoptosis in all samples (C-H respectively. The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

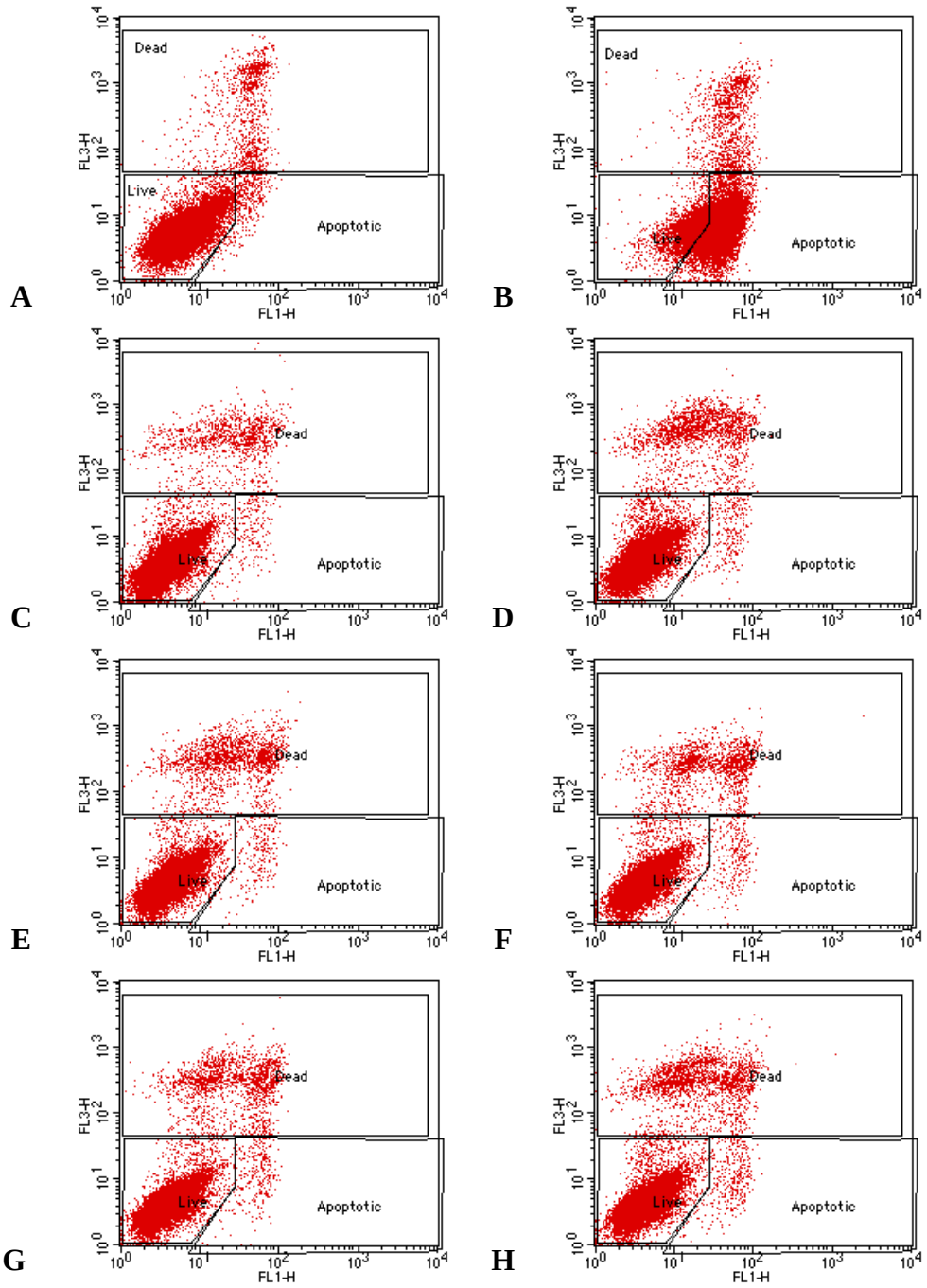

Figure 7.4.5.4The Annexin-V flow cytometry data showing apoptosis occurring in tumour DK-MG incubated with 20µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

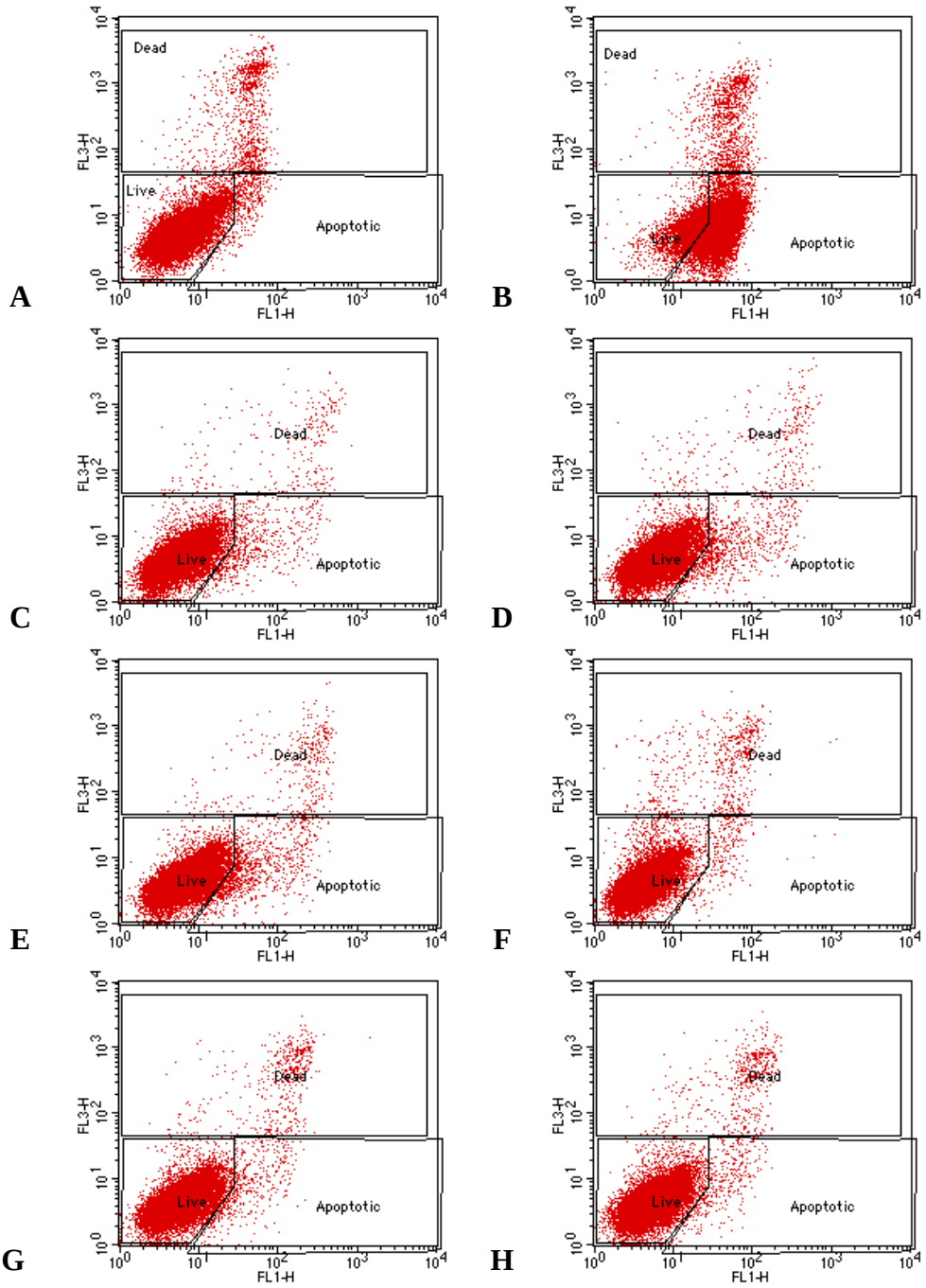

Figure 7.4.5.5The Annexin-V flow cytometry data showing apoptosis occurring in tumour DK-MG incubated with 40µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

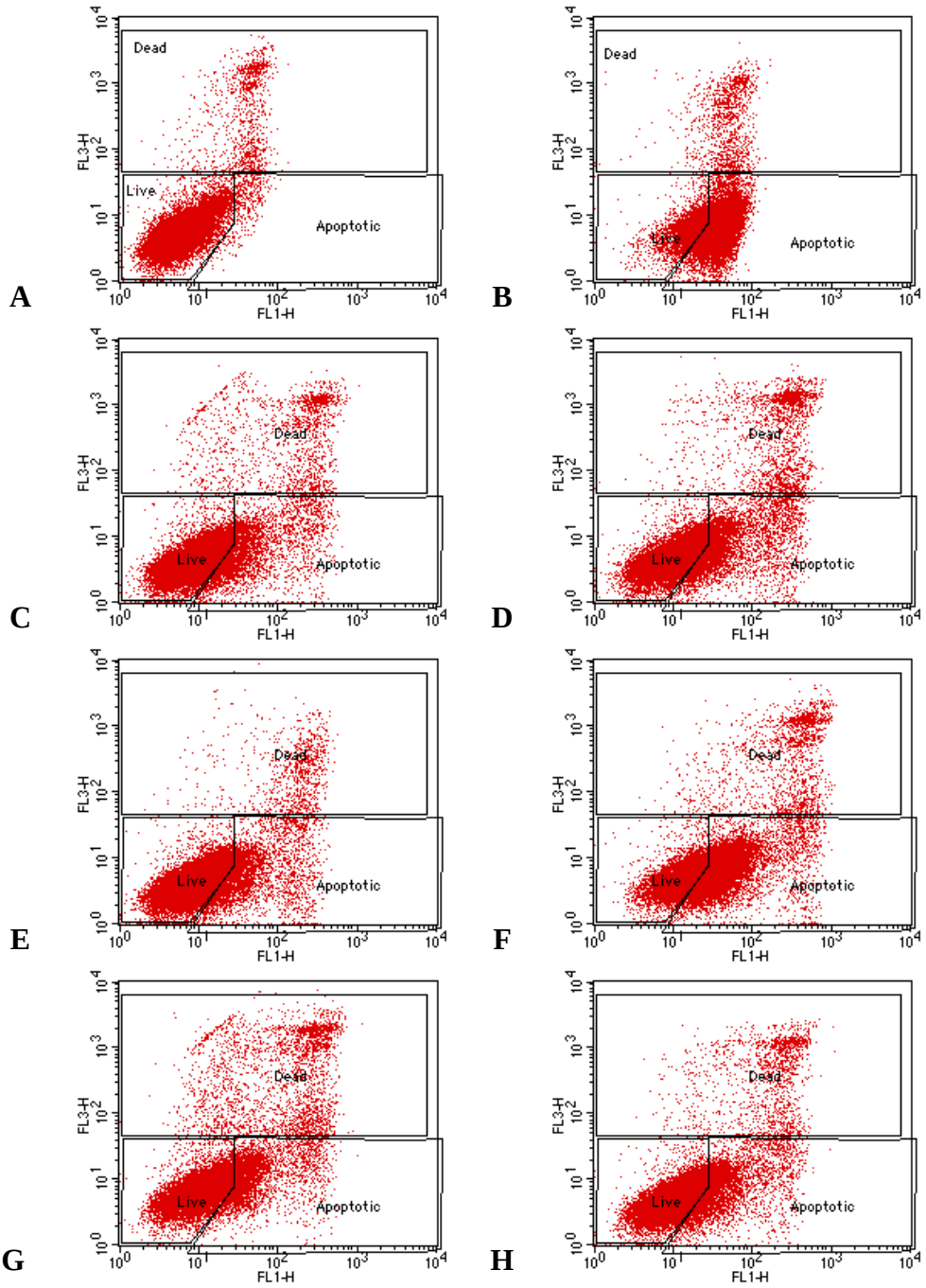

Figure 7.4.5.6The Annexin-V flow cytometry data showing apoptosis occurring in tumour DK-MG incubated with 60µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

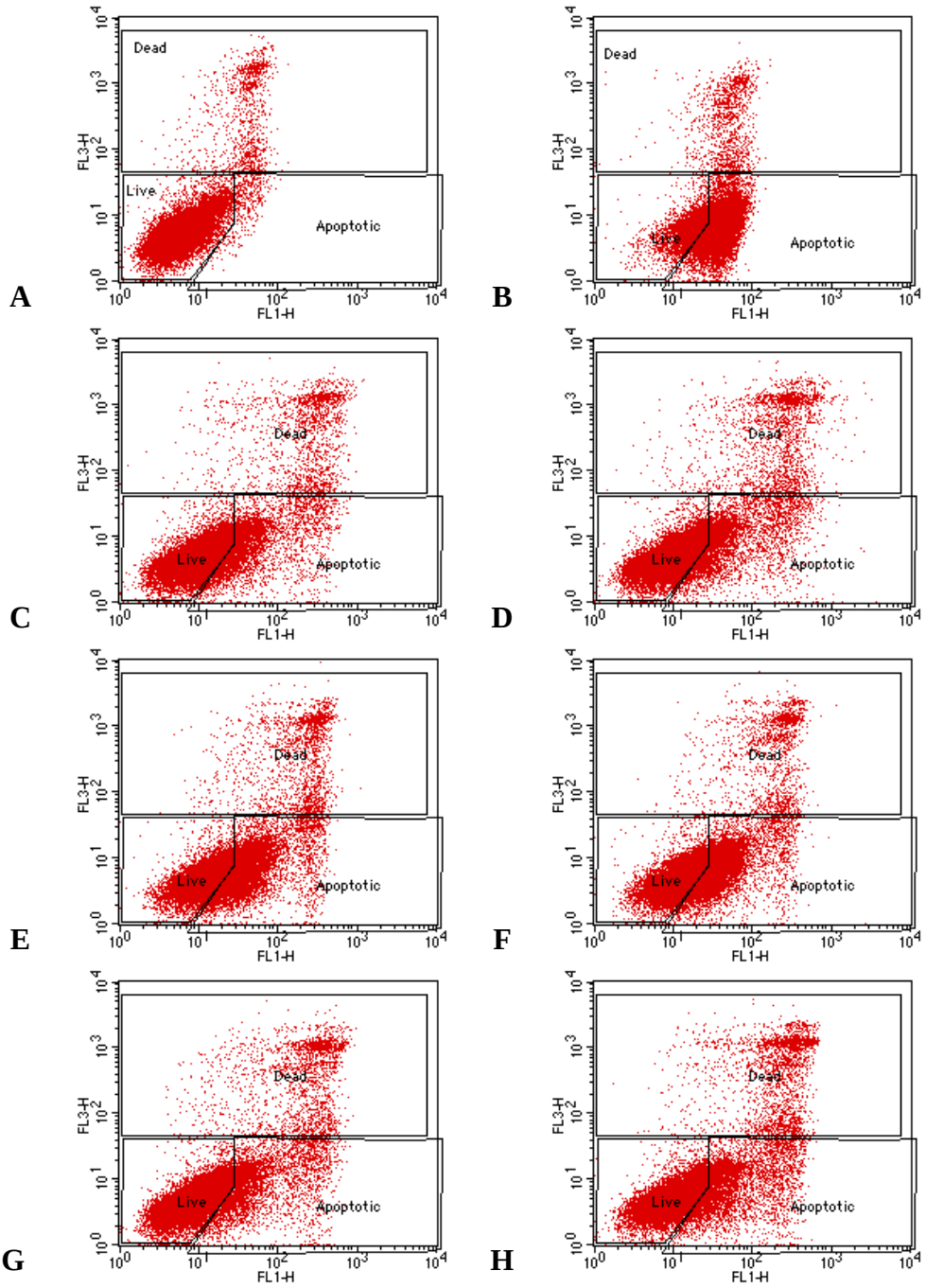

Figure 7.4.5.7The Annexin-V flow cytometry data showing apoptosis occurring in tumour DK-MG incubated with 80µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

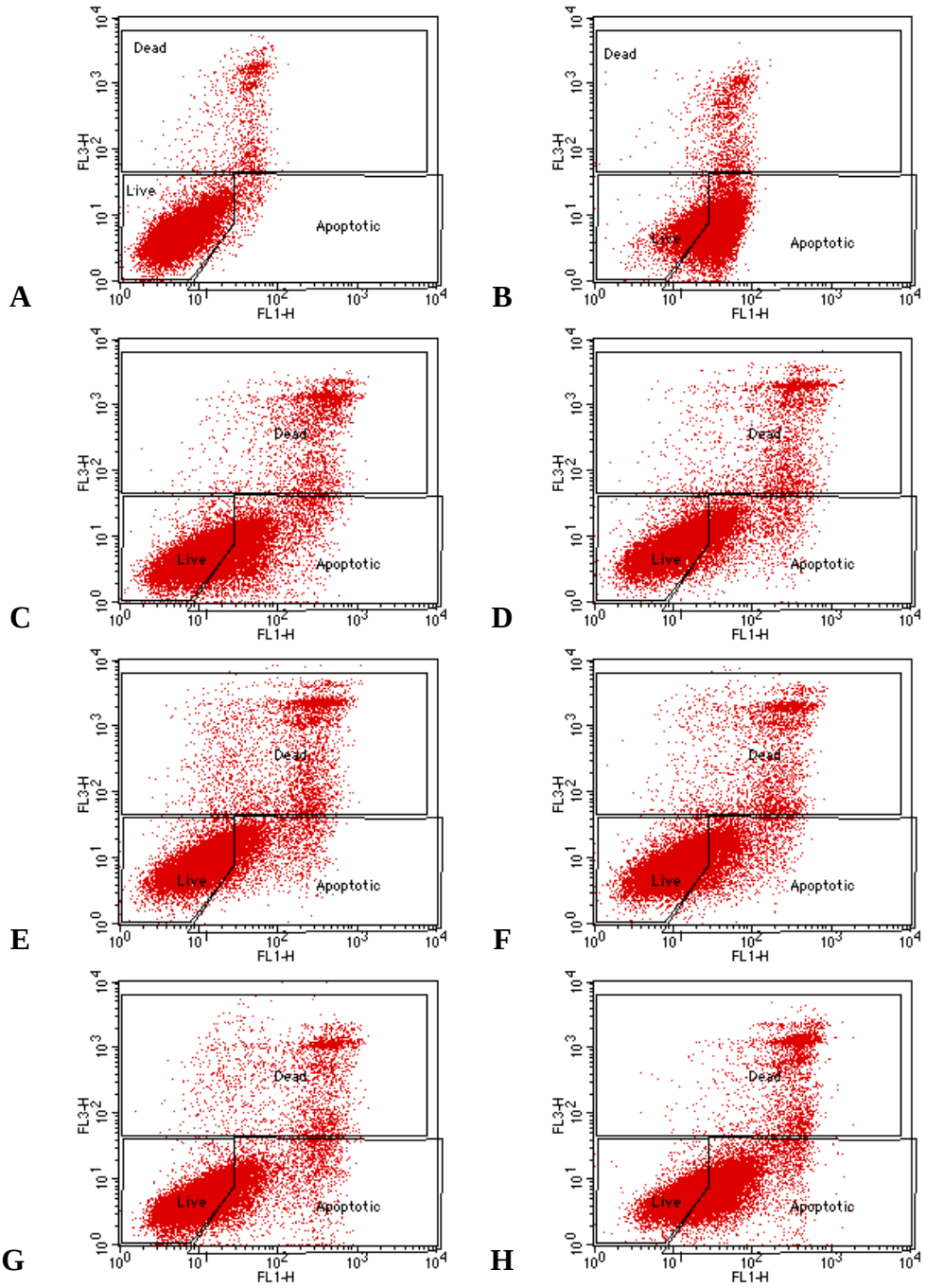

Figure 7.4.5.8The Annexin-V flow cytometry data showing apoptosis occurring in tumour DK-MG incubated with 100µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

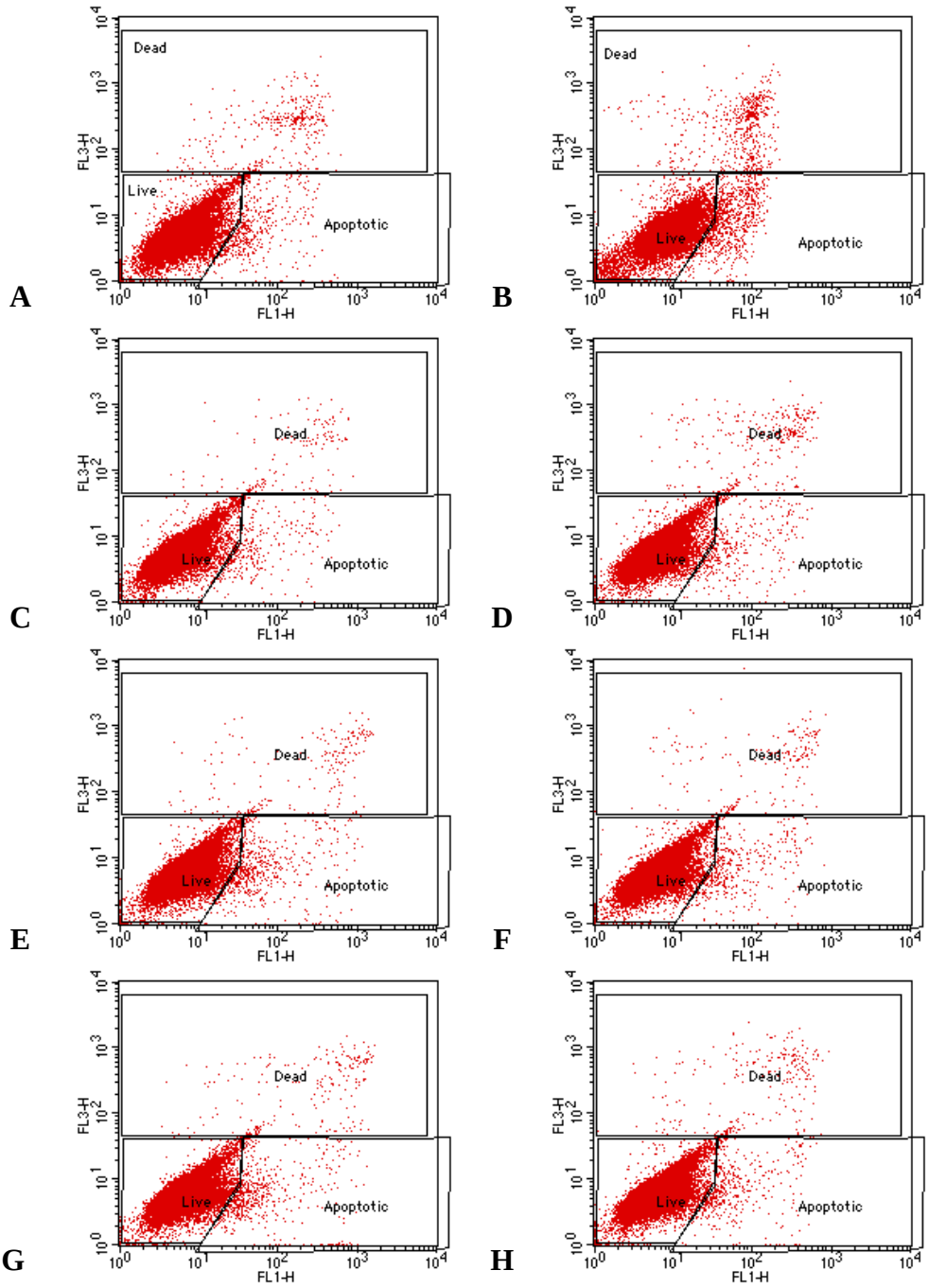

Figure 7.4.5.9The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPAB incubated with 20µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

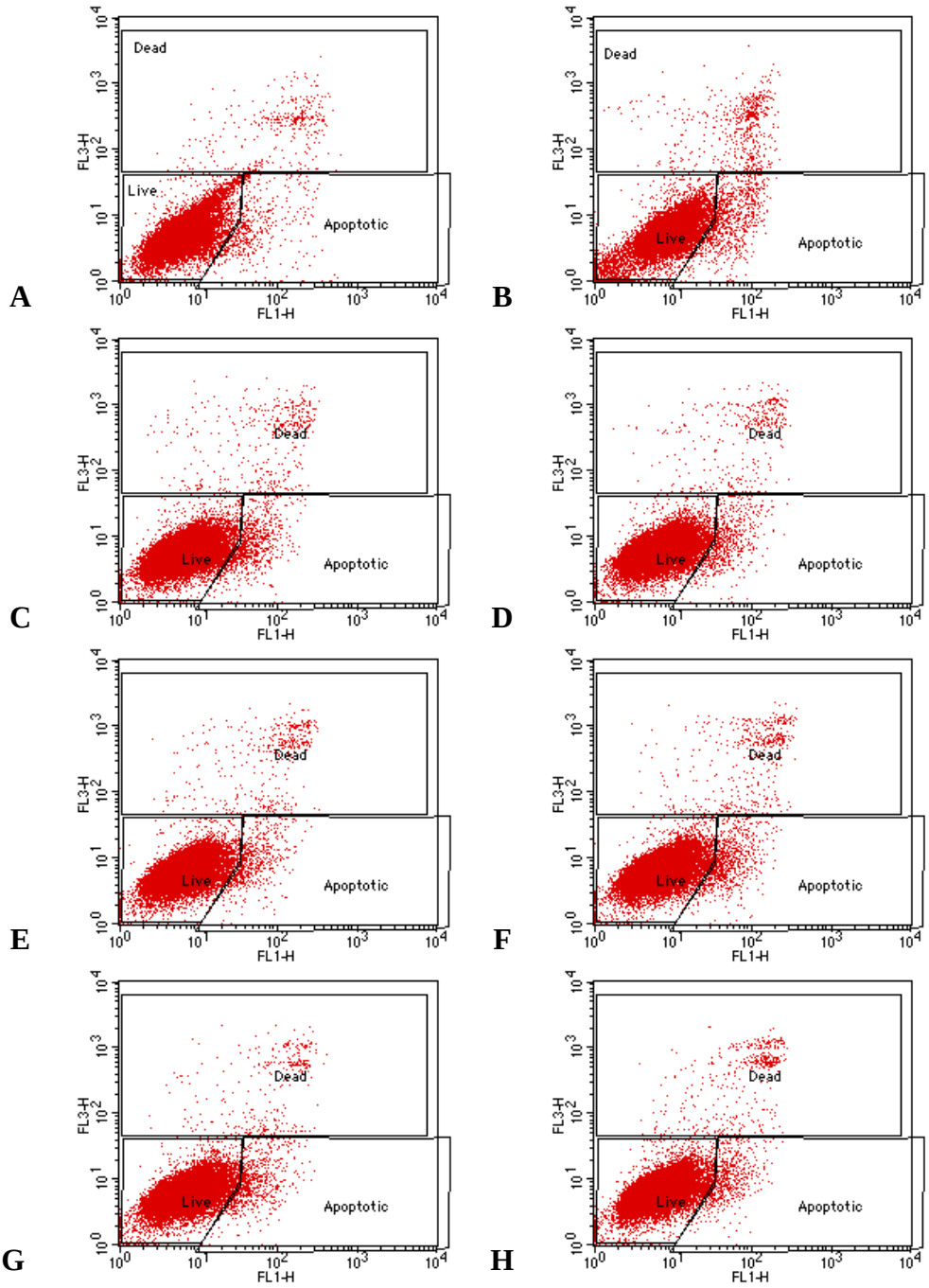

Figure 7.4.5.10The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPAB incubated with 40µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

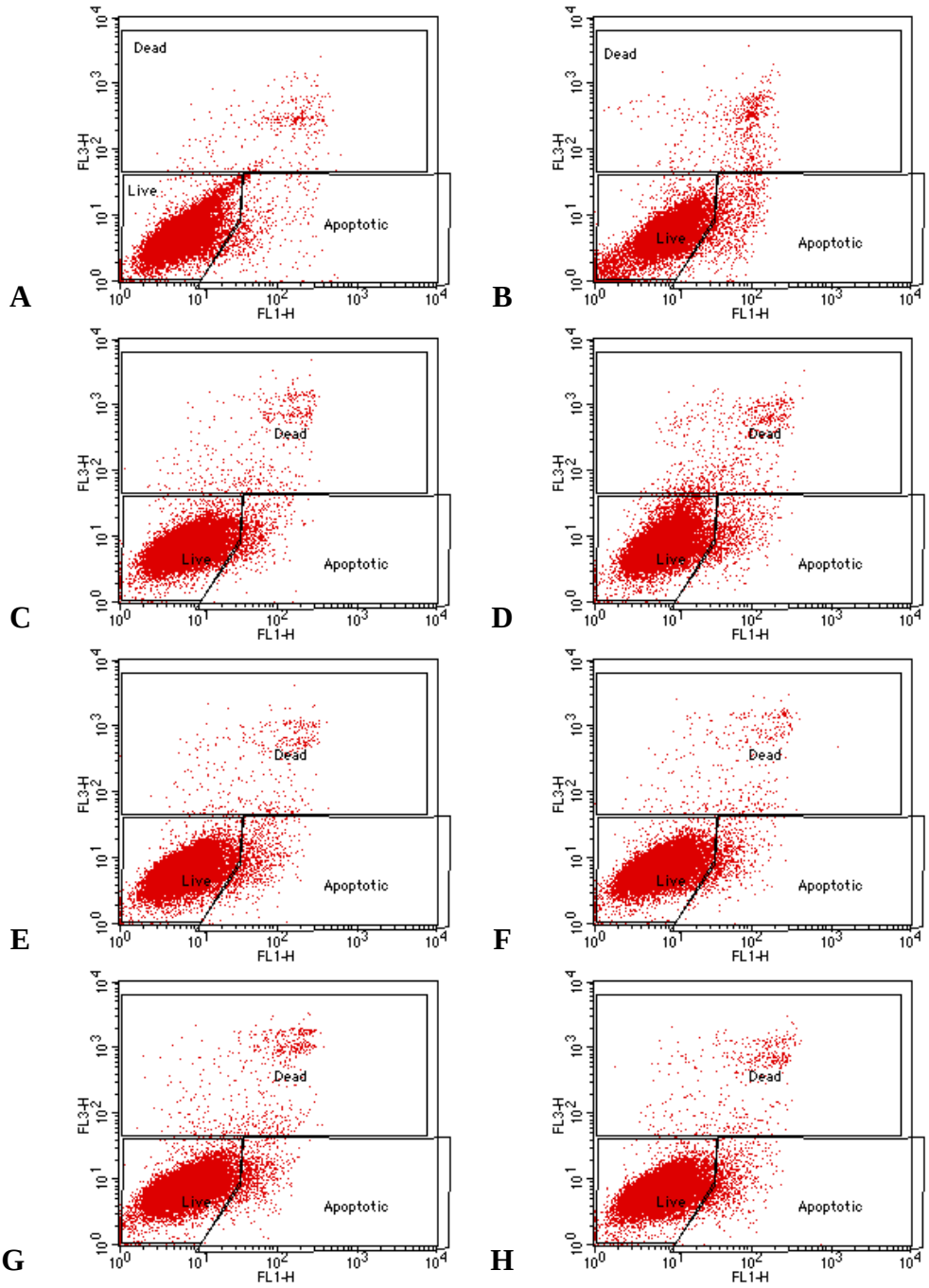

Figure 7.4.5.11The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPAB incubated with 60µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

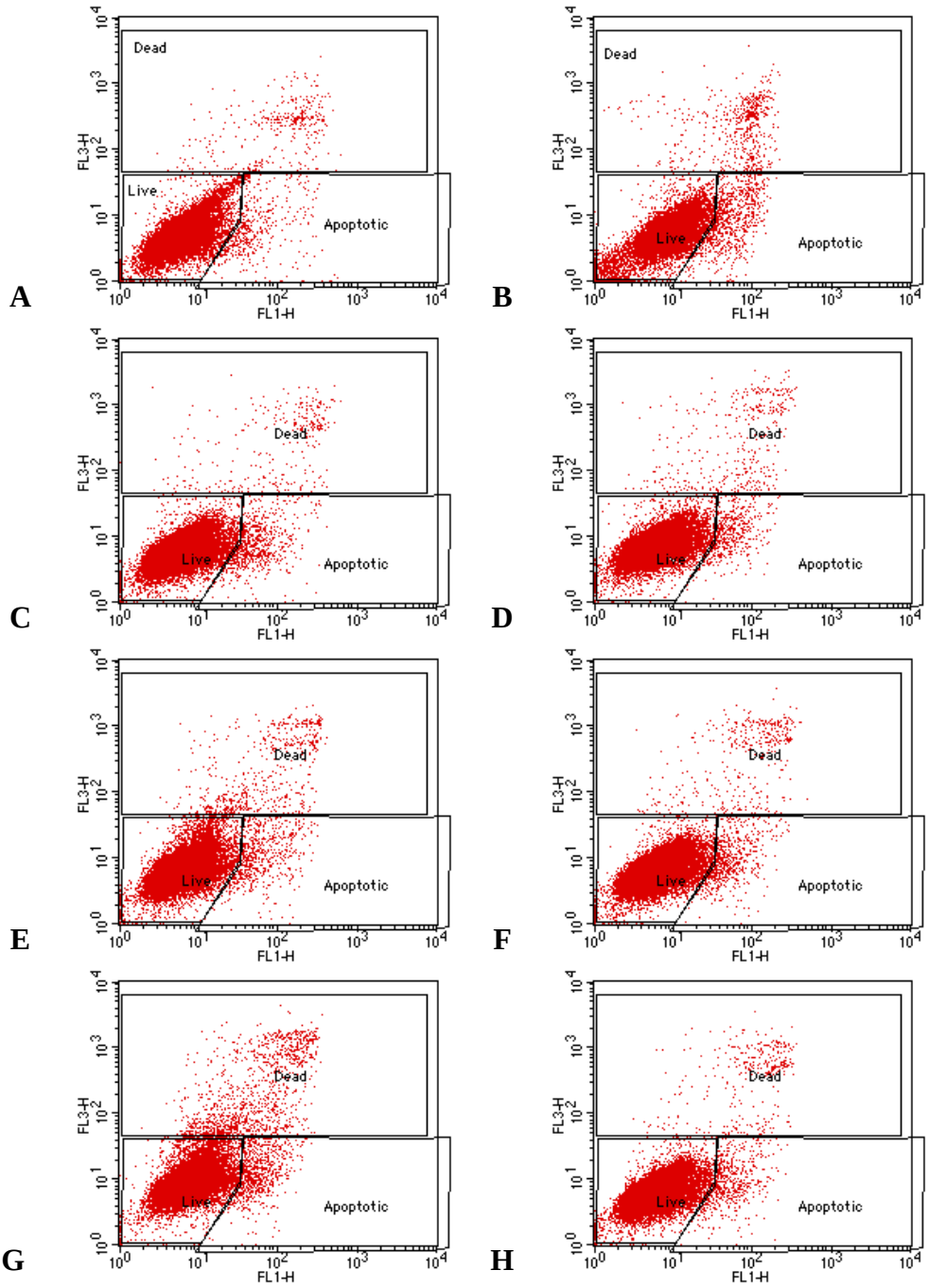

Figure 7.4.5.12The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPAB incubated with 80µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

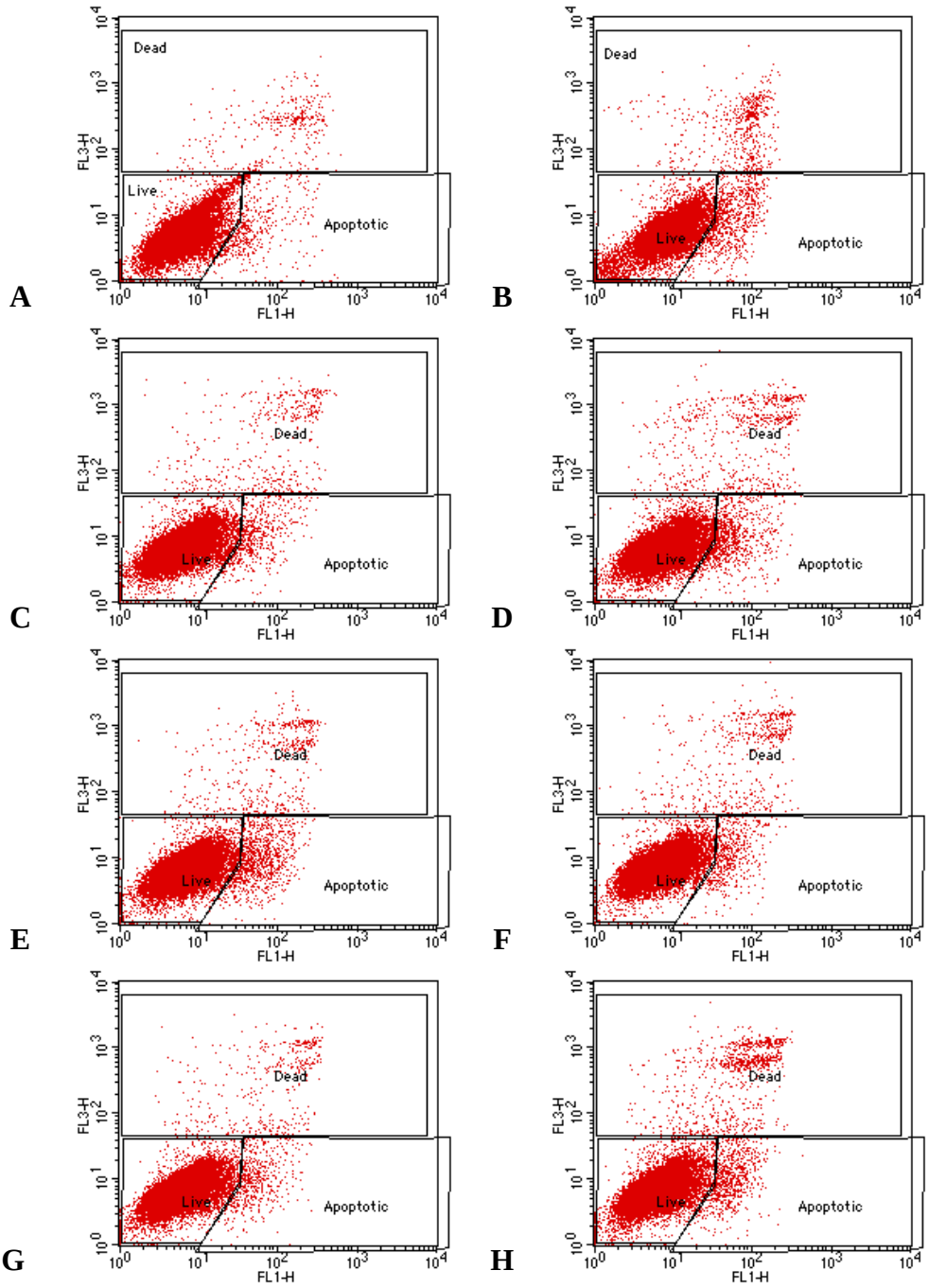

Figure 7.4.5.13The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPAB incubated with 100µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

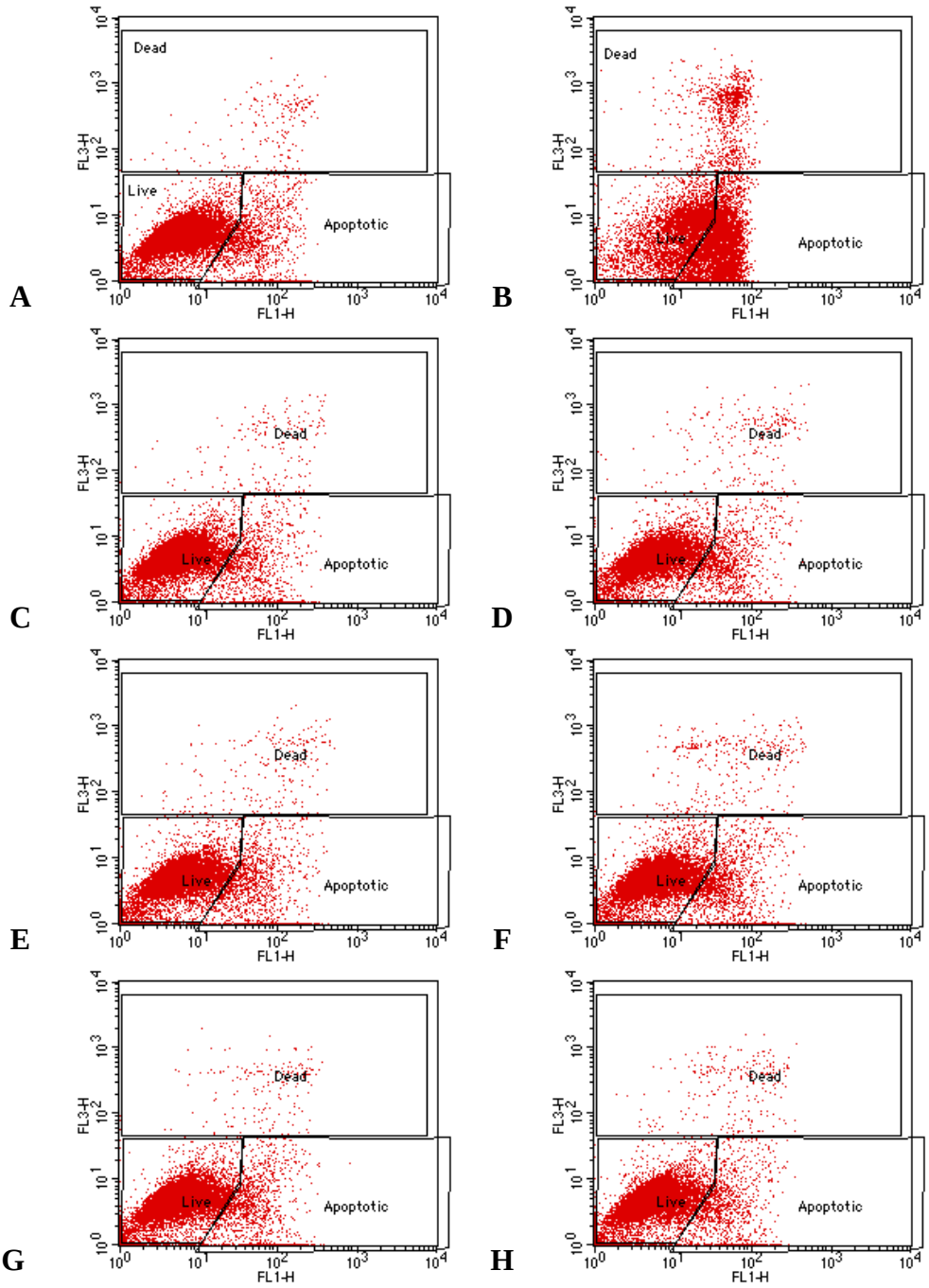

Figure 7.4.5.14The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPMC incubated with 20µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

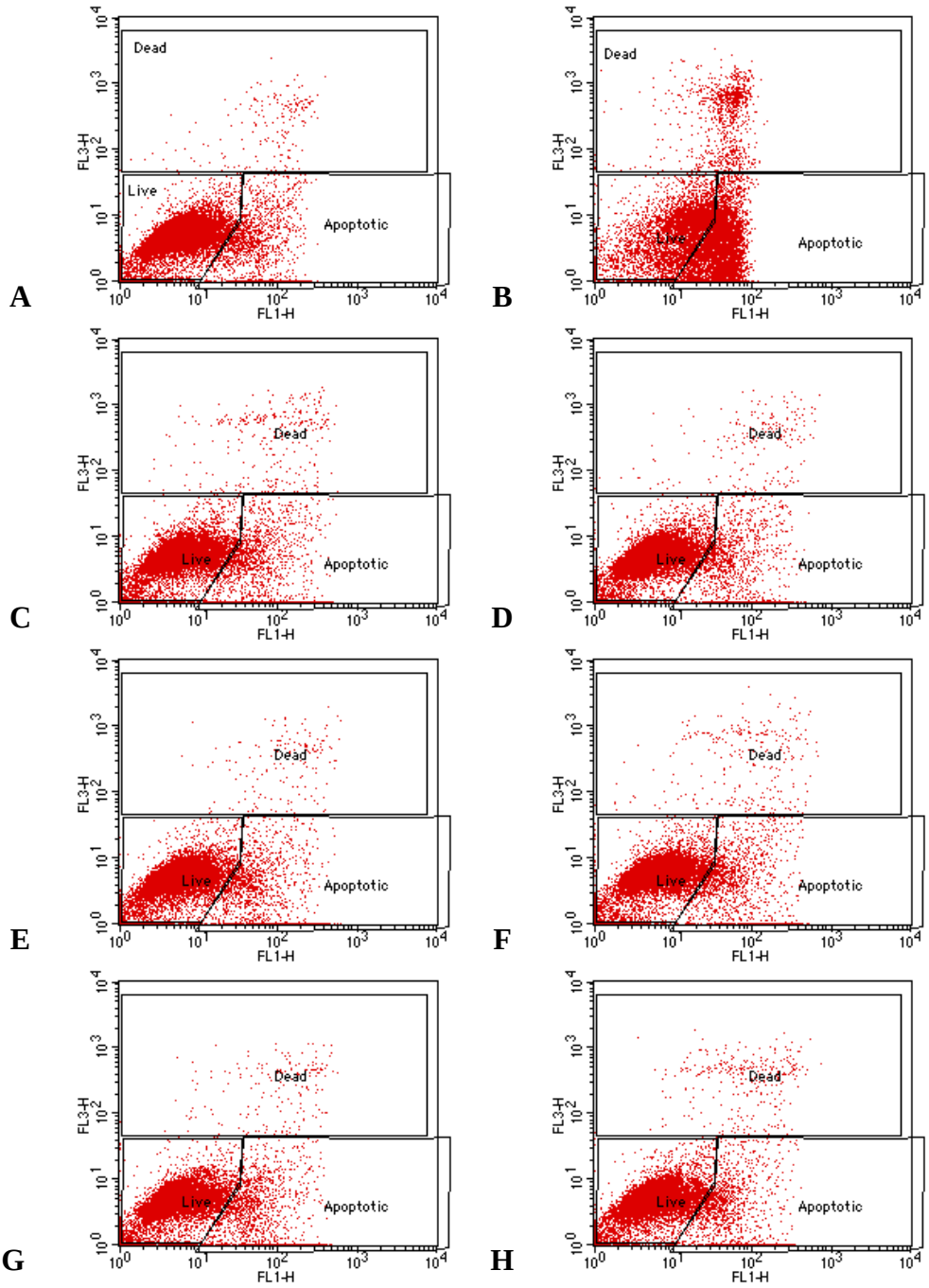

Figure 7.4.5.15The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPMC incubated with 40µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

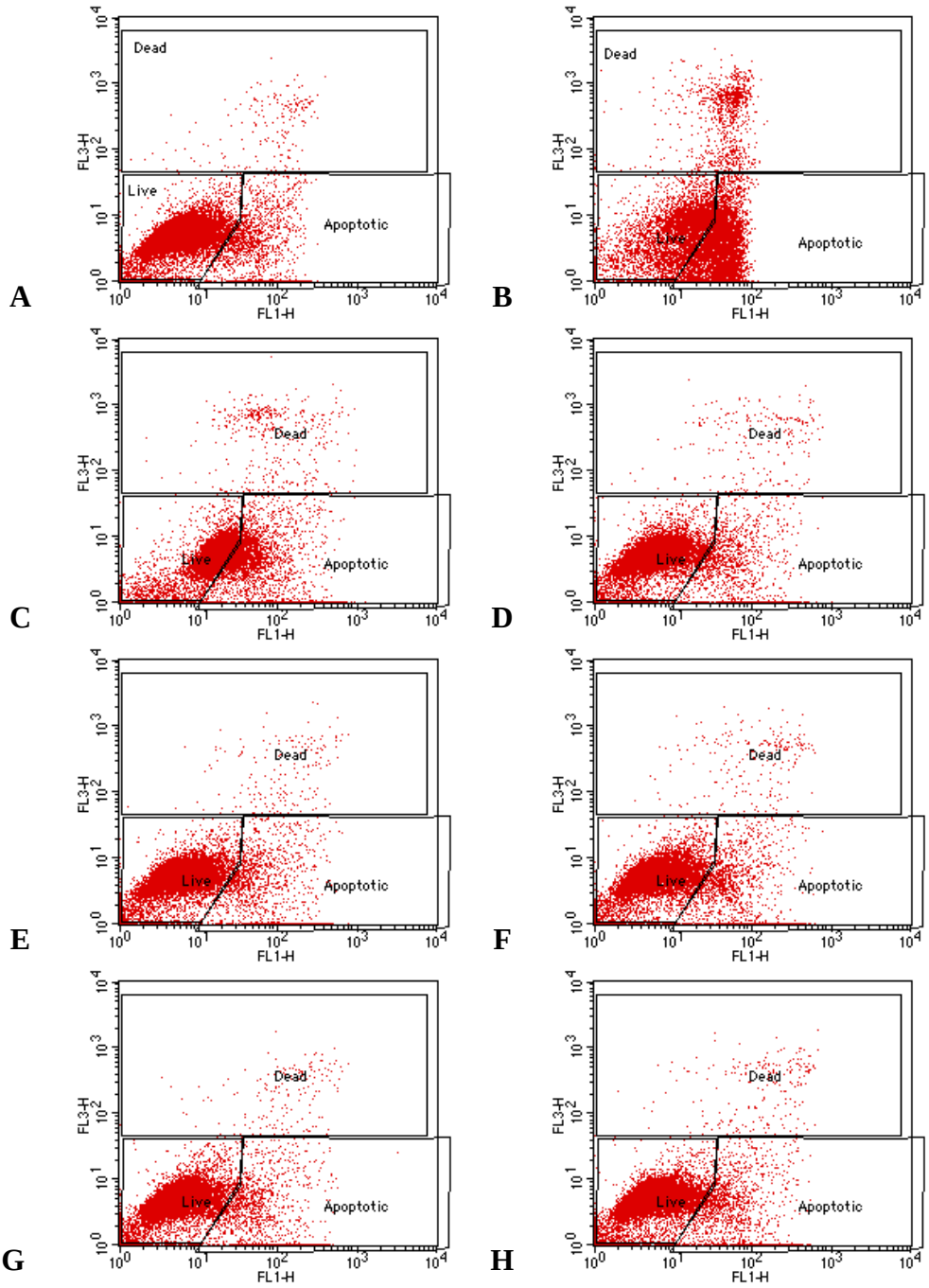

Figure 7.4.5.16The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPMC incubated with 60µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

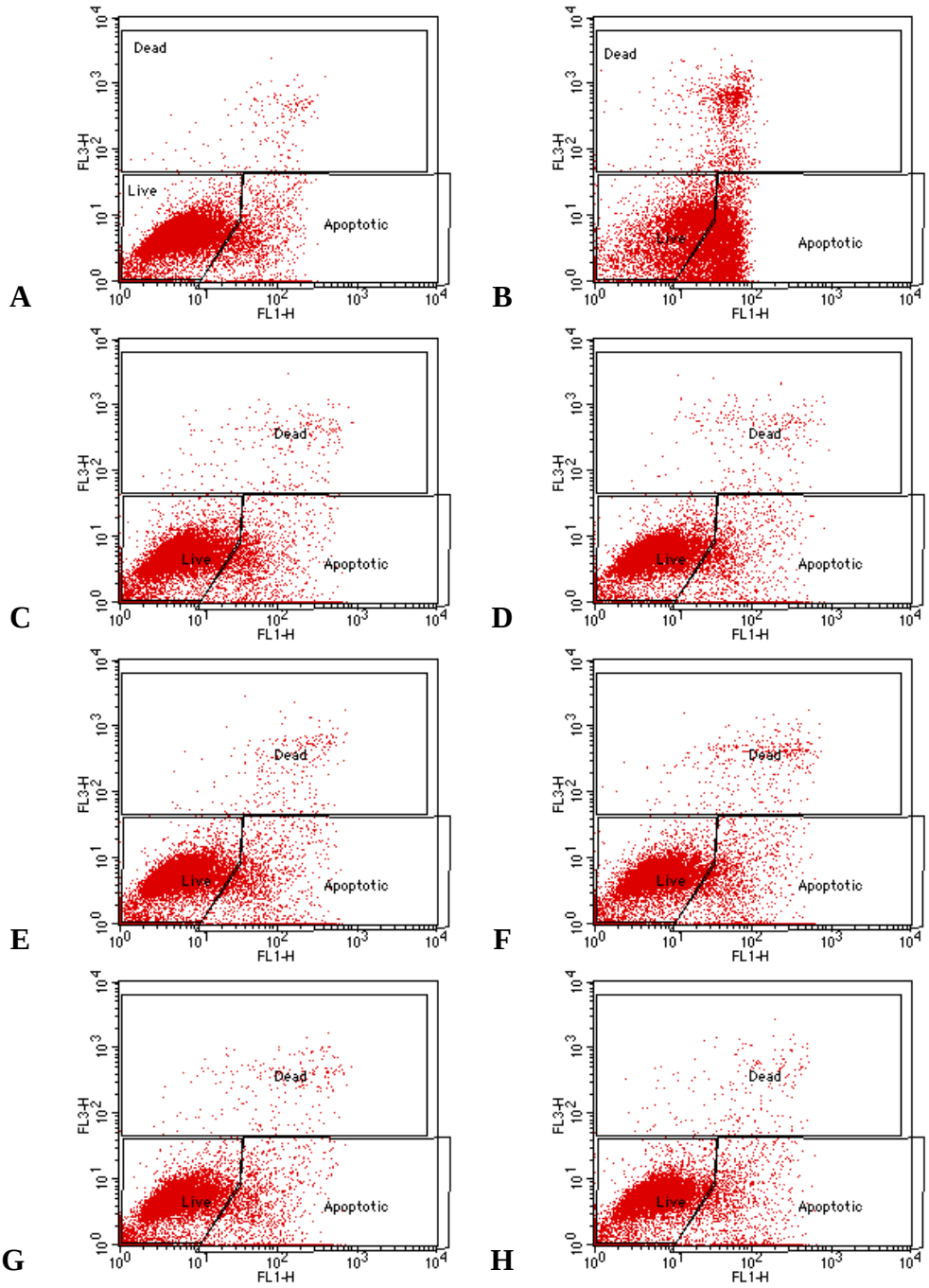

Figure 7.4.5.17The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPMC incubated with 80µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

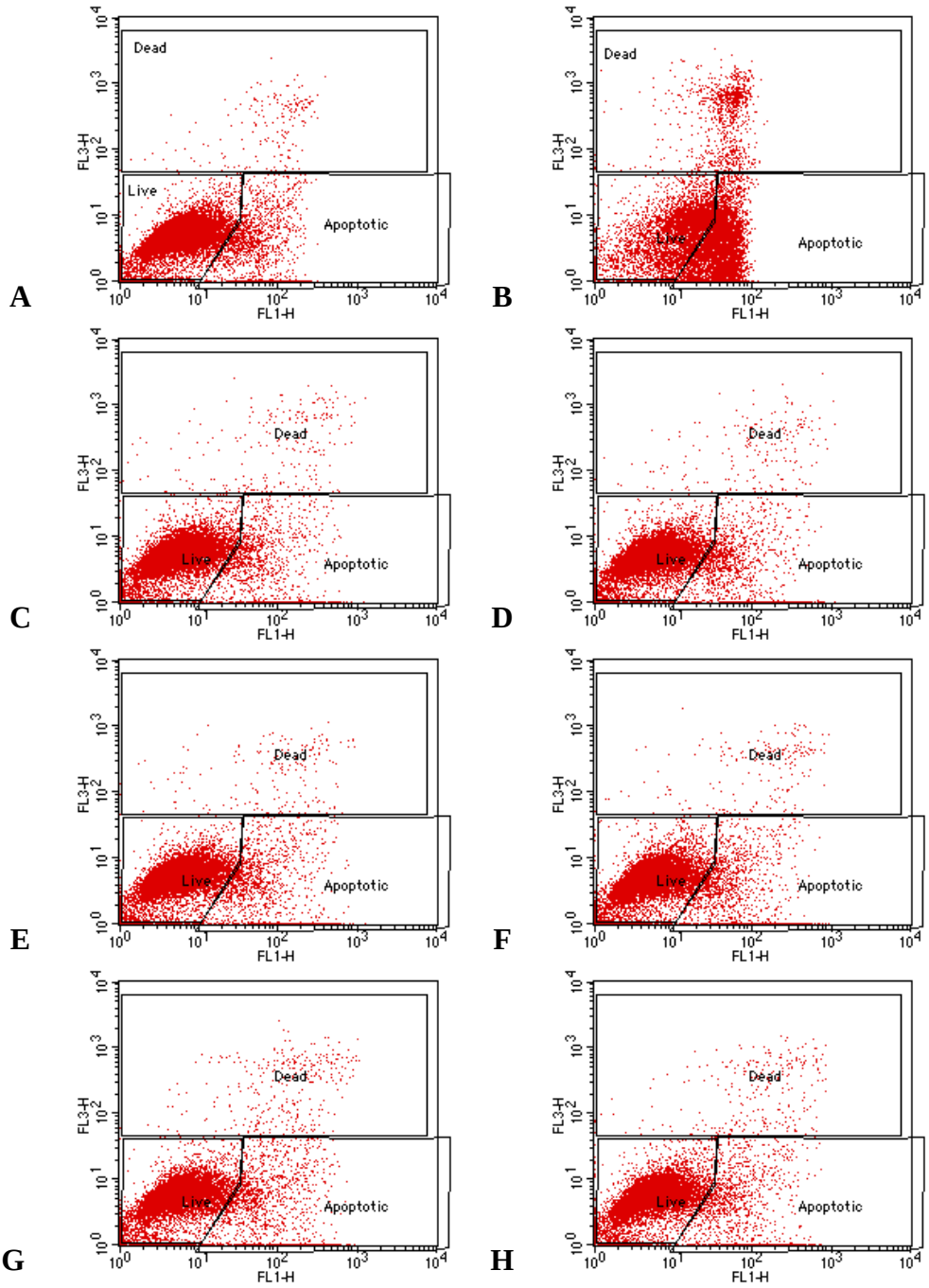

Figure 7.4.5.18The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPMC incubated with 100µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

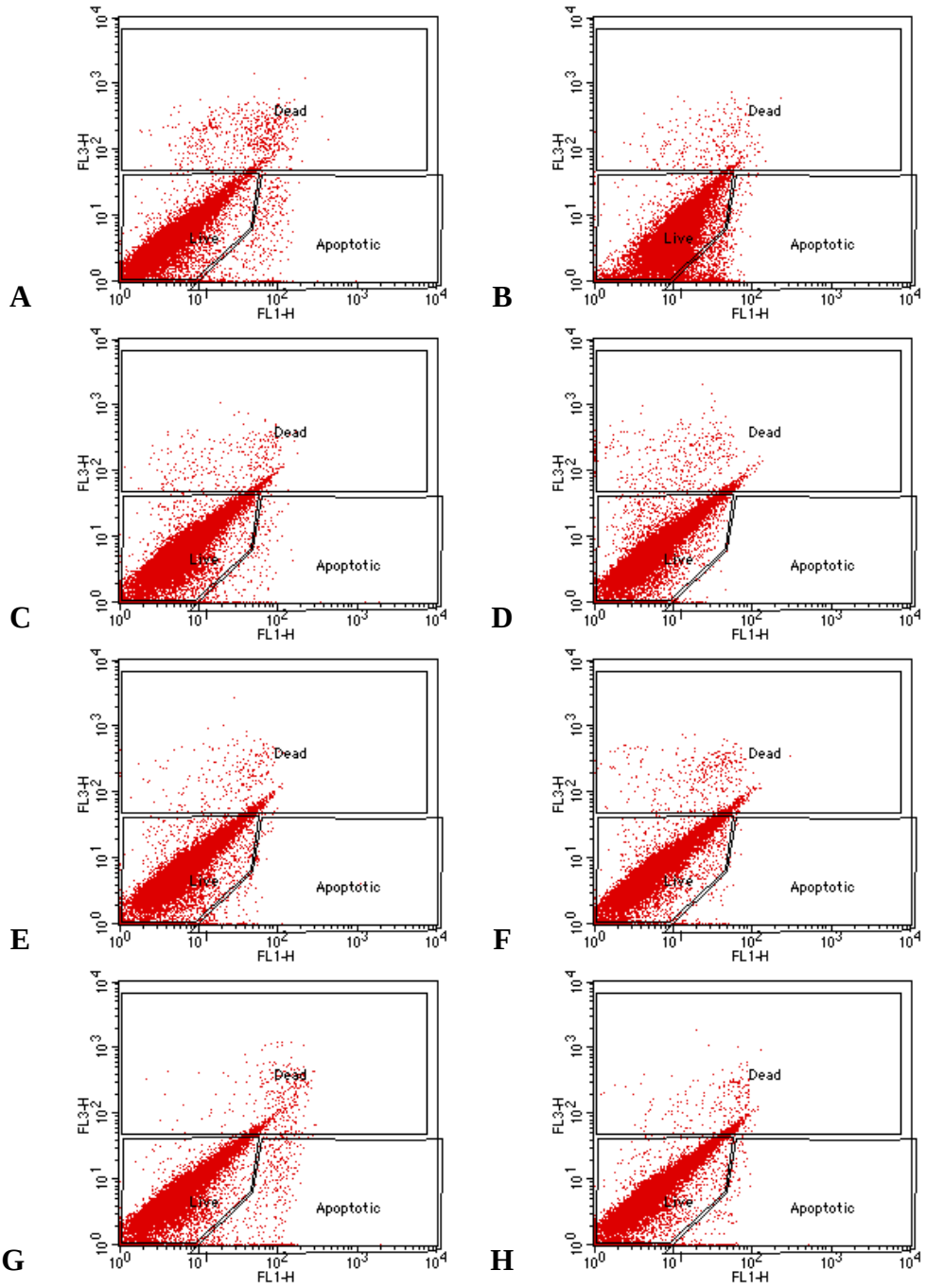

Figure 7.4.5.19The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPJM incubated with 20µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

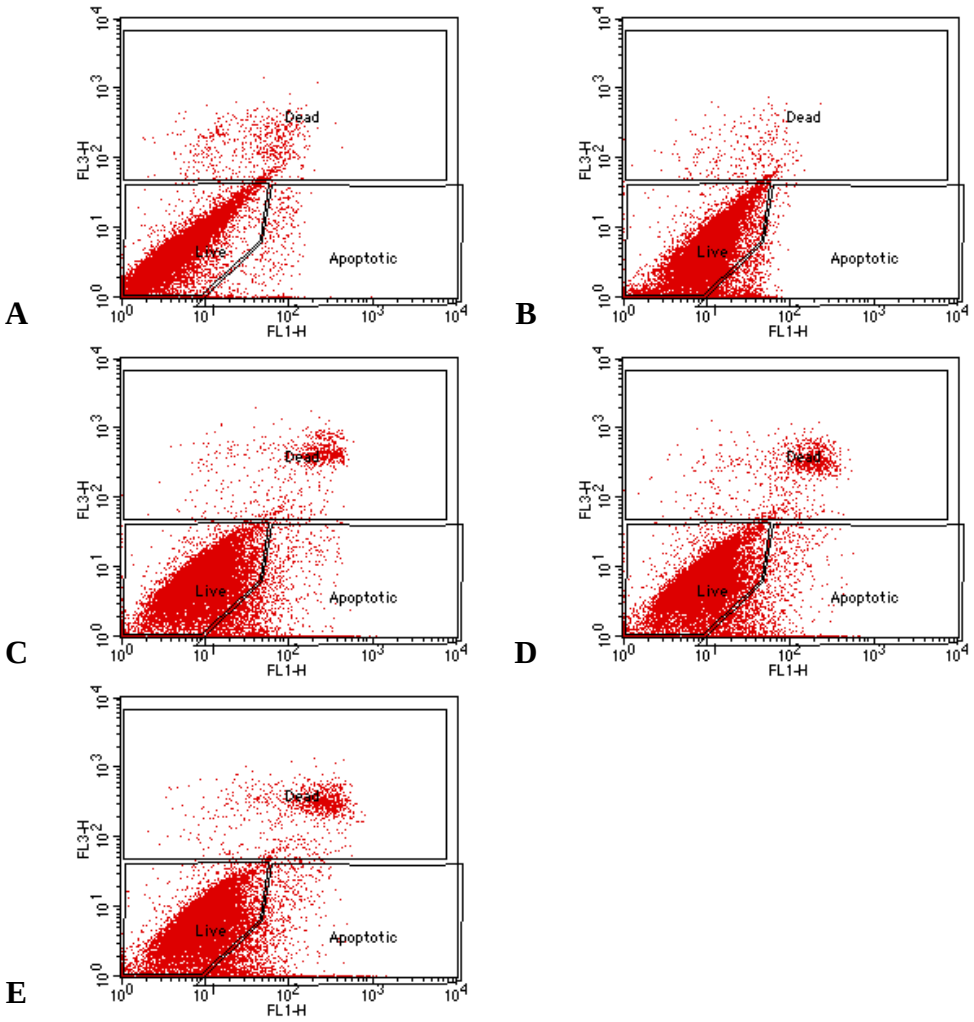

Figure 7.4.5.20The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPJM incubated with 40µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is apoptosis in the 4-6 hour samples (C-E respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

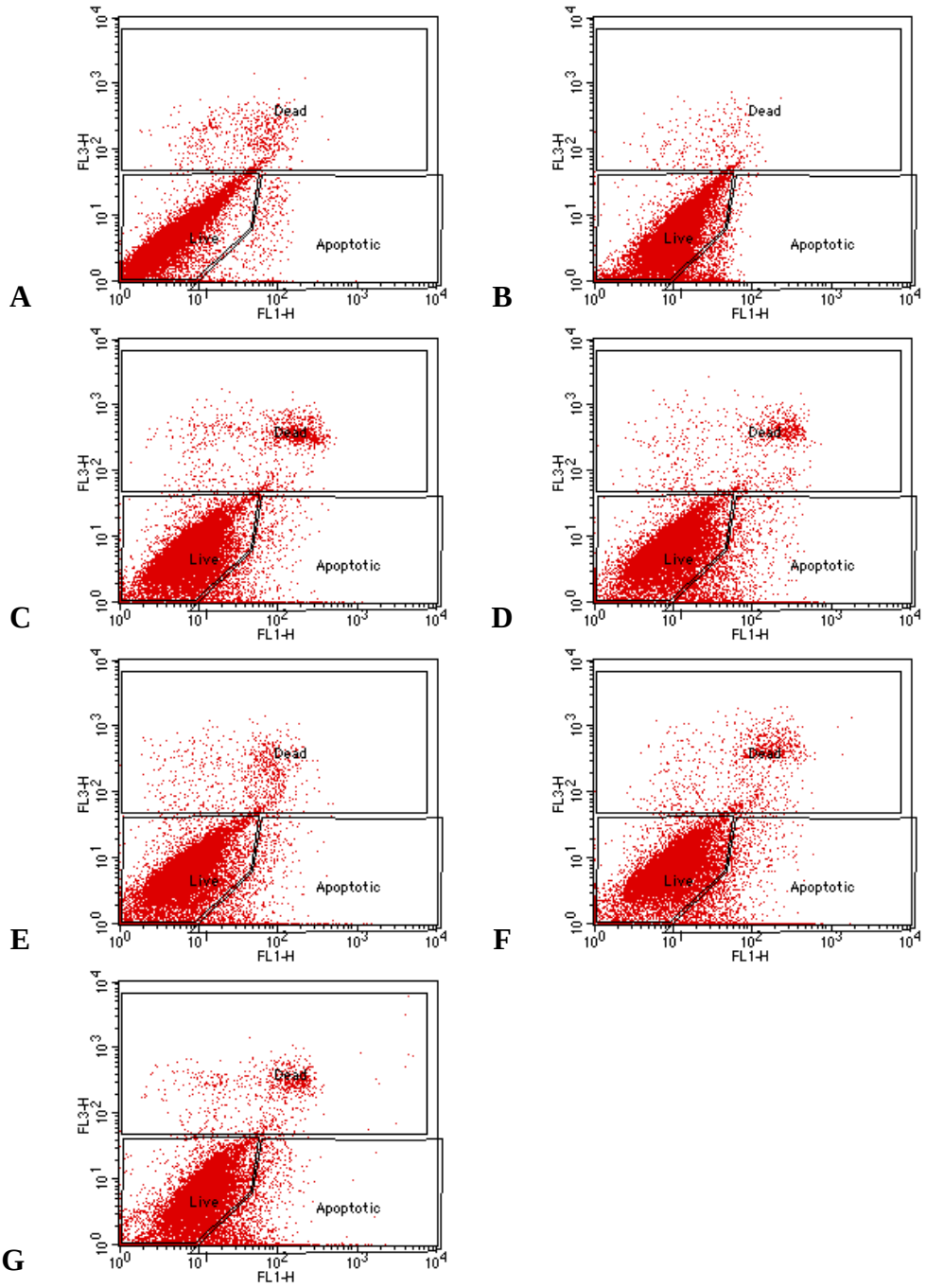

Figure 7.4.5.21The Annexin-V flow cytometry data showing apoptosis occurring in tumour UPJM incubated with 40µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is apoptosis in the 2-6 hour samples (C-G respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

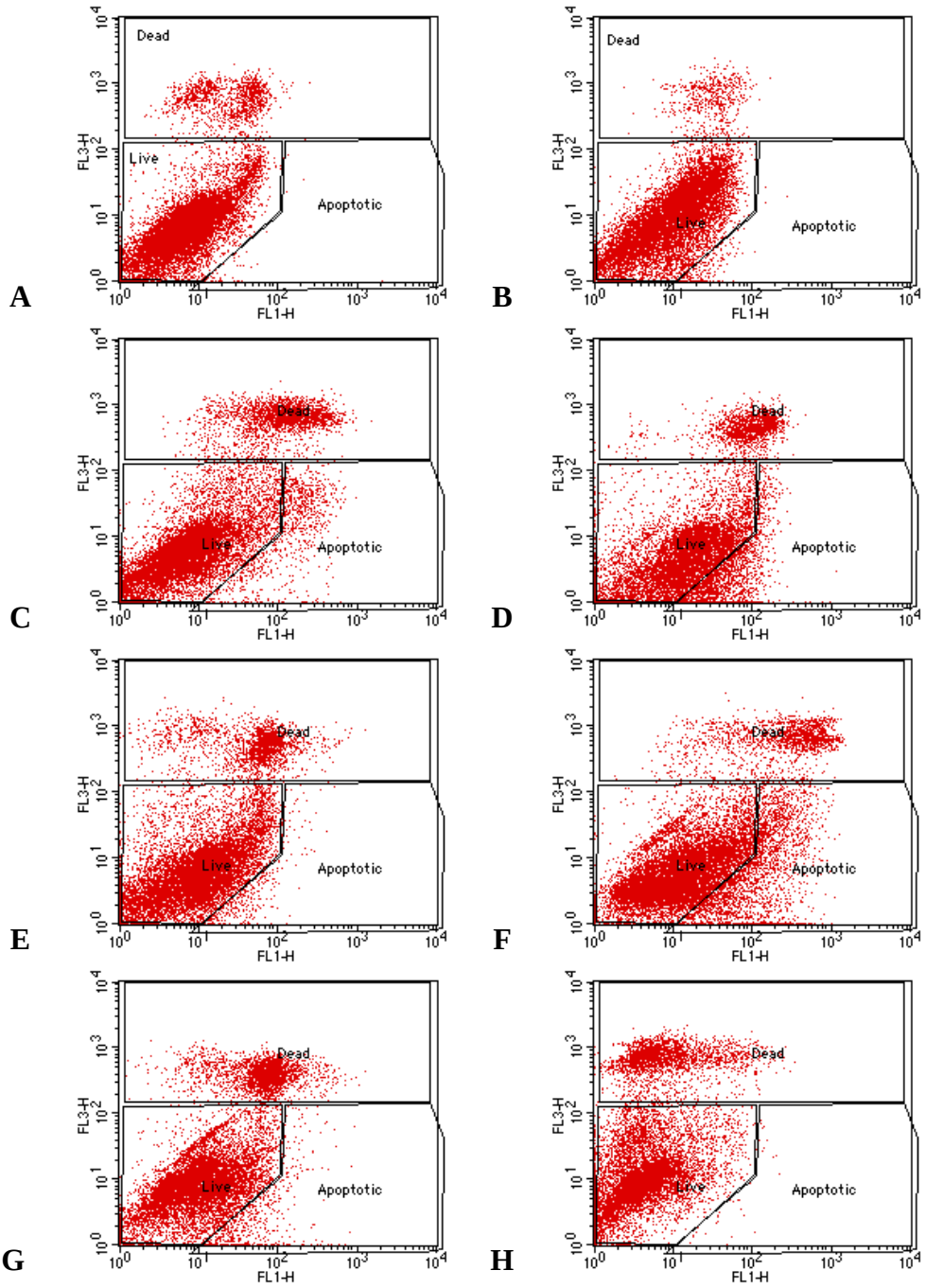

Figure 7.4.5.22The Annexin-V flow cytometry data showing apoptosis occurring in CC-2565 incubated with 20µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

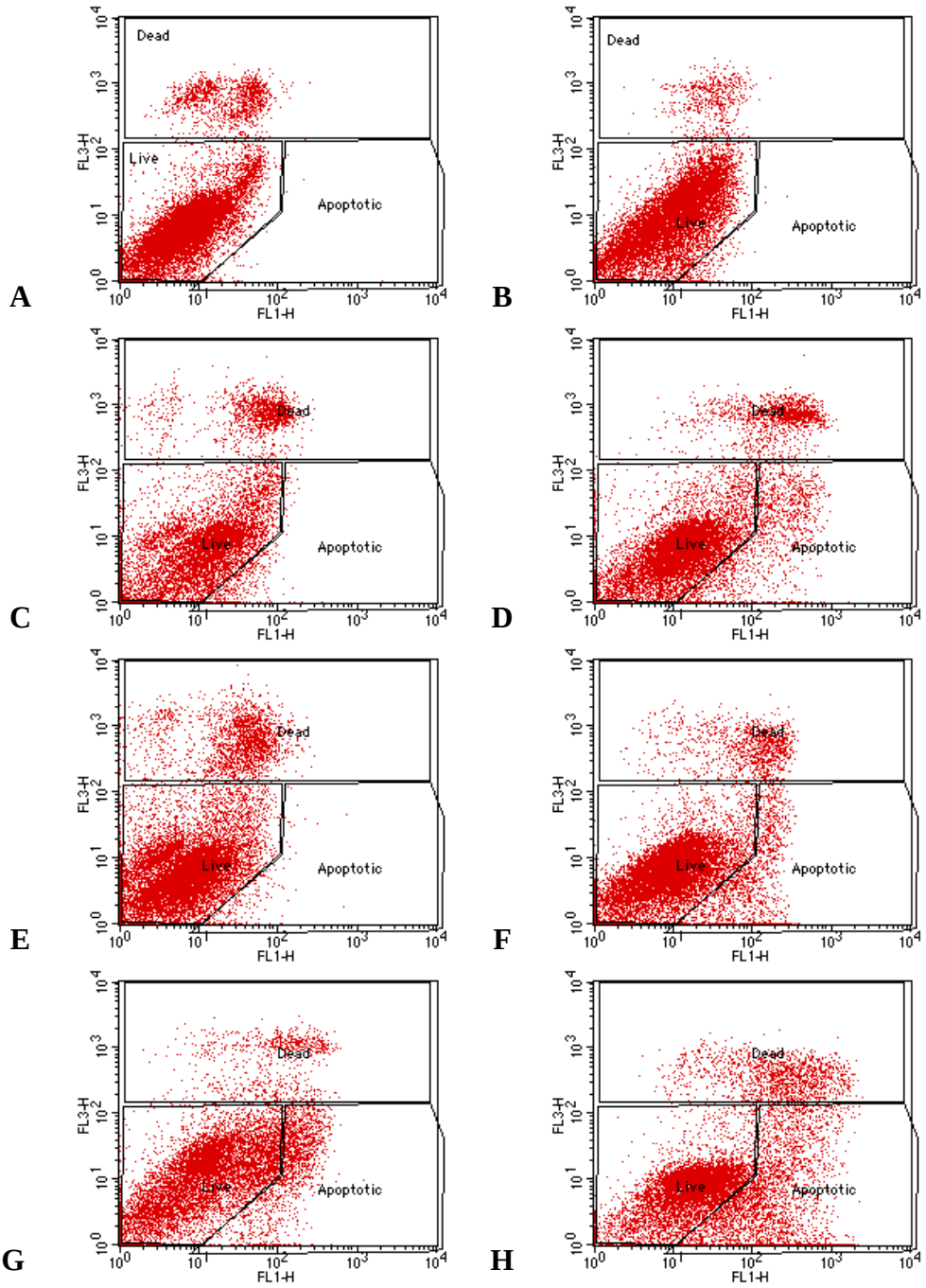

Figure 7.4.5.23The Annexin-V flow cytometry data showing apoptosis occurring in CC-2565 incubated with 40µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

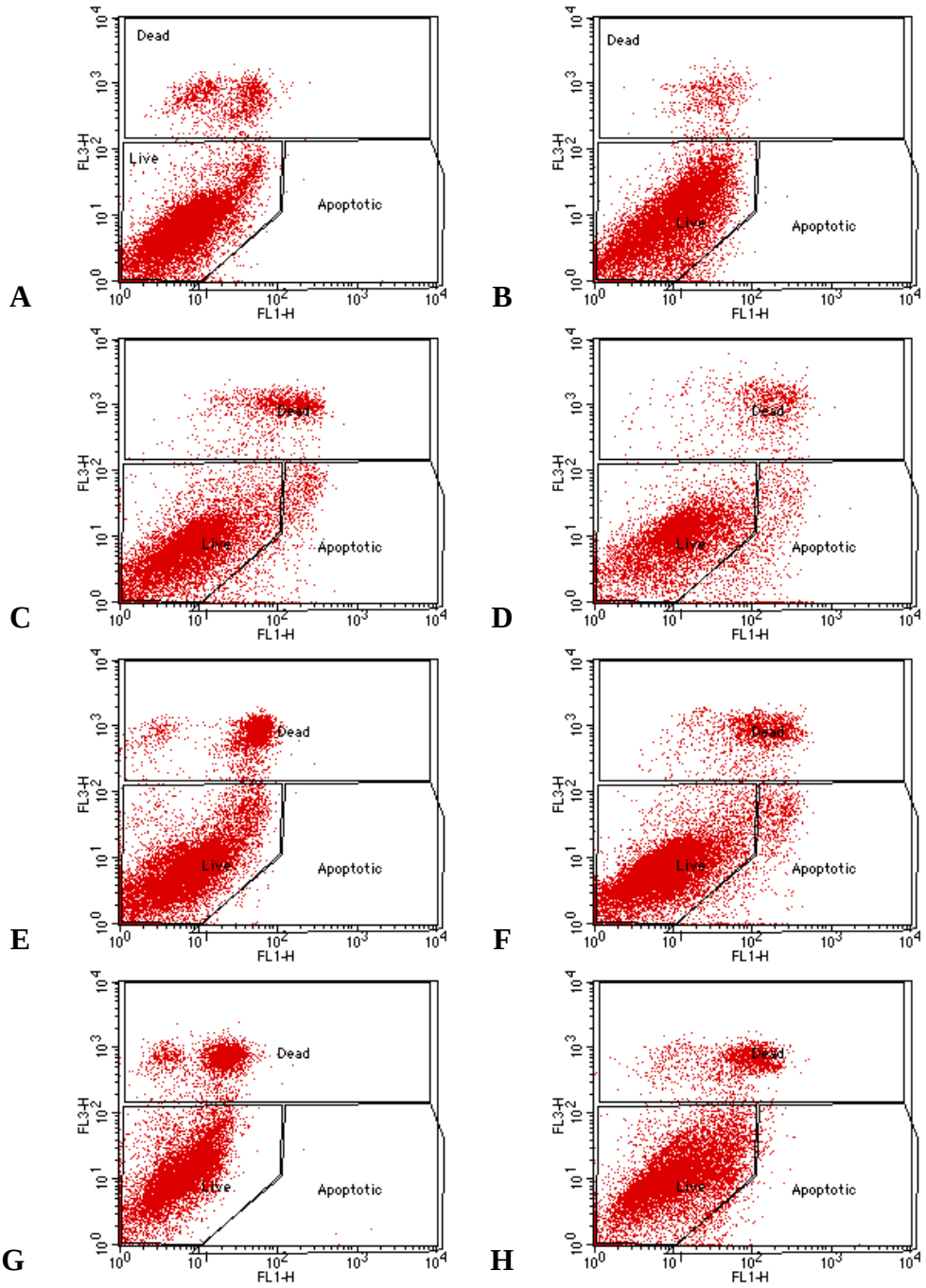

Figure 7.4.5.24The Annexin-V flow cytometry data showing apoptosis occurring in CC-2565 incubated with 60µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

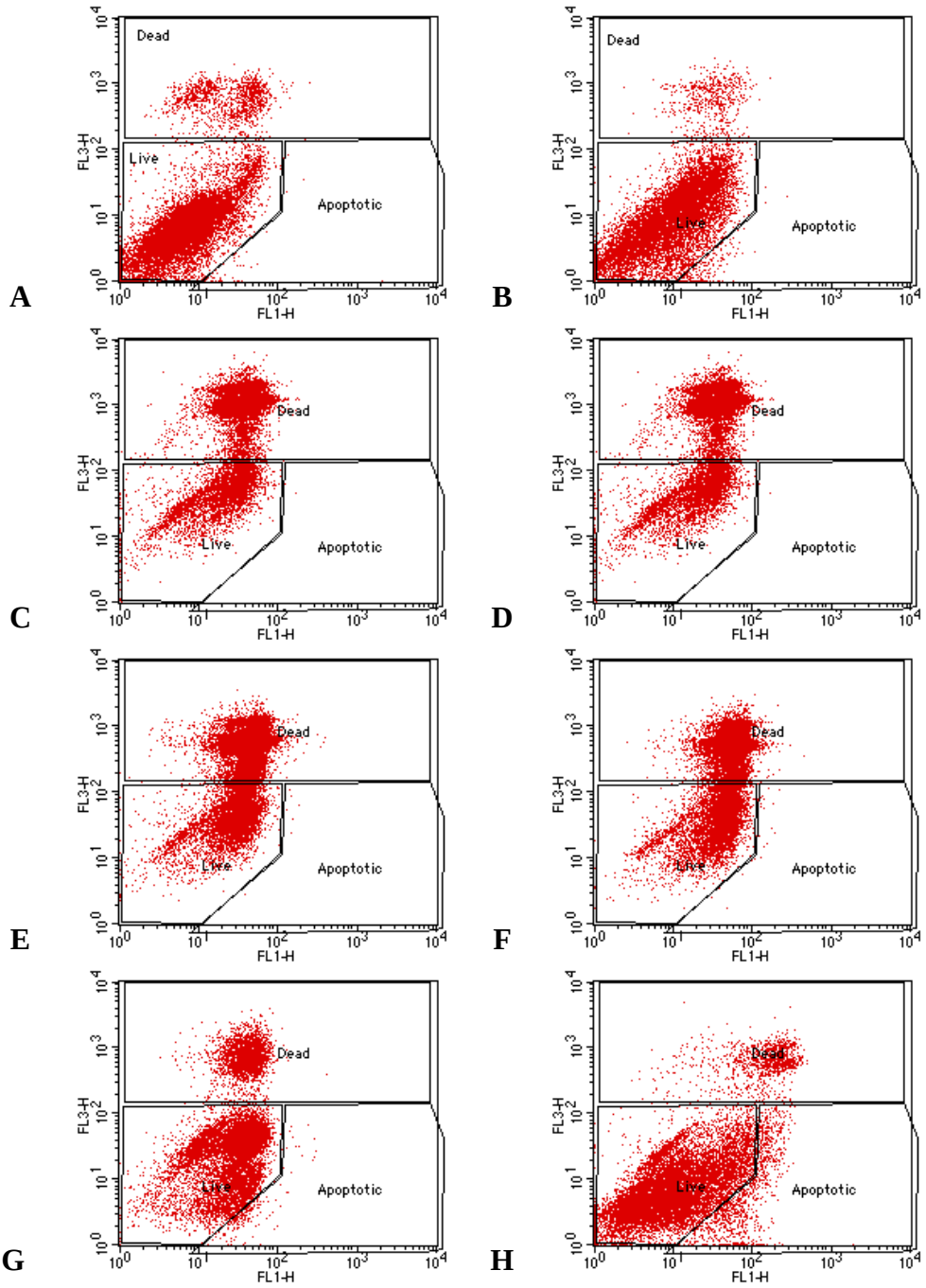

Figure 7.4.5.25The Annexin-V flow cytometry data showing apoptosis occurring in CC-2565 incubated with 80µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

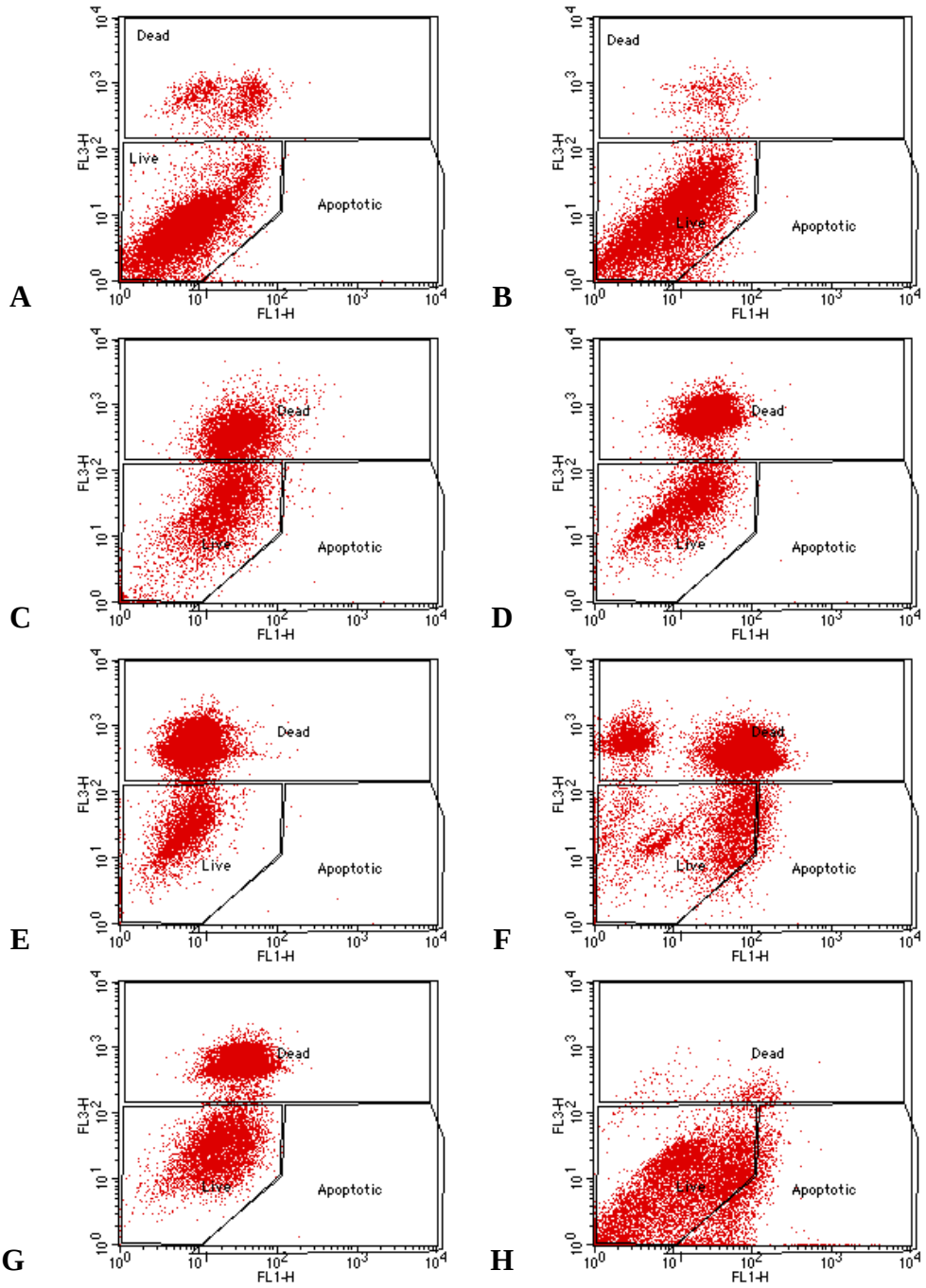

Figure 7.4.5.26The Annexin-V flow cytometry data showing apoptosis occurring in CC-2565 incubated with 100µM clomipramine

The graphs show that compared to the negative control (A) and the positive control (B) there is no apoptosis in the 1-6 hour samples (C-H respectively). The negative control histogram was divided into three sections, live, dead and apoptotic ensuring that <2% cells were contained within the apoptotic area and then the template grid was pasted onto all test histograms to obtain percentage values for apoptosis.

9.2 IC_{50}Macro (Excel)

Used in the ATP-TCA assay (see 2.3.10)

If $Inh_{6.25} < 49.99$ and $Inh_{12.5} > 50$ and $Inh_{25} > 50$ is true, then x = 6.25; if false, x = 0
If $Inh_{6.25} < 49.99$ and $Inh_{12.5} < 49.99$ and $Inh_{25} > 50$ and $Inh_{50} > 50$ is true, then x = 12.5; if false, x = 0
If $Inh_{12.5} < 49.99$ and $Inh_{25} < 49.99$ and $Inh_{50} > 50$ and $Inh_{100} > 50$ is true, then x = 25; if false, x = 0
If $Inh_{25} < 49.99$ and $Inh_{50} < 49.99$ and $Inh_{100} > 50$ and $Inh_{200} > 50$ is true, then x = 50; if false, x = 0
If $Inh_{50} < 49.99$ and $Inh_{100} < 49.99$ and $Inh_{200} > 50$ is true, then x = 100; if false, x = 0
If $Inh_{100} < 49.99$ and $Inh_{200} > 50$ is true, then x = 200; if false, x = 0
$y_1 = \Sigma(x)$
If $Inh_{6.25} < 49.99$ and $Inh_{12.5} > 50$ and $Inh_{25} > 50$ is true, then x = $Inh_{6.25}$; if false, x = 0
If $Inh_{6.25} < 49.99$ and $Inh_{12.5} < 49.99$ and $Inh_{25} > 50$ and $Inh_{50} > 50$ is true, then x = $Inh_{12.5}$; if false, x = 0
If $Inh_{12.5} < 49.99$ and $Inh_{25} < 49.99$ and $Inh_{50} > 50$ and $Inh_{100} > 50$ is true, then x = Inh_{25}; if false, x = 0
If $Inh_{25} < 49.99$ and $Inh_{50} < 49.99$ and $Inh_{100} > 50$ and $Inh_{200} > 50$ is true, then x = Inh_{50}; if false, x = 0
If $Inh_{50} < 49.99$ and $Inh_{100} < 49.99$ and $Inh_{200} > 50$ is true, then x = Inh_{100}; if false, x = 0
If $Inh_{100} < 49.99$ and $Inh_{200} > 50$ is true, then x = Inh_{200}; if false, x = 0
$y_2 = \Sigma(x)$
If $Inh_{6.25} > 50$ and $Inh_{12.5} > 50$ is true, then x = 6.25; if false, x = 0
If $Inh_{6.25} < 50$ and $Inh_{12.5} > 50$ and $Inh_{25} > 50$ is true, then x = 12.5; if false, x = 0
If $Inh_{6.25} < 50$ and $Inh_{12.5} < 50$ and $Inh_{25} > 50$ and $Inh_{50} > 50$ is true, then x = 25; if false, x = 0
If $Inh_{6.25} < 50$ and $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} > 50$ and $Inh_{100} > 50$ is true, then x = 50; if false, x = 0
If $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} < 50$ and $Inh_{100} > 50$ and $Inh_{200} > 50$ is true, then x = 100; if false, x = 0
If $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} < 50$ and $Inh_{100} < 50$ and $Inh_{200} > 50$ is true, then x = 100; if false, x = 0
$y_3 = \Sigma(x)$
If $Inh_{6.25} > 50$ and $Inh_{12.5} > 50$ is true, then x = $Inh_{6.25}$; if false, x = 0
If $Inh_{6.25} < 50$ and $Inh_{12.5} > 50$ and $Inh_{25} > 50$ is true, then x = $Inh_{12.5}$; if false, x = 0

If $Inh_{6.25} < 50$ and $Inh_{12.5} < 50$ and $Inh_{25} > 50$ and $Inh_{50} > 50$ is true, then $x = Inh_{25}$; if false, $x = 0$
If $Inh_{6.25} < 50$ and $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} > 50$ and $Inh_{100} > 50$ is true, then $x = Inh_{50}$; if false, $x = 0$
If $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} < 50$ and $Inh_{100} > 50$ and $Inh_{200} > 50$ is true, then $x = Inh_{100}$; if false, $x = 0$
If $Inh_{12.5} < 50$ and $Inh_{25} < 50$ and $Inh_{50} < 50$ and $Inh_{100} < 50$ and $Inh_{200} > 50$ is true, then $x = Inh_{200}$; if false, $x = 0$
$y_4 = \Sigma(x)$

$IC50 = ((50-y_4)/(y_2-y_4)*(y_1-y_3))+y_3$

9.3 Parker KA and Pilkington GJ: Morphological, Immunocytochemical and Flow Cytometric *In Vitro* Characterisation of a Surface-adherent Medulloblastoma