Evaluating The Potential Neurotoxicity of Hexanedione Isomers: An *In Vitro*

Approach

Thomas Roland Zilz

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ASTON UNIVERSITY

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2,5-hexanedione (2,5HD) is the neurotoxic metabolite of the aliphatic hydrocarbon n-Hexane. The isomers, 2,3-hexanedione (2,3HD) and 3,4-hexanedione (3,4HD) are used as food additives. Although the neurotoxicity of 2,5HD is well established, there are no human data of the possible toxicity of the 2,3- and 3,4- isomers.

MTT and flow cytometry were utilised to determine the cytotoxicity of hexanedione isomers in neuroblastoma cells. The neuroblastoma cell lines SK-N-SH and SH-SY5Y are sufficiently neuron-like to provide preliminary assessment of the neurotoxic potential of these isomers, in comparison with toxicity towards human non-neuronal cells.

Initial studies showed that 2,5HD was the least toxic in all cell lines at all times (4, 24 and 48h). Although considerably lower than for 2,5HD, in general the IC_{50}s for the α isomers were not significantly different from each other and, besides 4h exposure, the SH-SY5Y cells were significantly more sensitive to 2,3HD and 3,4HD than the SK-N-SH cells. All three isomers caused varying degrees of apoptosis in the neuroblastoma lines, with 3,4HD more potent than 2,3HD. Flow cytometry highlighted cell cycle arrest indicative of DNA damage with 2,3- and 3,4HD.

The toxicity of the isomers towards 3 non-neuronal cell lines (MCF7, HepG2 and CaCo-2) was assessed by MTT assay. All 3 hexanedione isomers proved to be cytotoxic in all non-neuronal cell lines at all time points.

These data suggest cytotoxicity of 2,3- and 3,4HD (mM range), but it is difficult to define this as specific neurotoxicity in the absence of specific neurotoxic endpoints. However, the neuroblastomas were significantly more susceptible to the cytotoxic effects of the α hexanedione isomers at exposures of 4 and 24 hours, compared to non-neuronal lines.

Finally, a mechanism of toxicity is suggested for the α HD isomers whereby inhibition of the oxoglutarate carrier (OGC) releases apoptosis inducing factor (AIF), causing apoptosis-like cell death.

Key Words: Apoptosis, Cytotoxicity, Flow Cytometry, Hexanedione, MTT

Acknowledgements

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Abbreviations

2,3HD	2,3-Hexanedione
2,5HD	2,5-Hexanedione
3,4HD	3,4-Hexanedione
AIF	Apoptosis Inducing Factor
ANOVA	Analysis Of Variance
ATP	Adenosine triphosphate
BBB	Blood Brain Barrier
CJD	Creutzfeldt-Jakob disease
CNS	Central Nervous System
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
EC	European Commission
ECACC	European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic Acid
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization of the United Nations
FBS	Foetal Bovine Serum
FS	Forward Scatter
G_1	Gap 1 phase of the cell cycle
G_2	Gap 2 phase of the cell cycle
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
HIV	Human Immunodeficiency Virus
I	Intermediate, neuroblastoma cell type

IC_{50}	Inhibition Concentration, causing 50% inhibition
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal Dose, causing 50% death
LDH	Lactate Dehydrogenase
M	Mitosis phase of the cell cycle
MAO-B	Monoamine Oxidase B
MDMA	3,4-methylenedioxy-N-methamphetamine
MPP^+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionpiperidine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger Ribonucleic Acid
MRP	Multi-drug Resistance Protein
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Neuroblastic, neuroblastoma cell type
NB	Neuroblastoma
NEAA	Non Essential Amino Acids
NF68	Neuro-Filament Protein 68kDa
NGF	Nerve Growth Factor
NGFR	Nerve Growth Factor Receptor
NSE	Neuron Specific Enolase
NT2	NTERA-2 cl.D1 cell line
NT2-N	NTERA-2 derived Neurons
OECD	Organisation for Economic Co-operation and Development
OGC	Oxoglutarate Carrier
OPPTS	Office of Prevention, Pesticides and Toxic Substances

P	Probability
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PFK	Phosphofructokinase
PI	Propidium Iodide
PNS	Peripheral Nervous System
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
S	Substrate adherent, neuroblastoma cell type
S-Phase	DNA Synthesis phase of the cell cycle
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SS	Side Scatter
TBS	Tris-buffered Saline
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
WHO	World Health Organisation

Chapter 1. General Introduction

The brain and nervous system are the seat of human consciousness, allowing us to experience our lives and thrive in our environment. Distortion of nervous system structure and function caused by toxic agents may have a huge impact on human behaviour and survival and the processes involved in neurotoxicity can be difficult to study. Despite the importance of nervous tissue and its vulnerability to toxins, the development of *in vitro* methods to study these toxins and possibly predict their effects in man are still in their infancy. This thesis will describe the application of techniques for the study of cellular health under pressure from possible neurotoxins and the use of cellular models of human neural tissue, neuroblastoma cell lines. Prior to detailed description of this work, it is necessary to summarize the main aspects of the human nervous system, current techniques and models intended to study it, as well as the effects of some neurotoxic agents, including the hexanediones.

1.1 The Nervous System

The nervous system is made up of several subdivisions. The two main divisions are the central nervous system (CNS) and the peripheral nervous system (PNS). The primary function of the nervous system is to maintain homeostasis through the control of other body systems. To maintain a relatively stable internal environment for optimum cell function, the nervous system manages the activities of other organ systems so that they function in cooperation with each other.

1.1.1 The Central Nervous System (CNS)

The CNS is made up of the brain and the spinal cord, which are located within the skull and the vertebral canal respectively, thus affording protection of these vital, but delicate structures (Seeley et al., 1998). The role of the CNS is to process, coordinate and integrate sensory information and motor commands. The CNS is also responsible for functions including personality, intelligence, memory, learning and emotions (Martini, 2001).

1.1.2 The Peripheral Nervous System (PNS)

The PNS refers to all neural tissue outside the CNS, which comprises neurones, ganglia and neuromuscular junctions. Neurons are bundles of axons/nerve fibres that extend out into the peripheral structures of the body, such as muscles and glands, from the CNS and from the sensory organs, such as the eyes and ears, to the CNS. In total there are 43 pairs of nerves originating in the CNS that project into the PNS. 12 pairs originate from the brain, (cranial nerves) and the other 31 pairs originate from the spinal cord, which are termed spinal nerves. Ganglia are clusters of neuron cell bodies that are situated outside of the CNS. The primary function of the PNS is the transmission of information in the form of action potentials between the CNS and the rest of the body (Seeley et al., 1998).

1.2 The Cells of the Nervous System

There are several different types of cell that make up the nervous system. These can be divided into neurons, the functional unit of the nervous system, and non-neuronal support cells known as neuroglia or glial cells.

1.2.1 Neurons

Neurons are responsible for receiving stimuli and transmitting action potentials to other neurons as well as directly to peripheral effector organs. There are two functional types of neuron, afferent (sensory) neurons and efferent (motor) neurons. Afferent neurons relay information, in the form of action potentials, from sensory organs to the CNS and efferent neurons convey stimuli from the CNS to peripheral muscles, glands and tissues in order to exert central control (Seeley et al., 1998; Martini, 2001).

Neurons consist of a cell body and cellular processes known as axons and dendrites. There are three structural types of neurons; these are unipolar, bipolar and multipolar neurons. A simple schematic of a typical multipolar neuron can be seen in figure 1.1. The majority of sensory neurons are unipolar (have one process, one axon and dendrite-like processes at the peripheral end of the axon), bipolar neurons (have two processes, one axon and one dendrite) are sensory neurons that make up parts of specialised sensory organs, such as the rods and cones of the eye. The majority of CNS neurons are multipolar (have many processes, one axon and many dendrites) (Seeley et al., 1998; Martini, 2001).

1.2.1.1 Neuron cell body

The cell body or soma of neurons, has a large centrally located nucleus with a prominent nucleolus. The perikaryon is the cytoplasm that surrounds the nucleus; the cytoskeleton of the perikaryon contains numerous neurofilaments and neurotubules, which are the equivalent of microfilaments and microtubules in other cell types.
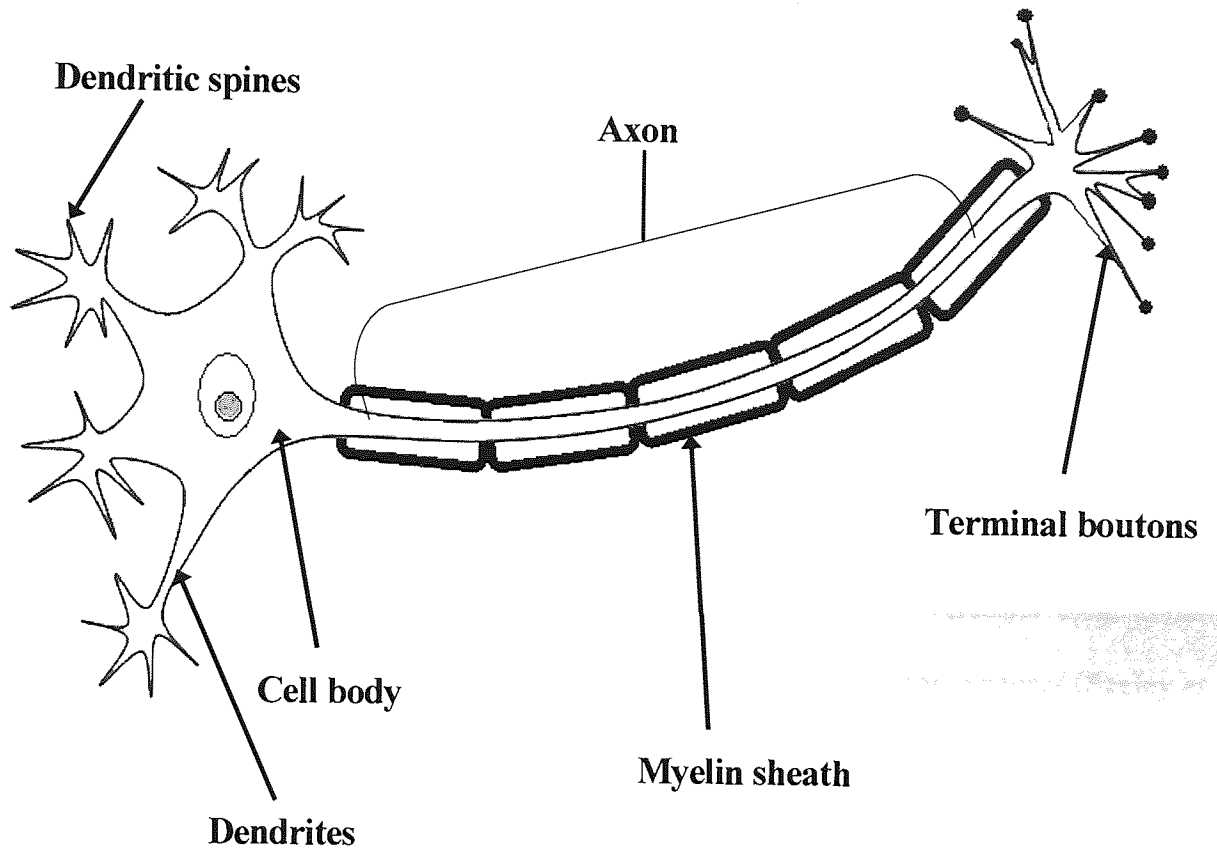
Figure 1.1: A typical multipolar neuron (Adapted from Seeley *et al.*, 1998).

Bundles of neurofilaments provide internal support for the narrow processes of the neuron by extending into the axon and dendrites. An extensive rough endoplasmic reticulum and numerous mitochondria are present. Indeed, large numbers of mitochondria are required for the production of ATP to meet the high energy demands of active neurons compared with other cellular types (Seeley *et al.*, 1998; Martini, 2001).

1.2.1.2 Dendrites

Extending from the neuron cell body there are small cytoplasmic processes known as dendrites. As described earlier, the number of dendrites can vary depending on the type of neuron. Usually dendrites are highly branched and each branch has very fine processes known as dendritic spines. These dendritic spines can represent 80 – 90% of the total surface area of a neuron. Dendrites respond to neurotransmitters released from other neurons, it is at the dendritic spines that axons from other neurons form synapses (Seeley *et al.*, 1998; Martini, 2001).

1.2.1.3 Axons

The main function of the axon is the propagation of electrical pulses called action potentials. The majority of neurons have only a single axon that extends from the cell body from the axon hillock. The axon can branch off forming collateral axons or just remain a single structure. The length of an axon can very from just a few millimetres to over a metre. At the end of the axon it terminates by branching to form small extensions known as presynaptic terminals or terminal boutons. In the CNS the axon can either be exposed to

the interstitial fluid or covered by the processes of various neuroglia (Seeley *et al* 1998; Martini, 2001).

Cytoskeletal proteins, organelles like mitochondria and neurohormone-containing vesicles can be propelled to the terminal boutons down the axon by axonal transport mechanisms. Also, damaged organelles, recycled plasma membrane and items taken up by endocytosis can be transported up the axon to the cell body. This movement of materials up and down the axon is essential to the normal function of the neuron, although it also provides a route for potentially harmful substances to gain access to neurons and be propagated to the cell body (Seeley *et al.*, 1998).

1.2.2 Neuroglia Cells

There are several fold more neuroglial cells for every neuron in the nervous system and these glial cells account for more than half the weight of the brain. Neuroglia play the major supporting role in the CNS including phagocytosing foreign substances, formation of myelin sheaths around neurons, the production of cerebrospinal fluid and participating in the formation of a permeability barrier between blood and neurons. There are five different type of neuroglial cell; astrocytes, ependymal cells, microglia, oligodendrocytes and Schwann cells. Each cell type possesses unique structural and functional characteristics (Seeley *et al.*, 1998).

1.2.2.1 Astrocytes

Astrocytes are the largest and most abundant type of neuroglial cell in the CNS. As their name suggests astrocytes are star shaped, due to cytoplasmic extensions of the cell body. These processes wrap the surfaces of blood vessels, neurons and pia mater (the membrane lining the brain and spinal cord) (Seeley *et al.*, 1998; Martini, 2001).

The functions of astrocytes are extremely varied and it is highly likely that many remain to be discovered and documented. Among their known functions are the maintenance of the blood brain barrier and the creation of a three-dimensional structural framework for the neurons of the brain and spinal cord. Astrocytes can also move into the area of neural damage and assist in repair and stabilisation of the damaged tissue to minimize further trauma. During initial embryonal development, they appear sometime after neurons, but they are thought to be involved in directing the later stages of embryonic neuronal development. The final key function of astrocytes is their role in regulating the composition of the interstitial fluid (Martini, 2001).

1.2.2.2 Ependymal cells

The ventricles of the brain and the central canal of the spinal cord are covered by cerebrospinal fluid filled cells called ependymal cells. These cells secrete cerebrospinal fluid that circulates around the ventricles and central canal. Often they possess cilia that assist with the movement of cerebrospinal fluid (Seeley *et al.*, 1998).

1.2.2.3 Microglia

Microglia are the smallest of the neuroglia and appear in relatively small numbers compared to some of the other neuroglial cells. One feature of these cells is their ability to migrate through neural tissue (Martini, 2001). They are specialised macrophages and in response to inflammation they become mobile and phagocytic. They essentially 'patrol' the CNS engulfing necrotic tissue, waste products and foreign bodies (Seeley et al., 1998).

1.2.2.4 Oligodendrocytes

Oligodendrocytes are similar to astrocytes in that they possess cytoplasmic processes, although their cell bodies are smaller and the number of processes they exhibit is lower. The oligodendrocyte processes wrap themselves around CNS axons and one oligodendrocyte is capable of surrounding portions of several axons (Seeley et al., 1998; Martini, 2001). The processes are modified to form myelin sheaths that facilitate action potential propagation along an axon (Seeley et al., 1998).

1.2.2.5 Schwann cells

Schwann cells are neuroglia cells found in the PNS. Like oligodendrocytes, Schwann cells form myelin sheaths around axons, although one Schwann cell is required to form a myelin sheath around a portion of only one axon. Specialised Schwann cells known as satellite cells, provide nutrients and support to neuron cell bodies in ganglia (Seeley et al., 1998; Martini, 2001).

1.3 Neurotoxicology

1.3.1 Vulnerability of the CNS to Toxicity

Whilst the consequences of toxicity to peripheral organs can range from trivial to life-threatening, the chief role of the CNS, through its detection of pain and systemic derangement, is to facilitate the removal of the whole organism from the source of the toxicity or take steps to ensure that the organ damage is ameliorated in some way. If the CNS itself is damaged due to chemical toxicity, then the consequences for the survival of the organism can be extremely serious due to damage inflicted on higher functions such as behaviour, personality and memory. Damage or destruction of memory leads to loss of identity and personal sense of self. These symptoms can be seen in human neurodegenerative disease and the consequences are often manifested in loss of independence and the requirement for constant care. Hence, the study of neurotoxicity is of particular importance in terms of its impact on the survival, viability and quality of life of the individual.

Neurotoxicity or a neurotoxic effect is defined as an adverse change in the structure or function of the nervous system following exposure to a chemical agent. For example at a molecular level toxins may interfere with protein synthesis within nerve cells, causing a reduction in the production of a neurotransmitter and brain dysfunction. At a cellular level toxins may affect the flow of ions across the cell membrane perturbing the transmission of information between nerves. Toxins that cause sensory or motor dysfunction, disturb learning and memory processes or cause detrimental behavioural effects are neurotoxic even if the underlying molecular and cellular effects are unknown. There are several features of the CNS and PNS and its cell populations that make these systems unique in

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terms of their susceptibility to toxins. One of these features, axons and their transport systems, has already been discussed earlier in section 1.2.1.3. Other features include; the presence of a biochemical barrier between the blood and the brain, the high-energy requirements of neural tissues, the need to maintain a lipid rich environment as part of normal neuronal function and the conduction of information across the extracellular space at the synapse (Anthony *et al.*, 2001).

1.3.1.1 The Blood-Brain Barrier

The blood brain barrier (BBB) is an anatomical barrier which has the dual function of isolating the brain from the rest of the body, as well as protecting the nervous system from ingress by some toxins. Ehrlich first observed the existence of a BBB in 1885 whilst studying the distribution of dyes in the body, when he noted that the brain and spinal cord did not stain with the dyes. The CNS and PNS maintain this barrier to the periphery and the basis of the BBB is considered to be specialised endothelial cells in the brain's microvasculature, aided by interactions with glia (Kniesel and Wolburg, 2000).

The gaps between normal endothelial cells outside of the nervous system are approximately 4-nm, however the existence of tight junctions with no measurable gap between cells is one of the unique properties of endothelial cells in the nervous system (Kniesel and Wolburg, 2000; Rubin and Staddon, 1999). Due to these tight junctions, molecules can only gain access to the nervous system if they are physicochemically capable of passing through the cell membrane of the endothelial cells. The BBB also contains xenobiotic transporters, such as the MRP series of transporters that pump many agents that do gain entry into the BBB back into the peripheral blood.

Systemic Capillaries   **Brain Capillaries**

Pericyte

Glial cells

Endothelial
cell

Vascular
Lumen

Vascular
Lumen

Tight junction

Pinocytosis   Lipid Soluble

Fenestration

Active Transport   Lipid Soluble
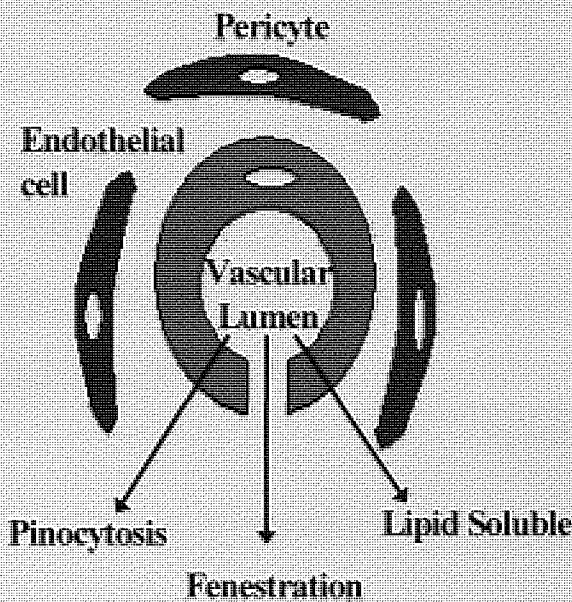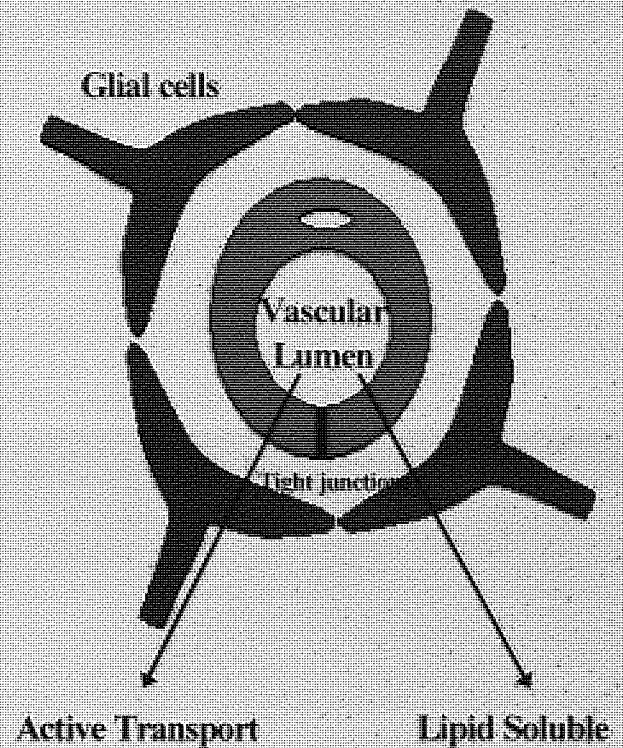
Figure 1.2: Schematic diagram of the blood-brain barrier: Systemic capillaries have intercellular gaps, or fenestrations, which allow the passage of molecules unable to cross the endothelial cell. Also pinocytosis is more abundant in systemic capillaries, as well as the transcellular passage of lipid soluble compounds. Due to the existence of tight junctions between endothelial cells and the lack of pinocytosis, transport in brain capillaries is limited to compounds with active transport mechanisms or those which can pass through cellular membranes due to their lipid solubility. (Adapted from Anthony *et al.*, 2001)

Some molecules can cross the cell membranes of the BBB into the nervous system by active transport mechanisms. However the ability of toxins to penetrate into the nervous system is largely dependent on their degree of lipid solubility, which determines whether they can cross the lipid-rich plasma membranes of the cells forming the barrier (Pardridge, 1999; Stewart, 2000).

At birth, the development of the BBB is incomplete and this is especially so in premature infants. This means that premature infants are predisposed to neural injury by toxins such as unconjugated bilirubin, for example, that are excluded from the nervous system in adults (Lucey et al., 1964). Aside from the BBB, there are several specialised cells that limit the access of molecules from adjacent tissue, and they completely line the brain spinal cord and peripheral nerves. This protective area is described as the meningeal surface in the CNS whilst in the PNS, perineural cells surround each fascicle of nerves.

1.3.1.2 Energy Requirements of the Nervous System

Neurons are heavily reliant on aerobic metabolism so they can propagate the conduction of electrical impulses and maintain repetitive reinstitution of ion gradients. These processes are extremely energy consuming. Adenosine triphosphate (ATP) is the main energy source for cells and is produced in the mitochondria through cellular respiration. Neurons have to be able to produce large quantities of ATP even whilst resting, as membrane depolarisation and repolarisation processes occur almost constantly (Anthony et al., 2001).

Being so dependent on continuity of energy supply makes neurons very vulnerable and sensitive to interruptions in the energy supply chain, such as the inhibition of enzymes and

processes involved in ATP production. This is reflected globally in the disproportionate relationship between the size and weight of the brain, which may be less than 2% of the body mass, and its normal consumption of up to 20% of cardiac output. This huge and essentially (from the point of view of the survival of the organism) uninterruptible energy demand gives considerable scope for toxic agents to cause both direct and indirect damage to neurons. Following systemic exposure to toxins such as cyanide, which inhibits aerobic respiration, or carbon monoxide, which causes hypoxia, some of the earliest signs of systemic dysfunction are observed at the cellular level in neurons (Anthony *et al.*, 2001).

1.3.1.3 Formation and Maintenance of Myelin

As mentioned briefly earlier oligodendrocytes and Schwann cells form myelin sheaths in the CNS and PNS respectively. This insulates the axons and increases the velocity at which action potentials are conducted down axons.

The cells eliminate cytoplasm from the cellular processes that attach and wrap themselves round axons. This forms the major dense line of myelin (Quarles *et al.*, 1997), the extracellular space is also reduced and the lipid membranes stack together forming concentric layers of lipid-rich myelin.

Metabolic and structural proteins that are unique to the CNS are required for the formation and maintenance of myelin. In the CNS myelin basic protein is an essential component of myelin that is associated with the intracellular space (Monuki and Lemke, 1995; Quarles *et al.*, 1997), the equivalent protein in the PNS is P1 protein. Proteolipid protein is located on the extracellular surface of the lipid bilayers in the CNS. Myelin in the CNS does not form

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properly if Proteolipid protein is mutated or over expressed (Pham-Dinh *et al.*, 1991; Readhead *et al.*, 1994). In terms of toxicity it is clear that some toxic agents can interfere with the formation and maintenance of myelin and lead to myelinopathies (Anthony *et al.*, 2001).

1.3.1.4 Neurotransmission

Neurotransmission is the intercellular communication system in the nervous system. Neurotransmitters are released from the terminal bouton end of one axon into the synapse. The neurotransmitter crosses the synapse and binds to the postsynaptic receptor of another cell. This then causes the modulation of an ion channel and the activation of a second messenger system, causing a change in the responding cell. For example, in neuromuscular transmission the neurotransmitter acetylcholine is released, crosses the synaptic cleft and binds to the cholinergic receptor of a myocyte and causes muscle contraction (Anthony *et al.*, 2001).

Various pharmaceutical drugs as well as toxins may modulate neurotransmission positively or negatively leading to local and general CNS symptoms. To a large extent with drugs and toxins, dose is usually the distinguishing feature between therapeutic effects and toxicity. Although an advantageous effect may be achieved with some agonist or antagonist binding at neurotransmitter receptors, toxicity may rapidly ensue leading to deleterious neural and astrocytic effects (Anthony *et al.*, 2001).

1.3.2 Specific implications of neurotoxicity

As outlined earlier, the study of neurotoxicity is of vital importance as the CNS controls all higher functions ranging from movement to memory, mood and emotions (Seeley *et al.*, 1998; Martini, 2001), so damage due to neurotoxicity can manifest as both psychological and physiological effects.

There are many specific effects on the manifestation of behaviour following CNS toxicity. Physiological manifestations include: impairment of speech, loss of feeling in the extremities, decreased reaction time as well as hand and body tremors. The psychological effects include: memory and cognitive defects, excessive mood swings and depression, panic attacks, personality and behavioural changes. More specifically, personality and behavioural manifestations of toxicity can include: increased aggression, increased obsessive/compulsiveness, irrationality, unusual, violent, or even criminal behaviour (Singer, 2002; Cole and Sumnall, 2003; Singer, 2003).

1.4 Assessing Neurotoxicity

The last 20 years has witnessed an increased pressure to identify neurotoxic, or potentially neurotoxic, substances in pharmaceuticals, industrial chemicals, foodstuffs and homecare products (Switzer, 1991). Pharmaceutical companies are currently heavily reliant upon the use of animal models for studying the toxicology of potentially neurotoxic compounds. The use of animals in medical and biological research dates back centuries and considerable knowledge of biosystems has been derived via such research. Many medical advances have been made through the use of animal experimentation. Currently, the

Medicines Act (1968) requires that the toxicity evaluation of new therapeutic entities must be carried out in at least two species of animal, one rodent and one non-rodent species, prior to clinical trials. However, using animals for assessing the toxicity of future pharmaceutical agents is increasingly contentious, not only from the moral and ethical standpoints, but from scientific perspectives also, with regards to differences between animals and humans and the resulting predictivity of animal models concidering these differences.

1.4.1 Animal models

The order of animal generally used in neurotoxicology studies are rodents, such as mice and rats with primates, principally marmosets and baboons, used to a far lesser extent. There are several animal models and tests specifically for assessing the neurotoxicity of any given compound (the general guidelines for these studies are described in OECD 424: Neurotoxicity Study in Rodents and EPA OPPTS 870.6200: Neurotoxicity Screening battery). Some of the more commonly employed tests include, animal behavioural/observation studies, which involve detailed observation and recording of changes in defined behavioural patterns. These effects can be poorly predictive of the situation in humans and are increasingly ethically contentious. For example, the 'landing foot splay test' is used extensively with rats, where the animals are held horizontally at about 20cm above a platform and then dropped. The distance between the hind feet upon landing is then measured. Depending on whether the toxin assessed has been dosed acutely or chronically, increased splay is observed with agents that cause peripheral neuropathy. Despite the popularity of this test and the fact that it is included in the EPA (EPA OPPTS 870.6200) guidelines for neurotoxicity testing, its relation to human neuropathies is

relatively poorly documented (Ross, 2002). Indeed, there is no practical equivalent test used by human neurologists and the anatomical basis for the test is poorly defined (Ross, 2000). Other neurotoxicity-directed animal tests include quantitative grip strength measurements, learning and memory tests such as water mazes and novel object recognition, brain morphometry and neuropathology studies.

1.4.2 Human predictivity of animal studies in neurotoxicity

To determine specific mechanisms of neurotoxicity at a cellular level, animal brain tissues are widely used *in vitro*. However, the extent of specific toxin susceptibility can differ significantly between animal and human tissues *in vitro* and *in vivo*. There have been well documented instances where neurotoxicity or lack of neurotoxicity in animal models, specifically rat models, has not been predictive of the effects observed in man. One of the most cited examples of this is the effect of the "designer" drug contaminant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which was inadvertantly formed during poorly controlled chemical synthesis of the illicit synthetic opiate, 1-methyl-4-phenyl-4-propionpiperidine (MPPP). Habitual users of MPTP-contaminated MPPP developed a severe motor disorder, which exhibited symptoms similar to advanced stage Parkinson's disease (Langston *et al.*, 1983). Although studies of MPTP in primates have since shown similar effects (Burns *et al.*, 1983; Langston and Ballard, 1984; Jenner *et al.*, 1984), rats and guinea pigs seem relatively invulnerable to the effects of MPTP (Kopin, 1987).

MPTP is metabolised by monoamine oxidase B (MAO-B) to the active neurotoxic metabolite 1-Methyl-4-phenyl-pyridine (MPP^+) (Chiba *et al.*, 1984). The localisation of MAO-B is crucial to the indentification of the respective loci of MPTP toxicity between

animal species and humans. In rats MAO-B is located at brain micro vessels where it is thought to form an enzymatic barrier, thus MPP^+ is formed outside the neurons, whereas in humans and other primates MAO-B is predominately located in astrocytes which facilitates the formation of MPP^+ within the BBB (Kopin, 1987). The mechanism of toxicity of MPP^+ has been proposed to be mediated through inhibition of mitochondrial complex I and oxidation of mitochondrial proteins. MPP^+ also causes massive dopamine release which promotes oxidative stress, eventually leading to destruction of dopaminergic cells in the substantia nigra (Ramachandiran et al., 2007).

As well as MPTP, humans are more sensitive to the neurotoxicity of 3,4-Methylenedioxymethamphetamine (MDMA) than rats and there are also regional differences in MDMA toxicity within human and rat brains (Reneman et al., 2002). Recent advances in the understanding of human brain biochemistry indicate that many brain disorders and the fate of the drugs designed to treat them depend upon biochemical systems unique to man (Ravindranath, 1998).

1.4.3 The 3 R's – Refinement Reduction Replacement

As described previously, although many advances have been made through the use of animals in research, their very use in the future for assessing chemical and drug toxicity in general is increasingly contentious from several standpoints. Issues of ethics, as well as animal rights and welfare are the most controversial. In 1959, William Russell and Rex Burch, defined the principle of the "Three R's" (Refinement, Reduction and Replacement) in their book *Principles of Humane Experimental Technique*. Refinements include any modifications in procedures that lead to a reduction in the frequency and/or severity of

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stress and discomfort to animals used in experimental procedures. Reduction involves the development of scientifically acceptable techniques to attain the same quality of information using fewer laboratory animals. Lastly, replacement is the implementation of new procedures that do not require the direct use of sentient animals in experimentation (Russell and Burch, 1959; Purchase *et al*, 1998). Although the vast majority of scientists are in agreement with refinement and reduction, there is far more controversy surrounding the idea of replacement (Coleman, 2006).

Regarding refinement and reduction, the use of large numbers of experimental animals in the classical LD_{50} test has long been superceded. This was mainly achieved by consideration of the scientific value of the test as well as ethical considerations. It was determined that differences in species, timing, formulation and many other factors essentially meant that the use of large quantities of animals in LD_{50} determinations gave relatively little useful information. The LD_{50} assay was replaced by the Fixed Dose Procedure, the 'up-and-down' method and other stepwise procedures (Ninomiya and Inomata, 1998), which used fewer animals.

Whilst reduction and refinement nevertheless retain animal models as their central feature, the object of the development of replacement systems is to produce systems that do not involve animal models, cells or structures at all and depend on entirely human-based systems. It is intended that these should eventually provide a high quality of reproducible and relevant information to human toxicity.

1.4.4 Criticisms of replacement

Replacement of animal tissues and cells with assays based on human cells undoubtedly has the potential for increasing scientific knowledge through the expansion of the armamentarium of methods available to study human toxicity. However, these studies share one of the main problems associated with all *in vitro* models, in that the effects of other cells, tissues and organs are not investigated contemporaneously. This lack of completeness can hamper the scaling of *in vitro* studies to man. Although any given *in vitro* model may not replace a complete animal, there is the potential to fill a specific niche where particular areas of cellular toxicity can be evaluated, such as possible neurotoxicity. Indeed, in the past five years, issues of numbers, relevance and practicality involved in recent European Union legislation ('REACH', see below) has actually accelerated the acceptance and development of niche *in vitro* animal and human-cell based models.

1.4.5 REACH

REACH is the acronym for the Registration, Evaluation, Authorisation and Restriction of Chemicals. REACH is the European Community regulation on chemicals (EC 1907/2006) which is intended to improve the protection of human health and the environment through earlier identification of the risks posed by the properties of chemical substances. One of the subsidiary aims of REACH is the promotion of alternative test methods and this is predicated on the idea that whole animal testing is essentially unsuitable for modern requirements in terms of the mass testing of very large numbers (in excess of 30,000) of potentially toxic chemicals. The original European Commission (EC) White Paper titled "Strategy for a future chemicals policy" (EC, 2001) proposed that all chemicals in

existence should have the same toxicity records as required since 1981 for all new chemical entities. It was estimated at the time that there were approximately 30,000 existing chemicals that would require toxicity testing, including neurotoxicity profiling.

Although international regulatory bodies, such as the Organisation for Economic Co-operation and Development (OECD) and Environmental Protection Agency (EPA), currently only accept animal *in vivo* toxicology data for the prediction of neurotoxicity in humans, the White Paper actually includes a specific clause which promotes the use of alternative test methods, such as the use of *in vitro* human cell based assays. Hence, to some extent there are political and practical pressures behind the promotion of the development of alternative, that is non-*in vivo* animal based, as well as human *in vitro*-based systems.

1.5 Alternatives to Animal Models

There are several strategies for the replacement of animals in scientific and specifically toxicological research. A number of physical and chemical techniques, such as metabolism assays, can be used to define and predict the potential of chemicals to have toxic effects and their possible modes of action. Also mathematical and *in silico* computer modelling can be used for assessing quantitative structure-activity relationships and molecular modelling; biochemical, physiological, pharmacological, toxicological and behavioural systems and processes can now be analysed rapidly and in detail (Broadhead and Bottrill, 1997). Although there are some successful *in silico* models for the prediction of specific organ toxicities such as hepatotoxicity, these systems are extremely dependent on the quality and breadth of their databases. In the niche area of human neurotoxicity, this is

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somewhat limited due to the relative paucity of experimental models. In addition, chemical agents do not always act *in vivo* as predicted by computer models, as the complexity of the impacting biological processes cannot always be incorporated within the model paradigms. Many animal-based *in vitro* models retain the disadvantage of animal DNA, whilst there are several invertebrate and vertebrate models used in toxicity assessments, such as various worm species and zebra fish, their predictivity and applicability towards humans remains controversial.

The use of human *in vitro* test systems, which may employ surgical waste, tissue slices, or cultures of primary cells and human derived transformed cell lines, theoretically possess many distinct advantages over animal models in terms of relevance to human systems. However, the predictivity of these models appears to vary according to the state of development of the model and the particular human tissue or system which is the subject of the study. For instance, the prospects for *in vitro* models which might model successfully the human immune system are believed to be remote at present (Ip and Uetrecht, 2006; Silliman and Wang, 2006), mainly due to the sheer diversity and complexity of this system. At the opposite pole, the availability of human tissue waste has allowed the successful development of models for the human eye (Wormstone *et al.*, 2006) and in recent years three-dimensional human skin models have been developed (Williams, 2006). In 2007 the scientific advisory committee of the European Centre for the Validation of Alternative Methods (ECVAM) endorsed the scientific validity of the EPISKIN test and the EpiDerm method. These alternative models will probably be the first *in vitro* toxicity testing methods used in Europe and internationally to replace the Draize rabbit skin irritation test (Spielmann *et al.*, 2007).

Sources of human tissue include living donors, cadavers and various tissue banks (Indech, 2000). However, there are several crucial issues that surround the use of human tissue that can severely impact their usefulness. There are a number of well-documented infections that are associated with human (as well as primate) tissues, both neural and non-neural. Handling of both types of tissues involve the risks of the various hepatitis viruses, as well as HIV. Neural tissues hold specialized risk factors such as human variant CJD. The level of infection containment required to handle these tissues with an acceptable risk factor may require high investment in training and facilities. Other directly scientific issues involve the quality and the reproducibility of the data derived from the tissue. These are strongly related to laboratory/operating theatre procedures and recent changes in current legislation related to the actual release of tissues/organs from the theatre. All of these factors directly impact on the condition and quality of each tissue sourced. Other problems include the difficulty in obtaining supplies of human tissue and the rules and regulations governing the laboratory use, transport and handling of this tissue (Indech, 2000; Suzuki *et al.*, 2002). Although the use of human tissues continues to be extremely valuable in research, various cell culture systems have become the most popular and commonly researched alternative to animals in research, partly as they circumvent many of the concerns listed above which pertain to human tissues and organs.

1.5.1 Cell culture

1.5.1.1 Primary cell culture

Tissue and cell culture was first used in the early 1900's to study animal cells free of systemic effects (Harrison, 1907; Carrel, 1912). Since the 1950's the use of these techniques has developed rapidly. This process was driven principally by the demands of

cancer and virology research. Now, virtually all types of animal and more importantly human cells can be cultured to some degree. Primary cultures involve the short-term maintenance of cells isolated from a tissue or organ. In blood cells, separating specific cell populations is easily achieved through differential centrifugation (Mason and Atwood, 1986), whilst separation of cells from other tissues and complete organs requires a more involved and specialized process of isolation from the cellular organic matrix. In the case of hepatocytes, perfusion of the liver with collagenases in calcium free media separates the cells from the main structure of the organ, leading to a high yield of healthy cellular entities (Seglen, 1976). The cells can be maintained in various physiological buffers supplemented with glucose and other carbohydrates. Although these cells may exist and demonstrate functionality in terms of various indices of viability for some hours, it was noticed as far back as the 1950's, that irreversible loss of phenotypic characteristics would occur relatively rapidly in primary culture. This was extensively reported with hepatocytes, with respect to reduction or loss of specific liver functions (Bissell and Guzelian, 1980; Guguen-Guillouzo et al., 1983). These effects do essentially reflect the situation in vivo, where different cell variants are strongly influenced by hormones, cytokines and other instructional molecules in terms of their phenotypic expression. To a large extent the loss of phenotype can be averted through co-culture or supplementation of medium with the appropriate additives and growth factors (Nieto, 2006; Jasmund et al., 2007).

1.5.1.2 Transformed cell lines

In stark contrast to primary cells, it was immediately apparent that cells derived from human tumours grew rapidly and often adapted to culture relatively quickly. They would also exhibit many aspects of their parent non-proliferative cell or tissue type, such as

specific enzyme systems and anatomical structures adapted to function. Most cell lines require a basic growth medium supplemented with foetal bovine serum (FBS; which contains many unspecified but potent growth factors) and various amino acid supplements. More advanced media use less FBS and are supplemented with various factors such as insulin. There are several main advantages of the use of continuous cell cultures in toxicity testing. Once cell lines are sub-cultured, they usually take on a homogeneous or uniform constitution (Augusti-Tocco and Sato, 1969). The lines can be stored in liquid Nitrogen for long periods of time and defrosted when required, without losing the characteristics of the cell line (Hay, 1992). When using a continuous cell line, cell proliferation results in a constant supply of cells for multiple experiments. Cell culture can be more economical for studies with reagents that are expensive or limited in availability or quantity than *in vivo* work as much less reagent is required for direct exposure to the cells than an *in vivo* injection where up to 90% of the compound is lost through excretion and tissue distribution. In addition, highly specific cell types can be studied in isolation of other cell variants. To the present day, many established transformed cell lines are either commercially available or available on request from generous fellow workers for virtually all tissue and cell types. From the experimental point of view, one of the most important advantages to cell culture is that all aspects of the physiological and physiochemical environment of the culture are fully controllable, allowing the design of highly mechanistic studies. In order to progress towards the more widespread usage of cell lines in toxicological research, the use of various well characterised cell lines allows for a combination of predictivity, uniformity and reproducibility between laboratories which can rival the homogeneity of animal models (Coecke *et al.*, 2006).

Although it remains that current cell culture *in vitro* systems cannot predict many complex *in vivo* responses, they are capable of modelling local responses in specific tissues and processes occurring at the cellular level which do essentially have a direct bearing on the aetiology of more complex *in vivo* processes, such as systemic toxicity.

1.6 Neuronal Cell Models

There are several human *in vitro* cell culture options for use in modelling neuronal cells for researching neuronal diseases and neurotoxicity. They include the use of embryonic stem cells, embryonal carcinoma cell lines and neuroblastoma cell lines.

1.6.1 Stem Cells

Stem cell research is still relatively new in terms of *in vitro* scientific research and human embryonic stem cells can be differentiated *in vitro* into normal human neurons. Various models have been developed; Zeng *et al* (2006) produced "An *in vitro* model of human dopaminergic neurons derived from embryonic Stem cells" to model the neurotoxicity of MPP$^+$. DeGroot *et al* (2007) were developing a "Stem cell based assay for an *in vitro* developmental neurotoxicity testing". The progress of human embryonic stem cell research depends on national legislation and requires compliance with these laws to proceed. Stem cell research is very costly to operate and can require highly specialized facilities. In addition the use of human embryonic stem cells is still considered highly controversial both ethically and scientifically.

1.6.2 Human Embryonic carcinoma cell Lines

Some human cell lines capable of stem cell-like differentiation into post-mitotic human cells have existed since the 1980's. A series of embryonal carcinoma cell lines, such as the NTERA-2 cl. D1 (NT2) cell line, can be differentiated by treatment with retinoic acid into human neurons (NT2-N), which demonstrate a close resemblance to human foetal neurons (Andrews, 1984; Lee and Andrews, 1986). These NT2-N cells have been used in several neuronal research areas and have been applied to neurotoxicity (Woehrling et al., 2006). However, the differentiation process is time consuming and labour intensive, taking approximately 8 weeks for complete post mitotic neurons. There are several cell purification steps which result in a considerable amount of cell waste as the number of resulting neurons is far fewer than the starting density of NT2 cells. This means that these cells do not really lend themselves to a high throughput method for assessing neurotoxicity.

1.6.3 Neuroblastoma cells

Neuroblastomas (NB) have a relatively high incidence in children under 15 years old (Young et al., 1986) and they account for approximately 9% of all childhood cancers. Samples have been taken from numerous tumours over the years and cultured in vitro to establish many in vitro cell lines for uses in research areas such as cancer research, modelling neurodegenerative disease and neurotoxicity assays.

Murray and Stout (1947) first used short term in vitro cultured NB tumours as a diagnostic tool. They found that plasma-clot cultures of tumours would readily produce axon-like

structures, however, culturing neuroblastoma tumours in the long term, as well as their complex differentiation properties expressed *in vitro* are under continuous study.

Neuroblastomas are thought to develop from postganglionic sympathetic neuroblasts of the embryonal neural crest. There is considerable evidence that NB cell lines are like their neural crest "parent" cells in their expression of neuronal properties. The ability to be induced to spontaneously generate neuritic processes is a characteristic of NB cell lines *in vitro*. They also express many other neuronal properties that include; *"the synthesis of neurotransmitter biosynthetic enzymes; expression of neurofilaments; opioid, muscarinic and neurotrophin receptors expression; dense core granules presumed sites of catecholamine storage; immunoreactivity to neuron specific enolase"* (Thiele, 1998).

The majority of established tumour derived NB cell lines are heterogeneous in nature and are usually made up from 3 morphological variants. These cell types are neuroblastic (N), substrate adherent (S) and intermediate (I). The N cells are the most common variety and they are small and round with neuritic processes. They contain the same neurotransmitter biosynthetic enzymes, cell surface receptors and uptake mechanisms as embryonic neuronal precursor cells (Ross and Biedler, 1985; DeClerck *et al.*, 1987; Rettig *et al.*, 1987; Ciccarone *et al.*, 1989). The S type cells are flat cells that are similar to neural crest derived non-neuronal precursors. These cells express similar properties, in their cytoplasmic and cell surface proteins, to embryonic Schwann, glial, melanoblasts, meningeal and ectomesenchymal smooth muscle cells (Ross and Biedler, 1985; DeClerck *et al.*, 1987; Rettig *et al.*, 1987; Sugimoto *et al.*, 1991). The I cells possess biochemical properties of both the N and S cells and are morphologically intermediates between the two (Biedler *et al.*, 1973; Ross and Biedler, 1985; Ciccarone *et al.*, 1989). It has also been

suggested that the I type cells are multipotent embryonic precursor cells of the peripheral nervous system, which are able to differentiate into neuronal or non-neuronal cells (Ross *et al.*, 1995).

There are many neuroblastoma lines currently available and the cell lines chosen for this study were the SK-N-SH and SH-SY5Y lines, as these cells are well established in the study of human neurotoxicity.

1.6.3.1 The SK-N-SH Cell Line

The SK-N-SH cell line is a heterogeneous human, Caucasian neuroblastoma line. The line was established from a bone marrow metastasis, from a 4-year-old female with a neuroblastoma of which the thorax was the primary site. They were first described by Spengler *et al* (1973) and developed by Biedler *et al* (1973, 1978). The SK-N-SH cell line consists of all three cell variants seen in NB cell lines namely, neuroblastic (N), substrate adherent (S) and intermediate (I) cells (Thiele, 1998). In 1975 there was initial evidence that suggested that the N and S cell types of the SK-N-SH line can switch morphological type from one to the other (Biedler *et al.*, 1975), this was supported by Ross *et al* (1983) who indicated a morphological and biochemical interconversion of the SK-N-SH cells.

The SK-N-SH cells have predominantly adrenergic, but multiple neurochemical characteristics and express a number neuronal properties and markers, such as neuron-specific enolase (NSE), to support their neuronal phenotype. They have been shown to exhibit substantial expression of dopamine-β-hydroxylase activity, which is comparable to that of superior cervical rat ganglia, however, choline acetyltransferase activity is low

(Biedler *et al.*, 1978). Tyrosine hydroxylase activity was shown to be present (Ross *et al.*, 1983) and glutamic acid decarboxylase activity was also found to be present in very low levels (West *et al.*, 1977).

The peptide hormone, nerve growth factor (NGF), is essential for the survival of neurons, as is the NGF receptor (NGFR) through which its effects are mediated (Thoenen and Barde, 1980; Vinores and Perez-Polo, 1983; Green and Greene, 1986). SK-N-SH cells were shown to be NGFR positive for NGFR mRNA and protein (Azar et al., 1990).

Evidence suggests that oestrogens are likely to be crucial for normal brain function (Simpkins *et al.*, 1994). Singh *et al* (1993, 1995) showed that the expression of neurotrophic factors such as NGF and brain derived neurotrophic factor can be induced by 17β-estradiol. It has also been shown that oestrogens protect neurons and glial cells from various toxic insults such as serum deprivation (Bishop and Simpkins, 1994). SK-N-SH cells are oestrogen responsive and express oestrogen receptor mRNA (Ratka *et al.*, 1991; Ratka *et al.*, 1995). SK-N-SH cells were effectively protected from the toxic effects of serum deprivation by 17β-oestradiol (Bishop and Simpkins, 1994).

The SK-N-SH cell line has been used extensively for various applications in scientific research. These include the *in vitro* neurotoxicity assessment of known toxins/neurotoxins, such as organophosphates (Katz and Marquis, 1992) and manganese (Stredrick *et al.*, 2004), as well as modelling mechanisms of toxicity *in vitro*, such as the cross linking of neurofilaments caused by the hexane metabolite 2,5-hexanedione (Heijink *et al.*, 1995; Heijink *et al.*, 2000). These cells have also been used in cancer research, where they were

employed to determine the possibility of enhancing the potency of chemotherapeutic agents (Chiu *et al.*, 2007).

1.6.3.2 The SH-SY5Y Cell Line

Due to their heterogeneous nature several sub lines have been cloned from the SK-N-SH line, they include; SH-SY5Y (N cell type), SH-EP (S cell type), SH-EPI (S cell type), SH-EPIE (S cell type) and SH-IN (I cell type) (Ciccarone *et al.*, 1989).

The SH-SY5Y line consists of homogeneous neuroblast-like cells which have been thrice cloned from SK-N-SH cells (SK-N-SH → SH-SY → SH-SY5 → SH-SY5Y) (Biedler *at al.*, 1978). The SY-SY5Y line exhibits a slightly more neuronal phenotype than the SK-N-SH line. They have small rounded cell bodies with a scarce cytoplasm and neuritic processes (Biedler *at al.*, 1978; Ross *et al.*, 1983). The line is nerve growth factor (NGF) responsive like its parent and in the presence of NGF the proportion of cells exhibiting neurite-like processes increases (Sonnenfeld and Ishii, 1982). Further evidence for their neuronal adrenergic phenotype includes the following observations: firstly, they exhibit reasonable dopamine-β-hydroxylase activity (Biedler *et al.*, 1978), however the levels are lower than seen in the SK-N-SH line. In addition, three forms of dopamine-β-hydroxylase have been identified in the line, membrane bound, soluble intracellular and constitutively secreted forms (Oyarce and Fleming, 1991). Secondly, tyrosine hydroxylase activity exists, although at a low level (Ross *et al.*, 1983). Thirdly, unlike the SK-N-SH line, they are able to fire injury induced or spontaneous action potentials (Kuramoto *et al.*, 1981). The resting membrane potential is between -40 and -80mV which is comparable to that of neurons *in vivo* (Perri *et al*, 1970).

Research into the SH-SY5Y line has identified the existence of opioid binding sites which are linked to adenylate cyclase, these were later shown to be μ and δ opioid receptors (Kazmi and Mishra, 1986). These receptors have also been identified in SK-N-SH cells (Yu *et al.*, 1986). Also, the presence of α_2 adrenergic receptor sites has been identified in SH-SY5Y cells (Kazmi and Mishra, 1989). Earlier studies have suggested a dynamic relationship exists between receptors such as opioid and α_2 adrenergic receptors, which modulate presynaptic neurotransmitter release (Aghajanian and Wang, 1986; Limberger *et al.*, 1988). As with the SK-N-SH line, SH-SY5Y cells are responsive to oestrogens (Lew, 1993).

Like their parental line, the SK-N-SH cells, the SH-SY5Y line has been extensively researched and used in neuronal research. Examples include the modelling of neurodegenerative diseases such as Parkinson's disease with the aim of screening potential therapeutic compounds (Zhao *et al.*, 2007), as well as the investigation of the biology of human neuroblastomas (Do *et al.*, 2007) and assessing the properties of neuroprotective compounds (Levites *et al.*, 2003; Iwashita *et al.*, 2004; Lin *et al.*, 2007).

1.7 Cell Death

Cell death by whichever pathway may have acute consequences for a tissue or organ during toxicity, or may exhibit chronic effects such as those seen in long-term degenerative disease. A wide variety of chemicals, ranging from pharmaceuticals to industrial or environmental agents can give rise to cell death. There are several cell death pathways from which a cell undergoing toxic insult is already known to perish. It is thought that there are many forms of cell death that are still to be defined (Majno and Joris, 1995),

however, there are two very distinct patterns of cell death which apply to non neuronal and neuronal cells that have been identified and documented to date; these are apoptosis and necrosis.

1.7.1 Apoptosis

Apoptosis has also been referred to as programmed cell death or cell 'suicide', as it occurs normally in the maintenance and renewal of tissues as well as being stimulated by toxic chemicals, such as 2,3,7,8-tetrachlorodibenzo-p-dioxins (TCDD; McConkey *et al.*, 1988). Apoptosis is a biochemically and morphologically distinct form of cell death in which cells die in a controlled manner. An early change in this active process is blebbing of the plasma and nuclear membranes. Apoptotic cells then shrink and become more dense and therefore lose contact with their neighbouring cells. Swelling of cellular organelles, such as mitochondria, is limited or non-existent. Changes also include nuclear and cytoplasmic condensation, mitochondrial release of cytochrome c, phosphatidyl serine externalisation on the plasma membrane and eventually DNA fragmentation and the formation of apoptotic bodies, which are then taken up and degraded by adjoining cells (Allen *et al.*, 1997). A family of proteins known as caspases mainly control apoptosis and caspase activation is a hallmark of this death process. There is also a caspase independent apoptosis-like cell death process, which involves the release of a mitochondrial intermembrane protein known as apoptosis inducing factor (AIF; for a review see Lu *et al* 2003). AIF enters the nucleus and acts directly on DNA leading to large-scale DNA fragmentation (Cande *et al.*, 2002).

1.7.2 Necrosis

Necrosis (accidental cell death) on the other hand is a passive form of cell death that is associated with inflammation – normally resulting from overwhelming cellular insults. The morphological characteristics of necrosis include the vacuolation of the cytoplasm, cell and organelle swelling, the breakdown of the plasma membrane, release of lysosomal enzymes, extracellular spillage of the cell contents and as a result of this, the induction of inflammation around the dying cell (Proskuryakov *et al.*, 2002).

1.8 Assay Selection

This thesis will describe the application of a two tiered toxicity regime for the assessment of potential neurotoxins. The first tier can lend itself to high throughput cytotoxicity assessment that provides reliable reproducible evaluation of apoptotic and necrotic effects, whilst the second, more detailed assessment is intended to determine effects on DNA and the cell cycle which will provide more detailed information about possible ultrastructural cell damage as well as cell death.

1.8.1 MTT assay

There are several *in vitro* high throughput methods for assessing cytotoxicity, the most common of these are trypan blue staining, the LDH (lactate dehydrogenase) assay, neutral red uptake and the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The trypan blue staining method is the simplest method, where a sample of cells are stained with the dye and counted using a haemocytometer. Live cells appear clear, whilst

dead cells take up the dye and appear blue and are frequently distorted and swollen. The LDH leakage assay involves the measurement of LDH activity within the cell culture medium and is a measure of cell membrane integrity; it is a fast and reliable assay (Decker and Lohman-Matthes, 1988). The neutral red assay is similar to the trypan blue assay, although living cells take up neutral red and the results can be read using a spectrophotometer, which makes it faster and more reliable than trypan blue. The MTT assay is a rapid quantitative colorimetric assay for cell survival and proliferation. It detects living cells on the basis of metabolic activity, so can be used to measure viability or cytotoxicity (Mosmann et al, 1983). The rationale of the assay is based on cleavage of the tetrazolium ring of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) by dehydrogenase enzymes (Slater et al., 1963) found in metabolically active mitochondria. A blue formazan product is formed that can be read at 590nm when in solution. (For method see section 2.3) It is a measure of cell viability and has been shown to be a fairly reliable measure of toxicity (Yang and Acosta, 1994).

In a study carried out by Fotakis and Timbrell (2006) the LDH, neutral red and MTT assays were compared. It was concluded that the MTT assay was more sensitive than the LDH assay in hepatoma cell lines. The MTT assay has also been widely used in neuroblastoma cell lines including the SK-N-SH line (Habtemariam, 1995; Dukhande et al., 2006) and the SH-SY5Y line (Willets et al., 1995; Datki et al., 2003), hence the MTT assay will be utilised as the first tier in this study.

The assays described above, including the MTT assay, are unable to distinguish between the type of cell death, i.e apoptosis or necrosis, just that the cells are no longer in a position to generate sufficient metabolic energy to reduce the dye and are likely to be dead or

dying. The ability to distinguish between apoptosis and necrosis yields insight into the processes that may be involved in how the toxins interact with cellular structures and their function. The second tier in this programme of study was intended to provide some perspective on the later stages of apoptosis. There are many assays that can detect apoptosis at various stages, such as the earliest caspase-based stages. However, as stated above not all apoptotic processes are dependent on caspase.

1.8.2 Flow Cytometric Analysis and the Cell Cycle

It was intended to utilise flow cytometry to provide the second tier of this neurotoxicity investigation process, as it is an extremely versatile tool that offers quantitative information relating to any cellular process that can be followed using fluorescent dyes. Propidium iodide (PI) is a DNA intercalating fluorescent dye that binds specifically and in a stoichiometric fashion to nucleic acids. This method works on nucleoid size to identify which stage of the cell cycle any given cell is currently in, or whether the DNA has been fragmented, which is a direct marker of apoptosis. As well as detecting the stage of apoptosis process, the information provided on the cell cycle may highlight several other effects a toxin may be exhibiting, including possible DNA damage. Although mature cells of the nervous system are post mitotic, information regarding any toxic effects on the cell cycle is important for identifying any developmental neurotoxicity and toxicity in neural stem cell populations.

Determination of nuclear DNA content via flow cytometric analysis of nucleoid associated PI fluorescence provides quantitative information on all stages of the cell cycle and

apoptosis. Such information is useful as it can be used to evaluate the effects of toxic agents upon the phases of the cell cycle and apoptosis.

The cell cycle can be split into two major stages, the interphase and mitosis. The interphase consists of three distinct, successive stages, called G1, S and G2 phases. As well as these specific stages, the G0 phase has been described for cells that exit the cell cycle and are in a quiescent, non-dividing state (Figure 1.3).

Once cell division is triggered by exposure to various cytokines, growth factors or mitogens, a cell is considered to be in the G1 phase of the cell cycle, where these factors induce growth, as well as synthesis of RNA and proteins required for DNA replication. Following this, the cell then enters the synthetic phase (S-phase) and replication of chromosomal DNA occurs. A G2 phase follows the S-phase. The cell continues to grow and prepare for mitosis, so this phase allows time to ensure DNA replication is complete before mitosis is initiated. The final phase of the cycle is the mitotic phase, where the cell divides into two daughter cells each containing the same genetic material as the parent cell.

For any control population of cells the majority will be in the G_0/G_1 phase of the cell cycle, with the normal single set of DNA Cells in the G_2/M phase of the cell cycle have two sets of DNA. PI binds stoichiometrically to DNA, therefore PI stained G_2/M nucleoids have double the fluorescence of those in the G_0/G_1 phase. Cells in the S phase of the cell cycle are undergoing DNA synthesis and therefore have more than a single set of DNA but not two complete sets consequently PI stained S phase nucleoids have a fluorescence that is intermediate of G_0/G_1 and G_2/M nucleoids and is represented on the flow cytometric histogram by the area between the G_0/G_1 and G_2/M peaks (Figure 1.4).
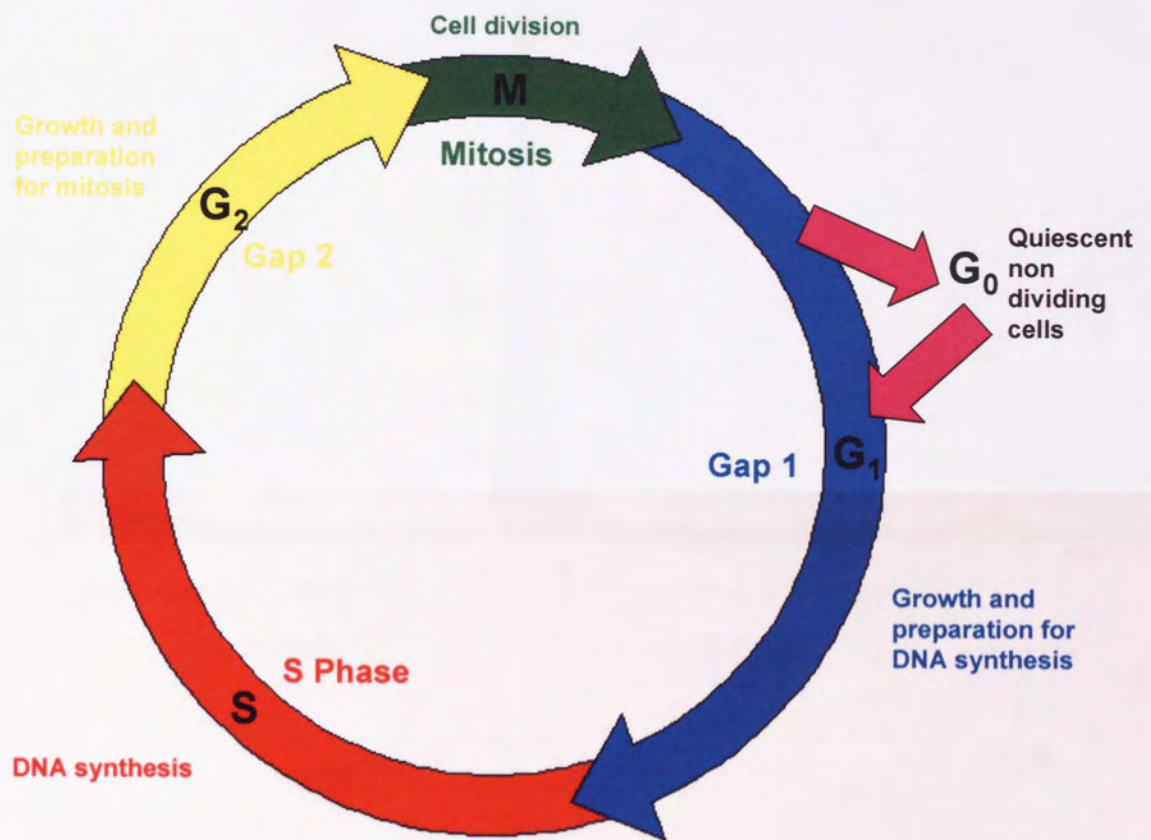
Figure 1.3: Some of the main features of typical Cell Cycle Phases in human cells.
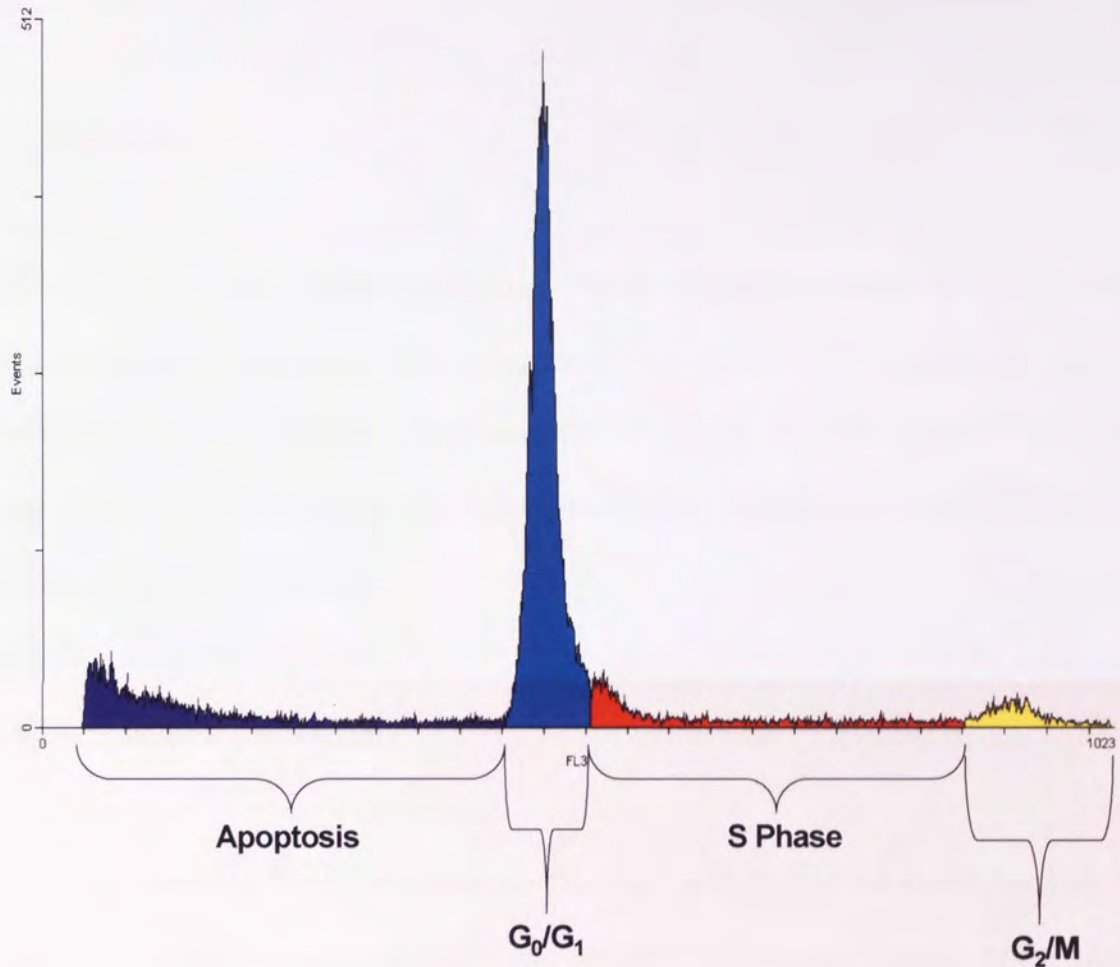
Figure 1.4: A flow cytometric DNA cycle histogram showing the corresponding phases of the cell cycle (x axis: Florescence; y axis: Number of events): The main peak observed in the histogram represents the cells in the G_0/G_1 phase of the cell cycle with a normal DNA content. The area to the left of the G_0/G_1 peak represents the apoptotic cells with a decreased DNA content. The cells that fall into the area immediately to the right of the G_0/G_1 peak are in the S phase of the cell cycle undergoing DNA synthesis. The peak that is observed at twice the florescence of the G_0/G_1 peak represents cells in the G_0/G_1 with two complete sets of DNA.

During apoptosis DNA is fragmented, these fragments bind less PI due to its smaller size (Wyllie, 1980; Cohen, 1993). For that reason apoptotic nucleoids yield reduced fluorescence when compared to aneuploid DNA, which gives rise to a peak to the left of the G_0/G_1 peak see figure 1.4.

1.9 Neurotoxins

Neurotoxins are toxic chemicals that specifically target the nervous system. There are many chemicals that are specifically neurotoxic, and some of these agents are capable of inflicting damage to different components of the human nervous system. Three of the major groups of these agents are organophosphates, metals and solvents and they are discussed below.

1.9.1 Organophosphates

One of the largest groups of pesticides are the organophosphates and these chemicals have been used in industry, as agricultural pesticides and also chemical warfare agents for over 60 years. Although in general their use has declined due in part to their toxicity to man, in 2001 organophosphate pesticides were still widely used in countries such as Taiwan, where organophosphate poisoning was a significant cause of human morbidity and mortality (Hsieh *et al.*, 2001). Pesticide poisonings are still a severe public health issue worldwide. The World Health Organization estimates, 3 million cases of pesticide poisoning occur every year, leading to more than 250,000 deaths (World Health Organization, 2004). Amongst the many pesticides whose toxicity can result in death, organophosphate insecticides are the most common culprits (Yang and Deng, 2007).

Organophosphates cause their toxicity by inhibiting acetylcholinesterase, which is responsible for catalysing the decomposition of acetylcholine. The inhibition of acetylcholinesterase results in the accumulation of acetylcholine at synapses and this leads to acute cholinergic crisis through constant neurotransmission. In severe cases death can occur within minutes due to repiratory muscle weakness, bronchospasm and bronchorrhea (Hsieh *et al.*, 2001).

The effects of organophosphates at the level of the neurone, manifests at the CNS as anxiety, restlessness, ataxia, convulsions, respiratory depression and coma (Hsieh *et al.*, 2001). In some cases paralysis of muscles in the proximal limbs, neck and respiratory system can occur up to 4 days after exposure and this is referred to as intermediate syndrome (Senanayake and Karalliedde, 1987). Delayed peripheral polyneuropathy can also occur several weeks after organophosphate poisoning, starting with paraesthesia and pain in the lower limbs, progressing to paresis (Senanayake and Johnson, 1982).

1.9.2 Metals

It is thought that metals are some of the oldest toxins known to man. A significant factor in the decline of the Roman Empire is considered to be the exposure of the population to excessive lead levels, probably through their consumption of acidic wines in pewter vessels (Gilfillan, 1965).

Metal toxicity is closely related to the physicochemical state, with the pure metal least toxic, whilst if organic forms such as methyl mercury are ingested, they are most bioavailable and cytotoxic. The majority of metals that exert toxicity exist as cations

(positively charged ions), and therefore can interact with many different ligands within cells, such as sulfhydryl, phosphate, amino and carboxyl groups (Clarkson, 1987). This means that potentially they have the ability to affect many different cellular processes. These effects include inhibition of enzymes, the disruption of cellular membranes and structural proteins and even affect the genetic code in nucleic acids.

The principal target site of toxicity for many metals is the nervous system. Inorganic compounds of many metals such as aluminium, arsenic, lead, manganese, mercury and thallium are known for their neurological and behavioural effects in man. Some of these effects include senile dementia in dialysis patients; this toxicity has been linked to aluminium (Alfrey *et al.*, 1976). Parkinsonian symptoms can be caused by damage to specific centres in the brain by manganese (Perl and Olanow, 2007). Metals such as thallium cause toxicity by mimicking potassium (Gehring and Hammond, 1967). The most severe neurotoxic metals are the organometals such as methymercury (Clarkson, 1983), tetramethyl lead (Walsh and Tilson, 1984) and the various organotins (Krigman and Silverman, 1984).

1.9.3 Solvents

A solvent is defined as *"a liquid that has the ability to dissolve, suspend or extract other materials, without chemical change to the material or solvent"* (HSE, 1999). Organic solvents are used extensively in industry worldwide. In fact, their use is so widespread they could be considered ubiquitous. Organic solvents are used in the manufacture of many products including pharmaceuticals, pesticides, cosmetics, paints, inks, degreasants, cleaning products, adhesives and foodstuffs. Some of the most commonly used solvents

include isopropanol, toluene, xylene, various ketones and hexane. Generally solvents are extremely volatile substances and toxic exposure to them is usually via inhalation of solvent vapour, although dermal exposure is also important in the painting and degreasing industries (Semple, 2004).

Some of the acute symptoms of solvent toxicity include headaches, dizziness, light-headedness, convulsions and death (White and Proctor, 1997). More long term effects of solvent exposure include leukaemia in the case of benzene (Sorahan et al., 2005) and scleroderma, associated with exposure to many solvents including; [benzene, bis(4-amino-3methyl-cyclohexyl) methane, dimethylbutylphenyldiamine, heptane, toluene, toluidene, trichloroethane, trichloroethylene, vinyl chloride, xylene, and xylidene (Silman and Hochberg, 1996; Maitre et al., 2004). With respect to the nervous system, other long term symptoms following general solvent toxicity are impaired memory, fatigue, depression, altered personality, impaired cognitive function and in some cases peripheral neuropathy (Dick, 2006).

There has been growing concern that prolonged workplace exposure to organic solvents are also associated with the development of several neurodegenerative diseases, such as Alzheimer's disease (Kukull et al., 1995), Parkinson's disease (Seidler et al., 1996; McDonnell et al., 2003) and multiple sclerosis (Landtblom et al., 1993; Riise et al., 2002).

The mechanism by which some solvents exert their neurotoxicity is not completely clear although it is thought that in many cases the metabolism of the parent solvent to a reactive metabolite, which is either the terminal toxin itself, or it forms reactive species which exert toxicity through oxidative stress.

1.9.3.1 n-Hexane and 2,5-Hexanedione

The simple aliphatic hydrocarbon n-Hexane is a volatile organic solvent whose use in industry was widespread until relatively recently and Hexane isomers are still present in many commercial products, such as glues and varnishes. Due to Hexane's presence in various blends of automotive petrols, low level environmental exposure is ubiquitous (Ikeda and Kasahara, 1986). Studies carried out by Spencer and Schaumburg (1976) showed that chronic n-Hexane exposure could cause a polyneuropathy that can be defined as a distal central-peripheral axonopathy. Chronic exposure to n-Hexane in humans, through occupational contact or solvent abuse, has been shown lead consistently to neurotoxic illness (Spencer *et al.*, 1980; Korobkin *et al.*, 1975). Neuropathy as a result of occupational exposure usually manifests itself as slow, progressive sensory and motor dysfunction, whereas solvent abuse often results in the neuropathy taking a more sub-acute course, with mainly motor symptoms (Herskowitz *et al.*, 1971; Smith and Albers, 1997; Kuwabara *et al.*, 1999).

Until the 1970's, ketone-related solvents were thought to be generally safe and it was believed that they presented little risk to health, thus they were widely used in industry (Couri and Milks, 1982). Perceptions of the toxicity of ketones as a class was restricted to irritation of mucous membranes, which was thought to limit further exposure and prevent systemic toxicity, although this is now known not to be true. Yamamura (1969) reported that n-Hexane caused neuropathy in both animals and humans, although at the time, these studies were widely disregarded.

It was determined the 1970's that n-Hexane metabolism is complex and it is likely to be oxidised by cytochrome P450 2E1 (Bolt *et al.*, 2003) to a variety of metabolites, which include 2-hexanone, 1-hexanol, 5-hydroxy-2-hexanone and 2,5-hexanedione. The diketone 2,5-hexanedione (2,5HD) has been shown to be the neurotoxic metabolite that causes injury to the nervous system seen in cases of n-Hexane exposure (Spencer and Schaumburg, 1975; Governa *et al.*, 1987; Perbellini *et al.*, 1990; Graham, 1999).

1.10 Aims and Objectives

1.10.1 Background

As described above, 2,5-hexanedione is the neurotoxic metabolite of the aliphatic hydrocarbon n-Hexane and there are also several other structural isomers of the solvent available commercially. Two of these isomers, 2,3-hexanedione and 3,4-hexanedione are currently used as food colourings and flavourings, although there is no evidence that they are formed *in vivo* from Hexane exposure. Although the neurotoxicity of 2,5-hexanedione is well researched and documented, there is no human data in the scientific literature of the possible toxicity of the 2,3- and 3,4- isomers of hexanedione, especially *in vitro*. This study will investigate the potential toxicities of these isomers through the development of a tiered *in vitro* test system.

1.10.2 Primary aims

- It is intended to use the MTT and flow cytometry endpoints to determine whether the 2,3- and 3,4-hexanedione isomers are cytotoxic to the neuroblastoma lines SK-

N-SH and SH-SY5Y human cells in relation to the acute toxicity of the known neurotoxic hexanedione isomer, the 2,5 variant.

- The neuroblastoma lines SK-N-SH and SH-SY5Y are sufficiently neuron-like to provide a preliminary assessment of the neurotoxic potential of these isomers. The toxicity of 2,5-, 2,3- and 3,4-hexanedione will also be evaluated in 3 non-neuronal cell lines and the data compared with the neuroblastoma lines to establish whether there is specific neurotoxicity or if the isomers are generally cytotoxic.

1.10.3 Specific objectives

- The neuroblastoma lines SK-N-SH and SH-SY5Y were to be established in continuous culture in the laboratory.

- There are several protocols listed regarding the basic MTT method, so the first objective of the study was to optimise the most consistent and appropriate protocol for analysis of the metabolic competence of the various neuronal and non-neuronal cell lines.

- Flow cytometry conditions were to be optimized regarding the potential problems linked with consistent analysis of the cell cycle in adherent cell lines, where conditions of detachment of the lines from plasticware can be crucial to reproducibility.

- The acute and intermediate toxicity (4-48h) of 2,5-hexanedione, followed by the 2,3 and 3,4 hexanedione isomers, was to be investigated using the MTT assay and flow cytometry in the SK-N-SH and SH-SY5Y human neuronal cell lines.

- Finally, using the MTT assay, the toxicity of the hexanedione isomers in the neuroblastoma lines was compared with toxicity in a series of non-neuronal cell lines which originated from human breast cancer (MCF-7), human hepatoma (HepG2) and human colon adenocarcinoma (CaCo-2) tissues.

Chapter 2. Materials and Methods

2.1 Materials

All reagents were of molecular biology grade unless otherwise stated. Standard solvents were from Fisher (Loughborough, UK) and all gases from BOC Ltd (Guildford, UK). All cell culture media, foetal bovine serum, non-essential amino acids (NEAA) and L-glutamine were purchased from Gibco Invitrogen (Paisley, UK). The mouse anti-NSE antibody was obtained from Chemicon (Watford, UK), the mouse anti-NF68 antibody was from Sigma Chemical Company (Poole, UK) and the rabbit anti-mouse HRP antibody was purchased from Cell Signalling Technology (Hitchin, UK). The SK-N-SH (Human Neuroblastoma) cells (ECACC No. 86012802) were kindly donated by Dr Neil Foster (Birmingham University), whilst SH-SY5Y (Human Neuroblastoma) cells (ECACC No. 94030304) and HepG2 (Human Hepatoma) cells (ECACC No. 85011430) were kindly donated by Dr M. Grant (Aston University). The CaCo-2 (Human adenocarcinoma) cells (ECACC No. 86010202) were kindly donated by Dr A. Vernallis (Aston University) and Dr E. Adams (Aston University) kindly provided the MCF7 (Human breast adenocarcinoma) cells (ECACC No. 86012803).

2.2 Cell Culture

SK-N-SH and HepG2 cells were maintained in Dulbecco's Minimum Essential Medium (DMEM), supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) Penicillin/Streptomycin. SH-SY5Y cells were maintained in RPMI 1640 containing Glutamax^{TM} I, supplemented with 10% (v/v) FBS, 1% NEAA and 1% (v/v)

Penicillin/Streptomycin. CaCo-2 cells were maintained in Dulbecco's Minimum Essential Medium (DMEM), supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine, 1% NEAA and 1% (v/v) Penicillin/Streptomycin. The MCF7 cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) containing Glutamax[TM] I, supplemented with 10% (v/v) FBS, 1% NEAA and 1% (v/v) Penicillin/Streptomycin. Cells were incubated at 37°C in a humid atmosphere of 5% CO_2 and 95% air, all cell lines were grown as mono-layer cultures in 75cm^2 and 162cm^2 tissue culture flasks (Corning Costar, Netherlands).

2.2.1 Subculture of Cell Lines

All cultures were monitored on a daily basis for their level of confluence, changes in morphology and signs of infection. Sub-culture was carried out for all cell lines once they had reached between 80% and 100% confluency. If cultures were not confluent within 3 days of seeding, the medium was aspirated and replaced. For confluent cultures, following aspiration of the medium from the cells and washing with 10ml PBS (Phosphate Buffered Saline), 0.25% trypsin-EDTA was added to the flasks to detach the cell monolayers. After 5 minutes incubation at 37°C, the flasks were gently knocked to aid cell detachment. Following detachment, fresh medium, which contains anti-trypsin agents, was added to the cells to stop the action of the trypsin, then the cell suspension was added to new flasks containing fresh medium. All cell lines were usually split between the ratios 1:2 and 1:6 depending on degree of confluence and speed of division

2.2.2 Cryopreservation of Cells in Liquid Nitrogen

In order to maintain adequate stocks of the different lines in long-term storage, cells were routinely frozen down and stored under liquid Nitrogen. The medium was aspirated and the cells washed with 10ml PBS (Phosphate Buffered Saline) to remove any dead cells and remaining media. PBS was then removed by aspiration. A suitable volume of fresh medium was then added and the cells were detached from the flask by gentle scraping. The cells were then transferred into sterile 15ml centrifuge tubes and then pelleted by centrifugation at 1000rpm for 5 minutes. The medium was aspirated and the pellet was resuspended in 1ml freezing media, which was prepared using 90% FBS and 10% DMSO, and transferred to a cryovial (Corning Costar, Netherlands). The cells were then placed overnight in a -70°C freezer before being placed into liquid Nitrogen.

2.2.3 Resuscitation of Cell Lines from Liquid Nitrogen

The cells were stored in cell freezing media (90% FBS, 10% DMSO) at an approximate concentration of 4×10^6 cell/ml in liquid nitrogen. When necessary, a cryovial of the required cell line was selected from the liquid Nitrogen cell bank and transferred to a waterbath at 37°C until fully thawed. The thawed cells were then added to 10ml of fresh, pre-warmed medium in a sterile 15ml centrifuge tube and the cells were pelleted by centrifugation at 1000rpm for 5 minutes. The medium was aspirated and the pellet was resuspended in 5ml fresh medium and transferred to a $75cm^2$ cell culture flask where more medium was added to a volume of 20ml.

2.3 MTT Assays

Although several variations of the assay have been published, the method used in this study is based on that of Tada *et al.*, 1986 and is described below.

Cells were detached from the flasks as described in section 2.2.1 and then counted using a haemocytometer, in conjunction with the vital stain trypan blue, to distinguish between viable and non-viable cells. Cells were then plated out into 96 well plates at the optimum density for each cell line, calculated from the calibration assay described below in section 2.3.1, and left overnight in a humidified chamber at 37°C in 5% CO_2 and 95% air. The medium was removed and replaced with 100μl fresh medium supplemented with increasing concentrations of the toxin being assessed, 5% methanol was used in one coloumn of the 96 well plate as a positive control for each experiment. The plates were then returned to the humidified chamber at 37°C in 5% CO_2 and 95% air for 4, 24 or 48 hours. After the incubation period the medium was removed and replaced with 100μl fresh medium. Then 30μl of MTT (5mg/ml in PBS) was added to all wells across the plate. The plate was then placed inside a humidified chamber and incubated for 4h at 37°C in 5% CO_2, 95% air. After incubation, to solubilize the formazan product, 100μl 10% SDS in 0.01M HCl was added to each well and incubated as before, overnight. The plate was then read at 590nm using a Thermo Multiskan EX 96 well micro plate reader (Thermo Electron Corporation).

2.3.1 MTT Calibration Assays

The optimum seeding density adopted for the MTT assay was established for each cell line. Increasing numbers of cells were seeded onto a 96 well plate and made up to 100µl with fresh medium. The cells were then incubated overnight to allow cell attachment and division. Then the MTT assay was carried out as described above.

Durring the initial experiments to optimise the MTT assay for each cell line it was noticed that to remove as much medium as possible from the plates between steps, such as adding and removing toxin supplemented medium, it was required to place the pipette on the surface of the wells. This could cause cells to become dislodged and/or damaged which could have led to inconsistent results, therefore the method was adapted so that the plates were blotted on to sterile absorbent paper to remove the medium when necessary.

2.4 Flow Cytometric DNA Cell Cycle Analysis

Initial experiments were carried out in 25cm^2 tissue culture flasks, however, this was not logistically efficient, and therefore 6 well plates were used instead. Pilot experiments were also carried out to determine whether cells should be detached from the 6 well plates prior to staining using trypsin or scraping. From these pilot experiments scraping the cells appeared to give more consistent results and a lower level of apoptosis in control cells than trypsinising; an example of this can be seen in the histograms seen in figure 2.1a and 2.1b below.

Cells were detached from the flasks as described in section 2.2.1 and then counted using a haemocytometer, in conjunction with the vital stain trypan blue, to distinguish between viable and non-viable cells. Cells were then plated out into 6 well plates at approximately 5 x 10^5 cell/ml and left overnight in a humidified chamber at 37°C in 5% CO_2 and 95% air to allow the cells to attach and become confluent. The medium was removed and replaced with 1ml fresh medium supplemented with increasing concentrations of the toxin being assessed. The plates were then returned to the humidified chamber at 37°C in 5% CO_2 and 95% air for 48 hours.

After the cells had been exposed to the toxins the cells were detached from the plates by scraping. Then they were centrifuged at 1000rpm for 5 minutes. The supernatant was removed and the pellet resuspended in PBS and centrifuged again at 1000rpm for 5 minutes to wash the cells. The supernatant was discarded and the cell pellet resuspended in 1ml hypotonic fluorochrome solution (50μg/ml PI in 0.1% sodium citrate and 0.1% Triton X-100) to extract and stain nucleoids (Nicoletti *et al.*, 1991). Samples were then incubated at 4°C in the dark overnight prior to flow cytometric cell cycle analysis. PI intercalated nuclei were excited by a 488nm Argon laser at a low flow rate. Forward scatter (FS) and side scatter (SS) of the nuclei were simultaneously measured in addition to peak and integral linear red fluorescence (FL3, bandwidth 605nm-635nm).
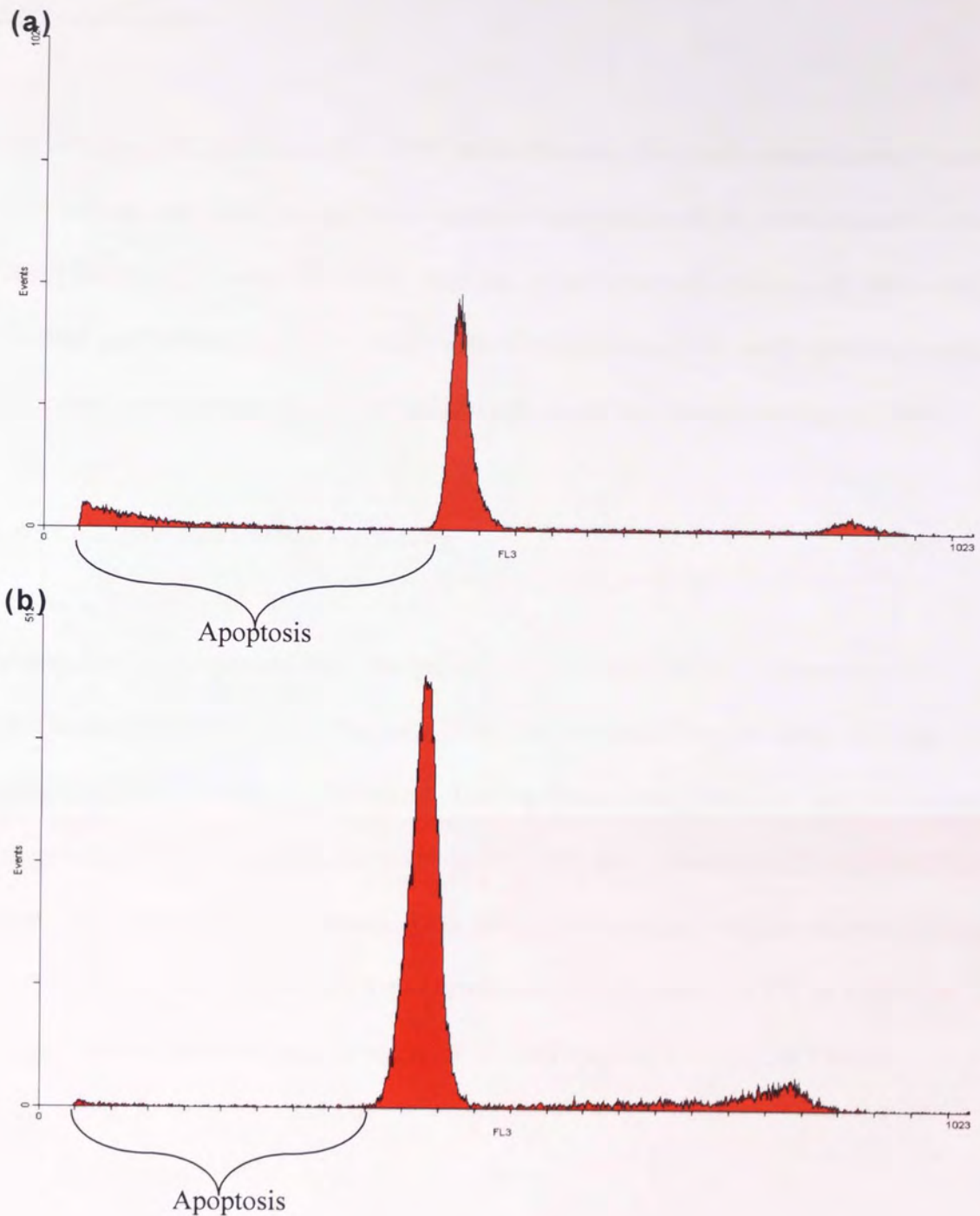
Figure 2.1: a) Flow cytometric histogram of cells detached using trypsin. b) Flow cytometric histogram of cells detached by scraping. Detaching the cells from the 6 well plates with trypsin led to a much higher level of background apoptosis when compared to detaching the cells with gentle scraping.

2.5 Protein Analysis

The cell lines SK-N-SH and SH-SY5Y neuroblastoma lines both possess some 'neuronal like' features and they express some neuronal markers, such as neuron-specific enolase (NSE) and neurofilament 68 (NF68). Samples of the cells used in this study were analysed for NSE and NF68 using SDS PAGE and Western blotting to verify their neuronal-like phenotype and to demonstrate a difference between the two neuroblastoma cell lines.

2.5.1 Cell Lysis and Protein Extraction

In order to extract protein from the cells, firstly the medium was removed and the cells were washed in PBS 3 times. The cells were then detached from the flask by scraping and centrifuged for 5 minutes at 1000rpm. The supernatant was discarded and the cell pellet resuspended in 0.5ml of RIPA (10mM Tris HCl (pH 8.0), 100mM NaCl, 1mM EDTA, 1% NP40, 0.5% Sodium deoxycholate, 0.1% SDS, plus protease inhibitor tablets (Complete mini, Roche)). The sample was then centrifuged for 30 minutes at 4°C at 15000rpm. The supernatant was removed and frozen at -20°C until examined using SDS PAGE.

2.5.2 SDS PAGE

Protein samples (from section 2.5.1) underwent SDS PAGE using the Mini Protean® 3 Cell (BioRad). 10% SDS gels were made according to the manufacturer's instructions. The samples were prepared in sample buffer (deionised H_2O, 5% glycerol, 12.5mM Tris HCl pH 6.8, 0.4% SDS, 0.002% bromophenol blue and 1% β-mercaptoethanol) and 5µg of cell

lystate added to each lane loaded onto the gel along with molecular weight markers. The gel was run at 200V, 60mA for 45 minutes.

2.5.3 Western Blot Transfer

Once protein samples had been separated by SDS PAGE, (Section 2.5.2) the gels were equilibrated in transfer buffer (25mM Tris, 192mM glycine, 10% methanol), before being transferred to methanol-pre-wetted PVDF transfer membrane (0.45µm) (Amersham Pharmacia). The protein gels and transfer membranes were then sandwiched between four pieces of Whatman filter paper and two pieces of sponge, which had been pre-soaked in transfer buffer. Transfers were performed using the mini trans-blot electrophoretic transfer cell (BioRad) at 30V, 90mA overnight.

2.5.4 Western Blot Analysis

PVDF membranes (Amersham Pharmacia) were blocked for 2 hours at room temperature in TBS (8g NaCl, 0.2g KCl, 3g Tris-base in 1L H_2O) 0.01% Tween with 5% powdered milk and then rinsed in TBS 0.01% Tween. Following this the membranes were incubated in TBS 0.01% Tween, 3% powdered milk containing the relevant primary antibody and incubated at 4°C overnight on a rolling platform. Mouse anti-NSE (Chemicon; 1:200 dilution) and mouse anti-NF68 (Sigma, 1:1000 dilution) were used as primary antibodies. The next day the membranes were washed for 5 minutes in TBS 0.01% Tween, this was repeated 6 times to completely remove unbound antibody. Following this washing phase the membranes were placed in TBS 0.01% Tween 3% powdered milk, containing rabbit anti-mouse HRP (Cell Signalling Technology) for 1 hour at room temperature on a rolling

platform. Then the membranes were washed extensively in TBS 0.01% Tween and then TBS alone. Membranes were placed in chemiluminescent substrate (Amersham) for 5 minutes before being immediately sandwiched between acetates and exposed to Hyperfilm (high performance chemiluminescence film; Amersham) in a developing cassette. To visualize the bound antibody, exposed films were developed using developer and after extensive washing fixed with fixer in a darkroom.

2.5.5 Western Blot Results

Samples of SK-N-SH and SH-SY5Y cells were analysed for the expression of the neuronal markers NSE and NF68. The resulting Western blots can be seen in figure 2.2.

Both cell lines express NSE (Thiele, 1998) and this can be seen in figure 2.2a. There was no distinct band for NSE in either cell line. Although the molecular weight of NSE is approximately 45kDa it has been shown to produce multiple bands between 45 to 56kDa (Whittaker *et al.*, 1993).

NF68 is usually expressed by the SH-SY5Y cell line (Walton *et al.*, 2004) but not the SK-N-SH cells, so it is it is a good marker for distinguishing between the two cell lines. As can be seen in figure 2.2b there is a clear band at the expected molecular weight of NF68 in lysates of SH-SY5Y cells which is absent from lysates of the SK-N-SH line.

a)

SK-N-SH   SH-SY5Y

45kDa
(NSE)

b)

SK-N-SH   SH-SY5Y

68kDa
(NF68)
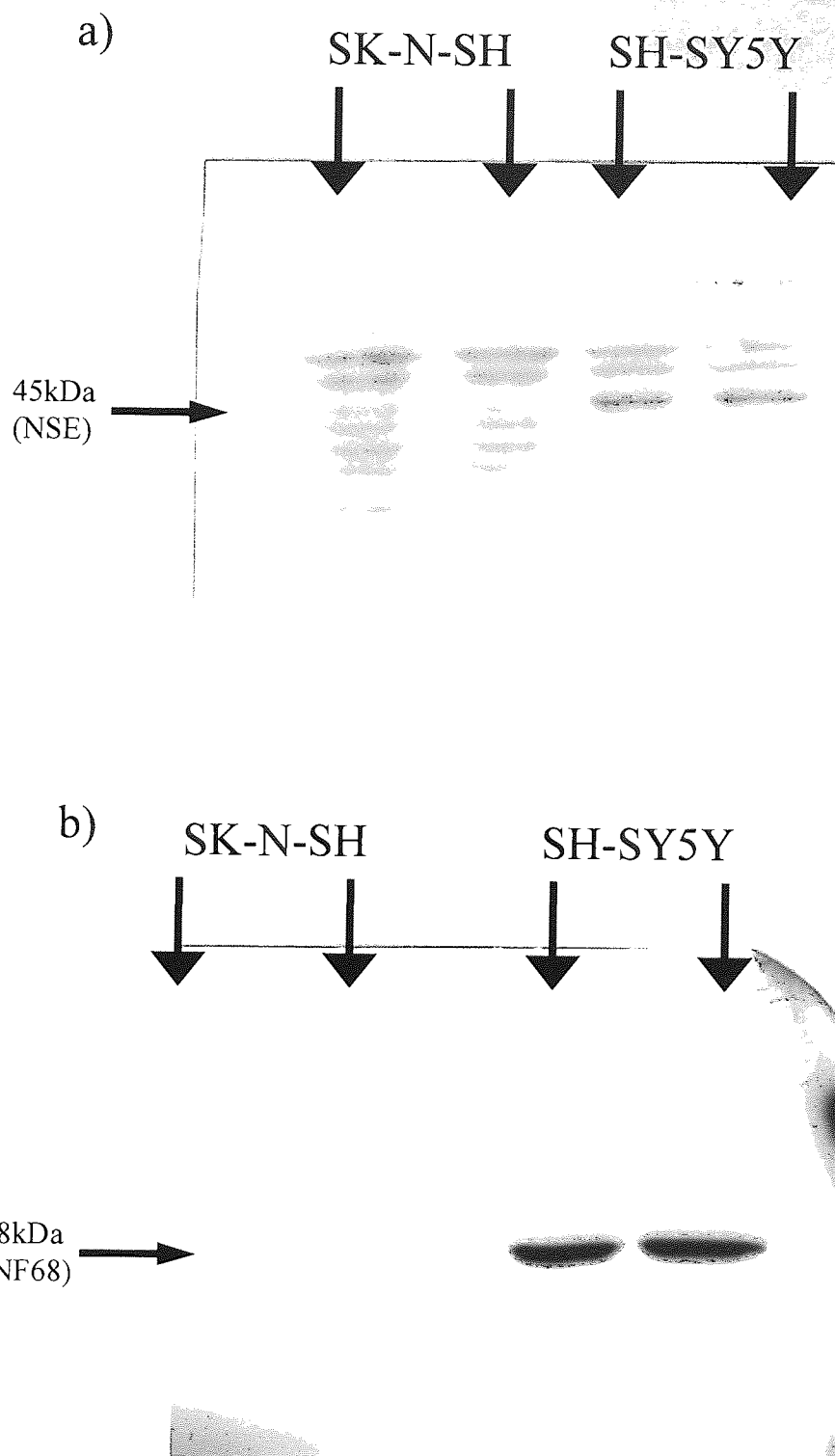
Figure 2.2: Western blot analysis of neuronal markers a) NSE and b) NF68 in both
SK-N-SH and SH-SY5Y lysates.

2.6 Statistics

MTT assays were carried out in 96 well plates with 8 repeats per plate and each experiment was repeated 3 times (n = 3). The flow cytometric cell cycle assays were carried out in 6 well plates with 6 repeats per plate and each experiment was repeated 3 times (n = 3).

All results are expressed as mean ± SEM and the values were compared using the Students unpaired t-test or one-way ANOVA followed by the Tukey post-test using Graphpad Instat. Groups of data were considered to be significantly different if $P<0.05$.

Chapter 3. Acute toxicity of 2,5-Hexanedione in human neuroblastoma cell lines

3.1 Introduction

2,5HD is a γ-diketone (Spencer and Schaumburg 1975; Perbellini et al., 1990; Graham, 1999), formed during a series of cytochrome P450 dependent ω-1 hydroxylation and oxidation reactions (DiVincenzo et al., 1976; DiVincenzo et al., 1977; Figure 3.1).

2,5HD's mechanism of neurotoxicity is still not fully understood; indeed, no single mechanism of neurotoxicity has been postulated which can offer a complete explanation of the structure/toxicity relationship with this agent, as well as its mediation of the slowing of nerve conduction velocities, eventual demyelination and degradation of exposed axons.

It is thought that it is the γ structure of 2,5 HD (Figure 3.2) that contributes to neurotoxicity as this configuration facilitates the formation of cross-links with neurofilaments. It is well established that focal accumulation of neurofilaments gives rise to axonal swelling, which precedes the peripheral neuropathy. The multifocal accumulations of the neurofilaments have been associated with the blockade of fast axonal transport (Mendell et al., 1977). Specifically, 2,5HD reacts covalently with the critical c-terminus neurofilament lysine ε-amines forming 2,5 dimethylpyrrole adducts (DeCaprio et al., 1982; DeCaprio and Fowke, 1992; DeCaprio et al., 1997). These alkylpyrroles undergo oxidation reactions that lead to neurofilament protein cross-linking (Graham et al., 1991; Zhu et al., 1994; Graham et al.,

N-Hexane

(ω-1)-Oxidation

2-Hexanol

(ω-1)-Oxidation

2,5-Hexanediol

5-Hydroxy-2-hexanone
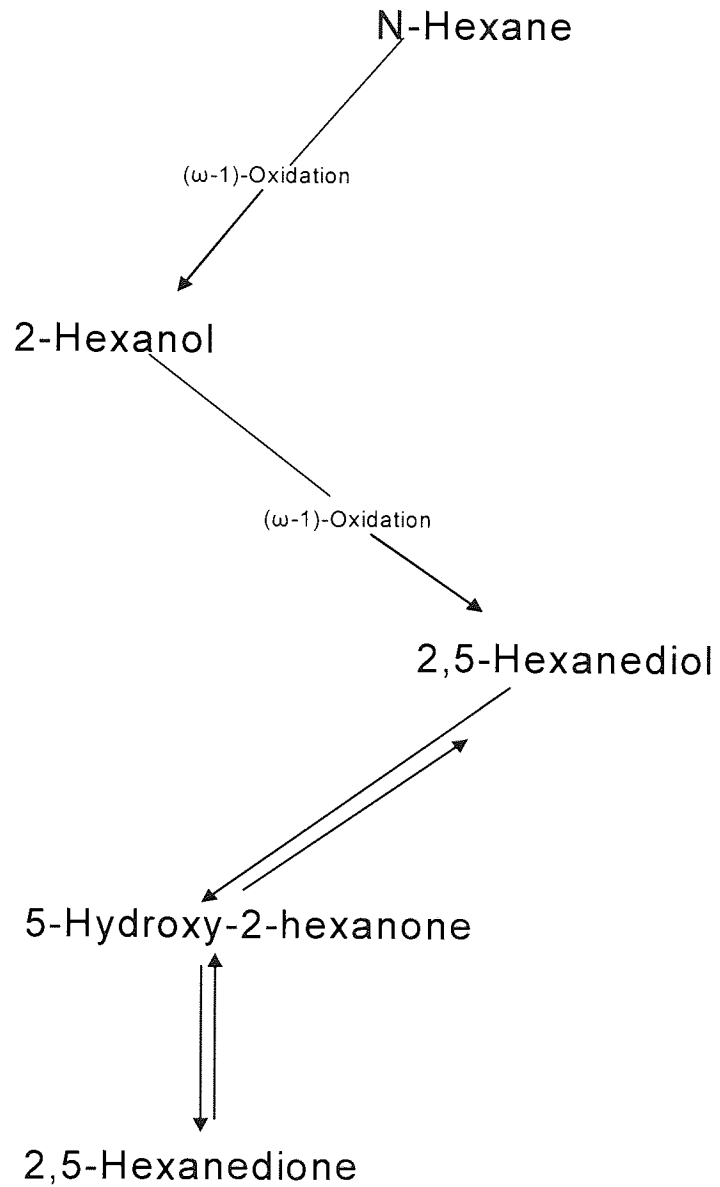
2,5-Hexanedione

Figure 3.1: The metabolism of n-Hexane giving rise to the neurotoxic metabolite 2,5 Hexanedione (Adapted from Couri and Milks, 1982).
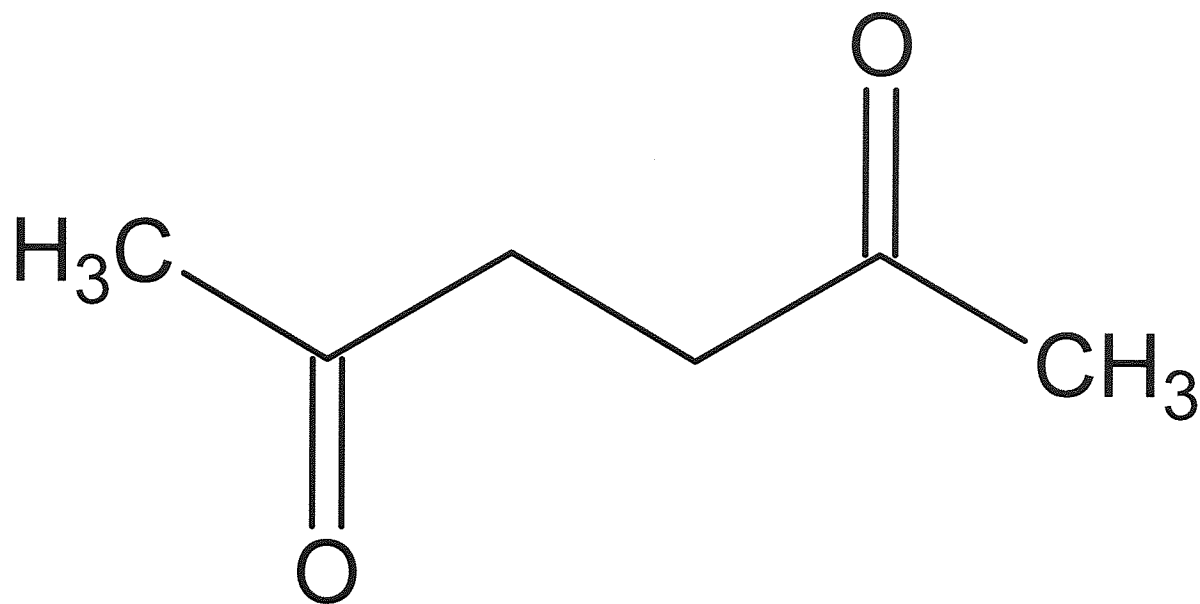
Figure 3.2: The structure of 2,5-Hexanedione.

1995; Heijink *et al.*, 2000). Interestingly, the importance of this neurofilament protein cross-linking step in 2,5HD mechanism of toxicity has not yet been fully established (reviewed in LoPachin & DeCaprio, 2005).

Another proposed mechanism of hexacarbon neurotoxicity in general, is impairment in energy metabolism (Spencer *et al.*, 1980). The basis of this hypothesis is that neurotoxic hydrocarbons are believed to inhibit the sulfhydryl enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphofructokinase (PFK) in a dose dependent manner. The resulting energy insufficiency would then reduce the axoplasmic supply of glycolytic enzymes from the perikaryon, leading to inadequate energy production in the axons, leading to their deterioration. Sabri & Ochs (1971; 1972) have shown that inhibition of GAPDH blocks fast axoplasmic transport and that 2,5HD inhibits GAPDH (Sabri *et al.*, 1979). Work carried out by Howland *et al* (1980) also showed that 2,5HD inhibits PFK and GAPDH and to some extent the glycolytic enzyme enolase. Another biochemical alteration following 2,5HD administration is an inhibition of sterol biosynthesis in rat sciatic nerve. Gillies *et al* (1980) suggested that the neurotoxicity of 2,5HD might be related to the reduced biosynthesis of ubiquinone, an essential link in the mitochondrial electron transport chain.

There are many case study reports and reviews of industrial workers exposed to n-Hexane, where evaluation of 2,5HD in urine is used as a marker of n-Hexane exposure levels (examples; dos Santos *et al.*, 2002; Prieto *et al.*, 2003). Other controlled n-Hexane exposure research has been carried out in human volunteers. However, these studies were directed at evaluating and comparing two methods of monitoring n-Hexane exposure, n-

Hexane concentrations in alveolar air and 2,5HD concentrations in urine, rather than the evaluation of 2,5HD neurotoxicity (Hamelin *et al.*, 2004; Hamelin *et al.*, 2005).

Relatively little research has been carried out on the effects of 2,5HD on human tissues *in vitro*, especially with regards to the first stages of acute exposure. As mentioned above, the majority of human studies are case studies of industrial workers exposed to hexane chronically. However, it has been shown that exposure to 25mM 2,5HD of both differentiated and undifferentiated SH-SY5Y human neuroblastoma cells causes the formation of perikaryal inclusion bodies after 48hrs and in the undifferentiated cell this concentration was also cytotoxic (Hartley et al., 1997). Also, work carried out by Heijink *et al.*, 2000, showed that the cross-linking of neurofilaments, a common effect of 2,5HD toxicity in humans and other animal models such as rats, occurred *in vitro* in the SK-N-SH neuroblastoma cell line after 72 hours exposure to 2,5HD.

As neuroblastoma lines are established models of human neurotoxicity, this chapter is intended to evaluate in detail the toxicity of 2,5HD at time points which range from acute (4h) to acute/intermediate/chronic exposure (24 & 48h) in both the SK-N-SH and SH-SY5Y neuroblastoma line clones, using both MTT assays and flow cytometry.

Utilisation of the rapid, quantitative colorimetric MTT assay casts light on the cytotoxic and necrotic effects of a given test compound. The end point of the assay is an indication of mitochondrial damage or the effects of other processes which may contribute to mitochondrial attrition which results in the mitochondria being unable to metabolise MTT (For more details on the method refer to section 2.3). The MTT assay could be regarded as a "triage" assessment at this level, in that a gross comparison of the toxicity of 2,5HD may

be made in terms of concentration and effect, as well as a comparison of the susceptibility of the different cell lines to the toxin.

The information provided from the MTT "triage" would then inform the estimation of appropriate concentration ranges for flow cytometry-based investigations, which were aimed at uncovering much more specific data on the effects of 2,5 HD on the cell cycle. This was facilitated by treating the cells with propidium iodide prior to flow cytometric analysis of the cell cycle and apoptosis (For more details on the method refer to section 2.4). Cell cycle analysis provides a detailed insight into the effects of a toxin on the processes which regulate DNA metabolism. It also shows whether any cell death caused is predominantly necrotic or apoptotic.

3.2 Methods

All cell culture was carried out as described previously (chapter 2). All 2,5HD solutions were made up in the corresponding media for the cell type being assayed. At the concentrations used 2,5HD was readily soluble at 37°C.

3.2.1 MTT Assays

Following calibration assays to decide the optimum seeding density, both SK-N-SH and SH-SY5Y were seeded into 96 well plates at $\approx 5 \times 10^4$ cells/well. The experiments were then carried out as described in section 2.3 with 2,5HD.

3.2.2 Flow Cytometric Assays

SK-N-SH and SH-SY5Y cells were seeded into 6 well plates at $\approx 5 \times 10^5$ cell/ml. The experiments were then carried out as described in section 2.4 with 2,5HD.

3.3 Results

3.3.1 MTT Assays

2,5HD reduced MTT turnover in a concentration dependent manner in both SK-N-SH and SH-SY5Y cell lines and at all exposure time points (4h, 12h, 24h and 48h). Figure 3.3 shows the concentration-response characteristics of cultures of the SK-N-SH and SH-SY5Y cells, after 4h exposure to 2,5HD with respect to MTT turnover. In both cell lines the level of MTT turnover initially increased slightly ($P<0.05$), between 43-213 mM for the SK-N-SH cells and 17-51 mM in the SH-SY5Y cells. MTT turnover fell steadily between 213-426 mM, with an IC_{50} of 303.6 ± 2.9mM in the SK-N-SH cells and between 68-298 mM, with an IC_{50} of 156.9 ± 2.5mM for the SH-SY5Y cells. These IC_{50}s show that the SH-SY5Y line is approximately twice as sensitive to the effects of 2,5HD at 4h exposure. Comparison between these IC_{50}s of 2,5HD revealed a significant difference between the two cell lines in terms of the effects of 2,5HD on the two cell lines ability to metabolise MTT ($P<0.001$).

Figure 3.4 shows the concentration-response characteristics of the SK-N-SH and SH-SY5Y cells, after 24h exposure to 2,5HD with respect to MTT turnover. In both cell lines MTT turnover fell steeply between 17-128 mM, with an IC_{50} of 62.8 ± 1.12mM for SK-N-SH cells and between 17-68 mM, with an IC_{50} of 48.2 ± 1.0mM for SH-SY5Y cells.
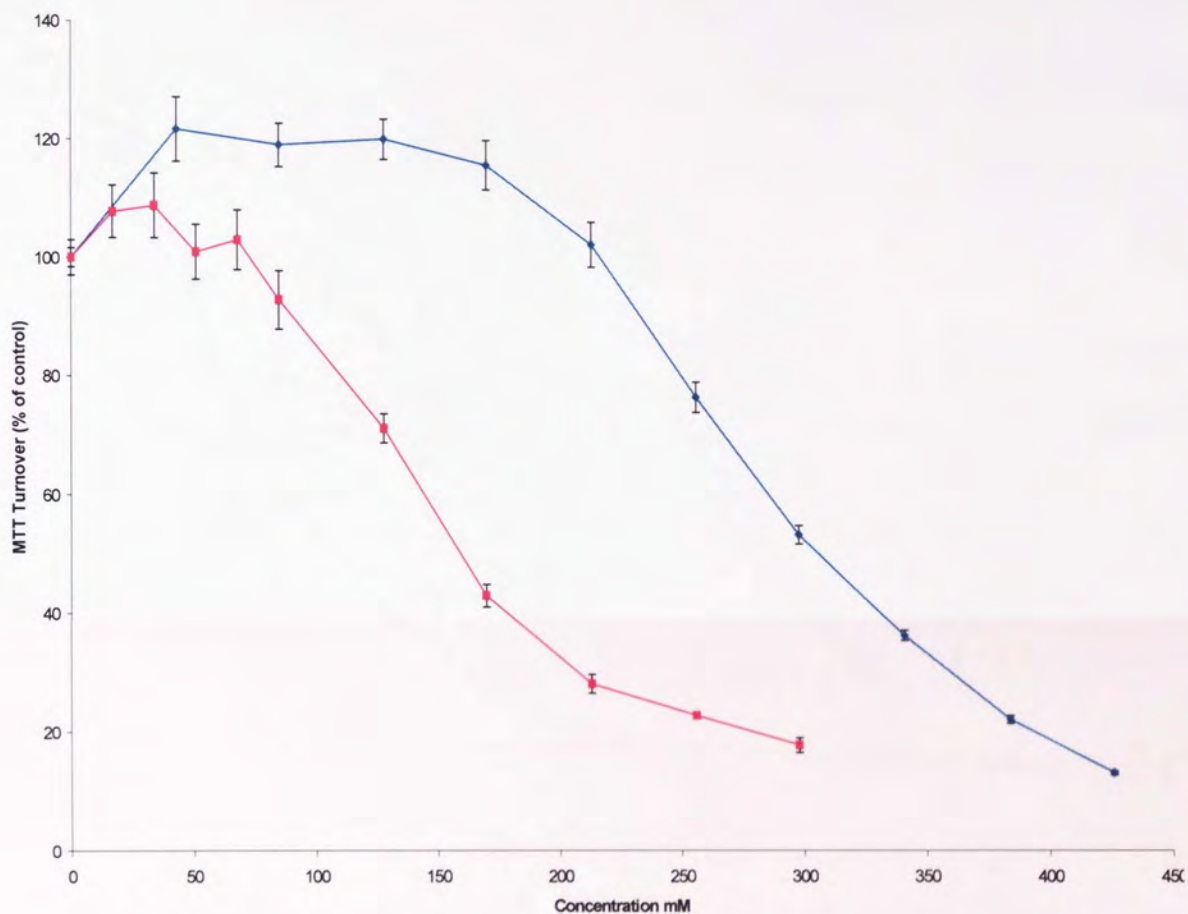
Figure 3.3: MTT assay on SK-N-SH (●) and SH-SY5Y (■) cells following 4h treatment with 2,5-hexanedione. Cells were treated with increasing concentrations of 2,5-hexanedione and incubated for 4h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).
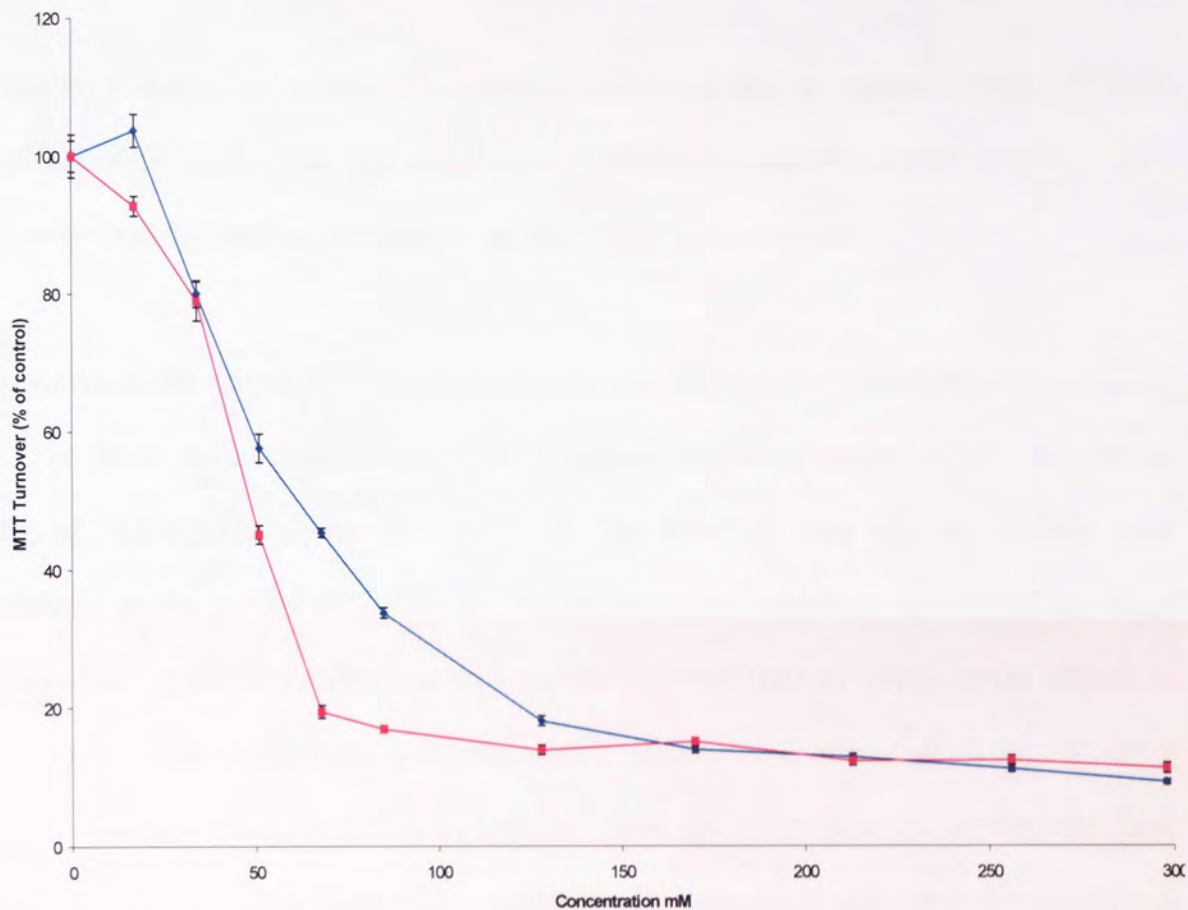
Figure 3.4: MTT assay on SK-N-SH (●) and SH-SY5Y (■) cells following 24h treatment with 2,5-hexanedione. Cells were treated with increasing concentrations of 2,5-hexanedione and incubated for 24h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).

The SH-SY5Y line was more sensitive to the effects of 2,5HD after 24h exposure and statistical analysis of the IC_{50}s showed significant differences between the two cell lines in terms of the effects of 2,5HD in terms of their relative abilities to metabolise MTT after 24h exposure (P<0.001).

Figure 3.5 shows the concentration-response characteristics of cultures of the SK-N-SH and SH-SY5Y cells, after 48h exposure to 2,5HD with respect to MTT turnover. MTT turnover initially increased slightly in the SK-N-SH cells, between 8.5-15 mM.

In the SK-N-SH cells MTT turnover decreased sharply between 17-30 mM, with an IC_{50} of 22.1±0.2mM. A steady reduction in MTT turnover occurred between 4-34 mM, with an IC_{50} of 20.3±0.3mM for the SH-SY5Y cells. The SH-SY5Y line was only slightly more sensitive to the effects of 2,5HD at 48h exposure and statistical analysis of the IC_{50}s showed no significant difference between the two cell lines in terms of the effects of 2,5HD on their ability to metabolise MTT after 48h exposure (P>0.05). Table 1 summarizes the IC_{50}s for 2,5HD in both cell lines and at all time points. Between time points for each cell line IC_{50}s were significantly different from each other (P<0.001), and apart from at 48h exposure the IC_{50}s were significantly lower (P<0.001) in the SH-SY5Y line than the SK-N-SH line.

Table 1: Summary table of IC_{50}s for 2,5HD on SK-N-SH and SH-SY5Y cell lines. Results are means ± SEM (n=3).

	SK-N-SH	SH-SY5Y
4h	303.6±2.9mM	156.9±2.5mM
24h	62.8±1.2mM	48.2±1.0mM
48h	22.1±0.2mM	20.3±0.3mM
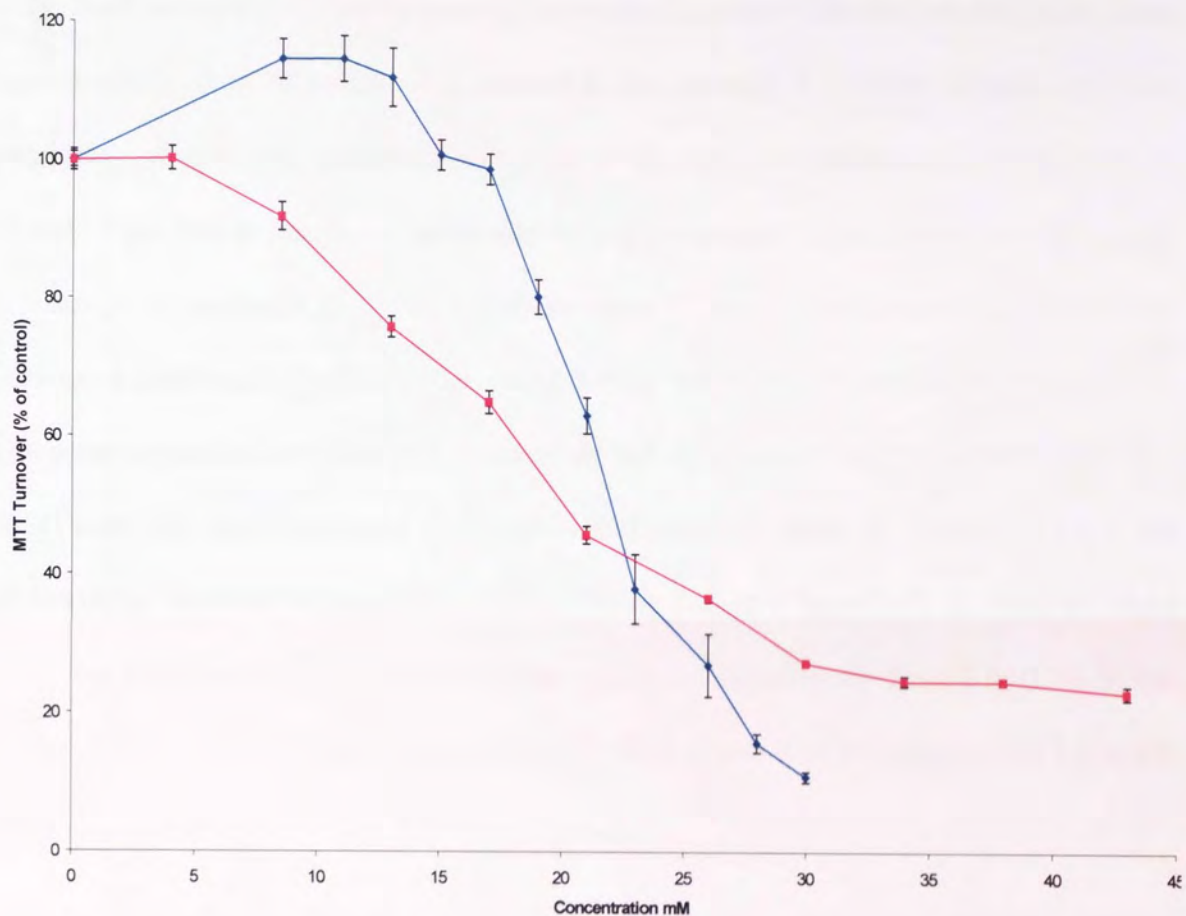
Figure 3.5: MTT assay on SK-N-SH (●) and SH-SY5Y (■) cells following 48h treatment with 2,5-hexanedione. Cells were treated with increasing concentrations of 2,5-hexanedione and incubated for 48h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).

3.3.2 Flow Cytometric Assays

In the both cell lines, 2,5HD induced increases in apoptosis within the respective concentration ranges of toxicity shown previously by MTT (Figure 3.6). In the SK-N-SH cells, there was a significant increase in apoptotic nucleoids over the concentration range from 4.4 ± 0.2mM to 18.3 ± 0.9mM compared to the controls ($P < 0.001$) (Figure 3.6). The percentage of apoptotic nucleoids increased to the observed maximum of $33.0\pm0.7\%$ at 34mM. This rise in apoptotic nucleoids was accompanied by a significant fall in the percentage of nucleoids in the G_0/G_1 phase from $72.4\pm0.3\%$ to $45.4\pm0.6\%$, ($P < 0.001$) between 8.5mM and 17mM and decreased further to $38.8\pm0.3\%$ by 34mM (Figure 3.7). The percentage of cells in the G_2M phase of the cell cycle was $7.1\pm0.3\%$ in the control SK-N-SH cells, this value increased significantly and almost doubled by 17mM to $13.6\pm0.4\%$ ($P < 0.001$), this then fell slightly to $11.3\pm0.3\%$ by 34mM (Figure 3.8). As with the G_2M phase the percentage of cells in the S phase increased significantly from $6.6\pm0.3\%$ in the control cells to $23.1\pm0.3\%$ at 17mM 2,5HD ($P < 0.001$) and then fell slightly to $17.5\pm0.4\%$ by 34mM (Figure 3.9).

In the SH-SY5Y cell line the level of apoptosis in the control was $19.1\pm0.8\%$ was significantly greater than that of SK-N-SH cells at $2.0\pm0.1\%$. With the SH-SY5Y cells the percentage of apoptotic nucleoids increased significantly ($P<0.001$) at every concentration between 8.5 and 30mM peaking at $81.7\pm1.2\%$ at 30mM (Figure 3.6). This rise in apoptotic nucleoids was accompanied by a significant fall in the percentage of nucleoids in the G_0/G_1 phase from 68.4 ± 0.8 at 8.5mM to $45.9\pm0.9\%$ at 13mM ($P<0.001$), where the level plateaued until 19mM when it decreased further from 43.1 ± 0.8 to $15.3\pm1.4\%$ by 30mM (Figure 3.7).

Figure 3.6: Apoptosis of SK-N-SH (●) and SH-SY5Y (■) cells following 48h treatment with 2,5-hexanedione. Cells were treated with increasing concentrations of 2,5-hexanedione and incubated for 48h. Cells were stained with propidium iodide and analysed using a flow cytometer. Results are means ± SEM (n=3).
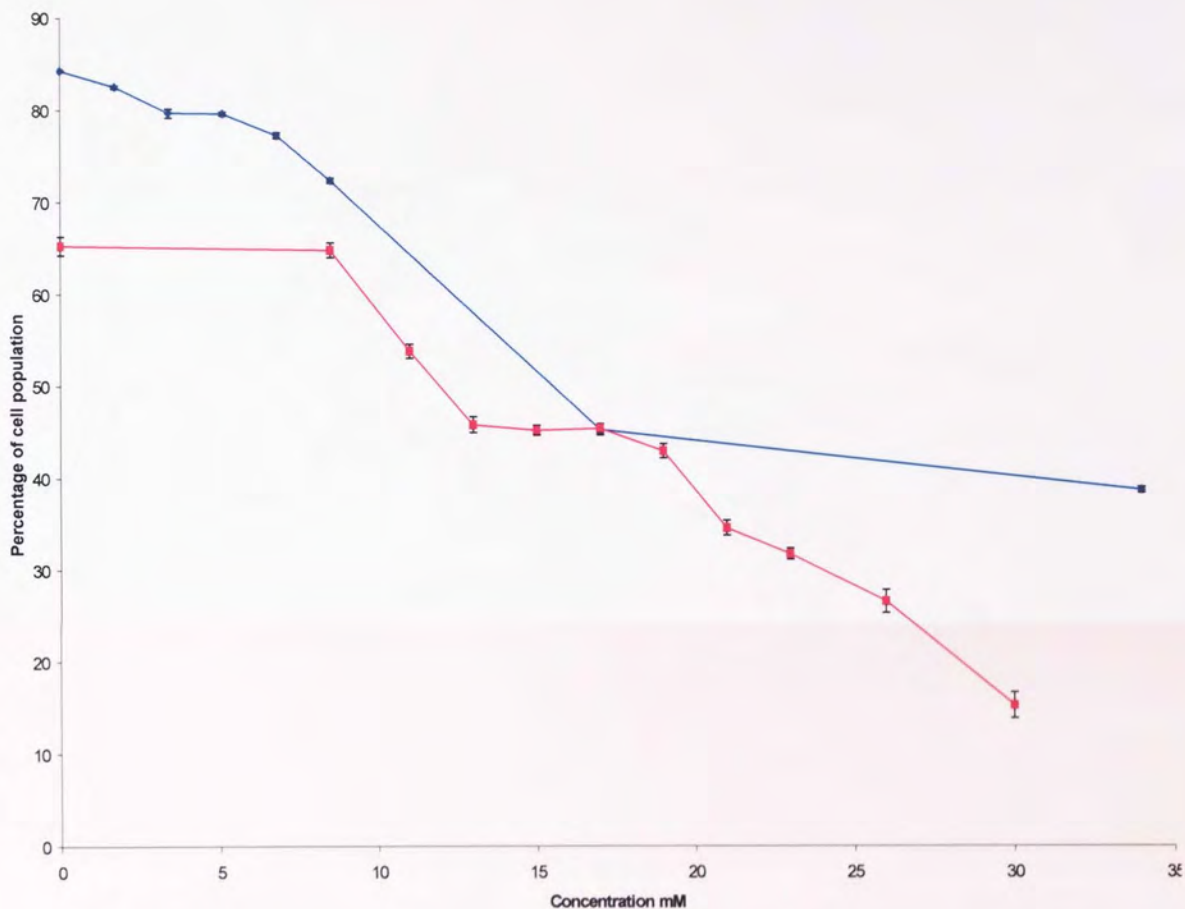
Figure 3.7: G_0/G_1 phase of flow cytometric analysis of SK-N-SH (●) and SH-SY5Y (■) cells following 48h treatment with 2,5-hexanedione. Cells were treated with increasing concentrations of 2,5-hexanedione and incubated for 48h. Cells were stained with propidium iodide and analysed using a flow cytometer. Results are means ± SEM (n=3).
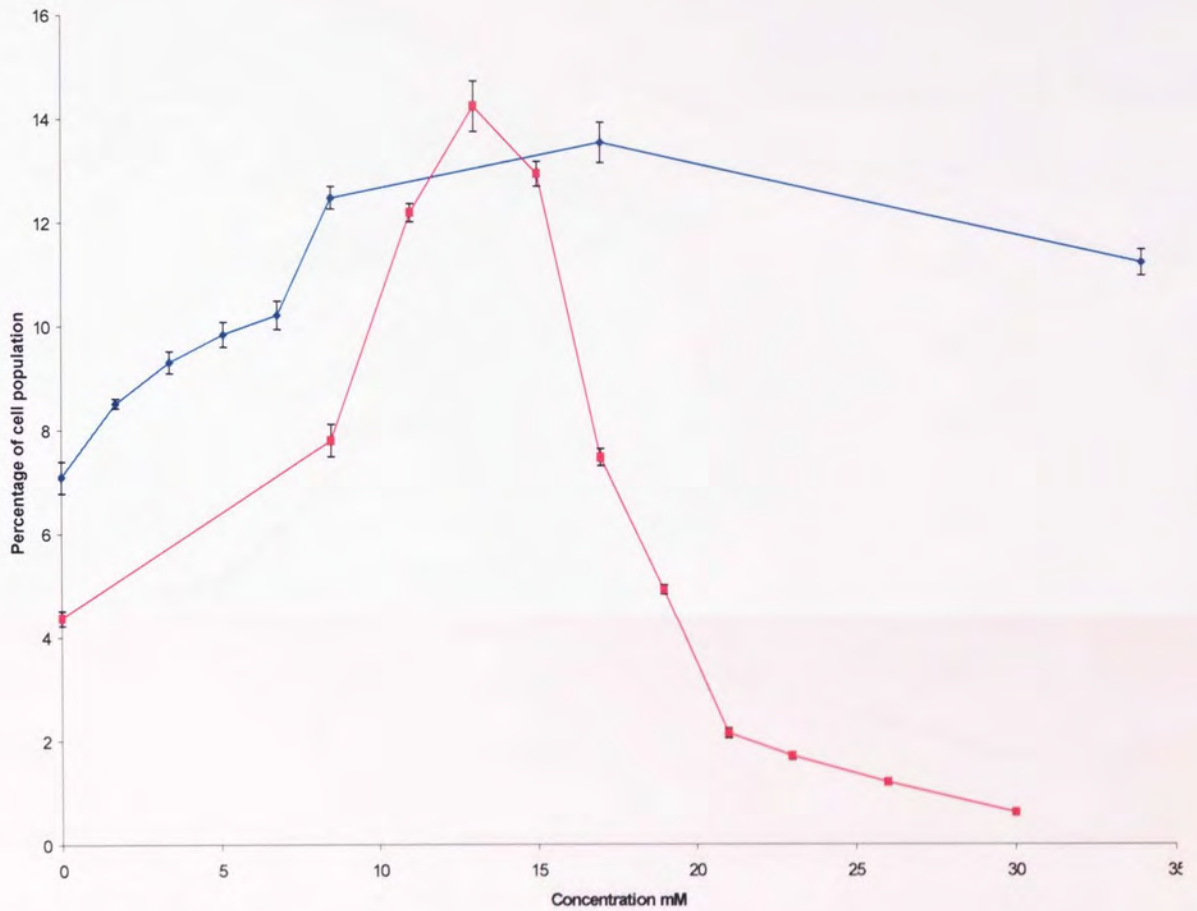
Figure 3.8: G$_2$/M phases of flow cytometric analysis of SK-N-SH (●) and SH-SY5Y (■) cells following 48h treatment with 2,5-hexanedione. Cells were treated with increasing concentrations of 2,5-hexanedione and incubated for 48h. Cells were stained with propidium iodide and analysed using a flow cytometer. Results are means ± SEM (n=3).
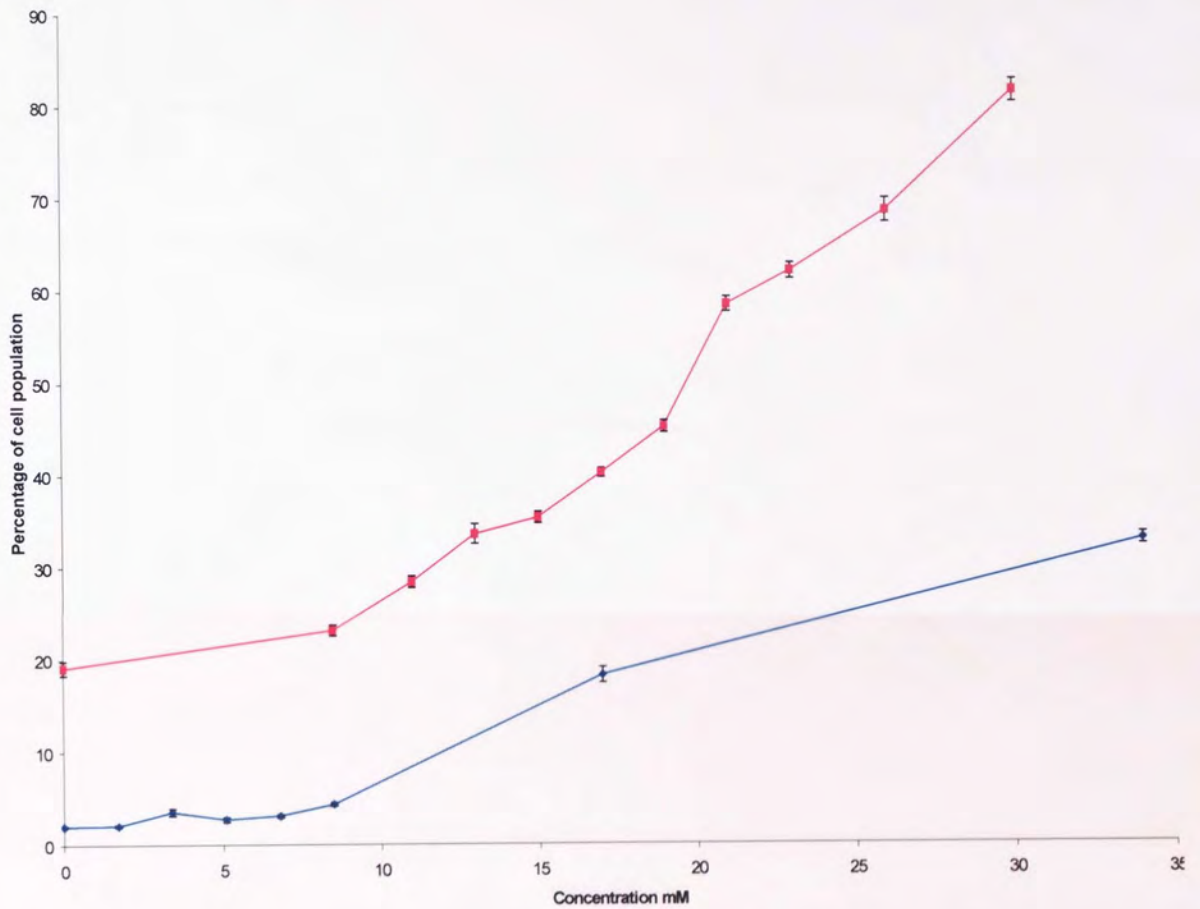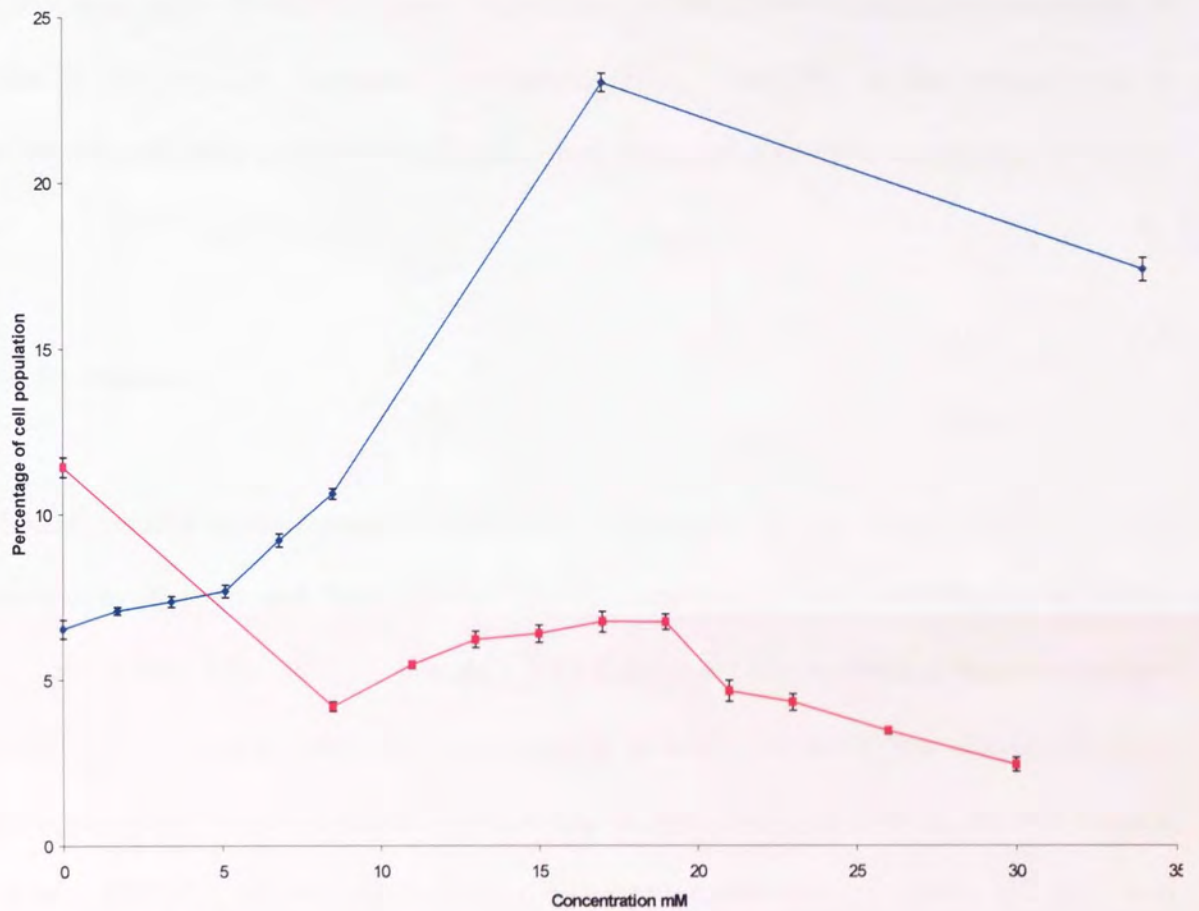
Figure 3.9: S phases of flow cytometric analysis of SK-N-SH (●) and SH-SY5Y (■) cells following 48h treatment with 2,5-hexanedione. Cells were treated with increasing concentrations of 2,5-hexanedione and incubated for 48h. Cells were stained with propidium iodide and analysed using a flow cytometer. Results are means ± SEM (n=3).

The percentage of cells in the G$_2$M phase of the cell cycle was 4.4±0.1% in the control SH-SY5Y cells, this value increased significantly at all concentrations of 2,5HD between 8.5 and 13mM where the percentage of these nucleoids peaked at 14.3±0.5% (P<0.001), this then fell sharply between 15 and 30 mM to 0.6±0.02% which is significantly lower than the control value (P<0.001; Figure 3.8). Unlike in the SK-N-SH cells, the percentage of cells in the S phase decreased significantly from 11.4±0.3% in the control cells to 4.2±0.1% at 8.5mM 2,5HD (P<0.001) and then fluctuated slightly to around this level then fell to 2.5±0.2% by 30mM (Figure 3.9).

3.4 Discussion

Hexane induces chronic neurotoxicity in rats and humans through the effects of its 2,5HD metabolite (Spencer and Schaumburg 1975; Governa et al., 1987; Perbellini et al., 1990; Graham, 1999). Most cases of human 2,5HD toxicity are due to chronic hexane exposure in industrial situations, such as in the handling of adhesives in the manufacture of shoes (dos Santos et al., 2002; Prieto et al., 2003). In this chapter the acute and sub chronic toxicity of 2,5HD has been examined in the human neuroblastoma cell lines SK-N-SH and SH-SY5Y. Several groups have carried out studies into 2,5HD neurotoxicity in vitro using different models, although over a similar timeframe to the work presented in this chapter. Heijink et al (1995, 2000) observed cross-linking of neurofilaments after 48h with 10mM 2,5HD in SK-N-SH cells. Kamijima et al (1996) investigated the toxic effects of hexane derivatives, including 2,5HD, on cultured rat Schwann cells and found some mild toxicity, including changes in morphology and inhibition of mitosis, using a concentration range between 0.67mM and 85.3mM over 24h. Also, Hartley et al (1997) observed neurofilament accumulation but no cytotoxicity (LDH assay) with 25mM 2,5HD after both

24 and 48h exposure in the SH-SY5Y cell line. These studies were used to help formulate the concentration ranges used in these experiments.

The MTT assay is an established cell viability assay that is commonly used as a measure of cytotoxicity following toxic insult in a large variety of cell lines (Habtemariam, 1995; Plumb, 2004). Fotakis and Timbrell (2006) found the MTT assay to be more sensitive than the LDH leakage assay and the protein assay in a comparison of cytotoxicity assays. On the whole, the MTT assay provided a simple high throughput method for accurately evaluating the cytotoxicity of 2,5HD and estimating IC_{50} values for the different time points.

With the MTT assay at 4h, 24h and 48h in the SK-N-SH cell and only at 4h in the SH-SY5Y there was a small, but significant, increase in MTT turnover, above that of the controls, at low concentrations of 2,5HD. The MTT assay assesses the ability of a cell to cleave the tetrazolium ring of MTT to form the formazan product. As cellular metabolic activity/viability is reduced and mitochondrial function decreases, its ability to convert MTT decreases. However, it is possible for toxins/chemical entities to cause an increase in metabolic activity within a cell through the enzyme succinate dehydrogenase; this enzyme is located in the mitochondrial inner membrane and it is sensitive to local changes in ion concentrations and flux. Thus, with appropriate ionic conditions, increased succinate dehydrogenase activity can lead to an increase in MTT turnover at a low level exposure to any particular toxin (Putnam et al., 2002). Therefore it is reasonable to suggest that 2,5HD may increase succinate dehydrogenase activity by causing changes in cellular ion concentrations or flux, causing the increased MTT turnover observed in this study.

In both cell lines at the majority of time points 2,5HD reduced MTT turnover, suggesting some degree of acute cytotoxicity occurred. The concentrations of 2,5HD employed were within similar ranges used by other groups in various cell lines (Heijink *et al.*, 1995; Kamijima *et al.*, 1996; Hartley *et al.*, 1997; Heijink *et al.*, 2000; Woehrling *et al.*, 2006), but are significantly higher than observed physiological levels of the metabolite (Heijink *et al.*, 2000). In this chapter at 4h 2,5HD exposure, the SH-SY5Y cells were nearly twice as sensitive to this toxin in comparison with the SK-N-SH cells. After 24h and 48h exposure, the sensitivity to 2,5HD increased progressively over each earlier time point in both cell lines. However, the 2-fold difference between the cells lines IC_{50}s after 4h was not sustained after 24h and 48h 2,5HD exposure. SH-SY5Y cells are a sub-clone of the SK-N-SH line and they display a more neurone-like phenotype compared with the parent line. This may have accounted for the greater vulnerability of the sub-clone compared with the parent at 4h although it does not necessarily account for the lack of differences in sensitivity seen in the later time points.

Fritz Haber initially developed "Haber's rule" in the early 20th century whilst evaluating the acute toxicity of chemical warfare agents (Witschi, 1999). Although originally applied to the toxicity of inhaled gaseous compounds, Haber's rule can effectively be applied to a variety of *in vitro* toxicity studies. The rule states that, for any given toxin;

$$C \times t = k$$

where C is concentration (in this case the IC_{50}), t is the exposure period and k is a constant.

Therefore, if the exposure period is doubled, from 24h to 48h for example, the IC_{50} should be halved. It has been suggested that the neurotoxicity resulting from 2,5HD exposure follows Haber's Rule (Rozman and Doull, 2000; LoPachin and DeCaprio, 2004; Woehrling et al., 2006). With the exception of the 4h data in the SH-SY5Y cells, the IC_{50}s observed in this study show some indication of conformity to this rule. If the equation is applied as below, the comparison between the IC_{50}s at 4h, 24h and 48h exposure in the SK-N-SH line shows that the values are roughly comparable and suggest some agreement with Haber's rule.

IC_{50} (mM) x duration of exposure (h) = constant

303.64±2.90mM x 4h = 1214.55±11.58

62.81±1.18mM x 24h = 1507.44±28.43

22.05±0.24mM x 48h = 1058.34±11.73

In general the IC_{50} values observed for 2,5HD in this study were quite high (high mM range), no directly comparable data could be found in the literature to see how these values compare to other similar neurotoxins in these cell lines.

A major advantage of flow cytometry is the rapid analysis of thousands of cells (Siegel, 1984). The use of DNA-specific fluorescent dyes, such as PI, can be utilised with flow cytometry to measure any changes to DNA content or the cell cycle caused by a potential toxin (Gray et al., 1975; Laerum & Farsund, 1981). In this chapter, determination of nuclear DNA content via flow cytometric analysis of nucleoid associated PI fluorescence provided quantitative information at several stages of the cell cycle and apoptosis.

The base line level of apoptosis in the SK-N-SH control cells was consistent at approximately 2%. However, in the SH-SY5Y control cells this level was consistently higher, at approximately 19% and has not been previously reported to the author's knowledge. There are a number of factors that may influence the predisposition of various neuroblastoma cell lines to enter apoptosis, these include effects on protein kinase C, Bcl-2 and $G\alpha_s$-L (Lombet *et al.*, 2001; Zhao *et al.*, 2006), as well as differences in responses to growth factors (da Motta *et al.*, 1997). The process of removing adherent cells from plasticware is likely to exert stress on the cells and trigger some background levels of apoptosis. It is probable that the heterogeneous SK-N-SH line was consistently more resistant to this process compared with the homogeneous SH-SY5Y cells.

After allowing for the higher base line level of apoptosis in the SH-SY5Y compared with the SK-N-SH cells, both cell lines followed a similar pattern in apoptosis over the early time points. However the maximum observed level of apoptosis was much higher in the SH-SY5Y, above and beyond the difference seen in the control groups. Again, this may be a reflection of the greater closeness to the neural phenotype displayed by the SH-SY5Y compared with the SK-N-SH line.

Paralleled with this increase in the percentage of cells in apoptosis, there was a corresponding decrease in the percentage of cells in the G_0/G_1 phase of the cell cycle, and an increase in G_2/M checkpoint arrest. This arrest at the G_0/G_1 and G_2/M phase was symptomatic of inhibition of the cell cycle at the stages of DNA replication and chromosome segregation, respectively (Yuan *et al.*, 2005). In the SH-SY5Y cells the level of G_2/M arrest fell below control, whilst this does not occur in the SK-N-SH cells. Also, there was an increase in S-phase arrest in the SK-N-SH cells, whereas in the SH-SY5Y

line, the percentage of cells in the S-phase fell below that of the controls at the lowest concentration of 2,5HD. Both cell lines were stimulated to enter the apoptosis by 2,5HD, however maximum observed level of apoptosis was considerably higher in the SH-SY5Y line compared with the SK-N-SH line. Among the possible explanations for this effect, may be that the SH-SY5Y cells are more sensitive to the effects of 2,5HD than the SK-N-SH cells and at the higher concentrations of 2,5HD the SK-N-SH cells do not enter into the cell cycle and commence apoptosis instead necrosis ensues due to acute cellular damage.

Possible differences between the two neuroblastoma lines with regards to their respective sensitivities to the apoptotic effects of 2,5HD may also be linked with energy metabolism. The primary pathway for glucose oxidation is glycolysis, therefore the inhibition of glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphofructokinase (PFK), can lead to a reduction in intracellular ATP which in turn would result in the blockade of the energy-dependent axonal transport system (Sabri and Ochs, 1972). 2,5HD has been shown to selectively inhibit GAPDH and PFK (Sabri, 1984a; Sabri, 1984b). Also, Sickles *et al* (1990), showed 2,5HD causes a progressive significant reduction in the rate of ATP formation and reduced ATP content of the mitochondrial fraction.

One major physiological difference between apoptotic and necrotic cells regards intracellular ATP levels, which are swiftly reduced in necrotic cell death, whereas in apoptotic cell death ATP levels are decreased very late in the process (Shimizu *et al.*, 1996). ATP is required for a cell to undergo apoptosis, as depletion in ATP levels causes apoptosis to be significantly blocked (Eguchi *et al.*, 1997) and inhibition of mitochondrial ATP production can act as a switch between apoptosis and necrosis (Leist *et al.*, 1999).

Therefore if the SK-N-SH cells were more susceptible to the inhibition of GAPDH and PFK by 2,5HD than the SH-SY5Y cells this could account for the differences observed in apoptosis as the SK-N-SH cells would be more likely to necrose due to the decreased ATP levels.

Human exposure to 2,5HD via the metabolism of hexane ultimately causes axonal atrophy. 2,5HD reacts with neurofilament lysine ε-amines, leading to the formation of 2,5-dimethylpyrrole adducts which in turn contributes to neurofilament cross linking (Zhu *et al.*, 1994; Heijink *et al.*, 2000; LoPachin & DeCaprio, 2005). *In vitro* and *in vivo* studies have shown a variety of effects of 2,5HD on cellular organelles and processes (Selkoe *et al.*, 1978; Heijink *et al.*, 2000; LoPachin & DeCaprio, 2004). To date, no studies have appeared in the literature showing the apoptotic effects of 2,5HD as observed in this chapter.

As the MTT assay operates solely through measurement of the product of tetrazolium ring cleavage, it cannot distinguish between necrotic or apoptotic cell death. The use of the fluorescent dye PI with flow cytometry clearly identifies the latter stages of the process of apoptosis. As the DNA of an apoptotic cell is fragmented, when it is stained with PI it binds less fluorochrome due to its smaller size (Wyllie, 1980; Cohen, 1993). Therefore, apoptotic nucleoids fluoresce at a less intense level when analysed compared to aneuploid DNA, which gives rise to a peak to the left of the G_0/G_1 peak. At 48h exposure the percentage of MTT turnover fell to approximately 10% of that of the controls in the SK-N-SH cells and 20% of that of the controls in the SH-SY5Y. This can be related to approximately 90% and 80% cell death respectively. This corresponds to the level of apoptosis observed in the SH-SY5Y, although in the SK-N-SH cell line this is significantly

higher than the percentage of apoptosis seen via flow cytometry. It could be suggested that as the cells had entered the apoptotic pathway their mitochondrial capacity may have deteriorated to the point that they were physically unable to metabolise the MTT. However, it is also possible that the process of DNA fragmentation may not have been complete at the point of staining. It is probable that there may have been a combination of necrosis and apoptosis occurring in these cell lines at different rates. .

2,5HD caused a concentration dependent reduction in MTT turnover in both cell lines at all time points, with the SH-SY5Y line showing a 2 fold greater sensitivity at 4h exposure. Apart from this difference at 4h, the pattern of toxicity between the two cell lines was very similar. The use of flow cytometry drew attention to several differences between the toxic responses to 2,5HD between the two cell lines that are not apparent with the basic MTT assay. The level of apoptosis in the SH-SY5Y line reached more than twice that of the SK-N-SH cells. The effect of 2,5HD exposure on the S-phase was very different between cell lines with an increase in S-phase arrest in the SK-N-SH cells and a decrease in the SH-SY5Y line.

In summary, the neurotoxic effects of 2,5HD were observed in the neuroblastoma cell lines SK-N-SH and SH-SY5Y. Although the MTT assay is a rapid, high throughput method for assessing cytotoxicity, the application of flow cytometry was necessary to determine more subtle toxicological responses to 2,5HD between the two cell lines.

Chapter 4. Acute toxicity of 2,3- and 3,4-Hexanedione in human neuroblastoma cell lines

4.1 Introduction

Although there is no evidence that the 2,3-hexanedione (2,3HD) and 3,4-hexanedione (3,4HD) isomers are formed by hexane metabolism *in vivo*, they remain in use commercially as food additives, where they are employed as colouring and flavouring agents (FAO/WHO, 1999). 2,3HD occurs in chicken, beer and coffee and it has been described as possessing a creamy, sweet and buttery aroma. It is also used in butterscotch, caramel, pineapple and custard flavours. The 3,4HD isomer occurs in foodstuffs ranging from coffees to cauliflower and has what has been described as a toasty, burnt, buttery and nutty aroma (Burdock and Fenaroli, 2004).

Both 2,3HD and 3,4HD are α-diketones (see figure 4.1) and due to steric hindrance are unable to form pyrrole adducts in the same way as 2,5HD, and have been classified previously as non-toxic and non-neurotoxic (FAO/WHO, 1999; Stone *et al.*, 1999; 2000).

Considerably less research has been carried out into the toxicity of 2,3HD and 3,4HD compared with 2,5HD. Spencer *et al*, (1978) conducted a chronic study in Sprague-Dawley rats, comparing the toxicity of several aliphatic hexacarbon compounds including 2,3- and 2,5HD. As well as normal weight gain and clinical signs, no change was observed to the histology of tissues removed from the nervous system of rats treated with 2,3HD, compared with the axonal swellings seen with 2,5HD.

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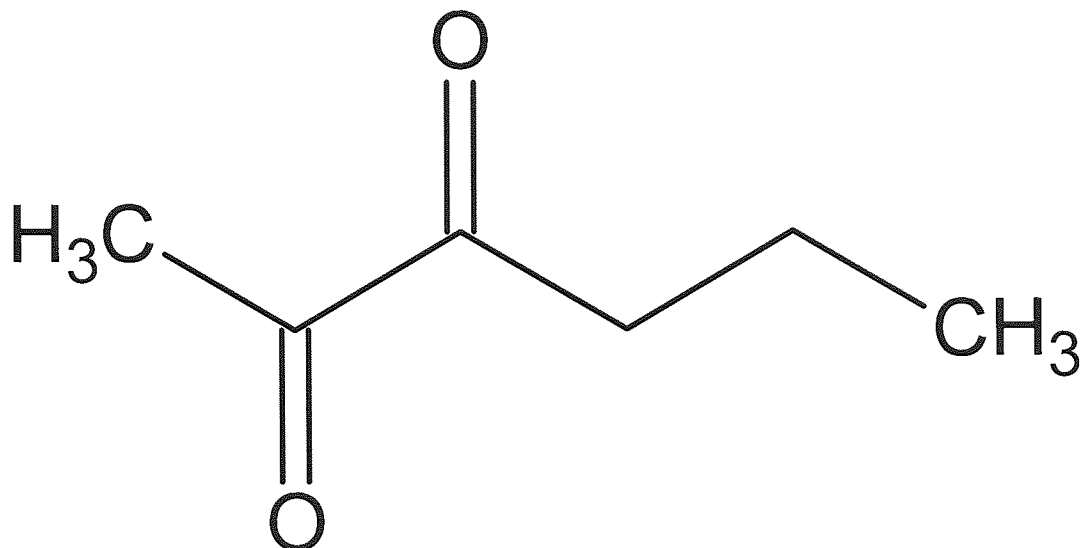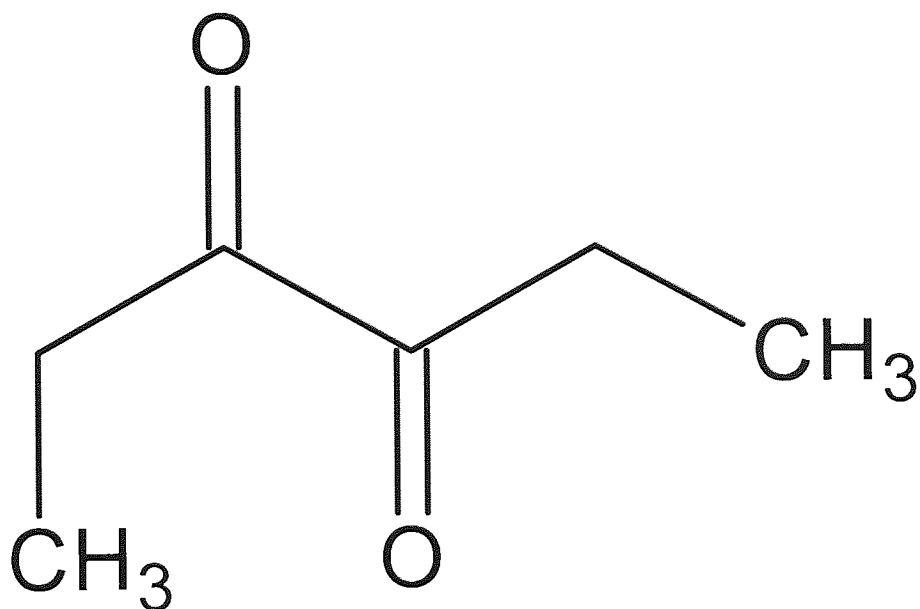
Figure 4.1: The structures of 2,3- and 3,4-Hexanedione

Zimmermann and Mohr (1992) looked at chromosome loss and chromosome malsegregation. Chromosome malsegregation can be caused by direct damage in a number of areas, such as DNA, chromatin structure, the centromere/kinetochore complex, as well as the mitotic spindle apparatus (Zimmermann and Mohr, 1992). The 2,3HD isomer induced chromosomal malsegregation in *Saccharomyces cerevisiae* strain D61.M. Interestingly, almost all the yeast colonies became respiratory deficient, indicating that 2,3HD induces mitochondrial, rather than nuclear mutation. In addition, 3,4HD was mildly mutagenic in the Ames test (Dorado *et al.*, 1992; Mellado and Montoya, 1994). The Ames test is a biological assay that can be used to assess both the mutagenic and carcinogenic potential of chemical compounds. Chen and Hee established EC_{50} values for 2,3HD in 1995 using the Microtox® Test System, which utilises the luminescent bacterium, *Vibrio fischeri* (strain NRRL B-11177), to measure toxicity. Luminescent bacteria produce light as a by-product of their cellular respiration and any toxicity results in a reduction in the rate of respiration and a corresponding decrease in luminescence. Strain 11177 was originally chosen for the acute and chronic tests because it displayed a high sensitivity to a broad range of chemicals. The EC_{50} values were 2.17mM, 1.81mM and 1.6mM at 5, 15 and 25 minutes respectively (Chen and Hee, 1995).

The inner mitochondrial membrane contains a transport protein, the oxoglutarate carrier (Walker and Runswick, 1993) that catalyzes a counter exchange between 2-oxoglutarate and malate. The oxoglutarate carrier is important for several metabolic processes including the malate-aspartate shuttle, the oxoglutarate-isocitrate shuttle and gluconeogenesis. 2,3HD caused a concentration dependent inhibition of the reconstituted oxoglutarate transport activity. Nearly complete inhibition of activity occurred with both 2,3HD and 3,4HD at 5mM in reconstituted OGC from bovine heart mitochondria. It was also shown that in

intact mitochondria, oxoglutarate transport is inhibited by 2,3HD at similar concentrations (Stipani *et al.*, 1996).

In this chapter it was intended to evaluate the acute as well as intermediate chronic toxicity of 2,3- and 3,4HD at time points ranging from 4-48h in both the SK-N-SH and SH-SY5Y neuroblastoma line clones, using both MTT assays and flow cytometry.

4.2 Methods

All cell culture was carried out as described in chapter 2. All 2,3HD and 3,4HD solutions were made up in the corresponding media for the cell type being assayed. At the concentrations used 2,3HD and 3,4HD were readily soluble at 37°C.

4.2.1 MTT Assays

Following calibration assays to decide the optimum seeding density, both SK-N-SH and SH-SY5Y were seeded into 96 well plates at $\approx 5 \times 10^4$ cells/well. The experiments were then carried out as described in section 2.3 with both 2,3HD and 3,4HD.

4.2.2 Flow Cytometric Assays

SK-N-SH and SH-SY5Y were seeded into 6 well plates at $\approx 5 \times 10^5$ cell/ml. The Experiments were then carried out as described in section 2.4 with both 2,3HD and 3,4HD.

4.3 Results

4.3.1 MTT Assays

As seen with 2,5HD in the previous chapter, 2,3HD and 3,4HD reduced MTT turnover in a concentration dependant manner in both SK-N-SH and SH-SY5Y cell lines and at all exposure time points (4h, 24h and 48h). Figure 4.2a shows the concentration-response characteristics of cultures of the SK-N-SH cells, after 4h exposure to 2,3HD and 3,4HD with respect to MTT turnover. Both 2,3HD and 3,4HD caused the level of MTT turnover to fall steeply between 8-33mM, with an IC_{50} of 18.6 ± 0.4mM for 2,3HD and of 19.6 ± 0.2mM for 3,4HD. Statistical analysis of these IC_{50}s showed no significant difference between the two hexanedione isomers in the SK-N-SH line after 4h exposure ($P>0.05$). The concentration-response characteristics of cultures of the SH-SY5Y cells, after 4h exposure to 2,3HD and 3,4HD with respect to MTT turnover can be seen in figure 4.2b. With both compounds the level of MTT turnover initially increased slightly, between 2-4 mM with 2,3HD and 2-8 mM with 3,4HD. MTT turnover fell steadily between 8-41 mM, with increasing concentrations of both isomers. The IC_{50} for 2,3 HD (25.7 ± 0.5mM) was significantly higher ($P<0.001$) than that of 3,4 HD (22.2 ± 0.4mM).

Figure 4.3a shows the concentration-response characteristics of cultures of the SK-N-SH cells, after 24h exposure to 2,3HD and 3,4HD with respect to MTT turnover. Over the concentration range of 1.6-8mM, both 2,3HD and 3,4HD caused a rapid reduction in MTT turnover. The IC_{50}s determined for the isomers of 4.4 ± 0.1mM for 2,3HD and 4.2 ± 0.1mM for 3,4HD were revealed not to be significantly different ($P>0.05$). The concentration-response characteristics of cultures of the SH-SY5Y cells, after 24h exposure to 2,3HD and 3,4HD with respect to MTT turnover can be seen in figure 4.3b.
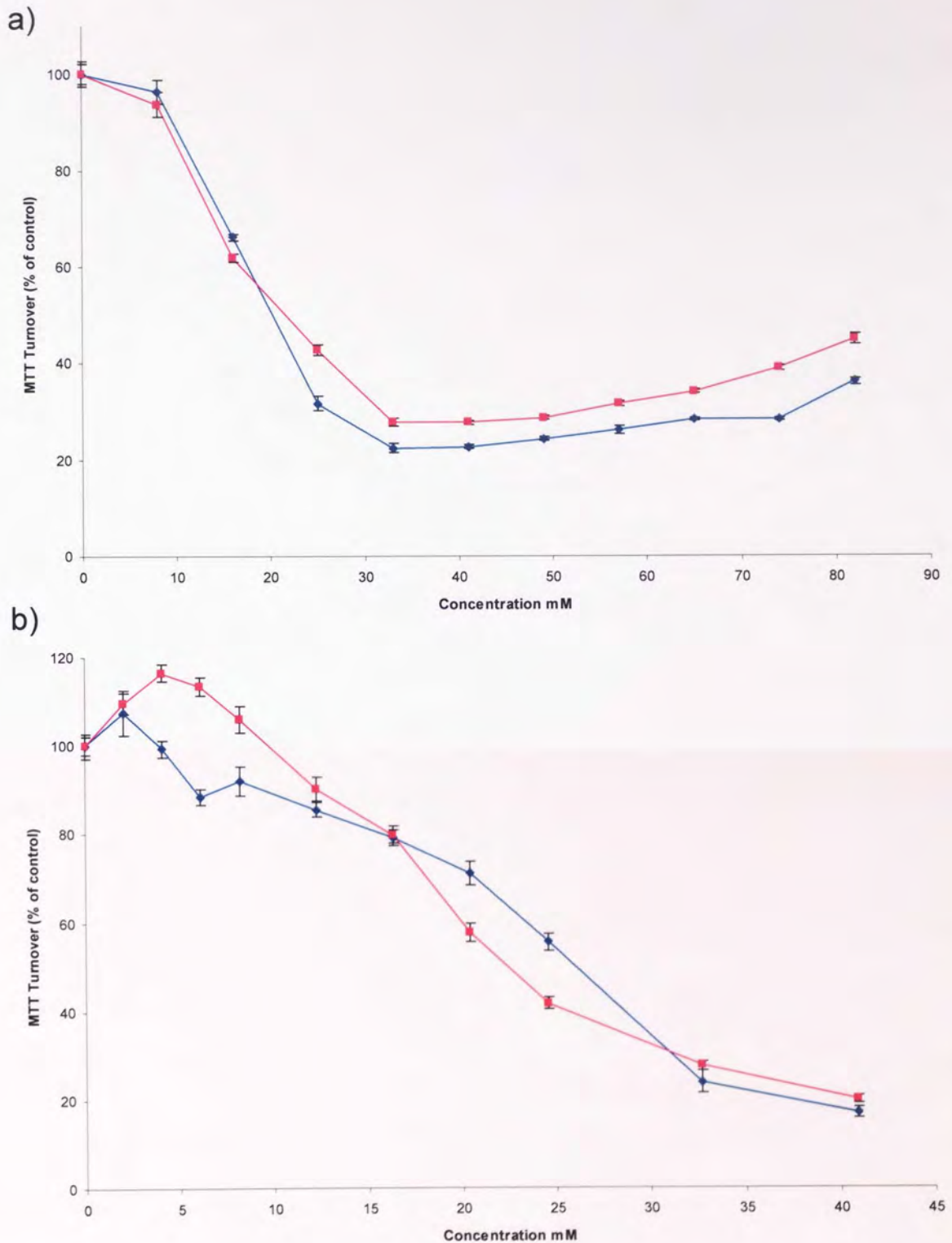
Figure 4.2: MTT assay on a) SK-N-SH and b) SH-SY5Y cells following 4h treatment with 2,3-hexanedione (•) or 3,4-hexanedione (▪). Cells were treated with increasing concentrations of 2,3-hexanedione or 3,4-hexanedione and incubated for 4h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).
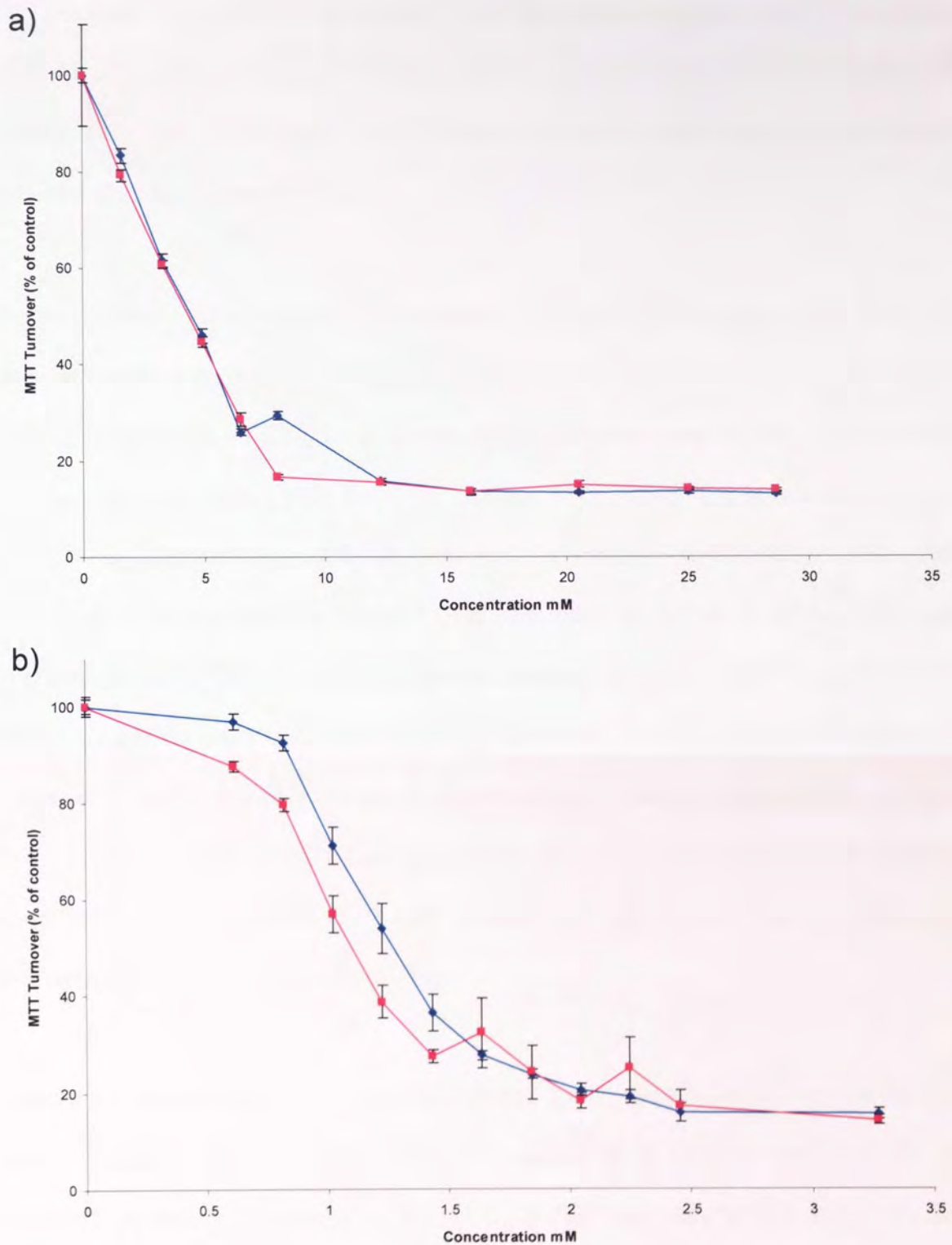
Figure 4.3: MTT assay on a) SK-N-SH and b) SH-SY5Y cells following 24h treatment with 2,3-hexanedione (•) or 3,4-hexanedione (■). Cells were treated with increasing concentrations of 2,3-hexanedione or 3,4-hexanedione and incubated for 24h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).

MTT turnover was reduced systematically with increasing concentrations of both isomers over the concentration range of 0.6-1.4 mM. IC_{50}s of 1.3±0.1mM and 1.2±0.1mM were estimated for the 2,3HD and 3,4HD isomers respectively and were not significantly different from each other (P>0.05).

Figure 4.4a shows the concentration-response characteristics of cultures of the SK-N-SH cells, after 48h exposure to 2,3HD and 3,4HD with respect to MTT turnover. With both compounds the level of MTT turnover over the concentration range of 0.8-1.6mM initially increased slightly. Both 2,3HD and 3,4HD caused MTT turnover to be reduced between 1.6-5.7mM. Calculated IC_{50}s of 3.3±0.1mM for 2,3HD and of 3.6±0.1mM for 3,4HD were shown not to be significantly different from each other in the SK-N-SH line after 48h exposure (P>0.05). The concentration-response characteristics of cultures of the SH-SY5Y cells, after 48h exposure to 2,3HD and 3,4HD with respect to MTT turnover can be seen in figure 4.4b. MTT turnover decreased slowly with increasing concentrations of both isomers between 0.16-0.98mM then fell sharply. IC_{50}s of 1.0±0.03mM and 0.9±0.03mM were estimated for 2,3HD and 3,4HD respectively; again, there was no significant difference between the isomers (P>0.05).

Table 2 is a summary table of IC_{50}s of 2,3HD and 3,4HD in both cell lines and at all time points. Statistical analysis showed that both compounds at all time points, except 4h, exhibited significantly lower IC_{50}s in the SH-SY5Y line than in the SK-N-SH line (P<0.001).

Figure 4.4: MTT assay on a) SK-N-SH and b) SH-SY5Y cells following 48h treatment with 2,3-hexanedione (•) or 3,4-hexanedione (■). Cells were treated with increasing concentrations of 2,3-hexanedione or 3,4-hexanedione and incubated for 48h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).

Table 2: Summary table of IC$_{50}$s of 2,3HD and 3,4HD in both SK-N-SH and SH-SY5Y cell lines. Results are means ± SEM (n=3).

	SK-N-SH		SH-SY5Y	
	2,3HD	3,4HD	2,3HD	3,4HD
4h	18.6±0.4mM	19.6±0.2mM	25.7±0.5mM	22.2±0.4mM
24h	4.4±0.1mM	4.2±0.1mM	1.3±0.1mM	1.2±0.1mM
48h	3.3±0.1mM	3.6±0.1mM	1.0±0.03mM	0.9±0.03mM

4.3.2 Flow Cytometric Assays

In the SK-N-SH cell line, with both 2,3HD and 3,4HD, increases in apoptosis were observed (Figure 4.5a), within their respective concentration ranges of toxicity demonstrated by the MTT studies. The increase in apoptosis initially was apparent at the same concentration of both HD isomers, 2.5mM, although the increase in the rate of apoptosis was much steeper with 3,4HD compared to 2,3HD. Between 1.6mM and 5.7mM the percentage of apoptosis caused by 3,4HD increased from 4.6±0.3% to 60.4±0.5%, a significant change of ≈56% (P<0.0001), whereas for 2,3HD over the same concentration range, the increase in apoptosis was only ≈8% from 3.3±0.5% to 10.9±1.6% (P<0.001). The percentage of cells that underwent apoptosis due to 3,4HD exposure decreased from this maximum of 60.4±0.5% to 45.2±1.3% between 6.5mM and 8.2mM (P<0.001). Exposure to the 2,3 isomer caused apoptosis to increase sharply between 5.7mM and 7.4mM towards 42.8±6.3%, where the level reached a plateau. Overall, the data sets for the two isomers were found to be significantly different from each other (P<0.01).

The changes in apoptosis in SH-SY5Y cells after exposure to both HD isomers can be seen in figure 4.5b.
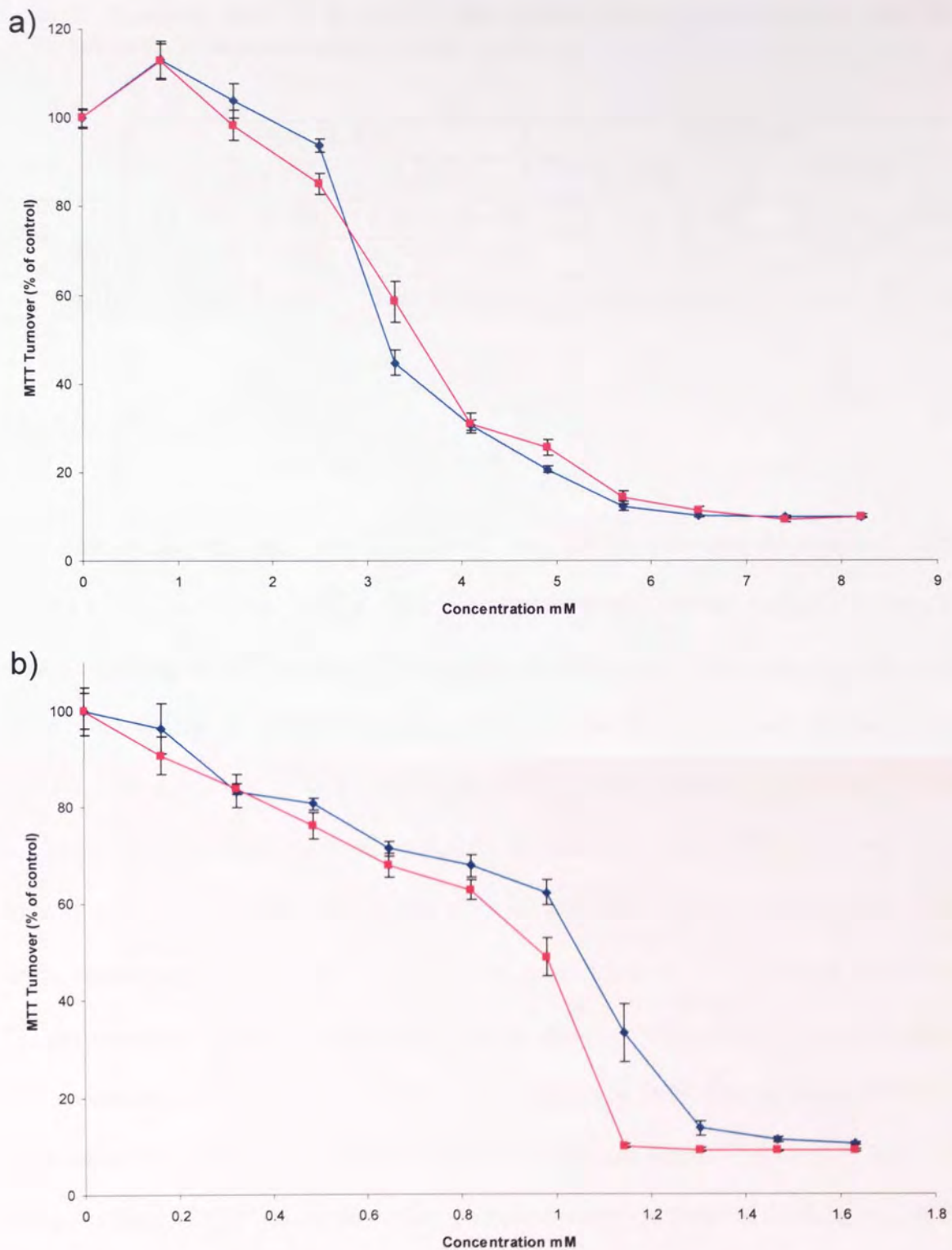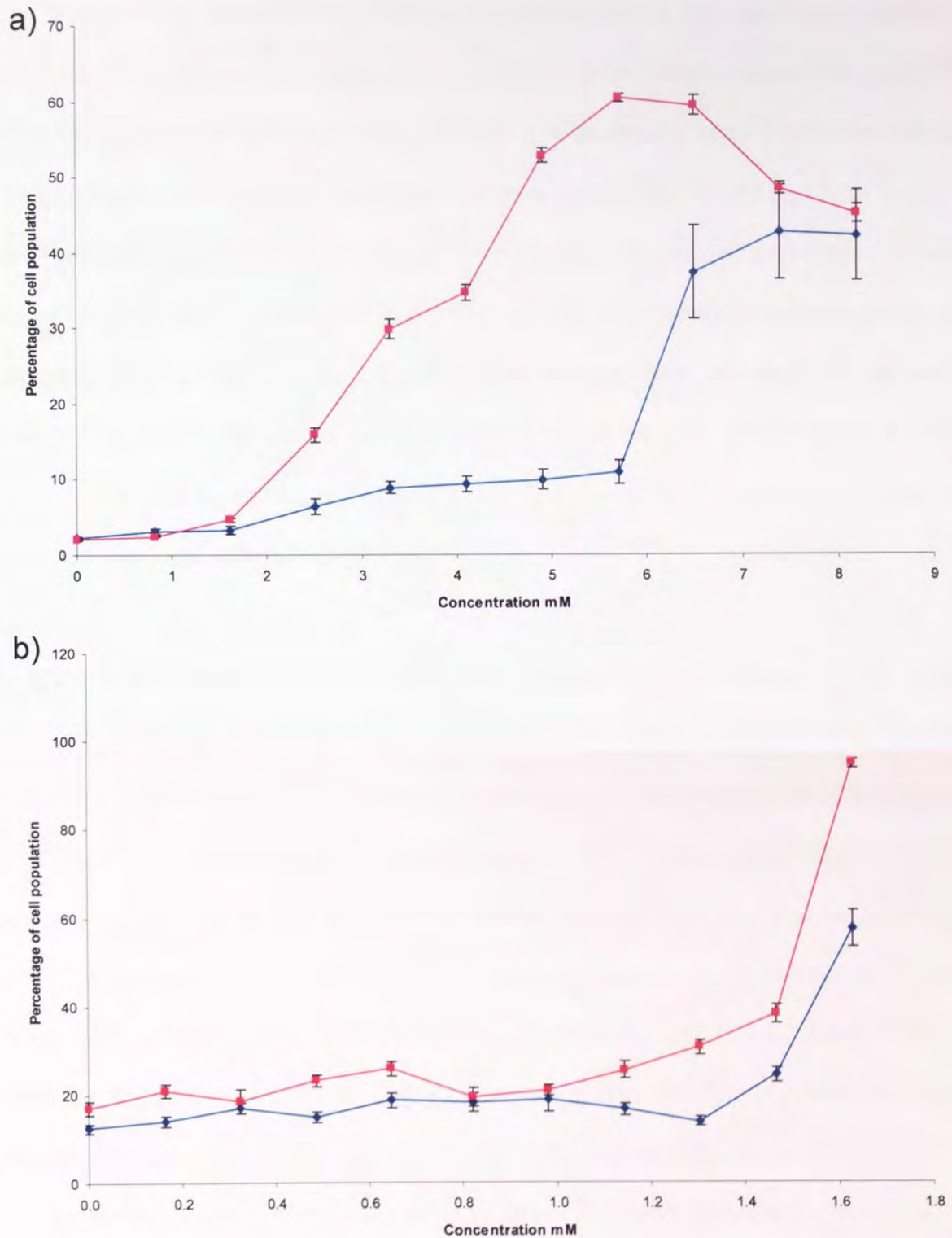
Figure 4.5: Apoptosis of a) SK-N-SH and b) SH-SY5Y cells following 48h treatment with 2,3-hexanedione (●) or 3,4-hexanedione (■). Cells were treated with increasing concentrations of 2,3-hexanedione or 3,4-hexanedione and incubated for 48h. Cells were stained with Propidium Iodide and analysed using a flow cytometer. Results are means ± SEM (n=3).

The baseline level of apoptotic nucleoids in the control groups was consistently higher in the SH-SY5Y cell line when compared to the SK-N-SH line. Initial increases in apoptosis after 3,4HD exposure were observed at 1.14mM. The percentage of apoptosis then rose at a steady rate with increasing concentrations from $25.4\pm1.9\%$ to $38.1\pm2.1\%$ at 1.5mM ($P<0.001$). Between 1.47mM and 1.63mM the percentage of apoptotic nucleoids increased dramatically to nearly 95% ($P<0.001$). With 2,3HD, significantly increased levels of apoptosis were not noted until 1.47mM, where the percentage of apoptosis increased rapidly from $24.4\pm1.7\%$ to $57.5\pm4.1\%$ ($P<0.001$). In both cell lines 3,4HD caused a more rapid increase in apoptosis, whilst in the SH-SY5Y cells the overall higher level of apoptosis compared with 2,3HD ($P<0.001$).

Along with the increase in apoptotic nucleoids, a decrease in the percentage of cells in the G_0/G_1 phase was observed with exposure to 2,3HD and 3,4HD, in both the SK-N-SH cell line (Figure 4.6a) and SH-SY5Y cell line (Figure 4.6b). In the SK-N-SH cells the pattern of this decrease in G_0/G_1 nucleoids followed closely the rise in apoptotic nucleoids for both isomers. As with the increase in apoptosis, the fall in G_0/G_1 nucleoids was much steeper with 3,4HD as compared to 2,3HD. The first significant decrease in the percentage of cells in the G_0/G_1 phase following 2,3HD exposure was between 2.5mM and 3.3mM, where it fell from $74.0\pm2.4\%$ to $62.9\pm2.0\%$ ($P<0.001$). However after this point, the level increased gradually between 3.3mM and 5.7mM to $70.6\pm2.5\%$ before falling again to its nadir of $45.2\pm5.3\%$ at 8.2mM, which is significantly lower ($P<0.001$) than the control level of G_0/G_1 nucleoids, $82.2\pm1.2\%$. The first significant fall ($P<0.001$) in the percentage of cells in the G_0/G_1 phase following 3,4HD exposure, compared to the control group, occurred at a slightly lower concentration, between 1.6mM and 2.5mM. The percentage of G_0/G_1 nucleoids fell by $\approx15\%$ from $76.2\pm0.5\%$ to $61.8\pm1.0\%$.
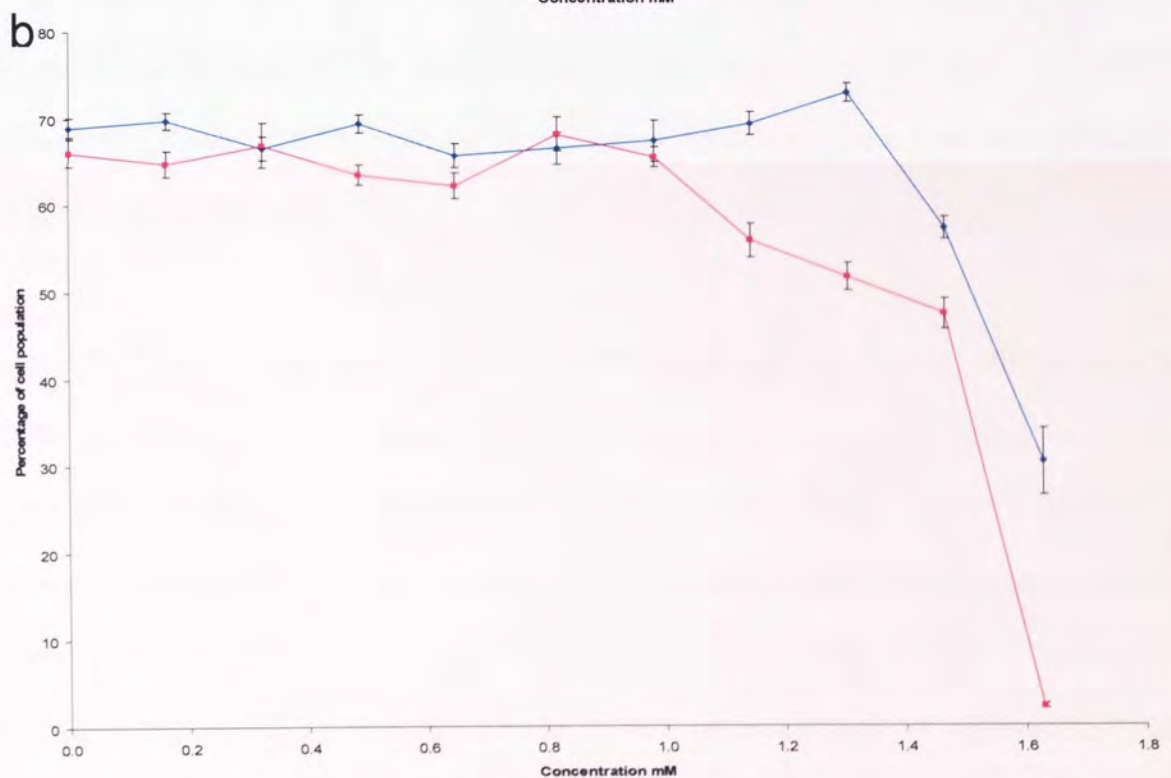
Figure 4.6: G_0/G_1 phase of flow cytometric analysis of a) SK-N-SH and b) SH-SY5Y cells following 48h treatment with 2,3-hexanedione (•) or 3,4-hexanedione (■). Cells were treated with increasing concentrations of 2,3-hexanedione or 3,4-hexanedione and incubated for 48h. Cells were stained with propidium iodide and analysed using a flow cytometer. Results are means ± SEM (n=3).

The lowest percentage of G_0/G_1 nucleoids, $26.4\pm0.9\%$, was observed at 6.5mM, whilst the value rose again to $35.0\pm1.0\%$ (P<0.001) at 8.2mM, which coincided with the peak and fall in apoptosis at these concentrations of 3,4HD mentioned earlier. Significant changes in the percentage of G_0/G_1 nucleoids in the SH-SY5Y cells were not seen until the higher concentration range (1.14mM - 1.63mM) of both 2,3HD and 3,4HD. Initial exposure of the cells to both isomers did not result in significant changes with respect to the control groups. Between 1.31mM and 1.63mM, 2,3HD caused a significant drop (P<0.001) in G_0/G_1 nucleoids from $72.9\pm1.0\%$ to $30.4\pm3.9\%$, which corresponds directly to the rise in apoptosis. With 3,4HD, the first significant decrease (P<0.01) in G_0/G_1 nucleoids occurred between 0.98mM and 1.14mM, with a decrease of $\approx10\%$ from $65.5\pm1.2\%$ to $55.9\pm2.0\%$. This latter value then fell at a steady rate with increasing concentration, reaching $47.4\pm1.8\%$ at 1.47mM, before abruptly falling significantly (P<0.001) to just $2.3\pm0.3\%$ following 1.63mM 3,4HD exposure; these values again coincided with the substantial increase seen in apoptosis.

In the SK-N-SH cell line, both 2,3HD and 3,4HD caused a similar pattern in the changes in G_2/M nucleoids, with both isomers causing an initial increase before falling to a level below that of the controls at the higher end of the concentration range (Figure 4.7a). 2,3HD caused a gradual but significant increase (P<0.01) in G_2/M nucleoids, reaching a peak at 3.30mM of $16.1\pm0.4\%$. This is significantly lower (P<0.01) than the peak caused by 4.1mM 3,4HD of $22.3\pm0.6\%$. At 7.4mM 2,3HD the percentage of cells with G_2/M nucleoids had fallen to $4.1\pm0.5\%$ significantly lower (P<0.001) than in the control cells. This was also the case with 6.5mM 3,4HD where the percentage of G_2/M nucleoids was $4.4\pm0.2\%$.

As can be seen in Figure 4.7b, both 2,3HD and 3,4HD caused an elevation in G_2/M nucleoids followed by a sharp decrease, which was in broad agreement with the results observed in the SK-N-SH line.

There was no effect with either isomer on this phase of the cell cycle at the lower end of the concentration range (0.16-0.82mM for 3,4HD and 0.16-1.31mM for 2,3HD). At the concentration range beginning with 1.14mM, with 3,4HD, the percentage of cells in the G_2/M phase rose significantly (P<0.001) to a peak of 11.9±0.3% before falling to 0.7±0.2% at 1.63mM, significantly lower than in the controls (P<0.001). After 1.47mM 2,3HD, the percentage of G_2/M nucleoids increased significantly (P<0.001) to a peak of 10.7±0.5% before falling to 7.6±0.4% at the highest concentration used, 1.63mM, still significantly higher than in the controls (P<0.01).

Figure 4.8 shows the effects of 2,3HD and 3,4HD on the S-phase of the cell cycle in (a) SK-N-SH cells and (b) SH-SY5Y cells. In the SK-N-SH line the percentage of cells of S-phase nucleoids was almost double that of the controls at 3.3mM 2,3HD, increasing from from 7.0±0.6% to 12.3±1.1% (P<0.001). This percentage then fell with increasing concentrations of 2,3HD back to approximately control levels at 6.5mM. 3,4HD had the opposite effect and the percentage of S-phase nucleoids fell to below (P<0.001) that of the controls to 7.9±0.2%. This level was maintained with increasing concentrations, until 6.5mM 3,4HD, where the S-phase nucleoid percentage gradually increased to levels seen in the controls.

As can be seen in figure 4.8, both HD isomers caused a similar, gradual but significant (P<0.01) decrease in S-phase nucleoids with increasing concentrations up to 0.98mM.

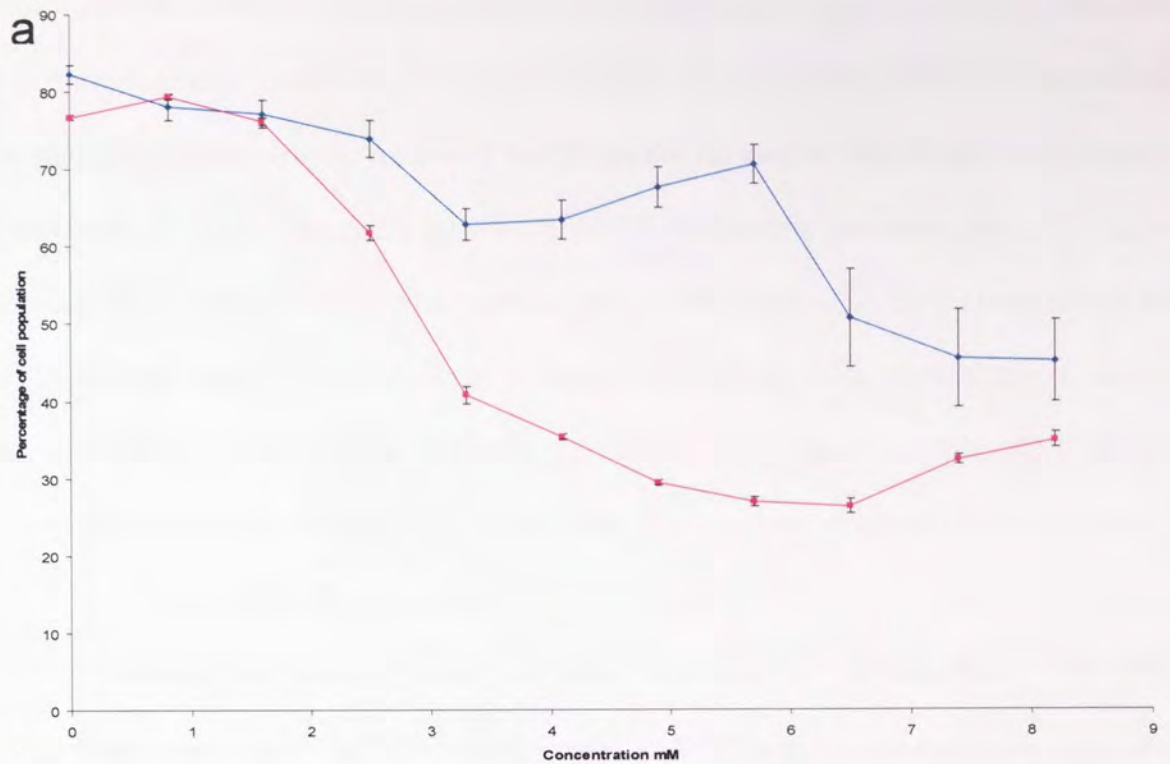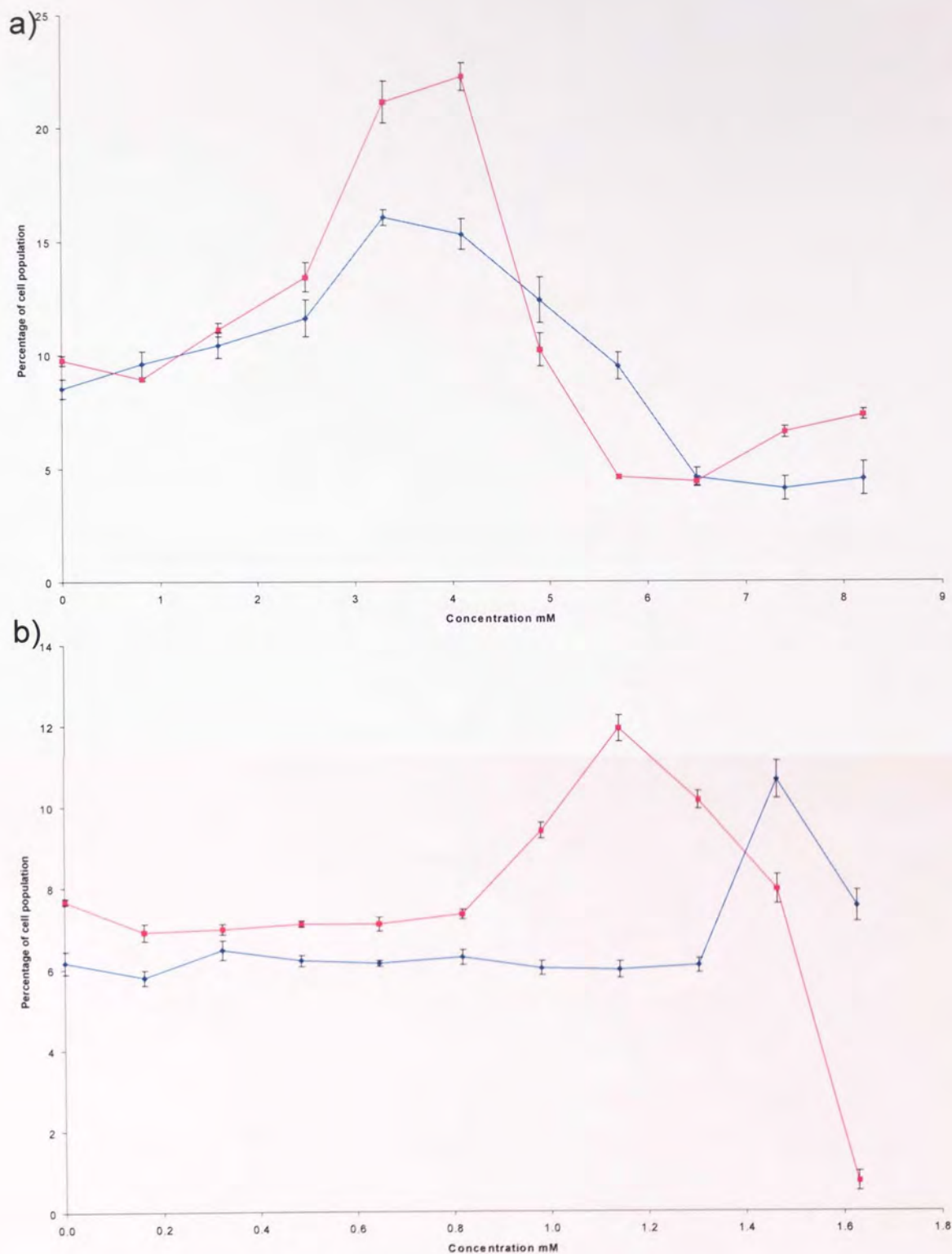**Figure 4.7: G$_2$/M phase of flow cytometric analysis of a) SK-N-SH and b) SH-SY5Y cells following 48h treatment with 2,3-hexanedione (●) or 3,4-hexanedione (■). Cells were treated with increasing concentrations of 2,3-hexanedione or 3,4-hexanedione and incubated for 48h. Cells were stained with Propidium Iodide and analysed using a flow cytometer. Results are means ± SEM (n=3).**
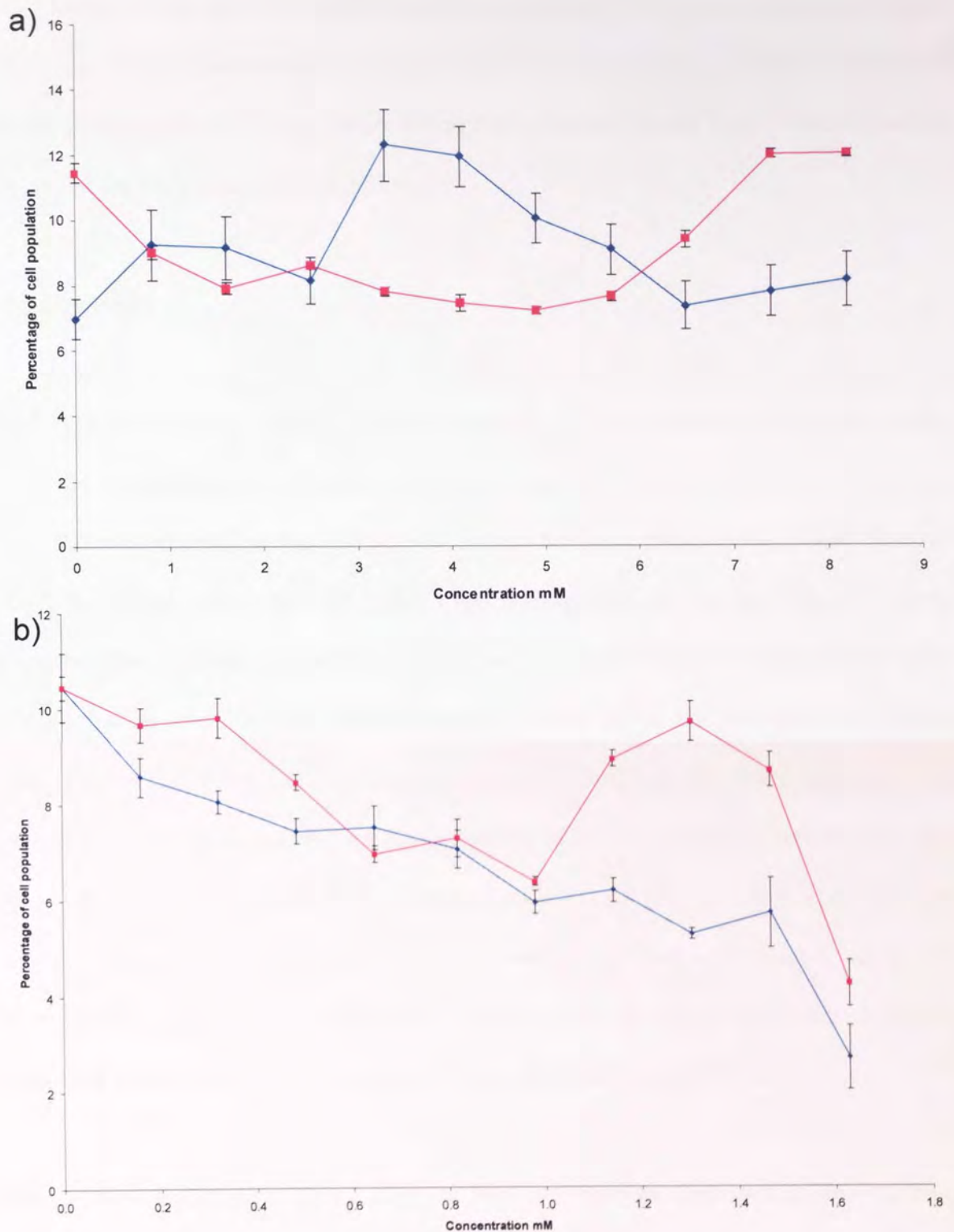
Figure 4.8: S phases of flow cytometric analysis of a) SK-N-SH and b) SH-SY5Y cells following 48h treatment with 2,3-hexanedione (●) or 3,4-hexanedione (■). Cells were treated with increasing concentrations of 2,3-hexanedione or 3,4-hexanedione and incubated for 48h. Cells were stained with Propidium Iodide and analysed using a flow cytometer. Results are means ± SEM (n=3). The data in graph b) was normalised by a factor of 0.021.

Increasing concentrations of 2,3HD from this point caused a continued decrease in S-phase nucleoids, whereas with 3,4HD the percentage of S-phase nucleoids returned to levels seen in the control cells at 1.31mM before falling back to a comparable level to that caused by 2,3HD at the highest concentration, 1.6mM.

4.4 Discussion

2,3HD and 3,4HD are both α diketone isomers of 2,5-hexanedione, the only proven neurotoxic metabolite of n-Hexane *in vivo*. Although the α diketone isomers (2,3HD and 3,4HD) have been defined as non-neurotoxic and non-toxic (FAO/WHO, 1999; Stone *et al.*, 1999; 2000), relatively little research has been carried out into the toxicity of these isomers. The decision to term these agents as non-toxic has rested mainly on studies carried out *in vivo*, in rats (2,3HD; Spencer *et al.*, 1978) and *in vitro*, in isolated mitochondria (2,3HD and 3,4HD; Stipani *et al.*, 1996), although they have been shown to be less benign in other models such as various bacterial assays (2,3HD; Zimmermann and Mohr, 1992; Chen and Hee, 1995; 3,4HD; Dorado *et al.*, 1992; Mellado and Montoya, 1994). However, to the author's knowledge, no studies have been carried out in human cell lines. In this chapter the acute and sub chronic toxicity of 2,3HD and 3,4HD has been examined in the human neuroblastoma cell lines SK-N-SH and SH-SY5Y.

2,3HD and 3,4HD reduced MTT turnover in a concentration dependent manner in both SK-N-SH and SH-SY5Y cell lines and at all exposure time points. The $IC_{50}s$ observed for both isomers were between 6 and 40 fold lower than seen for 2,5HD in the previous chapter at specific time points. As observed with 2,5HD in the previous chapter, there was a small, but significant, increase in MTT turnover, above that of the controls, at lower

concentrations of 2,3HD and 3,4HD, which could be explained by an increase in succinate dehydrogenase activity in response to low level exposure of toxins (Putnam et $al.$, 2002). Although the IC_{50} values for 2,3HD and 3,4HD are considerably lower than for 2,5HD they are relatively high (in the mM range), however a degree of cytotoxicity was shown in this study which was not observed previously in $vivo$ (FAO/WHO, 1999; Stone et $al.$, 1999; 2000). A possible explanation for this could be related to the absorbtion, distribution, metabolism and excreation characteristics of these compounds, in a whole body situation 2,3HD and 3,4HD may be metabolised and excreated before they are able to exert any toxicity.

Apart from at 4h exposure in the SH-SY5Y line, there was no significant difference between the IC_{50} values for 2,3HD and 3,4HD within each cell line observed with the MTT assay. The difference between the hexanedione isomers in the SH-SY5Y cells at 4h was approximately 10%, although there were no significant differences in IC_{50}s between the two isomers observed at the other time points or in the SK-N-SH cell line. It was apparent that aside from the 4h SH-SY5Y data, there was little overall difference between the data sets. It is also difficult to account for the differences at 4 h in the SH-SY5Y line, the causes of which remain unknown.

At 24h and 48h both the 2,3HD and 3,4HD isomers showed significantly lower IC_{50}s in the SH-SY5Y cell line when compared to the SK-N-SH line. This difference was extremely significant and equates to approximately 4 fold. The SK-N-SH cell line is a heterogeneous line, consisting of N, S and I cell types, whereas the thrice cloned sub line SH-SY5Y line is homogeneous, which may account for this significant difference observed in the sensitivity to the two hexanedione isomers between the two cell lines. The SH-SY5Y cells would

have displayed a common sensitivity to the toxins, whilst the heterogeneous nature of the SK-N-SH cells would cause each sub population of cells to display differential sensitivities to the toxins.

The graphical representation of the data from the MTT assays underlines the similarity in response in terms of cell death between the isomers and the two cell lines. The MTT assay was essentially unable to distinguish between the respective isomers' cytotoxic actions. However, by contrast, when the various phases of the cell cycle in the presence of the isomers were examined with flow cytometry, there were some noticeable differences between the effects of the two agents.

As previously stated both 2,3HD and 3,4HD are known to inhibit the inner mitochondrial membrane transport protein, the oxoglutarate carrier (OGC) (Stipani et al., 1996). This may provide a possible mechanism of cytotoxicity. From the flow cytometry data it is clear that both HD isomers caused apoptosis, or apoptosis-like effects, as the DNA was fragmented and this led to a peak to the left of the G_0/G_1 peak on the flow cytometry histogram. The OGC catalyzes the counter exchange of 2-oxoglutarate and malate (Stipani et al., 1996), and there is also evidence for the mitochondrial uptake of glutathione by the OGC (Chen and Lash, 1998; Lash, 2006). The OGC transport system is essential for several metabolic processes including gluconeogenesis. The production of glucose via gluconeogenesis is important for the production of ATP. Therefore inhibition of the OGC by 2,3HD and 3,4HD could lead to a decrease in cellular ATP. Although ATP is a requirement for caspase dependent apoptosis, decreased levels of ATP are sufficient to cause release of apoptosis inducing factor (AIF) from the mitochondria (Daugas et al., 2000). AIF is a mitochondrial flavoprotein that is set in the inner mitochondrial membrane

(Otera et al., 2005; Uren et al., 2005). Under cell stress conditions, such as ATP depletion, AIF is cleaved from the inner mitochondrial membrane through the activation of cathepsins and calpains (Yuste et al., 2005). Once cleaved and released into the cytosol, AIF is translocated to the nucleus and then acts directly on DNA within the nucleus leading to large-scale DNA fragmentation (Cande et al., 2002). This is a caspase-independent apoptosis-like cell death. Both the 2,3HD and 3,4HD isomers inhibit the OGC to a similar extent and if their mechanism of action is as described above, the difference between the two isomers in the concentrations leading to the initial rise in apoptosis and the difference in highest level of apoptosis observed may be linked with differences in the respective lipophilicities of 2,3HD and 3,4HD. The 3,4HD isomer in both cell lines required lower concentrations than 2,3HD to cause the initial rise in apoptosis and a higher percentage of apoptosis was observed with the 3,4 as opposed to the 2,3HD isomer. The 3,4 isomer is much more lipophilic (log P = 0.236) than 2,3HD (log P = 0.004). This would suggest that 3,4HD may have been taken up into the cells more rapidly than 2,3HD, thus accounting for these differences seen with flow cytometry at 48h exposure.

Differences in the effect of 2,3HD and 3,4HD on the G_2/M and S phases of the cell cycle were also observed with flow cytometry. In general, the percentage of G_2/M and S phase arrest was highest with exposure to 3,4HD. S and G_2/M phase arrest is symptomatic of inhibition of the cell cycle at the stages of DNA replication and chromosome segregation, respectively (Yuan et al., 2005). The 3,4HD isomer has been shown to be slightly mutagenic with the Ames test (Dorado et al., 1992; Mellado & Montoya, 1994) which could cause a cell to enter the early stages of apoptosis during DNA replication, hence, this might account for the rise in S and G_2/M phase arrest seen in this chapter.

In summary, the toxic effects of 2,3HD and 3,4HD were evaluated in the neuroblastoma cell lines SK-N-SH and SH-SY5Y. 2,3HD and 3,4HD caused concentration dependent reductions in MTT turnover in both cell lines at all time points, with the SH-SY5Y line ≈4 fold more sensitive at 24h and 48h exposure. The pattern in toxicity between the two HD isomers in both cell lines was very similar. The use of flow cytometry drew attention to several differences between the toxic responses of both cell lines to 2,3HD and 3,4HD that are not apparent with the basic MTT assay. 3,4HD caused an initial rise in apoptosis at a lower concentration than with 2,3HD and the highest level of apoptosis observed was also with 3,4HD. A possible mechanism of toxicity was suggested for the two α HD isomers involving the inhibition of the OGC resulting in the release of AIF and an apoptosis-like cell death.

Chapter 5. Acute toxicity of 2,5-, 2,3- and 3,4-Hexanedione in human neuroblastoma and non-neuronal cell lines

5.1 Introduction

The previous chapters have established the profile of toxicity of the 2,3HD and 3,4HD isomers in neuroblastoma lines, which are commonly used to model neurotoxicity. It is important to provide a preliminary assessment as to whether these agents are able to demonstrate any degree of specific neurotoxicity, such as in the case of 2,5HD (Spencer and Schaumburg, 1975; Governa et al., 1987; Perbellini et al., 1990; Heijink et al., 1995; Graham, 1999; Heijink et al., 2000). One possible method for indentifying any specific neurotoxicity would be to investigate specific neurotoxic endpoints such as, neurofilament cross-linking. However, without knowing the mechanism of toxicity of 2,3HD and 3,4HD selecting appropriate specific neurotoxic endpoints is very difficult. Therefore, examination of the effects of 2,3 and 3,4 HD isomers in non-neuronal cell lines may place their cytotoxicity in the general context of some 'mainstream' cell lines. Although there is a wide variety of transformed cell lines derived from many human tissues, three cell lines were chosen for this chapter that are well established examples of their tissue type.

The MCF7 line comprises human breast adenocarcinoma cells, established from a pleural effusion of a 69-year-old Caucasian woman. Growing *in vitro* as monolayers, their morphology is epithelial-like and they exhibit some features of differentiated mammary epithelial cells, such as oestradiol synthesis and dome formation. They are used as a model of breast epithelial tissue and are well documented as toxicity test models by other groups (Schlumpf et al., 2001; Kaur et al., 2005; Vanparys et al., 2006).

The HepG2 cell line is long established and is one of the most commonly used liver cell lines. They are human hepatocyte carcinoma cells that were isolated from the liver biopsy of a 15-year-old Caucasian boy suffering from a well differentiated hepatocellular carcinoma. The morphology of the HepG2 line is epithelial and they are again widely used as human hepatotoxicity models (Falk *et al.*, 1995; Rueff *et al.*, 1996; Morgan et al., 2002).

The final non-neuronal cell line selected for use in this chapter was the CaCo-2 line which originated from a colon adenocarcinoma. This line is again morphologically epithelial and it has attained wide application in studies of human gut function. Utilising the explant culture technique, this line was originally isolated from a primary colonic adenocarcinoma in a Caucasian male aged 72. As with the other two cell lines CaCo-2 cells have been extensively used in toxicity studies for many years (De Angelis *et al.*, 1994; Duncan & Izzo, 2005; Pohjala *et al.*, 2007).

In this chapter the acute toxicity of the known neurotoxin 2,5HD and its closely related isomers 2,3HD and 3,4HD were evaluated in the non-neuronal cell lines MCF7, HepG2 and CaCo-2.

5.2 Methods

All cell culture was carried out as described in chapter 2. All hexanedione solutions were made up in the corresponding media for the cell type being assayed. At the concentrations used all hexanedione isomers were readily soluble at 37°C.

5.2.1 MTT Assays

Following calibration assays to decide the optimum seeding density, all cell lines were seeded into 96 well plates at $\approx 5 \times 10^4$ cells/well. The experiments were then carried out as described in section 2.3 with 2,5HD, 2,3HD and 3,4HD.

5.3 Results

5.3.1 MTT Assays

2,5HD reduced MTT turnover in a concentration dependant manner in MCF7, HepG2 and CaCo-2 cell lines at all exposure time points (4h, 24h and 48h). Figure 5.1 shows the inhibition of MTT turnover caused by 2,5HD in MCF7, HepG2 and CaCo-2 cell lines following a) 4h, b) 24h and c) 48h exposure. At 4h exposure all three cell lines displayed relatively high resistance to the effects of 2,5HD with a very gradual decrease in MTT turnover with increasing concentration. The MCF7 line was the most sensitive and at the highest concentration of 2,5HD used MTT turnover was reduced to 15.1% compared to 45.5% and 60.1% for the CaCo-2 and HepG2 lines respectively. Sensitivity to 2,5HD in all three cell lines increased slightly over 24h exposure. As observed at 4h MTT turnover decreased gradually with increasing concentration in all cell lines, except for an initial sharp fall in the HepG2 line. The HepG2 cell line went from the least sensitive at 4h exposure to the most sensitive at 24h exposure. After 48h exposure to 2,5HD an initial increase in MTT turnover was observed at the lowest concentration in both the HepG2 and MCF7 lines, this was also seen in the neuroblastoma lines. MTT turnover decreased in a gradual fashion in the MCF7 cells with increasing concentration, whereas the fall in MTT turnover was considerably sharper in the CaCo-2 and HepG2 cell lines.

A comparison of the IC_{50}s of 2,5HD for the SK-N-SH, SH-SY5Y, MCF7, HepG2 and CaCo-2 cell lines can be seen in figure 5.2. At 4h exposure the SH-SY5Y line was the most sensitive to 2,5HD with the lowest IC_{50} of 156.9 ± 2.5mM, whilst all the other cell lines had significantly higher IC_{50} values ($P<0.001$). The IC_{50} for the MCF7 line (323.3 ± 10.7mM) was not significantly higher ($P>0.05$) than that of the SK-N-SH line (303.6 ± 2.9mM) whereas the value seen for the CaCo-2 cells of 392.7 ± 13.0mM was significantly higher ($P<0.001$). A true IC_{50} value was not estimated for the HepG2 cell line as MTT turnover was not inhibited to below 50% within the concentrations used (0 - 428mM) and therefore the IC_{50} is >428mM and represented on the graph as 428mM.

The SH-SY5Y cells were also the most sensitive to 2,5HD at 24h exposure with an IC_{50} of just 48.2 ± 1.0mM. In fact, all five lines showed increased sensitivity to 2,5HD at 24h. However, all three non-neuronal cell lines had significantly higher IC_{50}s than the two neuronal-like lines ($P<0.001$). The MCF7 line, which was the most sensitive of the three non-neuronal lines at 4h exposure, was the least sensitive at 24h with an IC_{50} of 234.5 ± 2.4mM. The HepG2 line went from being the least sensitive at 4h, not reaching 50% inhibition, to the most sensitive of the three non-neuronal lines at 24h with an IC_{50} of 94.0 ± 4.4mM, less than half that of the MCF7 line but twice that of the SH-SY5Y line. At 48h, all five cell lines showed a steep increase in sensitivity to 2,5HD. The CaCo-2 were the most sensitive (IC_{50} 12.7 ± 0.2mM), almost twice as sensitive as the SK-N-SH, SH-SY5Y and MCF7 lines (IC_{50}; 22.1 ± 0.2mM, 20.3 ± 0.3mM and 24.6 ± 1.0mM respectively). The values for the SK-N-SH and SH-SY5Y cells were not significantly different from each other ($P>0.05$), the IC_{50} for the MCF7 line was significantly higher than the SK-N-SH cells ($P<0.05$) and the SH-SY5Y cells ($P<0.001$). Both the HepG2 and CaCo-2 lines had significantly lower IC_{50}s compared to the neuroblastoma lines ($P<0.05$).

Figure 5.1: MTT assay on MCF7 (●), HepG2 (■) and CaCo-2 (▲) cells following a) 4h, b) 24h and c) 48h exposure to 2,5-hexanedione. Cells were treated with increasing concentrations of 2,5-hexanedione and incubated for 4, 24, or 48h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).

Figure 5.2: Bar chart of IC$_{50}$ values calculated from MTT assays of SK-N-SH (■), SH-SY5Y (■), MCF7 (■), HepG2 (■) and CaCo-2 (■) cell lines following 4, 24 and 48h exposure to 2,5-hexanedione. Values are means ± SEM (n=3).

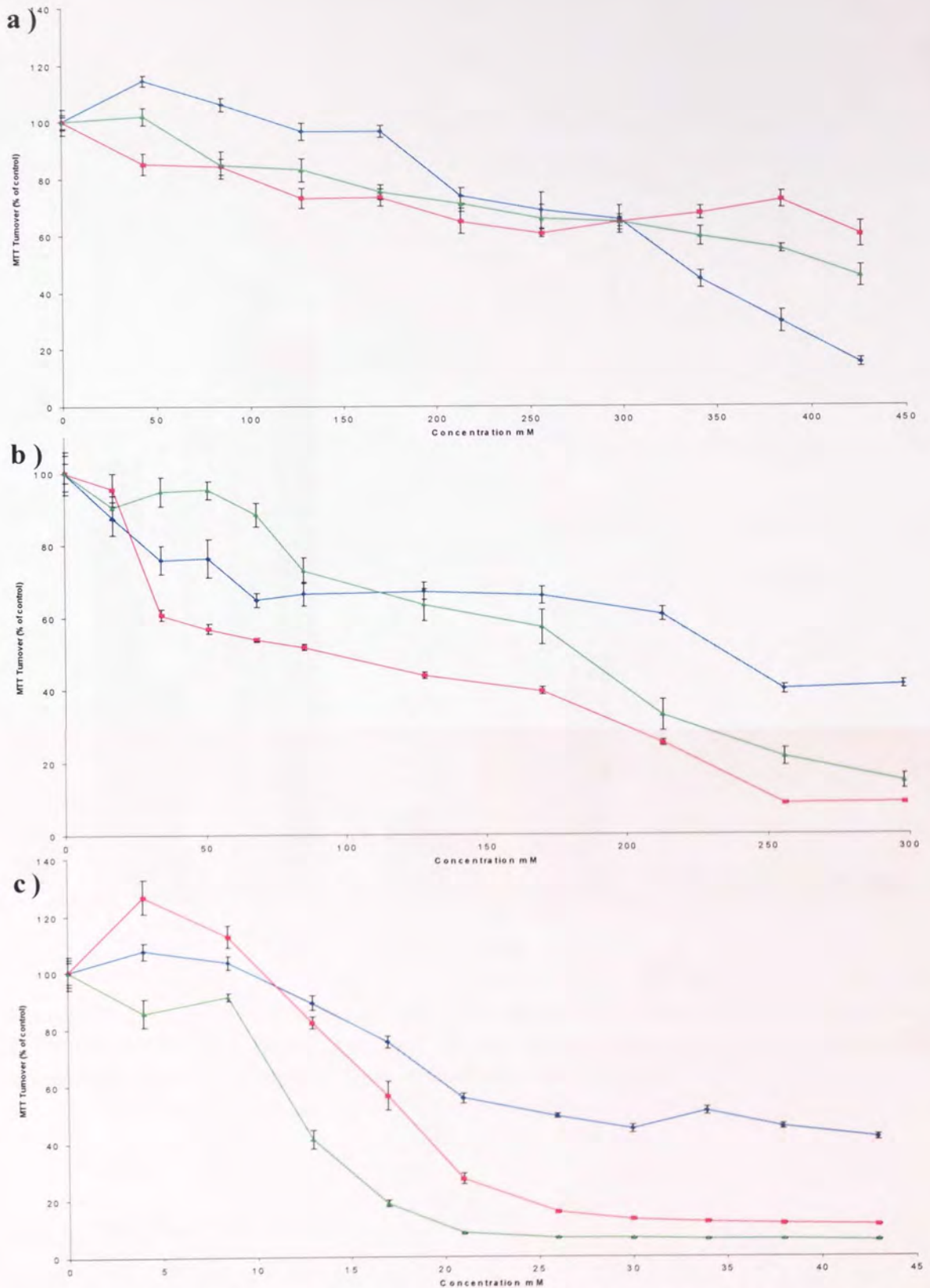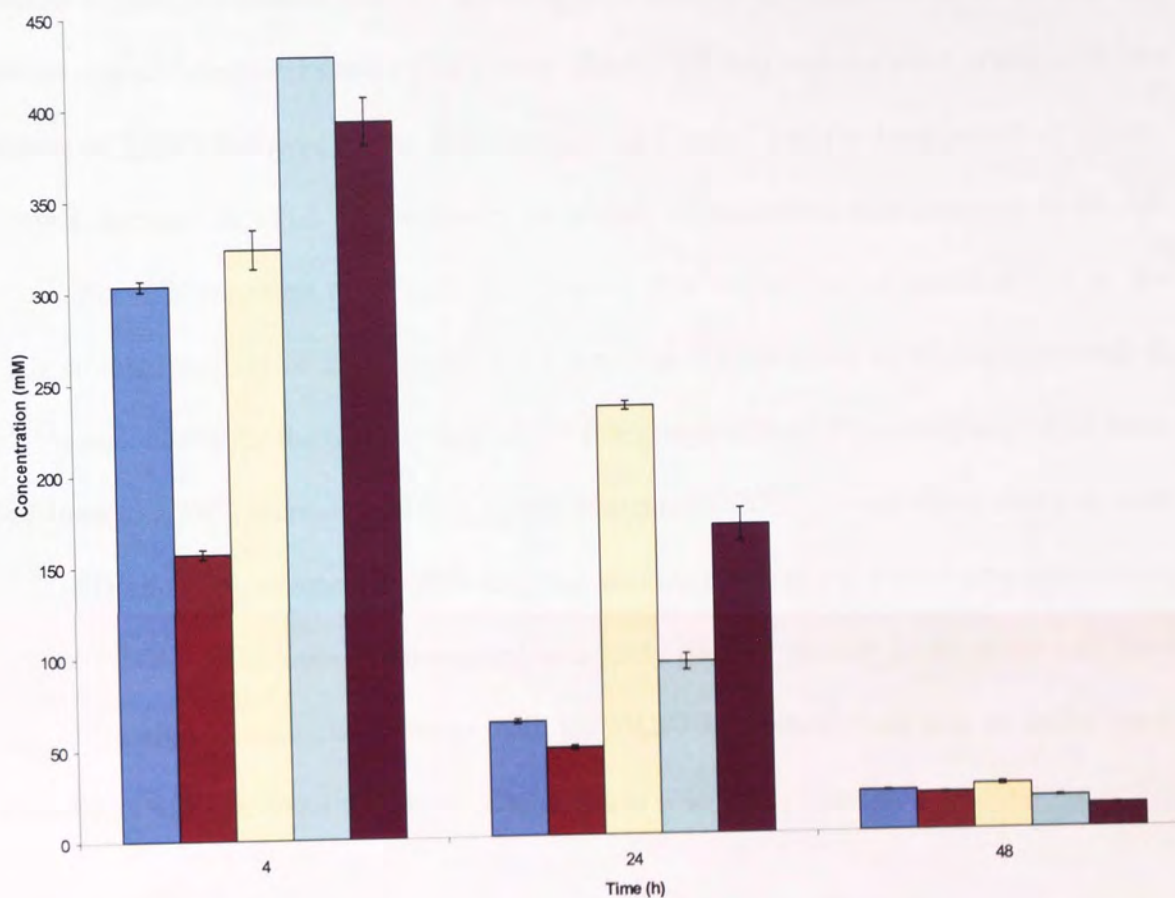The concentration-response characteristics of cultures of the MCF7, HepG2 and CaCo-2 cells following a) 4h b) 24h and c) 48h exposure to 2,3HD can be seen in figure 5.3. 2,3HD reduced MTT turnover in a concentration dependant manner in MCF7, HepG2 and CaCo-2 cell lines and at all exposure time points (4h, 24h and 48h). At 4h exposure 2,3HD initially caused a gradual decrease in MTT turnover with increasing concentration in all cell line before a much sharper decrease in all lines. The MCF7 line was the most sensitive to the effects of 2,3HD followed by the HepG2s with the CaCo-2 line the least sensitive. A very gradual decrease in MTT turnover with increasing concentration was observed in all cell lines after 24h exposure to 2,3HD. The HepG2 line was the most sensitive and at the highest concentration of 2,3HD used MTT turnover was reduced to 15.0% compared to 23.1% and 42.5% for the CaCo-2 and MCF7 lines respectively. The sensitivity of all three cell lines to 2,3HD increased 10 fold at 48h compared to 4h exposure. After 48h exposure to 2,3HD an initial increase in MTT turnover was observed at the lowest concentration in the MCF7 line. MTT turnover decreased in a fairly gradual fashion in all of the cell lines with increasing concentration, except with the MCF7 line where there was an initial steep decrease in MTT turnover to around 50% and then reached a plateaux.

Figure 5.4 shows a comparison of the IC_{50}s of 2,3HD for the SK-N-SH, SH-SY5Y, MCF7, HepG2 and CaCo-2 cell lines. 2,3HD was significantly more potent in all cell lines than 2,5HD ($P<0.001$). After 4h exposure, the lowest IC_{50} for 2,3 of 18.6±0.4mM was observed in the SK-N-SH cell line, which was significantly lower than all other cell lines at this time point ($P<0.001$). The CaCo-2 cell line was the least sensitive line to the effects of 2,3HD at 4h (IC_{50}; 42.2±1.3mM), the HepG2 line was significantly more sensitive than the CaCo-2 line ($P<0.001$) with an IC_{50} of 35.1±0.6mM and the MCF7 line more sensitive still ($P<0.001$) 26.3±1.2mM.

Figure 5.3: MTT assay on MCF7 (●), HepG2 (■) and CaCo-2 (▲) cells following a) 4h, b) 24h and c) 48h exposure to 2,3-hexanedione. Cells were treated with increasing concentrations of 2,3-hexanedione and incubated for 4, 24, or 48h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).
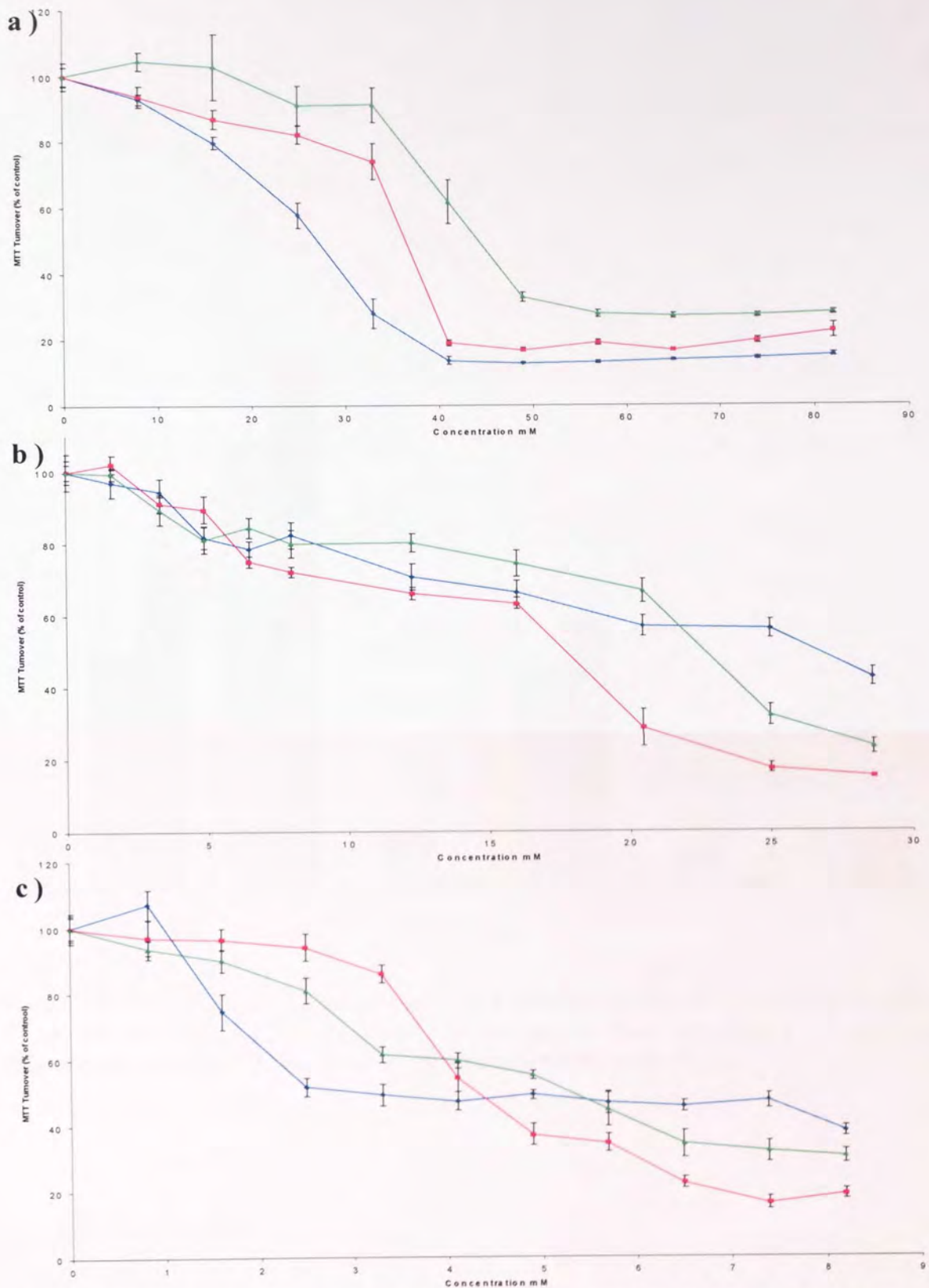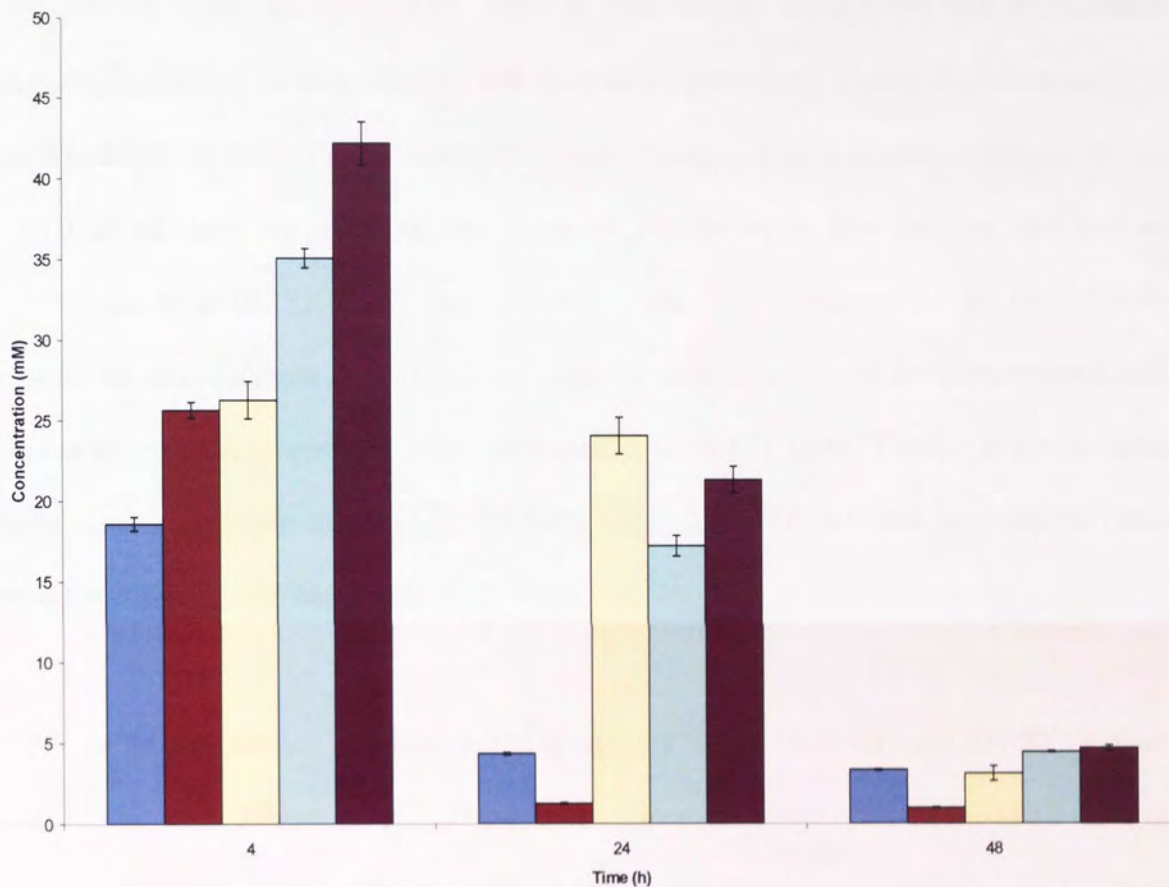
Figure 5.4: Bar chart of IC$_{50}$ values calculated from MTT assays of SK-N-SH (■), SH-SY5Y (■), MCF7 (■), HepG2 (■) and CaCo-2 (■) cell lines following 4, 24 and 48h exposure to 2,3-hexanedione. Values are means ± SEM (n=3).

The IC_{50} value observed in the SH-SY5Y line was 25.7±0.5mM which was not significantly different to that of the MCF7 line (P>0.05).

A steep increase in the sensitivity to the effects of 2,3HD on MTT turnover, was seen in both the SK-N-SH and SH-SY5Y lines at 24h (IC_{50}s; 4.4±0.1mM and 1.3±0.1mM respectively). All three non-neuronal cell lines had significantly higher IC_{50}s compared to the SK-N-SH and SH-SY5Y lines (P<0.001). There was an increase in sensitivity to 2,3HD in all three non-neuronal cell lines at 24h, however this increase was not as pronounced as in the SK-N-SH and SH-SY5Y cells. The sensitivity of the MCF7 cells showed the least increase (P>0.05) going from the most sensitive of the non-neuronal cell lines at 4h to the least sensitive at 24h with an IC_{50} of 24.1±1.1mM. The CaCo-2 cells were slightly more sensitive than the MCF7 cells (IC_{50}: 21.4±0.8mM) and the HepG2 more sensitive still (IC_{50}: 17.2±0.6mM)

Although little difference was seen in the sensitivity of the SK-N-SH and SH-SY5Y lines to 2,3HD between 24h and 48h, there was a steep increase in the sensitivities of all three of the non-neuronal lines with between 4 and 8 fold difference in IC_{50}s for 2,3HD between 24h and 48h. Again the MCF7 cells were the most sensitive with an IC_{50} of 3.1±0.5mM then the CaCo-2 cells and HepG2 cells were not significantly different from each other (P>0.05) with IC_{50} values of 4.7±0.2mM and 4.5±0.1mM respectively.

Figure 5.5 shows the concentration-response characteristics of cultures of the MCF7, HepG2 and CaCo-2 cells following a) 4h b) 24h and c) 48h exposure to 3,4HD. MTT turnover was reduced in a concentration dependant manner in MCF7, HepG2 and CaCo-2 cell lines and at all exposure time points (4h, 24h and 48h). After 4h exposure to 3,4HD

MTT turnover decreased with increasing concentration in all cell lines with the MCF7 cells the most sensitive line with the HepG2 line the next sensitive and the CaCo-2 cells the least sensitive. At 24h exposure the MCF7 cells went from being the most sensitive at 4h to the least sensitive to 3,4HD. The HepG2 line were by far the most sensitive cells to the effect of 3,4HD. As seen in some of the cell lines with the other isomers after 48h exposure 3,4HD cause and initial rise in MTT turnover, followed by a steep decrease in MTT turnover in the CaCo-2 and HepG2 lines and a more gradual decrease with increasing concentration in the MCF7 line. The CaCo-2 line was the most susceptible to 3,4HD at this time point followed by the HepG2 cells, with the MCF7 line the least sensitive.

The concentration of 3,4HD required to cause 50% reduction in MTT turnover in all cell lines were similar to those for 2,3HD. As with 2,3HD, at 4h the SK-N-SH cell line was the most sensitive with an IC_{50} of 19.6 ± 0.2mM, which was significantly lower than all other cell lines ($P<0.001$). The CaCo-2 cell line was the least sensitive line to the effects of 3,4HD at 4h (IC_{50}; 46.2 ± 1.7mM), the HepG2 line was significantly more sensitive than the CaCo-2 line ($P<0.001$) with an IC_{50} of 40.7 ± 0.4mM and the MCF7 line more sensitive still ($P<0.001$) 27.1 ± 1.0mM. The IC_{50}s of all three of the non-neuronal cell lines were significantly higher than both the SK-N-SH and SH-SY5Y cells ($P<0.001$).

As seen with 2,3HD at 24h, a steep increase in the sensitivity to the effects of 3,4HD on MTT turnover, was observed in both the SK-N-SH and SH-SY5Y lines at 24h (IC_{50}s; 4.2 ± 0.1mM and 1.2 ± 0.1mM respectively). All three non-neuronal cell lines had significantly higher IC_{50}s compared to the SK-N-SH and SH-SY5Y lines ($P<0.001$). There was an increase in sensitivity to 2,3HD in all three non-neuronal cell lines at 24h, however, this was not to the extent seen with the SK-N-SH and SH-SY5Y cells.
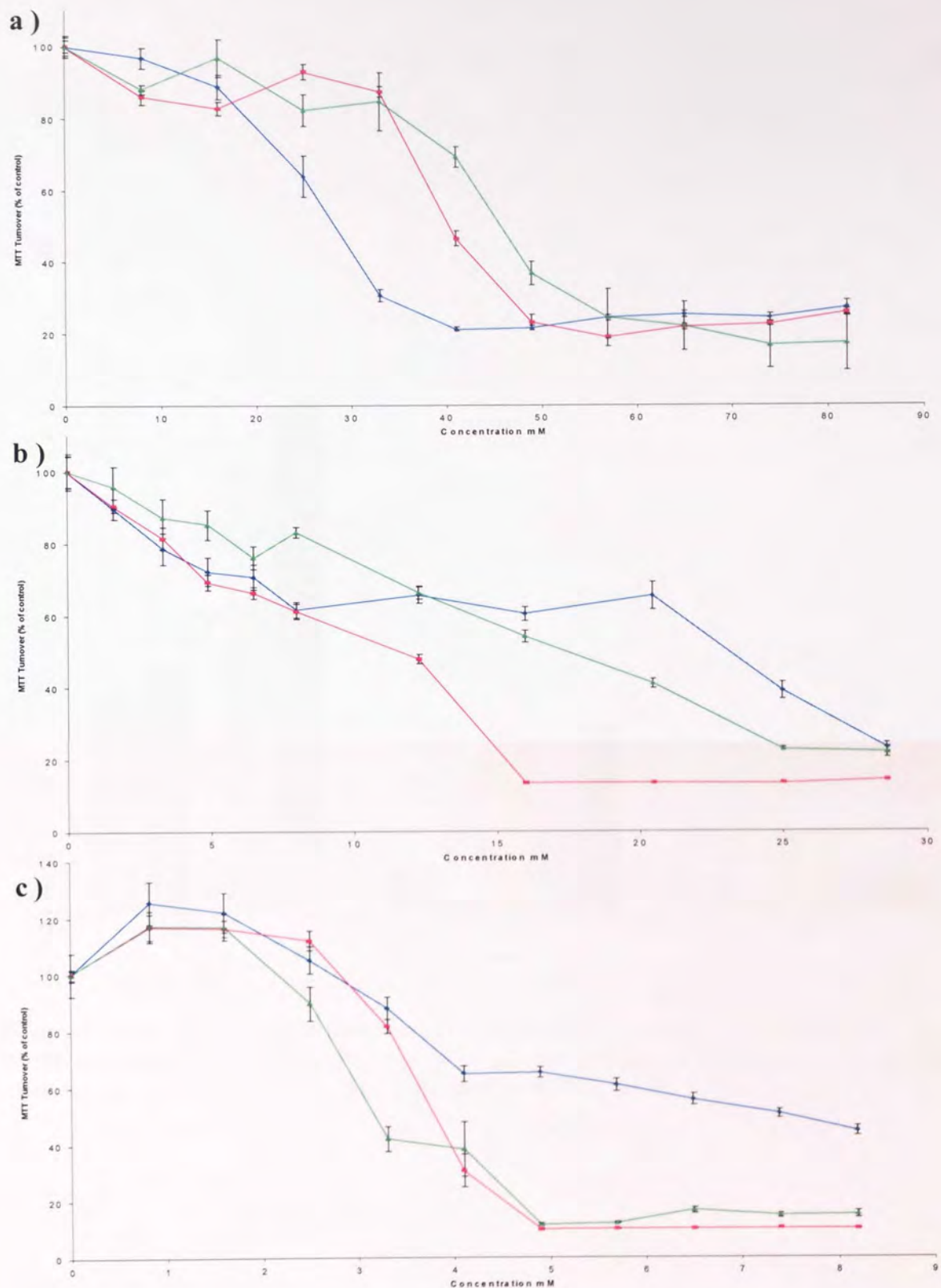
Figure 5.5: MTT assay on MCF7 (●), HepG2 (■) and CaCo-2 (▲) cells following a) 4h, b) 24h and c) 48h exposure to 3,4-hexanedione. Cells were treated with increasing concentrations of 3,4-hexanedione and incubated for 4, 24, or 48h. The MTT assay was carried out and absorbance was read at 590nm. Results are means ± SEM (n=3).
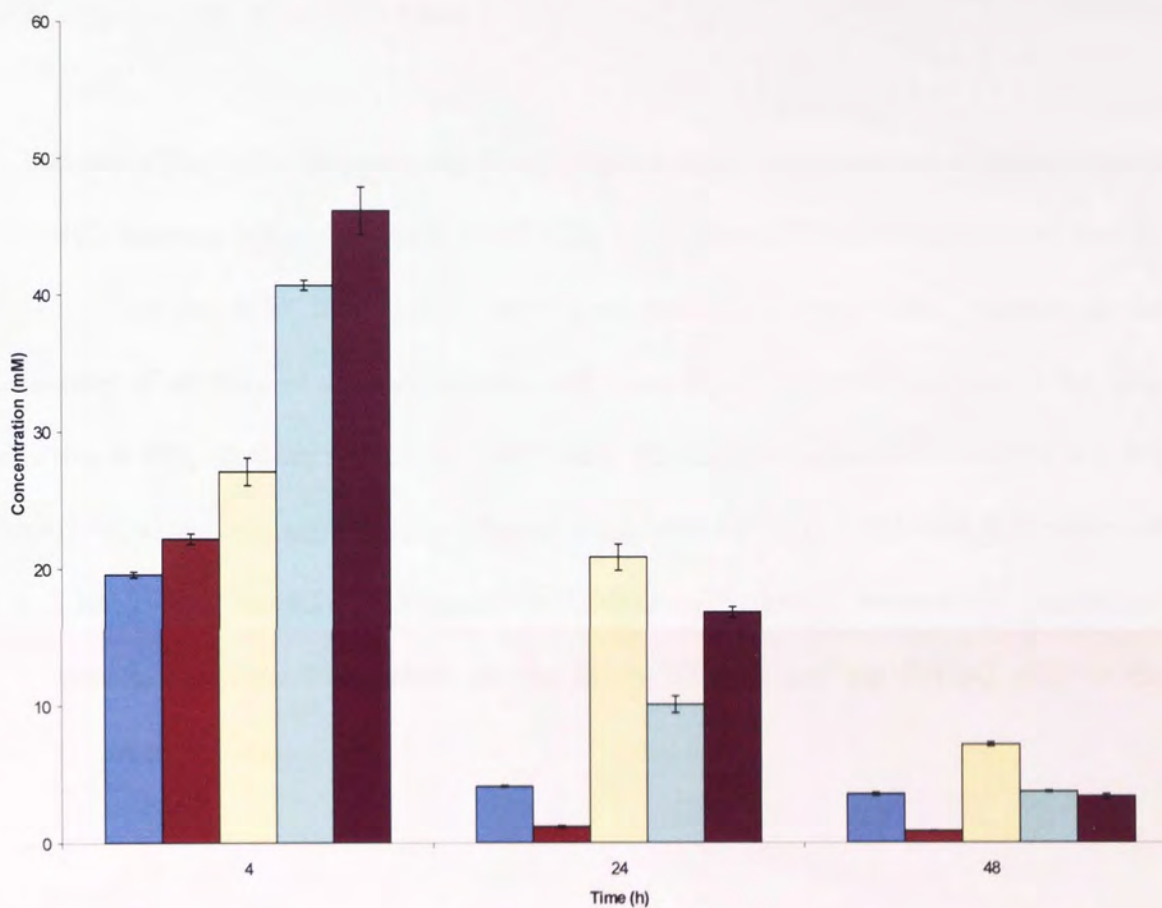
Figure 5.6: Bar chart of IC_{50} values calculated from MTT assays of SK-N-SH (■), SH-SY5Y (■), MCF7 (□), HepG2 (□) and CaCo-2 (■) cell lines following 4, 24 and 48h exposure to 3,4-hexanedione. Values are means ± SEM (n=3).

Again, as observed with 2,3HD, the sensitivity of the MCF7 line rose the least (P>0.05), meaning that these cells began as the most sensitive of the non-neuronal cell lines at 4h, but they became the least sensitive at 24h with an IC_{50} of 20.9 ± 1.0mM. The CaCo-2 cells were slightly more sensitive than the MCF7 line (IC_{50}: 16.9 ± 0.4mM) and the HepG2 cells more sensitive still (IC_{50}: 10.1 ± 0.6mM).

In both neuroblastoma cell lines only a very slight increase was observed in the sensitivity to 3,4HD between 24h and 48h (SK-N-SH IC_{50}: 3.6 ± 0.1mM; SH-SY5Y IC_{50}: 0.9 ± 0.1mM). However for the same time period there were between 3 and 5 fold increase in the sensitivity of all three of the non-neuronal cell lines. Again the MCF7 cells were the least sensitive at 48h, showing an IC_{50} of 7.2 ± 0.2mM, although the values from the CaCo-2 and HepG2 cells were not significantly different from each other (P>0.05) with IC_{50} values of 3.4 ± 0.2mM and 3.8 ± 0.1mM respectively. Statistical analysis showed no significant difference between the IC_{50} values for the SK-N-SH cells and the CaCo-2 cells or the HepG2 line (P>0.05).

5.4 Discussion

As discussed in detail previously, 2,5HD is the known neurotoxic metabolite of the aliphatic hydrocarbon n-Hexane (Perbellini *et al.*, 1990; Governa *et al.*, 1987; Spencer and Schaumburg 1975; Graham, 1999). In this chapter the acute and sub-chronic toxicity of 2,5 HD has been examined in 3 human non-neuronal cell lines, MCF7, HepG2 and CaCo-2; indeed, to the author's knowledge no similar studies have been carried out in human non-neuronal cell lines. 2,5HD caused a concentration dependent reduction in MTT turnover in all 3 cell lines. In general the IC_{50} values for 2,5HD were significantly higher in the non-

neuronal lines than in the neuroblastoma lines at 4h and 24h, however, at 48h, the values were not significantly different. One of the suggested mechanisms of neurotoxicity of 2,5HD in humans involves the cross-linking of neurofilaments (Graham *et al.*, 1991; Zhu *et al.*, 1994; Graham *et al.*, 1995; Heijink *et al.*, 2000), which leads to axonal atrophy. Although neurofilaments are not present in these non-neuronal cell lines, it is also known that 2,5HD is a 'soft electrophile' (electron deficient) and forms adducts with protein lysine residues which are related to several toxic effects such as oxidative stress (LoPachin and DeCaprio, 2005) and this may account for the broader, time-dependent cytotoxicity of 2,5HD observed in this study at 48h. A more recent study also identified the amino acids, β-alanine and glycine as reactive targets for 2,5HD (Pei *et al.*, 2007), which may also have contributed to the toxic effects of this hexanedione isomer seen in the results section.

Also in this chapter, the acute and sub chronic toxicities of 2,3HD and 3,4HD were examined in the 3 human non-neuronal cell lines, MCF7, HepG2 and CaCo-2. 2,3HD and 3,4HD showed toxicity in all 3 non-neuronal cell lines marked by a reduction in MTT turnover. As seen in the neuroblastoma lines the concentrations required to cause 50% reduction in MTT turnover were considerably lower in all cell lines than with the 2,5 isomer. As with 2,5HD, in general, the IC_{50} values for 2,3HD and 3,4HD were significantly higher in these lines than in the neuroblastoma lines at 4h and 24h, however at 48h the sensitivities of all cell lines were more comparable.

The neuronal-like neuroblastoma cell lines, SK-N-SH and SH-SY5Y, were considerably more susceptible to the effects of 2,3HD and 3,4HD at the more acute exposures, 4 and 24h, compared with the other non-neuronal lines. In fact the three non-neuronal lines showed some marked resistance to the toxicity of the hexanediones. The MCF7 line

showed particularly significant resistance to the toxic effects of all three hexanedione isomers. This may be linked to an enhanced ability of this multiple anti-cancer drug-resistant line to promote rapid cellular efflux of the ketones through its phenotypic overexpression of various efflux pump systems (Zampieri *et al.*, 2002; Woehlecke *et al.*, 2003).

The oxoglutarate carrier, which was the central factor in the suggested mechanism of toxicity for 2,3HD and 3,4HD outlined in the previous chapter, is found in all tissues and cells, so it would be expected that 2,3HD and 3,4HD would exert some broad cytotoxicity. The differences in sensitivities to the 2,3HD and 3,4HD isomers between cell lines could be due to differences in the amount of oxoglutarate carriers/mitochondria, the permeability of the cells to take up the compounds and the cellular demand for ATP.

In summary, all 3 hexanedione isomers proved to be cytotoxic, in all 3 non-neuronal cell lines at all time points. 2,5HD caused its toxicity through a neurofilament cross-linking free mechanism, possibly through the production of reactive oxygen species (oxidative stress). At 4h and 24h the neuroblastoma lines were considerably more sensitive to the effects of the hexanediones compared to the non-neuronal lines but at 48h the sensitivities of all cell lines were comparable.

Chapter 6. Final Discussion

6.1 Summary of Findings

The widespread use of n-Hexane in adhesives in the footwear industry in Non-EU countries, has led to reports of human hexane-induced neurotoxicity over the last 25 years (dos Santos *et al.*, 2002). It became apparent that the γ diketone 2,5-hexanedione (2,5HD) metabolite was responsible for the neurotoxicity of this alkane (Spencer and Schaumburg 1975; Governa *et al.*, 1987; Perbellini *et al.*, 1990; Graham, 1999) and although the cross linking of neurofilaments is thought to be involved, the complete mechanism of toxicity is still not fully understood (Graham *et al.*, 1991; Zhu *et al.*, 1994; Graham *et al.*, 1995; Heijink *et al.*, 2000; LoPachin & DeCaprio, 2005). 2,3-hexanedione (2,3HD) and 3,4-hexanedione (3,4HD) are both α diketone isomers of 2,5-hexanedione, used commercially as food additives and have been classified previously as non-toxic and non-neurotoxic in rats (FAO/WHO, 1999; Stone *et al.*, 1999; 2000).

To date, no work had been carried out in human cellular models on the cytotoxicity of the α diketone isomers and in the light of the known neurotoxicity of the 2,5- derivative, it was important to investigate initially if the 2,3HD and 3,4HD isomers were indeed cytotoxic to human cells and whether they showed any degree of toxicity in neural cell lines. As the studies reported in this thesis progressed, it was apparent that they showed a mild degree of cytotoxicity and some selectivity in their toxicity towards two human neuroblastoma cell lines.

6.1.1 MTT Assays

Initial studies with MTT turnover showed that the known neurotoxin 2,5-HD was the least toxic in all cell lines over the time periods 4, 24 and 48h exposure. These time points included an acute toxicity period (4h) as well as an acute/intermediate toxicity period (24-48h). In general the $IC_{50}s$ were not significantly different between the two α isomers, however, although not statistically different, there was a tendency for 3,4HD toxicity to slightly exceed that of the 2,3- derivative. Although 2,5HD is an established chronic neurotoxin, it has been shown to exert some toxicity at more acute exposure periods *in vitro* in cultured rat Schwann cells (Kamijima *et al.*, 1996) and SH-SY5Y cells (Hartley *et al.*, 1997) and neurofilament crosslinking, a likely mechanism of action, occurs within the concentration ranges used in the present study at 24h (Heijink *et al.*, 1995; Heijink *et al.*, 2000).

At the most acute exposure period, 4 hours, the SH-SY5Y line was the most sensitive to 2,5HD toxicity and this line was more sensitive than the other neuroblastoma line, the SK-N-SH cells. The next most sensitive cells at 4h were those of the MCF7 line, followed by the CaCo-2 cells and finally the HepG2 line, which was the least susceptible to 2,5HD toxicity at this time point. Alternatively, with both the 2,3HD and 3,4HD isomers at 4h exposure, the SK-N-SH line showed greater sensitivity compared with the SH-SY5Y cells. Indeed, both neuroblastoma lines were more sensitive to all isomers compared with the non-neuronal cells.

Regarding 24h exposure, the non-neuronal cell lines were again more resilient to 2,5HD than the neuroblastoma cells with the SH-SY5Y line being the most sensitive.

Interestingly, in contrast to data from 4h exposure, the 2,3HD and 3,4HD isomers showed the greatest toxicity to the SH-SY5Y line, followed by the SK-N-SH cells. Surprisingly at the 48h period of study, which borders on chronic exposure to 2,5HD, the CaCo-2 line was the most susceptible and there was very little difference in the IC_{50}s of the other four cell lines. As seen at 24h, the SH-SY5Y cell line was most vulnerable to the effects of both 2,3HD and 3,4HD at 48h exposure. The MTT studies suggested that the neuroblastoma lines were on the whole more sensitive to the alpha diketones compared with the other non-neuronal lines. Although the neuroblastomas are not close to human neurones in structure, they do display some neurone-like properties. However, the lack of sensitivity to α diketones in an established model of human neurones, the NT-2N differentiated human neuronal system (Woehrling et al., 2006), suggests that neuroblastomas do indeed display a marked sensitivity to these agents.

6.1.2 Flow Cytometry

Using flow cytometry it was determined that 2,5HD caused apoptosis in the SK-N-SH cell line after 48h exposure in the relatively high concentration range of 9-17mM. After allowing for the higher rate of background apoptosis in the SH-SY5Y cells compared with the SK-N-SH cells, the SH-SY5Y line showed a similar pattern in apoptosis and cell cycle checkpoint arrest in response to cellular damage compared with the SK-N-SH line. In both neuroblastoma cell lines, 3,4HD was a significantly more potent apoptosis inducer than the 2,3 isomer. Also, even allowing for higher rate of background apoptosis in the SH-SY5Y cells, both α isomers were at least five-fold more potent in the induction of apoptosis in the SH-SY5Y line compared with the SK-N-SH line.

Cell cycle arrest at G_0/G_1 caused by all three hexanedione isomers in both the SK-N-SH cells and SH-SY5Y line corresponded with the inhibition of the cell cycle at the DNA replication stage (S-phase). Interestingly, the effects of both α-diketones on the G_2/M chromosomal segregation stage in the SH-SY5Y line appeared to occur not only at 2-3 fold lower concentrations compared with the SK-N-SH line, but also in a different pattern. In the SK-N-SH cells the respective peaks for each isomer at G_2/M occurred over a similar concentration range, however, the G_2M peak for the 3,4 isomer in the SH-SY5Y line occurred between 1.1 and 1.3 mM and the 2,3 isomer peaked at the higher concentration range of 1.3 to 1.6 mM.

The differences in toxicity between the two α-diketones may be linked with lipophilicity, although 3,4HD has two ethyl groups either side of the ketone moieties, whilst 2,3HD possesses a methyl and a bulkier propyl group. It is possible that steric hindrance during interactions with vital cell macromolecules influenced the differential toxicity of these isomers.

6.1.3 General Conclusions of Findings

Overall at 4h and 24h exposure, the neuronal-like neuroblastoma cell lines were significantly more susceptible to the cytotoxicity of all hexanedione isomers, especially the α isomers 2,3HD and 3,4HD, compared with the non-neuronal cell lines. The SH-SY5Y cell line proved to be up to four-fold more sensitive to α-diketone toxicity compared to the SK-N-SH. The sensitivity of the SH-SY5Y cells could be linked with the relatively high but consistent background apoptosis seen in the SH-SY5Y cells with flow cytometry compared with the background apoptosis observed in the control groups of the SK-N-SH

cells. A number of factors may influence the predisposition of different neuroblastoma lines to enter apoptosis, such as effects on protein kinase C, Bcl-2 and G protein $G\alpha_s$-L (Lombet *et al.*, 2001; Zhao *et al.*, 2006), as well as different responses to growth factors present in FBS (da Motta *et al.*, 1997). Another factor that could account for the differences in sensitivity of the two neuroblastoma cell lines include the heterogeneous nature of the SK-N-SH cell line compared to the homogeneous line SH-SY5Y.

A mechanism of cytotoxicity for both 2,3HD and 3,4HD has been proposed based upon the knowledge that both α hexanedione isomers inhibit the inner mitochondrial membrane transport protein, the oxoglutarate carrier (OGC) (Stipani *et al.*, 1996). The principal behind the suggested mechanism is that inhibition of the OGC leads to inhibition of gluconeogenesis, which in turn leads to a reduction in the production of ATP. Decreased levels of cellular ATP are enough to cause the release of apoptosis inducing factor (AIF) from the mitochondria (Daugas *et al.*, 2000). AIF instigates an apoptotic-like cell death by acting directly on DNA within the nucleus leading to large-scale DNA fragmentation (Cande *et al.*, 2002). As neuronal cells are well known for their high energy demands this would suggest that they would be more sensitive to a reduction in the supply of cellular ATP than other cell types. This also may go some way to explaining the higher susceptibility of the neuronal-like neuroblastoma cell lines to the effects of the two α isomers compared to the non-neuronal cell lines utilised in this study.

6.2 Significance of Findings

6.2.1 Relevance of Findings with Regards to Human Exposure

In 1978 Cramer *et al.*, devised the "decision tree" procedure to assess potential toxic hazard and to prioritise compounds for the appropriate testing, based upon chemical structure and metabolism of a substance. The procedure uses recognised metabolic pathways, toxicity data and knowledge of the presence of the compound in "traditional" food and as an endogenous metabolite. This method is a useful tool for classifying flavour ingredients according to levels of concern. The outcome of the procedure is that the compound is classified into one of three categories, Class I, Class II or Class III:

- Class I compounds are substances with a simple chemical structure that are efficiently metabolised, which suggests a low order of oral toxicity. Examples include butyl alcohol and isoamyl butyrate.

- Class II substances have slightly more complex chemical structures. Their intermediate structures are less innocuous than those chemicals in Class I. Compounds that fall into Class II may possess reactive functional groups. Furfuryl alcohol, methyl 2-octynoate and allyl propionate are examples of chemicals in this class.

- Class III members have chemical structures that allow no presumption of safety and/or contain structural features suggestive of toxicity, such food additives are 2-phenyl-3-carbethoxy furan and benzoin.

Most flavouring substances found in foodstuffs are simple alcohols, aldehydes, ketones and acids etc that occur naturally and fall into Class I. Generally they are rapidly metabolised to innocuous metabolites such as acetic acid and water. 2,3- and 3,4-hexanedione have been designated Class II compounds. The majority of this class fall into one of two categories; one group consists of substances with similar but more reactive functional groups than the functional groups of substances in Class I; the other group is made up of substances with more complex chemical structures than Class I compounds, but are common components in food. Both 2,3- and 3,4-hexanedione belong to the latter group (FAO/WHO, 1999).

According to the 51st meeting of the joint FAO/WHO expert committee on food additives (JECFA) in 1999, the estimated average daily intake per person of 2,3HD and 3,4HD in Europe was 13µg or 0.22µg/Kg body weight and 33µg or 0.5µg/Kg body weight respectively and in the USA was 10µg or 0.2µg/Kg body weight and 0.76µg or 0.01µg/Kg body weight respectively (FAO/WHO, 1999). The intake threshold for Class II compounds is 540µg per person per day (FAO/WHO, 1999). The concentrations of the α-diketones required to cause cell death in this study equate to considerably higher levels than the estimated daily intake of these compounds in our diets when extrapolating from *in vitro* studies to man. The slightly more toxic of the two alpha-ketones, 3,4HD, began to cause apoptosis in the SY-SY5Y cell line at concentrations of approximately 800µM, which is more than two orders of magnitude higher than the average human intake of this agent. However, there are potentially thousands of chemicals in foodstuffs, either of plant, animal or eukaryotic origin which can mediate changes in cellular apoptosis processes and interact with DNA, beneficially and deleteriously (Chen *et al.*, 2007; Hirose *et al.*, 2004; Larrosa *et al.*, 2006; Chan *et al.*, 2007) and it remains to be determined whether additive or

synergistic effects on apoptosis could occur due to combinations of hexanediones and other DNA-interactive chemical agents in the diet.

Although it is probable that 2,3- and 3,4-hexanedione, in the concentrations consumed by the average individual, are unlikely to constitute a high risk of human toxicity, the sensitivity of the neuroblastoma cell lines in particular to the actions of 2,3- and 3,4-hexanedione over modest exposure periods compared to the other cell lines used in this study is worthy of investigation.

6.2.2 Relevance of Findings with Regards to Neuroblastoma

Neuroblastomas are neoplasms that arise from neuronal crest cells. Compared with other cancers, they have a relatively high incidence in children under 15 years old (Young *et al.*, 1986) and they account for approximately 9% of all childhood cancers, which makes them the most frequent extracranial solid tumours of childhood. Neuroblastomas exhibit a very broad range of clinical behaviour from spontaneous regression to rapid tumour progression and death (D'Angio *et al.*, 1971; Evans *et al.*, 1976; Haas *et al.*, 1988; Miller *et al.*, 1995). In many cases early stage neuroblastoma can be cured with surgery alone (Alvarado *et al.*, 2000; Perez *et al.*, 2000). Surgery combined with treatment with chemotherapy is known to show favourable results also (Bowman *et al.*, 1997; Schmidt *et al.*, 2000). Unfortunately, the majority of cases of advanced stage neuroblastoma in children over 1 year old results in death regardless of multimodality therapy (Matthay *et al.*, 1999). There are many different treatments other than surgery and radiotherapy that have been applied to the treatment of neuroblastoma and some of the newer therapeutic agents include cytotoxic agents, such as the topoisomerase I inhibitor topotecan (Nitschke *et al.*, 1998). Retinoid therapy, oral

administration of 13-cis-retinoic acid, has also been used, where the ability of retinoic acid to cause differentiation *in vitro* has been exploited to arrest tumour cell proliferation (Sidell, 1982; Pahlman *et al.*, 1984; Reynolds and Lemons, 2001). Unfortunately, initial clinical trials proved disappointing (Finklestein *et al.*, 1992; Villablanca *et al.*, 1995). Other therapies used in childhood neuroblastoma include immunotherapy and antiangiogenic therapy (Weinstein *et al.*, 2003).

The cytotoxic agent mentioned above, topotecan, has shown better activity against neuroblastomas when used in combination with another chemotherapy agent such as cyclophosphamide or carboplatin (Saylors *et al.*, 2001; Athale *et al.*, 2002). All these cytotoxins and antiproliferative agents carry the burden of high toxicity, which translates into short and long term impact on the patient, assuming they survive the therapy. In the short term, patient compliance is often severely compromised by systemic adverse reactions and in the longer term, there are consequences for the reproductive capacity of these children, whose developing tissues are exposed to DNA-reactive agents. Indeed, it is not uncommon that patients can develop subsequent lymphomas which probably resulted from chemotherapy. It would be highly desirable to supplement such treatment regimens with agents that would inhibit the growth of the neuroblastoma cells with relatively little impact on other cellular systems. Although 2,3HD and 3,4HD are clearly low in potency in their toxic effects on the neuroblastoma lines used in this thesis, they do appear to show some selectivity and they are relatively low in 'bystander' toxicity to other cell lines which originated in human tissues such as the liver, gut and breast. Therefore, it is conceivable that the 2,3HD and 3,4HD might be worthy of further *in vitro* investigation in terms of their use as an adjunct to conventional antiproliferative drugs. The low toxicity of these agents suggests that relatively high doses could be tolerated, which might offset their lack

of neuroblastoma inhibitory potency. Along with the potency issues the specificity of the two compounds for neuroblastomas is not fully know as, although in this study the neuroblastoma lines were statistically more susceptible than the other three lines, there are many cell types not tested in this study that could more vunerable to 2,3HD and 3,4HD. Another issue that has to be considered is that the absorbtion, distribution, metabolism and excreation characteristics of these compounds may mean that in a whole body situation even if 2,3HD and 3,4HD were highly specific for neuroblastomas they may never reach their intended target. However, hypothetically even if these studies only yielded a minor increase in the potency of combination tumour cell cytotoxicity, this might translate clinically to a reduction in the dose of the antiproliferative agents, thus reducing the impact of the side effects of chemotherapy. This would have the added bonuses of improving patient compliance and lessening the risks of subsequent iatrogenic neoplasm development.

6.2.3 The Suitability of the MTT Assay and Flow Cytometry for a tiered *in vitro* toxicity test system

The MTT assay is commonly used to assess cytotoxicity following toxic insult in a large variety of cell lines (Habtemariam, 1995; Plumb, 2004). As was reported by Fotakis and Timbrell (2006), the MTT assay proved to be a sensitive assay that produced reliable and repeatable results. On the whole, the MTT assay provided a simple high throughput method for evaluating accurately the cytotoxicity of the hexanedione isomers assayed in this study, ideal for a "triage" tier of a dual component toxicity test system. Flow cytometry allows for the rapid analysis of thousands of cells (Siegel, 1984). Throughout this study, determination of nuclear DNA content via flow cytometric analysis of nucleoid

associated PI fluorescence proved reliable and reproducible, providing quantitative information of several stages of the cell cycle and apoptosis. It allowed for differences between the effects of the two α hexanedione isomers to be identified, whereas with the more basic MTT assay, the two compounds were virtually indistinguishable. Essentially, the combination of the simple and high throughput method of the MMT assay, with the more detailed information provided by flow cytometry formed a useful basis for a preliminary, human-cell based multi tiered toxicity test regime. Although the *in vitro* cytotoxicity system developed and utilised in this study has its limitations and would need to undergo a lengthy validation process, a two-tiered approach for the initial screening of potential neurotoxins could be of use in REACH-inspired mass evaluation of various chemical agents. This process would be more relevant to man, as most current *in vitro* screens mooted for use as part of REACH are either animal cell–based, or have little or no neuronal, or neurone-like components (Coecke *et al.*, 2006). Although the neuroblastoma lines are far from a perfect model for human neurones, they are considerably closer to human neuronal susceptibility than many tests currently under development, such as those involving worms, fish and mice (Marvanova and Nichols 2007; Tiedeken and Ramsdall 2007; Lemeire *et al.*, 2007).

6.3 Future Work

For an initial assessment of whether 2,3HD and 3,4HD showed any specific neurotoxicity this study utilised 3 non-neuronal cell lines in comparison with the neuroblastoma cell lines, SK-N-SH and SH-SY5Y. Further investigation of more specific neurotoxic markers such as disruption of neurotransmitter synthesis and transmission of information between nerve cells or neurofilament cross linking would provide a more coprehensive assessment

of the isomers neurotoxic potential. Also it has been shown that 2,5HD causes neurofilament cross linking in the SK-N-SH line following 72h exposure (Heijink *et al.*, 2000), therefore it would be interesting to investigate whether this cross linking is apparent at the more acute exposure periods used in this study in both the SK-N-SH and SH-SY5Y lines.

In this thesis, on the basis of past literature, a possible mechanism of acute cytotoxicity was reported for 2,3- and 3,4-hexanedione that was based around the reduction of ATP production through the inhibition the oxoglutarate carrier. This may have led to the release of AIF from the mitochondria and an apoptotic-like cell death. Mechanistic studies would be required to reinforce this theory, such as cell death measurements could be carried out in the presence of increased concentrations of the endogenous "passengers" of the OGC, 2-oxoglutarate (Stipani *et al.*, 1996) and glutathione (Chen & Lash, 1998; Lash, 2006). If the hexanediones competitively inhibit the OGC then the presence of increased 2-oxoglutarate or glutathione levels may reduce the amount of OGC inhibition caused by 2,3- and 3,4-hexanedione and thus increase the concentrations required to cause toxicity. Other studies could include measurement of reduction in cellular ATP, there are many ATP assay kits available commercially. Also it would be important to check for the release of AIF, which could be identified by Western blotting. Once a mechanism of toxicity has been established it would then be interesting to investigate whether 2,3HD and 3,4HD are specifically neurotoxic through the examination of neurotoxic endpoints, such as effects on axonal transport, synapse function, myelination, cell-cell signaling, enzyme activities, and neurotransmitter uptake and metabolism.

In this study the food flavourings 2,3- and 3,4-hexanedione showed some acute *in vitro* cytotoxicity, in all cell lines tested, after single dose exposure. However the concentrations required to cause this toxicity were much higher than the estimated daily intake. Therefore it would be of interest to carry out chronic repeat dose studies to mimic the human exposure to these compounds. In the past, this type of chronic study has proved a complex area of research, as standard cell culture techniques do not readily lend themselves to long-term repetitive experiments (Koppelstaetter *et al.*, 2004). Recent advances in cell culture techniques has led to the use of perfusion culture systems, where there is a slow continuous flow of culture medium in and out of the culture vessel (Koppelstaetter *et al.*, 2004; Jennings *et al.*, 2004). Perfusion culture systems allow chronic or repeat dose administration of toxins to the cells in culture and the out flowing medium can be assayed for various biomarkers such as LDH and cellular metabolites. LDH is a soluble cytosolic enzyme that is only released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. Therefore LDH activity can be used as an indicator of cytotoxicity.

Due to the complexity of human diets, the 2,3- and 3,4-hexanedione when used as food additives are never consumed in the absence of other chemical agents. It could prove useful to investigate if there are any interactions between the hexanediones and any other commonly used potentially toxic food additives, perhaps from Class III. These additives may well be safe when used alone, but the potential for interaction and accumulations of combinations of food additives and the hexanediones could potentiate toxic effects. These experiments should include acute, chronic and repeat dose exposures.

Finally as stated earlier, further investigation into the specific toxicity of these compounds towards neuroblastomas would be required to suggest whether there is any scope for their potential use in the treatment of neuroblastoma. It would be important to investigate the effects of these isomers in a much larger range of neuroblastoma cell lines, of which there are over 100, established from different primary sites, metastatic sites and other origins. It would also necessary to work directly with surgically removed neuroblastoma tumours, to compare and contrast the sensitivity of these primary cultured cells with fully transformed which have been adapted to culture for a long period.

6.4 Concluding Remarks

2,5-Hexanedione showed some acute cytotoxicity in both the neuronal-like neuroblastoma cell lines, SK-N-SH and SH-SY5Y and the non-neuronal lines MCF7, CaCo-2 and HepG2, with the MTT assay although the concentrations required were very high with the lowest IC_{50}s observed in the SH-SY5Y cell line at all time points.

Flow cytometry highlighted cell cycle arrest indicative of DNA damage with 2,3- and 3,4-hexanedione. All the data suggests a degree of cytotoxicity exhibited by 2,3- and 3,4-hexanedione but it is difficult to say whether there is any specific neurotoxicity because although the study investigated the effects of these compounds in the neuronal-like neuroblastoma cell lines more specific neurotoxic endpoints would be required. However the neublastomas were statistically more susceptible to the cytotoxic effects of the α hexanedione isomers at the more acute exposures 4 and 24 hours than the non-neuronal cell lines MCF7, HepG2 and CaCo-2. The proposed mechanism of action would suggest

that cells that have a higher energy requirement such as nerve cells would be more sensitive to the effects of 2,3 and 3,4HD.

Finally as the neuroblastoma lines used in this research were considerably more susceptible to the cytotoxicity of 2,3- and 3,4-hexanedione than other cell lines and there is some evidence to suggest that their effects should be investigated in a wider variety of clinically isolated neuroblastoma cells and tissues.

Chapter 7. References

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Chapter 8. Publications Based on this Research

Coleman, M.D., Zilz, T.R., Griffiths, H.R. and Woehrling E. A. (2008). A comparison of the apoptotic and cytotoxic effects of hexanedione derivatives on human non-neuronal lines and the neuroblastoma line SH-SY5Y. *Basic Clin Pharmacol Toxicol* **102**, 25-29.

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