Investigation of Aspects of Reactive Gliosis in Human Astrocytoma Cell Lines: Application for Toxicity Assessment.

Lindsay Jane Holden

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<u>Aston University</u>

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Thesis Summary

The process of astrogliosis, or reactive gliosis, is a typical response of astrocytes to a wide range of physical and chemical injuries. The up-regulation of the astrocyte specific glial fibrillary acidic protein (GFAP) is a hallmark of reactive gliosis and is widely used as a marker to identify the response.

In order to develop a reliable, sensitive and high throughput astrocyte toxicity assay that is more relevant to the human response than existing animal cell based models, the U251-MG, U373-MG and CCF-STTG1 human astrocytoma cell lines were investigated for their ability to exhibit reactive-like changes following exposure to ethanol, chloroquine diphosphate, trimethyltin chloride and acrylamide.

Cytotoxicity analysis showed that the astrocytic cells were generally more resistant to the cytotoxic effects of the agents than the SH-SY5Y neuroblastoma cells. Retinoic acid induced differentiation of the SH-SY5Y line was also seen to confer some degree of resistance to toxicant exposure, particularly in the case of ethanol.

Using a cell based ELISA for GFAP together with concurrent assays for metabolic activity and cell number, each of the three cell lines responded to toxicant exposure by an increase in GFAP immunoreactivity (GFAP-IR), or by increased metabolic activity. Ethanol, chloroquine diphosphate, trimethyltin chloride and bacterial lipopolysaccharide all induced either GFAP or MTT increases depending upon the cell line, dose and exposure time. Preliminary investigations of additional aspects of astrocytic injury indicated that IL-6, but not TNF-α or nitric oxide, is released following exposure to each of the compounds, with the exception of acrylamide.

It is clear that these human astrocytoma cell lines are capable of responding to toxicant exposure in a manner typical of reactive gliosis and are therefore a valuable cellular model in the assessment of in vitro neurotoxicity.

Key words: Glial fibrillary acidic protein, Astrocyte, Astrogliosis, Toxicant

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Abbreviations

ABC transporter	ATP-binding cassette transporter
ACh	Acetylcholine
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP-1	Activator protein 1
AP-2	Activator protein 2
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BCRP	Breast cancer resistance protein
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cAMP	Cyclic adenine monophosphate
cELISA	Cellular enzyme-linked immunosorbent assay
cGMP	Guanosine 3'5'-cyclic monophosphate
CNS	Central nervous system
CRE	cAMP response element
CSF	Cerebrospinal fluid
dBcAMP	Dibutyryl cyclic AMP
DNA	Deoxyribonucleic acid
EC	European Commission
EC_{50}	Half maximal effective concentration
ECACC	European Collection of Cell Cultures

ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FGF	Fibroblast growth factor
GABA	Gamma-aminobutyric acid
GAP-43	Growth associated protein 43
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanylyl cyclase
GFAP	Glial fibrillary acidic protein
GFAP-IR	Glial fibrillary acidic protein immunoreactivity
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
GRP	Glucose regulated protein
GST	Glutathione-S-transferase
GSH	Glutathione
2,5 HD	2,5 hexanedione
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HSP70	Heat shock protein 70
IAP	Inhibitor of apoptosis
IC_{50}	Half maximal inhibitory concentration
IF	Intermediate filament
IFAP	Intermediate filament associated protein
IFN-γ	Interferon gamma
Ig	Immunoglobulin

IκB	Inhibitor of kappa B
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthetase
IP$_3$	Inositol trisphosphate
JAK	Janus kinase
LD$_{50}$	Lethal dose 50%
LIF	Leukaemia inhibitory factor
LogP	Log partition coefficient
LPS	Lipopolysaccharide
MAOB	Monoamine oxidase B
MAPK	Mitogen activated protein kinase
MBP	Myelin basic protein
MDMA	3,4-methylenedioxy-N-methylamphetamine (ecstasy)
MPP+	1-Methyl-4-phenylpyridinium
MPTP	1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MRP	multidrug resistance protein
MTT	3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide
NEDD	N-1-napthylethylenediamine dihydrochloride
NADH	Nicotinamide adenine dinucleotide (reduced)
NF-1	Nuclear factor 1
NFκB	Nuclear factor kappa B
NGF	Nerve growth factor

NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOAEL	No observed adverse effect level
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NSE	Neuron specific enolase
O_2^-	Superoxide anion
OECD	Organisation for economic co-operation and development
·OH	Hydroxyl radical
PBS	Phosphate buffered saline
PCD	Programmed cell death
PenStrep	Penicillin/Streptomycin
P-gp	P-glycoprotein
PKG	Protein kinase G
PNS	Peripheral nervous system
RA	Retinoic acid
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription

TGF-β	Transforming growth factor beta
TMT	Trimethyltin
TMTC	Trimethyltin chloride
TNF-α	Tumour necrosis factor alpha
TrkB	Tyrosine kinase receptor type 2

Chapter 1: Introduction.

1.1 The nervous system.

Weighing approximately one and a half kilograms, the human brain is made up of up to 100 billion neurons and a far greater number of supporting glial cells. As the control centre for the body, the brain functions in all aspects of movement, senses, thought and perception. This complexity of the brain has contributed historically to a lack of understanding of its functionality, as the brain's networks have been previously regarded as far too numerous and intricate to ever fully understand. However, major advances in the sophistication of methods in neurological research have improved understanding of many aspects of neuroscience, from brain organisation and physiology, to the processes of cognition and memory development. Such advances have also provided a wealth of information regarding the capabilities and dynamic functions of this organ.

The brain is the functional centre of the nervous system, of which there are two major anatomical divisions; the central nervous system (CNS) and the peripheral nervous system (PNS). The PNS is itself divided into the somatic and autonomic nervous systems, the first of which generally regulates activities under conscious control, such as body movement and co-ordination as well as receiving external stimuli via the afferent nerves. The autonomic nervous system regulates activities which are not under conscious control and is again split into three divisions; the sympathetic system which is responsible for the immediate danger or stress response, the parasympathetic system which has unconscious control of normal resting bodily function and lastly the enteric system which manages every aspect of digestion and movement of food through the digestive tract. The neural tissues of the PNS consist of nerve bundles, associated blood vessels and connective tissues and are collectively called peripheral nerves. These peripheral nerves either carry information from the CNS to muscles and glands (efferent nerves) or from the peripheral tissues to the CNS (afferent nerves) (Silverthorn 1998; Martini 2004).

The brain, along with the spinal cord, makes up the CNS that is responsible for integrating, processing and coordinating sensory data and motor commands. The spinal cord coordinates spinal reflex actions that are designed to deal quickly with impending danger, while the brain is associated with higher functions such as cognition, memory, emotion and conscious motor commands (Sherwood 2004). The CNS is a complex

network, not only of neurons, but also of a variety of different cells, each of which contribute to the support of the brain and the maintenance of homeostasis. Such supporting cells are termed neuroglia of which there are four types associated with the CNS; ependymal cells, oligodendrocytes, microglia and astrocytes (Brodal 1992). Before it is possible to relate each of these cell types to their supporting functions, it is necessary to first understand the structure and function of the neuron.

1.2 The neuron.

Neurons are largely regarded as the basic functional subunits of the nervous system, responsible for the relay of electrical information between the brain and other parts of the body. There are many different functional (sensory, motor, interneuron) and structural (anaxonic, bipolar, unipolar, multipolar) classifications of neurons (Martini 2004). Typically, the neuron structure comprises the cell body containing the nucleus surrounded by the perikaryon of the cell body. The cytoskeleton of the perikaryon contains both neurofilaments and neurotubules, similar to the microfilament and microtubules found in other cell types. These neurofilaments collect into bundles called neurofibrils that extend into the dendrites and the axon providing internal support for these processes. The dendrites of a neuron function to conduct electrical stimulation received from other neurons to the cell body. Each cell possesses several of these dendrites, located generally around the cell body and each may branch off ultimately into fine processes called dendritic spines (Martini 2004). This extensive receiving network collectively contributes to the high connectivity of neurons within the brain.

The axon, also known as the nerve fibre, is a long slender projection through which electrical impulses are transported away from the cell body. The length of an axon may vary from micrometres to centimetres with some particular axons of the PNS, for example those of the sciatic nerve, reaching up to one metre in length. Because all of the cellular components are synthesised in the cell body of the neuron, any organelles destined for the axon must be transported there, first by packaging into vesicles and then moved by a process called axonal transport. Axonal transport can either be slow (0.2-2.5 mm per day) where vesicles move along with the axoplasmic flow, or fast (up to 400 mm per day) where vesicles are "walked" down processes of stationary microtubules (Silverthorn 1998). The axon also transports electrical impulses by the propagation of an action potential. This action potential is a wave of electrical discharge caused by the

polarisation and depolarisation of the cell membrane as a result of changes in ion distributions inside and outside the cell (Silverthorn 1998).

The process of such action potential generation is based on the concept of achieving electrostatic potentials. Inactive cells are at a resting potential of -70mV. This negatively charged resting potential is maintained by the active transport of potassium (K+) and sodium (Na+) ions into and out of the cell by the K+/Na+ pumps, where for every two K+ ions transported into the cell, three Na+ ions are transported out, so that there are generally more K+ ions inside the cell than out and there are more Na+ ions outside than inside the cell. This results in a net negative charge inside the cell. The action potential is generated when a depolarization stimulus causes a localised depolarization of the cell membrane to threshold, at which point voltage gated Na+ channels open and Na+ enters the cell along its electrochemical gradient, helped by the negative charge inside the cell. This rapid entry of Na+ causes the cell to depolarise due to a net positive charge of approximately 40mV inside the cell which in turn results in the closure of Na+ channels and the opening of the voltage gated K+ channels. Potassium begins to move out of the cells along its concentration gradient, into the extracellular fluid causing the intracellular charge to move towards the negative resting potential. The rapid efflux of K+ ions, however, causes a hyperpolarisation of the cell to approximately -80mV at which point K+ channels close and some K+ moves into the cell through leak channels, returning the cell to resting ion permeability and resting membrane potential of -70mV. Achievement of an action potential in the axon is an "all or nothing" event as the depolarization must reach threshold before an action potential is generated. If the depolarising stimulus is not sufficient to cause depolarisation to threshold, the action potential fails to initiate. Movement of an action potential down the axon is the result of depolarisation of the membrane causing Na+ to enter the cell. This further depolarizes the membrane, opening adjacent sodium channels. The positive charge created moves to adjacent sections of the axon by local current flow, causing the next section of membrane to become depolarised, opening Na+ channels in that region of the axon, and so on (Silverthorn 1998).

In order to increase the rate of action potential conduction, each axon is wrapped by segments of insulating membranes collectively called the myelin sheath. Myelin is composed of approximately 80% lipid (Hu *et al.* 2004). This relatively high lipid content compared to other biological membranes is likely to contribute to its insulating function.

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Myelin acts to propagate an action potential by preventing the movement of ions through the axon membrane so that the only way the current can move is forward, until the myelin breaks at a segment of the axon named a node of Ranvier. It is here that the positive charge is free to depolarize the membrane and allow the influx of Na+ ions into the cell to maintain the positive charge and current flow until the next node of Ranvier. This process effectively speeds up the movement of an action potential along the axon (Nichols *et al.* 2001).

After travelling the whole length of the axon, an action potential will reach the axon terminal where the electrical signal is usually conveyed by neurotransmitter to an adjacent cell. Each synapse has three broad structural areas relevant to neurotransmission; the presynaptic axon terminal, the synaptic cleft and the membranes of the postsynaptic cell (usually at the dendrites or cell body). As an action potential reaches the axon terminal and depolarizes the cell membrane, voltage gated Ca^{2+} channels are opened and Ca^{2+} enters the cell. This calcium entry signals the synaptic vesicles to release neurotransmitter by exocytosis at the pre-synaptic membrane which subsequently diffuses across the synaptic cleft and binds to receptors on the post-synaptic membrane. These receptor-transmitter complexes on the post-synaptic membrane stimulate the generation of either a rapid, short acting fast synaptic potential through activation of ion channels or a slow synaptic potential by activating second messenger pathways. Remaining neurotransmitter in the synaptic cleft is normally removed, either by uptake into the presynaptic cell or degradation by enzymes in the synaptic cleft (Sherwood 2004). Some neurotransmitter, however, diffuses away from the synapse and is taken up by surrounding glial cells. There are several different types of neurotransmitter that can act at the synapse, each of which can be categorised by structure into one of five general classes; amino acids (glutamate, GABA and glycine), monoamines (dopamine, serotonin and norepinephrine), purines (adenosine triphosphate), peptides (neurotensin and somatostatin) and acetylcholine (ACh) which is in a class by itself. The main neurotransmitters in the peripheral nervous system are ACh, epinephrine and norepinephrine while in the CNS there is a much larger spectrum including glutamate which is the main excitatory transmitter and gamma-aminobutyric acid (GABA) and glycine which are the major inhibitory transmitters. These major excitatory and inhibitory transmitters are found generally throughout the CNS while many others have more specific effects (Silverthorn 1998).

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To summarise, the highly developed structure of the neuron, designed to maximise connectivity between cells in the CNS and PNS, as well as the specialised mechanisms of cell-to-cell communication, allow the nervous system to process information and initiate responses extremely efficiently. Unfortunately, such specialised mechanisms lead to a high degree of vulnerability to damage by trauma, disease and exposure to exogenous or endogenous toxins that might impact catastrophically the survival of the whole organism. Furthermore, most neurons are incapable of normal division due to their lack of centrioles. These barrel shaped microtubule structures play an important role in the movement of chromosomes during mitosis (Afanasiev and Kotovsky 1961). This incapacity for division means that neurons are unable to be replenished in the event of damage and it is therefore necessary for the CNS to employ various mechanisms to protect neurons and preserve the overall functionality of the nervous system. As such, the neuroglia of the nervous system acts to protect and preserve neurons and their intricate functions.

1.3 The neuroglia.

Neuroglia, also termed glial cells, outnumber neurons by approximately ten to one. The distinctive differences in neural tissue organisation between neurons of the CNS and PNS are reflected in the different types of glial populations found in each. As previously mentioned, each of the four types of neuroglia, the ependymal cells, oligodendrocytes, microglia and astrocytes, have their own specific functions.

1.3.1 Ependymal cells.

Of the major neuroglial cell types in the CNS, the ependymal cells are among the least understood. These partially ciliated cells make up the ependyma, an epithelial layer which lines the central canal of the spinal cord and ventricles of the brain, assisting in the production, circulation and monitoring of the cerebrospinal fluid (Sherwood 2004). This fluid occupies the subarachnoid space in the brain, surrounding the whole brain and spinal cord and acts to give the brain buoyancy and effectively make it lighter. This reduces the pressure on delicate structures such as the neural capillary beds as well as helping to attenuate compression damage in the event of head trauma. The CSF provides a closely regulated extracellular environment for neurons and other glial cells. In order to achieve this optimal environment, ependymal cells actively control the movement of ions and water into the ventricles (Silverthorn 1998).

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1.3.2 Oligodendrocytes.

The oligodendrocytes are responsible for forming and maintaining the myelin sheath along neuronal axons in the central nervous system. The oligodendrocytic myelin consists of a single sheet of plasma membrane containing no cytoplasm, which is wrapped tightly around the axonal segment, creating a layered bundle called an internode (Bunge *et al.* 1968). Each oligodendrocyte is capable of myelinating portions of several different neuronal axons. In the peripheral nervous system this same myelinating function is performed by Schwann cells, that in contrast to oligodendrocytes, myelinate only a small segment of a single axon. While the structures of oligodendrocytes and Schwann cells differ, the myelin produced by the two cell types shares a common wrapped plasma membrane conformation.

1.3.3 Microglia.

Microglia are the resident immune cells of the CNS, responsible for the phagocytic removal of damaged cells, cell debris, wastes, xenobiotic agents and pathogens (Martini 2004). Under normal conditions, resting microglia are evenly distributed throughout the CNS, however, upon activation by peripheral nerve injury, trauma or disease (Streit 2000), they adopt a phagocytic cell morphology, present surface antigens most typical of macrophages and chemotactically approach and accumulate at the site of the injury (Chan *et al.* 2007). Activated microglia are capable of producing inflammatory cytokines, nitric oxide and other reactive oxygen intermediates (Zielasek 1996). The reactive oxygen intermediates and nitrogen species are highly toxic and prolonged induction can result in chronic inflammation in the brain, leading potentially to neurodegeneration (Vilhardt 2005). While all other types of glia are derived from the neuroectoderm, adult microglia are considered to derive from mesodermal progenitors (Chan *et al.* 2007) and not from circulating monocytes as was originally suggested (Perry *et al.* 1985). Once inside the CNS, resting microglia can proliferate and are capable of self renewal, meaning further recruitment from outside the CNS is limited (Lassmann and Hickey 1993).

1.3.4 Astrocytes.

The last, and functionally most diverse of the neuroglia, are astrocytes. Astrocytes, so named because of their star-shaped morphology, are the most abundant type of glia in the central nervous system and are closely associated with neurons and other glial cell

types. Until relatively recently, astrocytes were regarded as merely connective tissue between neuronal cells in the CNS. Increasing research, however, has demonstrated that more and more intricate and important functions can be attributed to these cells, so that astrocytes are now widely regarded as a fundamental cell type in the CNS as they are integral to many processes originally thought to be governed solely by neuronal function.

1.3.4.1 Astrocyte functions.

1.3.4.1.1 Supportive role.

Astrocytes give structural support to the CNS by creating a three dimensional framework for neurons. The progenitors of astrocytes, radial glial cells, also play a key role in neurogenesis by guiding the growth and interconnection of neurons in the developing brain and spinal cord (Edmondson and Hatton 1987; Brittis *et al.* 1995). In fact, it is emerging that radial glial cells are also the precursor cells of neurons and that the generation of astrocytes commences once neurogenesis is ceased (for review see McDermott *et al.* 2005). As well as directly supporting the development of neurons, it is apparent that astrocytes may play a role in the control of myelination of neuronal axons by oligodendrocytes. It is thought that ATP released from neurons stimulates the release of leukemia inhibitory factor (LIF) by astrocytes, which in turn promotes myelination by mature oligodendrocytes (Ishibashi *et al.* 2006).

Astrocytes are also involved in the maintenance of the blood brain barrier (BBB); a tightly packed layer of endothelial cells surrounding capillaries supplying the brain, which effectively isolates neural tissue from the blood and its circulating products. The barrier consists of many complex tight junctions and a number of transport and enzyme systems which regulate the movement of molecules across the cells (Abbott 2002), thus inhibiting the entry of potentially disruptive hormones, xenobiotics and pathogens while allowing essential nutrients such as certain sugars and amino acids to pass through. As well as playing a part in the development of the blood brain barrier, astrocytes are closely associated with these endothelial cells, whereby the "endfeet" of astrocytic projections cover the epithelia forming rosette-like structures. These structures not only act as a secondary hindrance to lipophilic molecules, but also allow astrocytes to support and direct the functioning of the endothelial layer through the complex cell-cell exchange of chemical signals (for review see Abbott 2002). In this intimate position with the blood brain barrier, astrocytes are also able to satisfy the high energy demands of CNS neurons.

Bouzier-Sore *et al.* (2006) found that neuronal cultures consume lactate preferentially as an oxidative metabolism substrate. This feature may have a neuroprotective function against energy deprivation during pathological conditions such as stroke or during periods of high activity (Bergersen 2007). Astrocytes are intimately associated with this process, as they effectively act as a transit mechanism for several nutrients, ions and dissolved gases that are allowed to pass through the BBB to neuronal cells. During this process they convert blood glucose to lactate, which is thence supplied to neurons as a metabolic substrate, together with amino acids such as glutamine and alanine (Magistretti 1993; Tsacopoulos 2002; Schurr *et al.* 1999).

Astrocytes are also responsible for regulating the extracellular space between cells in the CNS in order to maintain a homeostatic environment. This includes the maintenance of extracellular ions such as potassium, as well as the removal and recycling of neurotransmitters from the synaptic cleft. By taking up neurotransmitters, astrocytes are able to prevent their excessive accumulation. Glutamate in particular is taken up by astrocytes by specific GLAST and GLT-1 transporters (Aschner 2000) where it is converted to glutamine and then cycled back to neurons (Albrecht *et al.* 2007).

1.3.4.1.2 Neuromodulatory role.
Whilst astrocytes were generally regarded as merely supportive of the neuronal cell system, recent studies into the role of astrocytes at the synapse have shown that they may play a more active role in neuronal functioning by influencing the activity of neurons. Complex astrocyte-neuronal signalling within the CNS is now known to occur with glutamate as the major signalling factor between the two cell types (Parpura *et al.* 1994), the release of which is mediated by tightly controlled intracellular Ca^{2+} concentrations (Dani *et al.* 1992; Pasti *et al.* 1997). It is thought that glutamate released from neurones at the synapse binds to G-coupled receptors on the astrocyte membrane causing an activation of phospholipase C, production of IP3, and successive activation of IP3 receptors, resulting in a release of Ca^{2+} from intracellular calcium pools at the endoplasmic reticulum (Carmingnoto 2000). The induction of intracellular Ca^{2+} increases is not restricted to glutamate but has been detected following receptor activation by several other neurotransmitters including GABA, norepinephrine, acetylcholine and histamine (Duffy and MacVicar 1995; Araque *et al.* 2002; Shelton and McCarthy 2000). It has been observed that stimulation of astrocytes in this way ultimately causes intracellular Ca^{2+}

oscillations that can vary in pattern depending upon the frequency/intensity of the original stimulus (Pasti *et al.* 1997). The Ca^{2+} oscillations in turn cause the release of glutamate by astrocytes, which is then taken up by neurons. This process may represent a feedback mechanism initiated by neurons to modulate neuronal excitability and synaptic transmission (Perea and Araque 2002). In addition to this reciprocal glutamate signalling, intracellular Ca^{2+} oscillations can propagate as waves between astrocytes by either extracellular ATP or gap junction signalling (Cornell-Bell *et al.* 1990). Such astrocyte-to-astrocyte communication may allow for the propagation of messages away from the original synapse and the signalling to neurons in other areas by the release of glutamate (Cornell-Bell *et al.* 1990).

In terms of CNS function, the role of glutamate signalling is yet to be defined. While links to neuronal modulation have been demonstrated (Perea and Araque 2002), previous research has also implicated intracellular Ca^{2+} changes in astrocytes, associated with elevated neuronal activity, with an increase in cerebral blood flow. As previously described, astrocytic end feet are intimately associated with the endothelial cells of the blood brain barrier surrounding brain capillaries. Zonta *et al.* (2003) found that blocking Ca^{2+} oscillations in glutamate-stimulated astrocytes resulted in the impairment of activity dependent vasodilation of capillaries in rat cortical slices and that similarly, activation of a single astrocyte in contact with arterioles triggered vessel relaxation. Such astrocyte mediated vasodilation would act to increase local cerebral blood flow, thus making metabolic substrates more available to the areas of increased neuronal activity (Jakovcevic and Harder 2007). Calcium waves and glutamate signalling have also been implicated in the memory process: an increase in oscillation frequency upon repeated stimulation has illustrated that calcium oscillations in astrocytes are potentiated with glutamate (Pasti *et al.* 1995). While long term potentiation of neuronal signalling has been associated with memory development (Bliss and Collingridge 1993), the potentiation seen in astrocytes following synaptic activity also suggests a role for these cells in the memory process (Zonta and Carmingnoto 2002).

1.3.4.1.3 Protective role.

In addition to these supportive and modulating functions, astrocytes also play an important protective role towards neurons. Data obtained from co-culture studies show that neurons respond differently to various stimuli in the presence or absence of

astrocytes. Brown (1999), Sass *et al.* (1993) and Rosenberg and Aizenman (1989) found that astrocytes protected neurons against the effects of glutamate excitotoxicity. Neuronal excitotoxicity is caused by the over activation of neurotransmitter receptors usually as a result of excessive neurotransmitter in the synaptic cleft. This process of excessive neurotransmitter activity can result in excess Ca^{2+} being allowed to enter the cell. Excessive intracellular Ca^{2+} can overwhelm the regulatory mechanisms of the cell and initiate cellular events which may lead to cell death, eventually resulting in widespread neurodegeneration (Salinska *et al.* 2005). As described previously, astrocytes are able to prevent the excessive accumulation of neurotransmitter in the synaptic cleft by uptake via specific neurotransmitter transporters (Aschner 2000). Brown (1999) also suggested that soluble factors released by astrocytes, such as the cytokine IL-6 may protect neurons from the excitotoxic effects of glutamate. Also using co-culture techniques, Tieu *et al.* (2001) and Yu and Zuo (1997) both demonstrated that astrocytes provided increased tolerance towards certain xenobiotic compounds. Similarly, Woehrling *et al.* (2007) demonstrated in a differentiated post-mitotic co-culture, that astrocytic cells exerted a protective effect on neurons during exposure to various human neurotoxins.

Astrocytes are an important site for the biotransformation of potentially toxic xenobiotics in the CNS and have been shown to express certain cytochrome P450s, (Hedlund *et al.* 1998). These cytochrome P450s are a diverse family of hemoproteins that are capable of metabolising many xenobiotics. Their expression in astrocytes, particularly in astrocytes associated with the blood brain barrier could act to protect neurons from the toxic action of the parent compound (Meyer 2007).

The protective capacity of astrocytes may also be attributed to the high levels of cellular protective systems found in astrocytes compared other cells in the brain (Pentreath and Slamon 2000). These systems act to tightly maintain normal levels of oxidative cell signalling molecules, as well as detoxifying chemicals and reducing the reactive oxygen species (ROS) produced by cells following exposure to many toxic compounds. Such ROS include hydrogen peroxide and nitric oxide, which are essential signalling molecules but which can cause damage at high levels, as well as other free radicals including the hydroxyl radical ($\cdot OH$) and superoxide anion (O_2^-). The production of ROS which exceeds normal reductive capacity of a cell can cause oxidative stress which can potentially cause structural damage and disrupt cellular processes. Cell systems

attempt to minimize the presence of ROS produced spontaneously as well as those formed by xenobiotics with the glutathione system. Glutathione is one of the most important cellular antioxidants, as it conjugates and detoxifies many xenobiotic compounds as well as reducing free radicals (Iwata-Ichikawa *et al.* 1999). The glutathione molecule has a thiol group that can conjugate and reduce chemicals, metabolites and ROS. Wang and Cynader (2000) suggested that neurons rely on astrocytes for their capacity to secrete thiols in the form of released glutathione, although the instability of this molecule dictates the constant astrocytic release of glutathione which is essential to maintain stable thiol levels in the CNS. Other protective systems include the antioxidants Vitamin C (ascorbate), Vitamin E, superoxide dismutase, catalase and the heme-oxygenase-1 system that liberates the antioxidants biliverdin and bilirubin from heme, as well as metal chelators such as the metallothioneins, selenoprotein P and apolipoproteins. The majority of these factors are present in higher levels in astrocytes than in neuronal or oligodendrocytic cells (Boyles *et al.* 1985, Makar *et al.* 1994, Aschner 1998, Lindenau *et al.* 2000, Dwyer *et al.* 1995) and have all been implicated in the neuron-protective role of astrocytes (Tanaka 1999; Halkes-Miller *et al.* 1986; Iwata-Ichikawa *et al.* 1999; Rosenberg 1991; Penkowa *et al.* 1999a; Desagher 1996; Yang 2000; Boyles *et al.* 1990; for review see Pentreath and Slamon 2000).

Not only do astrocytes protect against xenobiotic toxicity, excitotoxicity and oxidative stress by the expression and release of soluble factors, they also respond physically in a unique way to many forms of physical, chemical and disease induced trauma (Malhotra *et al.* 1990). Such a response is commonly termed astrogliosis but is also referred to as reactive astrogliosis or gliosis. The cells affected are similarly referred to as "reactive," "activated" or "gliotic." The response is typically characterised by hypertrophy of the cell body, together with the extension of fibrous processes and an increase in cellular levels of glial fibrillary acidic protein (GFAP); the major intermediate filament type in astrocytic cells (Eng and Ghirnikar 1994). These GFAP filaments form as bundles which can fill the entire cytoplasmic compartment (Bignami and Dahl 1976; Hozumi *et al.* 1990). In addition to these obvious morphological changes, reactive astrocytes undergo complex alterations in the expression of a large number of genes (for review see Eddleston and Mucke 1993); however, many of these alterations are yet to be characterised.

The purpose of the changes that occur in astrocytes when they become reactive are not yet fully understood, however, as suggested by Wu and Schwartz (1998) it is likely that such changes reflect alternations in functional status following injury. It has been noted that many of the normal protective mechanisms of astrocytes such as the glutathione and antioxidant systems are up-regulated following astrocyte activation (Osterberg and Wattenberg 1962; Rubinstein *et al.* 1962). Astrocytes are also capable of synthesizing a variety of growth factors, cytokines and neuropeptides. Many of these factors are expressed in low levels or not at all in resting astrocytes in the normal brain, although, following activation, their expression is induced or up-regulated. For example, nerve growth factor (NGF) and fibroblast growth factor (FGF), among others, are both increased following the onset of glial reactivity (Aubert *et al.* 1995). Similarly, increased levels of released cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα) have also been associated with astrogliosis (Ridet *et al.* 1997). The expression of growth factors following trauma has been shown to stimulate neuronal recovery and regeneration (Olson *et al.* 1994) and consequently many have been evaluated for their potential use in the therapy of neurodegenerative diseases (Hefti 1994, Lindsay *et al.* 1993).

A collective action of reactive astrocytes is manifested in the formation of a glial scar at the site of the CNS injury. This is a mature form of astrogliosis, whereby astrocytes orientate towards the margin of the wound, forming a network of cells and fibres, bound together by tight and gap junctions, which thickens progressively to form a tight protective physical barrier between affected and unaffected tissue (Malhotra *et al.* 1990). In general, the process of glial scar formation in the CNS from initiation of astrogliosis to full thickening of the glial scar can take up to eight weeks (Tedeschi 1970). The exact sequence of events, however, depends upon the size and nature of the injury (Fawcett and Asher 1999). It is thought that the glial scar acts to preserve tissue integrity following injury by isolating the affected area from the rest of the brain tissue, thus preventing any further damage from occurring. Any attempt by damaged neurons to repair their interconnections with other neurons may have catastrophic effects through development of dysfunctional neural networks. Instead, the regeneration of axons and axonal terminals after trauma is prevented through the secretion of various inhibitory factors by astrocytes and other glia types (Sandvig *et al.* 2004) and by the creation of axo-

glial terminals that resemble the terminals that axons make on target neurons during normal development (Liuzzi and Lasek 1987).

The manifestation of reactive astrogliosis is not rigid and can vary both qualitatively and quantitatively depending upon the type and severity of the trauma, including the degree of damage caused by mechanical injury, the type and stage of disease as well as the type of toxin and the concentration of exposure (Pentreath and Slamon 2000). For example, the formation of a glial scar is not always associated with reactive astrogliosis. While injuries caused by physical trauma or disease are generally associated with the formation of a glial scar, scar formation is not normally seen in chemically induced injury where such large and diffuse areas can be affected (Ridet *et al.* 1997).

This multitude of functions attributed to astrocytes, including maintaining homeostasis, providing structural support and eliciting neuroprotection both at rest and following injury, all illustrate the complexity and fundamentality of the astrocytic cell and its role in the CNS. These attributes, together with the emerging evidence that astrocytes have a complex reciprocal relationship with neurons, suggest that there would be major implications for the viability and functioning of the CNS if these roles were not fulfilled. This, together with the fact that astrocytes respond characteristically in the event of toxic exposure and act to protect neurons in such a situation, suggests that astrocytes have a much more valuable place in neurotoxicology research than has been appreciated to date.

1.4 Neurotoxicology.

Neurotoxicology is a specific discipline in toxicology concerned with the effects of toxic substances on the nervous system. Neurotoxicity itself is defined as "any adverse effect on the chemistry, structure and function of the nervous system during development or at maturity, induced by chemical or physical influences" (Ladefoged *et al.* 1995). Continued research in neurotoxicology and in the events following toxicant exposure is extremely important, as derangement of the brain has serious consequences for the organism and its survival.

1.4.1 REACH.

The impact of human exposure to potentially toxic agents in the environment and in foodstuffs is wide-ranging. Emerging evidence suggests there may be detrimental

cumulative effects of environmental chemicals on cancer and fertility rates (Wogan *et al.* 2004; Swan 2006). Similarly for neurotoxicology, as the cases of neurodegenerative diseases increase year upon year, it has been suggested that environmental toxins may be implicated in the aetiology of certain neurological disorders (Ly *et al.* 2007; Zuber and Alperovitch 1991). Despite these potential links between chemical exposure and pathology, the fact that relatively few of the chemicals to which we are exposed to have undergone rigorous toxicity assessment, let alone assessment for neurotoxicity, obviates the need for stringent and systematic evaluation of existing and new chemicals. In order to address this situation, on October 29th 2003, the European Commission proposed a new regulatory framework for the registration, evaluation and authorization of chemicals (REACH), with the aim of evaluating over 30 000 chemicals for toxicity in the next ten years (Coecke *et al.* 2006). This framework entered into European Union law on the 1st June 2007, requiring manufacturers and importers to gather information regarding the properties of their substances and to submit them to a central database managed by the European Chemicals Agency.

In order to aid the application of REACH, chemicals have been classified into two groups; existing and new chemicals. An existing chemical is one that has been on the European Community market between 1st January 1971 and 18th September 1981, whereas new chemicals are those introduced to the market after 1981. New chemicals are required to be tested before release on to the market yet there is no such requirement for existing chemicals. It is these existing chemicals that are targeted by the REACH framework in order to establish comprehensive toxicity data in relation to human health. This toxicity assessment includes the evaluation of a variety of different toxic responses including neurotoxicology and developmental neurotoxicity (Knight 2006).

1.4.2 Current testing regimes.

Current neurotoxicity testing is based mainly *in vivo*, involving the dosing of live animals, particularly rodents. Both the Organisation for Economic Co-operation and Development (OECD) and the European Commission (EC) have issued guidelines for the *in vivo* neurotoxicity assessment of chemicals. These guidelines involve initial testing for the determination of a compounds neurotoxic potential, including a variety of acute and chronic dermal, inhalation and oral toxicity assessments. Following these tests, more specific neurotoxicity studies in rodents and other animals are carried out, including the

assessment of neurobehaviour, neuropathology, neurophysiology and neurochemistry (Coecke *et al.* 2006). Current OECD and EC guidelines for neurotoxicity testing are both expensive and time consuming, which have practical implications when applied to the REACH framework. It is believed that using these current *in vivo* toxicity testing systems, the number of animals required to fulfil the targets set by REACH could exceed 3.9 million (Coecke *et al.* 2006). The registration of all substances covered by REACH has been estimated to take more than 11 years to complete at an estimated cost of up to 5.2 billion euros (European Commission 2006).

1.4.3 Disadvantages of *in vivo* testing.

There are several limitations to the use of animals in experimental research. Increasing legislative control and escalating costs associated with increased animal housing and welfare requirements result in the relatively low throughput of *in vivo* test systems by restricting the scale of work that can be performed within a limited laboratory budget. Similarly, restrictions on the use of animals for certain applications as well as limits on the numbers of animals used, consequently confers limits upon the research that can be performed.

Other limitations of *in vivo* models are the possible inaccuracies involved in the extrapolation of data from animal models to humans, with several species showing differential sensitivities and tissue specific effects following exposure to the same compound. Interspecies differences in physiology may begin to explain the variations of response to a compound, particularly in relation to toxicity. Different animal species have varying gastrointestinal absorbance efficiencies depending upon the route of exposure. The pH of the gastrointestinal tract varies among species and this has profound effects on the degree of ionization of weak electrolytes, which in turn influences their absorption. Absorption rates for drugs that are actively transported via the gastrointestinal tract also vary depending upon energy availability. Dermal absorption also depends upon the thickness of the stratum corneum, the level of hair covering and the extent of dermal vasculature, all of which vary between species (Calabrese 1983).

Similarly, the biotransformation of a compound may also differ between species. Xenobiotic metabolism within an organism is incredibly complex and involves a series of phase I and phase II reactions that modify and conjugate compounds respectively in order

to facilitate their excretion. The modification of compounds in phase I reactions is performed principally by the microsomal mono-oxygenase enzymes, such as the cytochrome P450 family. The distribution of these and many other biotransformational enzymes in organs of metabolism such as the liver, kidneys and gastrointestinal tract, as well as other organs such as the lungs and the brain is highly species dependent and may consequently determine the toxicity of a compound in a particular animal. For example, administration of the 'designer' drug MPTP has severely neurotoxic consequences to humans, causing permanent Parkinsonian effects, whereas it is harmless to several other species used in experimental studies such as the rat (Kopin 1987). This is due to the location of the enzyme monoamine oxidase B (MAOB) that metabolizes MPTP to its neurotoxic metabolite MPP+. MPTP is permeable to the human BBB whereas MPP+ is not. Once the parent compound is inside the human BBB it is metabolised by MAOB and can exert its toxic effects in brain tissue. In rats however, the compound is metabolised by high levels of MAOB outside the blood brain barrier rather than inside, and the hydrophilic MPP+ toxic metabolite cannot penetrate the CNS BBB to cause damage (Kopin 1987).

There are many other areas of difference that may confer sensitivity or resistance to a particular species, for example intestinal microflora levels, tissue distribution of xenobiotics, and rate of excretion. Consequently there have been many reported cases of differential toxicity of compounds between humans and other animals. The neurotoxic actions of MDMA ("ecstasy") are more pronounced in humans and primates than in rats. There are also differential regional sensitivities to the drug in the human and rat brain (Reneman *et al.* 2002). Alternatively, the drug Tamoxifen that is widely used in the treatment of breast cancer in humans has been found to have hepatotoxic effects in rats (Carthew *et al.* 1995). The failure of any rodent model to highlight a potential toxicity to humans may seriously compromise the safety of any human volunteer in clinical trials. On the other hand, it is entirely possible that potentially useful medicinal compounds could be eliminated as a result of a false-positive evaluation at the animal stage of toxicity testing.

Aside from the practical issues associated with animal use in toxicity testing, ethical considerations concerning the utilization and ultimately termination of live animals are also under increasing discussion in the public domain. While animal testing can be of great benefit to many areas of medical research and is likely to persist into the future,

there is increasing pressure to develop alternative models for toxicity testing which can improve predictivity for human exposure.

1.4.4 Development of alternative models to *in vivo* toxicity testing.

In 1959, Russell and Burch published their book "The Principles of Humane Experimental Technique" in which the concept of the three 'R's (Reduction, Refinement and Replacement) was originally coined. The concept has since had a major role in shaping current animal experimental research practices. In this context, refinement refers to "any alteration in procedures that leads to a decrease in the incidence and/or severity of stress or discomfort to animals used in experimental procedures." Reduction involves "the development of scientifically justifiable techniques to obtain the same quality of information using fewer experimental animals," whereas replacement is "the introduction of new methods that do not require the direct use of sentient animals in experimentation" (Purchase *et al.* 1998). While most efforts have focused on the reduction and refinement of animal research, the area of replacement is problematic for many researchers, mainly due to the inadequacy of current alternatives. However, considering the sheer scale of the *in vivo* requirements of the REACH framework as well as the limitations of the *in vivo* approach to toxicity testing and the associated ethical considerations, there is an obvious need to develop better alternative models that are simple, reproducible and relevant.

In order to be accepted as adequate, an alternative system must undergo a process of validation, whereby "the reliability and relevance of a procedure are established for a specific purpose" (Balls *et al.* 1995). The European Centre for the Validation of Alternative Methods (ECVAM) was set up in 1993 to coordinate the validation of alternative test methods as well as to set up and maintain a central database to facilitate information exchange in the development of alternative procedures. The five main stages of validation are; test development, prevalidation, validation, independent assessment and progression towards regulatory acceptance. The initial stage of test development is generally the most extensive. It is necessary for the method to be properly developed and so in order to submit a test for validation it is necessary to provide a case for its inclusion in validation studies, together with information regarding the purpose of the test and how it will fill a need in terms of toxicity testing, specifically in regards to a particular chemical spectrum. It is also necessary to provide a comprehensive protocol that is to be used in the prevalidation stage. In this second stage, informal, small-scale interlaboratory

studies are performed in order to identify any problems and optimise the protocol as well as establishing inter-laboratory transferability. Once a test has passed prevalidation, it goes into the main stage of validation where blind trials in elected laboratories are used to determine the relevance and reliability of the method. Following analysis and assessment of the outcomes of these validation studies, the method is then independently evaluated to determine whether the purpose and objectives of the validation study have been met together with an evaluation of the practicability, value and necessity of the alternative procedure in regards to existing and forthcoming tests. This independent assessment is crucial before any regulatory authority is asked to consider the validated method for formal acceptance into their regulatory framework for toxicity testing (Balls *et al.* 1995). The total time of the validation process from test development to regulatory acceptance is usually at least ten years from initial publication in scientific literature to the acceptance into OECD guidelines (Purchase 1997).

While scientific validation is generally regarded as an essential step in determining the adequacy of new alternative test methods, there is a clear double standard between the requirements of alternative and existing *in vivo* toxicity tests. While alternative systems are required to pass the rigorous validation process before they are considered for toxicity testing, validation is much less stringent for *in vivo* systems. This provides some advantages for alternative tests, such as the benefit of transparency, whereby the limitations and advantages are clearly identified and evaluated.

1.4.5 *In vitro* toxicity testing.

With the need for alternative toxicity methods evident and the necessary framework for their validation and acceptance in place, the potential for scientific advancement in this field is considerable. One of the main areas of alternatives research is in the development of *in vitro* test systems using tissue culture. Tissue culture is a generic term used to describe the *in vitro* cultivation of organs, tissues and cells as separate entities from the organism of origin. Pioneering work conducted by Harrison (1907) and Carrel (1912) has led the way to the massive development of *in vitro* tissue culture techniques and the ability to culture almost all types of animal and human cells. Organ culture involves the cultivation of part or whole organs as a means of maintaining their three dimensional architecture and functional characteristics. Similarly, the culture of tissue explants allows for the maintenance of tissue function *in vitro*. Some valuable *in*

vitro toxicity models have been derived from the culture of organs and tissues such as the alternatives to the Draize eye test, where chemicals were tested for ocular irritancy by application to the eyes of live rabbits. Instead, systems employing isolated rabbits eyes or isolated bovine corneas have been used for the same purpose (Cooper *et al.* 2001; Jester 2006). The culture of organ and tissue explants are however only considered to be "relative replacement" (Russell and Burch 1959) as animals must still be sacrificed to obtain the tissue. It is possible to use human sourced material although availability is predictably problematic, not only as there may be a lack of donated material but also the viability of such tissue should be safeguarded to high standards prior to inclusion in toxicity testing. This quality control problem means that human material can also show a great deal of experimental variation between cultures. In addition to these practical limitations there is also a high infection risk associated with working with human tissues as well as the potentially complex ethical implications. The infection risks are of particular concern as they relate to potentially lethal and debilitating viral and prion diseases such as the various hepatitises, HIV, as well as Creutzfeldt-Jakob disease (CJD).

The practical limitations of using human tissues can be reduced in cell cultures. Primary cell culture is the term given to freshly isolated cells in culture which have been enzymatically dissociated from living tissue. Primary cells are usually heterogeneous, consisting of a mixture of the cells present in the original tissue and as such retain to some degree their original characteristics and systemic properties. However, they have a low potential for growth and tend to change morphology or die in prolonged culture, therefore it is necessary to isolate fresh cells for each experiment. Also, the high infection risk, complex ethical complications and the practical limitations described for human organ and tissue culture can also be applied to human primary cell culture, meaning that these cells may not be suitable for the routine screening of toxicants.

The problems of disease, variation and scarcity of directly human-sourced tissue are effectively circumvented with continuous cell culture. Cell lines also have a much greater potential for division in culture. They can either be 'finite cell lines' which tend to die out after several subcultures or 'continuous cell lines' which can proliferate indefinitely provided they are maintained correctly. This prolonged life span means it is possible to store cells in liquid nitrogen for long periods of time while maintaining cellular characteristics (Hay 1992). Continuous cell lines in particular also have higher

growth rates and can produce a greater yield due to the ability to grow to higher cell densities. Another major benefit of cell lines is that they are typically of a constitution that facilitates the reproducibility of test systems, even between different laboratories.

The rapid and high throughput nature of cell line-based *in vitro* techniques should facilitate the ethical testing of large numbers of chemicals identified by REACH, as well as the testing of the virtually infinite number of chemical mixtures. The smaller scale of individual tests would also mean that much less of the test compound would be needed compared to *in vivo* tests for which the synthesis of large amounts of new compounds may be required. Even though the *in vitro* cell based systems cannot replicate the complexity of the entire organism or target organ, it is possible to focus on tissue specific toxicity using simple tests. By doing so it should be possible to obtain mechanistic data as to the toxic action of an agent, rather than simply observing the pathological consequences of exposure in whole animals. The subsequent application of a battery of tests with different endpoints could create a larger picture of the toxicological impact of a certain agent than can be seen in a single test. The simplicity of the cell line based toxicity models can also provide a platform upon which systemic mechanisms may be eventually integrated including absorption, distribution, metabolism and excretion. These practical advantages and the numerous potential applications provide a strong case in the use of continuous cell lines in the development of *in vitro* toxicity models.

1.4.6 *In vitro* neurotoxicity assessment.

At present there are no validated methods for the assessment of neurotoxicity (Coecke *et al.* 2006), however, there does exist a large amount of neurotoxicity data obtained using cell culture systems. Many current *in vitro* neurotoxicity tests focus on the use of primary cultures for assessing toxic insult. These cells are usually sourced from rodents (Coecke *et al.* 2006) with which genetic differences on a cellular level may create uncertainty during the extrapolation process. Similar to the problems associated with extrapolation from *in vivo* tests, differential sensitivities to toxicants have been reported between cells in culture (Peterson *et al.* 1997; Yong *et al.* 1992). These species-dependent toxicities suggest that the development of a human cell line model may be more relevant to the human condition than cells originating from other species (Sanfeliu *et al.* 1999). The current tendency to use primary cell culture also highlights the potential for the use of cell line based models in neurotoxicity testing.

1.4.6.1 Neuronal and astrocytic cell lines.

It is possible to source a variety of cell lines of CNS origin including those derived from neuronal, astrocytic or oligodendrocytic cell types. Many of these cell lines originate from excised tumour masses that would normally be discarded. For example, the human SH-SY5Y neuronal cell line, which is a sub-clone of the SK-N-SH line derived from the bone marrow biopsy of a four-year-old girl with metastatic neuroblastoma (Biedler *et al.* 1973), is widely used in neurotoxicity studies (Sandfeliu *et al.* 1999). Similarly, a variety of human astrocytoma cell lines are available which express typical astrocytic markers such as GFAP and glutamine synthetase and have the potential for use as *in vitro* astrocytic models in toxicity assessment.

Despite the wide array of different cell lines available, the majority of *in vitro* toxicity systems are focused on neuronal effects in monoculture. As astrocytes are intrinsically involved in the responses of the CNS to toxic insult, a system that would incorporate astrocytes would be more appropriate to closely represent the nervous system. The co-culture of neuronal and astrocytic cell lines can give an advantageous dynamic to *in vitro* neurotoxicity testing which is permissive of neuron-astrocyte interactions and any protective effects that astrocytes may confer upon neurons during toxicant exposure. Indeed, co-culture methods have been used to demonstrate the protection conferred by astrocytes to neurons from glutamate cytotoxicity as well as the enhancement of neuronal survival by astrocytic scavenging of extracellular ROS (Drukarch *et al.* 1998). A variety of different co-culture methods exist, each of which vary in complexity and applicability. Simple methods employing the use of conditioned media allow for the transfer of soluble molecules from one cell population to another as a way of assessing their effect on the latter. The same can be achieved by the growth of individual cell populations on separate cover-slips or in distinct culture compartments while allowing the same culture media to nourish each (Sass *et al.* 1993). Such methods have been used to illustrate the neuroprotective effect of molecules derived from astrocytes such as glutathione (Wang and Cynader 2000; Iwata-Ichikawa *et al.* 1999). Other methods such as the growth of one cell population upon another allow close cellular contact but do not allow easy separation and analysis. More recent efforts have seen the development of techniques employing the use of transwells that allow the very close culture of two cell populations while allowing for their eventual separation. Astrocytic and neuronal cell lines have been co-cultured in

these transwells to develop an *in vitro* blood brain barrier model (Toimela *et al.* 2004) and to investigate the astrocytic support of neurite extension (Walsh *et al.* 1992). Together with the benefits of permitting cellular interactions, co-culture methods are all amenable to relatively easy manipulation. Hence, there is huge potential for the manipulation of co-culture techniques for use in high-throughput neurotoxicity screening. While astrocytic/neuronal interactions are of particular interest in terms of toxicity, it would be beneficial to eventually develop a co-culture system that incorporates all the different cell types in the brain into one system.

1.4.6.2 Toxicity endpoints.

In any *in vitro* toxicity system, one of the main considerations is the choice of endpoint, or cellular event, which will be measured as an indicator of toxic effects. Many systems employ endpoints that measure cell lethality and which are generally regarded as "cytotoxicity assays." Cytotoxicity analysis is the measurement of events that occur when the basal level functions of a cell are severely compromised to the point of cell death, as a result of toxicant exposure (Ekwall 1983; Veronesi 1992). Cytotoxic cell death generally occurs via one of two pathways, necrosis or apoptosis. Necrosis, often termed "accidental cell death" is the unregulated lysis of damaged cells resulting from acute injury, identified by early swelling of the cell and cytoplasmic organelles, randomized DNA degradation and the release of lysosomal enzymes into the extracellular fluid (Holownia *et al.* 1997). Apoptosis, on the other hand, is a highly conserved process often referred to as programmed cell death (PCD) and occurs not only as a result of cell stress and damage, but also under normal physiological conditions including embryogenesis and cell turnover processes. It is typically characterised by cell shrinkage, membrane blebbing, DNA fragmentation and the formation of apoptotic bodies containing ribosomes, mitochondria and nuclear material (Pallardy *et al.* 1999). *In vivo*, these apoptotic bodies are readily phagocytised, whereas *in vitro* they undergo "secondary necrosis" whereby they swell and eventually lyse (Savill 1993). Cell lysis and the release of enzymes into the extracellular fluid associated with necrotic cell death can cause damage to local cells and have a knock-on effect for cytotoxicity. It is therefore clear why apoptotic cell death is the preferential pathway of cell death in maintaining tissue integrity following injury.

Vaux (2002) suggested a stimulus-response model for the induction of apoptosis following toxic exposure, whereby the compound does not directly act to cause apoptosis

but acts on the cell in a manner that causes stress, thus triggering events associated with the apoptotic pathway. It was also noted by Vaux (2002) that little is known about what integrates or connects various cell stress pathways and the cell death pathway and what determines the threshold above which defence against stress gives way to cell death.

It is difficult to determine conclusively whether lethal exposure to a particular compound might result in cell death by apoptotic or necrotic pathways. Even if the agent is seen to induce characteristic upstream events of apoptosis, there may also be simultaneous activation of apoptotic inhibitors such as members of the Bcl-2 family or the inhibitors of apoptosis (IAP) family that would prevent progression along the apoptotic pathways (Vaux 2002). It is not possible to determine the pathway of cell death from cytotoxicity assays but they do provide relative sensitivities of several cell types to a particular compound, making them particularly useful for comparative toxicology. Cytotoxicity assays are generally based upon one of three parameters; measurement of cell number, cell membrane integrity or metabolic activity, each of which are easily quantifiable and common to most cells. While the measurement of cytotoxicity is a valuable tool in evaluating the response of a cell population to external factors and is widely used in toxicological analysis to study the relative potencies of cytotoxic agents, cytotoxicity assays are limited to providing information regarding only the "basal" cytotoxicity of a compound and not how a compound may affect cells at low, non-cytotoxic concentrations. This is applicable particularly when investigating the effects of compounds upon astrocytes and cells of astrocytic origin, which can be highly sensitive to the subtle changes that small amounts of an agent may have on the microenvironment of the brain. Indeed, mild or transient alterations in cellular function may occur in response to low-level toxicant exposure. Identifying changes in protein and DNA synthesis, ion homeostasis, energy regulation, cell-cell communication, biosynthetic reactions, transport processes or specific enzyme changes may act as more sophisticated and sensitive markers of cell stress by identifying adverse toxic effects which may not have been identified by normal cytotoxicity analysis (Veronesi 1992) and which may be specific to only a certain cell type or system. Such transient alterations may also provide evidence for the mechanistic actions of certain toxicants.

In the development of a co-culture system, it is also important to be able to distinguish the response of one cell line from another. By using endpoints specific to

individual cell types, as opposed to non-specific cytotoxicity analysis, it may be possible to categorise the action of a toxicant as being specific to a cell population, as is one of the main objectives of first tier toxicity screening (Costa 1998). As different compounds can have a variety of different effects on the same cell, so too can cell types be differentially sensitive to the same toxicant. If a cell specific endpoint were affected only in a particular cell type or at concentrations below those required to cause alterations in cytotoxic endpoints, then the agent could be considered to have cell specific action. For example, while the term neurotoxicity is used generally to describe the effects of a toxicant on the nervous system, it is also widely used to refer to toxic effects specifically in neuronal cells. It may be possible to distinguish neurotoxicants from cytotoxicants by their effects on neurochemical, neuromorphological or neurotransmission functions (Veronesi 1992), which would obviously be distinct from non-neuronal cells that do not share the same morphology or functions. Similarly, specific astrotoxicants are those compounds that affect cellular processes specific to astrocytes or affect astrocytes at much lower concentrations than other cell types. As astrocytes have been established as a vital cell type in the CNS, their impairment in any of a number of ways may eventually lead to neurotoxic effects, for example, metals such as methylmercury and lead preferentially accumulate within astrocytes (Garman *et al.* 1975; Holtzman *et al.* 1984; Tiffany-Castiglioni *et al.* 1989), potentially affecting astrocyte homeostasis and causing the release of excitatory amino acids with possible consequential influences upon neuronal function (LoPachin and Aschner 1993). It was also suggested by LoPachin and Aschner (1993) that lead may act to perturb astrocyte signal transduction pathways, potentially disrupting intercellular communication. This would lead to a detrimental effect on homeostasis and eventually on neuronal function. Clearly it is necessary to investigate potential astrotoxicity in order to distinguish whether neurotoxicity observed following administration of an agent is due directly to the action on neurons or indirectly though the impairment of astrocytic function.

1.4.6.2.1 Astrocyte specific endpoints.

Astrocytes have unique features that could be exploited in the development of endpoints for such toxicity analysis, which in doing so would allow for astrotoxicity to be distinguished from neurotoxicity or general cytotoxicity. An important characteristic of astrocytes which is exclusive to this cell type and which also makes them ideal for looking at low-level exposure effects is their ability to respond characteristically to non-cytotoxic concentrations of chemicals and achieve a state of reactive gliosis. This reactive gliosis, or

astrocyte reactivity, was described earlier in this introduction as an important protective mechanism of astrocytes, following a variety of injuries or trauma including hypoxia, chemical or physically induced injury as well as the lesions observed in several neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease (Malhotra *et al.* 1990). Efforts to determine the relevance of astrocyte reactivity in the pathology of neurodegenerative disease and other injuries as well as in the development of treatments have focused on the identification and characterisation of the reactive astrocyte and the molecular events of its induction (Laping *et al.* 1994). The identification of reactive astrocytes is also of use as a method of assessing injury in the CNS as during astrocyte activation, there is up-regulated expression of many key proteins involved in the reactive response (Ridet *et al.* 1997). The identification and quantification of these proteins can provide an indication of the extent and location of injury *in vivo*. *In vitro*, following chemical exposure, quantification of these markers can also identify whether a compound is capable of causing reactivity in homogenous cultures of astrocytes, thus helping the classification of specific astrotoxins. Of the many up-regulated proteins, cytokines and growth factors, one of the most well recognised hallmarks of astrocyte reactivity and the most widely used marker to identify the response is the up-regulated expression of the astrocyte specific glial fibrillary acidic protein.

1.5 GFAP.

The glial fibrillary acidic protein (GFAP) was first discovered by Eng *et al.* in 1971, whilst researching the phenomenon of plaque formation in the white matter of Multiple Sclerosis sufferers. GFAP is a 50kDal cytoplasmic filamentous protein which, when assembled, makes up the main intermediate filament in cells of astrocytic origin. These Intermediate Filaments (IFs) along with microtubules and microfilaments make up the eukaryotic cytoskeleton. The cytoskeleton facilitates changes in cell morphology and motility as well as supporting intracellular movements. Each filament type has its specific role in the co-ordinated functions of the cytoskeleton.

1.5.1 Intermediate filaments.

The structures of IFs (so called because of their intermediate size between microtubules and microfilaments) can be highly variable between cell types, whereas those of microfilaments and microtubules are more conserved. Each IF monomer has a three-part structure of an amino-terminal head, a carboxyl terminal tail and an alpha helical central

rod domain (Parry and Steinert 1992). The central rod domain is common to all IF proteins whereas the head and tail regions are more variable between different IF types and are thought to attribute to the differential assembly and functional properties of intermediate filament types (Lazarides 1982). The structural variations of the head and tail regions allow for the typing of IFs into at least six general groups. There are the acidic and basic keratins (Type I and II), neurofilaments (Type IV), laminins (Type V) and nestin (Type VI). Type III IFs include vimentin, desmin, peripherin and as previously mentioned, GFAP. Each of these proteins from the six classes constitute the IFs in different cells or tissues; for example GFAP is the principle intermediate filament protein in cells of astrocyte origin while in neurons, one of the neurofilament proteins will be the constituting type (Steinert *et al.* 1984). A schematic diagram of the structure of GFAP can be seen in figure 1.1.

While GFAP is the major intermediate filament in mature astrocytes, in the developing brain, vimentin is the predominant IF type (Dahl 1981). The progression of maturation sees a shift in the prevailing IF to GFAP (Dahl 1994). A plateau in GFAP levels can then be observed in the brains of rodents during early to mid adult life, following which both GFAP mRNA and protein levels are greatly increased. These changes have been observed both in the hippocampus, striatum and cortex of both ageing rodents and humans (Goss *et al.* 1991; Nichols *et al.* 1993). More recently Sabbatini *et al.* (1999) investigated age related changes in GFAP immunoreactivity (GFAP-IR) in the cerebellar cortex of young (3 months), adult (12 months) and aged (24 months) rats using immunohistochemical staining. It was found that GFAP-IR decreases with age in the white matter of the cerebral cortex whereas in the granular layer GFAP-IR increases with age. It was suggested that these findings indicate differential regional sensitivities of cerebellar astrocytes to aging (Sabbatini *et al.* 1999).

1.5.2 Functions of GFAP.

The functions of individual intermediate filaments remain under much investigation yet the generally regarded function is to provide mechanical stability to cells and tissues. The structural diversity of intermediate filaments suggests, however, that additional variable functions could be attributed to individual IF in different cells (Steinert *et al.* 1984). The role of the GFAP IF in glial cells in particular has been subject to intensive investigation; as with the IF protein types in other cells, the principle role is thought to be in providing structural stability to glial cells and their processes by

N-terminal head   C-terminal tail
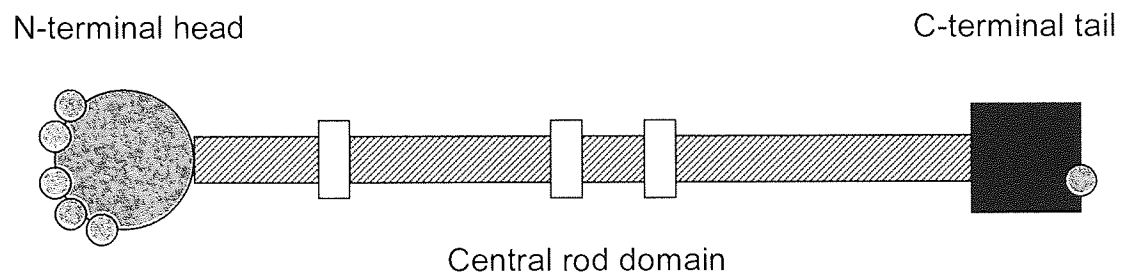
Central rod domain

Figure 1.1 Schematic diagram of the GFAP intermediate filament monomer. The GFAP, like other intermediate filaments consists of three main portions, the alpha helical central rod domain, the amino N-terminal head and the carboxyl C- terminal tail portion. The central rod domain which is highly conserved between intermediate filaments comprises of four main sections of heptad repeats, separated by three non-helical segments. The globular N-terminal domain of GFAP has 5 phosphorylation sites that play a key role in the assembly of the protein. Depending on the species, the globular C-terminal domain may also have a single phosphorylation site. Adapted from Rodnight et al (1997).

maintaining the integrity of the cytoskeleton. This is achieved through interactions between GFAP and both the nuclear and plasma membranes to stabilise the cytoskeleton of the cell (Goldman *et al.* 1990). These interactions are mediated by plectin, a 300kDa intermediate filament associated protein (IFAP) which links the GFAP filaments to the nuclear membrane by interacting with laminin B and to the plasma membrane by interactions with the actin microfilamentous system possibly through actin binding proteins (Seifert *et al.* 1992; for review see Rutka *et al.* 1997). The associations of GFAP with the plasma membrane via actin filaments and actin binding proteins, which in turn are associated with integrins responsible for extracellular adhesion, has led to suggestions of a role for GFAP in cell adhesion and the contact regulation of cell morphology (Rutka *et al.* 1994). GFAP is also involved in astrocyte mitosis. Certain cyclin-dependent protein kinases including protein kinase C, Ca^{2+}/calmodulin dependent protein kinase II, and cdc2 control the assembly/disassembly of the GFAP intermediate filaments by phosphorylation (Inagaki *et al.* 1994), modulating the remodelling of the cytoskeletal framework. GFAP has a number of phosphorylation sites at its N-terminal head domain that are thought to be responsible for GFAP filament formation (Inagaki *et al.* 1994). Work conducted by Takemura *et al.* (2002) using substitution mutant mice showed that blocking these phosphorylation sites in different combinations, resulted in differential astrocytic cell phenotypes. It was suggested that "each of the phosphorylation sites has a distinct impact on the dynamics of GFAP" and consequently astrocyte function (Takemura *et al.* 2002). Recent research has also implicated GFAP in exocytotic vesicle trafficking between astrocytes and neurons, as illustrated by Potokar (2007) where isolated cortical mouse astrocytes, deficient in GFAP, showed a reduction in directional mobility of vesicles. Extensive studies using GFAP-null mice has also alluded to a role of GFAP in the maintenance of the blood brain barrier (Pekny *et al.* 1998), the formation and maintenance of myelin (Liedtke *et al.* 1996) and in synaptic transmission (Shibuki *et al.* 1996; McCall *et al.* 1996). Weinstein *et al.* (1991) also found that process outgrowth in response to neurons in culture was inhibited in U251 human astrocytoma cells transfected with antisense GFAP constructs. Similarly Liedtke *et al.* (1996) found that the short and club-like processes of GFAP deficient astrocytes created insufficient contact between oligodendrocytes and the myelin sheaths. This implies that GFAP is necessary to maintain the integrity of astrocytic distal processes that are closely associated with neuronal cells and other glial cell types. Little is known about the exact function of GFAP in reactive astrocytes. It is thought that during reactive gliosis, as the cell undergoes hypertrophy, GFAP is an essential element in the modulation of glial scar

formation, the regulation of astrocyte permissivity and hence the obstruction of axonal regeneration following brain injury (Menet *et al.* 2001).

1.5.3 GFAP induction.

A variety of factors have been implicated in the regulation of GFAP gene expression and up-regulation of levels of the protein in astrocytes during astrocyte activation. Cytokines and growth factors such as interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), transforming growth factor beta (TGF-β) and basic fibroblast growth factor (bFGF) have been proposed as mediators of astrogliosis *in vivo*, each having been shown to induce increases in GFAP mRNA or protein following injection into the cortex of rodents. (Giulian *et al.* 1988; Balasingam *et al.* 1994; Laping *et al.* 1994b; Eclancher *et al.* 1990)

It has also been proposed that reactive oxygen intermediates such as nitric oxide (NO) may have important roles as inflammatory mediators and in the up-regulation of GFAP. Studies by Brahmachari *et al.* (2006) found that the induction of GFAP in mouse primary astrocytes exposed to bacterial LPS was nitric oxide dependent. Other inducers of NO and NO donors were also shown to cause elevations in GFAP expression, the effects of which could be blocked by NO scavengers or inhibitors of inducible nitric oxide synthase (iNOS).

It is apparent that there are multiple pathways of GFAP induction, whereby various mediators can induce or activate one of a number of transcription factors including the cAMP response element-binding protein (CRE), nuclear factor 1 (NF-1), nuclear factor kappa B (NFκB), activator protein 1 (AP-1) and activator protein 2 (AP-2). These transcription factors then bind to their specific binding sites or response elements on the promoter sequence of the GFAP gene to regulate GFAP mRNA expression (Laping *et al.* 1994). Interleukin signalling, for example, can cause an increase in levels of c-jun and c-fos early response genes which combine to make up the AP-1 transcription factor that binds to the AP-1 binding site on the GFAP promoter (Rutka *et al.* 1997). Nuclear factor-kappa B (NF-κB) has also been proposed as an important intracellular transcription factor in the induction of GFAP. Aspirin, which is a known inhibitor of NF-κB activity, has been shown to cause a reduction in GFAP protein and mRNA levels in the human astrocytoma A172 cell line and primary human astrocytes (Bae *et al.* 2006). Similarly, Brambilla *et al.*

(2005) showed that selective inhibition of NF-κB signalling also resulted in a reduction in pro-inflammatory cytokines and other factors involved in glial scar formation following spinal cord injury in mice. NF-κB is a "rapid acting" primary transcription factor that is present in the cell in its active state. The activation of NF-κB is normally sequestered by the association with an inhibitor of NF-κB (IκB). Activation involves the dissociation of IκB, leaving NF-κB free to translocate to the nucleus where it binds to its specific binding site on the GFAP gene promoter. There are many factors that can signal NF-κB activation including reactive oxygen species and free radicals (Kaltschmidt *et al.* 1999; Kim *et al.* 1999) as well as various cytokines, growth factors, pathogenic antigens, hormones and chemical exposure (Li and Stark 2002).

It is clear that the regulation of GFAP is a complex process that depends upon the action of several mediators, transcription factors and response elements on the GFAP gene. It has been inferred that these multiple regulators "may be integrated to optimise transcription according to local conditions and systemic hormones" (Laping *et al.* 1994). The extent of such integration and the exact pathways of GFAP induction by certain stimuli, particularly cellular and environmental changes caused by toxicant exposure, are still under investigation.

1.5.4 GFAP as a toxicity marker.

Following the discovery of GFAP, the majority of studies have focussed on the response of astrocytes and the dynamics of the protein following physical injury such as stab wounds or disease induced lesions *in vivo* (Cavanagh 1970; Latov *et al.* 1979; Malhotra *et al.* 1989). The use of GFAP as a marker for toxic effects in the CNS following chemical exposure was originally promoted by the work of O'Callaghan (1991a) where several conclusions were made regarding the response of GFAP following toxicant exposure *in vivo*. Firstly, it was observed that various toxicants have dose, time and region dependent effects on GFAP. Both neuropathic and non-neuropathic toxicants cause large increases in GFAP indicating that assays for GFAP should be "sensitive enough to reveal neurotoxicity in the absence of cytopathology" (O'Callaghan 1991a). Secondly, it was noted that increases in GFAP following toxicant exposure were not necessarily permanent but that "large increases in GFAP caused by both trimethyltin and MPTP abate with time to nearly control levels" and that "This decline in GFAP levels, however, differs markedly depending on the toxicant in question" (O'Callaghan 1991a). It was also found that

astrocytes typically respond by hypertrophy rather than hyperplasia, to toxicant exposure suggesting that observed increases in GFAP are not attributable to an increase in cell number. In respect to these findings, the glial fibrillary acidic protein was proposed as a valuable sensitive and specific biomarker in the analysis of neurotoxicology.

Many studies regarding the use of GFAP as such a biomarker have been focused on responses *in vivo* where toxicants were administered to experimental animals (typically rodent), then subsequently followed by the quantification of GFAP in the dissected brain (Brock and O'Callaghan 1987; Franke *et al.* 1997; Lam *et al.* 2000). While *in vivo* exposure has benefits of a multi-cellular response and the ability to distinguish region specific effects, it is not possible to determine whether the toxins act to increase GFAP by direct action on astrocytes or indirectly by signalling from injured neurons. As described earlier in this introduction, the use of sentient animals in toxicity assessment has many associated limitations including high cost, difficulties in extrapolation to humans, and ethical considerations. Such procedures are also not easily applicable to a high throughput assay procedure for toxicity screening.

Toxicant induced effects on GFAP have also been investigated in pure astrocyte cultures *in vitro*. Cookson and Pentreath (1994) and Cookson *et al.* (1994) investigated the effects of a battery of toxicants on the elevation of GFAP levels in rat primary astrocytes, successfully distinguishing the specific astrotoxicants from neurotoxicants. Mead and Pentreath (1998a) then went on to include the C6 rat glioma cell line in similar investigations. Whilst activation effects were inferred, these were not illustrated in the paper, which was focused primarily on results from the Neutral Red assay. Toimela and Tahti (1995) also used rat primary cerebellar astrocyte cultures to test the effects of mercuric chloride, methylmercury chloride and aluminium chloride on GFAP expression, each of which caused an increase in levels of the protein.

The above studies have largely focused on the use of animal sourced primary cells. As previously discussed, primary cells are not largely suited to high throughput assays and instead, cell lines may have several advantages for such applications providing that a reactive response can be achieved in the homotypic cell lines. Malhotra *et al.* (1995) proposed the rat 9L glioma cell line as a model for astrogliosis by investigating GFAP increases in response to mechanical trauma (scratching the monolayer with a plastic pipette

tip). It was concluded from positive results that the 9L astrocytic cell line was able to exhibit a reactive response in the absence of other cell types following mechanical trauma. Malhotra *et al.* (1997) then went on to investigate these effects in response to cadmium chloride exposure as well as combined chemical exposure and mechanical injury conditions. It was found that cadmium chloride could induce increases in GFAP immunoreactivity in the astrocytic cell line in the absence of other cell types normally found in the brain. This suggests that other toxicants may also be able to induce similar effects in GFAP in other astrocytic cell lines.

The vast majority of the previously used cell lines have been of non-human origin, usually sourced from the rat. In fact, most studies investigating the functions, structures and induction of GFAP have been conducted using non-human animal models. As previously discussed, animal cell lines may not be ideal to represent the human condition as closely as human cell lines. The use of human derived cells may simplify interpretation in such systems where extrapolation is a major consideration. To the author's knowledge there has been only a single study where human astrocytic cell lines were used to assess reactive gliosis associated elevations in GFAP by Pentreath and Mead (2004). This was to investigate the effects of amyloid β-peptide to model Alzheimer's disease using the human 1321N1 astrocytoma cell line. The effects of toxicant exposure, however, have not been investigated in human astrocytic cell lines and consequently there is scope for the potential development of such reactivity models using human cell lines.

As a consequence of the limited use of human cell lines in such assays there has been no comparison of the suitability of individual cell lines for the role. As individual cell lines of the same cell type can differ greatly in both phenotype and genotype it is worth assuming that their individual responses to toxicant exposure may also differ. There is always the possibility that effects observed by Malhotra *et al.* (1997) in the rat 9L cells may be unique to that line and may not occur in homotypic cultures of human astrocytic cell lines. Only further investigations in several human astrocytic cultures will determine whether this is the case or whether a variety of astrocytic cell lines can exhibit characteristics of a reactive response.

In summary, in order to permit the high through put and cost effective screening of potential toxicants, it is necessary to utilise endpoints with which their cellular specificity

can be distinguished. Reactive gliosis is an astrocyte specific event that occurs at very low levels of toxic exposure, below those that cause any discernable cytotoxicity. Determining the incidence of reactive gliosis in toxicant exposed astrocytic cells by assessing for the characteristic up-regulation of the glial fibrillary acidic protein associated with the response, would allow for the identification of specific astrotoxins. It would therefore be of benefit to investigate this response in human astrocytoma cell lines that are easily manipulated to potentially achieve a high-throughput, cost effective assay that is equally sensitive and applicable to the human condition.

1.6 Aims and objectives.

1.6.1 Primary rationale.

Determine whether the employment of various toxicant stimuli is able to induce a reproducible response in human astrocytoma cell lines which mimics reactive gliosis. Such a response system would be essential for the future design of a neuronal/astrocytic model where cell–specific endpoints could be studied under toxic pressure.

1.6.2 Specific objectives.

1. Identify candidate cell lines for investigation.

2. Identify appropriate toxicants that have been used previously to induce reactivity and increases in GFAP in previous *in vivo* and *in vitro* models.

3. Determine the relative cytotoxicity of these compounds in each of the cell lines and compare the relative sensitivities of astrocytic and neuronal cells to these toxicants.

4. Use the cytotoxicity data to establish appropriate exposure ranges for GFAP assays and as reference for interpreting non-cytotoxic effects.

5. Using the existing literature, identify an appropriate method for the quantification of GFAP levels in the astrocytoma cell lines following toxicant exposure that permits concurrent control assays.

6. Determine the effects of each toxicant on GFAP levels following toxicant exposure and relate these effects to coinciding metabolic assays and cell number controls.

7. Begin to investigate the relevance of exposure time on the incidence and onset of reactive changes in these astrocytic cells.

8. Carry out preliminary investigations into other aspects of astrocyte reactivity following exposure to the same toxicants.

It should be possible to conclude from these studies whether events typical of reactive astrocytes *in vivo* or in primary culture are similarly observed in human astrocytic cell lines following toxicant exposures. It should then be possible to determine the sensitivity of GFAP as a marker of low level toxicity compared to standard cytotoxicity assays and also to determine the suitability of each cell line for further inclusion in reactivity investigations.

Chapter 2: Cell characterisation and differentiation.

2.1 Introduction.

Choosing a suitable cellular vehicle is one of the first steps in the development of any *in vitro* toxicity model. As mentioned in Chapter 1, cell lines have many advantages when applied to screening techniques including the capacity to provide high throughput, cost effective and reliable models. As such, neuroblastoma and glioma cell lines have been widely used to model neuronal and astrocyte functions in toxicity studies, although to date these cells have almost exclusively been derived from non-human sources.

The establishment of glial fibrillary acidic protein (GFAP) expression is a prerequisite for the selection of appropriate glioma cell lines for the investigations into the effects of chemical injury on the expression of this marker. The level at which some glioma cells express GFAP is largely dependent upon their state of differentiation, as increasing astrocytic malignancy is associated with a progressive loss in GFAP expression in both glioma tumour cells and cell lines (Cairncross *et al.* 1982; Van der Meulen *et al.* 1978; Duffy *et al.* 1982; Jacques *et al.* 1981; Rutka *et al.* 1994). Stable transfection of the normally GFAP negative human SF-126 astrocytoma cell line with the GFAP gene results in a change in morphology, with cells exhibiting more astrocytic characteristics (Rutka and Smith 1993). The C6 rat glioma cell line has also been used as an astrocyte model of gliosis (Mead and Pentreath 1998), however, this line exhibits relatively low basal levels of GFAP (Cravioto 1986) and requires pre-treatment with dibutyryl cyclic AMP (dBcAMP) in order to induce a more differentiated state from which an astrogliosis-like response can be induced (Cookson *et al.* 1995). Another non-human glioma cell line, the rat glioma 9L line, has also been used to study the reactive response following sub-lethal injury from both physical (Malhotra *et al.* 1995) and chemical insult (Malhotra *et al.* 1997), although the problem of species dependent toxicity may again limit applicability in regard to the human response to a particular toxicant.

The lengthy process of treatment required by some cell lines to induce GFAP expression, as well as the predominant use of non-human cell lines obviates the need to find alternative human derived cell lines that constitutively express GFAP. There are a number of GFAP expressing human glioma cell lines, for example the U251-MG, U373-

MG and CCF-STTG1 human astrocytoma lines which have all repeatedly been shown to be positive for the protein and these lines have been used as models for astrocytic function in several contexts (Westermark 1973; Tlhyama *et al.* 1993; Wang *et al.* 1984; Barna *et al.* 1985).

The U251-MG human astrocytoma cell line was originally derived from a temporal lobe glioblastoma of a 62 year old male (Mark *et al.* 1977) and has since been used as an *in vitro* astrocyte model in neuroprotection studies (Weinstein *et al.* 1991) as well as specifically for the study of GFAP and its regulation (Rutka *et al.* 1998). Similarly, the human astrocytoma cell line U373-MG has also been used for the investigation of GFAP expression in relation to glial tumour behaviour (Murphy *et al.* 1998). This latter line was initially isolated from a grade III glioblastoma of a 61-year-old Caucasian male and has been used for the study of cytokines and their induction (Palma *et al.* 1995). According to Cairncross *et al.* (1982), both the U251-MG and U373-MG cell lines exhibit patterns of protein expression characteristic of the most differentiated glioma cell lines, including high levels of GFAP expression. The third cell line to be used is the CCF-STTG1 human astrocytoma cell line, established from a specimen of Grade IV astrocytoma from a 68-year-old Caucasian female. GFAP has been documented to be present in 70-80% of these cells in culture (Barna *et al.* 1985). The line has been used previously as an astrocyte model to investigate the effects of essential oils on heat shock induced apoptosis (Koo *et al.* 2001; Koo *et al.* 2002; Na *et al.* 2001) and also as a model to study apolipoprotein E production in astrocytes and its implications in Alzheimer's disease (Krul and Tang 1992; Starck *et al.* 2000; Gueguen *et al.* 2001).

Despite the wide use of each of these cell lines, to the author's knowledge there is no data regarding their use in the investigation of GFAP following chemical injury, consequently, following confirmation of GFAP expression by western blot analysis, the three cell lines may provide valuable human derived alternatives to existing animal cell models currently used for this purpose.

In order to allow for the comparison of cytotoxicity between cells of astrocytic and neuronal origin in relation to each of the test compounds, the human SH-SY5Y neuroblastoma cell line has been chosen for analysis. This cell line, which is one of the most widely used lines for the investigation of *in vitro* human neuronal toxicity, is a thrice

cloned sub line of the neuroepithelioma (peripheral neuroblastoma) cell line SK-N-SH which was originally established in 1970 from the bone marrow biopsy of a four year old girl with metastatic neuroblastoma (Biedler *et al.* 1973). The SH-SY5Y sub line is characterised as having a neuroblast-like morphology (Biedler 1978), exhibiting neuronal properties including positive staining for the neuron specific enolase (NSE) (Odelstad *et al.* 1981).

The SH-SY5Y neuroblastoma cell line is commonly differentiated by treatment with the vitamin A derivative retinoic acid (RA), to achieve a more neuronal phenotype. Following retinoic acid treatment SH-SY5Y cells are withdrawn from the cell cycle, extend neuronal-like processes and express many typical neuronal markers including the increased expression of the neuronal specific enolase (Pahlman 1984). The differentiating potential of retinoic acid was originally of interest regarding the application of such differentiation inducers in the therapy of neuroblastoma. The compound has since been used to induce the appearance of more neuronal characteristics in neuroblastoma cell lines for use as *in vitro* neuronal models for a wide range of applications including neuroblastoma therapy (Voigt 2003) and research into Alzheimer's disease (Jamsa *et al.* 2004). Both differentiated and undifferentiated SH-SY5Y cells have also been used widely in toxicological studies (Sanfeliu 1999; Hartley 1997; Hong *et al.* 2003). Utilising both would allow for the determination of the effects of neuroblastoma differentiation on the differential cytotoxicity of the tested compounds as well as providing an additional point of comparison with the astrocytoma cell lines.

Several methods for retinoic acid induced SH-SY5Y differentiation currently exist. There are variations in the period of retinoic acid treatment from two days (Voigt 2003) to fourteen days (Hartley 1997) as well as post treatments with various neurotrophic agents including nerve growth factor (NGF) (Hong *et al.* 2003) and brain derived neurotrophic factor (BDNF) (Encinas *et al.* 2000). This particular method developed by Encinas *et al.* (2000) involved the sequential treatment of SH-SY5Y cells with retinoic acid and BDNF in serum free medium. This treatment resulted in a homogenous population of cells with neuronal morphology which were withdrawn from the cell cycle and which expressed neuronal markers including neurofilaments, NSE and growth-associated protein-43 (GAP-43). Due to the ability of this method to yield such well-characterised, differentiated neuronal populations, it is the chosen method for SH-SY5Y differentiation in this study. In

addition to Encinas (2000), several other studies have reported effects of induced differentiation on the up-regulation of the neuron specific enolase protein (Marangos *et al.* 1978; Pahlman 1984). This common marker for neuronal differentiation will be assessed in this study in order to observe the differentiation state through the treatment process.

In summary, by confirming the GFAP positive phenotype of the chosen astrocytoma cell lines as well as establishing both undifferentiated and differentiated neuroblastoma populations using established methods, it should ultimately be possible to confirm these lines as suitable cellular models for cytotoxicity and sub-cytotoxic astrotoxicity analysis.

2.2 Methods.

2.2.1 Chemicals, reagents and tissue culture materials.

All cell culture reagents were purchased from Gibco Invitrogen UK unless stated otherwise. All other chemicals and reagents were purchased from Sigma, UK unless otherwise stated. All tissue culture plastics were from Corning, UK.

2.2.2 Preparation, maintenance and storage of cultures.

The CCF-STTG1 human astrocytoma cell line was purchased from the European Collection of Cell Cultures (ECACC, #90021502, Salisbury, UK). The U373-MG and U251-MG human astrocytoma cell lines were both obtained from the University of Wolverhampton, UK and the SH-SY5Y human neuroblastoma cell line was kindly supplied by Dr Melissa Grant at Aston University, UK.

The U373-MG cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) with Glutamax^{TM} supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1% Penicillin/Streptomycin (PenStrep) antibiotic solution (Sigma). U251-MG cells were maintained in Hams F10 medium with 25 mM HEPES, supplemented with 10% heat-inactivated FBS, 2 mM L-Glutamine and 1% PenStrep solution. CCF-STTG1 cells were maintained in RPMI 1640 medium supplemented with 10% non-heat inactivated FBS, 2 mM L-Glutamine and 1% PenStrep solution. SH-SY5Y cells were maintained in RPMI 1640 medium supplemented with 10% non-heat inactivated FBS, 2 mM L-Glutamine, 0.1 mM MEM non-essential amino acids (Gibco) and 1 % PenStrep solution. All cells were maintained by incubation at 37 °C in a humidified atmosphere of 5 % CO_2.

When approximately 80-90 % confluent, cells were passaged by trypsinisation with 0.25 % Trypsin, 0.1 mM EDTA for two minutes. Cells were pelleted by centrifugation at 179g for 5 minutes, then re-suspended in fresh medium (to the dilution required) and transferred to clean culture flasks. In order to determine the number of cells in suspension, cells were counted using the Trypan Blue exclusion technique. Briefly, an aliquot of cells was diluted 50:50 in trypan blue dye and the number of cells counted using a haemocytometer microscope slide. The viable cell count (those which were able to exclude the Trypan Blue dye) was multiplied by two to correct for the dilution with trypan blue

solution and then multiplied by 10^4 to determine the cell number per millilitre of suspension. For storage, all cells were kept below -70°C in the liquid phase of nitrogen. In order to do so, cells were initially washed with 10 ml of fresh medium then treated with 10 ml of 0.25% Trypsin, 1 mM EDTA solution for two minutes until detached from the culture vessel. The trypsin was denatured by the addition of 10 ml of fresh media to the cell suspension and the cells pelleted by centrifugation at 179g for 5 minutes. The cell pellet was resuspended in 1 ml of freezing medium (90% FBS, 10% Dimethyl Sulfoxide (DMSO)) and transferred to a 1 ml cryovial. Cells were cooled to -70 °C overnight before storage in liquid nitrogen. To resuscitate frozen cells, cryovials were removed from liquid nitrogen and allowed to stand at room temperature for one minute before being placed in waterbath at 37°C. Once thawed the cell suspension was transferred directly to a 25cm^2 cell culture flask containing 10ml of pre-warmed medium. After cell attachment the medium was replaced to remove any traces of DMSO.

2.2.3. Differentiation of SH-SY5Y cell line.

SH-SY5Y human neuroblastoma cells were differentiated according to the method outlined by Encinas *et al.* (2000). Briefly, cells were seeded in culture flasks at 10^6 cells per 25 cm^2 flask and allowed to attach overnight. The media was removed and fresh media containing 10 µM of pre-filtered all-*trans*-retinoic acid was added to the cells. Culture flasks were incubated for five days, changing the media every two days. The retinoic acid medium was then aspirated and the monolayer washed with fresh RPMI 1640 without FBS. Cells were then incubated in serum free medium supplemented with 50 ng/ml pre-filtered human BDNF (Sigma) for 5 days. Cells at various stages of differentiation were either used for protein analysis to determine levels of neuron specific enolase or photographed under phase contrast microscopy using a Nikon Coolpix995 digital camera.

2.2.4 Protein analysis.
2.2.4.1 Protein lysate preparation.

Cells were initially washed in 10ml of PBS, pH7.2 (Gibco) and collected by scraping in 10ml fresh PBS. Cells were then pelleted by centrifugation at 179g for 5 minutes and re-suspended in a further 1ml of PBS and similarly washed twice more. The cell pellet was dissolved on ice in approximately 200µl RIPA buffer (100mM Tris-HCL pH8, 100mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 1 protease inhibitor

cocktail tablet (Roche), and then samples were centrifuged at 15000g for 20 minutes at 4°C to remove any insoluble cellular debris. The cytosolic fraction of the lysate (supernatant) was collected for protein concentration assay and SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.4.2 Protein concentration determination by BCA assay.

Protein concentrations of each lysate were estimated using the colorimetric bicinchoninic acid (BCA) assay (Pierce) according to the manufacturer's guidelines. Briefly, 25µl of samples and pre-diluted bovine serum albumin (BSA) standards (125-2000 µg/ml) were mixed with 200µl of working reagent (50 parts Reagent A to 1 part Reagent B) in triplicate wells of a microplate (Corning). Plates were incubated at 37°C for 30 minutes, then allowed to cool to room temperature before reading the absorbance at 562nm using the Thermo Multiskan EX 96-well microplate reader (Thermo Electron Corporation). Protein concentrations of each sample were determined using the BSA standard curve (see Appendix 1).

2.2.4.3 SDS-PAGE.

Polyacrylamide gel casting and electrophoresis was performed using the TV100 Mini Vertical Gel Unit and Casting System (Scie-Plas) according to the manufacturer's guidelines. Samples were prepared by mixing with an equal volume of double strength sample buffer (deionised H_2O, 12.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-Mercapto-ethanol and 0.1% bromophenol blue) and heated for 5 minutes at 95°C. Samples were then cooled and centrifuged at 179g for 10 seconds to remove any particulates. Protein aliquots were loaded onto the gel alongside high-range rainbow molecular weight markers (Amersham). In initial experiments, 15ng of recombinant human GFAP protein (Calbiochem Cat# 345996) was also loaded onto the gel as a positive control. Gels were electrophoresed in running buffer pH 8.3 (25mM Tris, 192mM glycine, 0.1% SDS) at 180V for approximately fifty minutes until the bromophenol blue had reached the bottom of the resolving gel. Gels were then carefully removed from the electrophoresis unit ready for protein transfer to nitrocellulose.

2.2.4.4 Western blot transfer.

Following the separation of protein by SDS-PAGE (section 2.2.4.3), gels were carefully assembled next to a section of nitrocellulose membrane and which had been

trimmed to the dimensions of the gel and pre-soaked in transfer buffer pH 8.3 (25mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol). The gel and nitrocellulose membrane were then sandwiched between four pieces of Whatman filter paper and two pieces of sponge all pre-soaked in transfer buffer and placed into the transfer cassette (Scie-Plas). Protein gels were transferred using the TV100 system Mini Compact Electro-Blotting Unit (Scie-Plas) at 80 Volts for two hours.

2.2.4.5 Western blot analysis.

Following transfer, membranes were blocked with 5% powdered milk and 0.1% Tween-20 in phosphate buffered saline (PBS tablets from Sigma) for 1 hour at room temperature and then hung to dry overnight. Membranes were wet with 0.5% Tween-20 in PBS for 5 minutes and then incubated in 0.1% Tween-20 in PBS containing primary antibody for 2 hours at room temperature on an oscillating shaker. Mouse anti-GFAP 6F2 monoclonal antibodies (Abcam Cat# ab8975) diluted 1:1000, were used as primary antibody to detect the human glial fibrillary acidic protein epitope. Mouse anti-NSE monoclonal antibodies (Chemicon Cat# MAB324) diluted 1:5000, were used as primary antibody to detect the human neuron specific enolase epitope. Following primary incubation, blots were washed four times in 0.1% Tween in PBS for 15 minutes each wash to remove any residual unbound primary antibody and then incubated with anti-mouse IgG HRP-linked secondary antibody (Cell Signalling Cat# 7076) diluted 1:5000 in 0.1% Tween in PBS for one hour to detect the primary antibodies. Following secondary incubation, blots were washed four times in 0.1% Tween in PBS for 15 minutes each wash to remove any residual unbound secondary antibody. Membranes were placed in ECL^{TM} western blotting chemiluminescent detection reagent (Amersham) for two minutes, then wrapped in cellophane and placed in a developing cassette. Membranes were exposed to ECL Hyperfilm (Amersham) which were then developed and fixed in the darkroom. When appropriate, membranes were re-probed for the housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. To do so, imaged blots were stripped in 0.5% Tween-20 in PBS for 24 hours before re-probing with mouse anti-GAPDH monoclonal antibody (Abcam Cat# ab9484) diluted 1:10000 in 0.1% Tween-20 in PBS, for one hour. The remaining western blot analysis was performed as above with the exception that the anti-mouse immunoglobulin horseradish peroxidise-linked secondary antibody was diluted 1:20000 in 0.1% Tween-20 in PBS.

2.3 Results.

2.3.1 GFAP expression in the human glioma and neuroblastoma cell lines.

To determine the glial fibrillary acidic protein and neuron specific enolase expression of each of the cell lines, equal amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose membrane by Western blotting. The glial fibrillary acidic protein specific antibody revealed bands at approximately 50 kDa molecular weight from lysates of all three glioma cell lines but not from the neuroblastoma line (Figure 2.1) These 50kDa bands corresponded in size with that of human GFAP which was run alongside cell lysates in initial studies as a positive control. Similar levels of immunoreactivity for GFAP were observed for corresponding U251-MG and U373-MG bands when 5μg of protein of each cell type was analysed. However, lysate from the CCF-STTG1 cell line showed a much lower immunoreactivity for the GFAP protein as 5μg of protein was insufficient to produce an observable band. Instead the process was repeated using 25μg of protein, at which point a distinct band could be detected at 50kDa, albeit with a slight degree of non-specific background binding.

2.3.2. SH-SY5Y differentiation.

Under the light microscope, undifferentiated SH-SY5Y cells were highly adherent, flattened cells which exhibited very short neuritic extensions (figure 2.2A) and when differentiated with 10μM retinoic acid for 5 days followed by 5 days treatment with 50ng/ml BDNF in serum free medium, cells appeared to form aggregates which were interconnected by bundles of elongated neurites (figure 2.2B). There was also a decrease in cell proliferation compared to control cells allowed to grow untreated for ten days in culture which became overgrown and started to detach from the culture vessel (data not shown). Evaluation of neuron specific enolase levels by Western blot analysis showed a progressive increase in the immunoreactivity for the protein as the differentiation procedure progressed (figure 2.3). With GAPDH as a loading control reference, there was no obvious change in the intensity of the NSE band following an initial 2 days incubation with retinoic acid although after five days retinoic acid incubation and also after 5 days subsequent incubation in the presence of BDNF in serum free medium, levels of the protein were seen to be elevated.
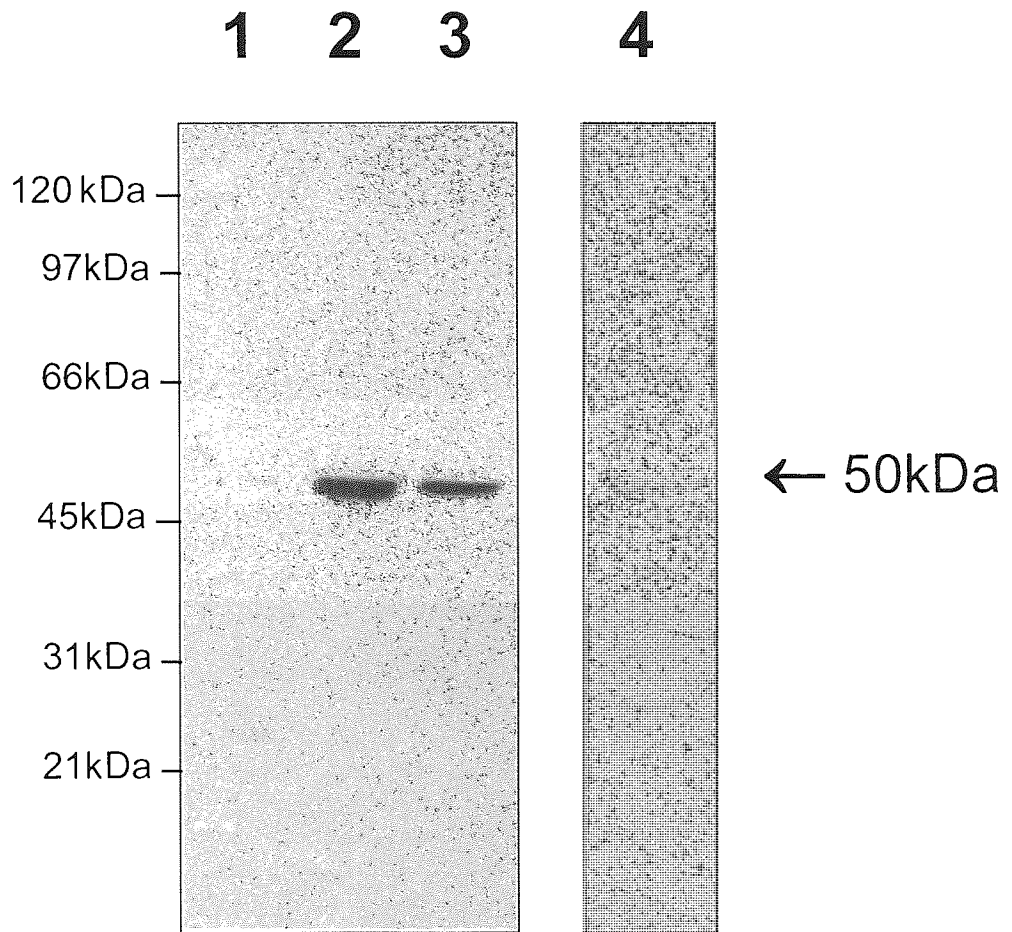
Figure 2.1 GFAP expression in human neuroblastoma and astrocytoma cell lines. Immunoblotting using human anti-GFAP antibody was performed with protein isolated from cell lysates from 1) undifferentiated SH-SY5Y neuroblastoma, 2) U251-MG astrocytoma, 3) U373-MG astrocytoma and 4) CCF-STTG1 astrocytoma cell lines.
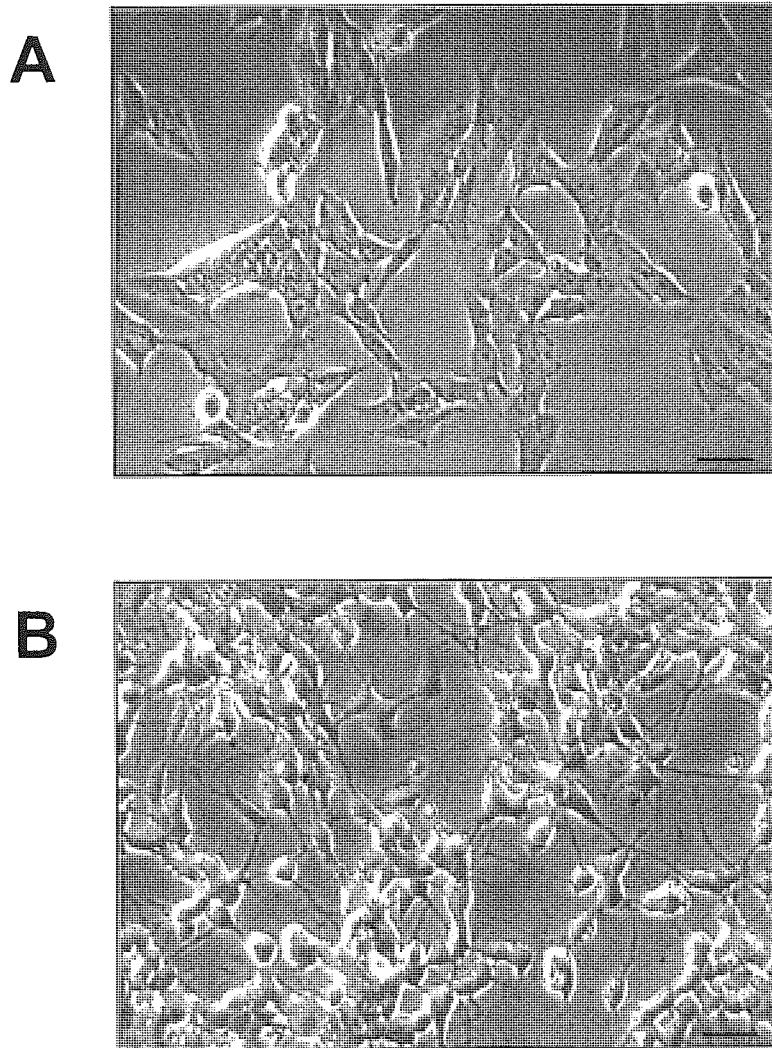
Figure 2.2 Effect of retinoic acid induced differentiation on the cellular morphology of human SH-SY5Y neuroblastoma cells. Phase contrast microscopic images of A) undifferentiated SH-SY5Y cells and B) cells differentiated with $10\mu M$ retinoic acid for 5 days followed by 5 days maintenance in serum free media supplemented with 50ng/ml BDNF. Scale bar = $25\mu m$.
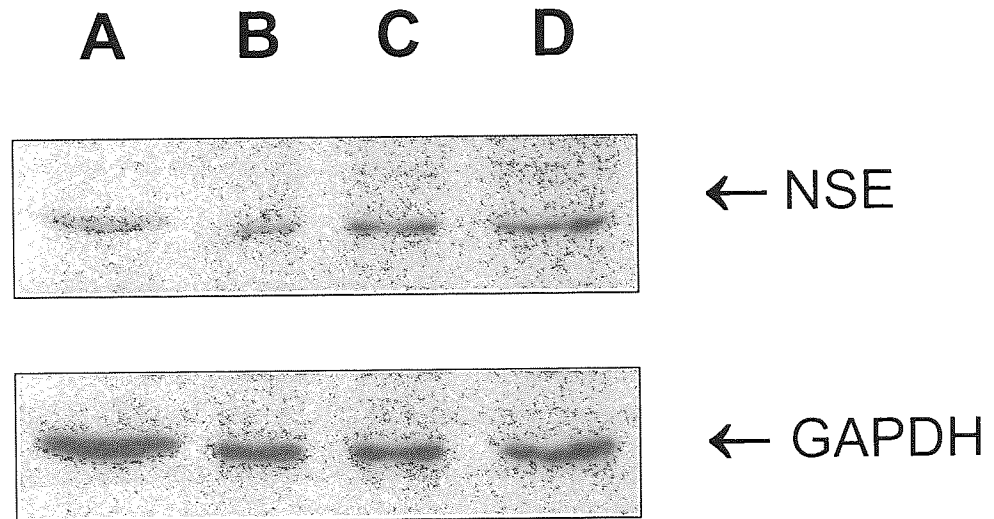
Figure 2.3 Effect of retinoic acid induced differentiation on neuron specific enolase expression of human SH-SY5Y neuroblastoma cells. Immunoblotting using mouse anti-human NSE antibody was performed with equal amounts of protein isolated from A) undifferentiated cells, B) cells treated with $10\mu M$ retinoic acid for 2 days, C) cells treated with $10\mu M$ retinoic acid for 5 days and D) cells treated with $10\mu M$ retinoic acid for 5 days followed by treatment with 50ng/ml BDNF for 5 days. Lysates were similarly probed with anti-GAPDH antibody as a loading control.

2.4 Discussion.

Each of the astrocytoma cell lines used in these studies was found to be positive for GFAP by Western blot analysis. According to the relative band intensities, however, expression levels of the protein were variable between cell lines, specifically the CCF-STTG1 lysate which contained much lower levels of the protein than lysates from the other two lines. It is possible that these cell line specific variations in GFAP content may be due to the level of differentiation of the cell line, as it has been noted that increasing malignancy is inversely proportional to the level of expression of GFAP (Cairncross 1982; Rutka 1997). As such, loss of the protein would be indicative of a reduced astrocytic phenotype.

Cairncross (1982) described three antigens, AJ8, AO10 and GFAP, which in various combinations indicate different levels of differentiation among human glioma lines. That study showed the AO10 antigen was present on GFAP positive tumours whereas tumours positive for the AJ8 antigen were GFAP negative. It was suggested that cultured astrocytomas can be divided into three groups on the basis of the expression of these three antigens. Cultured astrocytomas that are AJ8 negative, AO10 positive and GFAP positive represent more differentiated cell lines whereas AJ8 positive, AO10 negative and GFAP negative variants are the least differentiated cells. Those cell lines that are AJ8 negative, AO10 positive and GFAP negative are in an intermediate differentiation stage. Using this system, Cairncross showed that the U251-MG and U373-MG lines were amongst the most differentiated and bear the closest resemblance to human astrocytes of the many astrocytoma lines tested. This makes them ideal cell lines for application in this study where the benefits of a continuous cell line are coupled with the exhibition of one of the highest states of differentiation among continuous human glioma cell lines. While, to the author's knowledge, no studies have been conducted looking at the level of GFAP expression in the CCF-STTG1 cells compared with other astrocytoma lines, it is possible that the lower level of expression may indicate a phenotype which is less astrocytic than the other two lines used in this study. It would therefore be of added interest to include the CCF-STTG1 line in these studies in order to determine whether initial GFAP expression level has any bearing on the ability of a cell line to display a reactive-like response following injury.

Retinoic acid induced, BDNF supplemented differentiation of the SH-SY5Y human neuroblastoma cell line resulted in the development of a more neuronal morphology including the development of elongated neuritic processes, rounded cells and an increased expression of the neuron specific enolase protein. Neuroblastoma cells have cytoplasmic retinoic acid binding proteins (Seeger *et al.* 1982) via which retinoic acid can induce growth inhibition and morphologic differentiation of human neuroblastoma cells *in vitro* (Sidell 1982). It has been found that treatment of SH-SY5Y cells with retinoic acid induces the expression of TrkB which makes the cells responsive to brain derived neurotrophic factor (Kaplan *et al.* 1993) which has been shown to enhance the effects of retinoic acid differentiation (Arcangeli *et al.* 1999).

Neuroblastomas represent an early stage in neuronal development where cells in an arrested differentiation state can express a variety of phenotypes. Similarly in some neuroblastoma cell lines such as the SH-SY5Y line, there is normally a mix of cell types of either the neuronal-like N-type which are rounded with neuronal like processes, the flat growing, highly adherent S-type cells or the intermediate I-type, all of which are capable of interconversion (Ross *et al.* 1983). Following retinoic acid differentiation, generally, N-type cells become the dominant cell type and develop a more neuronal morphology whereas the S-type cells lose adherence and become apoptotic (Voigt 2003). These changes in cell morphology are represented in this study and may explain the observed reduction in cell proliferation when compared to the undifferentiated control.

The increase in the expression of neuron specific enolase following the differentiation procedure is typical of the changes in levels of the protein as described in previous studies of differentiation. Marangos *et al.* (1978) described how neuronal specific enolase in neuroblastoma cells is expressed in very low levels compared to brain tissue and instead the non-neuronal form of enolase predominates in these lines. It was then described how following differentiation, the enolase profile of the cells was shifted toward the neuronal type. This same pattern of expression upon differentiation has since been reported in the SH-SY5Y line (Odelstad *et al.* 1981). These findings support the notion that the increase in NSE levels as recorded in this chapter together with the observed morphological changes are conjunctional with the development of a more differentiated state.

In conclusion, Western blot analysis for the astrocyte specific glial fibrillary acidic protein determined that all three astrocytoma cell lines tested were positive for the protein making them suitable for use in this investigation. The ability to also achieve a population of SH-SY5Y cells of a more neuronal phenotype by retinoic acid induced differentiation, allows for a second neuronal model along with the undifferentiated line, when comparing the relative cytotoxicity of the chosen compounds in the astrocytic and neuronal-like cells.

Chapter 3: Evaluation of cytotoxicity in human astrocytic and neuronal cell lines.

3.1 Introduction.

In order to determine whether a reactive response, as measured by an increase in glial fibrillary acidic protein (GFAP), is achievable in the three chosen human astrocytic cell lines following toxic insult, it is necessary to choose agents which have previously been shown to induce GFAP expression in astrocytes. This should allow for a suitable comparison. Ethanol, trimethyltin and chloroquine have all been well documented for their ability to induce GFAP both *in vivo* and *in vitro* and have thus been chosen for use in this study. Each of these compounds has been widely investigated regarding their effects in the central nervous system and there exists a large amount of evidence on their physiological actions in humans as a result of recreational, medical or occupational exposure. Some of this evidence is summarised below.

Ethanol is one of the most widely researched agents consumed habitually by humans, primarily due to the worldwide scale of exposure, as well as its known toxicological consequences. Although the reasons for the consumption of ethanol across cultures vary widely, the purpose of the majority of ethanol consumption is for recreational intoxication. The brain is a major target for ethanol-induced toxicity and as such the compound has long been linked with damage to the central nervous system (Lamarche *et al.* 2003). Acute ethanol exposure can result in the depression of areas in the brain responsible for the control of consciousness, breathing and heart rate, potentially leading to coma and death (Brick 2003). Chronic exposure to ethanol can cause damage to the liver, heart and kidneys as well as the degeneration of areas in the brain and the peripheral nervous system, often leading to serious neurological disorders. Prenatal exposure to ethanol can have major implications for foetal brain development. Ethanol exposure during pregnancy has been linked to a variety of birth defects, one of the extremes of which is foetal alcohol syndrome (FAS) (Jones and Smith 1975). Features of the syndrome include craniofacial malformations, growth deficiency and CNS abnormalities including brain malformations and mental, motor and behavioural deficits (Little and Streissguth 1981). Such CNS abnormalities are thought to be irreversible, with effects persisting into adulthood (Streissguth *et al.* 1991). In addition to suggestions that glia may be a primary target of ethanol toxicity during brain development (Guerri *et al.* 2001), ethanol has been

described as having a variety of astrocytic effects in the adult nervous system (Snyder 1992) including the induction of astrocyte reactivity as evaluated by up-regulation of GFAP. This has been observed both *in vivo* (O'Callaghan *et al.* 1995) and *in vitro* (Cookson *et al.* 1994) in animal models, so this agent will be used to determine whether similar effects can be observed in human astrocytoma cell lines.

The second of the chosen toxins, trimethyltin, belongs to a series of synthetic agents known as the organotins, which do not occur naturally in the environment. Trimethyltin in particular is created as a by-product in dimethyltin dichloride production (Rohl and Sievers 2005). The organotins are known to be extremely toxic, particularly to cells of the nervous and immune systems and while each of the organotin compounds has a unique toxicological profile, trimethyltin is regarded as one of the most potently neurotoxic (Gunasekar *et al.* 2001). Poisoning with trimethyltin is generally limited to occupational industrial exposure or through accidental exposure in the laboratory (Saary 2002) which results in the manifestation of an acute neurological syndrome, with symptoms including aggression, confusion, deficits in learning and memory, delirium, tremors, seizures and occasionally death (Besser *et al.* 1987; Feldman *et al.* 1993). Trimethyltin has been shown to cause activation of microglia and astroglia *in vivo* (McCann *et al.* 1996; Brock and O'Callaghan 1987; Fiedorowicz *et al.* 2001), as well as glial activation *in vitro* using animal models (Monnet-Tschudi *et al.* 1995; Figiel and Fiedorowicz 2002). To the knowledge of the author there is no data regarding the effect of the compound on activation responses in human astrocytic cell lines in the scientific literature to date.

The third compound selected for study is chloroquine, a 4-aminoquinoline that is still effective in some areas in the prevention and treatment of malaria. Despite malarial parasite resistance, chloroquine remains one of the most widely used drugs in the world (Foster 1994). Chloroquine also possesses anti-inflammatory properties and is used for the treatment of autoimmune diseases such as rheumatoid arthritis (Fox 1993) and some aspects of the HIV condition (Romanelli *et al.* 2004). Despite its widespread use, chloroquine can be toxic to mammals, particularly after chronic exposure (Zurita *et al.* 2005). Following chronic exposure in humans, a particular target of chloroquine is the retinal pigment epithelium and the neural retina (Bruinink *et al.* 1991) together with other target organs including the heart, adrenal glands and liver (Jaeger *et al.* 1987). Contrary to its use as an anti-inflammatory agent, chloroquine has also been shown to induce a pro-

inflammatory response in several astroglioma cell lines including both the U251 and U373 cell lines used in this study (Park *et al.* 2003) The drug has also been shown to cause an increase in GFAP in primary rat glial cell cultures *in vitro* (Cookson *et al.* 1994).

It is also important to select an agent which is not generally recognised as an astrocytic toxin as a negative, specifically neurotoxic control. Acrylamide is a vinyl monomer formed from the hydration of acrylonitrile (Friedman 2003). The polymer of acrylamide has many industrial and commercial applications, including the manufacture of paper, textile and cosmetic products, as well as in water, soil and sand treatment processes. Acrylamide is also widely used in the making of polyacrylamide gels for the separation of proteins by electrophoresis and other molecular biology applications in laboratory research. The monomer of acrylamide is considered to be a potent neurotoxin whilst conflicting evidence exists as to its genotoxic (Dearfield *et al.* 1995) and carcinogenic (Schultz *et al.* 2001; Marsh *et al.* 1999; Mucci *et al.* 2003) effects. Polymers of acrylamide are generally non-toxic, although degradation of polyacrylamide to acrylamide can occur, particularly upon exposure to light and heat conditions (Smith *et al.* 1996). The incidence of human poisonings has largely been restricted to factory and construction workers chronically exposed to the compound or to laboratory workers in the scientific industries following accidental acute exposure. The formation of acrylamide by the heat treatment of high starch-content foods has also been implicated in widespread dietary acrylamide exposure. This link was first described by Tareke *et al.* (2000) who observed that rats fed on a fried chow diet exhibited elevated levels of the haemoglobin adduct of acrylamide, N-(2-carbamoylethyl)valine, compared with animals fed a non-fried control diet. This fried chow diet was subsequently found to contain higher acrylamide levels than the control diet. The formation of acrylamide in fried foods is thought to be a product of the heating of glucose and amino acids, particularly asparagine (Stadler *et al.* 2002), through a process described by Friedman (2003).

Acute exposure to acrylamide results initially in a symptom-free period of a few hours followed by the progressive onset of symptoms including seizures, hypotension and acute respiratory distress (Donovan and Pearson 1987). Delayed onset of peripheral neuropathy was also observed in these cases. Reported cases of chronic low-level exposure to acrylamide presented symptoms including truncal ataxia, mental confusion, tingling and numbness and peeling of the hands and feet and in most cases, delayed peripheral

neuropathy (Igisu *et al.* 1975; Hagmar *et al.* 2001; He *et al.* 1989). Due to the neurological nature of the symptoms, acrylamide has been widely researched specifically in regards to its neurotoxic mechanisms (Miller and Spencer 1985; LoPachin *et al.* 2002) including investigation using the SH-SY5Y neuroblastoma cell line chosen for use in this study (Hartley *et al.* 1997). There have been various hypotheses for the primary neurodegenerative effect of acrylamide from axonal transport impairment and axonopathy (Spencer and Schaumburg 1978; Sickles *et al.* 1996) to nerve terminal degeneration (LoPachin *et al.* 2002). It has been argued that nerve terminal effects and disruption of nerve terminals are the necessary event for acrylamide neurotoxicity and that axonopathy is of secondary importance and is "conditionally expressed and therefore, is an epiphenomenon related to low-dose, long-term induction of acrylamide neurotoxicity" (LoPachin *et al.* 2002). The compound is widely regarded as a specific neurotoxin, and has previously been used in studies of reactive gliosis as a negative control for the induction of GFAP (Cookson and Pentreath 1994; Cookson *et al.* 1994). As such the compound will be used for the same purpose in this study.

There has been a great deal of research regarding the toxicities of these four compounds in neurons. As such, *in vitro* toxicity assessment largely focuses on the use of neuroblastoma cell lines and other cells of neuronal origin. There is a lack of data regarding the cytotoxic potential of these compounds on human astroglial cell lines such as U251-MG, U373-MG and CCF-STTG1 and consequently, little comparative data exists regarding the cytotoxicity of these compounds on different human neural and astrocytic cell lines. Cytotoxicity assessments using these cell lines may provide evidence of differing specificities of particular toxins towards particular cell types in the human CNS.

The impairment of metabolic function gives an early indication of cellular damage and can be standardised to facilitate comparison of toxicity between different cell lines. One of the most widely used methods for the analysis of metabolic function is the MTT assay, which is a spectrophotometric assay based on the reduction of the tetrazolium salt MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) into a blue formazan product, mainly by the mitochondrial enzyme succinate-dehydrogenase (Slater *et al.* 1963) and this test is a sensitive marker of metabolic function. The amount of formazan produced is proportional to the number of living cells in the culture therefore the assay is used widely to quantify cell survival and proliferation. The MTT assay was originally

developed by Mossman (1983) and has since been modified and adapted (Denizot and Lang 1986; Tada *et al.* 1986; Hansen *et al.* 1989; Carmichael *et al.* 1987). The assay has been widely used in research for a variety of applications and is particularly regarded as an invaluable and convenient method for evaluating the response of a cell population to external factors either in terms of proliferation or cell death. The method is safe and easy to use and the necessary equipment is accessible to most laboratories. The method also allows for low cost, high throughput, processing and so is the method of choice for this study to measure the cytotoxic potential of each of the compounds to be tested.

While cytotoxicity analyses, such as the MTT assay, do not define a specific cellular death mechanism, they are valuable in terms of the information they do provide, particularly the IC_{50} (the concentration shown to cause a 50% inhibitory effect) and NOAEL (the no observed adverse effect level) of a particular compound, both of which facilitate the determination of relative toxicity in several different target cell lines. Due to the limited available data comparing the toxicities of the chosen compounds in both human astroglial and human neuronal cell lines, results achieved will give valuable insight into the cell type specific toxicity of the compounds as well as the applicability of glioma and neuroblastoma cell lines as models of relative toxicity. As discussed, the MTT assay will be performed to establish this IC_{50} and NOAEL data for comparison of cell line sensitivity and also for reference when assessing sub-cytotoxic effects of the compounds on GFAP immunoreactivity.

3.2 Methods.

3.2.1 Chemicals, reagents and tissue culture materials.

Acrylamide, chloroquine diphosphate, trimethyltin chloride and HPLC-grade ethanol were all purchased from Sigma. Cell culture reagents were purchased from Gibco, Invitrogen unless otherwise stated. All other chemicals and reagents were purchased from Sigma unless stated otherwise. All tissue culture plastics were from Corning.

3.2.2 Cell cultures.

Cells were maintained as described in section 2.2.2. When seeding cells for assay, cells were washed with 10 ml of fresh medium and treated with 10ml of 0.25% trypsin EDTA for two minutes until they detached from the culture vessel. 10ml of media was then added to the trypsin /cell suspension and spun at 179g for 5 minutes to pellet the cells. The supernatant was discarded and the pellet resuspended in 10ml of fresh medium. The cell concentration was determined and the suspension diluted accordingly in fresh medium to give the desired seeding concentration. The suspension was then transferred into a sterile reagent reservoir and 100μl pipetted into each appropriate well of a tissue culture treated 96-well plate using a multichannel pipette. Cells were allowed to attach to the plate overnight before use. SH-SY5Y cells were differentiated in tissue culture treated 96-well microplates according to the procedure described in section 2.2.3.

3.2.3 Experimental treatment.

For the determination of cytotoxicity, 96-well plates were seeded at the desired concentration of cells per well in accordance with the results of the cell seeding optimisation experiments (see section 3.4.1). Cells were initially seeded in 100μl of the appropriate cell culture media (see section 2.2.2) and allowed to stabilize overnight in a humid atmosphere at 37°C, 5% CO_2. Media was aspirated from the cells and replaced with 100μl of fresh media containing the test compound at sequential concentrations. Working solutions of each toxin were prepared in the appropriate supplemented culture medium according to the cell line being exposed. Solutions for use with the differentiated SH-SY5Y cell line were prepared in serum free media without BDNF supplementation. U251-MG, U373-MG, CCF-STTG1, differentiated and undifferentiated SH-SY5Y cells were all exposed to acrylamide (100μM to 80mM), ethanol (920μM to 1.84M), chloroquine

diphosphate (1μM to 4mM) or trimethyltin chloride (1μM to 5mM) for 24 hours and assayed for viability using the MTT assay.

3.2.4 Cytotoxicity assay.

Cytotoxicity was established as a measure of cell viability and was determined by the MTT reduction assay. The variant on the basic method used in this study is that of Tada *et al.* (1986). Briefly, following toxin exposure the media was aspirated and 100μl of fresh media and 30μl of 5mg/ml MTT was added to each well and incubated at 37°C, 5% CO_2 for 4 hours. 100μl of 10 % SDS in 0.1 M HCl was added to each well and incubated overnight. The released formazane product was quantified by spectrophotometric analysis using a Thermo Multiskan EX 96-well microplate reader (Thermo Electron Corporation), reading absorbance at 590nm. Appropriate cell free controls were included on each assay plate to determine the absorbance of the reagents alone. These values were then subtracted from all other test values before further analysis. MTT turnover per well was subsequently expressed as a percentage of the non-toxicant treated control wells from the same experiment.

For each cell line the optimal cell seeding concentration was determined by performing the MTT assay on sequential two-fold dilutions of cells from 200 000 to 200 cells per well, including a cell free control. The results were plotted as log cell concentration against MTT turnover as absorbance at 590nm. The optimal cell seeding concentration should fall within the linear portion of the resulting curve and give an absorbance value of approximately 1 by a standard microplate reader. To determine the effect of the SH-SY5Y differentiation procedure on cell proliferation and viability and to determine any change in the optimal seeding density, the MTT assay was performed as above comparing cell populations initially seeded at approximately 500 000 cells per well, before and after the differentiation procedure. Data were expressed as the absorbance at 590nm.

3.2.5 Data and statistical analysis.

Non-linear regression analysis was used to determine the IC_{50} values (± standard error of the mean) from the concentration curves using GraphPad Prism software (Version 3.02). The IC_{50} is defined as the concentration of compound required to cause a 50% inhibition in MTT turnover. Levels of significance for comparisons between IC_{50} values

were determined using one-way ANOVA followed by Tukey's multiple comparison post-test. The NOAEL of each compound for each cell line was determined as the highest concentration tested to have no significant inhibitory effect on MTT turnover. NOAELs for the compounds in each cell line were determined using ANOVA followed by Dunnet's post-test where all data points were compared with a non-treated control. GraphPad Prism software (Version 3.02) was used for this purpose.

3.3 Results.

3.3.1 Calibration of the MTT assay.

MTT turnover increased with increasing cell number (figure 3.1). Optimum seeding densities were determined from the graph as the concentration of cells giving an MTT reading at A_{590} which is the closest to 1.0 and which must also fall within the linear portion of the graph. In the case of the four cell lines used, the U251-MG and CCF-STTG1 showed similar rates of MTT turnover and the optimum seeding densities for each estimated at 50000 cells per well. U373-MG cells exhibited a higher rate of MTT turnover and the optimum seeding density estimated at 20 000 cells per well. The seeding density closest to giving an absorbance reading of 1 for the SH-SY5Y cell line was 100 000 cells per well; however, the seeding density chosen was 50 000 cells per well, which is still within the linear portion of the graph, due to the difficulty in achieving such a high yield in culture for seeding.

The effect of the differentiation procedure on cell proliferation and viability was determined by MTT assay (figure 3.2). The results showed no significant difference between undifferentiated SH-SY5Y and cells seeded at the same density which were subsequently differentiated by treatment with 10μM retinoic acid for 5 days followed by 5 days treatment with 50ng/ml brain derived neurotrophic factor (p>0.05).

3.3.2 Cytotoxicity analysis.

Human astrocytoma and neuroblastoma cell lines were exposed to several concentrations of ethanol, acrylamide, chloroquine diphosphate and trimethyltin chloride for 24 hours. Figures 3.3 to 3.6 show the concentration response curves of each cell line for each of these compounds respectively, measured by MTT reduction. Readings for pH were taken for each compound at each concentration and were found not to deviate notably from that of a non-toxin containing control substrate.

3.3.2.1 Ethanol.

All 5 cell types showed relatively high resistance to ethanol compared to the other toxins. The IC_{50} of ethanol was 312.0 (± 18.94) mM in the U251-MG cell line, 382.6 (± 36.64) mM in the U373-MG cell line and 369.9 (± 31.90) mM in the CCF-STTG1 cell line. For the differentiated and undifferentiated SH-SY5Y cells, IC_{50}s were 372.4 (± 8.66) mM
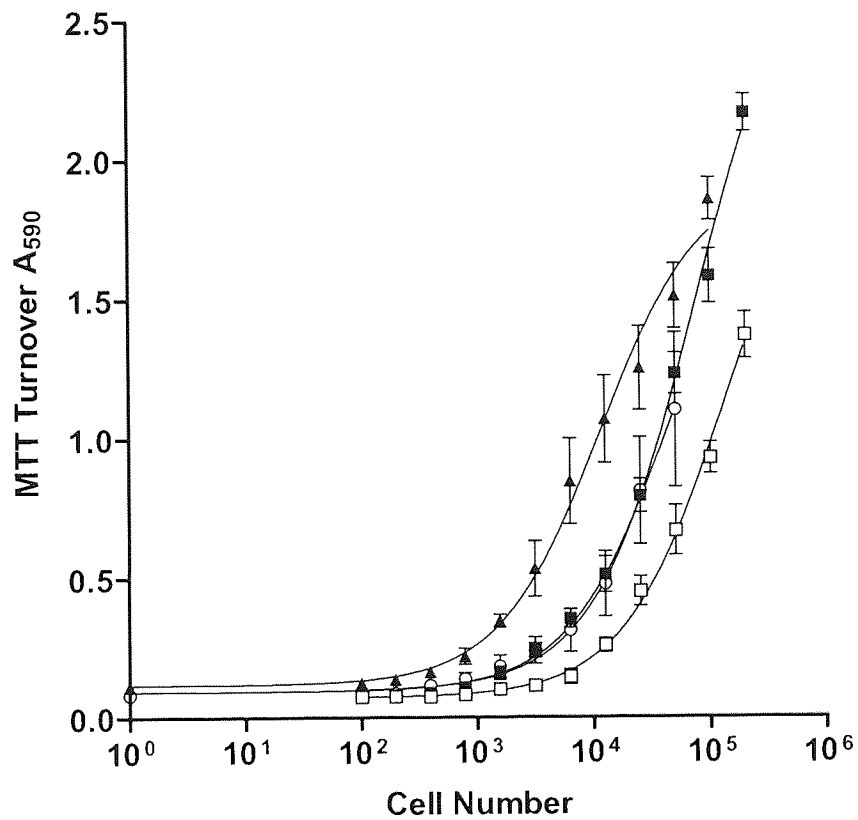
Figure 3.1 Cell seeding calibration of the MTT assay. MTT turnover in SH-SY5Y (□), U373-MG (▲), U251-MG (■) and CCF-STTG1 (○) cell populations of increasing density from 0 to 100 000 cells per well. Data are expressed as mean absorbance at 590nm ± SEM (n=8 from duplicate experiments).
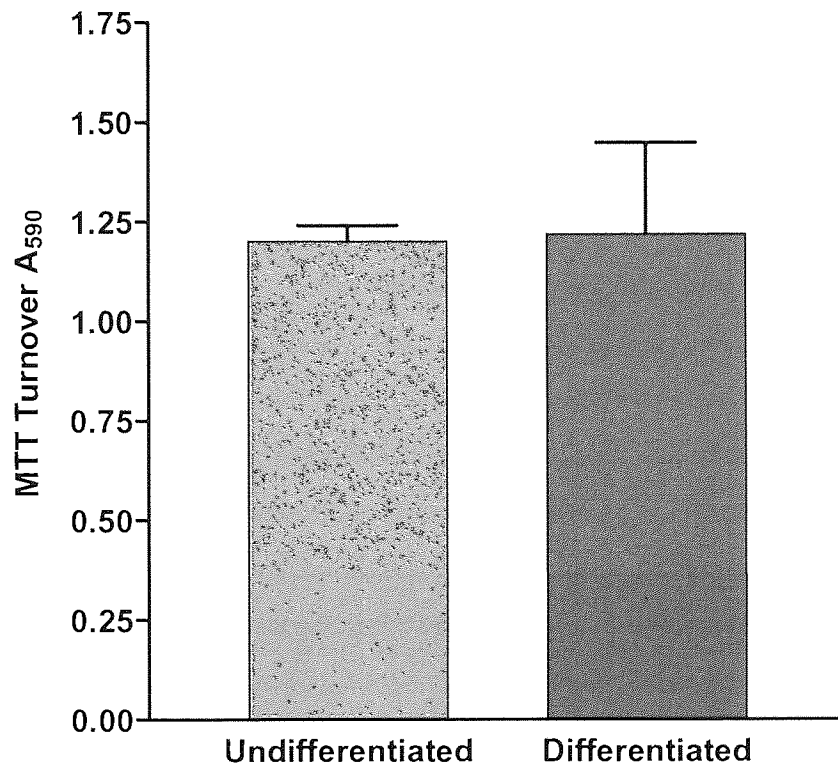
Figure 3.2 Effect of retinoic acid differentiation on SH-SY5Y cell proliferation. MTT turnover in SH-SY5Y cells seeded at 50000 cells per well, before and after retinoic acid differentiation. Data are expressed as mean absorbance at 590nm ± SEM (n=8 from duplicate experiments).

and 277.5 (± 5.65) mM respectively. The undifferentiated SH-SY5Y line was the most sensitive cell line to ethanol toxicity, being significantly more sensitive to ethanol toxicity than the differentiated SH-SY5Y cells ($p<0.05$) and the U373-MG cell line ($p<0.05$) which was the most resistant to ethanol. The NOAEL of ethanol in both the U251-MG and U373-MG glioma cell lines was 92mM, while in the CCF-STTG1 cell line the lowest NOAEL was observed at 9.2mM. In the differentiated SH-SY5Y line, the NOAEL for ethanol was 92mM, while the undifferentiated SH-SY5Ys, which were the most sensitive cells to ethanol according to comparative IC_{50} values, interestingly had the highest NOAEL of 184mM.

3.3.2.2 Acrylamide.

The reduction of MTT turnover by acrylamide in glioma cell lines presented IC_{50}s of 15.14 (± 0.71) mM for U251-MG, 19.16 (± 1.68) mM for U373-MG and 17.36 (± 0.77) mM for the CCF-STTG1 cell line at 24 hours of exposure. The differentiated and undifferentiated SH-SY5Y neuroblastoma cell lines showed greater sensitivity to acrylamide, with IC_{50}s of 9.36 (± 0.17) mM and 5.98 (± 0.28) mM respectively following 24 hours exposure. There was no significant difference between the IC_{50}s for the glioma cell lines when exposed to acrylamide, or between the differentiated and undifferentiated neuroblastoma cell types. The IC_{50} of acrylamide for the undifferentiated and differentiated SH-SY5Y cells were however both significantly lower than the U251-MG ($p<0.001$), U373-MG ($p<0.001$) and CCF-STTG1 ($p<0.001$) glioma cell lines. Despite the neuronal cell lines being most sensitive to acrylamide in terms of IC50 values, the NOAEL of 4mM and 2mM in the differentiated and undifferentiated SH-SY5Y cells respectively were greater than the NOAEL of 1mM in both the U251-MG and U373-MG glioma lines. The NOAEL for acrylamide in the CCF-STTG1 cell line was observed to be between 5 and 10 mM. Following 5mM exposure (the concentration preceding the first significant reduction of MTT turnover in the CCF-STTG1 cell line at 10mM), a significant elevation in turnover to 9.5 (±1.5) percent greater than control values was observed.

3.3.2.3 Chloroquine diphosphate.

The cytotoxic potency of chloroquine diphosphate was similar for the U373-MG, CCF-STTG1, differentiated SH-SY5Y and undifferentiated SH-SY5Y cell lines, with IC_{50} values of 0.169 (± 0.03) mM, 0.224 (± 0.02) mM, 0.255 (± 0.014) mM and 0.109 (± 0.01) mM respectively. Statistical analysis showed there was no significant difference between
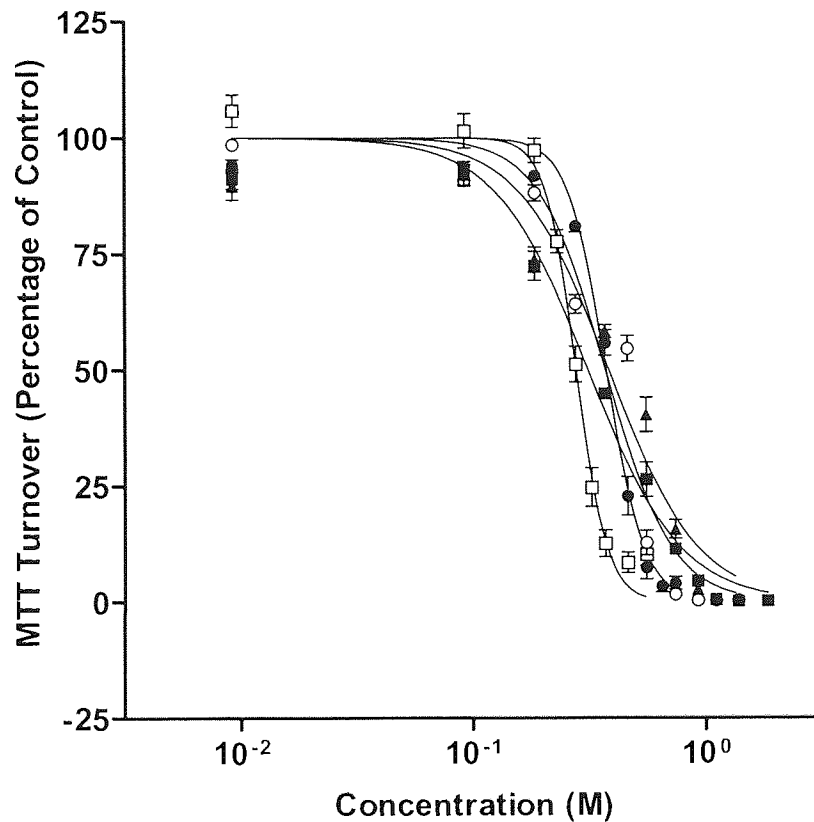
Figure 3.3 The effect of ethanol on MTT turnover in human glioma and neuroblastoma cell lines. Sigmoidal dose response curve (variable slope) in the presence of ethanol for U251-MG (■), U373-MG (▲), CCF-STTG1 (○), SH-SY5Y (□) and retinoic acid differentiated SH-SY5Y (●) cell lines following 24 hours incubation. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=24 from 3 individual experiments).
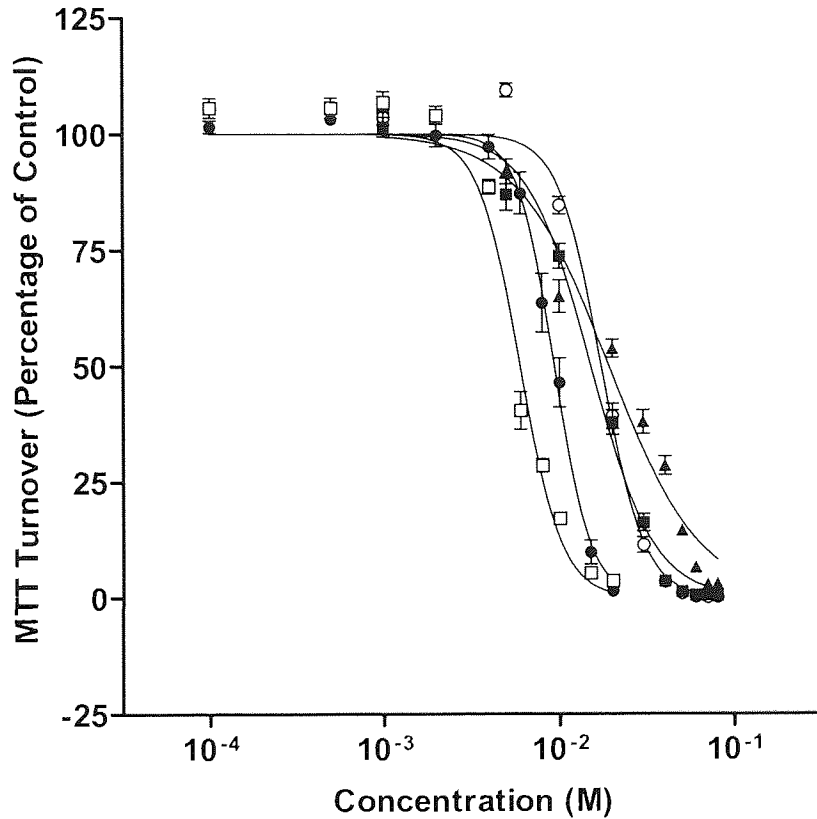
Figure 3.4 The effect of acrylamide on MTT turnover in human glioma and neuroblastoma cell lines. Sigmoidal dose response curve (variable slope) in the presence of acrylamide for U251-MG (■), U373-MG (▲), CCF-STTG1 (○), SH-SY5Y (□) and retinoic acid differentiated SH-SY5Y (●) cell lines following 24 hours incubation. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=24 from 3 individual experiments).
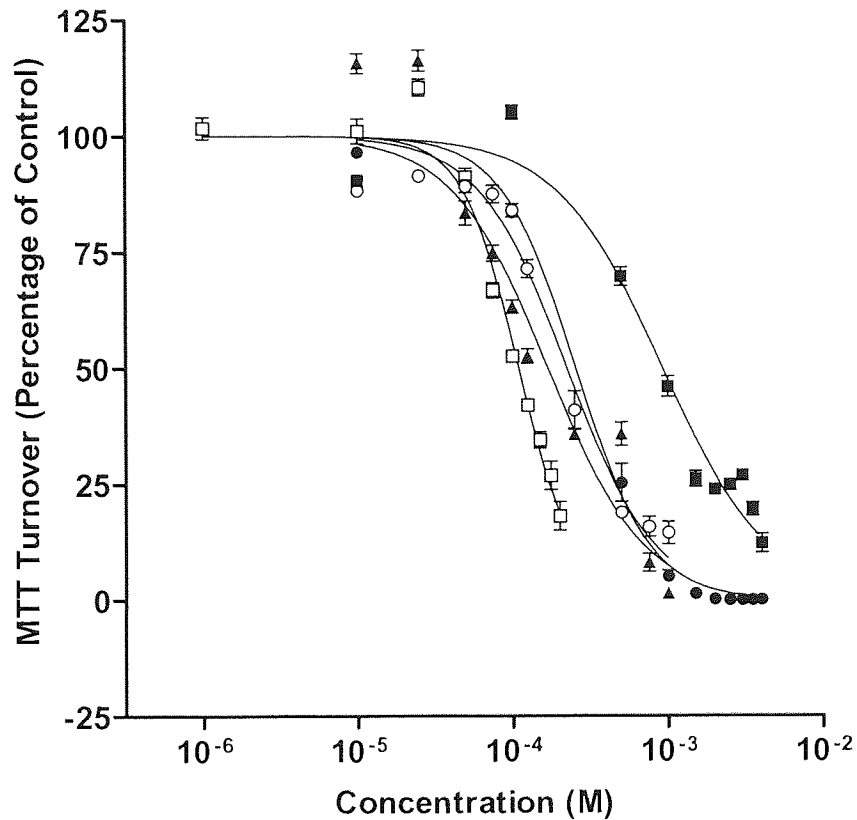
Figure 3.5 The effect of chloroquine diphosphate on MTT turnover in human glioma and neuroblastoma cell lines. Sigmoidal dose response curve (variable slope) in the presence of chloroquine diphosphate for U251-MG (■), U373-MG (▲), CCF-STTG1 (○), SH-SY5Y (□) and retinoic acid differentiated SH-SY5Y (●) cell lines following 24 hours incubation. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=24 from 3 individual experiments).
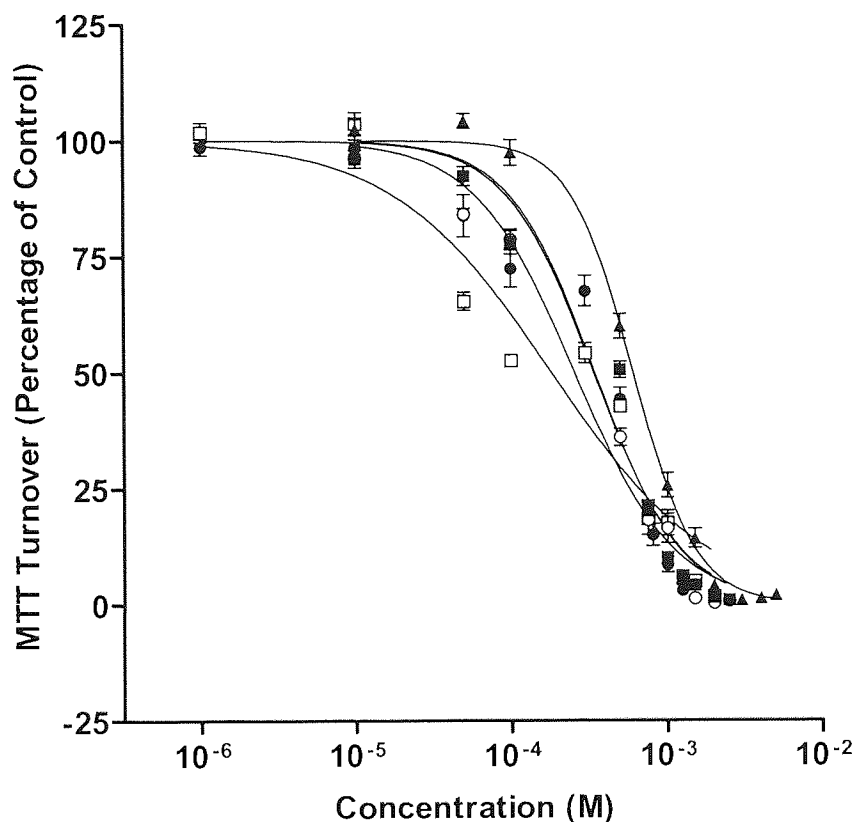
Figure 3.6 The effect of trimethyltin chloride on MTT turnover in human glioma and neuroblastoma cell lines. Sigmoidal dose response curve (variable slope) in the presence of trimethyltin chloride for U251-MG (■), U373-MG (▲), CCF-STTG1 (○), SH-SY5Y (□) and retinoic acid differentiated SH-SY5Y (●) cell lines following 24 hours incubation. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=24 from 3 individual experiments).

the IC_{50}s for each of these cell lines. The U251-MG cell line however showed a marked resistance to chloroquine diphosphate compared to any other cell line (p<0.001), with an IC_{50} of 0.9253 (± 0.11) mM. This pattern of sensitivity was also seen in the NOAEL for chloroquine diphosphate in each cell line. The CCF-STTG1 cell lines had a NOAEL of less than 0.01 mM while the NOAEL in differentiated and undifferentiated cell lines was 0.01 mM. The NOAEL of chloroquine diphosphate in the U373-MG line was between 0.025 and 0.05mM while the higher resistance of the U251-MG cell line is also illustrated by the highest NOAEL of 0.1mM. Similar to acrylamide exposure, there were again incidences of elevations in MTT turnover following both 0.01mM and 0.025mM chloroquine diphosphate exposure in the U373-MG cell line, 0.025mM in the undifferentiated SH-SY5Y line to 15.7(±2.2), 16.3(±2.3) and 10.5(±1.8) percent greater than control values respectively. All of these concentrations were again lower than the NOAEL in the same cell lines

3.3.2.4 Trimethyltin chloride.

There was no significant difference between the cytotoxicity of trimethyltin chloride on the U251-MG, CCF-STTG1, SH-SY5Y and differentiated SH-SY5Y cell lines, exhibiting IC_{50}s of 0.35 (± 0.06) mM, 0.26 (± 0.03) mM, 0.18 (± 0.05) mM and 0.34 (± 0.05) mM respectively. The U373-MG cell line was significantly more resistant to the compound than any other cell line (p<0.001) with an IC_{50} of 0.61 (± 0.02) mM. The relative sensitivities are reflected in the NOAEL for the compound, which were 0.1mM for the U373-MG cell line and 0.01mM for all others.

3.3.2.5 General comparison of cytotoxicity of compounds in all cell lines.

IC_{50} values indicated that ethanol was the least toxic compound to all cell lines, followed by acrylamide, trimethyltin chloride and finally chloroquine diphosphate, apart from in the U251-MG cell line which exhibited a marked resistance to chloroquine diphosphate and thus trimethyltin chloride was the most toxic compound for this cell line. The NOAELs for the compounds reflect this same pattern of sensitivity with ethanol exhibiting the highest NOAELs of 9.2mM to 184mM and acrylamide the next highest with 1 to 4mM. The CCF-STTG1 and both neuronal cell types exhibited the same chloroquine diphosphate and trimethyltin chloride NOAELs of 0.01mM whereas the marked resistance to chloroquine diphosphate by the U251-MG line was seen in the NOAEL of 0.1mM as opposed to 0.01mM trimethyltin chloride. Conversely, the U373-MG cell line showed the

highest trimethyltin chloride NOAEL of 0.1mM in comparison with 0.01mM for chloroquine diphosphate.

3.4 Discussion.

Each of the four compounds tested in these experiments caused a concentration dependent decrease in MTT turnover in all of the tested cell lines indicating a loss of cellular viability. Differential potencies between toxicants were also observed as determined by relative IC_{50} values for each toxicant in each cell line. Of the compounds tested, trimethyltin chloride and chloroquine diphosphate were more toxic compared with acrylamide and ethanol was the least toxic. This pattern was shown in the EC_{50} values for MTT turnover following studies with the same compounds with rat primary astrocytes and C6 glioma cell lines in work by Cookson *et al.* (1995). There are a number of cellular factors as well as characteristics of the respective toxins that may have influenced the differential toxicities observed in this chapter.

The relationship between ethanol consumption and neurodegeneration has been explored exhaustively in many different models. While ethanol toxicity in the CNS has largely focused on the identification of effects in neuronal cells, there is also evidence to suggest that ethanol has detrimental effects on other cell types in the brain, particularly astrocytes (Allansson *et al.* 2001). Acute ethanol exposure in the present study resulted in a loss of viability in cells of both astrocytic and neuronal origin in a concentration dependent manner with IC_{50} values within the range of 270 and 380mM, however, the astrocytic cells were more resistant to the cytotoxic effects of ethanol than the undifferentiated neuronal cells, as identified by higher IC_{50} values. This is consistent with published studies that show that neural stem cells are more sensitive to ethanol exposure than astrocytes when exposed to 10mM ethanol, as observed by Hao *et al.* (2003).

It has been previously reported that the cell type specificity of ethanol is dependent upon exposure concentration (Tapani *et al.* 1996). Very high concentrations of ethanol (between approximately 1.1 and 11M) were shown to be equally toxic to several clonal cell lines and isolated hepatocytes. In the present study, however, the results indicate that at lower concentrations between 200 and 400mM, cell line specific effects can be observed. With exposure to very high concentrations of ethanol, cell death is likely to be due to cell lysis caused by solubilisation of membrane lipids and the consequent loss of cell membrane integrity. At lower concentrations, ethanol induced cytotoxicity has been linked to oxidative stress brought about by the excessive generation of ROS (Wu and Cederbaum

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2005). Baker and Kramer (1999) suggested that the cell type differences in the toxicity of ethanol are not related just to concentration in any one type, but rather to the relative sensitivities of each cell line to the compound. It is possible that a higher degree of sensitivity to ethanol exhibited by a certain cell line is due to the perturbation of an essential function unique to that cell type (Baker and Kramer 1999).

Acrylamide is widely regarded as a specific neurotoxin capable of causing peripheral neuropathy in humans (Friedman 2003). Being regarded as a specific neurotoxicant, most attention has focused on the effects of acrylamide in neuronal cultures with few studies comparing acrylamide toxicity in astrocyte and neuronal cells, particularly in continuous cell lines. Previous work conducted by Presad (1991) showed that NBP2 neuroblastoma and C6 glioma were equally sensitive to the growth inhibitory effects of acrylamide. However, in the present study it was found that both the undifferentiated and differentiated SH-SY5Y cells were significantly more sensitive to the cytotoxic effects of acrylamide than the three astrocytoma lines as measured by MTT turnover, clearly shown with IC_{50} values of 9.4 and 6.0mM in differentiated and undifferentiated SH-SY5Ys respectively, compared to 15.1, 19.2 and 17.4mM in the astrocytoma cell lines. These values are similar to results achieved by Cookson et al. (1995) using C6 rat glioma cells where the acrylamide IC_{50} following 24 hour exposure was approximately 14.1mM, whereas in the same study primary rat astrocytes were found to be more resistant. To the author's knowledge there have been no similar studies assessing MTT turnover in neuronal cells as to allow for direct comparison.

Acrylamide is a soft electrophile known to form adducts with proteins and reduced glutathione by binding to active-hydrogen-bearing functional groups including sulfhydryl groups of cysteine and N terminal amino acid residues of proteins (Cavins and Friedman 1968; Friedman 2003). For example, adduction with haemoglobin has been observed in blood samples from persons exposed to the compound, the levels of which directly correlated to the level of exposure (Hagmar et al. 2001). Toxicity may manifest when the formed adduct disrupts the normal structure and functioning of the affected protein which may consequently disrupt the functioning of the cell and its processes (LoPachin and DeCaprio 2005). It has been described how different sulphydryl groups on different proteins can have varying degrees of reactivity with the same agent and has such been suggested to be the case in acrylamide adduct formation (Friedman 2003). It is possible

that certain neuronal proteins may have a higher affinity for acrylamide adduct formation than those in astrocytes, thus adversely affecting neuronal cells preferentially.

The greater neuronal sensitivity to acrylamide compared to astrocytes may be linked with the differential thiol content of the cells. The metabolism of acrylamide is principally by glutathione conjugation catalysed by glutathione-S-transferase (GST) (Friedman 2003). Higher levels of GSH or a greater capacity of its synthesis in astrocytes may confer a degree of resistance to the toxicity of acrylamide compared to neuronal cultures. Odland *et al.* (1994) found that acrylamide exposure resulted in a depletion of GSH levels in neuroblastoma cells. Similarly, the compound was shown by Srivastava *et al.* (1986) to reduce levels of GST and GSH in the brain. This may be attributable to the acrylamide adduction of glutathione, consequently preventing acrylamide detoxification. The higher levels of glutathione in astrocytes may confer some protection from irreversible acrylamide toxicity.

It had been widely reported that acrylamide also causes the collapse, redistribution and accumulation of intermediate filaments in different cell types (Durham 1983; Sager and Matheson 1988; Sager 1989) including neurofilaments in the SH-SY5Y cell line (Hartley *et al.* 1997) and GFAP in astrocytes (Cordo and Candurra 2003). It was proposed by Hartley *et al.* (1997) that this could be due a disruption in the phosphorylation of these proteins or other post-translational changes which, as described previously for GFAP in the main introduction, are important for intermediate filament organisation. It is possible that acrylamide differentially affects the intermediate filament components of different cell types; for example, neurofilaments in neuronal cells, GFAP in astrocytes, and vimentin in other cell types such as fibroblasts and immature astrocytes. A similar neurotoxin 2-5 hexanedione (2-5 HD) which has been suggested to share the same common mechanism as acrylamide (Sager and Matheson 1988; Hartley *et al.* 1997), also causes intermediate filament collapse and aggregation has been shown to be more specific for neuronal cells compared to astrocytes (Durham 1988). Here, the threshold concentration of 2-5 HD causing aggregation of GFAP in astrocytes was twice that capable of causing accumulation of neurofilaments. It is possible that the intermediate filaments of the neuronal and astrocytic cell lines used in the present study may exhibit this same pattern of sensitivity when exposed to acrylamide.

It is well known that chloroquine is toxic to humans (Jaeger et $al.$ 1987) and so the dose should be carefully considered when administered therapeutically, particularly in infants (Smith and Klein-Schwartz 2005). While the major clinical manifestation of chloroquine toxicity is retinopathy (Wilson 1961) the compound can affect other cell types according to their individual susceptibilities (Bruinink et $al.$ 1991). When comparing neuronal and astroglial cells in brain cell cultures, Bruinink et $al.$ (1991) described how nerve cells were more sensitive to chloroquine than astroglia. In regard to astrocytes, Cookson et $al.$ (1995) found that primary rat astrocytes were more sensitive to chloroquine than the C6 rat glioma cell line with $EC_{50}s$ of 0.1mM and 0.39mM respectively. These values were similar to the $IC_{50}s$ obtained for the CCF-STTG1 and U373-MG cell lines in this chapter. Also in this chapter, no significant difference in sensitivity to chloroquine diphosphate was observed between the two neuroblastoma cell types and the CCF-STTG1 and U373-MG cell line, however, the U251-MG cell line showed a marked resistance to the compound. Illustrating an IC_{50} value of 0.93mM, this cell line was approximately 4-fold more resistant than the other astrocytoma lines and approximately 9-fold more resistant than the undifferentiated SH-SY5Y cell line.

Lysosomal dysfunction has been proposed as a possible mechanism for chloroquine toxicity (Fredman et $al.$ 1987). Chloroquine is a lipophilic, diprotic weak base that can diffuse freely through the cell membrane into the cell. Once inside the acidic environment of the cell, the chloroquine molecule becomes protonated and accumulates in acidic compartments such as the lysosomes, raising the intravacuolar pH (Park et $al.$ 2004) causing lysosome swelling (lysosomotrophism) and release of contents into the cytoplasm, ultimately resulting in cell death. While lysosomotrophism has been regarded previously as the reason for the ultimate lysosome membrane rupture, alternatively, attack by ROS has also been implicated. Chloroquine is well known to cause oxidative stress by the stimulation of ROS production in a variety of cells including human astroglial cells (Park et $al.$ 2003; Park et $al.$ 2004). It has been proposed that the lysosomal membrane rupture and subsequent cell death by necrosis may be the result of ROS mediated lipid peroxidation of the membrane (Ollinger and Brunk 1995) the extent of which may depend on the target cell. Despite the clear effects of chloroquine on lysosomes and their function, Bruinink et $al.$ (1991) suggested that lysosomal disruption may not be the primary cytotoxic action of chloroquine as there were no notable differences between the effects of the compound on MTT turnover and Neutral Red uptake. It is possible that ROS induced

lipid peroxidation affects various cellular organelles that would eventually lead to cell death.

The cytotoxic effects of trimethyltin (TMT) have been shown to be highly selective depending upon cell type. It has been previously demonstrated that neuronal cells are consistently the most sensitive cell type to the toxic effects of TMT, for example, Christofol *et al.* (2004) found that following 24 hours exposure, variable levels of TMT were required to cause a 50% reduction in MTT turnover in a variety of cells including rat cerebellar granule cells, rat hippocampal neurons, individual cultures of human foetal primary neurons and astrocytes and human neuroblastoma cell lines. The most sensitive of these cell types to TMT toxicity were the rat hippocampal neurons, requiring only 1.45μM to cause a 50% reduction in cell viability, whereas the most resistant were the human astrocytes with which an LD_{50} of 609.7μM TMT was detected. The same relative sensitivities of neuronal and astrocytic cells to TMT toxicity have also been observed by Thompson *et al.* (1996). This suggests that neuronal cells are generally more sensitive to the cytotoxic effects of TMT than astrocytes. Contrary to this, the neuroblastoma cell lines in the present study did not exhibit a marked sensitivity to trimethyltin chloride compared to the majority of the glioma cell lines, apart from the U373-MG cell line that was found to be more resistant than the rest.

The lack of any differential sensitivity of the undifferentiated and differentiated SH-SY5Y, U251-MG and CCF-STTG1 cell lines could perhaps be due to an increase in the resistance of the SH-SY5Y neuroblastoma cell line compared to neuronal cells *in vivo*. Such resistance of neuroblastoma cell lines to potentially lethal concentrations in hippocampal cells was reported by Christofol *et al.* (2004). This group described an intermediate cytotoxic response in the two neuroblastoma cell lines between those of the rat hippocampal cells and human primary astrocytes. Similarly, Thompson *et al.* (1996) observed that neuroblastoma cell lines required up to 1000 times more TMT in order to exhibit the same level of toxic effects as measured in rat hippocampal cells (2-8μM compared to just 1-10nM) suggesting that the mechanism or factor by which this sensitivity is incurred to neurons is to some degree lost in immortalized neuronal cell cultures.

A possible reason for the sensitivity of neurons to TMT is the presence of Stannin in neuronal cells. Stannin is a mitochondrial membrane protein involved in several key signalling systems including growth regulation and apoptosis. Organotins such as trimethyltin can interact with stannin and may act to induce apoptosis (Buck-Koehntop *et al.* 2005). Stannin expression has been found to directly correlate with the sensitivities of various cell lines to the cytotoxic effects of TMT (Thompson *et al.* 1996). Similarly, Thompson *et al.* (1996) also found that blocking Stannin expression significantly protected neurons from TMT toxicity and apoptosis. It is possible that the neuroblastoma cell lines used in the present study do not express stannin thus explaining their relative resistance to TMT. The identification of this protein in these cells would be of interest to investigate levels in the various cell types in relation to comparative sensitivity to TMT. It is unclear whether the lack of differential toxicity of neuroblastomas and two of the astrocytomas is due to a loss of such a mechanism in neuroblastoma that confers sensitivity or due to a lack of protective mechanisms in the two astrocytoma cell lines. Additionally the significant level of resistance exhibited by the U373-MG cells compared to any other line may be attributable to a mechanism specific to that cell line.

Not only may the specific actions of each of the above compounds indirectly affect MTT turnover by causing a reduction in viable cells through mechanisms distinct from metabolic function, it is also possible that the compounds may act directly or indirectly to affect metabolic processes and cause the gradual reduction of MTT turnover. MTT enters the cell by endocytosis and is reduced by NADH reductase and other enzymes. MTT reduction to its formazan product has been largely attributed to the mitochondrial reductase enzyme succinate-dehydrogenase (Slater *et al.* 1963). Succinate-dehydrogenase is a membrane bound mitochondrial enzyme involved in the mitochondrial respiratory chain (electron transport chain) and contributes to the ultimate generation of ATP. There is also a strong body of evidence, however, to suggest that turnover of MTT may not necessarily be linked solely to mitochondrial activity but may also be due to the activity of reductase enzymes outside the mitochondria. Studies by Liu *et al.* (1997) provided evidence for the role of lysosomes and endosomes as an additional site of MTT reduction suggesting that mitochondria are not the exclusive site of MTT turnover.

Many of the enzyme systems responsible for MTT turnover are sensitive to the deleterious effects caused by toxicant exposure. The viability of the mitochondrial

dehydrogenase enzymes in particular is essential to facilitate the flow of electrons in the electron transport chain. Damage to these enzymes results in the leaking of electrons and the subsequent generation of reactive oxygen species. Ethanol, chloroquine and trimethyltin chloride have all been shown to induce oxidative stress through the formation of reactive oxygen species (Wu and Cederbaum 2005; Park *et al.* 2003; Park *et al.* 2004; Gunasekar *et al.* 2001). These ROS are unstable, requiring an additional electron to increase their stability. This electron is normally abstracted from its nearest source, thus causing oxidation damage to cellular proteins, lipids and DNA with deleterious consequences to their functions, compromising cellular organelles and leading eventually to cell death. In terms of the mitochondrial dehydrogenases, the released ROS and the subsequent oxidative damage to these local enzymes may cause further impairment of enzyme function and an increase in released levels of ROS, creating a cycle of cause and effect. The enzyme impairment would also mean that the electron transport chain would be disrupted and the generation of ATP abated. As ATP is necessary as an energy source for biological processes, it is likely that decreased levels will result in a reduction in the activity of other enzyme systems and ultimately a loss of cellular viability.

It is possible that the differential sensitivity of astrocytes and neurons to the compounds in the present study is due to a varying capability of each cell type to withstand toxicant induced oxidative stress. As mentioned previously, astrocytes are generally considered more resistant than neurons to oxidative stress (Gonthier *et al.* 2004) due to their up-regulated antioxidant systems (Boyles *et al.* 1985; Makar *et al.* 1994; Aschner 1998; Lindenau *et al.* 2000; Dwyer *et al.* 1995). TMT-induced increases in ROS levels in particular, were more pronounced in cortical neuron cultures compared to those of astrocytes (Gunasekar *et al.* 2001). In addition, in the same study, neurons were found to be more susceptible to glutathione depletion than the astrocytes, suggesting that glutathione depletion may be an important factor in TMT induced cytotoxicity. Indeed, it was also illustrated in the same study that multiple oxidative species including superoxide, nitric oxide and hydrogen peroxide are generated following TMT exposure and that pre-treatment with glutathione or nitric oxide scavengers protected against TMT cytotoxicity Gunasekar *et al.* (2001). Extreme ROS attack can overload the antioxidant systems resulting in cellular damage and DNA strand breaks, albeit at higher concentrations compared to cells without these comprehensive protective mechanisms. The level of resistance of each of the cell lines in the present study may be attributable to the degree of

oxidative stress experienced by the line and the capability of cellular antioxidant defence. It is likely that the cell lines used in this study still possess some, if not all of the antioxidant systems present in cells *in vivo* and that they may be present at higher levels in the astrocytoma cell lines than in the undifferentiated neuroblastomas, thus conferring commensurate resistance. It is difficult to generalise on the antioxidant status of the cell lines used in this chapter. Previous studies have shown that some of these lines express a wide range of endogenous antioxidant systems. In particular there is evidence that catalase, superoxide dismutase, glutathione and enzymes of the glutathione system are expressed in the U251-MG line (Lee *et al.* 2004). U251-MG cells were shown to respond to oxidative stress caused by radiation by the induction of antioxidant systems including a two-fold increase in superoxide dismutase and a five-fold increase in glutathione peroxidase (Lee et al. 2004). Little is known, however, of the antioxidant capability of the CCF-STTG1 and U373-MG cell lines.

It is also possible that differential resistance may also be conferred by the ability to express stress proteins. Stress proteins such as those from the heat shock protein 70 (HSP70) and glucose regulated protein (GRP) families are expressed constitutively in cells in very low levels until they are induced by stress conditions. These stress proteins act as cytoplasmic and endoplasmic reticulum molecular chaperones respectively and have a "protective stabilising effect on oxidative stress-induced cell injury" (Russo *et al.* 2001). Ethanol exposure has been shown to increase HSP70 expression in astrocytic cells (Russo *et al.* 2001; Sanchez-Moreno *et al.* 2003) and GRP in neuronal cells (Miles *et al.* 1994). Russo *et al.* (2001) found that inhibition of HSP70 resulted in a loss of viability in ethanol treated cells. Whether or not the cell lines used in this study are capable of up-regulating these stress proteins may be implicated in their differential sensitivities to the toxicants.

In addition to the energy availability and antioxidant status of the cells, it is possible that the differential sensitivities of the cell lines to each toxin could be attributed to the pharmacokinetics of the compound itself. Not only does the solubility of a compound influence its distribution in the body, the cytotoxic potential of a drug is largely dependent on its ability to penetrate through the cell membrane. While lipophilicity is beneficial for a molecule to penetrate through the lipid bilayer of biological membranes, those molecules of high lipophilicity may become trapped in the membrane. The degree of lipophilicity and hydrophilicity exhibited by the compounds used in this chapter was likely

to have influenced their cellular penetration and ultimately their toxic potency. For example, ethanol, with a partition coefficient of -0.14 Log P, is mildly hydrophilic having a hydrophilic OH group and a short hydrophobic hydrocarbon chain. This combination makes ethanol highly water soluble while the short chain still allows for some degree of lipophilic attraction. Similarly, acrylamide also has a low partition coefficient of -0.81 Log P making it more water soluble than ethanol. On the other hand trimethyltin chloride is mildly lipophilic with a partition coefficient of 0.28 Log P suggesting that the compound passes easily through biological membranes. Chloroquine diphosphate, however, has a relatively high coefficient of 4.49 Log P, meaning the compound is highly lipophilic and potentially rendering it difficult to pass through biological membranes into the cells.

Once a compound is inside a cell, there is always the possibility that the cell will act to eliminate the compound via ATP-binding cassette (ABC) transporters. These are transmembrane binding proteins that are responsible for the efflux of a wide variety of endogenous and exogenous substrates from the cell. One member of the ABC transporter family, P-glycoprotein (P-gp), acts as an energy dependent efflux pump at the plasma membrane and can interact with and transport a variety of structurally and functionally different amphiphilic compounds (Germann 1993). Many tumour cells over-express these ABC transporter proteins (O'Connor 2007). The over-expression of P-gp and the multidrug resistance protein-1 (MRP-1), which have been shown to eliminate anticancer drugs, has been associated with negative treatment responses in cancer therapy (O'Connor 2007). While there is a dearth of studies identifying the presence of ABC transporters in cell lines used in this study, the U373-MG line has been identified as expressing several of these transporters, including MRP-2, MRP-3 and breast cancer resistance protein-1 (BCRP-1) (Liu *et al.* 2005). It is therefore possible that the relative sensitivities of the cell lines in the present study to individual compounds may be attributable to the level of expression of the various ABC transporter proteins and to the level of clearance of that particular compound that they confer.

Whichever the mechanisms that confer differential sensitivity of cells to a particular compound, it is clear that the actions and potencies of toxicants are variable depending upon the cellular substrate. The results from these experiments illustrate the importance of using cells of different origins when investigating toxicity in the CNS, all of which may be differentially affected.

Retinoic acid differentiation of the SH-SY5Y cell line has been widely used to induce the appearance of more neuronal characteristics than the parent cell line, including a more neuronal phenotype and the increased expression of many neuronal markers (Encinas *et al.* 2000). The effect of retinoic acid induced differentiation on susceptibility to toxicant induced inhibition of MTT turnover was determined in these studies. It was found that the differentiation of the SH-SY5Y cells had no effect on their sensitivity to trimethyltin, chloroquine diphosphate or acrylamide exposure. This is in contrast to a report by Hartley *et al.* (1997) where differentiated SH-SY5Y cells were more sensitive to acrylamide compared with undifferentiated cells. Of the four compounds tested, only ethanol was shown to have a significantly different level of cytotoxicity in the differentiated SH-SY5Y cells compared to the undifferentiated cells. The undifferentiated cells were significantly more sensitive to ethanol than the differentiated cells suggesting that retinoic acid differentiation confers a marked increase in resistance to ethanol in the SH-SY5Y cell line.

There is conflicting evidence regarding whether retinoic acid induced differentiation results in an increased or decreased level of sensitivity to toxicant exposure. Similar to the increased resistance conferred to the cells in response to ethanol in the present study, Sakai *et al.* (2005) also found that differentiated cells were more resistant to cytotoxicity induced by 100mM ethanol. It was suggested by Sakai *et al.* (2005) that BDNF, which was used in a similar post-treatment manner like in the current study, may have a protective effect, with similar protection being observed in primary culture. It has also been suggested that the differences in sensitivity pre- and post-differentiation may be attributable to the changing antioxidant status of the cells. RA differentiation of primary cultures from neonatal rat hippocampus has been shown to make cells more resistant to oxidative stress, preventing a decrease in protein levels of SOD-1 and SOD-2 (Ahlemeyer *et al.* 2001) and glutathione depletion (Ahlemeyer and Kriegstein 2000) resulting in an enhanced level of neuronal survival. On the other hand, however, Erlejman and Oteiza (2002) illustrated that while retinoic acid differentiation of IMR-32 human neuroblastoma cells resulted in a similar increase in SOD-1 and SOD-2 activities, higher levels of glutathione peroxidase activity were also observed together with a reduced concentration of total glutathione and a higher ratio of oxidised/reduced glutathione content compared to undifferentiated cells. It seems, therefore, that RA differentiation alters the antioxidant status of neuronal cells, the pattern of which may be dependant upon the cell type. This

altered antioxidant status may affect the cell's reponse to different compounds depending upon the type and level of ROS generation.

The NOAELs for each of the compounds in each cell line were estimated in order to determine the levels of compound tolerable before adverse effects on cell viability were manifested (as measured by MTT turnover). It was found that the NOAELs did not always correspond with the relative sensitivities of the compounds as determined by IC_{50} values but that some compounds had a greater threshold for tolerance, after which the onset of cytotoxicity was more rapid with a steeper gradient to the slope of the dose response curve. These effects could be attributable to the mechanism of toxic action and again the relative capabilities of the cell lines at dealing with oxidative stress or the deleterious effects that the compounds may have on structural cellular components and organelle viability.

It was observed following exposure to acrylamide and chloroquine that elevations in the turnover in MTT occurred in the CCF-STTG1, U373-MG or SH-SY5Y cell lines. These elevations in MTT turnover are thought to be metabolic responses to injury (Mead and Pentreath 1998a) to either eliminate the compound, replace factors lost including any structural proteins or to replenish antioxidant systems depleted in the reduction of ROS produced. It will be of interest to determine whether metabolic activation effects are concurrent with elevations in GFAP as a feature of astrocyte reactivity and so will be investigated alongside GFAP in the next chapter.

To summarise, the five cell lines used in this study each showed varying sensitivities to each of the four compounds tested. While there is a great deal of variation between the patterns of sensitivity to individual compounds, in the case of all compounds, the most resistant cell line tended to be an astrocytoma line. It is likely that the resistance observed by the astrocytoma cell lines is either due to a specific action of a toxicant in a particular cell type or the capacity of individual cell lines to protect themselves against the cellular stresses that toxicant exposure can cause. It has also been shown that retinoic acid induced differentiation of the SH-SY5Y neuroblastoma cell lines confers some degree of resistance to toxicant exposure, particularly in the case of ethanol.

While it is only possible to suggest mechanisms of cell death and potential reasons for differential sensitivity of the cell lines following exposure to each compound, by

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illustrating the relative cytotoxic potencies of each compound, the results of experiments in this chapter have provided the basis for choosing appropriate exposure ranges of each compound for the sub-cytotoxicity experiments as well as providing a point of comparison when interpreting reactive response data in successive chapters.

Chapter 4: Glial fibrillary acidic protein as a marker of toxicity in astrocytoma cell lines.

4.1 Introduction.

Among their many functions, astrocytes have an integral role in the maintenance of homeostasis within the CNS. Any deficit in astrocyte numbers, brought about by the cytotoxic effect of a compound specifically upon astrocytes, is likely to impair normal neurological function. In addition to cytotoxic effects, it is important to consider how sub-cytotoxic levels of compounds might affect normal CNS behaviour by subtle alteration of astrocyte function and whether such exposure would trigger the reactive response of astrocytes in vitro.

As mentioned previously, the up-regulation of glial fibrillary acidic protein (GFAP) is a major hallmark of astrocyte reactivity, occurring in response to a variety of disease and lesion-induced injuries (Eng 1988). Specifically, the protein is a sensitive marker for detecting disturbances in tissues following low-level chemical exposure that would not normally be observed by conventional cytotoxicity analysis (O'Callaghan *et al.* 1995). Consequently, the measurement of GFAP has been used widely for the quantitative study of reactive astrogliosis in response to chemical insult in both *in vivo* and *in vitro* almost exclusively using animal models (O'Callaghan and Miller 1985; Brock and O'Callaghan 1987; O'Callaghan and Jensen 1992; Toimela and Tahti 1995; Lam *et al.* 2000; Cookson and Pentreath 1994; Cookson *et al.* 1994; Malhotra *et al.* 1995 and 1997; Mead and Pentreath 1998). As outlined in the introduction, human astrocytic and neuronal systems differ in many respects to those of animals, although to date, there has been a dearth of human derived cellular astrocytic models. Hence, this work is intended to develop an astrocytic system where potential reactive changes in GFAP will be detected following toxic insult. The three astrocytoma cell lines chosen for this study will form the basis of this human-derived cellular system.

The development of techniques for the identification of GFAP and its quantification following injury has advanced considerably in the past thirty years following the development of monoclonal antibody technology. Initially, the assessment of neuronal injury and the astrogliotic response was limited to *in vivo* neuroanatomical

evaluation. This approach allowed the detailed assessment of any alterations in the structure of intact neural tissues, although there are disadvantages. The pressures of time and cost associated with detailed anatomical assessment limit the quantity of tissue sections observed to numbers which may not be adequate for determining specific effects in certain regions of the brain. Histological staining techniques such as cupric silver staining (Carlsen and De Olmos 1981) may go some way to highlighting specific targets of degeneration, however, they are largely restricted to neuronal aspects of degeneration; this means that the intimate and subtle responses of astrocytes can be neglected. Furthermore, for the most part, anatomical assessment and staining methods allow only qualitative evaluation. The application of monoclonal antibodies and the development of immunological methods for the detection of specific biomarkers of injury such as GFAP have provided opportunities for the quantification of toxicity.

Immunohistological staining for GFAP in tissue sections is practised widely for the assessment of astrogliosis and has proven extremely useful for assessing regional variations in expression in the intact brain (O'Callaghan 1991b). The method is, however, restricted mainly to *in vivo* tissue slices and as such is subject to the same limitations of time and cost as neuroanatomical evaluation. Development of immunocytochemistry techniques has allowed for the detection of specific proteins in cells grown in culture such as primary cultures or continuous cell lines, and as such may alleviate some of these limitations.

An alternative to immunoprobing intact cells is to determine GFAP levels from the lysates of pre-exposed cellular material by Western blot analysis (Towbin 1979). Proteins from tissue or cell lysates can be separated by SDS-PAGE, then transferred to nitrocellulose membranes and the isolated GFAP band detected using antibodies coupled to enzymatic or radioactive substrates. Such detection may allow for the semi-quantitative assessment of GFAP levels. Variations of this immunoblotting technique have been developed to identify and quantify GFAP in a variety of cell types. O'Callaghan and Miller (1985) described a nitrocellulose-based dot-blot immunoassay for GFAP based on the method by Jahn *et al.* (1984) originally used for the identification and quantification of synapsin proteins. The procedure involves the initial spotting of detergent/protein mixture onto nitrocellulose membrane followed by the incubation of these dot blots with anti-GFAP primary antibody. Bound antibody was quantified using a radioactive detection

reagent (^{125}I-protein A) that was then detected by gamma spectrometry. Several improvements were made to this original assay, ultimately developing a slot-blot assay (Brock and O'Callaghan 1987) where manual dotting was replaced with template guided spotting. These immunoblotting procedures can be applied to both brain sections dosed *in vivo* or to lysates of cells dosed *in vitro*. However, the procedure can be time consuming, expensive and reproducibility between blots can be problematic (personal observations). The use of radioactive substrates by O'Callaghan *et al.* for the dot-blot and slot-blot assays also increased the safety risk of the procedures and as such, a method employing safer enzyme based reagent systems may minimise the risk. The productivity of both the Western and slot-blot methods is also limited by the number of samples that can be tested at any one time. In order to test a large number of chemicals for potential toxicity it is necessary to employ a high throughput immunoassay that can be applied to a multiple sample format.

The use of an Enzyme-linked Immunosorbent Assay (ELISA) technique for identifying GFAP has many advantages compared with the limitations of immunoblotting methods. Using the ELISA technique, quantitative immunoanalysis of GFAP is possible and can be applied to a 96 well microplate format to achieve rapid, simple and cost effective screening of a large number of samples. The development of automated ELISA procedure systems means further ease of performance can also be achieved. Consequently, an ELISA for GFAP was developed by O'Callaghan (1991b) to overcome the limitations of the slot- blot assay.

There are several different types of ELISA that have been adapted and employed for the detection of GFAP protein in various substrates such as bodily fluids, tissue samples or cell culture monolayers. One example is the direct ELISA, involving the binding of homogenate proteins to the cell culture surface and subsequent detection using a single enzyme conjugated antibody. The direct ELISA is a straightforward ELISA technique and the use of only a single antibody makes it both rapid and cost effective. However, difficulties experienced in achieving effective binding of GFAP to the culture plate from prepared cellular homogenates (O'Callaghan 1991b) suggest that an alternative sandwich ELISA may be more efficient for detection of GFAP in aqueous or detergent based samples. This sandwich ELISA is so named because protein from preparations of tissue homogenates or bodily fluids is "sandwiched" between a capture antibody bound to

the plastic plate surface and a detection antibody with an enzyme conjugate for detection. The dual antibody allows for more sensitive detection of individual protein levels (O'Callaghan 1991b) and when performed to include a standard curve, can provide accurate quantitative measurements of a particular protein in each sample. The sandwich ELISA for GFAP has been used predominantly to test lysates of sample preparations from tissue sections and bodily fluids for the diagnosis of disease or states of trauma (Ross *et al.* 2003; Van Geel *et al.* 2002; Gurnett 2003) and has also been employed for toxicity analysis (Toimela *et al.* 1994; O'Callaghan *et al.* 1995). The procedure can, however, prove very costly as it is necessary to either go through the time consuming preparation of all of the reagents, buffers and expensive epitope specific antibodies needed for the construction of assays in the laboratory or to purchase sandwich ELISA kits which are also very expensive and variable in efficacy between suppliers.

While useful for testing GFAP in aqueous samples such as cerebrospinal fluid, when using dosed cell cultures, as with this investigation, the sandwich ELISA requires the lysis of dosed cells in order to produce free GFAP monomer in solution for detection. This means considerable sample preparation, which when testing large numbers of individual cell samples would be extremely laborious. This would not be an appropriate method for use in this investigation where several repeats of each dosing concentration are to be performed with cells lines in a 96 well format. Consequently, it was deemed more appropriate to use a cell based ELISA (cELISA) for the high-throughput detection of GFAP in numerous individually treated cell cultures.

The cELISA is based on the principles of immunocytochemistry, whereby following treatment, cells are fixed in the same microplate, permeabilized, blocked to prevent non-specific binding and probed for the specific antigen using a single primary antibody. A secondary antibody with an enzyme conjugate is then added to bind to the primary antibody. Incubation with a substrate appropriate for the enzyme conjugate and subsequent spectrophotometric analysis allows for the quantitative assessment of antigen-antibody binding. The success of the procedure depends on the use of a good antibody and adequate blocking of non-specific binding sites to ensure specificity to the target antigen. Thorough yet gentle washing between incubation stages is also essential. If the procedure is performed correctly it is possible to achieve a semi-quantitative assessment of GFAP

content within the cells, where levels can be compared to a non-treated control and any increases or decreases from this control value can be quantified.

The cell based ELISA has been previously applied for the *in vitro* detection of GFAP in primary astrocyte cultures and some continuous cell lines in response to a variety of known neurotoxicants (Mead and Pentreath 1998b; Cookson and Pentreath 1994). The procedure has the main advantage of being much cheaper than the sandwich ELISA as it requires only a single antibody to bind GFAP. The assay also requires minimal reagents and buffers and as the same plate is used for culturing, dosing and detection, plasticware costs are kept to a minimum. The assay can also be applied to an automated system should the equipment be available. As the cELISA measures intracellular GFAP in its natural state, it does not involve the lengthy preparation of cellular fractions or lysates and consequently the speed of the assay is increased. By measuring GFAP in an assay employing intact cells in this way, it is possible to conduct parallel assays for other biological endpoints using the same number of cells and thus providing continuity throughout assays and endpoints. As such the cell based ELISA will be used in this investigation for the semi-quantification of GFAP levels.

As is easily permitted with this procedure, parallel assays for mitochondrial dehydrogenase activity and proliferation will also be performed using the same cell seeding concentrations as the GFAP assay. While the MTT reduction assay is normally used for the assessment of cell viability as a measure of cytotoxicity, it has also been employed previously to measure cell activation (Gerlier 1986). MTT turnover increased markedly in primary astrocyte cultures and the dBcAMP pre-treated rat C6 glioma cell line in response to a variety of toxic compounds (Cookson *et al.* 1995). In chapter 3 of this present study, elevations were also observed following acrylamide exposure in the CCF-STTG1 astrocytoma line and chloroquine diphosphate in the U373-MG astrocytoma and SH-SY5Y neuroblastoma lines. While metabolic activation responses are not restricted to astrocytes, responses in cultured astrocytes have been found to be particularly sensitive (Cookson and Pentreath 1994; Mead and Pentreath 1998). By assaying for MTT turnover following low-level exposure in this investigation, it may be possible to determine whether a correlation exists between metabolic activation and an increase in GFAP expression.

The effects of toxin exposure on GFAP immunoreactivity (GFAP-IR) and metabolic activation can only be accurately assessed if controls are performed for proliferation to determine whether any biological effects observed are not just due to changes in cell number. In order to determine any proliferative effects, a method must be chosen which is both suitable for an assay format and applicable to astrocyte activation studies. While a biological assay procedure such as the neutral red uptake assay would have been the most time and cost effective option, the fact that activation effects have been observed in astrocytes when assaying for mitochondrial function (Mead and Pentreath 1998) means it is difficult to rule out the possibility of similar activation effects on other metabolic processes. Similarly, increases in protein expression in cells following activation, particularly GFAP, suggests that determination of total protein is also not a suitable method for the evaluation of proliferation. The determination of cell number by counting is one of the simplest methods of assessing proliferation. By doing so it is possible to determine total cell number and not just viable cell counts. In addition, metabolic activation is not a limiting factor and thus cell counting was selected as the preferred method to assess cell numbers. While manual counting of cells using Trypan blue staining and a haemocytometer is extremely time-consuming, an automated, Coulter particle counter can expedite this process.

When counting cells using the Coulter counter, particles suspended in a weak electrolyte solution are drawn through a small aperture, separating two electrodes between which an electric current flows. The voltage applied across the aperture creates a "sensing zone". As particles pass through this sensing zone they displace their own volume of electrolyte, momentarily increasing the impedance of the aperture. This impedance creates a pulse that is detected by the machine and recorded in real time. According to the Coulter principle, the size of the pulse is directly proportional to the tri-dimensional volume of the particle that produced it. Analyzing these pulses enables the sizing of particles within suspension and by setting the apparatus to count only particles of a certain size, the concentration of cells in the sample can be determined. By preparing suspensions of the treated cells from each well it will be possible to determine the total number of cells in each well, and consequently whether cell numbers are affected by the different compounds and concentrations being assessed.

Cells will be exposed to the four chosen compounds previously investigated for cytotoxicity in Chapter 3; ethanol, trimethyltin chloride, chloroquine diphosphate and acrylamide. As previously mentioned, the first three of these compounds have all been shown to induce changes in GFAP levels following treatment in non-human animal models (Cookson and Pentreath 1994). Acrylamide, a known specific neurotoxicant, has no such effects (Cookson *et al.* 1994) and has consequently been chosen for use as a negative control. In addition to these four compounds, cells will also be exposed to chosen concentrations of bacterial lipopolysaccharide (LPS), a major constituent of the cell wall of most gram-negative bacteria that can be easily isolated and is widely used in life science research.

Lipopolysaccharide, as the name suggests, is made up of polysaccharide and lipid. As can be seen in figure 4.1, the polysaccharide component consists of the core polysaccharide and distal O-polysaccharide which is highly variable among bacterial strains and is the portion of the outer membrane which is recognised by host antibodies. Bound to the core polysaccharide by a specific sugar called 2-keto-3-deoxyoctulosonic acid is the hydrophobic lipid A portion (Rietschel 1982) which serves to anchor the LPS molecule to the outer bacterial membrane and is regarded as the region responsible for the toxic effects observed when LPS is released from the outer membrane.

LPS, also referred to as bacterial endotoxin, is a highly immunogenic antigen that is able to induce an immune response in a variety of cell types, including cells of astrocytic origin, as has been illustrated in investigations showing that LPS induces the expression of proinflammatory cytokines in astrocytes both *in vivo* (Lemke *et al.* 1999) and *in vitro* using human astrocytoma cell lines (Park *et al.* 2003 and Palma *et al.* 1995). LPS is also able to stimulate the release of pro-inflammatory chemokines, which promote the migration of immune system cells such as leukocytes to the site of injury (Laing and Secombes 2004). Of particular interest are studies by Oh *et al.* (1999) where LPS was found to induce chemokine release in the U251-MG and U373-MG cell lines to be used in this study. There exists a wealth of data to also suggest that LPS causes an increase in GFAP expression in astrocytic cells following both *in vivo* administration in rodents (Sugaya *et al.* 1998; Cai *et al.* 2003; Kaya 2004; Iravani *et al.* 2005; Herber 2006) and after treatment in primary glial cells (Boyen 2004) and in the rat C6 glioma cell line (Pentreath *et al.* 1994). The effects of LPS on GFAP in human glioma cell lines has not, to date, been investigated and so

Cytoplasm | Outer cell membrane | Extracellular space

O-specific Polysaccharide

Lipid A — Core Oligosaccharide — ☐☐☐☐☐☐
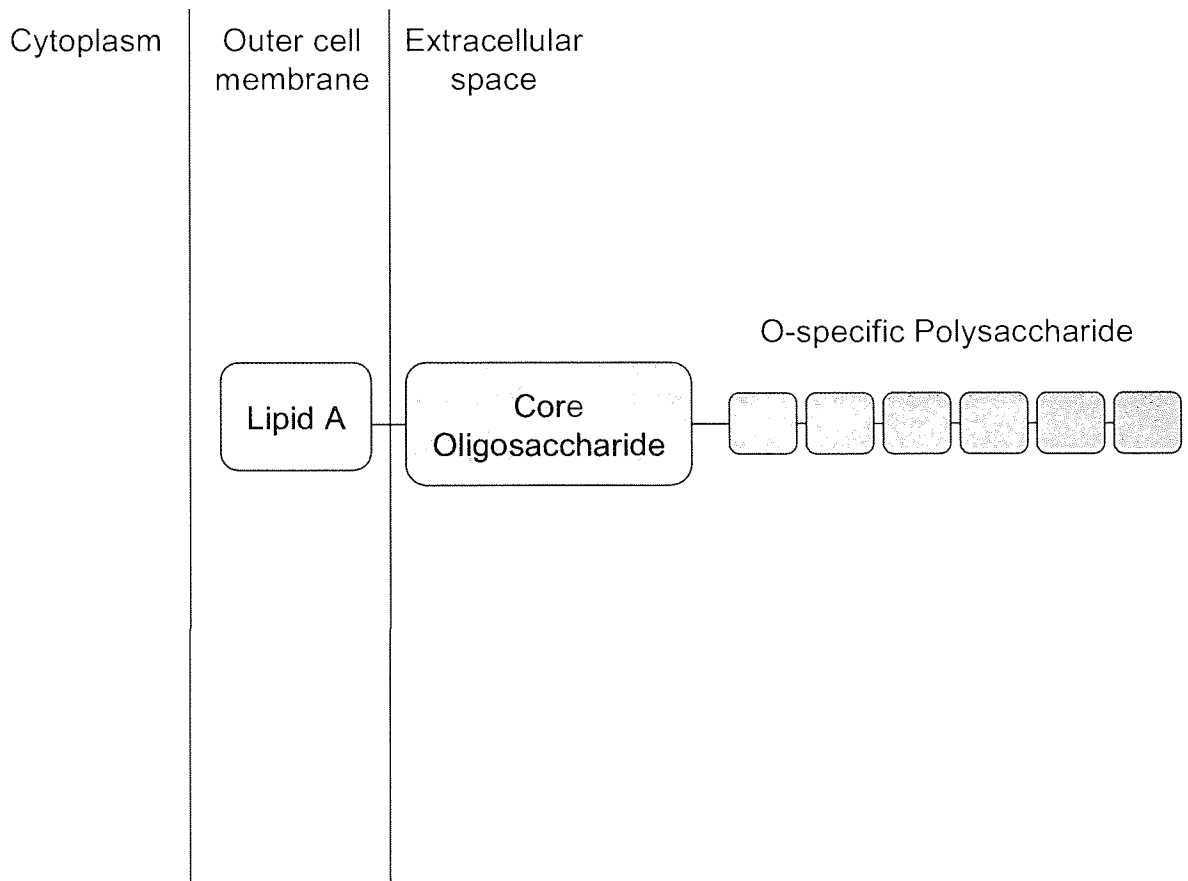
Figure 4.1 Schematic diagram of a typical bacterial lipopolysaccharide structure.
The lipopolysaccharide molecule consists of the membrane bound Lipid A portion that
is responsible for the toxic properties of the molecule once it becomes dissociated from
the membrane, the immunogenic core oligosaccharide and the outermost antigenic O-
polysaccharides.

consequently LPS was chosen to determine whether a reactive response, as characterised by an increase in GFAP expression, can be achieved in the three human astrocytoma cell lines when exposed to a biological inducer as opposed to toxicant exposure.

As with the cytotoxicity analysis in Chapter 3, toxicant exposure will be for an initial period of 24 hours. This time point coincides with similar experiments conducted using rodent primary cultures and glioma cell lines (Cookson and Pentreath 1994; Cookson *et al.* 1995; Mead and Pentreath 1998) and was thus chosen to allow comparison with existing data. *In vitro* treatment using cultured cells has largely been limited to 24 hours exposure or greater. To the author's knowledge there has been no investigation into the time course of onset of gliotic changes in cultured cells, despite increases in GFAP being of the earliest hallmarks of reactive gliosis (Eng *et al.* 2000). Consequently, a further time point of 4 hours has been chosen to provide a representation of the initial toxic response as well as an intermediate time of 16 hours.

It is hoped that by using these chosen methods, it will be possible to evaluate whether a reactive response, as characterised by increases in GFAP and/or metabolic activity, is achievable in single cell cultures of human astrocytic cell lines following exposure to known gliosis causing compounds. Comparing results at several time points may help to develop an idea of the time course of onset of any reactivity responses. It will also be of interest to observe any toxicant specific or cell line specific effects that may occur at each of these time points.

4.2 Methods.

4.2.1 Chemicals, reagents and tissue culture materials.

Cell culture reagents and saline buffers were purchased from Gibco. Toxicants were sourced as in section 3.2.1. LPS from *Escherichia coli* 0111:B4 was purchased from Sigma and reconstituted in PBS (pH 7.4) in accordance to the manufacturer's instructions before storage at -20°C. Dried skimmed milk powder was of the Marvel® brand and was purchased locally. All other chemicals and reagents were purchased from Sigma unless otherwise stated. All tissue culture plastics were from Corning.

4.2.2 Cell cultures.

Cell lines were maintained as outlined in section 2.2.2. Cells were seeded for assay in tissue culture treated 96 well microplates as described in section 3.2.2 at the desired seeding densities in accordance with the results of the cell seeding optimisation experiments (see section 4.3.1.2). Cells were allowed to attach to the culture plate and stabilise overnight at 37°C before treatment.

4.2.3 Experimental treatment.

Glioma cell lines were exposed to logarithmic dilutions of chloroquine diphosphate and trimethyltin chloride (TMTC) from 0.1nM to 10mM and ethanol and acrylamide from 1nM to 100mM. The different concentrations were employed to encompass a range of sub-cytotoxic and cytotoxic concentrations for each toxicant. Cells were also exposed to bacterial lipopolysaccharide at concentrations of 0.5, 1 and 5μg/ml. Using pre-filtered sterile stock solutions of each compound, working dilutions were prepared in the appropriate tissue culture medium according to the cell line being exposed (see section 2.2.2). Cells were exposed to toxicants for an initial time period of 24 hours followed by additional time points of 4 and 16 hours. Following exposure, cell reactions to the toxicants were determined by assaying for GFAP content using a cell based ELISA and for mitochondrial dehydrogenase activity using the MTT assay. Cell counts were also taken for each concentration to control for cell proliferation.

4.2.4 Immunological assay for GFAP.

Cellular levels of the astrocytic glial fibrillary acidic protein were determined by cell based enzyme-linked immunosorbent assay. The method used in this investigation was

provided kindly by Dr Agnieszka Kinsner at ECVAM (European Centre for the Validation of Alternative Methods), (Ispra, Italy). Modifications have been made to this original method in order to optimise the antibody concentrations and to minimise non-specific binding by the further addition of blocking agents. The evolution of these modifications including the original experimental design, the measures taken to optimise certain steps of the assay and the final experimental protocol are as follows:

4.2.4.1 Development of the cELISA protocol.

4.2.4.1.1 Original cELISA experimental design; before modifications.

Following toxicant exposure, the medium is removed from the wells of the 96 well microtitre plate and each well washed briefly with PBS (pH 7.4) at room temperature. Cells are fixed in 200μl of ice cold 100% ethanol for 30 minutes at -20°C and then washed a further 3 times in PBS for 5 minutes each time at room temperature. The plates are then blocked with 100μl of PBS containing 0.1% Triton X-100 (PBS-Tx) and 1% BSA (PBS-TxB) for 1 hour at room temperature, then incubated with mouse anti-GFAP antibody, clone GA5 (Chemicon), diluted 1:1000 in PBS-TxB, for 2 days at 4°C. After the incubation period, the antibodies are aspirated and each well washed three times for 3 minutes each time in PBS-Tx. The anti-mouse IgG horseradish peroxidise-conjugated secondary antibody is then diluted (Santa Cruz Biotechnology) 1:1000 in PBS-TxB and 100μl added to each well. The plates are then incubated at room temperature with gentle shaking for 1 hour. The secondary antibody solution is then aspirated and the wells washed 5 times for 3 minutes each time with PBS-Tx, then 100μl of ABTS (2,2'-AZINO-bis[3-ethylbenziazoline-6-sulfonic acid]) horseradish peroxidise substrate is added (Chemicon) to each well while protecting from the light. The plate is then covered in foil and incubated for 30 minutes at room temperature with gentle shaking. Finally, the colour change caused by the HRP conjugate reacting with ABTS in each well is analysed using a microplate reader, set at 405nm.

4.2.4.1.2 Modifications to the blocking stage of the GFAP ELISA.

In order to minimise non-specific binding by the GFAP primary antibody, additional blocking steps were evaluated. Initially, the original cELISA was performed as described above on wells of a 96 well culture plate in the absence of cells. Test wells were incubated with or without an additional 5% dried skimmed milk in both the blocking buffer and the primary antibody diluents. Following these modified stages, the cELISA was

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completed as in the original protocol and the level of immunoreactivity per well determined as the absorbance at 405nm. Any significant effects of the modifications were determined using unpaired two-tailed Students t-tests.

4.2.4.1.3 Cell Number calibration and antibody optimisation of GFAP ELISA.

Optimum cell seeding concentrations of each glioma cell line for the ELISA assay were determined by performing the cELISA procedure (with additional blocking modifications) on sequential two-fold dilutions of cells from 100 up to 100000 cells per well, including a cell free control. At the primary incubation stage, different wells of each cell density were incubated with three different antibody dilutions of 1:1000, 1:2000 and 1:5000 in order to determine optimum antibody concentrations, where minimal non-specific binding was observed without impairing the ability to detect maximal GFAP. A primary antibody-free control was also included at each cell density. Absorbance at 405nm was plotted against log cell number and the optimal seeding density was taken from the linear portion of the curve produced. As a negative control, the above procedure was repeated using the SH-SY5Y neuroblastoma cell line, using only the highest antibody dilution of 1:1000, in order to determine the levels of non-specific binding of the GFAP antibody to this non-astrocytic GFAP negative cell line.

4.2.4.2 Modified cELISA for GFAP as performed in this study.

Following the above modifications, the final methods for the cell based ELISA for GFAP are as follows: Subsequent to toxicant exposure, the medium was removed from the wells of the 96 well microtitre plate and each well was washed briefly with PBS (pH 7.4) at room temperature. The cells were fixed in 200μl of ice cold 100% ethanol for 30 minutes at -20°C and then washed a further 3 times in PBS for 5 minutes each time at room temperature. The wells were blocked with 100μl of PBS containing 0.1% Triton X-100 (PBS-Tx), 1% BSA and 5% dried skimmed milk (PBS-TxBM) for 1 hour at room temperature. Wells were then incubated with mouse anti-GFAP antibody, clone GA5 (Chemicon), diluted 1:5000 in PBS-TBM for assays testing U251-MG and U373-MG cell lines and 1:1000 for the CCF-STTG1 line, for 2 days at 4°C. After the incubation period, the antibody was aspirated and each well washed three times for 3 minutes each time in PBS-Tx. Anti-mouse IgG horseradish peroxidise conjugated secondary antibody (Santa Cruz Biotechnology) was diluted 1:1000 in PBS-TxBM and 100μl added to each well. Plates were wrapped in foil and incubated at room temperature with gentle shaking for 1

hour. The secondary antibody solution was aspirated and cells washed 5 times for 3 minutes each time with PBS-Tx, then 100μl of ABTS (2,2'-AZINO-bis[3-ethylbenziazoline-6-sulfonic acid]) horseradish peroxidise substrate (Chemicon) was added to each well while protected from the light. The plate was then covered in foil and incubated for 30 minutes at room temperature with gentle shaking. Each well was then analysed for colour changes using a Thermo Multiskan EX 96-well microplate reader (Thermo Electron Corporation), reading absorbance at 405 nm. Appropriate control wells were also included on each assay plate in which either the primary antibody or cells were omitted. Background antibody binding as represented by cell free control values were subtracted from all other test values before further analysis. The level of GFAP immunoreactivity per well was subsequently expressed as a percentage of the non-toxicant treated control wells from the same experiment.

4.2.5 Assay for metabolic activity.

Metabolic function of the treated cells was evaluated using the MTT assay as described in section 3.2.4. Briefly, following exposure, media was aspirated from the cells and 100μl of fresh media and 30μl of 5mg/ml MTT was added to each well. Following 4 hours incubation 100μl of 10 % SDS in 0.1 M HCl was also added and plates were incubated overnight. Absorbance readings were taken at 590 nm. Appropriate cell free controls were included on each assay plate to determine the absorbance of the reagents alone. These values were then subtracted from all other test values before further analysis. MTT turnover per well was subsequently expressed as a percentage of the non-toxicant treated control wells from the same experiment.

4.2.6 Determination of cell number using the Coulter counter.

In order to determine any change in cell number due to a proliferative effect of the test compound, cells were counted using a Coulter Z1 particle counter (Beckman Coulter) Briefly, following exposure, media containing the test compound was aspirated and the cells were treated with 100μl of trypsin EDTA solution to detach cells from the tissue culture surface. 100μl of fresh media was added to each well to give 200μl of cell suspension that was then added to clean Coulter vessels together with 10ml of Isoton II buffer (Beckman Coulter). The vessels were assembled within the coulter counter, which by drawing a known volume of the particle suspension through the aperture electronically counted the number of cells. To coincide with results from both ELISA and MTT assays,

cell counts in each well were expressed as a percentage of non-toxicant treated controls from the same experiment.

4.2.7 Data and statistical analysis.

For blocking experiments, cell number and antibody concentration calibrations, data are expressed as mean absorbance (± SEM). For GFAP, MTT and cell number assays data are expressed as a mean percentage of non-treated control wells ± SEM. Any significant changes in GFAP immunoreactivity (GFAP-IR), MTT turnover or cell numbers in response to toxicant exposure were evaluated using one-way ANOVA followed by Dunnet's post-test where all data points were compared with a non-treated control. GraphPad Prism software (Version 3.02) was used for this purpose.

4.3 Results.

4.3.1 Assay modifications and optimisations.

4.3.1.1 Blocking modifications.

It was observed that the original cELISA protocol resulted in a high level of non-specific antibody binding, even to the culture plate in the absence of cells. The choice was made to evaluate the ability of dried skimmed milk powder as an additional blocking agent in reducing this initial background binding. Figure 4.2 shows that the addition of 5% dried skimmed milk to both the blocking buffer and primary antibody diluent resulted in a significant decrease in non-specific binding to the culture plate ($p<0.01$). Consequently, these modifications were employed in all subsequent assays.

4.3.1.2 GFAP assay antibody optimisation and cell number calibration.

Prior to implementing the cELISA assay for the detection of GFAP, it was necessary to optimise the assay for the specifications of the cells used. This involved choosing appropriate primary antibody dilutions and cell seeding densities in order to achieve optimal results from the assay. In order to do this, the cELISA assay was performed on sequential two-fold cell concentrations using three different primary antibody dilutions from 1:1000 to 1:5000.

Optimum antibody dilutions were determined as the lowest concentration needed to produce the lowest background signal whilst still achieving an appropriate sigmoidal curve with which the range of the linear portion is maintained as with higher concentrations. Likewise to results in section 4.3.1.1, figures 4.3 to 4.5 illustrate how the more diluted the primary antibody, the less non-specific binding was observed, particularly at low cell seeding densities. For the U251-MG cell line the linear portion ranges of the curves for each antibody concentration were the same; from 800 cells per well to 6000 cells per well, at which point all three curves plateau, indicating maximal binding or that the assay has reached the limits of detection (figure 4.3). This same pattern can be observed for the U373-MG cell line in figure 4.4, where the range of the linear portions, produced using all three antibody concentrations, was 1600 to 25000 cells per well, again at which point a plateau was observed in all curves. The lack of any effect of lowering primary antibody concentrations in both the U251-MG and U373-MG cell lines on the ability to detect GFAP led to the choice that the highest dilution of 1:5000 would be applied to these two
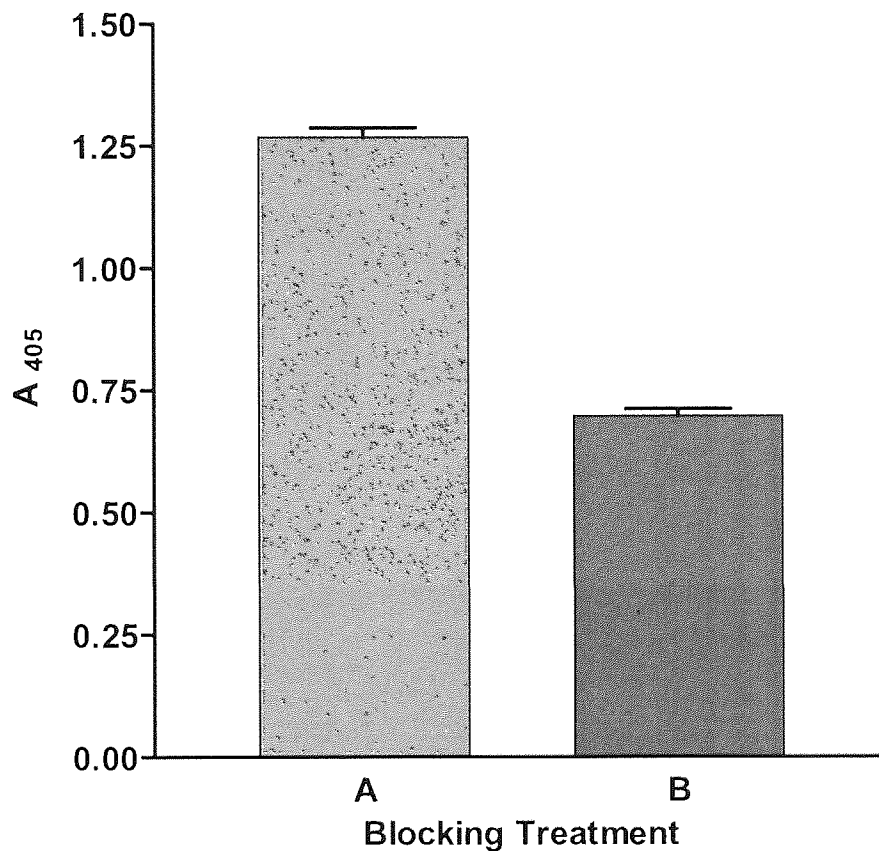
Figure 4.2 Comparison of non-specific binding of the GA5 anti-GFAP primary IgG following varying degrees of blocking. Comparable absorbance values following the cELISA assay using A) the original blocking procedure or B) with an additional 5% dried skimmed milk in the blocking solution and primary antibody diluent. Data are expressed as mean absorbance at 405nm ± SEM (n=2 from a single experiment).
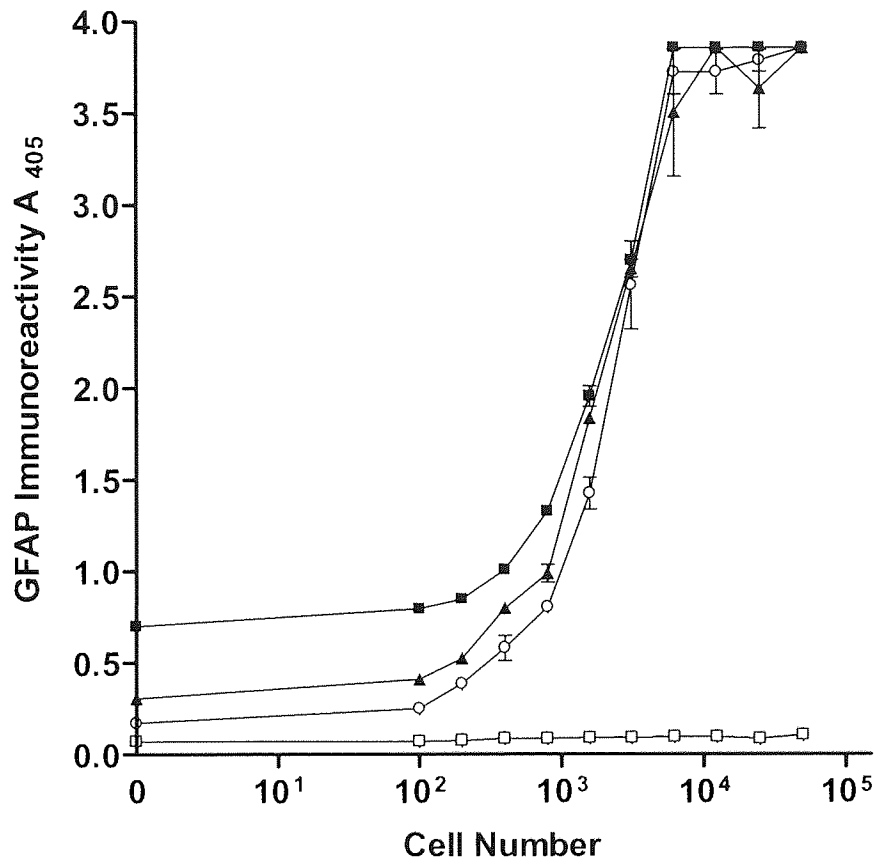
Figure 4.3 Cell seeding density and antibody concentration calibration of the GFAP ELISA for the U251-MG human astrocytoma cell line. GFAP Immunoreactivity in U251-MG cell populations of increasing density from 0 to 50 000 cells per well using anti-GFAP primary antibody at dilutions of 1:1000 (■), 1:2000 (▲) and 1:5000 (○) as well as a no antibody control (□). Data are expressed as mean absorbance at 405nm ± SEM (n=2 from a single experiment).
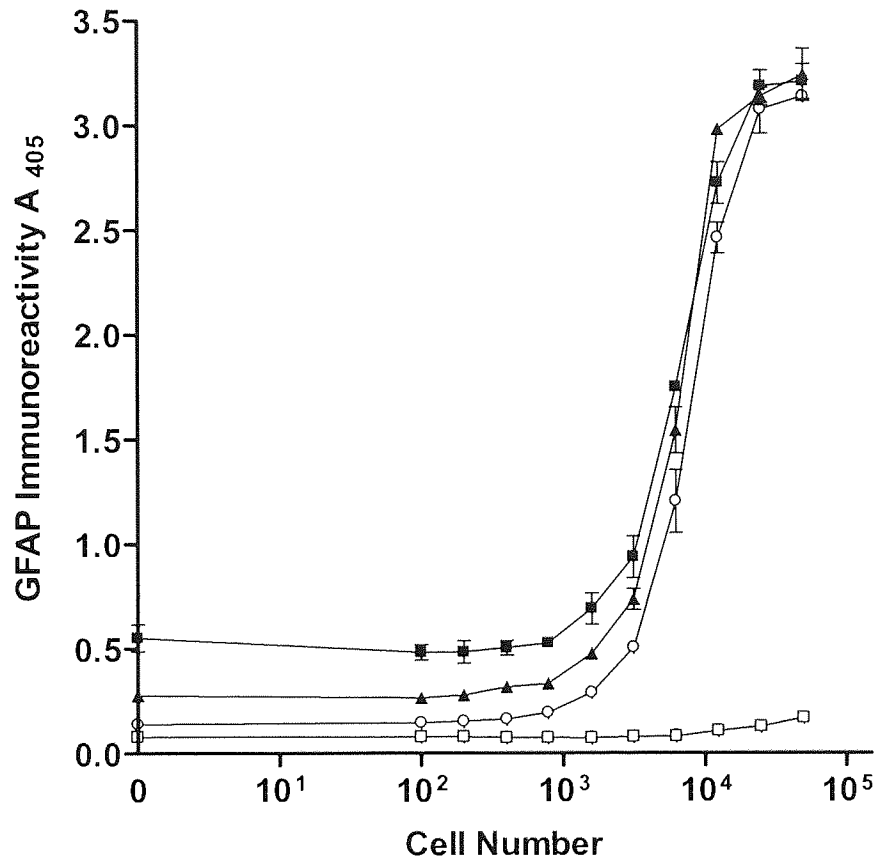
Figure 4.4 Cell seeding density and antibody concentration calibration of the GFAP ELISA for the U373-MG human astrocytoma cell line. GFAP Immunoreactivity in U373-MG cell populations of increasing density from 0 to 50 000 cells per well using anti-GFAP primary antibody at dilutions of 1:1000 (■), 1:2000 (▲) and 1:5000 (○) as well as a no antibody control (□). Data are expressed as mean absorbance at 405nm ± SEM (n=2 from a single experiment).
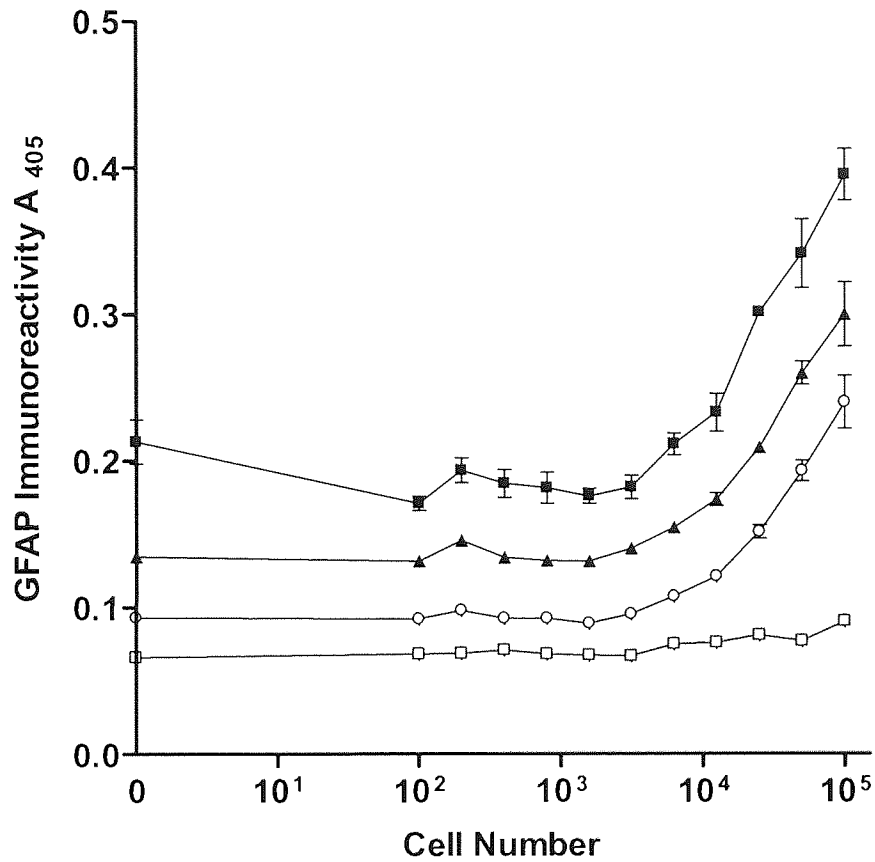
Figure 4.5 Cell seeding density and antibody concentration calibration of the GFAP ELISA for the CCF-STTG1 human astrocytoma cell line. GFAP Immunoreactivity in CCF-STTG1 cell populations of increasing density from 0 to 100 000 cells per well using anti-GFAP primary antibody at dilutions of 1:1000 (■), 1:2000 (▲) and 1:5000 (○) as well as a no antibody control (□). Data are expressed as mean absorbance at 405nm ± SEM (n=2 from a single experiment).
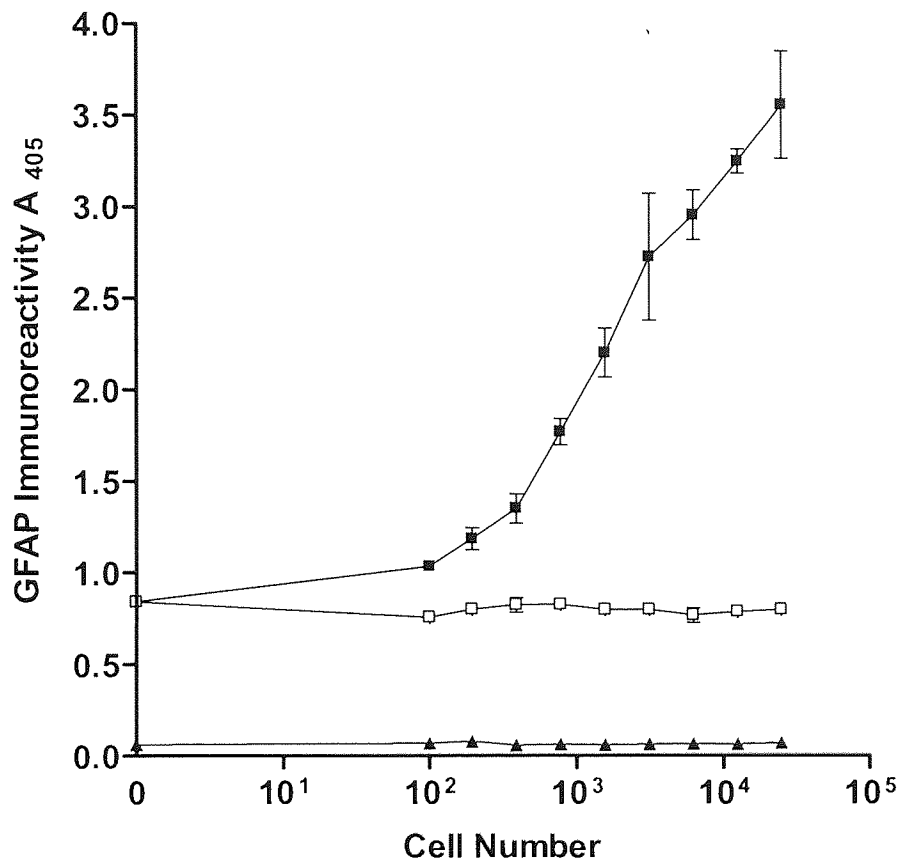
Figure 4.6 Comparison of GFAP-IR in the U251-MG astrocytoma and the SH-SY5Y neuroblastoma cell line as applied to the cell based ELISA for GFAP. A representative graph of comparable GFAP immunoreactivity in the U251-MG (■) and SH-SY5Y (□) cell populations of increasing density from 0 to 25 000 cells per well. anti-GFAP primary antibody, clone GA5 was used at dilutions of 1:1000 including a no antibody control (▲). Data are expressed as mean absorbance at 405nm ± SEM (n=2 from a single experiment).

cell lines in this study. Optimum seeding densities of both the U251-MG and U373-MG cell lines were determined from the linear portions of the curves achieved using 1:5000 primary antibody dilution. As GFAP binding was extremely high in these two lines of up to A_{405} values of 3.8 (± 0.013), much higher than the ideal starting absorbance value of 1, the optimal seeding density was chosen to be the lowest concentration of cells that still remained well within the linear portion of the curve. These seeding concentrations were determined to be 2000 and 6000 cells per well for the U251-MG and U373-MG cell lines respectively. The CCF-STTG1 cell line, on the other hand, exhibited much lower GFAP-IR than the other two astrocytoma cell lines as detected by the cELISA (figure 4.5). This was reflected in the inability to reach absorbance levels, even at 100 000 cells per well, of those achieved with the other lines. As the use of all antibody dilutions resulted in a similar increase in GFAP-IR with increasing CCF-STTG1 cell number, albeit still at relatively low levels, the highest concentration of primary antibody (1:1000 dilution) was chosen for use along with a relatively large number of 25 000 cells per well in order to achieve an appropriate level of signal that could be detected in this assay.

The GFAP negative SH-SY5Y human neuroblastoma cell line exhibited no increase in immunoreactivity with increasing cell numbers when subjected to the same anti-GFAP cELISA as the astrocytoma cell lines. Figure 4.6 shows a comparison of the cell number calibration curves of the SH-SY5Y cell line and the U251-MG astrocytoma cell line, as well as a no primary antibody control. In contrast to a large increase in GFAP-IR with increasing U251-MG cell numbers, immunoreactivity with increasing SH-SY5Y cell numbers remains at control levels. It can also be seen that there is a degree of non-specific binding of the primary antibody in the wells of the SH-SY5Y assay but that this is unrelated to the presence of cells.

4.3.2 Experimental toxicant exposure.

The three human astrocytoma cell lines were treated with increasing concentrations of ethanol, chloroquine diphosphate, trimethyltin chloride and acrylamide from sub-cytotoxic to known cytotoxic levels, as well as concentrations of bacterial lipopolysaccharide. In order to determine the effect of these compounds on the potential astrogliotic responses of the three cell lines, levels of intracellular GFAP were assessed using a cell based ELISA. Cells were also assayed for their mitochondrial dehydrogenase activity by MTT assay to determine any increase in mitochondrial function as an indicator

of metabolic reactivity. Cell counts were also taken post exposure as a control for cell proliferation. These parameters were assessed following toxicant and LPS exposure for an initial period of 24 hours followed by subsequent investigation at 4 and 16 hours.

4.3.2.1 Effect of bacterial lipopolysaccharide on GFAP-IR, MTT turnover and cell numbers.

24 hours incubation with LPS had no elevating effect on GFAP-IR in any of the three glioma cell lines tested (figure 4.7A), however, when compared to control values, a significant reduction in GFAP-IR was observed in the U251-MG cells at 0.5μg/ml (p<0.001) and 5μg/ml (p<0.05) and in the U373-MG cells at all three concentrations tested (p<0.05), however, GFAP-IR in the CCF-STTG1 cells was unaffected. Differential effects of LPS on MTT turnover were observed for each cell line following 24 hours exposure (figure 4.7B). In the U251-MG cell line, MTT turnover was 10.5(±2.61) and 12.0(±2.99) percent higher than control values in cells exposed to LPS concentrations of 1 and 5μg/ml respectively (p<0.001), but not at the lowest concentration tested of 0.5μg/ml. The contrary was observed in the U373-MG line where, compared to controls, MTT turnover was significantly increased by 8.4(±2.34) percent at the lowest concentration of 0.5μg/ml (p<0.05), but was significantly reduced at a higher concentration of 5μg/ml (p<0.05). In the CCF-STTG1 cell line, no change in MTT turnover was observed. Cell Counts were also unaffected in all three cell lines following 24-hour exposure to LPS (figure 4.7C).

Following subsequent investigation at reduced incubation times, 16 hours treatment with LPS had no significant effect on GFAP-IR in any of the three glioma cell lines tested (figure 4.8A). MTT turnover, however, was significantly elevated with a 6.8(±1.63) percent increase, compared to control values, observed in the U251-MG cell line following exposure with 0.5μg/ml LPS (p<0.05) (figure 4.8B). Similarly the U373-MG line exhibited 9.5(±2.14) and 8.4(±2.39) percent increases at 1 and 5μg/ml, respectively (p<0.05). No increase in MTT turnover was observed in the CCF-STTG1 cell line. Similar to 24 hours incubation, cell numbers were unaffected by LPS in each of the three glioma cell lines following 16 hours exposure (figure 4.8C).

Unlike both 24 and 16 hours exposure, 4 hours treatment with LPS did have an elevating effect on GFAP-IR in the U251-MG cell line (figure 4.9A) where levels were seen to be significantly increased to 17.0(± 5.02) percent above control levels when treated

Figure 4.7 Effect of bacterial lipopolysaccharide on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 24 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 2 individual experiments, for cell counts, n=6 from 2 individual experiments).

Figure 4.8 Effect of bacterial lipopolysaccharide on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 16 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 2 individual experiments, for cell counts, n=6 from 2 individual experiments).
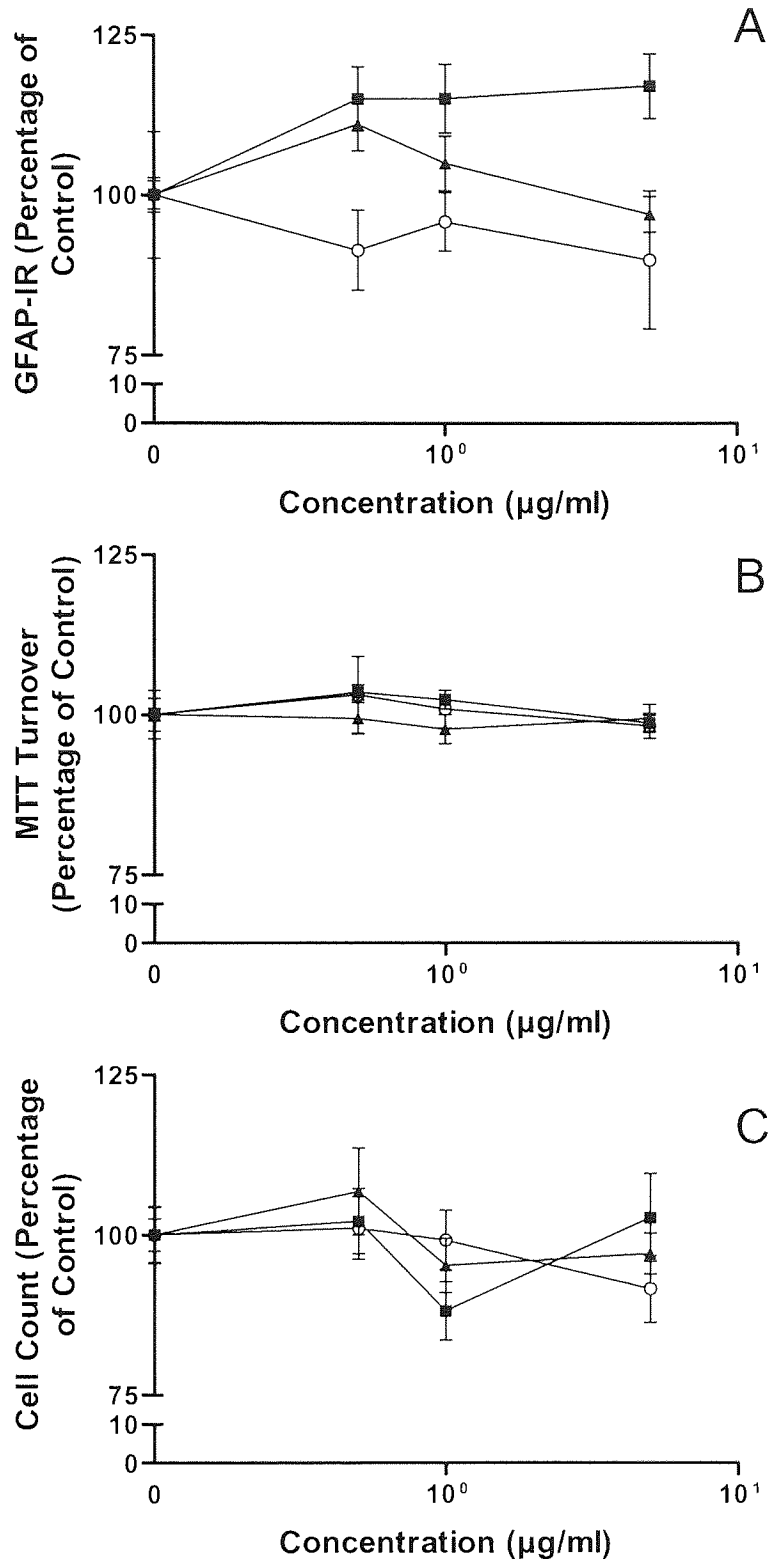
Figure 4.9 Effect of bacterial lipopolysaccharide on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 4 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 2 individual experiments, for cell counts, n=6 from 2 individual experiments).

with the highest concentration tested of 5µg/ml (p<0.05). There was no effect, however, on GFAP-IR in either the U373-MG or CCF-STTG1 cell lines. 4 hours incubation also saw no effect on MTT turnover (figure 4.9B), nor was cell numbers affected in any of the three cell lines (figure 4.9C).

4.3.2.2 Effect of ethanol on GFAP-IR, MTT turnover and cell numbers.

Treatment with ethanol for 24 hours resulted in significant increases in GFAP-IR in all three glioma cell lines (figure 4.10A). In the U251-MG cell line, GFAP-IR levels were 24.7(±5.17) and 21.2(±2.78) percent higher than control values at ethanol concentrations of 1 and 10mM respectively (p<0.01). Likewise in the U373-MG cells, significant elevations in GFAP-IR to 39.4(±8.4) percent more than control values were seen at 10mM (p<0.01). The same increase was also observed in the CCF-STTG1 cell line at even lower ethanol concentrations of 0.1 and 1mM (p<0.05), at which GFAP–IR levels were 56.8(±15.7) and 58.9(±11.5) percent greater than controls. All concentrations of ethanol tested did not cause any significant decrease in GFAP-IR. In each of the three cell lines, MTT turnover was unaffected following 24-hour exposure to ethanol in the concentration range of the experiment (figure 4.10B). Ethanol also had no effect on cell number at the same time of exposure apart from a decrease in the CCF-STTG1 cells at the highest concentration tested (100mM) (p<0.01) (figure 4.10C).

Following shorter ethanol exposure periods of 16 and 4 hours, no effect on GFAP-IR was observed in any of the three cell lines at either time point (figures 4.11A and 4.12A). Like-wise, no effect was seen on MTT turnover apart from a slight significant decrease in the CCF-STTG1 line following both 16 and 4 hours exposure to 100mM ethanol (p<0.05) (figures 4.11B and 4.12B) as was seen at 24 hours. Also akin to results of 24 hours, there was no significant effect on cell number following ethanol exposure for either 16 or 4 hours (figures 4.11C and 4.12C).

4.3.2.3 Effect of chloroquine diphosphate on GFAP-IR, MTT turnover and cell numbers.

Incubation with chloroquine diphosphate for 24 hours resulted in a significant increase in GFAP-IR in the CCF-STTG1 cell line to 25.7(±6.04) and 32.5(±10.07) percent higher than control values at concentrations of 1µM (p<0.05) and 10µM (p<0.01) respectively (figure 4.13A). In the U251 and U373-MG cell lines, however, the compound

Figure 4.10 Effect of ethanol on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 24 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of a non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 3 individual experiments, for cell counts, n=6 from 3 individual experiments).
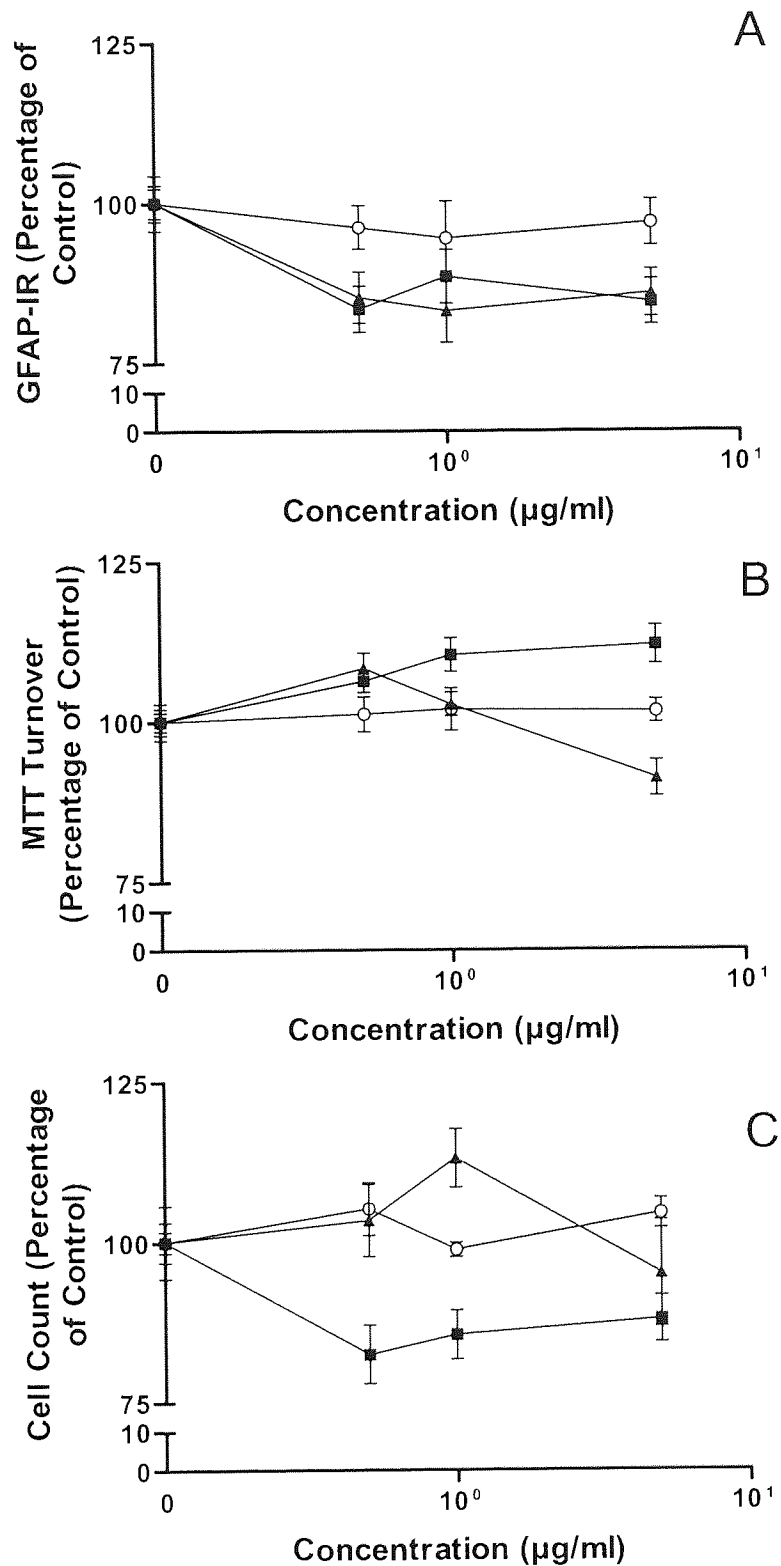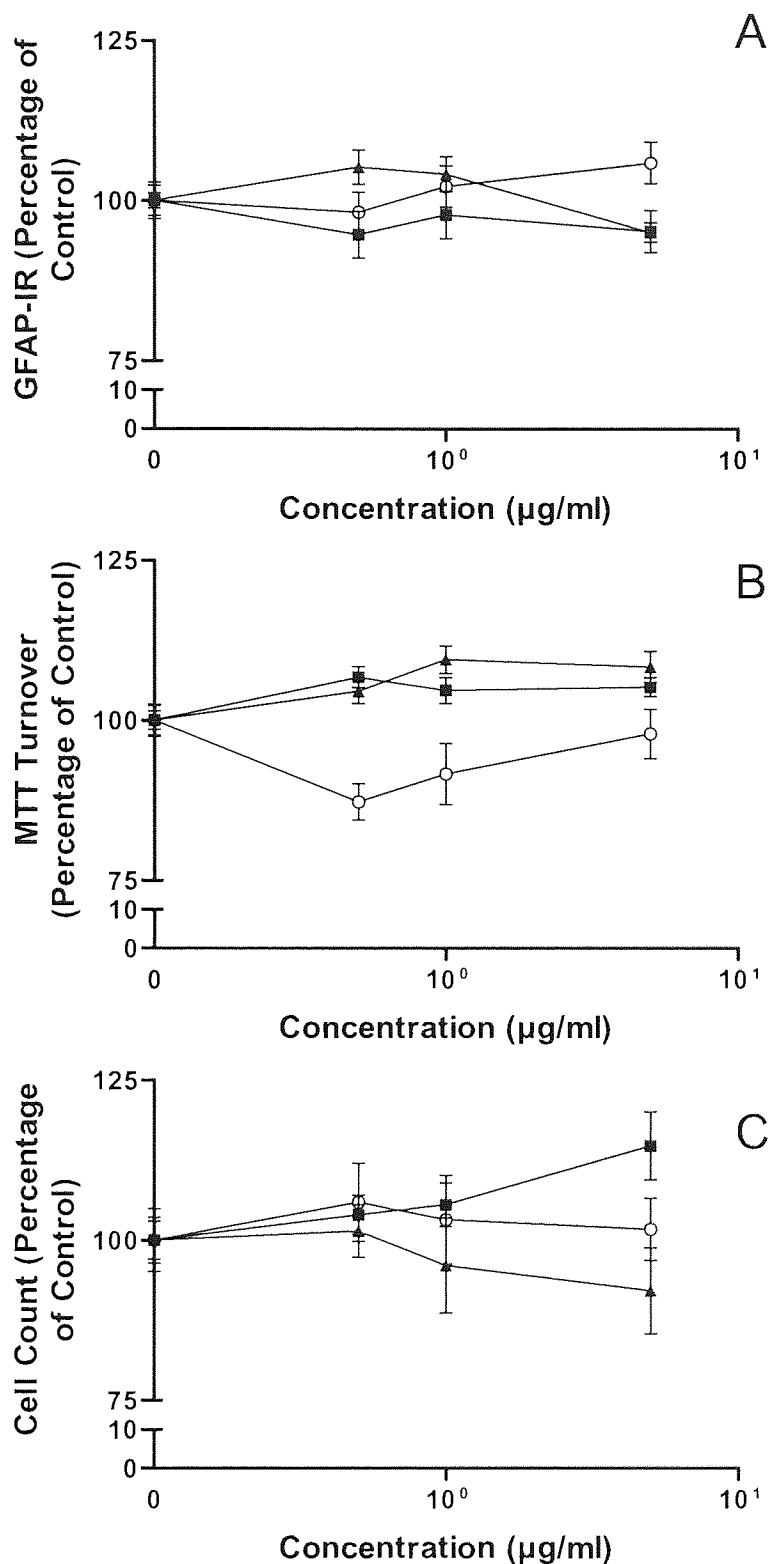
Figure 4.11 Effect of ethanol on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 16 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 3 individual experiments, for cell counts, n=6 from 3 individual experiments).

Figure 4.12 Effect of ethanol on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 4 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 3 individual experiments, for cell counts, n=6 from 3 individual experiments).

had no elevating effect on GFAP-IR. GFAP-IR was subsequently reduced at concentrations of and above 1mM in the U251-MG and CCF-STTG1 cell lines (p<0.01) and the lower concentration of 10μM in the U373-MG cells. There was no increase in MTT turnover following chloroquine diphosphate exposure for 24 hours in any of the glioma cell lines (figure 4.13B), however, significant reductions were observed at concentrations of and above 1μM in the CCF-STTG1 cells (p<0.01), and 10μM in the U373-MG (p<0.01) and U251-MG (p<0.05) cell lines. Significant reductions were also observed in cell counts of all cell lines at and above 1mM chloroquine diphosphate (p<0.01) (figure 4.13C), However, significant increases in U373-MG cell number to 39.5(±13.23) and 38.1(± 14.14) percent greater than controls, were also observed at very low levels of 10 and 100nM, much lower than any cytotoxic concentration observed in this study (p<0.05). This cell number increase was not, however, reflected by an increase in GFAP-IR or MTT turnover in the U373-MG cell line at these concentrations as described above.

Following a reduced incubation period of 16 hours chloroquine diphosphate had no elevating effect on GFAP-IR in the CCF-STTG1 cell line (figure 4.14A). Preliminary results for the U251-MG and U373-MG cell lines showed the same. In the U251 and CCF-STTg1, significantly decreased GFAP-IR was observed with concentrations at and above 1mM (P<0.01) and in the U373-MG at the lower concentration of 0.1mM (P<0.05). There was no elevating effect on MTT turnover in any of the cell lines (figure 4.14B). Turnover was decreased at much lower levels than at 24 hours in all three cell lines, at 1μM, 0.1μM and 10nM (P<0.05) in the U251-MG, U373-MG and CCF-STTG1 cell lines respectively. Cell counts were also reduced at and above 1mM in all three cell lines (P<0.01) (figure 4.14C). No cell number increases were observed as a result of 16 hours chloroquine diphosphate exposure.

Chloroquine diphosphate had no effect on GFAP-IR in the CCF-STTG1 cell line (figure 4.15A), however, preliminary results for the U251-MG line showed that unlike incubations for 24 and 16 hours, 4 hours treatment resulted in an increase in GFAP-IR to 20.3(±4.21) percent and 21.1(±4.13) percent above control levels 0.1μM (p<0.05) and 1μM (P<0.05) respectively. In the U373-MG line, no elevating effect was seen. The lowest concentration of chloroquine diphosphate that caused a significant decrease in GFAP-IR was 1mM in the U373-MG and CCFSTTG1 cell lines and 10mM in the U251-MG line. No

Figure 4.13 Effect of chloroquine diphosphate on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 24 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 3 individual experiments, for cell counts, n=6 from 3 individual experiments).
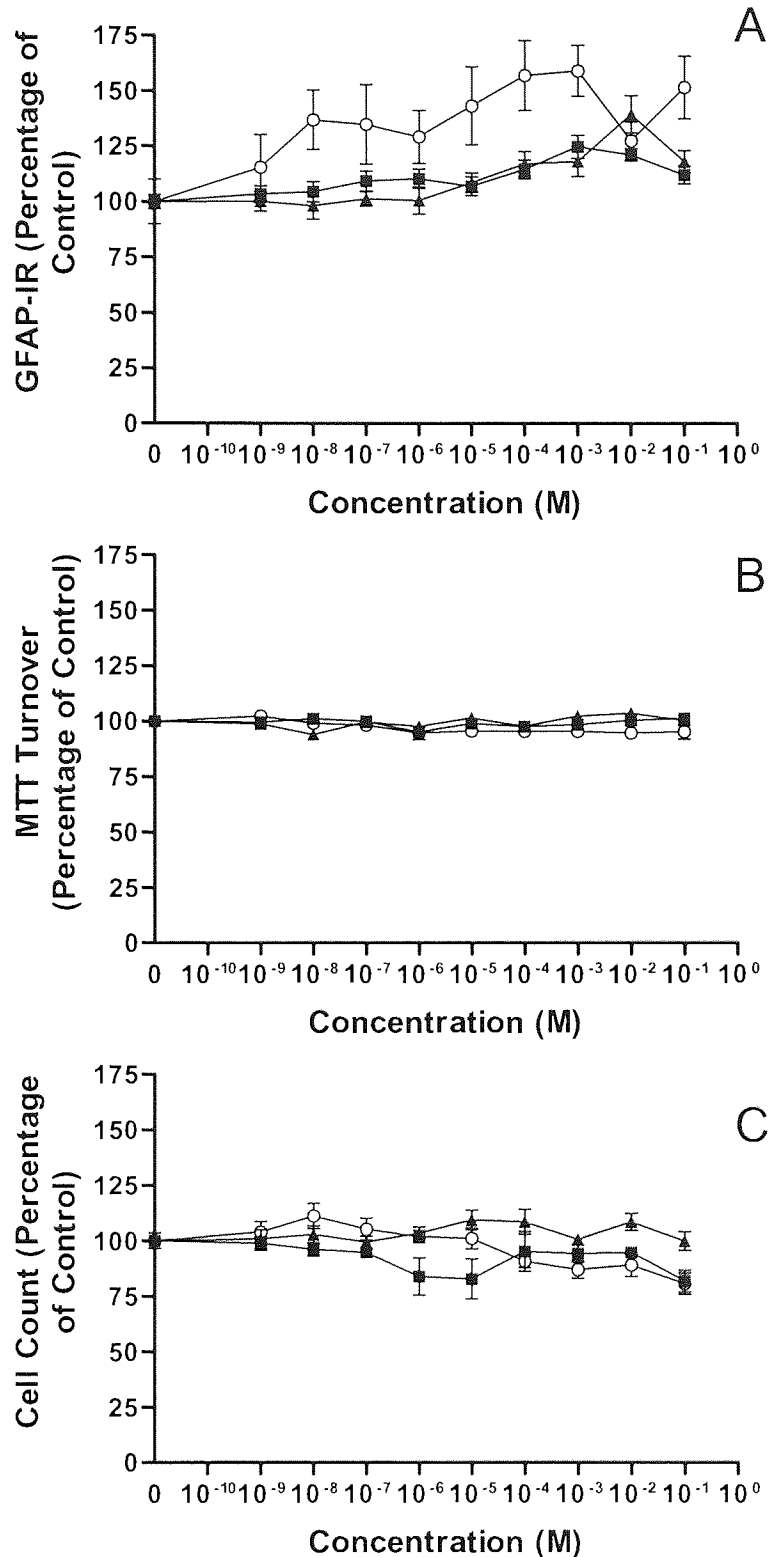
Figure 4.14 Effect of chloroquine diphosphate on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 16 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover in the CCF-STTG1 line, n=12 from three individual experiments and in the U251-MG and U373-MG lines n=4 from a single experiment. For cell counts, in the CCF-STTG1 line, n=6 from three individual experiments and in the U251-MG and U373-MG lines n=2 from a single experiment).
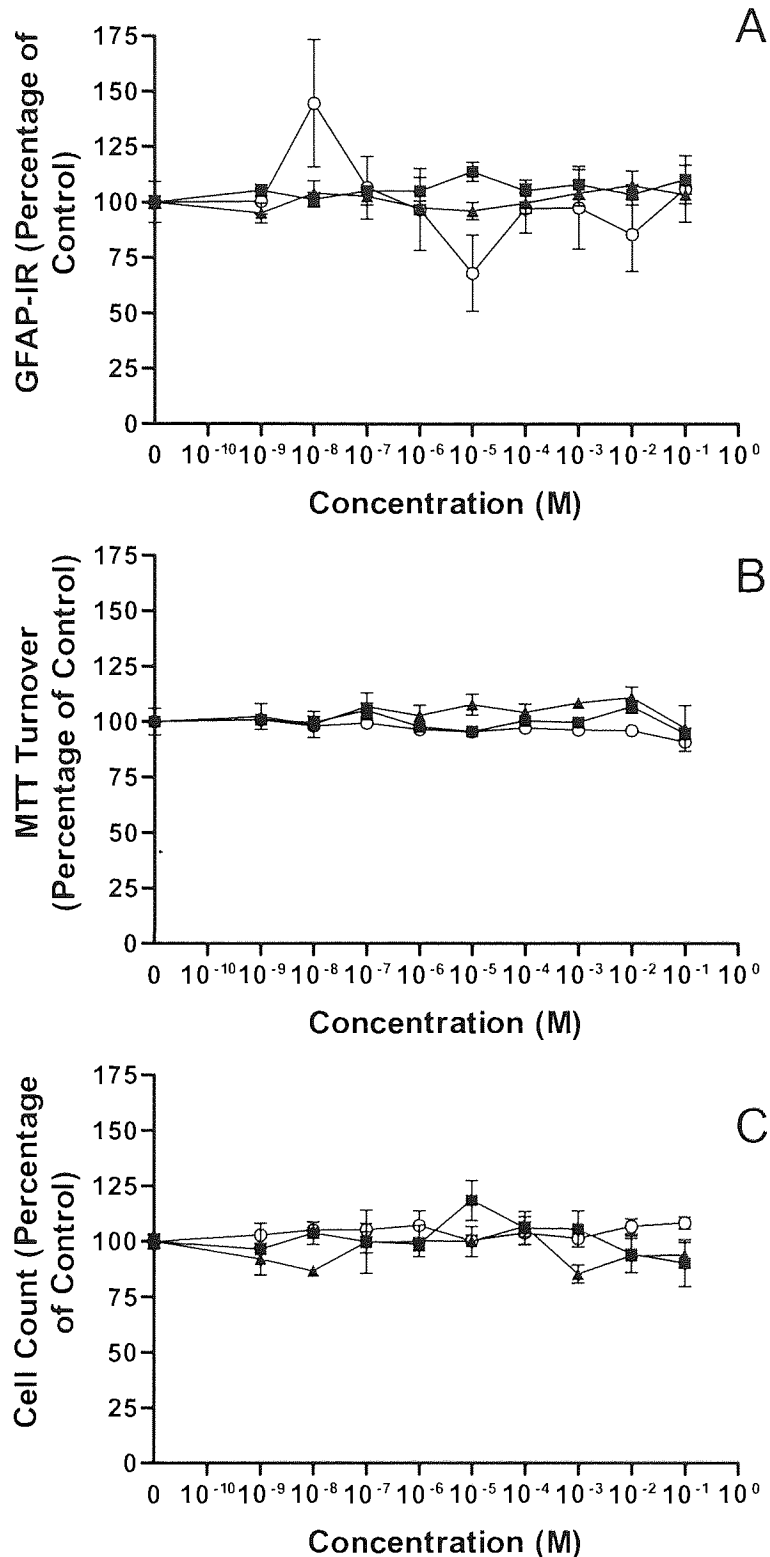
Figure 4.15 Effect of chloroquine diphosphate on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 4 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover in the CCF-STTG1 line, n=12 from three individual experiments and in the U251-MG and U373-MG lines n=4 from a single experiment. For cell counts, in the CCF-STTG1 line, n=6 from three individual experiments and in the U251-MG and U373-MG lines n=2 from a single experiment).

increase was observed in MTT turnover in any of the cell lines (figure 4.15B). The lowest concentrations to cause a significant reduction in MTT turnover were 10nM in the U251-MG line (P<0.05) and 1μM in the U373-MG (p<0.01) and CCF-STTG1 (P<0.01) cell lines. Chloroquine similarly had no effect on cell counts following 4 hours exposure (figure 4.15C), nor was a decrease seen in the U251-MG cell line at any of the concentrations tested. The U373-MG and CCF-STTG1 cell numbers, however, were significantly reduced, at concentrations above 1mM in both cases (P<0.01).

4.3.2.4 Effect of trimethyltin chloride on GFAP-IR, MTT turnover and cell numbers.

Initial treatment for 24 hours with trimethyltin chloride (TMTC) resulted in no significant increase in GFAP immunoreactivity at any toxicant concentration in all three glioma cell lines tested (figure 4.16A). At concentrations above 0.1mM in the U251-MG and U373-MG cell lines and 1mM in the CCF-STTG1 line, TMTC caused a significant reduction in GFAP-IR following 24 hour incubation (p<0.01). MTT turnover was similarly reduced at concentrations of and above 0.1mM in all three cell lines (p<0.01) (figure 4.16B). There was, however, a significant increase in MTT turnover in the U373-MG cell line following exposure to 1μM of TMTC (p<0.05) to 5.2(±1.37) percent above control levels. Significant increases in cell numbers to 34.9(±7.3) percent above control levels, were observed in the CCF-STTG1 line following 10μM TMTC exposure (p<0.01) but not in any other cell line (figure 4.16C). At higher concentrations, cell counts were significantly reduced in all three of the glioma cell lines at 100μM in the U373-MG line (p<0.01) and at 1mM in both the U251-MG and CCF-STTG1 lines (p<0.01).

Subsequent preliminary investigation at 16 hours exposure time to trimethyltin chloride again saw no significant increase in GFAP-IR in any of the three cell lines (figure 4.17A). The lowest concentrations to cause a significant decrease in GFAP-IR were identical to those observed at 24 hours, being 0.1mM for U251-MG and U373-MG cell lines and 1mM for the CCF-STTG1 cell line. Preliminary results showed that 10μM TMTC caused a significant increase in MTT turnover in both the U373-MG and CCF-STTG1 cell lines with turnover increasing to 58.3(±3.02) and 15.3(±3.82) percent of control values respectively (figure 4.17B). MTT turnover in the U251-MG line was not elevated. Reductions in MTT turnover were observed at 0.1mM in both the U251-MG and CCF-STTG1 cell lines and at a higher concentration of 1mM in the U373-MG cell line. Cell counts for each cell line were not increased at any TMTC concentration following 16
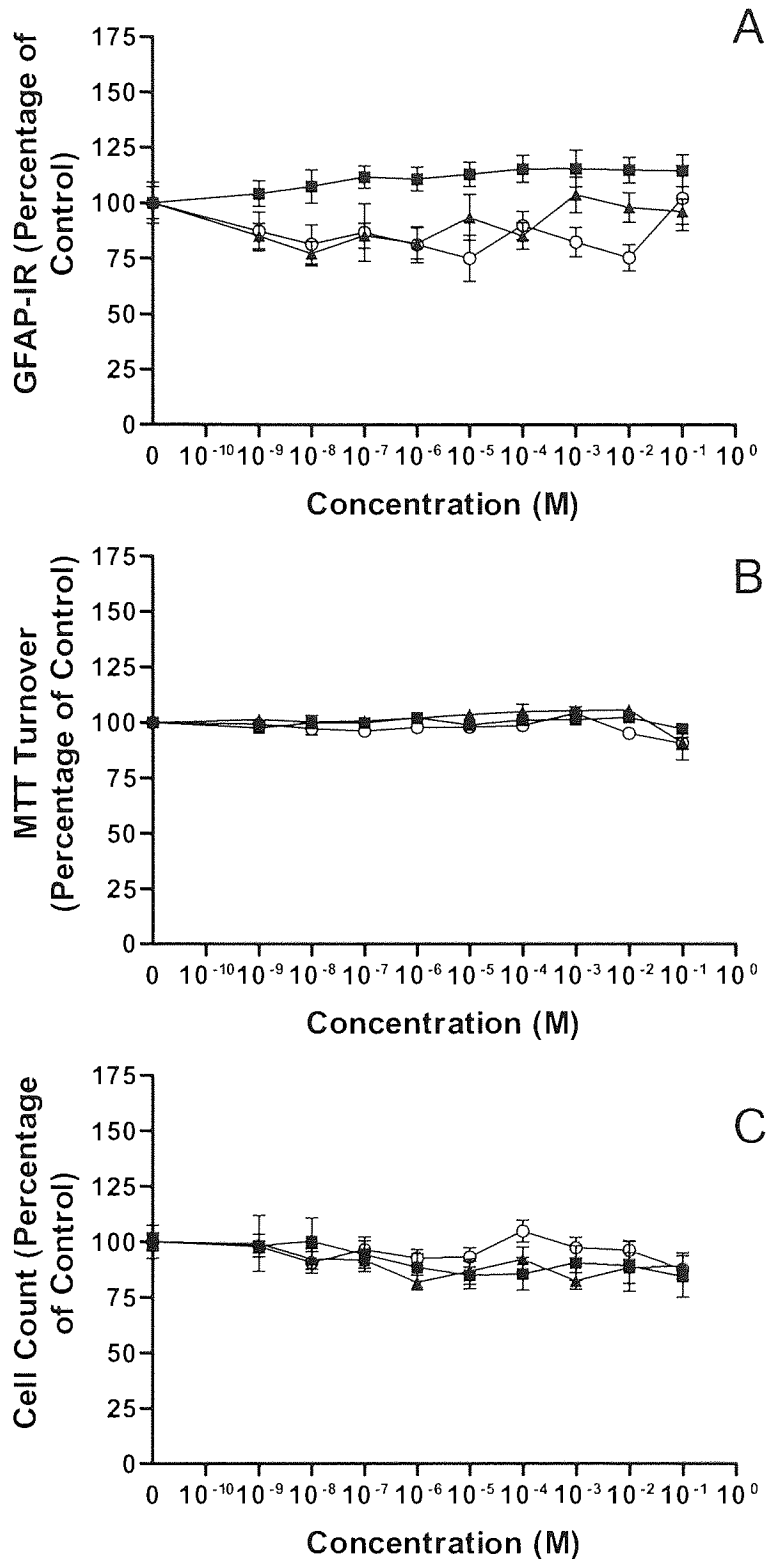
Figure 4.16 Effect of trimethyltin chloride on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 24 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 3 individual experiments, for cell counts, n=6 from 3 individual experiments).
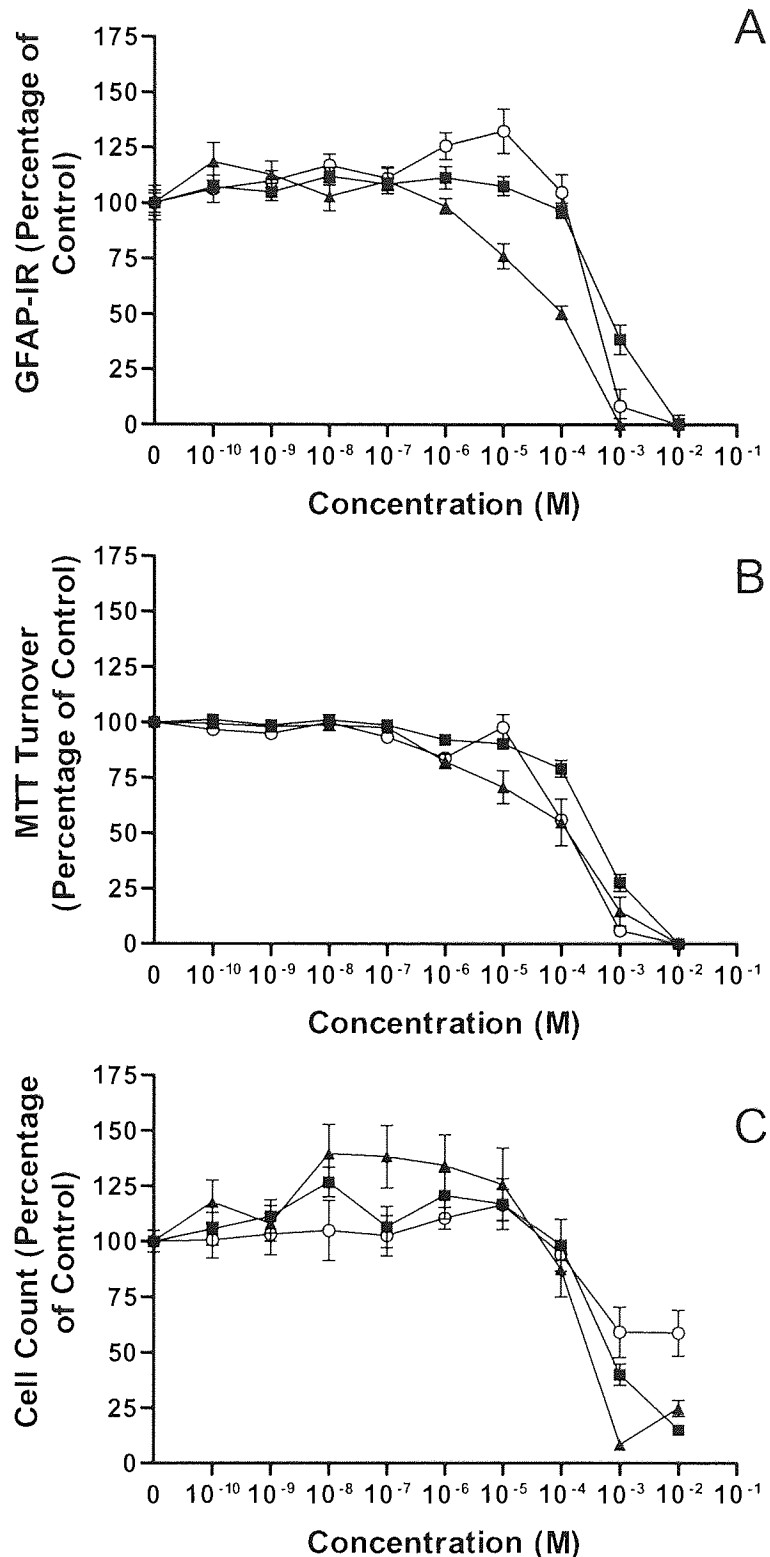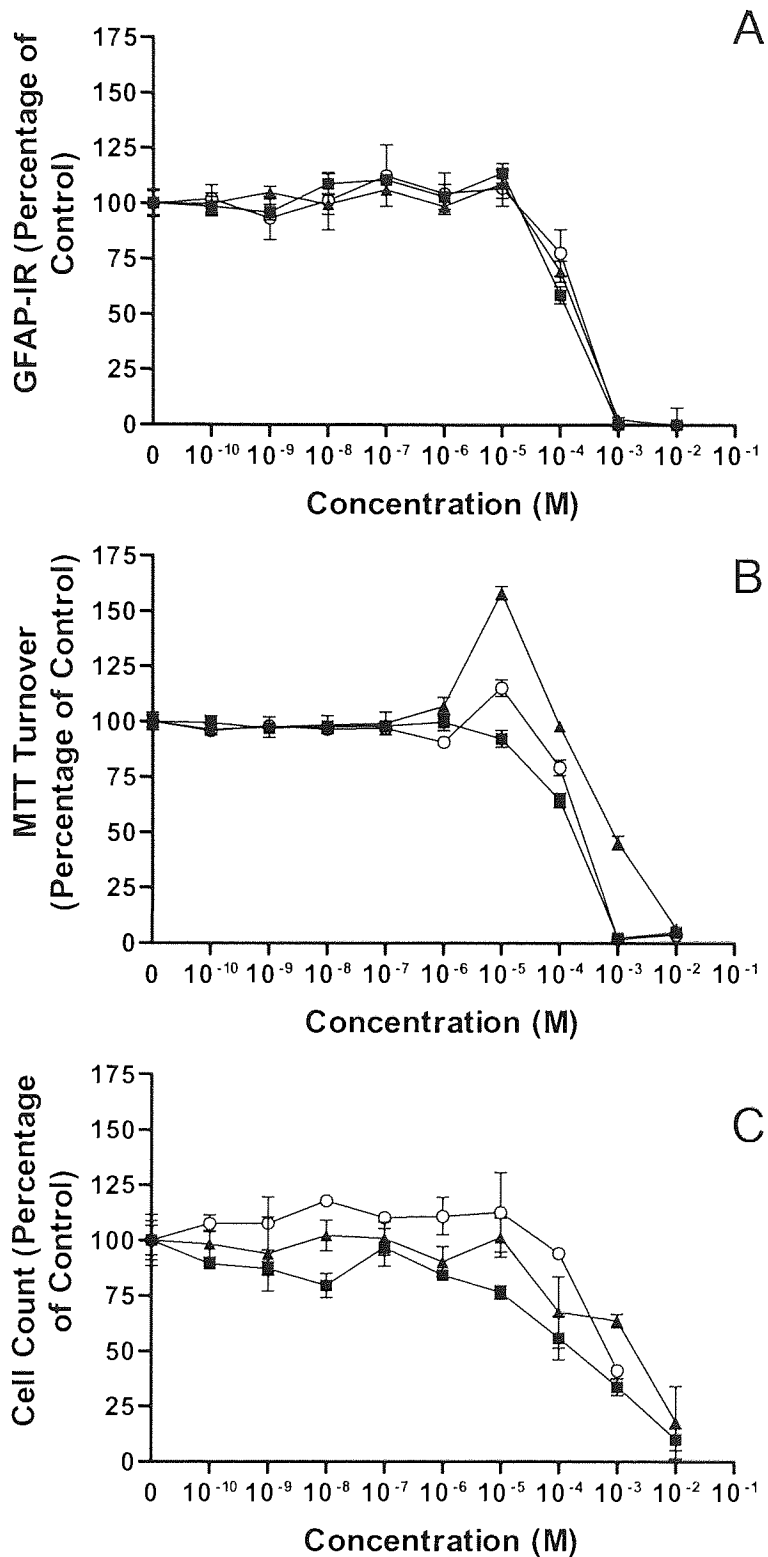
Figure 4.17 Effect of trimethyltin chloride on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 16 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=4 from a single experiment, for cell counts, n=2 from a single experiment).
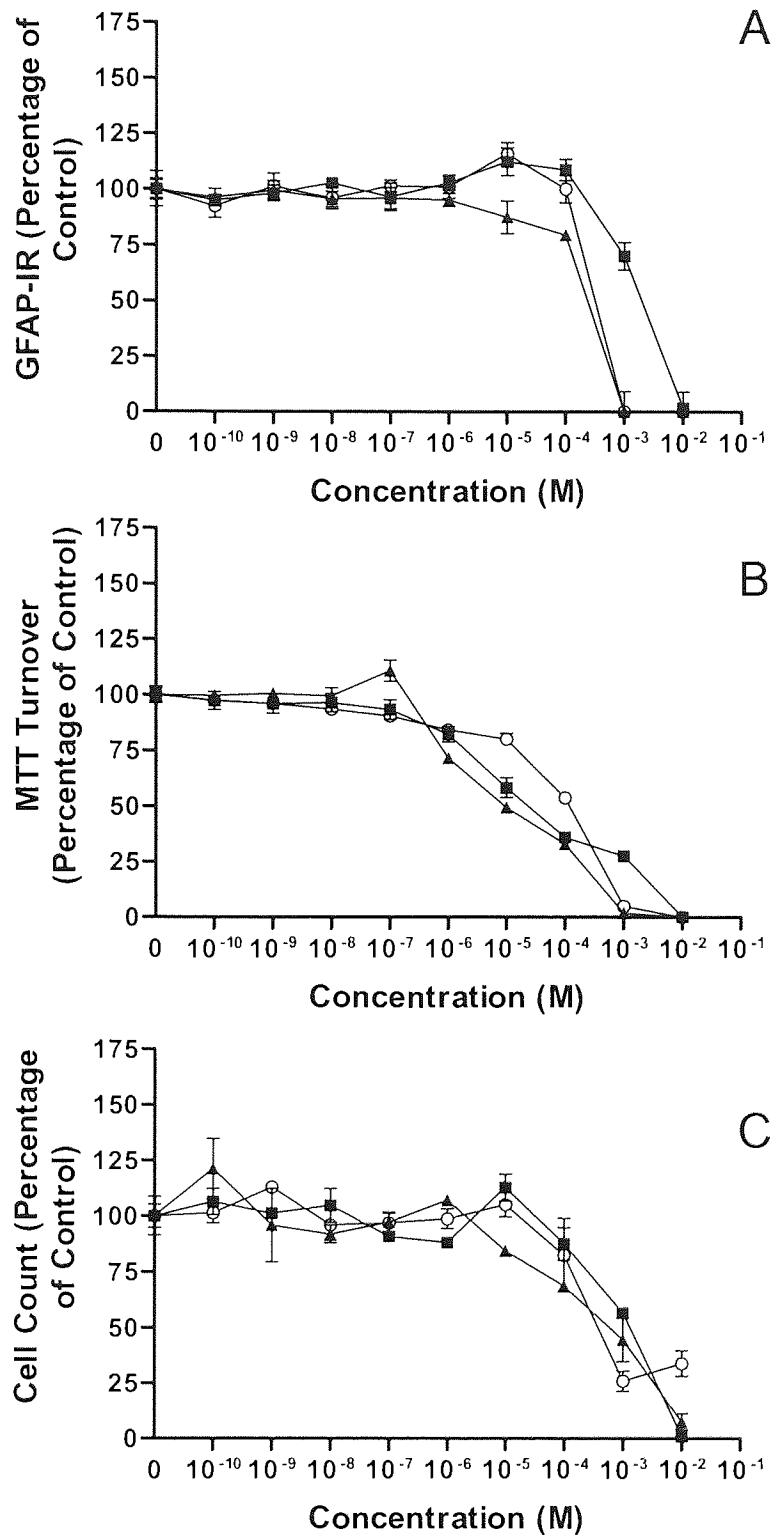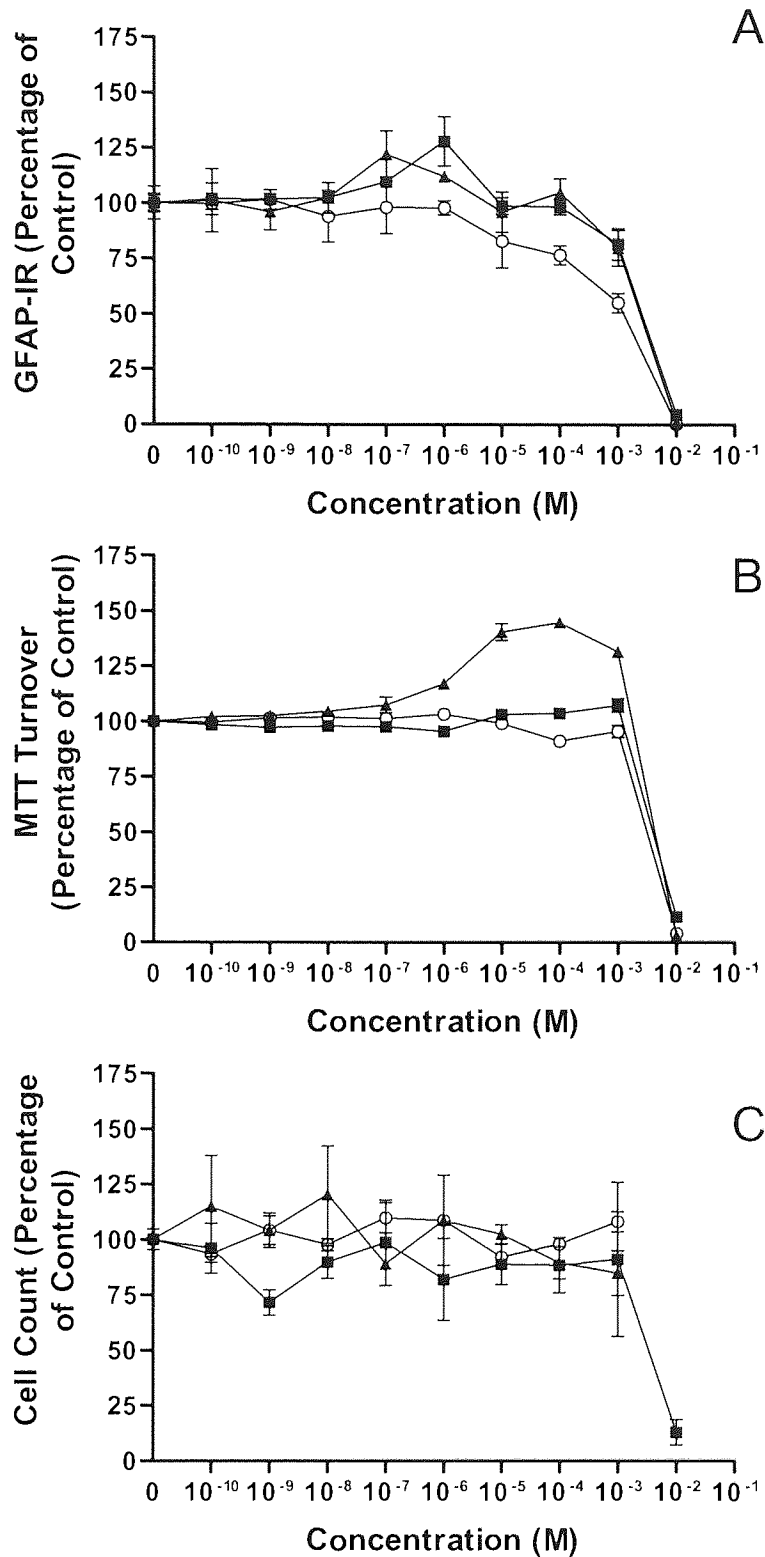
Figure 4.18 Effect of trimethyltin chloride on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 4 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=4 from a single experiment, for cell counts, n=2 from a single experiment).

hours treatment (figure 4.17C). Reductions in cell counts were seen at concentrations of and above 0.1mM in the U251-MG cell line and 10mM in both the U373-MG and CCF-STTG1 lines.

Unlike both 24 and 16 hours exposure, 4 hours preliminary treatment with TMTC was shown to have an elevating effect on GFAP-IR in the U251-MG cell line (figure 4.18A) where levels were seen to be significantly increased to $28.0(\pm15.73)$ percent above control levels when treated with $1\mu M$ of the compound ($p<0.01$). There was no elevating effect, however, on GFAP-IR in either the U373-MG or CCF-STTG1 cell lines. Compared to 24 and 16 hours incubation, higher concentrations of TMTC were required at 4 hours treatment in order to significantly reduce GFAP-IR. In the U251-MG and U373-MG lines 10mM of TMTC was required ($p<0.01$) and in the CCF-STTG1 line, 1mM ($P<0.01$). At 4 hours, MTT turnover was markedly increased (figure 4.18B), particularly in the U373-MG cell line at concentrations of $1\mu M$, $10\mu M$, 0.1mM and 1mM ($p<0.01$) resulting in respective elevations to $17.1(\pm2.31)$, $40.4(\pm3.83)$, $44.7(\pm1.32)$ and $31.5(\pm1.57)$ percent higher than control values. Elevations were also seen in the U251-MG cell line to $7.3(\pm3.11)$ percent above control values following 1mM exposure ($P<0.05$). There was no significant increase in MTT turnover above control levels in the CCF-STTG1 cell line. The lowest concentrations of TMTC to cause a significant decrease in MTT turnover in both the U251-MG and U373-MG lines at 4 hours was 10mM ($P<0.01$). In the CCF-STTG1 cell line MTT turnover was significantly reduced at 0.1mM ($P<0.01$), however, following 1mM exposure, MTT turnover was not significantly lower than control values, suggesting a rise in MTT turnover. At 10mM, turnover levels were again significantly reduced ($p<0.01$). Cell counts were unaffected by 4 hours TMTC exposure (figure 4.18C), apart from a decrease in the U251-MG cell line at the highest concentration tested of 10mM ($P<0.01$).

4.3.2.5 Effect of Acrylamide on GFAP-IR, MTT turnover and cell numbers.

Figures 4.19A, 4.20A and 4.21A show that no increases in GFAP-IR, MTT turnover or cell numbers were observed in any cell line following 24 hour treatment with acrylamide. At 10mM GFAP-IR and MTT turnover levels were significantly reduced in each line ($p<0.01$). Similarly 10mM acrylamide was the lowest concentration to cause a significant reduction in cell number in the U251-MG and CCF- STTG1 cell lines ($p<0.01$) while the lowest concentration for the U373-MG line was 100mM ($p<0.05$).
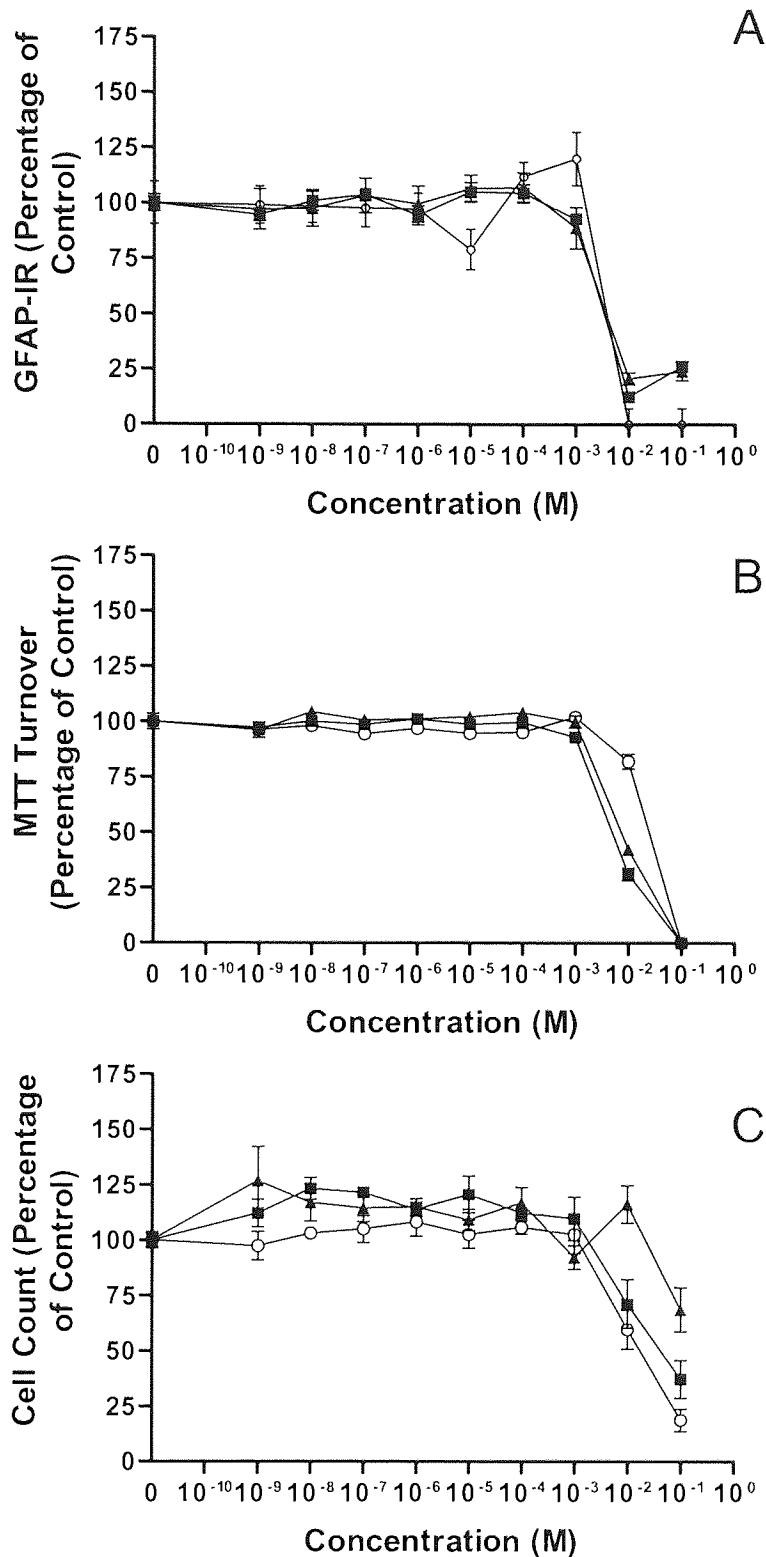
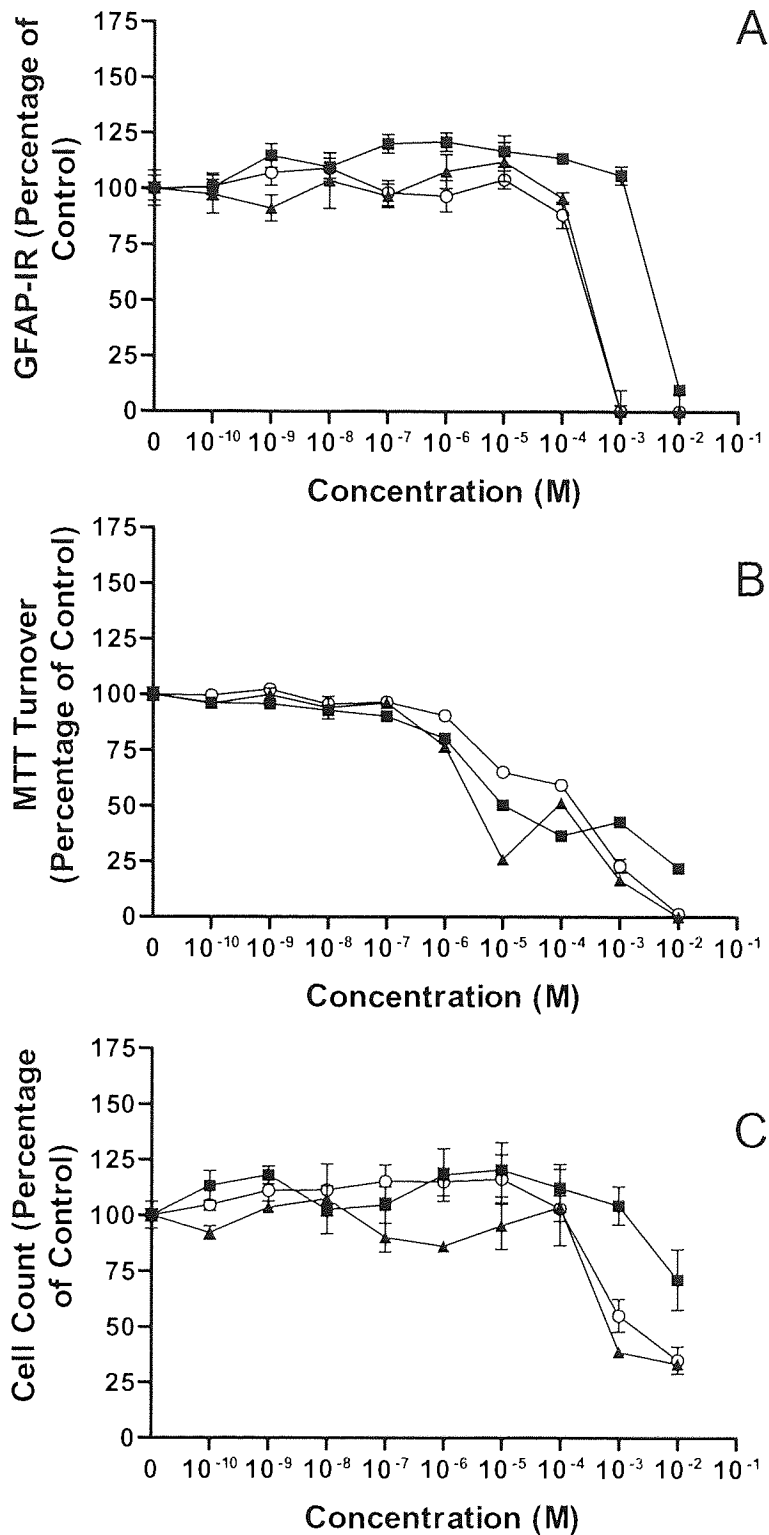**Figure 4.19 Effect of acrylamide on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 24 hours of exposure.** Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=12 from 3 individual experiments, for cell counts, n=6 from 3 individual experiments).
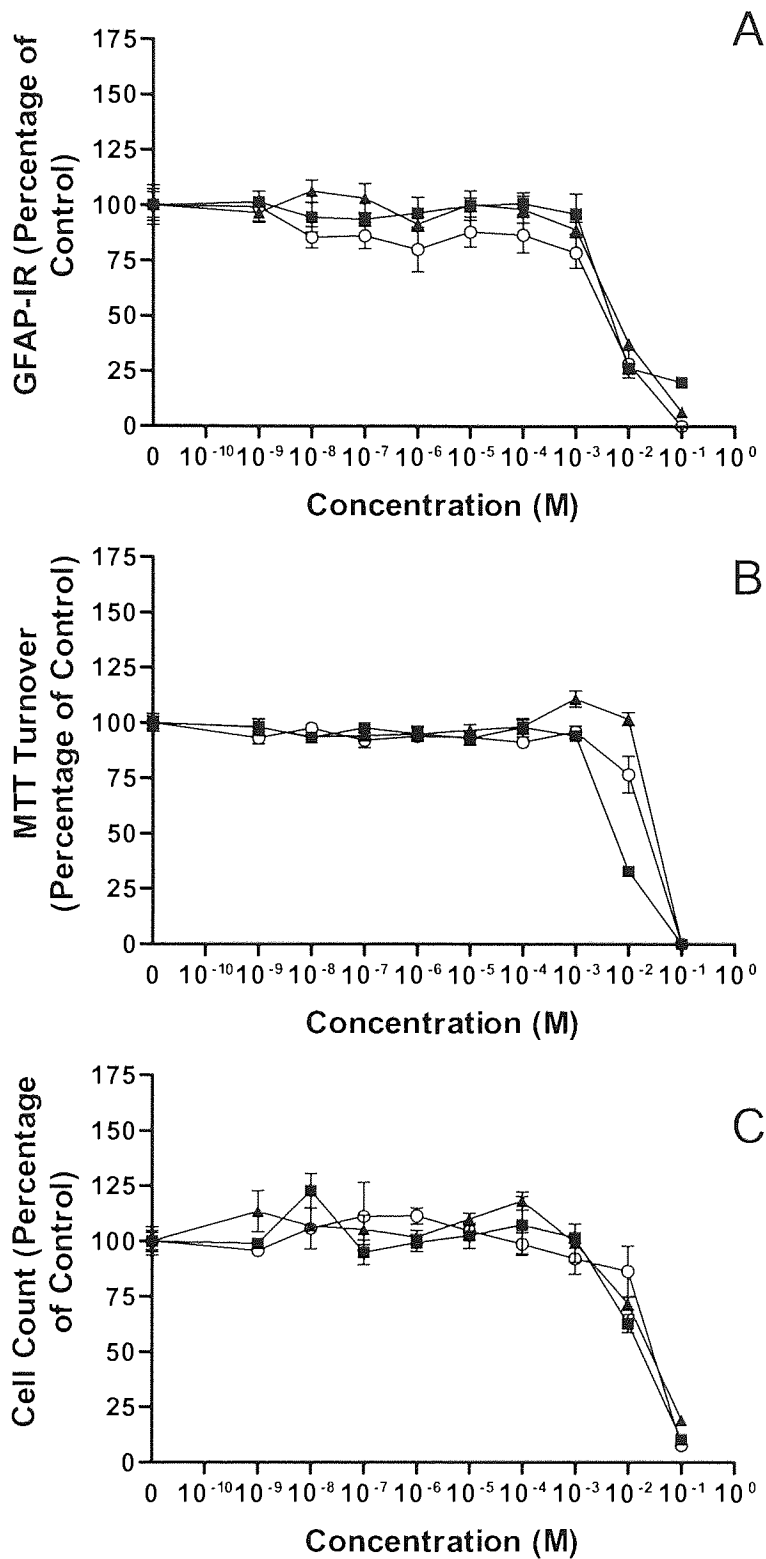
Figure 4.20 Effect of acrylamide on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 16 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=4 from a single experiment, for cell counts, n=2 from a single experiment).
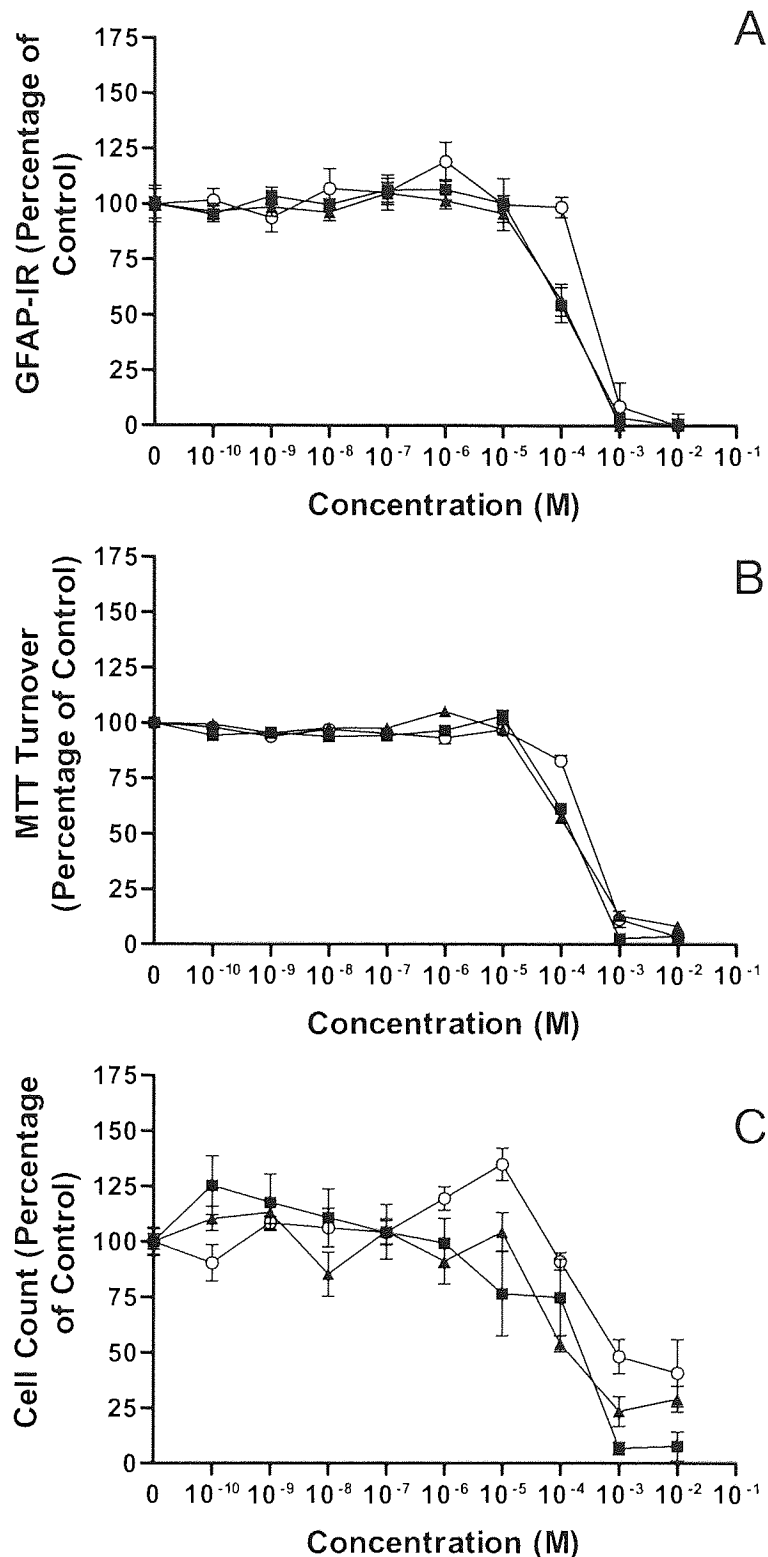
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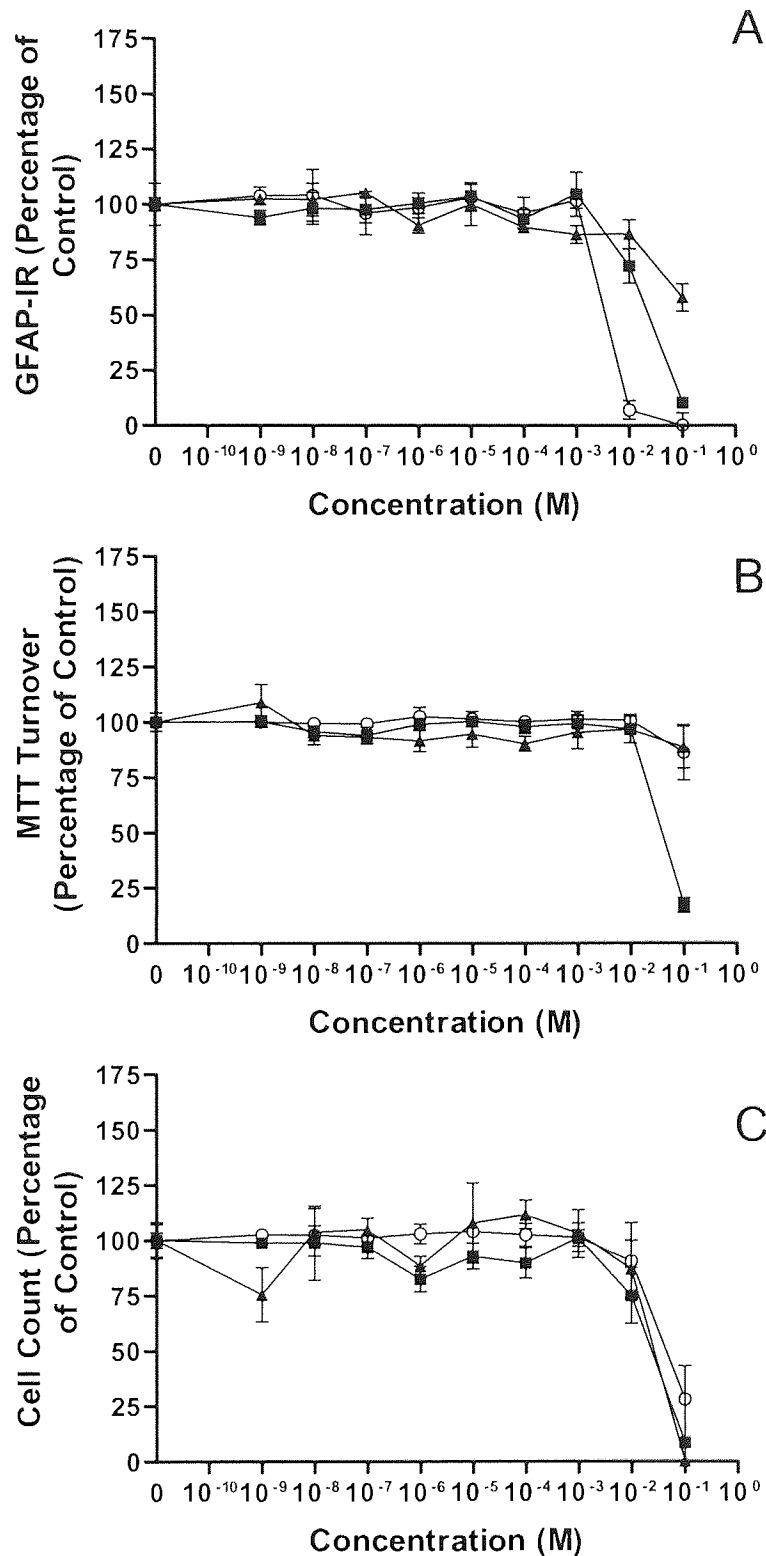
Figure 4.21 Effect of acrylamide on A) glial fibrillary acidic protein immunoreactivity, B) MTT turnover and C) cell proliferation in the cultured human glioma cells following 4 hours of exposure. Endpoints were assessed in U251-MG (■), U373-MG (▲) and CCF-STTG1 (○) cell lines. Data are expressed as a mean percentage of non-treated control wells ± SEM (for GFAP-IR and MTT turnover, n=4 from a single experiment, for cell counts, n=2 from a single experiment).

Preliminary investigations reducing exposure time to 16 hours also saw no elevations in GFAP-IR, MTT turnover or cell number in any cell line (figures 4.19B, 4.20B and 4.21B). As with 24 hours exposure, GFAP-IR levels were significantly reduced in each line (p<0.01) following exposure to and above 10mM acrylamide. MTT turnover was also significantly reduced in the U251-MG and U373-MG when exposed to 10mM (p<0.01) while 100mM was required to cause a significant reduction in turnover in the U373-MG line (p<0.01). Again, 10mM acrylamide was the lowest concentration of acrylamide to cause a significant reduction in cell number in the U251-MG and U373-MG cell lines (p<0.01) while the lowest concentration for the CCF-STTG1 line was 100mM (p<0.01).

As with the results following both 24 and 16 hours exposure, there were also no observed increases in any of the three tested parameters following only 4 hours preliminary treatment (figures 4.19C, 4.20C and 4.21C). The lowest concentrations to cause a significant reduction in GFAP-IR in the U251-MG and CCF-STTG1 cell lines were the same as 24 and 16 hours exposure at 10mM (P<0.01). In the U373-MG line, this was increased to 100mM (p<0.01). The lowest observed adverse effect levels for reduction in MTT turnover were much higher than those for longer exposure times as the only significant decrease observed was in the U251-MG cell line exposed to 100mM acrylamide (p<0.05). Cell counts were significantly reduced in all cell lines at 100mM (p<0.01). This concentration was again higher than the no adverse effect levels recorded at 16 and 24 hours exposure.

4.4 Discussion.

The glial fibrillary acidic protein is widely used as a quantitative marker for astrocyte reactivity. Toxicant exposure is known to cause reactive responses in astrocytes *in vivo* (Brock and O'Callaghan 1987; O'Callaghan and Jensen 1992) and *in vitro* using non-human primary cell cultures and continuous cell lines (Cookson and Pentreath 1994; Malhotra *et al.* 1995). However, the effect of these toxicants on cells of human origin has been largely neglected, thus the responses of each of the three human astrocytoma cell lines U251-MG, U373-MG and CCF-STTG1 were determined following exposure to several compounds known to cause astrogliosis.

Four of the five compounds tested, LPS, ethanol, chloroquine diphosphate and trimethyltin chloride were found to induce increases in GFAP-IR in certain cell lines depending on the exposure period. These increases in GFAP-IR were part of a dual phase response in the glioma cell lines whereby the increase was subsequently followed by attenuation at higher concentrations indicating cytotoxicity. Increases in GFAP were observed to be between 20 and 60 percent greater than control values that were similar to levels achieved in primary rat astrocytes by Cookson and Pentreath (1994) where GFAP elevations approximately 20 to 40 percent greater than controls were observed. The concentrations seen to induce GFAP-IR increases in the three cell lines in this study were all below those required to induce cytotoxic effects in the same cell lines according to the cytotoxicity data in Chapter 2. This suggests that the increase in GFAP-IR, indicating a reactive response, is a more sensitive marker for the effects of a compound than the attenuation associated with cytotoxicity.

To the author's knowledge this is the first time that LPS has been investigated for its ability to induce increases in GFAP in human cell lines. It was found that significant increases in GFAP-IR were observed at 4 hours in the U251-MG but not at greater time points. In previous studies, LPS has been found to similarly induce GFAP expression in mouse primary astrocytes (Brahmachari *et al.* 2006), as well as inducing an increase in GFAP-IR in rat astrocytes following *in vivo* acute (24 hour) and chronic (30 days) supranigral LPS administration (Iravani *et al.* 2005). Contrasting results were, however, found by Letournel-Boulland *et al.* (1994), whereby LPS caused a down regulation of GFAP mRNA in mouse astroglial primary cultures. These differing effects of bacterial

LPS exposure may be attributed to the type of LPS molecule employed. The biological consequences of LPS exposure can be highly variable depending on the strain of bacteria from which it was originally expressed and can be linked to variations in the structure of the Lipid A portion of the LPS molecule (Netea *et al.* 2002). It is this Lipid A portion, which is regarded as the region responsible for the toxic effects of LPS and for the induction of cytokine and other signalling molecule expression involved in the inflammatory response (Netea *et al.* 2002). While LPS from some strains act to induce an inflammatory response by causing the expression of cytokines, the LPS from other bacterial species such as *Rhodobacter capsulatum* actually act to antagonise cytokine expression (Loppnow *et al.* 1990). For those that do induce pro-inflammatory cytokines, the bacterial strain also influences the severity of the response, for example, the LPS of *Escherichia coli* as used in this study is a much more potent cytokine inducer than the LPS of other species such as *Borditella pertussis* (Netea *et al.* 2002). Despite the clear variation in effects, it is not always reported which strain of LPS is used in published studies. The use of the general term "LPS" without stating the strain, creates confusion when conflicting results are achieved and therefore the bacterial origin of the LPS used should be clearly reported when publishing data. LPS was partly chosen for these experiments because it is a potent inducer of inflammation. The GFAP-IR increases in astrocytoma cell lines following exposure to LPS may be of significance when considering astrocytes as a potential target of the gram-negative bacterial endotoxin during infection.

Ethanol was the only toxicant to consistently show the same elevating effect in GFAP-IR in all three cell lines at the same time point of 24 hours and correspond to similar elevations seen in rat primary astrocytes following 24 hours exposure (Cookson and Pentreath 1994) and in the astrocytes of Wistar rats chronically exposed to the compound (Franke *et al.* 1997). The three cell lines seemed to be variably sensitive to the compound with significant elevations appearing at 0.1mM, 1mM and 10mM for the CCF-STTG1, U251-MG and U373-MG cell lines respectively. The increases in GFAP-IR at these low levels suggest that even a relatively small amount of alcohol may exert a considerable effect on the underlying function of the cells in the CNS. In contrast to the GFAP-IR elevating effects of LPS at 4 hours, ethanol caused a significant increase in GFAP-IR at 24 hours but not at any shorter period of exposure. These effects at 24 hours could be compared to responses *in vivo* following moderately sustained or sub-chronic exposure

patterns akin to those with the modern social phenomenon of binge drinking (Goodlett *et al.* 1998; Eaves and Nixon 2007).

The responses of human astrocytoma cell lines were varied in response to chloroquine diphosphate. Increases in GFAP-IR were observed following 24 hours 1µM exposure in the CCF-STTG1 cell line and 4 hours 0.1µM exposure in the U251-MG cell line, indicating that the U251-MG cell line is the more sensitive to chloroquine diphosphate. Similar to the CCF-STTG1 cell line, concentrations of 1µM chloroquine were also seen by Cookson and Pentreath (1994) to have an elevating effect in primary astrocyte cultures following 24 hours exposure. While chloroquine is used as an anti-inflammatory agent in the treatment of autoimmune diseases, it has also been suggested by Park *et al.* (2003) that chloroquine may also have pro-inflammatory properties depending on the cellular context. Here, Park *et al.* demonstrated that chloroquine induced the production of pro-inflammatory cytokines associated with astrocyte activation, in CRT-MG human astroglial cells. It is possible that chloroquine may be inducing a similar response in these astrocytic cell lines. The detection of any released pro-inflammatory cytokines may go further to clarify this.

Trimethyltin has well known neurotoxic effects when administered *in vivo* (Bouldin *et al.* 1981). In addition, the compound is also known to induce an inflammatory response in the CNS, as well as increases in GFAP in both rat astrocytes and microglia *in vivo* (McCann *et al.* 1996; Brock and O'Callaghan 1987). However, *in vitro*, Rohl *et al.* (2001) found that TMT has no effect on GFAP expression in rat primary astrocytes after 40 hours exposure. Similarly, Cookson and Pentreath (1994) showed that TMT had no GFAP elevating effects in rat primary astrocytes after 24 hours exposure. It was suggested by Rohl *et al.* (2001) that the presence of neurones was necessary for TMT to induce an increase in GFAP expression. In the present study, a lack of effect was also observed after 24 and 16 hours exposure to trimethyltin chloride, however, 1µM of the compound was found to induce an increase in GFAP-IR in the U251-MG cell line following only 4 hours exposure. This indicates and that the effects of TMT may be time dependent, as suggested by Seibert *et al.* (2004). Despite the lack of effects of TMT on rat primary cultures, Reali *et al.* (2005) demonstrated that the compound was able to induce increases in GFAP expression and GFAP filament reorganisation in primary human foetal astrocytes at 10^{-5} M concentrations. This, together with the positive results from this study in human cell lines,

suggests that the presence of neurons may not be necessary for the induction of the protein and that the effects of TMT may be species specific.

Exposure to acrylamide did not cause an increase in GFAP-IR or MTT turnover in any of the three glioma cell lines at any time point. The compound was used specifically as a negative control and confirms similar results achieved by Cookson *et al.* (1994) when using primary astrocyte cultures. The results for each of these five compounds begin to provide promising evidence that the GFAP assay system employing human astrocytoma cell lines in this study may be able to discriminate between known neurotoxicants and astrotoxicants previously described in the literature when using more complex astrocytic cell systems. Results following 4 and 16 hours exposure time to chloroquine diphosphate in the U251-MG and U373-MG cell lines and trimethyltin chloride and acrylamide in all three astrocytoma lines were somewhat preliminary and further investigation is required to determine whether the increases in GFAP-IR and MTT turnover observed at these times are fully reproducible.

The differential capabilities of each of the compounds to cause an elevation in GFAP-IR suggests that there may be a mechanism or mechanisms for the induction of GFAP through which only certain compounds can initiate a response. The exact molecular mechanisms by which chemical exposure cause up-regulation of GFAP expression, either *in vivo* or in vitro, are still elusive due to the complex multi-factorial nature of the response. Malhotra *et al.* (1997) illustrated that simultaneous mechanical and chemical injury enhanced the induction of GFAP to levels higher than those achieved with either treatment alone; supporting the likelihood that more than one transcriptional mechanism is involved in the activation of astrocytes. It was also suggested that different injuries may affect different receptors and second and third-messenger pathways involved in GFAP induction (Malhotra *et al.* 1997). It is therefore conceivable that different toxicants may also induce GFAP through distinct mechanisms. It is largely unknown why some compounds affect GFAP expression and others do not. It is possible those that do not, are unable to induce key mediators involved in the process. It is also possible that certain compounds may affect the cellular architecture in such a way that the up-regulation of GFAP is not possible. For example, there is evidence that acrylamide, which is generally regarded as a specific neurotoxin, possibly exerts its toxic effects by forming adducts with target proteins (LoPachin and DiCaprio 2005). It has been particularly noted that

acrylamide causes collapse and aggregation of intermediate filaments (Sager *et al.* 1989; Hartley *et al.* 1997), including GFAP in astrocytic cells (Cordo and Candurra 2003). It is possible that acrylamide effects on GFAP may actually interfere with the up-regulation and rearrangement of the protein itself, as associated with reactive astrocytes. It is also possible that other compounds that fail to induce GFAP may also have an adverse effect on the cells in a manner that prevents such a response.

Along with these observed elevations in GFAP-IR, similar dual phase responses at sub-cytotoxic concentrations were also seen in MTT turnover in the human cell lines following exposure to bacterial LPS and TMTC; indeed, MTT levels were seen to exceed control values by almost 60% following a 16 hour exposure to TMTC in the U373-MG cell line. Again, increases were seen to be time and cell line dependent. For example, while LPS caused an increase in MTT turnover at 24 and 16 hours in the U251-MG and U373-MG cell lines, no elevating effect was seen following 4 hours incubation in these cell lines or at any time point in the CCF-STTG1 cells. Similarly, TMTC caused elevations at all three time points in the U373-MG cell line but only at 4 hours in the U251-MG line and 16 hours in the CCF-STTG1 cell line. Comparable increases in MTT turnover following trimethyltin exposure were observed in rat primary astrocytes by Cookson *et al.* (1995), however, to the author's knowledge no such effects have been reported following LPS exposure.

It was observed in the present study that a rise in MTT turnover was not always associated with a similar rise in GFAP levels and visa versa. For example, GFAP increases without an accompanying rise in MTT were observed in all three cell lines following 24 hours exposure to ethanol. On the other hand, MTT increases were observed without corresponding increases in GFAP-IR following LPS exposure in both the U251-MG and U373-MG cell lines at 24 and 16 hours exposure. There was only one instance of GFAP and MTT increases occurring in the same cell line at the same time point. This was following 4-hour trimethyltin chloride exposure in the U251-MG cell line. The differences in GFAP and MTT responses observed in a certain cell line have also been observed in other studies (Cookson *et al.* 1994; Cookson *et al.* 1995). From the nature of both these assays, it is believed that they each measure different aspects of cellular function (Cookson *et al.* 1994); indeed, the lack of synchrony between the two markers in this study indicates that the two events are independent. It is likely that increases in GFAP are linked solely to

reactive gliosis and that elevations in MTT turnover are merely a marker for increased metabolic activity. Indeed, unlike GFAP effects, elevations in MTT turnover are not restricted to astrocytes and have been observed in other cell types such as neurons (Johnston *et al.* 1993) including neuroblastoma cells as illustrated in Chapter 3 of the present study. It has been suggested that increases in metabolism may be a compensatory process before repair capacities are exceeded and turnover levels are reduced (Mead and Pentreath 1998). Despite the discrepancies over the enzyme responsible for MTT reduction and whether the assay can be used as a measure of purely mitochondrial function, as discussed in the previous chapter, it is clear from the results of this study that the MTT assay is still a valuable assay for assessing cellular responses following sub-cytotoxic chemical injury.

Cell counts were taken alongside MTT and GFAP assays in order to control for any proliferative effects of chemical exposure. Exposure to any of the toxins at any time had very little significant effect on the proliferation of the three cell lines apart from elevations in CCF-STTG1 cell number following 24 hour exposure to 10μM trimethyltin chloride and in U373-MG cell number following 24 hour exposure to 10 and 100nM chloroquine diphosphate. The fact that neither GFAP nor MTT levels were increased at these same concentrations, as would be expected if there were an increase in proliferation, suggests that these results are not reflecting true proliferative effects.

Both the GFAP and MTT assays used in this study showed variable sensitivities to the sub-cytotoxic and cytotoxic effects of the tested compounds. The reductions in each of the endpoints indicated cytotoxicity. For some compounds such as ethanol and acrylamide these respective sensitivities of the MTT and GFAP assays were quite similar, with cell count generally being reduced at higher concentrations. However, following TMTC and chloroquine diphosphate exposure, MTT turnover was reduced at lower concentrations than those required to cause a significant reduction in GFAP-IR. This was particularly evident for chloroquine diphosphate after 4, 16 and 24 hours exposure, in all three cell lines, indicating that MTT turnover is the most sensitive indicator of cytotoxicity. Alternatively, elevations in MTT turnover or GFAP-IR always occurred at sub-cytotoxic concentrations. Because it was uncommon for both GFAP-IR and MTT to be elevated at the same time point, it is difficult to determine which of the assays is the most sensitive indicator of sub-cytotoxic activation effects. An exception to this was the simultaneous

elevation in both GFAP-IR and MTT turnover in the U251-MG cell line following 4-hour TMTC exposure. Here, GFAP-IR was elevated at 1μM whereas MTT turnover was elevated at a higher concentration of 1mM, indicating that increases in GFAP-IR are the most sensitive indicator of the sub-cytotoxicity of TMTC. The differing sensitivities of both these endpoints when applied to cytotoxicity and sub-cytotoxicity assessment gives support for the use of both in subsequent investigations of astrocytic toxicity.

As described above, the results for both GFAP and MTT turnover indicate a clear effect of exposure time on the response of the cell lines. The majority of research performed *in vitro* to investigating the effects of toxicants on GFAP is limited to 24 hours exposure or more. This is the first study using continuous cell lines to look at activation responses at additional lesser time points of 4 and 16 hours. It was observed that three compounds (LPS, Chloroquine diphosphate and TMTC) that had GFAP-IR elevating effects at 4 hours in the U251-MG had no such effect at 24 hours in this same cell line. Conversely, however, chloroquine diphosphate caused elevations in GFAP-IR after only 4 hours in the U251-MG cell line but took 24 hours for the same response to be observed in the CCF-STTG1 cell line may be associated with the relative sensitivities of the cell lines to the same compound. Time dependent differences were also observed in MTT turnover. 4 hours exposure to LPS was not sufficient to cause an increase in MTT turnover whereas elevations were seen only after 16 and 24 hours. The magnitude of MTT elevations in the U373-MG line in response to TMTC were greater at lower time points of 4 and 16 hours compared to only a small increase at 24 hours. This implies that a longer incubation period may result in the MTT elevating response being masked by an overlapping cytotoxicity. Considering the time dependent effects observed in the present study it is proposed that a variety of different time points, including 4 hours exposure, be included for any future assays for astrotoxicity. A single exposure time of 24 hours, as applied as the major time point in many *in vitro* GFAP systems, may not be sufficient to investigate the responses of these cell lines whereby initial responses at 4 hours like those seen with TMTC would be missed .

Considering the ability of the human cell lines used in this study to achieve reactive-like responses similar to those observed in other *in vitro* cell systems using human and non-human primary cells, it is clear that the use of human astrocytic cell lines may have potentially valuable applications in the assessment of *in vitro* neurotoxicity, being not

only easier to maintain than primary cultures, but the human lines may be better suited to high through-put screening compared with human primary cultures. However, the responses of the lines to the toxins employed in this study were not uniform. The U373-MG cell line showed the most responses at 24 hours overall for both GFAP and MTT, whereas the U251-MG line was more responsive at early timepoints, showing more responses at 4 hours, in comparison with the other lines. CCF-STTG1 cells were the most sensitive to ethanol showing the most intense responses at the lowest concentrations of the compound, however, the results for this cell line were admittedly more variable than the other lines. The similar basal levels of GFAP immunoreactivity detected in both the U251-MG and U373-MG cell lines and the lower levels observed for the CCF-STTG1 cell line when performing the ELISA reflect the findings in chapter 2, in that GFAP was expressed at consistently higher levels in the U373-MG and U251-MG lines than in the CCF-STTG1 line in the authors hands. To the author's knowledge this is the first time that GFAP expression has been compared in these particular cell lines.

It is possible that the variation between cell lines in regards to their respective GFAP and MTT responses following toxicant exposure may be attributable to differences in key signals in the activation of metabolic enzymes and signal transduction pathways. Conceivably, as each of the lines was originally derived from cancer cells, key genes involved with the reactive response may have mutated or the cells may now be lacking in essential signalling factors or their corresponding response elements. To the author's knowledge there has been no previous research comparing the relative expression, release or activation of astrogliosis mediators in astrocytic cell lines following toxicant exposure and is a potential area for further investigation.

To summarise, from the results in this chapter it is clear that the three astrocytic cell lines are responding to toxicant exposure at concentrations below those required to have any cytotoxic effect. The elevations in GFAP-IR and MTT turnover therefore appear to be more sensitive markers for astrocytic response than the attenuations associated with cytotoxicity. The similarity of responses between primary astrocytes used in previous studies and the astrocytic cell lines used in this study following exposure to individual toxicants indicate that human astrocytoma cell lines have potentially valuable applications in the assessment of *in vitro* neurotoxicity. Time dependent, cell line dependent and toxicant dependent effects highlight the need to consider these factors in future studies.

Reactivity and astrogliosis is a complex multi-factorial astrocytic response. While GFAP induction is a characteristic marker in these circumstances, it is necessary to investigate further whether the elevations in GFAP-IR observed in these astrocytoma cell lines are indeed associated with the induction of gliosis. In order to begin to do this, the next chapter will begin to investigate the incidence of other events associated with gliosis, such as the up-regulated expression of certain cytokines.

Chapter 5: Toxicant induced cytokine and nitric oxide release.

5.1 Introduction.

Astrocyte reactivity is a multi-factorial response, the manifestation and severity of which can vary depending upon the induction by differing stimuli. While the up-regulation of glial fibrillary acidic protein (GFAP) is a widely used and sensitive marker for astrocyte toxicity, it is important to develop other endpoint markers to assess astrocyte reactivity that are also specific to the response. As described in Chapter 1, many factors, along with GFAP elevation, have been associated with the astrocytic response following trauma or injury *in vitro*. These include the up-regulated expression or secretion of a variety of cytokines and other soluble signalling molecules (Ridet *et al.* 1997). The cytokines produced by reactive astrocytes, such as interleukins, chemokines and tumour necrosis factors, may act as key mediators of inflammation in the CNS; some possessing neurodegenerative functions whilst others play a neuroprotective role (Struzynska *et al.* 2007).

One cytokine researched particularly for its role in CNS inflammation is interleukin-6 (IL-6). Various cell types in the CNS are capable of expressing IL-6, yet astrocytes seem to be the dominant source of this cytokine, particularly during brain injury, inflammation and disease states (Wagoner and Benveniste 1999). Elevated levels of IL-6 have been observed in the compromised CNS as a result of several conditions including Alzheimer's disease, multiple sclerosis, HIV and *Borrelia Burgdorferi* (Lyme's Disease, or Borreliosis) infections, traumatic brain injury and malignancy among many others (Wagoner and Benveniste 1999). IL-6 has a multitude of functions in the CNS. There is evidence to suggest that the cytokine is involved in the protection and differentiation of neurons (Hirota *et al.* 1996; Loddick *et al.* 1998; Satoh *et al.* 1998) as well as promoting proliferation in astrocytes (Selmaj *et al.* 1990). IL-6 production and astrogliosis have been associated in several studies. Penkowa *et al.* (1999b) demonstrated that IL-6 deficient mice showed depressed astrogliosis, compared to IL-6 positive mice following injury to the fronto-parietal cortex, together with a decreased presence of activated brain macrophages. In accordance with this, IL-6 is thought to be involved in the orchestration of CNS inflammation, modulating the expression of a number of participating factors (Van Wagoner and Benveniste 1999). The dysregulation or over-expression of IL-6 can have destructive effects in the CNS, potentially causing the increased expression of other

inflammatory cytokines that could participate in neurodegenerative processes (DiSanto *et al.* 1996).

Another cytokine released by activated astrocytes is tumour necrosis factor alpha (TNF-α) (Ridet *et al.* 1997). This cytokine is released from astrocytes in response to LPS, and combined exposure to cytokines IL-1β and interferon-γ (Liberman *et al.* 1989; Chung and Benveniste 1990). In addition to releasing the cytokine, astrocytes also express receptors for TNF-α, suggesting a possible autocrine pathway for stimulation in these cells (Chung *et al.* 1991). TNF-α has strong proinflammatory and immunostimulatory properties (Wajant *et al.* 2003), and along with IL-6, has a multitude of functions that can be both beneficial and destructive to the target cell. For example, TNF-α has a primary role in the modulation of the inflammatory response as an acute phase protein. In the CNS in particular, it is known to stimulate astrocyte proliferation and the expression of the proinflammatory cytokine IL-6, adhesion molecules and other antigens in astrocytes (Selmaj *et al.* 1990; Benveniste *et al.* 1990; Mauerhoff *et al.* 1988; Lavi *et al.* 1988; Frohman *et al.* 1989). Alternatively, TNF-α is also known to have potent cytotoxic properties, causing a degradation of myelin and lysis of oligodendrocytes (Robbins *et al.* 1987; Selmaj *et al.* 1988). TNF-α is also pro-apoptotic, causing the activation of caspases and the induction of the apoptotic pathway; however, the cytokine can also promote cell survival through anti-apoptotic signals (Gaur and Aggarwal 2003). Given the functional duality of TNF-α, the net effect of its action seems to depend upon several parameters including tissue type, cellular context, timing and duration of exposure and receptor type activation (Wajant *et al.* 2003; Fontaine *et al.* 2002).

Apart from LPS which has been shown to induce the expression of both IL-6 and TNF-α in the U373-MG cell line (Espevik *et al.* 1993; Palma *et al.* 1995) and IL-6, but not TNF-α, in the U251-MG line (Vitkovic *et al.* 1991), to the author's knowledge there is no evidence to show whether exposure to the particular toxicants used in this study cause the release of IL-6 or TNF-α in any of the U373-MG, U251-MG or CCF-STTG1 human astrocytoma cell lines. It would therefore be of interest to determine whether such signalling factors are released in these lines following toxicant exposure and whether there is any correlation between their induction and the increases in GFAP-IR and MTT turnover observed in the previous experimental chapter.

As discussed in Chapter 3, with the exception of acrylamide, free radical production has been proposed as a factor in the cause of cell death from each of the toxicants used in previous chapters. It may be coincidental that the three compounds chosen for their ability to induce astrocyte reactivity have been shown to induce reactive oxygen species production; however, it is possible that ROS may play a role in the induction of increases in GFAP-IR as observed in Chapter 4. In fact, as described in the main introduction, the reactive oxygen intermediate and biological mediator, nitric oxide (NO), has been proposed as a potential mediator in the induction of GFAP as associated with astrogliosis. (Brahmachari *et al.* 2006).

Nitric oxide is lipid soluble and diffuses freely across cellular membranes. It is produced together with citrulline from L-arginine, NADPH and O_2 via a reaction catalysed by enzymes of the nitric oxide synthase (NOS) family (Palmer *et al.* 1988). There are three members of the NOS family; nNOS, eNOS and iNOS. Both nNOS and eNOS are expressed constitutively, not only in neuronal and endothelial cells respectively, but also in a variety of other cell types and their expression is regulated by calcium and calmodulin (Alderton *et al.* 2001). The third member of the NOS family, iNOS, or inducible NOS, can be expressed in a wide variety of cell types including several types of immune system cells and glial cells (Bogdan *et al.* 2001; Saha and Pehan 2006). iNOS has an insensitivity to calcium and as the name suggests its expression is induced by a variety of stimuli including endotoxin and inflammatory cytokines (Alderton *et al.* 2001).

Nitric oxide has a variety of cellular effects including an involvement in the formation of the second messenger guanosine 3'5'-cyclic monophosphate (cGMP) by activating the enzyme guanylyl cyclase (GC) which catalyses its formation. cGMP in turn regulates process such as cell proliferation (Hood and Granger 1998), neuronal synaptic plasticity (Arancio *et al.* 1996) and inflammation (Guzik *et al.* 2003). Boran and Garcia (2007) provided evidence for the involvement of NO-dependent cGMP formation in the induction of reactive changes induced by lipopolysaccharide (LPS), whereby direct treatment with dibutyryl-cGMP was able to alter GFAP distribution in cultured rat cerebellar astroglia. Subsequent inhibition of protein kinase G prevented this cytoskeleton rearrangement. It is therefore possible that factors which stimulate the production of reactive oxygen species such as nitric oxide may induce GFAP through the GC-cGMP-PKG pathway and not exclusively via NF-κB.

Given the proposed role of nitric oxide in the induction of GFAP, it would be of interest in this chapter to determine whether there is an induced expression of nitric oxide in the astrocytic cell lines following toxicant and endotoxin exposure, in order to explore the possible role of this signalling molecule in the chemical stimulation of GFAP-IR increases as observed in chapter 4.

As a way of beginning to determine the potential expression of cytokines and the generation of nitric oxide in human astrocytic cell lines following toxicant and endotoxin exposure, this chapter will employ commercially available assays in order to quantify the levels of such released factors. Cytokines are normally released into the extracellular space in order to facilitate their signalling to adjacent cells. Released cytokine can therefore be detected in the cell culture supernatant of cultured cells. For this purpose the sandwich ELISA, as described in the introduction to Chapter 4, is an established method used to capture, detect and quantify these cytokine molecules in solution and will therefore be used to identify levels of IL-6 and TNF-α in the cell culture supernatant of cells exposed to the same toxicants and endotoxin used in previous experimental chapters.

As nitric oxide is a highly unstable oxygen metabolite, it is difficult to assess accurately levels released into biological substrates such as culture media. NO is quickly reduced to its metabolites nitrite (NO_2^-) and nitrate (NO_3^-). Assaying for these more stable derivatives, particularly nitrite, has therefore been widely used as a simple method to indirectly measure relative NO release from cells. The nitrite assay relies on a diazotization reaction that was originally described by Griess in 1879. Many modifications to this original reaction have been described over the years yet the principle remains the same; in the first step of the reaction, nitrite is converted to a nitrosating agent under the acidic (phosphoric acid) conditions of the sulphanilamide solution. This nitrosating agent then binds to sulphanilamide to produce the diazonium ion. This ion is then coupled to N-1-napthylethylenediamine dihydrochloride (NEDD) to form the chromophoric azo-derivative which absorbs light at 540-570nm. The formed product can be easily quantified by spectrophotometric analysis. This method of nitrite determination will therefore be used to determine changes in nitric oxide release in treated cells.

IL-6 and TNF-α release are to be assayed in the U373-MG cell line, as this line exhibited the most activation-like responses in GFAP-IR levels and MTT turnover in Chapter 4. There is also evidence that the U373-MG line is able to release the IL-6 cytokine in response to bacterial LPS (Palma *et al.* 1995) that would serve as a positive control and for comparison purposes. The nitrite assay was performed for each of the three astrocytoma cell lines. A single preliminary exposure period of 24 hours was also chosen for all assays to coincide with the major time point of these investigations with previous cytotoxicity (Chapter 3) and astrocyte activation (Chapter 4) studies.

It is hoped that by using these assay systems to identify levels of released cytokines and nitrite from exposed cells, it will be possible to begin to create a more comprehensive picture of the effect of sub-cytotoxic concentrations of the test compounds on the reactive response of the astrocytic cell lines in this study.

5.2 Methods.

5.2.1 Chemicals and reagents.

Cell culture reagents and saline buffers were purchased from Gibco unless otherwise stated. Toxicants were sourced as in section 3.2.1. All other chemicals and reagents were purchased from Sigma unless otherwise stated. All tissue culture plastics were from Corning.

5.2.2 Cell cultures.

Cell lines were maintained as outlined in section 2.2.2. Cells were seeded for assay in tissue culture treated 96 well microplates as described in section 3.2.2 at the seeding densities consistent with those used in the GFAP assays in Chapter 4. Cells were allowed to attach to the culture plate and stabilise overnight at 37°C before treatment.

5.2.3 Experimental treatment.

Astrocytoma cell lines were exposed to logarithmic dilutions of chloroquine diphosphate and trimethyltin chloride (TMTC) (from 0.1nM to 100µM) and ethanol (from 100nM to 100mM) and acrylamide (from 10nM to 10mM). Cells were also exposed to bacterial lipopolysaccharide at concentrations of 0.5, 1 and 5µg/ml. LPS from E.coli 0111:B4 was purchased from Sigma and reconstituted in PBS (pH 7.4) in accordance to the manufacturer's instructions before storage at -20°C. Using pre-filtered sterile stock solutions of each compound, working dilutions were prepared in the appropriate tissue culture medium according to the cell line being exposed (see section 2.2.2). Cells were exposed to toxicants for 24 hours.

5.2.4 Cell culture supernatant preparation.

Following exposure, the supernatants were collected from each test well and centrifuged at 179g for 10 minutes to remove any debris. The supernatants from each sample were then analysed for levels of human IL-6, human TNF-α and nitrite.

5.2.5 Cytokine detection assays.

5.2.5.1 Human IL-6 ELISA.

The human IL-6 ELISA Ready-SET-Go!® kit, purchased from eBioscience (San Diego USA), was used to quantify levels of the human interleukin-6 cytokine in the cell

culture supernatant of treated cells. All reagents unless otherwise stated were included in the kit and were prepared in accordance with the manufacturer's protocol. The ELISA method was performed in accordance with the manufacturer's guidelines, briefly; high affinity binding plates were coated with purified anti-human IL-6 capture antibody diluted in coating buffer by incubation overnight at $4^{\circ}C$. Wells were subsequently washed three times using $300\mu l$ of wash buffer (PBS, 0.05% Tween-20 pH 7.2), leaving to soak for 1 minute between washes. Wells were then blocked by incubation with $200\mu l$ of 1x assay diluent for 1 hour at room temperature and washed 3 times as before with $300\mu l$ of wash buffer. $100\mu l$ of recombinant human IL-6 standard dilutions and experimental samples were added to the appropriate wells in duplicate and incubated at room temperature for 2 hours. Wells were washed as before for a total of 5 washes and incubated with $100\mu l$ Biotin-conjugate anti-human IL-6 detection antibody at room temperature for 1 hour. Wells were again aspirated and washed 5 times and incubated with $100\mu l$ of avidin-HRP enzyme solution for 30 minutes at room temperature. Wells were then aspirated and washed 7 times leaving to soak for 2 minutes between washes. $100\mu l$ of substrate solution was then added to each well and incubated for 15 minutes at room temperature, after which $50\mu l$ per well of stop solution (2N sulphuric acid) was added to each well. Plates were then read at 450nm using a Thermo Multiskan EX 96-well microplate reader (Thermo Electron Corporation) with the absorbance set at 450nm. The IL-6 content of each sample was calculated using values from the recombinant human IL-6 standard curve (see Appendix 2).

5.2.5.2 Human TNF-α ELISA.

The human TNF-α ELISA Ready-SET-Go!® kit purchased from eBioscience (San Diego USA), was also used to quantify levels of the human tumour necrosis factor alpha cytokine in the cell culture supernatant of treated cells. All reagents unless otherwise stated were included in the kit and were prepared in accordance with the manufacturer's protocol. The ELISA method was performed in accordance with the manufacturer's guidelines, briefly; high affinity binding plates were coated with purified anti-human TNF-α capture antibody diluted in coating buffer by incubation overnight at $4^{\circ}C$. Wells were subsequently washed three times using $300\mu l$ of wash buffer (PBS, 0.05% Tween-20 pH 7.2), leaving to soak for 1 minute between washes. Wells were then blocked by incubation with $200\mu l$ of 1x assay diluent for 1 hour at room temperature and washed 3 times as before with $300\mu l$ of wash buffer. $100\mu l$ of recombinant human TNF-α standard dilutions and experimental samples were added to the appropriate wells in duplicate and incubated at room

temperature for 2 hours. Wells were washed as before for a total of 5 washes and incubated with 100µl of Biotin-conjugate anti-human TNF-α detection antibody at room temperature for 1 hour. Wells were again aspirated and washed 5 times and incubated with 100µl of avidin-HRP enzyme solution for 30 minutes at room temperature. Wells were then aspirated and washed 7 times, leaving to soak for 2 minutes between washes. 100µl of substrate solution was then added to each well and incubated for 15 minutes at room temperature, after which 50µl per well of stop solution (2N sulphuric acid) was added to each well. Each well was then analysed using a Thermo Multiskan EX 96-well microplate reader (Thermo Electron Corporation) with the absorbance set at 450nm. The TNF-α content of each sample was calculated using values from the recombinant human TNF-α standard curve (see appendix 3).

5.2.5 Nitrite assay.

NO produced during toxin treatment was estimated spectrophotometrically as formed nitrite (NO_2). In order to determine the nitrite content of the cell culture supernatant from treated cells, the Griess nitrite assay system (Promega) was employed. Briefly, following toxicant exposure, 50µl of cell culture supernatant was taken from each well and added to 50µl of the Griess reagent 1 (1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 minutes. 50µl of Griess Reagent B (0.1% NEDD) was then added and again incubated at room temperature for 10 minutes. Each well was then analysed at 550nm using a Thermo Multiskan EX 96-well microplate reader (Thermo Electron Corporation). The nitrite content of each sample was calculated using values from the standard curve constructed using $NaNO_2$ as standard (see appendix 4).

5.2.6 Data and statistical analysis.

In order to be consistent with other data analysis in this thesis as well as to aid comparison between results, data from both cytokine and nitrite assays are expressed as a mean percentage (± SEM) of non-toxicant treated control wells from the same experiment. Any significant changes in nitrite or cytokines levels in response to toxicant exposure were evaluated using one-way ANOVA followed by Dunnet's post-test where all data points were compared with a non-treated control. GraphPad Prism software (Version 3.02) was used for this purpose.

5.3 Results.

5.3.1 Interleukin-6 assay.

A sandwich ELISA for human IL-6 with a sensitivity of 2pg/ml and a standard curve range of 2 -200pg/ml was used in order to determine levels of released IL-6 by the U373-MG human astrocytoma cells following toxicant exposure. Using recombinant human IL-6, duplicate wells of sequential dilutions of the protein were included for each assay as standard. Increases in absorbance were directly proportional to the IL-6 concentration (R^2 values were greater than 0.989 for all constructed standard curves (see Appendix 2)), indicating that the assay was able to successfully identify the presence of IL-6 within the range of the standard curve.

IL-6 levels in the media of U373-MG cells were found to be significantly increased following 24 hours exposure to ethanol, trimethyltin chloride, chloroquine diphosphate and LPS. No significant increase was observed following acrylamide exposure (Figure 5.1). Treatment with 10 and 100mM ethanol for 24 hours resulted in detectable IL-6 levels of 29.9 ($\pm$2.10) and 29.74 ($\pm$3.73) pg/ml respectively. These values indicated a significant rise in levels of the cytokine, being 60.4 ($\pm$11.28) and 59.6 ($\pm$20.04) percent greater than control values respectively ($p<0.05$) (figure 5.2). Treatment with trimethyltin chloride also resulted in a significant increase in detected IL-6 levels in the media following 1μM exposure ($p<0.001$), achieving levels of 46.9 ($\pm$5.12) pg/ml compared with 19.1 ($\pm$0.14) pg/ml of controls, thus demonstrating a level of release that was 146.36 ($\pm$26.82) percent greater than controls (figure 5.3). Exposure of the U373-MG cells to the highest tested concentration of chloroquine diphosphate of 0.1mM resulted in a dramatic and significant elevation in the concentration of IL-6 in the cell culture supernatant (figure 5.4). Absorbance readings at this treatment concentration were above the range of the standard curve; in excess of the maximal highest value of 200pg/ml detectable by the assay, while the control value was 35.3 ($\pm$3.24) pg/ml thus demonstrating a significant increase at this maximal value of 566.5 percent ($p<0.001$).

A similarly dramatic increase in IL-6 was observed following 24-hour exposure to bacterial lipopolysaccharide (figure 5.5). For all three concentrations tested, IL-6 levels were elevated to above the 200pg/ml range of the standard curve and were thus
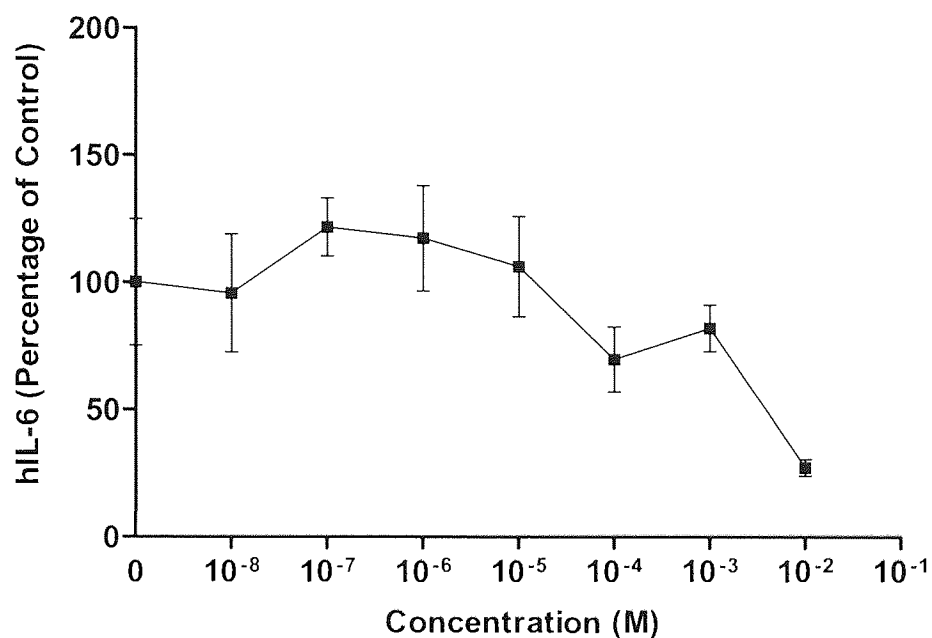
Figure 5.1 Effect of 24 hours exposure to acrylamide on released IL-6 levels from cultured U373-MG human astrocytoma cells. Levels of IL-6 in the cell culture supernatant of treated cells were determined by sandwich ELISA. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=4 from duplicate experiments).
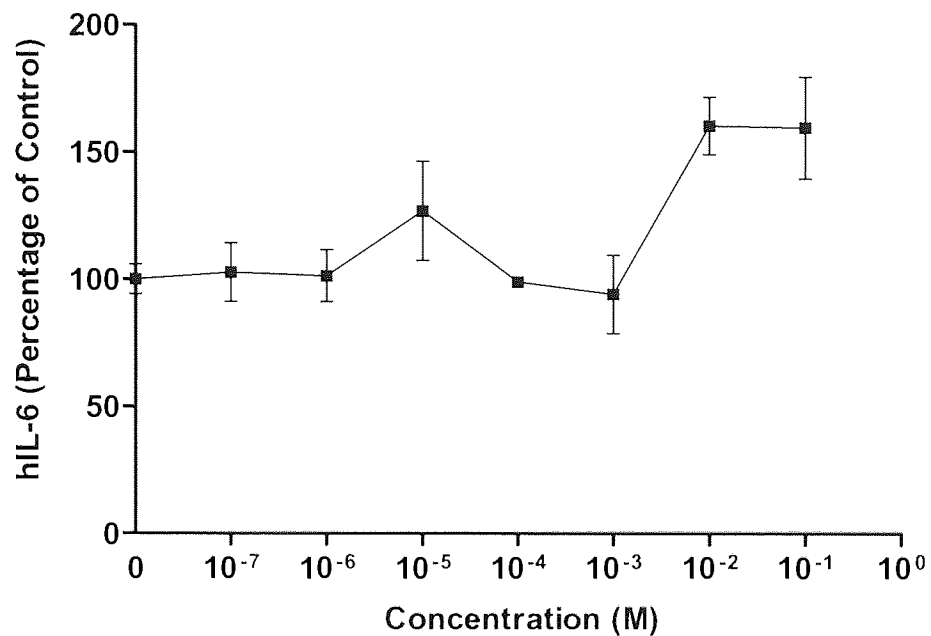
Figure 5.2 Effect of 24 hours exposure to ethanol on released IL-6 levels from cultured U373-MG human astrocytoma cells. Levels of IL-6 in the cell culture supernatant of treated cells were determined by sandwich ELISA. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=4 from duplicate experiments).
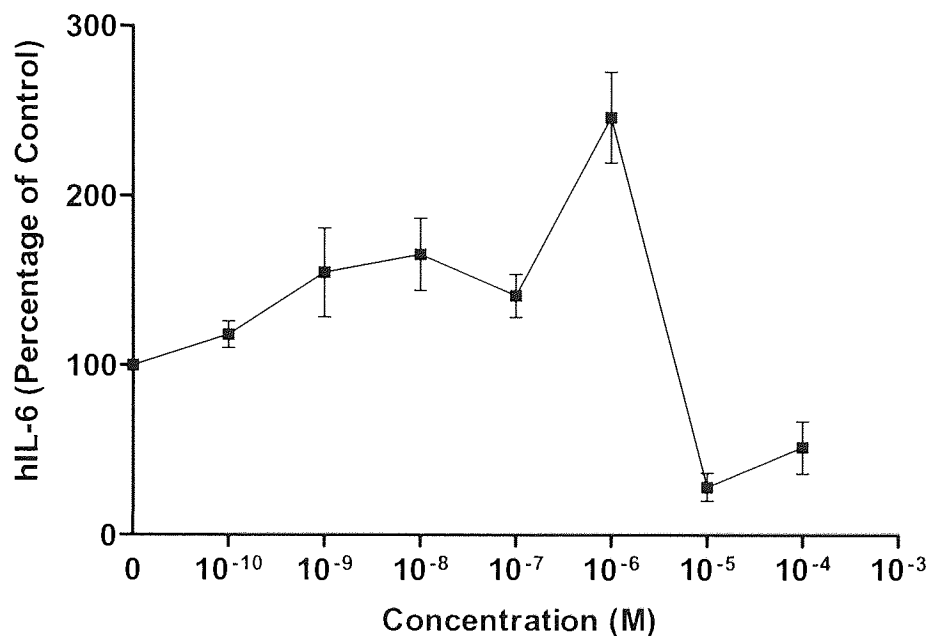
Figure 5.3 Effect of 24 hours exposure to trimethyltin chloride on released IL-6 levels from cultured U373-MG human astrocytoma cells. Levels of IL-6 in the cell culture supernatant of treated cells were determined by sandwich ELISA. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=4 from duplicate experiments).
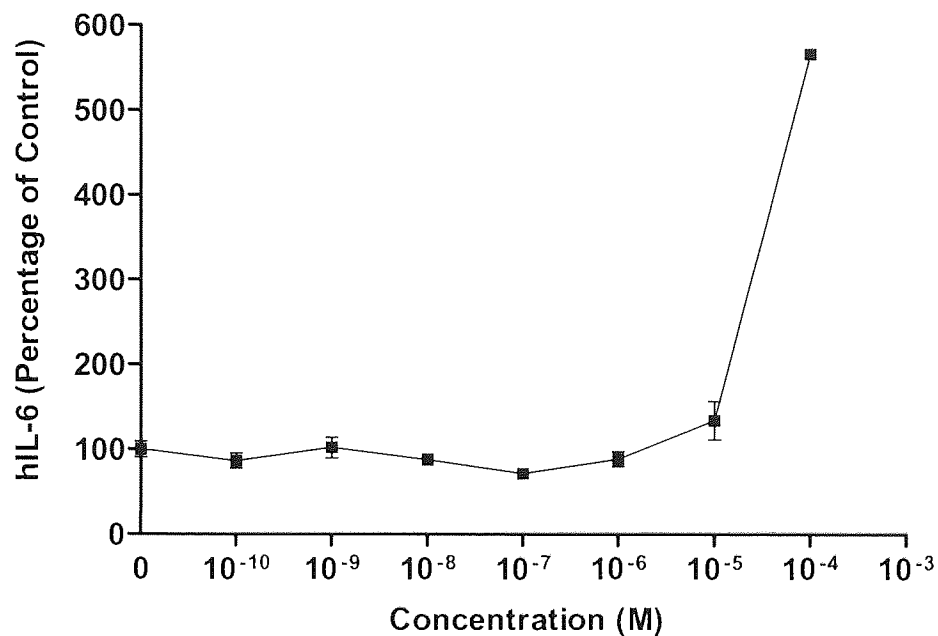
Figure 5.4 Effect of 24 hours exposure to chloroquine diphosphate on released IL-6 levels from cultured U373-MG human astrocytoma cells. Levels of IL-6 in the cell culture supernatant of treated cells were determined by sandwich ELISA. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=4 from duplicate experiments).
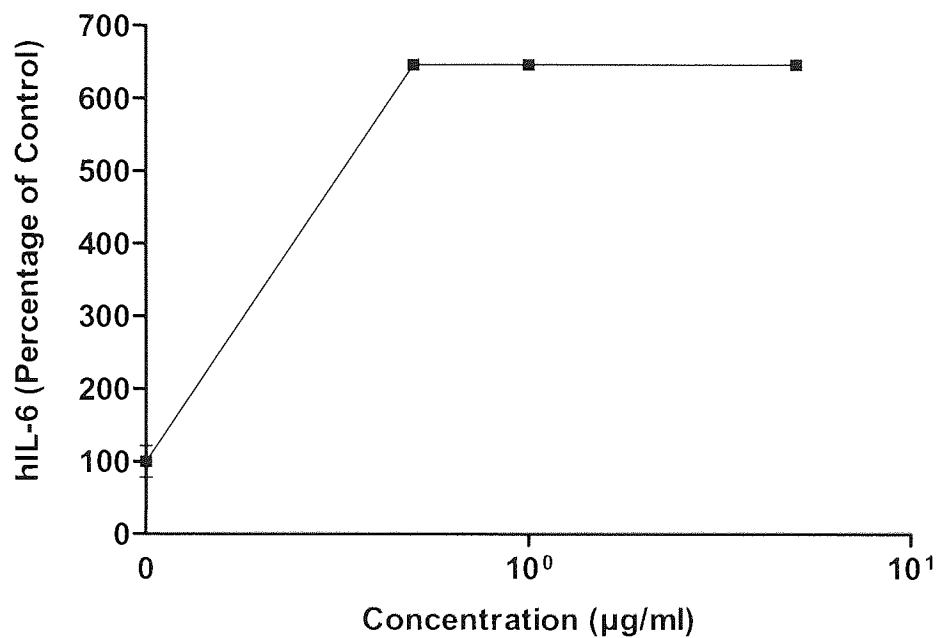
Figure 5.5 Effect of 24 hours exposure to bacterial lipopolysaccharide on released IL-6 levels from cultured U373-MG human astrocytoma cells. Levels of IL-6 in the cell culture supernatant of treated cells were determined by sandwich ELISA. Data are expressed as a mean percentage of non-treated control wells ± SEM (n=4 from duplicate experiments).

significantly greater than control values of 30.9 (±6.73) pg/ml, thus demonstrating a significant increase at this point of 647 percent (p<0.001).

5.3.2 Tumour necrosis factor-alpha assay

Levels of human TNF-α were also determined in the cell culture medium of U373-MG cells exposed to the individual toxicants using a commercially available ELISA kit with a sensitivity of 4pg/ml and a standard curve range of 4 -500pg/ml. Duplicate wells of the diluted standards of recombinant TNF-α were included on each assay plate. These standard wells showed positive for the protein in a concentration dependent manner (The R^2 values for each of the standard curves performed were greater than 0.996 (see Appendix 3), thus indicating that the assay was successful at identifying the presence of TNF-α within the range of the standard curve. Using the standard curve to calculate the concentration of TNF-α in the cell culture supernatants, no TNF-α was identified in the non-treated control wells, nor was there any TNF-α identified from the cells exposed for 24 hours to any of the experimental compounds, including LPS, at any concentration.

5.3.3 Nitrite

The Nitrite assay was performed to determine levels of this nitric oxide metabolite in the culture medium of the experimentally treated U373-MG, U251-MG and CCF-STTG1 cells. Sequential two fold dilutions of sodium nitrite from 100-1.56µM were used as standard, for which an increase in absorbance was observed in direct proportion with the sodium nitrite concentration (R^2 values were consistently greater than 0.998 for all constructed standard curves (see Appendix 4)), indicating that the assay was able to successfully identify the presence of nitrite within the range of the standard curve. Using the standard curve to calculate nitrite concentration of the cell culture supernatant, no nitrite was detected in the non-treated control wells for each cell line. No significant elevation in nitrite was detected in the cell culture supernatant of each of the three astrocytoma cell lines following 24 hours exposure to any of the experimental compounds, including LPS, at any concentration.

5.4 Discussion.

It is clear from results in this chapter that toxicant exposure evokes a cytokine response in the U373-MG astrocytic cell line. The significant increases in IL-6 release caused by 24 hours exposure to ethanol, chloroquine diphosphate, trimethyltin chloride and bacterial lipopolysaccharide highlight the capacity of the U373-MG cell line to exhibit aspects of the astrocytic inflammatory response.

Bacterial lipopolysaccharide is widely used to experimentally induce astrocytic expression of proinflammatory cytokines including IL-6 in primary culture (Sharif *et al.* 1993; Grimaldi *et al.* 1998). In the present study, LPS was found to have one of the most dramatic effects on IL-6 release from the human astroglial U373-MG cells, causing increases above the maximal levels detectable by the assay following exposure to all three concentrations tested, the lowest being 0.5µg/ml. These results are similar to those of Espevik *et al.* (1993) whereby LPS at concentrations above or equal to 32ng/ml resulted in dose related IL-6 production. Similar LPS stimulation of IL-6 induction was observed in U373-MG and U251-MG by Palma *et al.* (1995) and Vitkovic *et al.* (1991) respectively.

Chloroquine diphosphate exposure was also shown to cause elevations in IL-6 release to levels exceeding the maximal limit of the assay. While chloroquine has many well known anti-inflammatory properties, reflected in its use to treat a variety of autoimmune diseases, it is also known to have pro-inflammatory properties specifically in astrocytes, stimulating the production of pro-inflammatory cytokines in these cells (Park *et al.* 2003) while inhibiting their production in other cells of the human immune system (Jeong and Jue 1997; van den Borne *et al.* 1997; Karres *et al.* 1998, Park *et al.* 2003). Indeed, in the present study, while 24-hour chloroquine diphosphate exposure was shown to have no elevating effect on GFAP or MTT turnover in the U373-MG cell line, there was a dramatic elevation in the amount of IL-6 released into the cell culture medium following exposure to 100µM of the compound. This concentration is only marginally greater than the lowest observed concentration to a cause significant reduction in cell viability according to results in Chapter 3. Park *et al.* (2003) also demonstrated that this same concentration of chloroquine was able to induce IL-6 mRNA and protein expression in the CRT-MG human astroglial cells, commenting also that increases in IL-6 mRNA were also

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seen upon chloroquine treatment in the U251-MG and U373-MG cells used in this study as well as in primary human and rat astrocytes.

Significant elevations in IL-6 release were also observed following 24 hours of 1μM TMTC exposure, the same concentration seen to induce a significant increase in MTT turnover in this cell line at the same time point and which is also lower than the NOAEL for MTT turnover in chapter 3. The majority of research regarding the effect of trimethyltin on IL-6 cytokine release has focused mainly around *in vivo* studies. The main findings of these studies are that the compound induces the production of proinflammatory cytokines including IL-6 in the hippocampus of effected animals (Maier *et al.* 1995; Bruccoleri *et al.* 1998; Harry *et al.* 2001; Jean Harry *et al.* 2003). However, it is not clear in these studies the exact cellular source of these cytokines. Of the limited studies conducted specifically using astrocytic cell cultures, the findings were similar to *in vivo* studies. Harry *et al.* (2002) described how 24 hour exposure to 10μM TMT induced an increase in IL-6 mRNA and protein levels in mixed cortical glial cultures. The increases in IL-6 levels observed in the present study using human astrocytoma cell lines therefore seem to be consistent with *in vivo* and primary cell studies.

Ethanol exposure was shown to induce IL-6 release at concentrations of 10 and 100mM. The former of these concentrations was the same as that shown to induce increases in GFAP-IR in chapter 4 while both are within the non-cytotoxic range according to cytotoxicity results of chapter 3. While ethanol has been implicated in the production of several pro-inflammatory cytokines in a variety of cell types and this study demonstrates that ethanol also causes the induction of IL-6 release from astrocytoma cell lines at non-cytotoxic concentrations, a fact which may have implications in regards to the proinflammatory properties of ethanol in the brain following recreational exposure.

The fact that the astrocytoma cells showed no response to acrylamide again substantiates its continued use as a negative control. From the repeated lack of response shown by the astrocytoma cells throughout this study it can be proposed that the compound does not affect the reactive response in astrocytic cells in culture.

There have been several suggested pathways via which the above compounds act to cause the induction of IL-6. There are a great number of proinflammatory agents,

neurotransmitters and second messengers that can modulate IL-6 expression (for review see Van Wagoner and Benveniste 1999). The particular modulators involved in the toxicant induced expression of IL-6, as demonstrated in this study, are still to be fully elucidated. It has been suggested by Van Wagoner and Benveniste (1999) that the mechanism by which LPS induces astrocytic IL-6 is likely to involve LPS induced astrocytic expression of TNF-α and IL-1β, both of which are known to have a central role in modulating the transcription of IL-6 via their activation of the transcription factor NF-κB (Shimizu *et al.* 1990; Zhang *et al.* 1990). In the case of chloroquine, Park *et al.* (2003) similarly investigated the possibility that chloroquine induced IL-6 induction is due to the production of mediators such as IL-1β or TNF-α. While it was reported that chloroquine induced the pro-inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α in human astroglial cells, it was also found that blocking the actions of IL-1β and TNF-α did not stop the chloroquine induced expression of IL-6, suggesting that IL-6 induction is independent these factors. In the same study, NF-κB studies indicated that chloroquine induced the activation of NF-κB and the subsequent induction of cytokines. It was also shown that NF-κB activation is a result of the proteosomal degradation of its inhibitor IκB (Park *et al.* 2003). Alternatively to the involvement of NF-κB, other transcription factors such as AP-1, which has also been associated with IL-6 synthesis in astrocytes (Lieb *et al.* 1997), may also be involved in toxicant induced IL-6 expression. Determination of the exact pathways and mediators involved in the IL-6 induction, specifically in astrocytic cell lines, by each individual compound used in this study requires further investigation.

In contrast with the dramatic elevation in IL-6 release, there were no detectable levels of TNF-α in the cell culture media of treated cells following exposure to any of the experimental toxicants. While Eskes *et al.* (2003) demonstrated that co-culture with neurons is necessary for TMT induced TNF-α release from astrocytes, potentially explaining the lack of induction in our monotypic astrocytic cultures, this is in contrast with the findings of Park *et al.* (2003) who reported that monotypic cultures of human astrocytic cell lines exposed to chloroquine were induced to express TNF-α in a time and dose dependent manner. Similarly, 24 hours exposure to 10μM trimethyltin was also shown to induce both TNF-α along with IL-6 in mixed cortical glial cell cultures by Harry *et al.* (2002). As the same does not seem to be the case in this study, it is possible that these conflicting findings may be attributed to differences in cell line and type as well as exposure times used for the respective studies. While Park *et al.* (2003) demonstrated

increases in TNF-α mRNA and protein in the CRT-MG human astroglial cell line following 16 hours exposure, only increases in mRNA for the cytokine were reported in the U251-MG and U373-MG cell lines at this time point. As the present study assays for cytokine protein in the cell culture supernatant it is possible that the U373-MG cell line in particular may require a longer period of exposure before levels of released TNF-α are detectable in the media. Alternatively, it is also possible that potential posttranslational modification of TNF-α mRNA may mean protein levels are not actually elevated in this cell line.

The cytokine assays in this study were performed merely as a preliminary assessment to begin to determine whether factors of the astrocytic activation response were presented following toxicant exposure in human astrocytoma cell lines. As a consequence of time and financial constraints, it was decided to initially assay IL-6 and TNF-α release in just the U373-MG cell line. Further investigations using the U251-MG and CCF-STTG1 cell lines will help build a more complete picture of cytokine release which can be applied over a number of different cell lines. This may also shed some light on the differential capacity of astrocytoma lines to releasing TNF-α in response to toxicant exposure. Similarly only the 24 hour time point was used as a preliminary assessment. It would be of interest to further investigate 4 and 16 hours expression as well as greater time points. This would allow comparison with the GFAP data and determine any exposure time link between the onset of expression of certain cytokines and the increases in GFAP-IR.

In order to determine whether exposure to the experimental toxicants resulted in the increased expression of the astrogliosis associated signalling molecule, NO, its stable derivative, nitrite, was measured. Previous studies have shown that LPS induces the expression of NO in mouse and rat primary astrocytes (Brahmachari *et al.* 2006; Kozuka *et al.* 2005). In the present study, however, there was no detectable nitrite in the media taken from cultures of any of the three astrocytoma cell lines, following exposure to each of the tested compounds. This indicates that NO is not released from the three astrocytoma cell lines following exposure to the compounds, including LPS. Despite previous evidence to suggest that it is possible to induce iNOS in U373-MG, U251-MG and CCF-STTG1 cells (Nanetti *et al.* 2005; Kawakami *et al.* 2002; Kim *et al.* 2002), Janabi *et al.* 2004 found that in each of these same cell lines, IFNγ+IL-1β (together a potent inducer of iNOS expression) failed to induce the expression of iNOS protein or mRNA. It was suggested

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that this lack on iNOS induction could possibly be due to a reduction in NF-κB activation inherent to glioma cells, particularly as iNOS activation depends strongly upon NF-κB activation (Taylor and Geller 2000). This does not, however, explain the reported induction of iNOS and the release of NO in the other studies using these same cell lines as described above. It is possible that the reason for such disparity between results following LPS exposure is that different stains of bacterial LPS were being used. Similarly, this could also explain the lack of any LPS induced TNF-α release in the present study. While it is possible that the astrocytoma cell lines used in the present study are not capable of iNOS expression and subsequent elevations in NO, the lack of detectable nitrite in the cell culture media of exposed cells in this study does not rule out nitric oxide as a mediator of GFAP expression. It is possible that while nitric oxide expression is induced, it is not released from the cell and acts purely as an intracellular signalling molecule. It is also possible that NO was released, but in such low amounts that it was undetectable by the assay meaning that higher cell seeding densities may be required. It would be possible to further elucidate the effect of toxicant exposure on NO induction by using a more sensitive assay such as the identification of potential increases in iNOS mRNA or protein levels.

To summarise the results in this chapter; each of the experimental toxicants, with the exception of acrylamide, caused the induction of IL-6 release from the U373-MG cell line indicating that a cytokine response can be achieved in this cell line. The lack of any induction in TNF-α expression may be highlighting the limitations of monotypic glioma cell cultures or may be indicative of the plasticity of the astrocytic response to various stimuli. No elevations in nitrite were detected in the culture medium from each of the three astrocytoma cell lines following exposure to any of the toxicants. This suggests that nitric oxide may not be released as an extracellular signalling molecule following toxicant exposure in these astrocytic cell lines, however, further work investigating the effects of each compound on iNOS expression is required to determine the involvement of NO in GFAP induction.

Chapter 6: Discussion and future directions.

The purpose of these investigations has been to begin the development of a human cell line based model for the accurate assessment of toxicity which was both specific to astrocytes and sensitive to low level toxic insult. The assessment of the astrocyte specific glial fibrillary acidic protein (GFAP), the up-regulation of which is a hallmark of astrogliosis, was chosen as a sensitive and specific marker of astrocytic response to chemical injury. The main aim was therefore to determine whether a reactive-like response as demonstrated by an increase in GFAP is achievable in human astrocytic cell lines.

The specific objectives were to firstly identify candidate cell lines for use in these studies. In Chapter 2 the U251-MG, U373-MG and CCF-STTG1 human astrocytoma cell lines were chosen for investigation due to their previous use as astrocytic models for a variety of purposes other than reactive studies. It was determined that all three of these lines were positive for GFAP and so were deemed suitable for use in the studies. Both the U251-MG and U373-MG lines were shown to strongly express the protein while the CCF-STTG1 cell line exhibited a lower level of expression. Despite the lower level of expression, it was decided to include this cell line in order to determine whether the initial level of GFAP expression had any bearing on the ability of the cell line to display a reactive-like response following injury. In addition to astrocytic cells, the SH-SY5Y human neuroblastoma cell line was chosen as a neuronal point of comparison. As this cell line is capable of differentiation to a more neuronal like phenotype upon treatment with retinoic acid, it was decided that both undifferentiated and differentiated cells would be used for comparison with the astrocytic cell lines. The differentiation procedure was confirmed in these cells through the visual detection of a more neuronal phenotype, including the rounding of cells and the extension of neuritic processes, as well as a detected up-regulation of the neuron specific enolase protein by Western blotting analysis.

The objective was then to determine the relative sensitivities of these cell lines in terms of cytotoxicity to various toxicants chosen for their previously reported abilities to cause astrocyte reactivity and increases in GFAP. These compounds were ethanol, chloroquine diphosphate, trimethyltin chloride and acrylamide, the latter of which was chosen as a negative control with the expectation that it would not cause elevations in GFAP when used in following experiments. Using the MTT assay, each compound caused

a dose dependent decrease in cellular viability with chloroquine diphosphate and trimethyltin chloride being similarly the most potent, followed by acrylamide and finally ethanol. While each of the five cell lines showed varying sensitivities to the compounds, the astrocytic cell lines were generally more resistant to the compounds than the neuronal lines. It was suggested that this resistance may be due either to the specific neurotoxic action of the compounds at cytotoxic levels or the enhanced protective systems inherent to the astrocytoma lines. When considering the effect of retinoic acid differentiation on the sensitivity of SH-SY5Y cells, differentiation was seen to confer some degree of resistance to toxicant exposure, particularly in the case of ethanol. Using the data from Chapter 3, it was possible to determine appropriate concentration ranges for each of the compounds which would be used when treating cells for the assessment of GFAP elevating effects. These ranges differed between compound in order to encompass both cytotoxic and sub-cytotoxic concentrations.

The experiments in both Chapter 2 and Chapter 3 set the ground work for meeting the main objective of the study which was to determine whether each of the chosen compounds were able to induce an up-regulation in GFAP levels in each of the astrocytoma cell lines. This was investigated in Chapter 4, whereby each of the compounds from Chapter 3, as well as bacterial lipopolysaccharide (LPS), were assessed. By performing a cell based ELISA for GFAP as well as concurrent assays for metabolic activity and cell number, it was found that each of the three cell lines were capable of responding either by up-regulated GFAP expression, as determined by an increase in GFAP immunoreactivity (GFAP-IR), or by increased metabolic activity, depending upon the toxicant type, dose and exposure time. Elevations in these parameters occurred at concentrations below those to have any significant effect on cytotoxicity according to the results of Chapter 3 making these activation responses more sensitive markers of astrocytic response to chemical injury than cytotoxicity analysis. When comparing both markers of activation, GFAP-IR is the more sensitive marker than MTT turnover at the times when elevations in both occur after exposure to the same compound for the same period of time. Exposure time is an important factor in the induction of these responses caused by different compounds in different cell lines, attributable possibly to the differential potencies and actions of the compounds combined with the differential sensitivities of the cell lines.

Because as with any toxicological screening procedure it is necessary not to rely upon one single endpoint to determine low level toxicity in the astrocytic cell lines, the final experimental objective of these studies was to begin to investigate other events typical of the astrocytic response to injury. It was therefore decided to preliminarily investigate the effect of toxicant exposure upon the release of cell signalling molecules associated with reactive astrocytes, such as interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α) and nitric oxide (NO). Of these three molecules only IL-6 was found to be released from the U373-MG cells following toxicant exposure. Indeed all experimental compounds apart from the negative control acrylamide were capable of inducing IL-6 released from the cells, indicating that these compounds are capable of invoking a cytokine response from the astrocytoma cell line. Further work, however, is required to justify the lack of increased TNF-α and NO release and to determine whether the same responses observed in this chapter for the U373-MG cell line are also typical of the U251-MG and CCF-STTG1 astrocytoma cell lines.

The completion of these studies calls for the evaluation of which cell lines are the most appropriate for use in further studies. It was decided in Chapter 2 that despite low basal levels of GFAP, the CCF-STTG1 cell line would be included in order to determine whether the initial level of GFAP expression had any bearing on the ability of the cell line to display a reactive-like response following injury. The significance of basal levels of GFAP expression with regard to increased cellular expression of the protein following chemical exposure is largely unknown. Malhotra et al. (1995) suggested that the ability of a glioma cell line to exhibit a reactive response as characterised by an increase in GFAP, may depend on their level of differentiation. Interestingly, basal GFAP expression is regarded as a reliable marker for astrocyte differentiation (Rutka et al. 1997); it is generally regarded that the more tumorigenic a cell line, the lower the basal expression of GFAP. This was illustrated by Murphy et al. (1998) where several clones of the U373-MG cell line were compared and contrasted in regards to their GFAP expression. That report indicated that high GFAP expression correlated strongly with lower tumorigenic aggression, as shown by high contact inhibition, low invasiveness and reduced migratory rate compared with low-GFAP expressing clones. The CCF-STTG1 cell line by these standards should be the least differentiated of the astrocytoma cell lines and thus according to Malhotra et al. (1995), the least likely to achieve a reactive response. Contradictory to this however, is that in the case of ethanol, the CCF-STTG1 cell line was the most

sensitive and exhibited the greatest increases in GFAP-IR. It therefore seems that low basal expression of GFAP has little bearing upon whether increases in GFAP can be induced in a particular cell line. Considering the variation in responses seen between each cell line, rather than select a single suitable cell line for continued use in similar studies, it would seem beneficial to facilitate the continued assessment of several cell lines in order to create a comprehensive picture that may permit generalisations in regards to typical responses.

It was important in these studies to choose compounds that had been previously identified as causative agents of astrogliosis *in vivo* or in primary cultures in order for there to be a point of comparison for the results in this study. In the present studies, ethanol, which was one of the least potently cytotoxic compounds, was found to cause an increase in GFAP-IR in all three astrocytoma cell lines following 24 hour exposure at concentrations between 0.1mM to 10mM depending upon the cell line. Specifically in the U373-MG cell line, the 10mM concentration was the same as that also shown to induce significant increases in IL-6 release. This indicates that increases in GFAP-IR and induced release of IL-6 occur simultaneously upon ethanol exposure at these concentrations in the U373-MG astrocytoma cell line. Not only does the former result confirm similar findings by Cookson and Pentreath (1994), suggesting that these astrocytoma cell lines respond in a similar manner to cells in rat primary cultures, both results also highlight the potential implications of recreational ethanol intoxication on the cells in the brain. These concentrations are all below the recognised legal limit for intoxication, which is currently 22mM in countries such as the US (Weinberg *et al.* 1998). This suggests that astrocytic effects can occur at levels of ethanol exposure below those of intoxication and which are easily achievable from social drinking. Also of particular interest are the cellular responses to chloroquine diphosphate exposure. Not only did the compound cause elevations in GFAP-IR after 4 hours in the U251-MG line and 24 hours in the CCF-STTG1 cell line, dramatic elevations in released IL-6 were also observed following 24 hours exposure in the U373-MG cell line. The capacity of chloroquine to cause such elevations in both GFAP-IR and cytokine release is particularly interesting as the agent is commonly used medicinally as an anti-inflammatory agent to treat conditions such as rheumatoid arthritis. Park *et al.* (2003) demonstrated differences in the immuno-modulatory effects of chloroquine between monocytes, microglia and astroglial cells. It was suggested that the differential response of astrocytes compared to other cells of the immune system may be attributable to differences in NF-κB activation and other signalling pathways in each of these cell types

(Park *et al.* 2003). With similar astrocytic effects observed in the present study, astrocytic cells are identified as a specific target of chloroquine toxicity. Trimethyltin chloride, like chloroquine diphosphate was one of the most potently cytotoxic of the tested compounds as determined in Chapter 3. While no effect was seen on GFAP-IR following 24 hours exposure, preliminary studies at 4 hours show significant increases in GFAP-IR following 1 μM exposure. While the U251-MG line was not included in the cytokine experiments, this same concentration was shown to induce significant increases in released IL-6 in the U373-MG cells. No elevations in GFAP-IR were observed in the U373-MG cells but interestingly, at each time point significant elevations in MTT turnover are observed at similar concentrations to those causing elevations in IL-6. This indicates that at similar exposure concentrations, both metabolic activation and IL-6 cytokine release are increased. The choice to use acrylamide as a negative control has been substantiated by the results in these studies. Not only were the neuronal cell lines significantly more sensitive to the compound than the astrocytic lines, acrylamide was the only agent not to cause elevations in GFAP-IR, MTT turnover, confirming results by Cookson *et al.* (1994) and also highlighting the similarity of responses in astrocytic cell lines and astrocytes in primary culture. From the similar lack of effect on IL-6 release, it is clear that the compound is a specific neurotoxicant and has little effect on astrocytic responses.

As previously discussed, both IL-6 and TNF-α have been associated with the activated astrocyte. These are only two of the many cytokines which have been proposed to have potential associations with astrocytic activation (Ridet *et al.* 1997). Due to the complexity and plasticity of astrocytic activation, not all of these factors may be involved in every pathway of induction. In fact whether cytokine expression or signalling is a necessity for astrocyte reactivity and the subsequent elevation in GFAP to occur is contentious. Little and O'Callaghan (2001) questioned whether cytokine expression or signalling is a necessity for astrocyte reactivity and the subsequent elevation in GFAP. By evaluating the current literature in this area Little and O'Callaghan (2001) deduced that not only does it seem that gliosis can occur in the absence of cytokine induction, but conversely, elevations in cytokines can occur without the induction of gliosis. It was therefore proposed that the traditional theory that links cytokine expression and astrogliosis induction is circumstantial and that while proinflammatory cytokines are seen to be induced in astrocytes alongside astrocyte activation, there is little evidence to suggest that they are involved in the induction of the process. In the present studies, in the case of

chloroquine diphosphate exposure, increases in GFAP-IR occur at concentrations lower than those shown to cause any significant increase in IL-6. From this it would seem that the cells respond initially by increases in GFAP followed by the expression of proinflammatory cytokines. This supports the idea that proinflammatory cytokines are the effect and not the cause of astrocyte activation; however, the assays for IL-6 release were performed only after 24 hours exposure and the nature of the assay means that IL-6 content of the media is cumulative and so may occur at any time up until this point. It is therefore necessary that cytokine expression is investigated at earlier time points, as in the GFAP assays, in order to elucidate the pattern of cytokine and GFAP-IR induction following exposure to a particular toxicant.

6.1 Further studies and future directions.

Some of the results in these studies are gathered from preliminary data, particularly the effects of TMTC and acrylamide exposure on GFAP-IR at 4 and 16 hour time points. Further repeats of these experiments are required to confirm the results in preliminary studies. As discussed in Chapter 5, the assays for cytokine release were also performed as an initial assessment and require further investigation using the U251-MG and CCF-STTG1 cell line. Similarly, cytokine and nitrite assays were performed only at 24 hours exposure as initial studies. The induction of GFAP up-regulation seems to be dependent upon the time of exposure in these studies, suggesting that the timing of onset from stimulation to expression of protein and the subsequent reduction of protein with cell recovery is crucial. The full exploration of time points would therefore be beneficial. While this study focuses on the effect of acute exposure to high doses of ethanol, *in vitro* chronic exposure to ethanol for example, even at low levels, has been shown by Lamarche *et al.* (2003) to be more harmful than acute exposure. It would therefore be of interest to explore the effects of prolonged exposure on both cytotoxicity and astrocytic responses by adapting the assay systems used in these studies for more chronic exposure conditions.

While detection of protein levels is necessary as the end product and hallmark of astrocyte reactivity, it would be of added interest to investigate the onset of mRNA expression for the protein, not only as a more immediate marker of astrocytic response but also to determine whether the elevations in GFAP-IR are due to an increase in transcription of the protein meaning new RNA/protein synthesis or whether the changes are due to an "activation" of GFAP as suggested by Eng and Ghirnikar (1994) and Wu and Schwartz

(1998) whereby the specific epitopes recognised by anti-GFAP antibodies are made immunohistochemically more accessible. There may also be the added dimension of possible increases in GFAP mRNA without subsequent increases in the protein. Investigation of these factors may help to create a comprehensive picture of the astrocytic response following toxicant exposure.

To this point, the current studies have provided the ground work in the development of a simple assay system utilising human astrocytic cell lines for the high throughput screening of toxicants. Using these assays, astrocytic responses can be distinguished by those of other cell types by assessing levels of the specifically astrocytic marker GFAP. The simplicity means that there are many possibilities in regards to future directions that the research could take by building upon this assay system. As described previously, the ultimate aim of this course of investigation is to develop a co-culture model of cells in the CNS, specifically neurons and astrocytes. The introduction of a neuronal component in these test systems would create a more accurate representative of the intricate interactions between different cell types in the CNS and how this impacts on the individual responses of each cell type to toxicant exposure.

If assaying for the effect of co-culture on the reactive response of astrocytes, it would be possible to use even the simplest methods of co-culture, such as the growth of one cell population upon another. In this respect, the cELISA assay used in the present studies is ideal for use as a basis in co-culture experiments, as all cells can be fixed post treatment and only the cells of interest can be identified by utilising appropriate cell specific markers. In regards to detecting only astrocytes or quantifying GFAP levels, anti-GFAP antibodies would specifically detect this astrocytic marker. It would be necessary, however, to control for the ratios of one cell type from another as co-culture may be more permissive of the growth of one cell line than another. If investigating the specific responses of neuronal cells in co-culture, it would be necessary to employ a neuronal specific marker of toxicity, the suitability of which would have to be evaluated. Alternatively to mixed culture, if non cell type specific endpoints were to be assessed, for example the MTT assay for cellular viability, a method for the separation of the cell types would be required which would not excessively disrupt the cells and distort the results. The use of transwells may be ideal for this purpose whereby very close culture (separated by a

porous membrane) of two cell populations is permitted while allowing for their eventual, easy separation.

It is likely that regardless of any toxicant exposure, the cell types will respond to being grown with others. Morphological changes have been observed in cells grown in co-culture, for example, when cultured with neurons, U251-MG cells produce highly GFAP positive processes. (Weinstein *et al.* 1991) while in the absence of neurons, the same cells still express significant levels of GFAP but do not extend such processes. Similar results were also found by Chen *et al.* (1994). While Weinstein *et al.* (1991) observed that the extended astrocytic processes were not directly involved in neurite outgrowth or neuronal survival, it is possible that the processes will at some point come into contact with the neuronal cells and potentially result in the altered phenotypes and behaviours of both cell types. The determination of such alterations would be of particular interest in relation to basal expression of GFAP protein in astrocytic cells as well as to any changes in cellular sensitivity to cytotoxic injury resulting from changes in the capacity of the cell to withstand toxicant induced injury. It is possible that the co-culture of both astrocytes and neurons would confer some degree of resistance against toxicant exposure upon the neuronal cells, particularly as previous studies employing co-culture of astrocytes and neurons have demonstrated such neuroprotection. Woehrling *et al.* (2007) found that by comparing co-cultures of mixed astrocyte and neuronal cells with purely neuronal cultures, the astrocytic cells exerted a protective effect on neurons during exposure to various human neurotoxins. These studies were conducted using NTera2D1 neuroblastoma cells, experimentally differentiated using retinoic acid and combinations of mitotic inhibitors in order to produce either solely neuronal cells or mixed cultures of these neurons and astrocytic cells. It would be of interest to determine whether similar protection can be conferred by co-culturing undifferentiated astrocytoma and neuroblastoma cell lines to facilitate the ongoing high-throughput nature of the assays.

As well as the mechanisms by astrocytes confer protection to neurotoxic insult such as the increased intracellular antioxidant systems and the glutamate mediated protection against excitotoxicity, it would also be of interest to determine whether astrocyte activation incurs an even greater level of protection to neurons, particularly as several antioxidant systems are up-regulated during astrocyte reactivity (Pentreath and Slamon 2000). It is possible to induce gliosis either using an astrocyte specific agent such as LPS which should

have little effect on neurons and then subsequently expose neurons to toxicants in the presence of these activated astrocytes. Alternatively, if investigating a particular agent for its neurotoxic effects but which is also known to cause gliosis *in vitro*, simultaneous treatment could be performed.

While GFAP is seen to increase in the astrocytoma cell lines following toxic insult, it cannot be ruled out that GFAP increases *in vivo* may be a result of prior damage to neurons and that the incorporation of neurons into the *in vitro* system may result in a greater GFAP response. Astrocytes respond not only to direct injury but to injury in surrounding cell types including neurons. Canady and Rubel (1992) showed how astrocytes respond by an increase in GFAP to even mild neuronal dysfunction and impairment. Co-culture with neurons may result in increases in GFAP following toxicant exposure which would not be seen in astrocyte cultures. If this is the case it would also be of interest to also identify the factors released by injured neurons which cause the up-regulation of GFAP.

As well as astrocytic co-culture with neurons, it may be of added interest to co-culture with additional cells types such as microglia. It was found that microglial activation precedes astrogliosis following TMT exposure (McCann *et al.* 1996) therefore investigation of microglial markers may be an even more sensitive marker for neurotoxicant induced brain injury. It is possible to source human microglial cell lines, such as the HM06 (Nagai *et al.* 2001) and CHME3 (Lindberg *et al.* 2005) cell lines, that can be incorporated into a co-culture system with astrocytes and neurons to simulate a more complete inflammatory response as could be observed *in vivo*. Additionally, human oligodendrocytic cell lines HOG, MO3.13 and KG-13 (Buntinx *et al.* 2003) may also be included in these systems, adding to the diversity of cell types in the culture. There may be complications in developing a multi-cell culture system, for example with the formulation of a culture medium that will support the growth of all cell types in the culture. If, however, the difficulties in developing such a system could be overcome, the potential benefits and applications of a human, *in vitro*, multi-cellular system for toxicity would be great.

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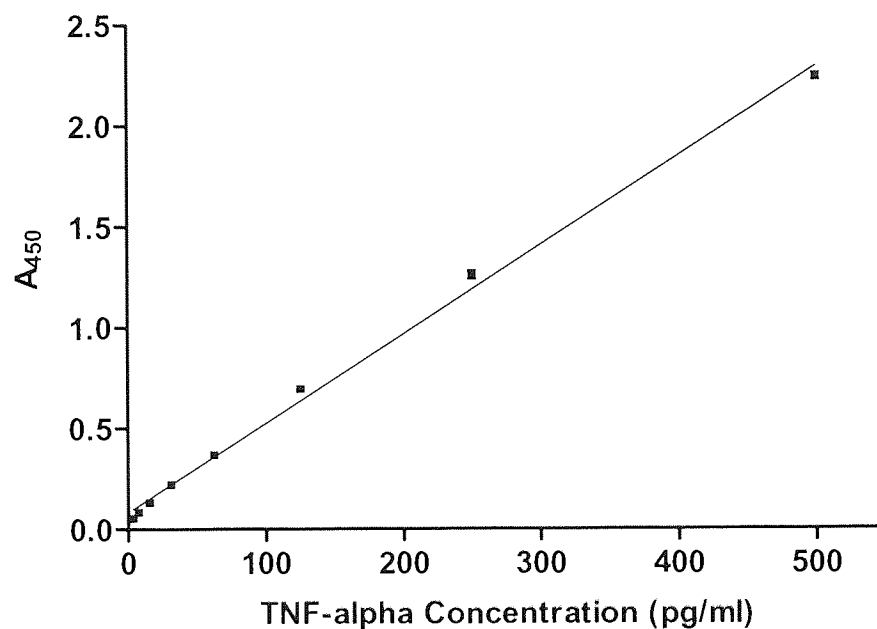
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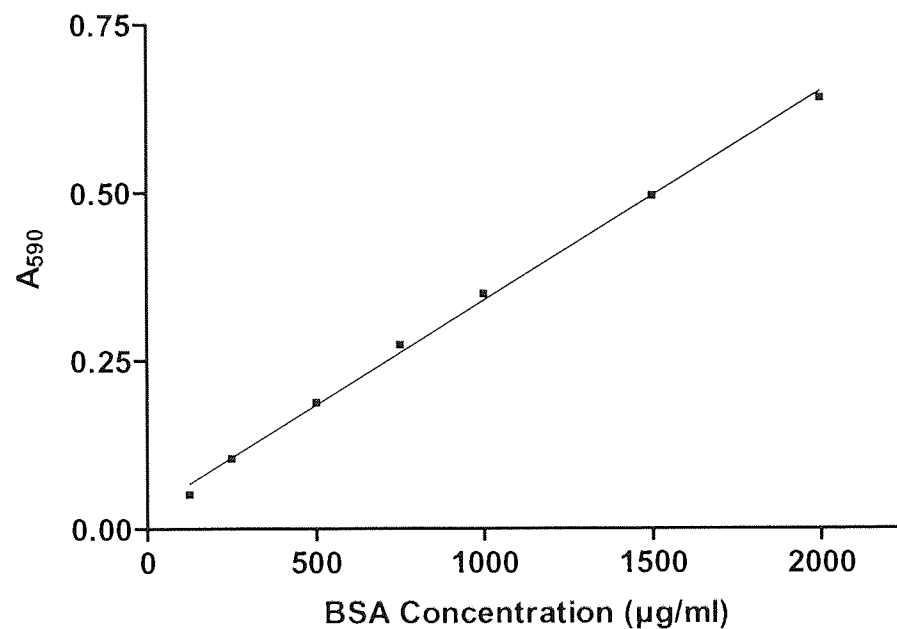
Appendix 1 Pierce bovine serum albumin BCA assay representative standard curve. Linear regression of detected BSA. $R^2=0.998$ calculated by GraphPad Prism software (version 3.02). Data are expressed as mean absorbance at 590nm $\pm$ SEM (n=3 from a single experiment).
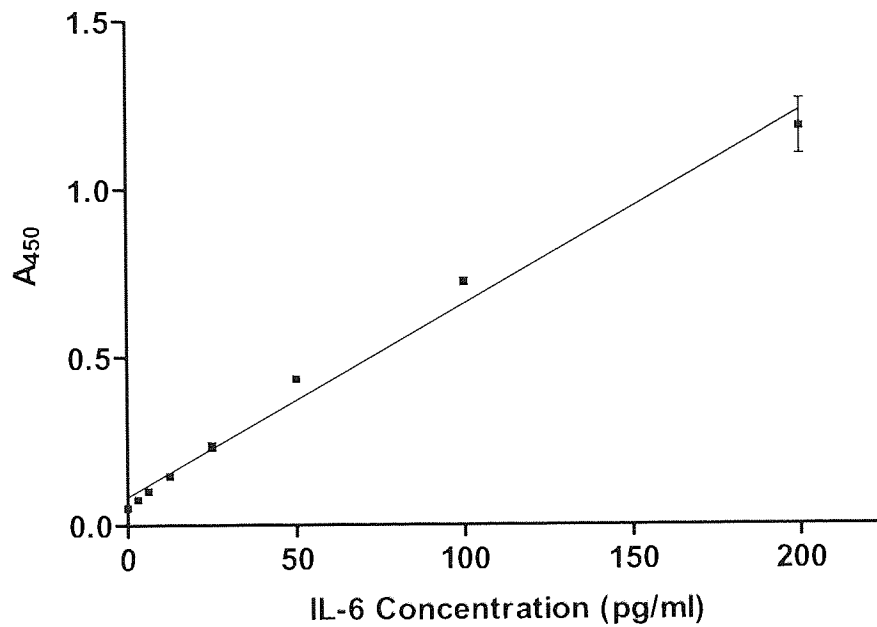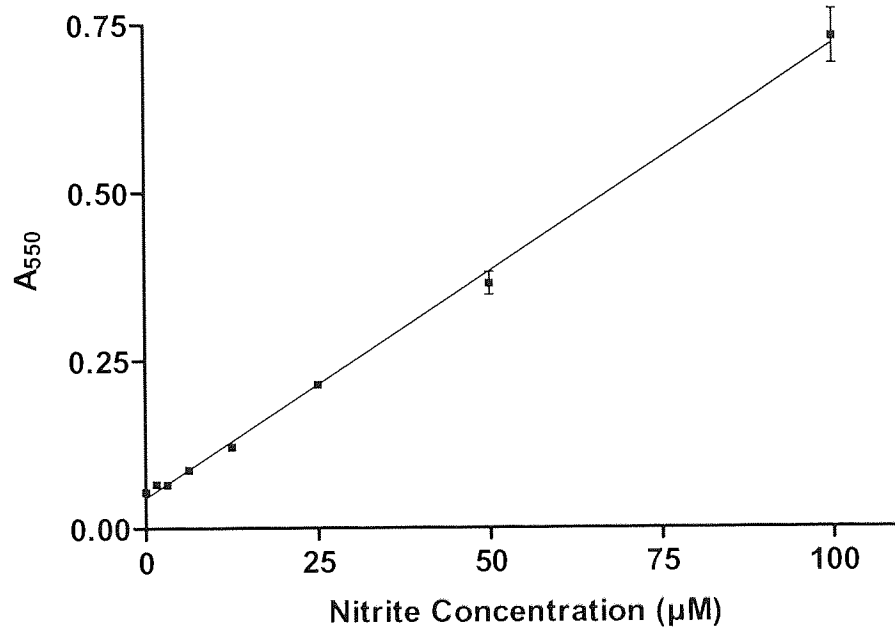
Appendix 2 eBioscience Ready-SET-Go! human IL-6 ELISA representative standard curve. Linear regression of detected human IL-6. $R^2=0.989$ calculated by GraphPad Prism software (version 3.02). Data are expressed as mean absorbance at 450nm $\pm$ SEM (n=2 from a single experiment).

Appendix 3 eBioscience Ready-SET-Go! human TNF-α ELISA representative standard curve. Linear regression of detected human TNF-α. $R^2=0.996$ calculated by GraphPad Prism software (version 3.02). Data are expressed as mean absorbance at 450nm ± SEM (n=2 from a single experiment).

Appendix 4 Promega Griess Reagent System, nitrite assay representative standard curve. Linear regression of detected nitrite $R^2=0.998$ calculated by GraphPad Prism software (version 3.02). Data are expressed as mean absorbance at 550nm ± SEM (n=2 from a single experiment).